UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Gymnodinium catenatum strains Isolated from the Portuguese coast: physiological and genetic characterization

Teresa Lopes Silva

MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

2013

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Gymnodinium catenatum strains isolated from the Portuguese coast: physiological and genetic characterization

Teresa Lopes Silva

Dissertação orientada pela Prof. Dra.. Ana Amorim (Departamento de Biologia Vegetal, Faculdade de Ciências da Universidade de Lisboa) e Dr. Pedro Reis Costa (Instituto Português do Mar e da Atmosfera) especialmente elaborada para a obtenção do grau de Mestre em Biologia Molecular e Genética.

2013

Abstract

Gymnodinium catenatum is a toxic bloom forming dinoflagellate, which has been linked to paralytic shellfish poisoning (PSP) outbreaks in humans. Along the Portuguese coast (NE Atlantic), *G. catenatum* is ubiquitous but shows a complex bloom pattern, raising questions about the origin and affinities of each bloom population. Toxin and genetic signatures can discriminate phenotypic and genotypic variability among *G. catenatum* isolates from different geographical regions. In this work, the variability within six cultured strains of *G. catenatum* isolated from Portuguese coastal waters (S coast, W coast and NW coast), between 1999 and 2011, was investigated. The strains were analyzed for physiological parameters, such as growth patterns and toxin profiling and for intra-specific genetic diversity.

Regarding the toxin profile, differences recorded between strains could not be assigned to the time of isolation or geographical origin. The parameter that most influenced the toxin profile was the life-cycle stage that originated the culture: vegetative cell *versus* hypnozygote (resting cyst). These differences were also registered for other physiological parameters such as growth curves and pigment profile.

At the genetic level, all strains showed similar partial large subunit ribosomal DNA (D1-D2 LSU rDNA) sequences and shared up to 100% identity with strains from Spain, Algeria China and Australia. However, we did not find a total identity match for the ITS-5.8S rDNA fragment. After sequence analysis, a guanine/adenine single nucleotide polymorphism (SNP) was detected near the end of the ITS1 region, for all strains. *G. catenatum* has previously been reported to present very conservative LSU and ITS rDNA regions. These polymorphisms, not yet described, may represent a distinctive mark of the Portuguese population

. Results obtained in the present work, show high homogeneity in toxin profile and at the genetic level for the analyzed strains. This may suggest an expansion of the North limit distribution of *G. catenatum* in the NE Atlantic rather than an anthropogenic introduction.

In addition, we have applied for the first time DNA BOX-PCR fingerprinting to microalgae and found that it is a valuable taxonomic tool discriminating at the species level.

Key words**:** *G. catenatum*; intra-specific variability; Physiology; Toxins; DNA markers; life-cycle; Portugal.

Resumo

A occorência de proliferações de algas nocivas (HAB's -harmuful algal blooms) nas zonas costeiras reveste-se de extrema importância pela ameaça que representam para a saúde pública e por serem a causa de consideráveis perdas económicas à escala mundial, para o sector das pescas, restauração e turismo.

Gymnodinium catenatum é um dinoflagelado, que durante o seu ciclo de vida passa por uma fase de dormência (hipnozigoto), normalmente referida como quisto de resistência, que lhe permite sobreviver a condições adversas e desta forma contribuir para o reestabelecimento das populações vegetativas. Produz neurotoxinas, da família das saxitoxinas (STXs), tóxicas para alguns animais marinhos e para o homem. *G. catenatum* está descrito como uma das principais espécies de dinoflagelados responsáveis pela occorrência de episódios de intoxicação paralisante por ingestão de marisco (PSP-paralytic shellfish poisoning) nos humanos, que nos casos mais graves provoca morte por paralisia respiratória. Em Portugal e durante última a década, a proibição da apanha de marisco devido a PSTs (paralytic shellfish toxins) tem resultado exclusivamente da contaminação por esta espécie. As PSTs mais comuns são frequentemente divididas em três grupos com base na estrutura química da cadeia lateral que apresentem e no seu potencial de neurotoxicidade. Toxicidades superiores estão associadas ao grupo carbamato [STX, neosaxitoxina (NeoSTX) e gonyautoxinas (GTX 1-4)], seguidas pelas toxinas decarbamato (dcSTX, dcGTX1-4 e dcNeoSTX) e por fim o grupo sulfamato (N- sulfocarbamoílo), considerado o menos tóxico (C1, C2, C3, C4, GTX5 e GTX6). Em *G. catenatum* o perfil de toxinas é muitas vezes dominado pelastoxinas do grupo sulfamato.

Devido à crescente consciencialização científica sobre a existência de microalgas com perfil tóxico, o conhecimento sobre a distribuição geográfica de *G. catenatum*, aumentou nos últimos anos e é hoje reportada nas águas costeiras de regiões temperadas de todos os continentes. Contudo, para além da sua distribuição biogeográfica natural, a distribuição actual de *G. catenatum* está associada à acção do homem como por exemplo, à dispersão da espécie pelo transporte de quistos de resistência nas águas de lastro dos navios.

Desde os anos 80 que em Portugal, *G. catenatum* é descrito como a principal espécie produtora de PSTs. Esta problemática tem sido alvo de estudo por parte de várias entidades, de entre as quais o laboratório de referência para biotoxinas marinhas IPMA (Intituto Português do Mar e da Atmosfera), que reporta a recorrência intermitente, anual e decadal, de blooms de *G. catenatum* na nossa costa.

Nos últimos anos o conhecimento sobre a ecologia e dinâmica destes episódios tem aumentado consideravelmente mas, ainda não foi possível compreender os mecanismos responsáveis pela variabilidade inter-anual e decadal observada. Embora se saiba que ao

longo da costa Portuguesa (NE Atlântico) *G. catenatum* é ubíquo, o padrão complexo de blooms que é observado levanta questões sobre a origem e as afinidades das populações inóculo.

Estudos sobre o registo fóssil de *G. catenatum*, indicam a colonização recente desta espécie na Península Ibérica, a partir do final do século XIX. Esta colonização recente foi inicialmente interpretada como uma possível evidência da introdução antropogénica de *G. catenatum* ao NE Atlântico. No entanto, trabalhos ulteriores indicam que a espécie já estava presente no Norte de África há pelo menos 3000 anos e sugerem que a colonização da Península Ibérica se deve à expansão natural do limite Norte de distribuição da espécie. Estas duas rotas de introdução originariam, presumivelmente, populações regionais com diferentes níveis de diversidade intra-específica. A primeira hipótese originaria uma população Ibérica com maior variabilidade, dada a sua origem em várias regiões geográficas, enquanto que a segunda geraria uma população de menor variabilidade intra-específica.

Um dos aspectos ainda por analisar em populações de *G. catenatum* da costa Portuguesa é o da variabilidade intra-específica. É sabido que a análise do perfil de toxinas e o uso de marcadores genéticos podem servir para discriminar a variabilidade fenotípica e genotípica existente entre isolados de *G. catenatum* de diferentes regiões geográficas. Neste sentido, no presente trabalho investigámos a variabilidade fisiológica, padrões de crescimento e perfis de toxinas, e a variabilidade genética. de 6 culturas de *G. catenatum* obtidas a partir de isolados colhidos em diferentes locais da costa Portuguesa (costa S, costa W e costa NW), entre 1999 e 2011. Estudámos dois tipos de culturas, umas provenientes do isolamento e germinação em laboratório de quistos de resistência e outras resultantes de células vegetativas planctónicas isoladas da natureza.

No que diz respeito aos resultados obtidos para os perfis de toxinas, as diferenças verificadas entre as estirpes não podem ser atribuídas à sua origem geográfica ou data de isolamento. A análise dos resultados evidencia que o perfil de toxinas é sobretudo condicionado pela fase do ciclo de vida que originou a cultura: célula vegetativa *versus* hipnozigoto (quisto de resistência). Esta evidência também foi registada noutros parâmetros fisiológicos, tais como nas curvas de crescimento e no perfil de pigmentos das estirpes. Estes resultados parecem sugerir que a expressão de alguns caracteres fisiológicos em *G. catenatum* depende de factores ambientais que não são reprodutíveis em laboratorio.

Não obstante, o padrão de toxinas evidenciado pelas estirpes que derivaram da germinação de quistos, enquadram-se com o que já foi descrito para a costa NE do Atlântico (Portugal e Espanha) e que é caracterizado pela produção mais abundante de C1-4, GTX5 e GTX6 e valores baixos de dcSTX. Curiosamente as estirpes resultantes do isolamento de

células vegetativas mostraram perfis de toxinas diferentes dos anteriormente descritos com valores muito superiores de dcSTX, ausência de C3+4 e GTX6 e presença de dcGTX2+3.

Ao nível genético, todas as esirpes evidenciaram sequências parciais idênticas no ADN ribossomico (ADNr) que codifica a subunidade maior do ribossoma (LSU-Large Sub-Unit). Em relação ao fragmento da LSU analisado (D1-D2), as nossas estirpes partilharam sequências idênticas com estirpes do GeneBank de Espanha, Argélia, China e Austrália. No entanto, para uma outra região do mesmo ADNr – região ITS-5.8S – a identidade genética entre as estirpes não foi total. Após a análise de sequências, foi detetado um polimorfismo no final da região ITS1, com uma sobreposição guanina/adenina. De acordo com trabalhos anteriores, *G. catenatum* apresenta regiões LSU e ITS-5.8S muito conservadas, pelo que este polimorfismo, ainda não descrito por outros autores, poderá vir a revelar-se um marcador das populações de *G. catenatum* da costa Portuguesa e da Península Ibérica.

Os resultados obtidos mostram que as populações de *G. catenatum* que ocorrem na costa Portuguesa são bastante semelhantes em relação ao perfil de toxinas e à assinatura genética. Esta observação sugere que a introdução da espécie em águas Ibéricas tenha ocorrido a partir de uma mesma população e não a partir de múltiplas introduções com possível origem em populações geográficas diferentes, como se esperaria se a introdução fosse via águas de lastro de navios. Nesse sentido, este trabalho suporta a teoria mais recente que aponta para a colonização da Península Ibérica ter ocorrido em resultado da expansão do limite Norte de distribuição da espécie no Atlântico NE.

Ainda no que respeita à análise de ADN, aplicámos neste trabalho pela primeira vez a técnica do BOX-PCR a microlagas, organismos unicelulares eucariotas. Esta técnica de análise do ADN total, tem sido apenas usada em bactérias e fungos. Os resultados indicam que em dinoflagelados esta técnica não permite descriminar a variabilidade intra-específica, no entanto, verificamos que poderá vir a constituir uma ferramenta valiosa em estudos taxonómicos de microlagas permitindo a discriminação ao nível de espécie.

Palavras-chave**:** *G. catenatum*; Variabilidade intra-específica; Fisiologia; Toxinas; marcadores de DNA; Ciclo de vida; Portugal.

Agradecimentos / Acknowledgements

Nas próximas linhas, gostaria de expressar a minha gratidão a todos os que de alguma forma contribuíram para a elaboração desta tese.

Agradeço à Professora Dra. Ana Amorim pela confiança que depositou em mim, por me ter recebido tão prontamente na sua equipe de trabalho e por me ter orientado num dos maiores desafios profissionais que enfrentei até ao momento.

Agradeço igualmente ao Dr. Pedro Reis Costa, por me ter acolhido no laboratório de toxinas marinhas do IPMA, pela disponibilidade e simpatia, e por me ter dado a oportunidade de realizar um projeto multidisciplinar que em muito contribuiu para a robustez da dissertação.

Um agradecimento especial à Professora Dra. Filomena Caeiro, pelo apoio incondicional que me dedicou e cuja experiência como geneticista sempre me condiziu por caminhos de descoberta. Muito obrigada pela sua bondade e por ser uma fonte de inspiração.

Aos meus colegas do Centro de Oceanografia, um gesto de carinho; Ana Brito, Carolina Sá, Lourenço, Carolina Beltrán, Joaninha, e mais outro carinho à Vera Veloso, por me ter dado a conhecer o maravilhoso mundo das microalgas à luz dos seus olhos.

À Professora Dra Vanda Brotas, por me ter proporcionado, mais que uma vez, continuar a fazer parte de toda esta equipe (CO). Por me ter dado a oportunidade de estar envolvida em projectos que me permitiram adquirir conhecimentos complementares e essenciais para o desenvolvimento das minhas aptidões científicas.

Gostaria também de enviar uma palavra de apreço à Dra. Teresa Moita e a todos os colegas do HABSpot, especialmente àqueles que partilharam comigo horas de expectativa, alegria e de trabalho intenso, enquanto no mar.

Quero expressar a minha mais sincera gratidão à Maria João R. Teles, pela sempre presente amizade e pelo apoio decisivo nesta fase da minha vida.

Por último, às minhas queridas amigas Fátima e Rita Azevedo e à minha famila, um muito obrigada por tudo.

Index

1. Introduction

Harmful algal blooms (HABs) are increasing in frequency, distribution and intensity worldwide (Hallegraeff 2012). These events are the cause of biotoxin contamination and threaten the environment, public health and economics of the affected areas. One of the major concerns for scientists is that blooms can extend into previously unaffected areas, through anthropogenic or natural conditions, and some of the transported species will adapt to foreign environments and become well established invasive populations (Bolch et al. 1999; Bolch and Salas, 2007)

Gymnodinium catenatum Graham is a bloom forming dinoflagellate, which has been linked to paralytic shellfish poisoning (PSP) outbreaks, in temperate to equatorial tropical waters affecting 23 countries (Hallegraeff et al. 2012). Vegetative cells are distributed throughout the water column, while resting cysts (a stage of its life-cycle) settle in bottom sediments (Anderson et al. 1988; Bolch and Reynolds, 2002) (Fig. 1). *G. catenatum* produces a great range of neurotoxins, which accumulate in shellfish and may affect humans (Oshima et al. 1987).

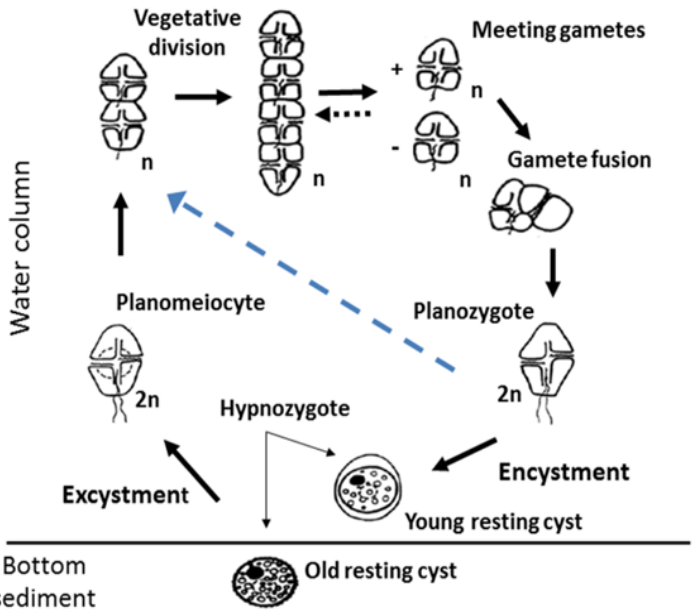

Figure 1- Schematic representation modified from Hallegraeff et al. (2012) of *G. catenatum* life-cycle, evidencing resting cyst formation after sexual reproduction (gamete production and fusion) and cyst bottom deposit.

Paralytic shellfish toxins (PSTs) produced by *G. catenatum* include saxitoxin (STX) and its natural occurring analogues (Lawrence and Niedzwiadek, 2001). The PST family can be divided into three groups, based on their side chain chemical structure and neurotoxicity potential. Higher toxicity is linked to the carbamoyl group [STX, neosaxitoxin (NeoSTX) and gonyautoxins (GTX 1-4)], followed by the decarbamoyl toxins (decarbamoyl derivatives of STX, GTX1-4 and Neo) and the less toxic sulfamate group (C1, C2, C3, C4 and GTX5 plus GTX6). In *G. catenatum* the toxin content is commonly dominated by the N-sulfocarbamoyl sub-group (Oshima et al. 1993; Ordás et al. 2004; Negri et al. 2007).

It is important to understand *G. catenatum* global distribution and how the distinct geographically populations relate. Evidence so far suggests that *G. catenatum* toxin and genetic profiles are relatively conservative and function as markers of regional populations.

Global *G. catenatum* populations and cultures exhibit variation in PST profiles, but with consistent regional patterns (Oshima et al. 1993). For instance, most Australian strains produce a high proportion of C3 and C4 (Negri et al. 2001, 2007) while strains from the Gulf of California are characterized by a high proportion of neoSTX (Band-Schmidt et al. 2006). Japanese isolates produce C1 and C2 derivatives but not C3 and C4 (Hallegraeff et al. 2012), while Negri et al. (2001) found Iberian strains (Portugal and Spain) to produce all C1-4 range and GTX5 and GTX6. Recently, hydroxybenzoate toxins analogues (GC1-3) have been identified and found to be common in worldwide *G. catenatum* strains (Negri et al. 2007). In addition, when present, the relative abundance of this toxin group is considered a differentiating factor between regions (Negri et al. 2007).

Despite the geographical signature in toxin profiles, *G. catenatum* seems to exhibit low intra-specific genetic variability. Polymerase chain reaction (PCR) based methods coupled with sequencing targeting ribosomal DNA regions (rDNA), allowed the identification of single nucleotide polymorphisms (SNPs) (Bolch and Salas, 2007). These authors found a SNP cytosine/thymine (C/T) on the start of the 5.8S rDNA region, which allowed the separation of Australian and New Zealand isolates (T-nucleotide), from strains originating from the rest of the world (C-nucleotide); the only exception concerns the strains from Japan that show both nucleotides, suggesting phylogenetic affinities with the Australian strains as well. Later, Band-Schmidt et al. (2008) detected a unique profile for Mexican isolates (Gulf of California) in the D1-D2 region of the large subunit (LSU) rDNA where a C-nucleotide is substituted by a guanine (G-nucleotide). Attempts to resolve genotypic differences within regional populations turned out to be ineffective with these procedures. In contrast, total DNA fingerprinting (RAPD-PCR analysis) indicated high genetic variation within local strains (Bolch et al. 1999; Camino-Ordás et al. 2004).

The coastal region of Portugal is affected by seasonal blooms of *Gymnodinium catenatum*. Since the first bloom record in 1986 (Franca and Almeida, 1989), blooms were

annually recurrent until 1995. Despite the regular monitoring program for toxic algae species in Portuguese coastal waters (IPMA – Instituto Portugês do Mar e da Atmosfera), blooms of *G. catenatum* were not recorded between 1996 and 2005. Since 2005 and until the present, blooms became again annually recurrent.

The distribution and population dynamics of *G. catenatum* have been studied along the Portuguese coast (Amorim et al. 2001; Moita et al. 2003, 2006), but still little is known about toxin and genotypic variability among *G. catenatum* strains within regional populations.

The aim of this study was the characterization of *G. catenatum* populations present in Portuguese coastal waters. Cultured strains isolated from local populations representing distinct bloom periods, were analyzed for toxin profiling. In addition, sequencing of several genomic regions with a potential polymorphic differentiating value was performed.

2. Materials and Methods

2.1. Algal cultures

Studied cultures were obtained by germination of single wild cysts, isolated from surface sediments, or established from single vegetative cells isolated from plankton net samples (20 µm mesh) (Table 1). For cyst germination, sediment samples were collected between 1999 and 2011 at different locations, along the Portuguese coast (S-N) where *G. catenatum* blooms had occurred (Fig. 2). Samples were kept in the dark at 4°C until further treatment, to avoid cyst germination during storage.

Table 1 – Collection date, Isolation date and sampling location of *G. catenatum* cysts or plankton cells used to obtain the studied cultures.

Strain	Source	Sampling date	Isolation date	Coordinates	Location
IO13-01	Cyst	Sep. 1999	Jan. 2003	37° 00’ N / 08°55’ W	Algarve
IO13-02	Cyst	March 2003	April 2003	38° 41’ N / 09°25’ W	Lisbon
IO13-06	Plankton	Sept. 2005	Sept. 2005	38° 41’ N / 09°25’ W	Lisbon
IO13-17	Plankton	July 2008	July 2008	39° 22’ N / 09°26’ W	Algarve
IO13-22	Cyst	Sep. 2010	March 2011	38° 25’ N / 09°09’ W	Aveiro
IO13-24	Cyst	Sep. 2011	Sept. 2011	38° 25’ N / 09°09’ W	Aveiro

Live cysts were isolated using a density step gradient (Amorim et al. 2001) and transferred into culture plates (Nunc^{TM} 176740) filled with sterilized seawater. Cysts were maintained in a culture chamber at 19 ± 1 °C (Fitoclima 750E, Aralab, Portugal), under cool white fluorescent light at 40 µmol photons m^{-2} s^{-1} (18 Watts, Osram, Germany), on 14:10 light:dark cycle. After germination, the motile cells were transferred to GSe medium (Doblin et al. 1999) (15 mL Petri dishes), salinity 35, in the same conditions as described for cyst incubation.

Resulting cultures (IO13-01, 02, 06, 17, 22 and 24), are currently maintained in the Algae culture collection at Lisbon University (ALISU), Center of Oceanography. Cultures with origin in resting cysts presumably result from meiosis and are assumed to be non-clonal.

Figure 2 - Maps of the Iberian Peninsula and Portugal, showing sampling locations in the Portuguese coast, NE Atlantic. Aveiro (NW Portugal), Lisbon (Central Portugal) and Algarve (South Portugal).

2.2. Growth curves

For scale-up procedure, all strains were grown on GSe medium but with salinity 28, as described above. Growth was followed in 100 mL medium batch cultures (250 mL SCHOTT flasks) for at least 30 days. Triplicate cultures were started with an initial cell concentration of 200 cells mL^{-1} from a common inoculum in exponential growth phase. For each replicate subsamples (1mL) were collected every two days and fixed in Lugol's solution for cell counting.

Cells were counted at 100X magnification by light microscopy (Olympus BX50, Japan) using a Palmer-Maloney counting chamber (Guillard and Sieracki, 2005). A minimum of 300 cells were counted to ensure statistical significance. Growth rate, k (divisions day^{-1}) and doubling time, T2, were calculated according to Wood et al. (2005).

2.3. Pigment analysis

Pigments were analyzed by high performance liquid chromatography (HPLC). Samples were collected during the stationary growth phase. Variable volumes (depending on cell density) of each culture were filtered onto 25 mm Whatman GF/F filters with a nominal pore size of 0.7 µm. To ensure pigment integrity filters were immediately frozen and kept at -80°C.

Photosynthetic pigments were extracted with 95 % cold-buffered methanol (2 % ammonium acetate) enriched with a known concentration of internal standard (trans-beta-apo-8'-carotenal pigment) for 30 min at -20 °C in the dark. Samples were sonicated (Bransonic model 1210, w:80, Hz:47) for 5 min at the beginning of the extraction period then centrifuged at 2800 xg for 15 min at 4 °C. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 µm pore size) immediately before injection in HPLC to remove cell and filter residues.

Pigment extracts were analyzed using an HPLC system (Shimadzu, Japan) composed by a solvent delivery module (LC-10ADVP) with system controller (SCL-10AVP), a photodiode array (SPD-M10ADVP) and a fluorescence detector (RF-10AXL).The chromatographic separation of pigments was achieved using a C8 column and an injection volume of 100 µL following the method described by Zapata et al. (2000). Pigments were identified and quantified by comparing the absorbance spectra and retention times with the HPLC calibration results carried out with pigment standards from DHI (Institute for Water and Environment, Denmark). Pigment concentrations were calculated from the signals in the photodiode array detector or fluorescence detector. Concentrations of pigments for which a standard was not available could not be quantified (e.g. peridiniol, diadinochrome and dinoxanthin).

2.4. Toxin analysis

Once the growth curves were established, new cultures were prepared in the same conditions as described above, using three replicates. A volume of 600 mL of late exponential growing cultures were filtered onto 47 mm Whatman GF/C with a nominal pore size of 1.2 µm, under low vacuum.

Toxins were extracted in 4 mL of 0.05 M acetic acid and sonicated for 4 min at 25 W, 50 % pulse duty cycle (Vibracell, Sonic & Materials, Newtown, CT, USA) in an ice bath. Cell

lysis was confirmed with light microscopy. The extract was then centrifuged (4000 ×g) for 10 min and an aliquot of the supernatant was used for determination of PSTs. Extracts were purified using a solid-phase extraction (SPE) clean-up with an octadecyl bonded phase silica (Supelclean LC-18 SPE cartridge, 3 ml, Supelco, USA). Periodate and peroxide oxidations of PSTs were carried out and samples containing N-1-hydroxylated PSTs (NEO, dcNEO, GTX1+4, C3+4 and GTX6) were further separated using an SPE ion exchange cartridge with carboxylic acidsiliane (COOH) bonded to silica gel (Bakerbond COOH, 3 ml, J.T. Baker, USA).

The PSTs were determined using high performance liquid chromatography with fluorescence detection (HPLC-FLD) based on the pre-column oxidation method developed by Lawrence and Niedzwiadek (2001). The HPLC-FLD equipment consisted of a Hewlett-Packard/Agilent Model 1050 quaternary pump, Model 1100 in-line degasser, auto-sampler, column oven, and Model 1200 fluorescence detector. The PST oxidation products were separated using a reversed-phase Supelcosil LC-18, 15 × 4.6, 5 µm column (Supelco, USA). The mobile phase gradient consisted of 0–5 % B (0.1 M ammonium formate in 5% acetonitrile, pH 6) in the first 5 min, 5–70 % B for the next 4 min and back to 0% B in the next 2 min. Then 100% mobile phase A (0.1 M ammonium formate, pH 6) was used for 3 min before the next injection. Flow rate was 1 mL min^{-1} and the detection wavelength set to 340 nm for excitation and 395 nm for emission. Instrument limits of detection (S/N = 3) ranged from 0.002 µM dcSTX to 0.02 µM for dcNeo.

The toxicity equivalent factors stated by EFSA (2009) were used for calculation of PSTs in terms of saxitoxin equivalents. Toxins that co-elute were determined together (C1 + 2, dcGTX2 + 3, GTX1 + 4 and GTX2 + 3) the higher toxicity equivalent factor of the co-eluted compound was used. Due to the lack of analytical standards for C3+4 and GTX6, the concentrations were estimated after a hydrochloric acid hydrolysis conversion into their carbamate analogues GTX1+4 and NeoSTX, respectively. Certified calibration solutions for PSTs were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council, Canada (STX-e, NEO-b, GTX2&3-b, GTX1&4-b, dcSTX, dcGTX2&3, GTX5-b (B1), C1&2 and dcNEO-b).

The toxin content of the strains was expressed as molar percentages of single toxins or epimer pairs (e.g. C1+2) and the total cellular content expressed in fmol per cell. Euclidean distances among strains were calculated and a cluster analysis was constructed using the unweight pair group mean average (UPGMA) algorithm using Statistica 6.0.

2.5. DNA analysis

2.5.1. DNA Extraction

Aliquots of the same xenic cultures processed for the growth curves and toxin analyses were used for DNA extraction. Cell pellets of the cultures were obtained by a two-step centrifugation process at 4600 x*g* (20 min) followed by 17600 x*g* (10 min). Eppendorf vials containing cell deposits were immediately frozen and kept at -80°C to avoid DNA degradation.

For DNA extraction, the cell pellets were flicked with a pipette tip to remove a small inoculum (pilot sample) and DNA was extracted by resuspending the samples in 500 µL of a preheated (60 °C) mixture containing: 100 mM TrisHCl (pH 8.8), 50 mM KCl and 0.25mg/mL Proteinase K (1/80 of a 20mg/mL solution). Samples were then incubated from 1 hour up to 3 hours at 60 °C (with shaking for 5 seconds every 2 min during the first 15 min). The samples were then transferred into ice for 5 min, heated in a dry bath at 95-100°C for 5 min and brought back to ice for another 10 min. The DNA containing supernatant was recovered by centrifugation at 12000 x*g* (5 min) and kept at -20°C. DNA extraction was confirmed by electrophoresis on 0.7% agarose gels in TBE buffer, and stained with ethidium bromide (5µg/mL).

2.5.2. DNA Amplification

The D1–D2 fragment of the large subunit (LSU) of ribosomal DNA (rDNA) and the internal transcribed spacers 1 and 2 (ITS 1 and ITS2), including the 5.8S rDNA gene, were amplified for all strains by polymerase chain reaction (PCR). In addition, amplification of repetitive sequences presumably present in total DNA (BOX elements) was also performed by BOX-PCR. *Gymnodinium microreticulatum* (IO12-05) and *Gonyaulax* sp. (IO104-3) were used as outgroups for PCR screening. Amplified regions and respective primer sets are shown in Table 2.

Table 2 - List of primers used to amplify target DNA regions of several Portuguese strains of dinoflagellates: *G. catenatum*, *G. microreticulatum* and *Gonyaulax sp*.

Primer	Sequence (5'→ 3')	Amplificon size (pb)	Reference
SSU(p)- ITS1,2-5,8S- LSU(p) region			
18d (Forward)	CACACCGCCCGTCGCTACTACCGATTG	1500	Hillis & Dixon (1991)
28z (Reverse)	AGACTCCTTGGTCCGTGTTTCAAGAC		
D1-D2 LSU region			
DinoLSU210F (Forward)	CGAGACCGATAGCAAACAAGTA	400	Patil et al. (2005)
28z (Reverse)			
ITS1-5.8S-ITS2			
ITSA (Forward)	TCGTAACAAGGHTCCGTAGGT	610	Adachi et al. (1994)
ITSB (Reverse)	AKATGCTTAARTTCAGCRGG		
BOX-PCR			
BOX1R	CTACGGCAAGGCGACGCTGACG	Variable patterns	Louws et al. (1994)

Wobbles: R=A+G; H=A+C+T; K=G+T. (p) = partial sequence.

D1-D2 LSU and ITS1-5.8S-ITS2 regions were amplified by a two-steps PCR procedure starting from a larger fragment: SSU(partial)-ITS1-5,8S-ITS2-LSU(partial) (Fig. 3). The first PCR was performed with the universal eukaryotic primer pair 18d and 28z. The second PCRs were carried out using 1/10 volume of the first reaction as template. D1-D2 LSU region was amplified by semi-nested PCR using the specific dinoflagellate primer DinoLSU210F instead of primer 18d. For the ITS1-5.8S-ITS2 region, nested PCR was conducted with the degenerate primers ITSA and ITSB adapted from Adachi et al. (1994) that originally contained an extra sequence with restriction sites which were not necessary for our work. BOX-PCR was conducted with the universal primer BOX1R. The BOX-PCR assay was repeated three times to ensure its reproducibility.

PCR reactions were prepared using My Taq® Red Mix (Bioline®) kit and the set-up of the reactions were made accordingly to the manufacturer's instructions except for the reaction volume which was 5 µL (1 µL template DNA + 4 µL mix) for strain screening PCRs, or 20 µL for sequence analysis. All reactions were performed in a T personal® (Biometra®) cycler.

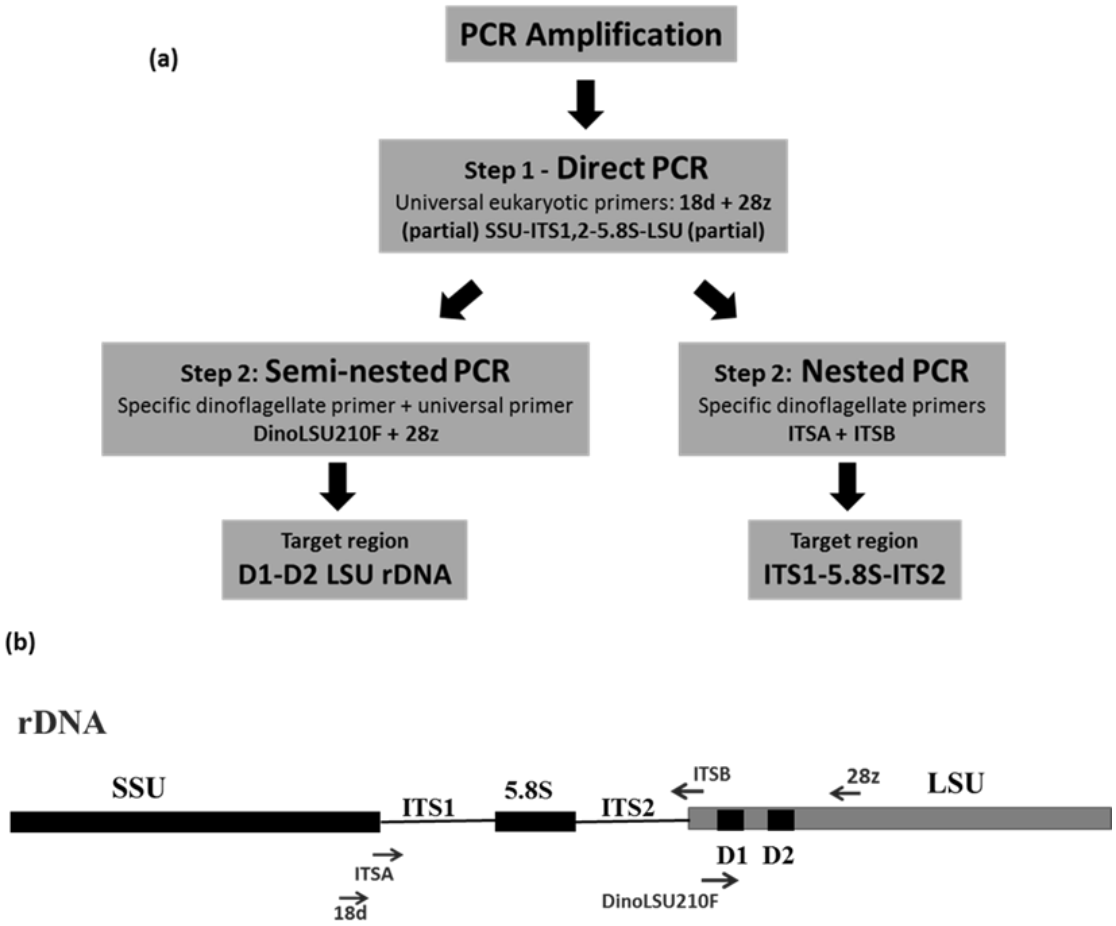

Figure 3 - Schematic representation of (a) the two-step PCR amplification used to amplify target rDNA regions in the analyzed strains. Direct, semi-nested and nested PCRs were performed based on the use of universal and dinoflagellate-specific primers. (b) One transcriptional unit of the nuclear-encoded ribosomal DNA (rDNA). The scheme also shows the annealing sites of the employed primers.

PCR cycling conditions for the primer set 18d/28z were a 95°C preheating step, initial denaturation at 95 °C for 1 min, followed by 36 cycles at 95 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 45 s; a final extension was performed at 72ºC for 2 min before the samples were kept at 4°C. For the primer sets DinoLSU210F/28z and ITSA/ITSB, cycling conditions were identical with a 95°C preheating step, initial denaturation at 95°C for 3 min, followed by 36 cycles of 15 s at 95°C, annealing at 45°C for 15 s, extension at 72°C for 30 s and final extension at 72ºC for 10 min. Box-PCR with primer BOX1R was performed as described for DinoLSU210F/28z, except for the extension time that was increased up to 2 min. The PCR products were resolved on 1.5% agarose gel, in TBE buffer and stained with ethidium bromide (5µg/mL). The 1kb plus DNA ladder (Invitrogen®) or HyperLadder I™ 1kb (Bioline®) were also included on the gel.

The bands with sizes corresponding to the expected PCR products were excised from the gels and were purified with the Zymoclean™ Gel DNA Recovery kit (Zymoresearch®)

following the gel purification procedure as described in the product's manual. For sequencing, all purified samples were sent to a commercial lab (StabVida®, Lisbon, Portugal). The retrieved sequences were then analyzed using the FinchTV® (Geospiza®) program for the chromatogram analysis, for the sequence alignment, BioEdit ® (Hall T, 2011) for sequence and alignment analysis, and blastn® (National Library of Medicine, USA) for database search.

3. Results

3.1. Growth patterns

Growth patterns could only be studied for the strains that originated from cysts. The strains with origin in vegetative plankton cells had very low growth rates and never reached cell densities above 500cell.mL^{-1} (data not shown). Maximum cell densities, growth rates and doubling time for the studied strains are shown in Table 3. Cyst strains exhibited differences in maximum cell density values, despite having the same order of magnitude. Strain IO13-22 presented the highest maximum cell density (2.8 x10^3 cells.mL^{-1}) and IO13-24 the lowest value (1.4 x10^3 cells.mL^{-1}). These two cultures were obtained from the same sampling site (Aveiro) but in different years (2010 and 2011 respectively). Growth rates ranged between 0.144 (IO13-24) and 0.281 (IO13-01) divisions day^{-1}**.** The latter strain, despite having the highest growth rate did not achieve the highest cell yield.

Table 3 – Maximum cell densities, growth rates (k) and doubling time (T2) in the four cultures of *G. catenatum* strains originated from cysts.

Strain	Maximum cell densities (cell.mL^{-1})	k (div.day^{-1})	T_2 (day)
IO13-01	1.52 X 10^3	0.281	3.55
IO13-02	1.64 X 10^3	0.202	4.94
IO13-22	2.82 X 10^3	0.212	4.71
IO13-24	1.38 X 10^3	0.144	6.96

As illustrated in Figure 4, the strains IO13-01, IO13-02 and IO13-24 revealed similar chain-structure with time (2-4 cell-chain through growth), differing from strain IO13-22, the culture with longer chains (> 6 cell-chain through the growth curve) and for which the highest cell density was recorded (Table 3).

3.2. Photosynthetic pigment composition

A total of 12 pigments were separated and identified for all 6 strains studied (Fig.5). Pigment concentrations (pg.cell^{-1}) are summarized in Table 4. The pigment profile, as expected, showed a predominance of chlorophyll *a* (Chl *a*), chlorophyll c_2 (Chl c_2), diadinoxanthin and peridinin, the signature pigments for peridinin containing dinoflagellates (Zapata et al. 2012).

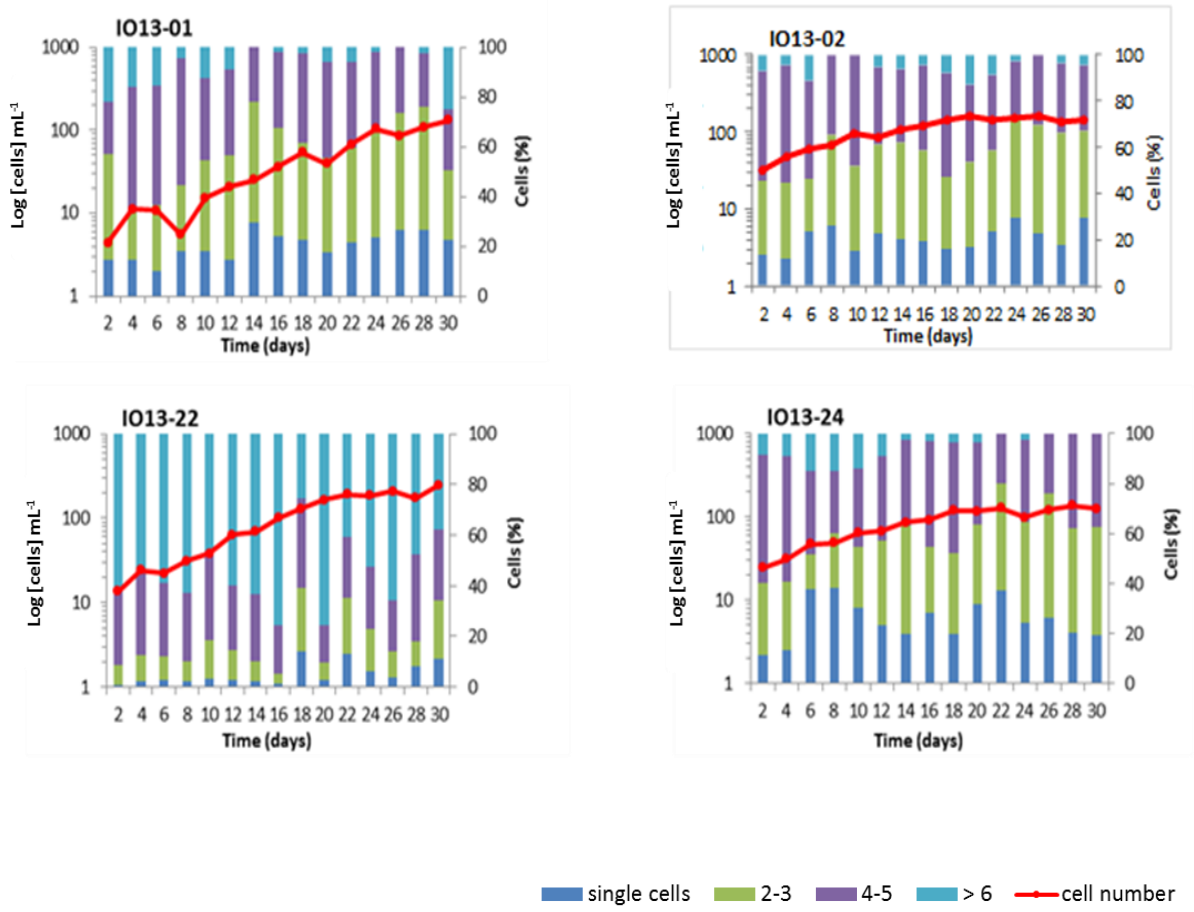

Figure 4 – Growth curves - cell density (Log [cells].mL^{-1}) and percentage of single cells, 2-3 cell chains, 4-5 cell chains and >6 cell chains in the studied strains.

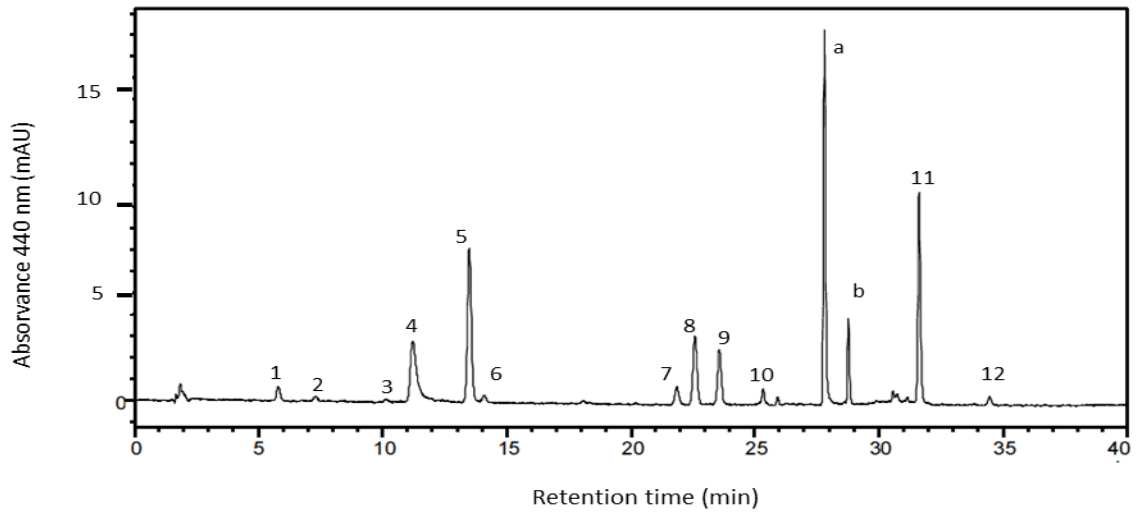

Figure 5 - HPLC-UV chromatogram at 440 nm for *G. catenatum* at stationary growth phase. (1) peridiniol, (2) unknown carotenoid, (3) clorophyllide *a*, (4) chlorophyll c_2, (5) peridinin, (6) peridinin-like, (7) diadinochrome, (8) diadinoxanthin, (9) dinoxanthin, (10) diatoxanthin, (11) chlorophyll *a*, (12) β-carotene. (a) internal standard (trans-β-apo-8'-carotenal pigment), (b) internal standard degradation product.

Strains originating in the laboratory by cyst germination showed very similar cell pigment concentrations among them (Table 4). Strains originating from vegetative cells showed higher cell pigment concentrations when compared to the cyst cultures, particularly strain IO13-17, despite being subjected to the same light regime and culturing conditions.

Table 4 – Pigment values (pg cell^{-1}) at the stationary phase for 6 strains analyzed from different regions.

Region	Algarve		Cascais Bay		Aveiro	
Strain	IO13-01^c	IO13-17^p	IO13-02^c	IO13-06^p	IO13-22^c	IO13-24^c
Pigments (pg cell^{-1})						
Peridinol	+	-	+	+	+	+
Unknown carotenoid	+	+	+	+	+	+
Clorophyllide *a*	0.255	-	0.563	0.438	0.087	0.796
Chl C_2	2.783	6.504	2.363	4.429	3.084	4.571
Peridinin	11.387	43.863	11.343	20.857	10.912	19.049
peridinin-like	+	-	-	-	+	+
Diadinochrome	+	+	+	+	+	+
Diadinoxanthin	2.468	13.474	2.785	4.314	2.651	4.743
Dinoxanthin	+	+	+	+	+	+
Diatoxanthin	0.469	2.215	0.422	0.177	0.276	0.588
Chl *a*	12.494	53.852	12.031	28.370	12.799	18.307
β-Carotene	0.256	0.993	0.229	0.490	0.252	0.392
Chl C_2./Chl *a*	0.224	0.121	0.200	0.155	0.242	2.251
Peridinin/ Chl *a*	0.911	0.815	0.943	0.735	0.853	1.041
Diadinoxanthin/ Chl *a*	0.198	0.250	0.231	0.152	0.207	0.259
Diatoxanthin/Chl *a*	0.038	0.041	0.035	0.006	0.022	0.032
β-Carotene/Chl *a*	0.020	0.018	0.019	0.017	0.020	0.021

Cultures established from isolation of (c) germinated cysts and (p) plankton cells. (+) = detected but not quantified; (-) = not detected

3.3. Toxin analysis

The molar percentage of each toxin in the *G. catenatum* strains included in this study is shown in Figure 6. The PST profiles showed marked differences between strains obtained from laboratory cyst germination and from natural vegetative populations.

Cyst germinated strains showed the same toxin profile between them but with differences in toxin concentration and proportion. The PSTs profile was mostly constituted (> 97.50 %) by N-sulfocarbamoyl derivatives (C1+2, C3+4, GTX5 and GTX6). Only minor amounts of decarbamoylsaxitoxin (dcSTX) were found, ranging from 0.9 to 2.5 %. By contrast, strains from wild planktonic cells had high percentage of dcSTX (29.9 % for IO13-16 and 31.1 % for IO13-17). For these two strains the toxin dcGTX1+2 was also detected, while C3+4 and GTX6 toxins could not be detected. Due to the low growth rates and cell yield obtained for cultures derived from vegetative plankton cells, these were analyzed as pooled samples and standard deviation is not presented in Figure 6.

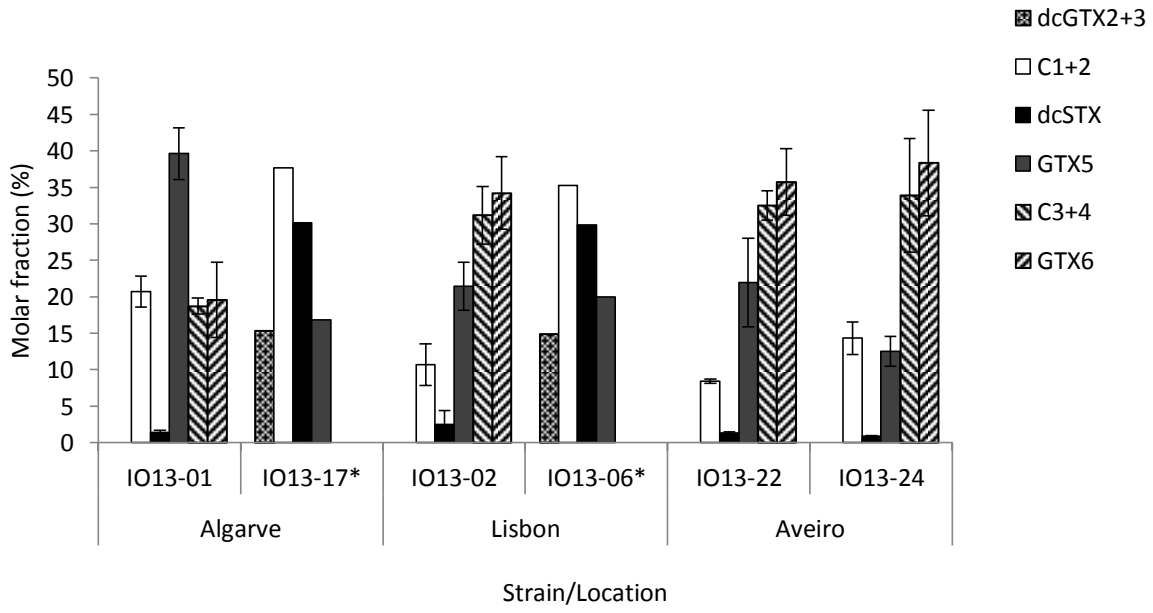

Figure 6- Species-specific paralytic shellfish toxins (PSTs) profiles in 6 studied cultures of *G. catenatum*. (*) - plankton cell originated cultures

In terms of toxin content, strain IO13-01 presented the highest amount, reaching approximately 84 fmol cell^{-1}. For the remaining strains the sum of all toxin analogues ranged from 33 fmol.cell^{-1} to 38 fmol.cell^{-1}. No marked differences were observed among cell cultures derived from cyst isolates, from different locations and bloom periods. However strains from the same region, IO13-01 and IO13-17 from S coast (Algarve) and IO13-02 and IO13-06 from the W coast (Lisbon) but from different life-stage origin, showed divergent dcSTX content. In the first case, the strain originated from a vegetative cell showed 20-fold dcSTX content relative to the cyst derived culture, while in the second case the strain from vegetative origin had 3-fold dcSTX content relative to the cyst derived culture. These observations are well illustrated in a cluster analysis, using a similarity test based on the toxin composition, where strains from the same location did not cluster together while all cyst strains grouped in one cluster and plankton strains in another (Fig. 7). In the cyst cluster, the strain from Algarve, IO13-01 recorded the highest linkage distance.

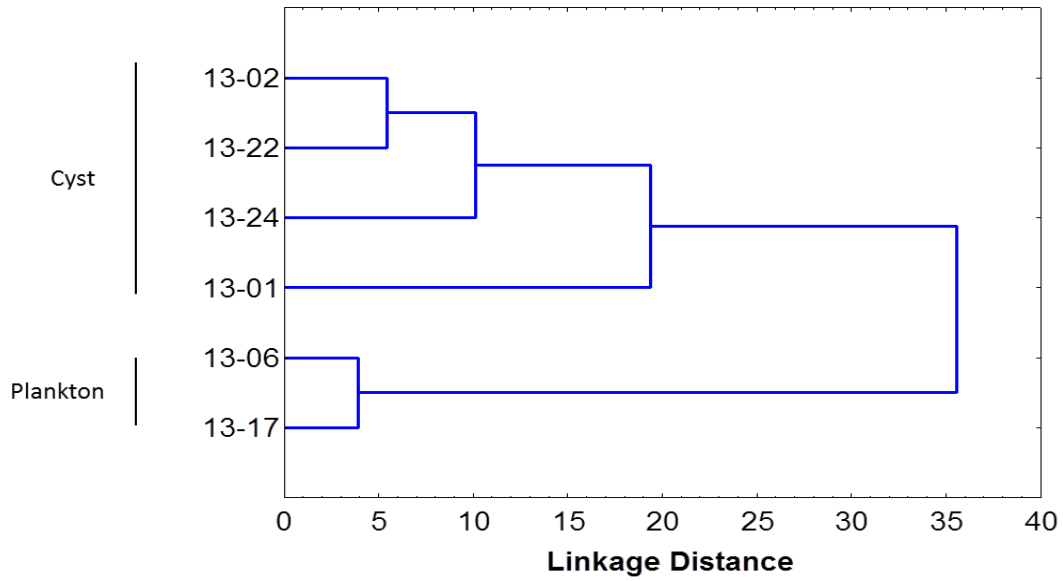

Figure 7 – Similarity dendrogram for regional Portuguese isolates of *G. catenatum* based on toxin compositions.

Despite of the lower toxin content when compared to strain IO13-01, strains originating from plankton cells were more toxic (Fig.8). This, results from the presence of significantly higher concentration of decarbamate compounds, such as dcSTX and dcGTX2+3, and the lack of the two less potent sulfamate derivatives, C3+4 and GTX6. Cell toxicity of these plankton strains is markedly higher, differing in 3 orders of magnitude from cyst derived strains (note different scales in Fig.8). Concerning the toxicity levels, within each group, the

two strains from the South coast, Algarve, were the most toxic (plankton strain IO13-17 and cyst strain IO13-01) (Fig. 8).

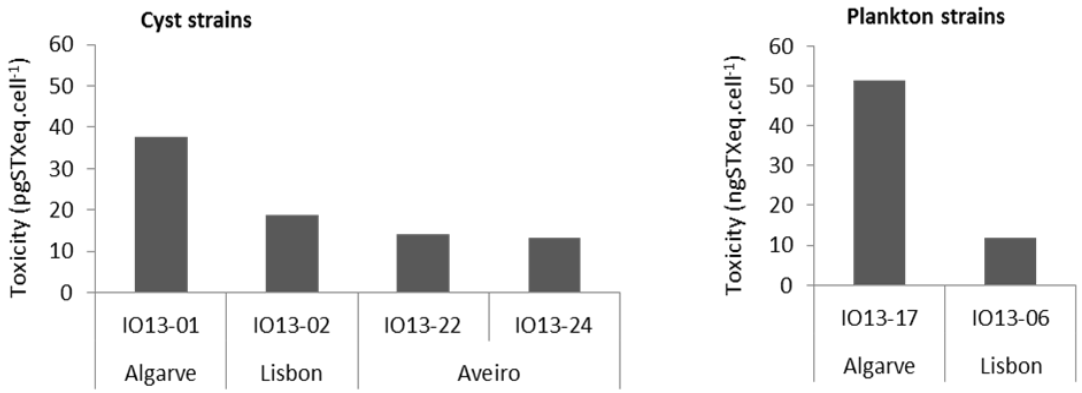

Figure 8 – *G. catenatum* cell toxicity (STXeq = saxitoxin equals) for 6 cultures obtained from resting cysts and plankton cells. Note different scales.

3.4. Amplification and DNA sequencing

Direct, semi-nested and nested PCR for *G. catenatum* strains were successfully accomplished for all strains.

3.4.1. Ribosomal DNA analysis

Direct PCR of the SSUp-ITS1-5,8S-ITS2-LSUp rDNA region with the primer set 18d/28z, resulted in a fragment of ~1500bp (Fig. 9). This step was followed by semi-nested and nested PCR. Amplification of the D1-D2 LSU region with the primer set DinoLSU210F/28z, resulted in a product of ~400 bp for *G. catenatum* and *G. microreticulatum* and in a ~380 bp product for *Gonyaulax* sp. (Fig. 10). PCR amplification of the ITS-5.8S rDNA with ITSA/ITSB resulted in a ~600 bp for *G. catenatum* species, as expected for dinoflagellates according to Adachi et al. (1994).

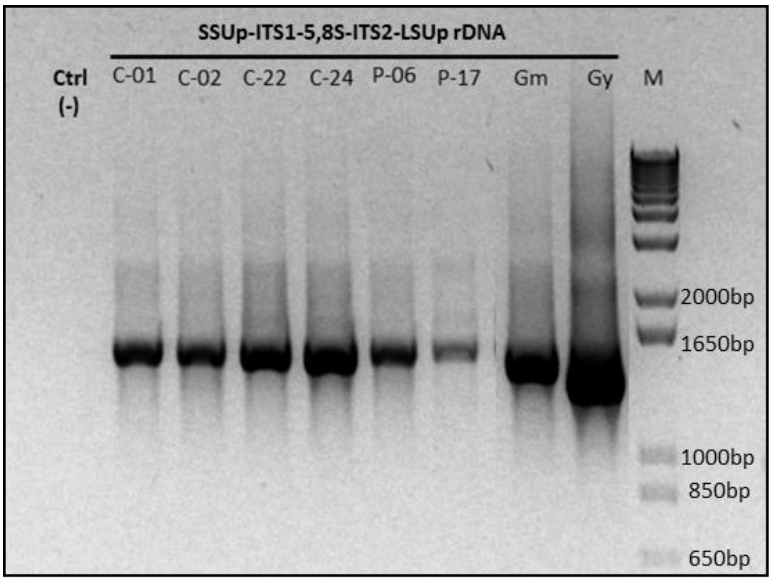

Figure 9– Photograph of a 1.5% agarose gel with PCR products obtained for SSUp-ITS1-5,8S-ITS2-LSUp rDNA, using primer pair 18d/28z. M – Marker (1kb plus DNA ladder, Invitrogen); Ctrl (-) – Negative control; C (01, 02, 22 and 24) – *G. catenatum* cyst strains; P (06 and 07) – *G. catenatum* plankton strains; Gm - *G. microreticulatum*; Gy - *Gonyaulax* sp.

Some PCR products showed additional bands, besides de main product with the expected size which was necessary to purify in order to obtain only the target rDNA region for sequence analyses.

Sequencing of the rDNA regions was performed with the same primers used for nested or semi-nested PCRs and rendered sequences of similar length (~400 bp) for D1-D2 LSU region, but shorter sequences (~180 bp) for the ITS-5.8S rDNA. The resulting sequences were then aligned with sequences already published in databases.

3.4.2. Sequence analysis

Blast alignments of the partial LSU rDNA and ITS-5.8S rDNA sequences were performed both between the sequences analyzed in this study and sequences retrieved from GenBank. Our sequences are identical to those of *G. catenatum* strains and clearly different from the sequence obtained for the *Gonyaulax* sp. isolate. *Gymnodinium catenatum* and *G. microreticulatum* exhibit a closer similarity. Identical (100% similarity) LSU rDNA sequences were found within the analyzed *G. catenatum* strains and it was detected a G nucleotide in the SNP of the D1-D2 LSU fragment described by Band-Schmidt et al. (2008).

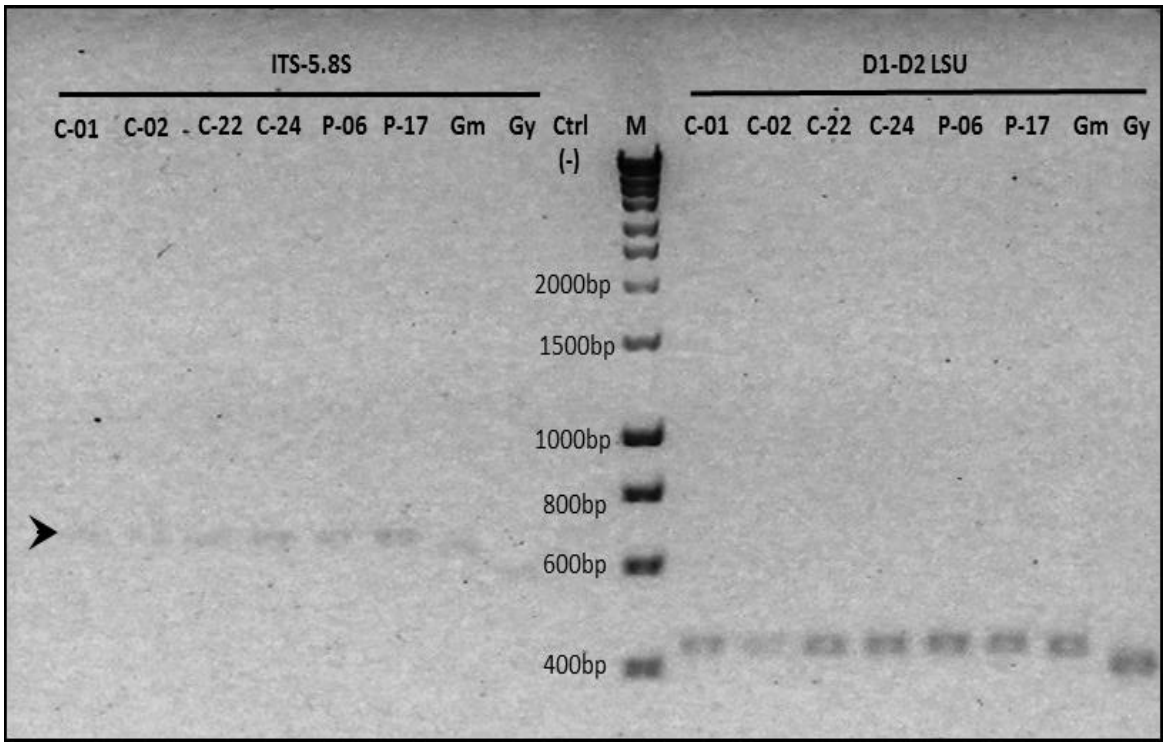

Figure 10 – Photograph of a 1.5% agarose gel, with purified PCR products obtained for ITS-5.8S rDNA (left) and D1-D2 LSU rDNA regions (right), using primer pairs ITSA/ITSB and DinoLSU210F/28z, respectively. M – Marker (HyperLadder I, Bioline); Ctrl (-) – Negative control; C (01, 02, 22 and 24) – *G. catenatum* cyst strains; P (06 and 07) – *G. catenatum* plankton strains; Gm - *G. microreticulatum*; Gy - *Gonyaulax* sp.

However, we did not find a total identity match for the ITS-5.8S rDNA region. A single nucleotide polymorphism (SNP) was detected near the end of the ITS1 region (Fig. 11). Figure 12 summarizes the analyzed chromatograms, evidencing the overlap of guanine (G) and adenine (A) peaks corresponding, to the detected SNP, with guanine corresponding to the main peak among all the analyzed strains, except for strain IO13-24 which evidenced a main peak of adenine instead of guanine. In addition, our strains showed to possess a C-gene in the SNP found in the beginning of the 5.8S rDNA region by Bolch and Salas (2007) (Fig.11).

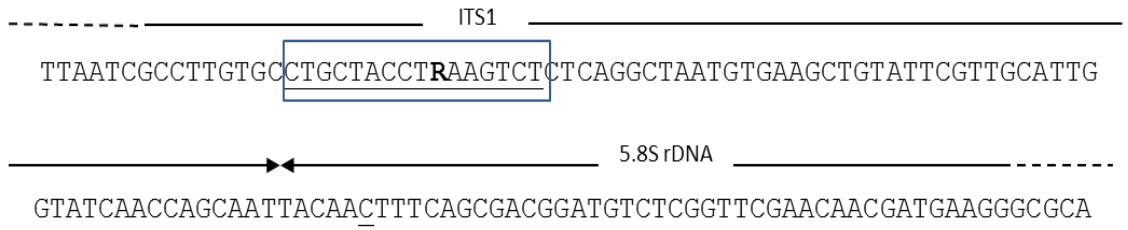

Figure 11 - Schematic representation of the partial segment ITS1-5.8S rDNA, showing the position and flanking DNA sequences of the single nucleotide polymorphisms (SNP's) detected among the analyzed *G. catenatum* strains. **R** corresponds to the SNP (G/A) found in the Portuguese isolates (this study) and C for SNP demonstrated by Bolch and Salas (2007).

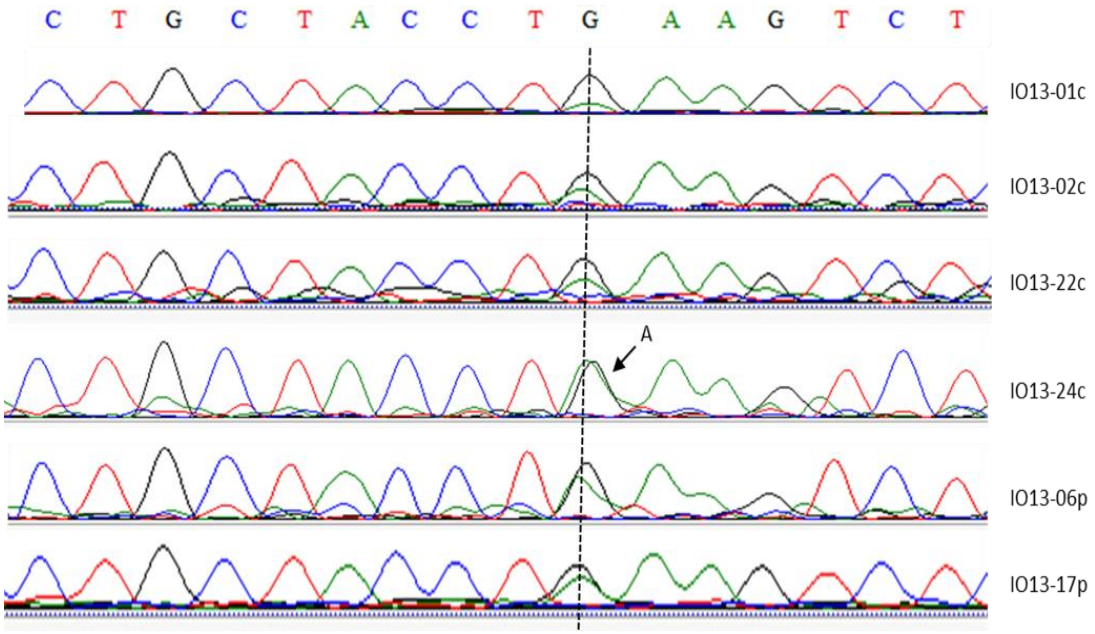

Figure 12 – Alignment of ABI Chromatograms of partial ITS1 rDNA sequences obtained after sequencing of *G. catenatum* strains. In the figure is evidenced the overlap of guanine (G) and adenine (A) peaks corresponding to the SNP. The arrow linked to the letter A indicates that for the strain IO13-24 the sequencing device assumed adenine instead of guanine.

3.4.3. DNA fingerprinting

In order to complementary resolve intra-specific variability BOX-PCR was assayed. Total DNA fingerprinting resulted in similar band patterns for all the analyzed *G. catenatum* strains indicating that this method cannot discriminate at the intra-specific level. However it

discriminated well the different studied species, giving distinct band patterns even for species within the same genus (*G. microreticulatum* and *G. catenatum*) (Fig.13).

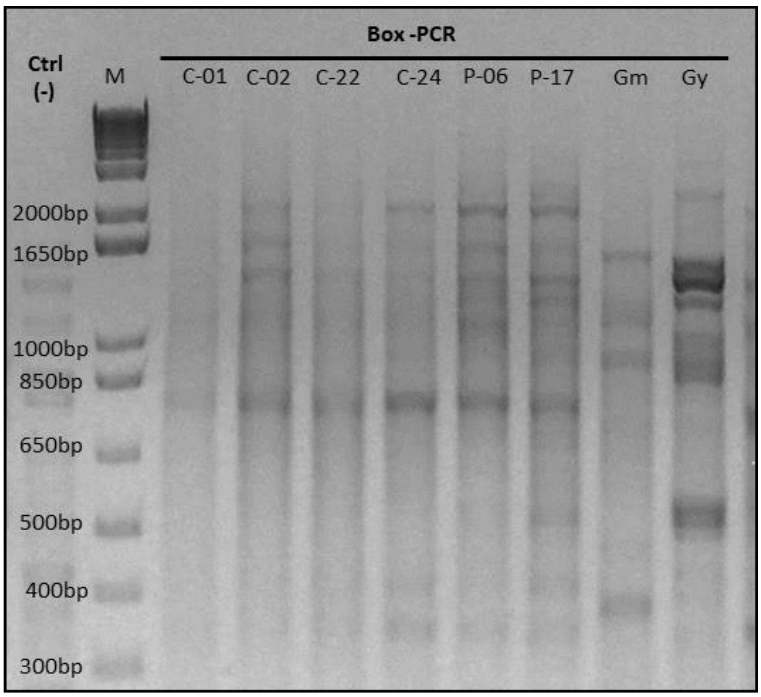

Figure 13 – Photograph of a 1.5% agarose gel with PCR products obtained for BOX-PCR amplification, using primer BOX1R. M – Marker (1kb plus DNA ladder, Invitrogen); Ctrl (-) – Negative control; C (01, 02, 22 and 24) – *G. catenatum* cyst strains; P (06 and 07) – *G. catenatum* plankton stains; Gm - *G. microreticulatum*; Gy - *Gonyaulax* sp.

4. Discussion

Since 1986, *Gymnodinium catenatum* is the dinoflagellate species responsible for the most serious events of PSP in the Portuguese coast (Franca and Almeida 1989; Vale et al. 2008). It occurs along the whole coast but shows a complex geographical and inter-annual bloom pattern raising questions about the origin of the bloom inocula. Evidence from the fossil record suggests this species only colonized the Iberian Peninsula from the end of the XIX century (Amorim and Dale 2006, Ribeiro et al. 2011). This recent colonization was first interpreted as a possible evidence of the man mediated introduction of *G. catenatum* to the NE Atlantic (Amorim and Dale, 2006). However, more recent work supports a natural northward expansion of its distribution range from NW Africa (Ribeiro et al. 2011). These two introduction routes would presumably generate regional populations with different levels of intra-specific diversity. The first hypothesis would originate a diverse Iberian population from multiple geographical sources while the latter would generate a less diverse population. In our work, toxin profiling and genetic markers were used in order to ascertain intra-specific

diversity in Iberian populations of *G. catenatum* and contribute to a better understanding of the ecology and global biogeographical distribution of this species.

4.1. Toxin profile

Results from the toxin profile did not show differences between strains from distinct sites along the coast and from different sampling periods. The most significant differences were observed between strains that originated from different life-cycle stages, namely motile cells obtained from the wild and cysts (hypnozygotes) incubated under laboratory conditions (Figs. 6 and 7). The concentration of the most potent decarbamate compound, dcSTX, was substantially higher in strains that originated from motile cells than in those obtained from cysts despite being from the same sampling area. In addition, two less potent sulfamate derivatives, C3+4 and GTX6, that were predominant in the PST profile of cyst strains were not detected in strains with a planktonic origin. Cluster analysis, using a similarity test based on the toxin composition, supported this interpretation (Fig. 7), strains from different locations and isolation date grouped together while two clear separate clusters corresponded to cyst *vs.* plankton originated cultures.

Relevance of the life stage origin of cultures was also observed regarding other physiological parameters, such as growth patterns and pigment profiling. Four cultured cyst-derived strains, isolated from different locations along the coast, showed similar growth patterns (growth rates, maximum cell yields and chain-length) without noticeable variability, except for strain IO13-22 that showed different chain morphology (Table 3 and Fig.4). Plankton derived strains showed a different behavior since they were not able to grow under the same culturing conditions. Cultured plankton cells had very low growth rates never reaching cell densities above 500 cell.mL^{-1}. Regarding photosynthetic and other accessory pigments, plankton derived cultures presented higher pigment concentration per cell, particularly strain IO13-17, possibly indicating differences in their photophysiology (Table 4).

All cyst strains exhibited PST profiles similar to those already described by several authors for the Portuguese coast (see Annex 1), with predominant presence of N-sulfocarbamoyl derivatives, such as C1-4, GTX5 and GTX6, and much lower values of dcSTX (Franca et al. 1996, Negri et al. 2001, 2007; Vale 2008 and Costa et al. 2012). This toxin profile is characteristic for the NE Atlantic region, including Portugal and Spain (Negri et al. 2001, 2007). Most of these authors have only analyzed plankton derived strains. Interestingly our results regarding plankton strains showed much higher values of dcSTX following the most abundant C1+2 toxins and the presence of dcGTX2+3. (Fig. 6).

These results lead us to suggest that when growing in the laboratory, the physiology of *G. catenatum* is mainly conditioned by the life-cycle stage that originated the culture (cyst *vs.* plankton isolates).

Similar observations have been recorded by other authors who reported cultures which express abnormally low amounts of PST per cell when originated from laboratory incubation of cysts (Oshima et al. 1993; Bolch et al. 2001; Negri et al. 2001 and Green et al. 2004). In previous studies, Negri et al. (2007) assumed that this variability was due to culture conditions and/or to cyst isolation techniques from sediment samples, which includes sonication and a sterile seawater washing step, changing *G. catenatum* natural bacterial consortia and consequently the biosynthesis of PSTs. Additionally, Green and collaborators (2004) concluded that key bacteria are important for the induction of PST production by *G. catenatum* following excystment/germination. The reason behind our observations still needs investigation but is out of the scope of the present work.

4.2. DNA analysis

All isolates (IO13-01, 02, 06, 17, 22, 24) presented identical partial large subunit ribosomal DNA (D1-D2 LSU rDNA) sequences. The recoded homogeneity among the Portuguese strains was expected since this is considered a highly conserved region. In addition, our strains shared up to 100% similarity with another Portuguese strain (AY916536) (Patil et al. 2005), strains from Spain, Andalucía (AF375857) and Vigo (AF375855) (Ordás et al. 2004), from Algeria (FN647679) (Sampedro et al. 2010), from China (KF234066) (Haifeng et al. 2013) and from Australia (AY036072) (Bolch et al. 2001) among others. With the use of this genetic marker, we have confirmed the presence of a G nucleotide for the analyzed strains, regarding the cytosine/guanine (C/G) polymorphism described by Band-Schmidt et al. (2008) for the D1-D2 LSU rDNA region.

In the present work we report for the first time a single nucleotide polymorphism (SNP) corresponding to guanine/adenine (G/A), for the ITS-5.8S rDNA near the end of the ITS1 region (Fig. 11 and 12). This SNP has not yet been described, being guanine (G) the nucleotide present in all *G. catenatum* sequences found in GenBank, including a Portuguese strain (FJ823541) from Aguda (NW Portugal) (Stern el at., 2011). From the six analyzed strains in our work, five of them (IO13-01, 02, 06, 17 and 22) showed a G nucleotide in the main peak while one (IO13-24) showed an A nucleotide. Strain IO13-24 was isolated in Aveiro, and it would be important to screen this region in order to clarify these results. Still, the SNP is evident for all our strains, demonstrated by the G/A peaks overlap (Fig. 12).

The significance of this SNP in the genetic diversity of *G. catenatum* occurring populations depends on the cultures origin (monoclonal *versus* polyclonal) and implies different interpretations. If the same cell line (monoclonal) possesses this SNP, it means that it is found in the rDNA multigene family. This may also be true if the SNP is found in a polyclonal culture origin but, in this case, it may also be originated by the existence of two kinds of genotypes in circulating *G. catenatum* strains.

Our results should be further investigated since some of the cultures used in this study, namely those resulting from cyst germination were possibly polyclonal. *G. catenatum* has a haploid (n) life-cycle, with the only diploid (2n) stage being the zygote (resting cyst (hypnozygote or the planozygote) (Fig. 1) (Figueroa, 2005). The cultures obtained from the germination of sexual cysts, which is followed by meiosis, may thus be polyclonal and cell lines with different rDNA sequences may co-occur in the culture. In this case, the observed SNP could result from the presence of two distinct cell lines and not from a true polymorphism. On the other hand, the SNP was also detected in cultures originating from planktonic vegetative cells giving strong support for a true polymorphism. In addition, two ITS rDNA sequences presenting G/A SNPs, in other positions, have already been recorded in GenBank for *G. catenatum*: a strain from Algeria (AM998536) (Penna et al. 2010) and another one from Australia (FJ823543) (Stern et al. 2011) (Fig.14).

Future work with clonal cultures obtained by single cell re-isolation is needed to assess the true nature of this SNP. On a geographical basis, these SNP patterns are important to outline population affinities and origins. Intra-strain diversity raises questions about whether a polymorphic strain is the result of crossings between different population types.

The G/A polymorphism, here reported for the first time, may be a distinctive of the *G. catenatum* populations from Portuguese waters, it will be important to investigate this SNP in the ITS-5.8S rDNA in strains from other geographical locations. The SNP found in all our sequences, may represent a significant difference in this highly conserved region among the numerous strains already sequenced. Therefore, we must be cautious when comparing our data with the published literature since different approaches, depending on what is intended, have been applied for DNA analysis. Many authors chose to employ cloning procedures in order to target unique DNA sequences. This approach implies a loss of polymorphic diversity, specifically within the same strain since unique DNA sequences are cloned before being is analyzed. Consequently, the use of non-cloned DNA for sequencing may evidence the existence of polymorphisms otherwise not detected.

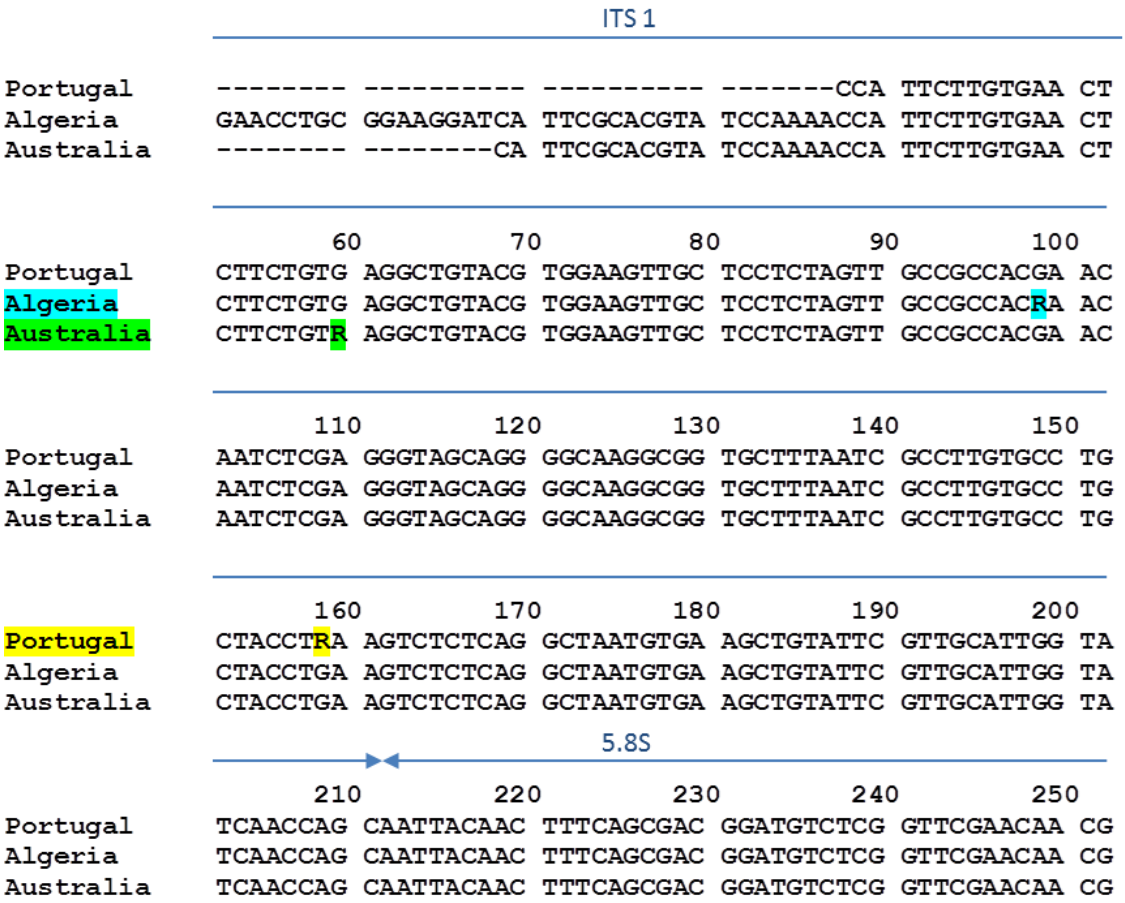

Figure 14 – Graphic image of the sequence alignment considering the ITS1- 5.8S rDNA region for *G. catenatum* strains, from Portugal (this work), Algeria (Penna et al. 2010) and Australia (Stern et al. 2011). In the image is evidenced the detected (R) polymorphisms in three different locations within the ITS1 region.

Also based in the ITS-5.8S rDNA region, several authors (Bolch and Salas, 2007 and Band-Schmidt et al. 2008) have managed to successfully distinguish between worldwide populations. Our results concerning this SNP allowed to find out that our strains share comparable ITS regions with strains from Europe, South America, and South-east Asia, and can be classified as 5.8S C-gene type according to (Bolch and Salas, 2007) (Figure 11).

BOX-PCR is a Rep-PCR which is based on the fact that repetitive and conserved DNA sequences are found in the genome of the majority of eukaryotes and prokaryotes. When subjected to PCR, these sequences produce characteristic patterns after separation on agarose gels, and thus can constitute a method to fingerprint genomes. Until now, the presence of BOX elements had only been described for bacteria and fungi (Gillings and Holley, 1997). We had the opportunity to assay BOX1R primer with strains of microalgae. Although just with 8 samples (6 *G. catenatum* + 2 outgroup), we could discriminate at the

species level. We have applied for the first time BOX-PCR to microalgae and found out that the discrimination patterns are similar to those obtained for the RAPD technique applied by other authors to dinoflagellates, including *G. catenatum* (Camino-Ordás, 2004; Bolch and Salas, 2007).

Identical DNA patterns were observed for the analyzed *G. catenatum* strains and different from those obtained for *G. microreticulatum* and *Gonyaulax* sp. (Figure 13). This method, so far, does not allow the identification of intra-specific variability but it proved to be a valuable tool to discriminate at species level, since it has produced consistent results, applying a single primer (BOX1R). This method may in the future be used, in combination with other markers, for taxonomic studies.

5. Concluding remarks and Further studies

The present work highlights that *G. catenatum* populations occurring in the Portuguese coast are very similar in terms of toxins and genetic profile. This observation suggests that the introduction of *G. catenatum* in Iberian waters in late XIX century has occurred from the same geographic region (a single origin) and not from multiple introductions, with different geographical origins, as would be expected if it had occurred via ships ballast water.

Considering that previous studies in the North coast of Africa (Holzwarth et al. 2010) indicate the presence of this species for more than 3000 years in that region, and that subsequent studies in Iberia indicate a propagation of the species in the Portuguese coast from S to N (Ribeiro et al. 2011), our data support the theory that argues the colonization of the Iberian region by *G. catenatum* has resulted from a natural expansion of the North distribution limit of the species in the NE Atlantic. Further work on the genetic variability of *G. catenatum*, such as clarification of the ITS1 guanine/adenine SNP detected in this study, may bring further insight into the phytogeography of this species.

Results from this work have raised several questions regarding the significance of environmental conditioning in the expression of certain physiological traits in *G. catenatum*, namely the toxin profile. Natural environmental factors, both biotic and abiotic, cannot be fully recreated in the laboratory. Our results indicate that the main factor determining differences between the toxin profiles of different *G. catenatum* strains isolated from the coast of Portugal was the life-cycle stage of the cell that originated the cultures. This factor was more important than the date of isolation or the geographical origin of the inocula.

If environmental conditioning of the cells (wild cells vs laboratory germinated populations), defines quantitatively the toxin expression, future research should consider the following topics: (1) analyze more cultures derived from wild plankton cell isolates to confirm

our observations, (2) investigate the influence of wild cell culture medium in the expression of the PST of laboratory incubated cultures (e.g. study the associated bacterial community), (3) compare the proteome of cultures originated from wild populations and from laboratory incubations.

Understanding what determines the expression of the toxin profile in *G. catenatum* wild populations is essential for a better management of marine resources and public health.

6. References

Adachi M, Sako Y and Ishida Y (1994). Restriction Fragment Length Polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese Alexandrium species (Dinophyceae). J. Phycol., 30: 857–863.

Amorim A, Dale B, Godinho R and Brotas V (2001). *Gymnodinium catenatum*-like cysts (Dinophyceae) in recent sediments from the coast of Portugal. Phycologia 40: 572-582.

Amorim A, Dale B (2006). Historical record as an evidence for the recent introduction of the dinoflagellate Gymnodinium catenatum in the north-eastern Atlantic. Afr. J. Mar. Sci. 28, 193–197.

Anderson DM, Jacobson DM, Bravo I, Wrenn JH (1988). The unique microreticulate cyst of the naked dinoflagellate Gymnodinium catenatum. Journal of Phycology 24: 255–262

Artigas ML, Vale P, Gomes SS, Botelho MJ, Rodrigues SM, Amorim A (2007). Profiles of paralytic shellfish poisoning toxins in shellfish from Portugal explained by carbamoylase activity. J Chromatography A (1160), pp 99-105

Band-Schmidt C, Rojas-Posadas D, Morquecho L and Hernadéz-Saavedra N (2008). Heterogeneity of LSU rDNA sequences and morphology of *Gymnodinium catenatum* dinoflagellate strains in Bahía Concepción, Gulf of California, Mexico. J. Plank Research, 30(7): 755-763.

Bolch C, Blackburn S, Hallegraef G and Vaillancourt R (1999). Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). J Phycol 35: 356-367.

Bolch C, Salas M (2007). A review of the molecular evidence for ballast water introduction of toxic dinoflagellates *Gymnodinium catenatum* and the *Alexandrium* "*tamaresis* complex" to Austrália. Harmful Algae 6: 465-485.

Costa PR, Pereira P., Guilherme S, Barata M, Nicolau L, Santos MA, Pacheco M, Pousão-Ferreira P (2012). Biotransformation modulation and genotoxicity in white seabream upon exposure to paralytic shellfish toxins produced by *Gymnodinium catenatum*. Aquat. Toxicol. 106e107, 42e47.

EFSA (European Food Safety Authority), 2009. Scientific opinion of the panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish-saxitoxin group. EFSA J. 1019, 1–76.

Doblin, M. A., Blackburn, S. I. and Hallegraeff, G. M. (1999). Comparative study of selenium requirements of three phytoplankton species: *Gymnodinium catenatum*, *Alexandrium minutum* (Dinophyta) and *Chaetoceros* cf. *tenuissimus* (Bacillariophyta). J. Plankton Res., 21, 1153–1169.

Figueroa RI (2005). The significance of sexuality and cyst formation in the life-cycles of four marine dinoflagellate species. *Dissertation*. Lund University, Sweden.
Franca S, Alvito P, Sousa I, Gago A, Rodríguez- Vásquez JA, Leaão JM, Comesaña M, Thibault P, Burdaspal P, Bustos J & Legarda T (1996). The toxin profile of some PSP toxin producing dinoflagellates occurring in Portuguese coastal waters as determined by alternative analytical methods. In Yasumoto, T., Y. Oshima & Y. Fukuyo (eds), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris: 519–522.

Franca S, Almeida JF (1989) Paralytic shellfish poisons in bivalve mollusks on the Portuguese coast caused by a bloom of the dinoflagellate Gymnodinium catenatum. In: Okaichi T, Anderson DM, Nemoto T (eds) Red Tides: Biology, Environmental Science and Toxicology. Elsevier Science Publishing, New York, pp 93–96

Graham HW, (1943). Gymnodinium catenatum, a new dinoflagellate from the Gulf of California. Transactions of the American Microscopical Society 62, 259–261.

Guillard, R R L and Sieracki, M S (2005). Counting cells in cultures with the light microscope. In Andersen, R. A. (ed.), Algal Culturing Techniques. Academic Press, New York, pp. 239–252.

Green D H, Lyndon E L, Negri A P, Blackburn S I, Bolch C J S (2004). Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. FEMS Microbiology Ecology 47: 345-357

Gillings M and Holley M (1997). Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. Lett Appl Microbiol., 25(1):17-21

Guillard, R R L and Sieracki, M S (2005) Counting cells in cultures with the light microscope. In Andersen, R. A. (ed.), Algal Culturing Techniques. Academic Press, New York, pp. 239–252.

Hallegraeff GM, Anderson DM and Cembella AD (2003). Manual on harmful marine microalgae. Unesco (eds). France.

Haifeng Gu, Tingting Liu, Paulo Vale and Zhaohe Luo Morphology (2013). Morphology, Phylogeny and toxin profiles of *Gymnodinium inusitatum* sp. nov., *Gymnodinium catenatum* and *Gymnodinium microreticulatum* (Dinophyceae) from the Yellow Sea, China. Harmful Algae - in press.

Hall, T. BioEdit: Biological Sequence Alignment Editor for Win95/98/NT/2K/XP/7. Ibis Biosciences - A division of Abbott Laboratories. Available at http://www.mbio.ncsu.edu/bioedit/bioedit.html (2011).

Hallegraeff GM, Blackburn SI, Doblin MA, Bolch CJS (2012). Global toxicology, ecophysiology and population relationships of the chainforming PST dinoflagellate *Gymnodinium catenatum*. Harmful Algae, 14: 130-143.

Hillis DM, Dixon MT (1991). Ribosomal DNA: Molecular evolution and phylogenetic inference. Q Rev Biol. 1991;66:411–53.

Holzwarth U, Meggers H, Esper O, Kuhlmann H, Freudental T, Hensen C, Zonneveld KAF (2010). NW African climate variations during the last 47, 000 years: Evidence from organic-walled dinoflagellate cysts. Palaeogeogr Palaeoclimatol Palaeoecol 291:443–455

Lawrence JF and Niedzwiadek, B., 2001. Quantitative determination of paralytic shellfish liquid chromatography with fluorescence detection. J. AOAC Int. 84, 1099–1108.

Louws F J, Fulbright DW, Stephens CT and de Bruijn J. (1994) Specific genomic fingerprints of phytopathogenic Xanthomonas and *Pseudomonas pathovars* and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286–2295.

Moita, T. M., P. B. Oliveira, J. C. Mendes & A. S. Palma (2003). Distribution of chlorophyll a and *Gymnodinium catenatum* associated with coastal upwelling plumes off central Portugal. Acta Oecologica 24: S125–S132.
Moita MT, Palma AS, Oliveira PB, Vidal T, Silva A, Vilarinho MG (2006) . The return of Gymnodinium catenatum after 10 years: bloom initiation and transport off the Portuguese coast. Proceedings of the 12th International Conference on Harmful Algae. 4-8 September, Copenhagen, Denmark.

Negri, AP, Bolch, C, Geier, S, Green, DH, Park, T G, Blackburn, SI, (2007). Widespread presence of hydrophobic paralytic shellfish toxins in *Gymnodinium catenatum*. Harmful Algae 6, 774–780.

Negri AP, Bolch C, Blackburn S, Dickman M, Llewellyn L, Mendez S, (2001). Paralytic shellfish toxins in Gymnodinium catenatum strains from six countries. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J. (Eds.), Harmful Algal Blooms 2000. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 210–213.

Ordás MC, Fraga S, Franco J, Ordás A and Figueras A (2004). Toxin and molecular analysis of *Gymnodinium catenatum* (Dinophyceae) strains from Galicia (NW Spain) and Andalucía (S Spain). J Plank Research, 26(3): 341-349.

Oshima, Y., Buckley, L.J., Alam, M. and Shimizu, Y. (1993) Toxin production by the dinoflagellate Gymnodinium catenatum. In: Toxic Phytoplankton Blooms in the Sea (Smayda, T.J. and Shimizu, Y. Eds.), pp. 907-912. Elsevier, Amsterdam.

Oshima, Y., Hasegawa, K., Yasumoto, T., Hallegraeff, G.M., Blackburn, S.I., (1987). Dinoflagellate Gymnodinium catenatum as the source of paralytic shellfish toxins in Tasmanian shellfish. Toxicon 25, 1105–1111.

Patil J G, Gunasekera R M, Deagle B E, Bax N J and Blackburn S I (2005). Development and evalution of a PCR based assay for detection of the toxic dinoflagellate, Gymnodinium catenatum (Graham) in ballast water and environmental samples. Biol. Invasions, 7: 983-994

Penna A, Battocchi C, , Garcés E, Anglés S, Cucchiari E, Totti C, Kremp A, Satta C, Grazia Giacobbe M, Bravo I and Bastianini M (2010). Detection of microalgal resting cysts in European coastal sediments using a PCR-based assay. Deep-Sea Research II, (57):288–300

Penna A, Fraga S, Masó M, Giacobbe M G, Bravo I, Garcés E, Vila M (2008). Phylogenetic relationships among the Mediterranean *Alexandrium* (Dinophyceae) species based on sequences of 5.8S gene and internal transcript spacers of the rRNA operon. Eur. J. Phycol. 43:163–78.

Ribeiro S, Amorim A, Andersen TJ, Abrantes F, Ellegaard M (2011). Reconstructing the history of an invasion: the toxic phytoplankton species *Gymnodinium catenatum* in the Northeast Atlantic. Biol Invasions, 14:969–985.

Sampedro N, Fraga S, Penna A, Casabianca S, Zapata M, Grunewald C F, Riob P and 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

Stern R F, Andersen, R A , Jameson I, Kupper FC, Coffroth MA, Vaulot D, Le Gall F, Veron B, Brand, JJ, Skelton H, Kasai F, Lilly EL and Keeling PJ (2012). Evaluating the Ribosomal Internal Transcribed Spacer (ITS) as a Candidate Dinoflagellate Barcode Marker. PLoS ONE 7 (8), e42780.

Vale, P., Botelho, M.J., Rodrigues, S.M., Gomes, S.S., Sampayo, M.A.M., (2008). Two decades of marine biotoxin monitoring in bivalves from Portugal (1986–2006): a review of exposure assessment. Harmful Algae 7, 11–25.

Wood, A. M., Everroad, R. C. and Wingard, L. M. (2005). Measuring growth rates in microalgal cultures. In Andersen, R. A. (ed.), Culturing Techniques. Academic Press, New York, pp. 269–285.

Zapata, M., Rodríguez, F. and Garrido, J. L. (2000). Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridinecontaining mobile phases. Mar. Ecol. Prog. Ser., 195, 29–45.

Zappata M, Rodríguez F and Garrido JL (2000). Separation of chlorophylls and carotenoids from marine phytoplankton: A new HPLC method using a reverse phase C_8 column and pyridine-containing mobile phases. Mar Ecol Prog Ser, 195: 29-45.

7. Annex 1

Table 5 – Summary of mean (mol%) composition of distinct PST profiles of *G. catenatum* strains of populations in the Iberian Peninsula. Source refers to cultures established from plankton cells (P) or isolated single cysts (C).

Region	Location	Year	N	Source	Saxitoxin (STX) derivates present (mol%)												References
					C1+2	C3+4	GTX1+4	GTX2+3	GTX5	GTX6	dcGTX2,3	dcSTX	dcNeo	STX	neoSTX	doSTX	GC1-3	
NE Atlântic																		
Portugal																		
	Algarve	1999	1	C	20.7	18.7	-	-	39.6	19.6	-	1.4		-	-	-		This work
		2008	1	P	37.7	-	-	-	16.9	-	15.4	30.1		-	-	-		
	Aveiro	2010	1	C	8.4	32.5	-		22.0	35.7	-	1.34		-	-	-		
		2011	1	C	14.3	33.9	-	-	12.5	38.4	-	0.86		-	-	-		
	Lisbon	2003	1	C	10.7	31.2	-	-	21.4	34.2	-	2.50		-	-	-		
		2005	1	P	35.3	-	-	-	20.0	-	14.9	29.9		-	-	-		
		2007	1	P	34.3	17.1	-	-	23.6	16.2	3.2	4.1	1.5	-	-	-	+	Costa et al (2012)
		1999	1	C	+	nd	+	+	+	nd	+	+		+	-	-		Artigas et al (2007)
	Atlantic	1989	3	P	31.8	10.4	-	1.8	26.9	15.1	8.1	5.5		0.6	-	-		Negri et al (2001)
	Porto	1989	1	P	30.8	12.7	-	2.1	27.4	12.5	8.5	5.3		0.7	-	-	+	Negri et al (2007)
	Espinho	2005	1	P	+	nd	-	+	+	nd	+	+		-	-	-	+	Vale (2008)
			1		+	nd	+	-	+	nd	+	+		+				Franca (1996)
Spain																		
	Vigo		5	P	18.9	10.5	0.2	1.4	28.0	30.4	3.7	3.3		0.3	-	3.3	+	Negri et al.(2007)
	Vigo	85/693	5	P	23.6	7.6	0.2	0.7	21.6	16.0	2.0	3.5		4.0	2.8	18.0		Ordás et al (2004)
	Andalucia	1999	1	P	27.9	10.5	-	0.4	11.2	14.8	1.6	0.6		-	6.7	26.2		Ordás et al (2004)

(-) = fraction absent; (+) = fraction present but not quantified; (nd)= not determinated; N= nº of analyzed isolates.

8. Annex 2

Table 6 – Sequences and list of primers used to amplify target rDNA regions of several Portuguese strains of dinoflagellates: *G. catenatum* (IO13-01, 02, 22, 24, 06 and17), *G. microreticulatum* (Gm) and *Gonyaulax* sp. (Gy).

	Strain	Primer	DNA sequence	Sequence size (bp)	PCR Product size (bp)
LSU	IO13-01	28z	CCATGACATTTCGTCCATTAGCTTGTAAGGAAGGTGCCACGCGTGTGAAGCTCGCCAGAGCACGCAACACRAAGCAACGCAGC CCCACAAAGGTTGAACTGTTTGTTGGTGGGTCCCGCAAGGCACATTCACTCACCCAGGGGCGAGCTACCAGGTCCCAAGAGTT GTCCCCACCTCCAAACTGATGATGACACACAAGGCAAGAAACATCACACCRAACAAATCACAGCCCACCGCCCCGCTTTCGCTG GAATAGAAGGTGATCATTGCTGCGTGCGACAATCTCACCAAGCAAAACTGGTTCCATCCGCTTTCCTTTCAGCAAGTTCAGGCAC TTTTAACTCTCTTTTCAAAGTCCTTTTCATCTTTCCCTCATGGTACTTGTTTGCTATCGGTCTCGA	401	~400
		Dino210F	na		
	IO13-02	28z	Identical to IO13-01		
		Dino210F	na		
	IO13-22	28z	Identical to IO13-01		
		Dino210F	na		
	IO13-24	28z	Identical to IO13-01		
		Dino210F	AAGGACTTTGAAGAGAGTTAAGTGCCTGAACTTGCTGAAAGGAAAGCGGATGGAACCAGTTTTGCTTGGTGAGATTGTCGCACG CAGCAA AGTGCCTGAACTTGCTGAAAGGAAAGCGGATGGAACCAGTTTTGCTTGGTGAGATTGTCGCACGCAGCAATGATCACCTTCTATT CCAGCGAAAGCGGGGCGGTGGGCTGTGATTTGTTCGGTGTGATGTTTCTTGCCTTGTGTGTCATCATCAGTTTGGAGGTGGGGA CAACTCTTGGGACCTGGTAGCTC	90 192	
	IO13-06	28z	GGTGCCACGCGTGTGAAGCTCGCCAGAGCACGCAACACGAAGCAACGCAGCCCCACAAAGGTTGAACTGTTTGTTGGTGGGTC CCGCAAGGCACATTCACTCACCCAGGGGCGAGCTACCAGGTCCCAAGAGTTGTCCCCACCTCCAAACTGATGATGACACACAA GGCAAGAAACATCACACCGAACAAATCACAGCCCACCGCCCCGCTTTCGCTGGAATAGAAGGTGATCATTGCTGCGTGCGACAA TCTCACCAAGCAAAACTGGTTCCATCCGCTTTCCTTTCAGCAAGTTCAGGCACTTTTAACTCTCTTTTCAAAGTCCTTTTCATCTTT CCCTCATGGTACTTGTTTGCTATCG	362	
		Dino210F	na		
	IO13-17	28z	Identical to IO13-01		
		Dino210F	na		
	Gm	28z	CATAGATTGTAGGGAGGTGCCCGTGCTCTGAGCCACAAGGACAGCAGAGTACAGCAGCGCAGCCAAACAAAAGAGTGCAGTAC ACAAGCGAGTCCTACAAGGCACATTCACCTACCCGAAGATGGGCTACCATGTCCCAAGAGTTATCCTCGCCTGCCAACTGACAG TGACTGACAAGGCAAGAAACATCACGCGCGAACAAATCAAAGACTACAAGCACGCTTACGCTGAGCAAGCAGACAATCAATGCA GCACGTGACAATCTCACCAAGCAGACTGGTTCCATCCGCTTTCGTTTCAGCAAGTTCAGGCACTTTTAACTCTCTTTTCAAAGTCC TTTTCATCTTTCCCTCATGGTACTTGTTTGCTATCGGTC	376	
		Dino210F	na		
	Gy	28z	CAGCACTCAGCATCACCATGCACACCCACGCGCAACCATAAGGGGAAGGTATGCCAACTGGCAACAGCAGCAGGTTGAATGTC AATGCATTGCAGCAGGTAATCAATCAATGACCAAGGTAGCATGCACGCCATGTTGACAATGCAACCTGGTTGCAGCAAGGCAGC AAGACACGAAGTATACACAGCACGCGCCAAGGATGCTCAAAGCACCAGCCAGCACGTGTCTGTACACGTCAATCTTGCTAACAT GCTAAGAACCATTTGCTTTAATTGCAGCAATGTCAAGTACTTTTAACTTTCTTTTCAAAGTCCTTTTCACATTTCCCTCATGGTACTT GTTTGCTATCGGTCTCGA	357	

		Dino210F	na		
ITS	IO13-01	ITSA	CGTATCCAACCATTCTTGTGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTCGAGG GTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAATTCTCTCAGGCTAATGTGAACCTGTATTCGTTGCAT TGGTATCAACCAGCAATTACAA**C**TTTCACCGCCGGATGTCTGGGTTCGAACTTCCATGAGTGT TTCGCACGTATCANNCATTCTTGTGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTC GAGGGTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAGCTGTATTCGT TGCATTGGTATCAACCAGCAATTACAACTTTCAGCGACGGATGTCTCGGTTCGAACAACGATGAAGGGCGCAGCGAAGTGCGAT AAGCATTGTGAATTGCAGAATTCCGTGAACCGACAGAAACTTGAACGCATATTGTGCTTTTGGGATATCCCTGAAAGCATGCCTG CTTCAGTGTCTATGTTGTTATTTCCAGCAGTTGTCATTCTTCCTCTCGAAGGTGCGTTCTGCTGTGATTATGCGTGCTTCAAGACG CGCGTCCTCTGACGAGAGGGTGTGTGGCGCTCTNNGCGGCATTC CATT	232 472 R	610
		ITSB	ACTCTGTCTTAGCTGGGCGCAGCCACAGTTGATCGATGCAGAATGGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTT CAATCAGTTGGCAGCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCT TGAAGCACGCATAATCACAGCAGAACGCACCTTCGAGAGGAAGAATGACAACTGCTGGAAATAACAACATAGACACTGAAGCAG GCATGCTTTCAGGGATATCCCAAAAGCACAATATGCGTTCAAGTTTCTGTCGGTTCACGGAATTCTGCAATTCACAATGCTTATCG CACTTCGCTGCGCCCTTCATCGTTGTTCGAACCGAGACATCCGTCGCTGAAAGTTGTAATTGCTGGTTGATACCAATGCAACGAA TACAGCTTCACATTAGCCTGAGAGACTTCAGGTAGCAGGCACAAGGCGATTA GCTGGGCGCAGCCACAGTTGATCGATGCGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATCAGTTGGCA GCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAAGCACGCATA ATCACAGCAGAACGCACCTTCGAGAGGAAGAATGACAACTGCTGGAAATAACAACATAGACACTGAAGCAGGCATGCTTTCAGG GATATCCCAAAAGCACAATATGCGTTCAAGTTTCTGTCGGTTCACGGAATTCTGCAATTCA	474 312 R	
		Consensus	TAATCGCCTTGTGCCTGCTACCTGAATTCTCTCAGGCTAATGTGAACCTGTATTCGTTGCATTGGTATCAACCAGCAATTACAACT TTCAGCGACGGATGTCTGGGTTCGAAC	113	
	I O13-02	ITSA	TCGCACGTATCCAACCATTCTTGTGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTC GAGGGTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAGC AAACCATTCTTGTGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTCGAGGGTAGCA GGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAGCTGTATTCNTTGCATTGGTAT CAACCAGCAATTACAACTTTCAGCGACGGATG	160 201 R	
		ITSB	ACTACTCTGTCTTAGCTGGGCGCAGCCACAGTTGATCGATGCGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTT CAATCAGTTGGCAGCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCT TGAAGCACGCATAATCACAGCAGAACGCACCTTCGAGAGGAAGAATGACAGCTGCTGGAAATAACAACATGGACTCTGAAGCAT GTATGCTTTCAGGGATCTCTGAAGGCGCCCGTATCGATCCCAGCCCAACCCCACCCGGTAAATT CTGGGCGCAGCCACAGTTGATCGATGCGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATCAGTTGGCAG CGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAAGCACGCATAAT CACAGCAGAACGCACCTTCGAGAGGAAGAATGACAACTGCTGGAAATAACAACATTAAACCTGATGCACGCATGCCTCTGCGGA TATCCTTCTGGCACCACATGCGTTCACCGG	316 282 R	
	IO13-22	ITSA	CATTCTTGTGACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTCGAGGGTAGCAGGGGC AAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAGCTGNNTTCCTTGCATT GTATCCAACCATTCTTGTGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTCGAGGGT AGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAGCTGTATTCGTTGCATT GGTATCAACCAGCAATTACAACTTTCAGCGACGGATGTCTCGGTTCGAACAACGATGAAGGGCGCAGC	159 R 237	
		ITSB	TCTGTCTTAGCTGGGCGCAGCCACAGTTGATCGATGCGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATC	229	

			AGTTGGCAGCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAAG CACGCATAATCACAGCAGAACGCACCTTCGAGAGGAAGAATGACAGCTGCTGGAAATAACA TCGATGCGAATAGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATCAGTTGGCAGCGT GACAACACCTTGTCAA TCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAAGCACG CATAATCACAGCAGAACGCACCT TCGAGAGGAAGAATGACAACTGCTGGAAATAACAACATAGACACTGAAGCAG CCATGCTTTCAGGGATATCCCAAAAGGGCAC CATCCGTNCAANTTG	264 R	
	IO13-24	ITSA	ACGTATCCAAAACCATTCTTGTAGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACRAACAATCTCG AGGGTAGCARGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTRAAGTCTCTCAGGCTAGTGTGA ACGTATCCAAAACCATTCTTGTAGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACAAACAATCTCG AGGGTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTAAAGTCTCTCAGGCTAGTGTGAATCTGTATTCTTT GAATTGCTAACAATCC	156 185 R
		ITSB	GCTGGGCGCAGCCACAGTTGATCGATGCGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATCAGTTGGCA GCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAATCACGCATAA TCACTGCAAAACCCACCTTCGACA	192
	IO13-06	ITSA	ACGTATCCAAAACCATTCTTGTAGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTACTTGCCGCCACAAACAATCTCG AGGGTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAT GC ACGTATCCAAAACCATTCTTGTAGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTACTTGCCGCCACAAACAATC TC GAGGGTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGA	159 R 156
		ITSB	GCTGGGCGCAGCCACAGTTAGATCAGATGCAGAATAGAAACATTGACACGTCGTGCGCAACTAGTTAATTCGCTTCAATCAGTT GGCAGCGTGACAACACCTTGTCAATCAAATGCCTCAAGAGCGCCACACACCATCTCGTAAGAGGACGCGCGTCGTGCG	162
	IO13-17	ITSA	TTCGCCGTATCCAACCATTCTTGTGAACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTC GAGGGTAGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAGCTGTATTCGT TGCATTGGTATCAACCAGCAATTACAACTTTCAGCGACGGATGTCTCGGTTCGAACAACGATGAAGGGCGCAGCGAAGTGCGAT AATCGTTGTGACTTGGAAAATTCCGTGCAGCGCCCCACACACCCTTTACATTGTATTTTTTAGATTTTCC CACGTATCAACATTCTTGTGACTCTTCTGTGAGGCTGTACGTGGAAGTTGCTCCTCTAGTTGCCGCCACGAACAATCTCGAGGGT AGCAGGGGCAAGGCGGTGCTTTAATCGCCTTGTGCCTGCTACCTGAAGTCTCTCAGGCTAATGTGAAG	323 153 R
		ITSB	GCTGGGCGCAGCCACAGTTGATCGATGCAGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATCAGTTGGC AGCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAAGCACGCAT AATCACAGCAGAACGCACCTTCGAGAGGAAGAATGAAACTGTTGGAAAACCAACAAAGACACTGAATTTATAAAT GCTGGGCGCAGCCACAGTTGATCGATGCGAATGAAACATTGACACGTCGTGCGCAACTAGTTGATTCGCTTCAATCAGTTGGCA GCGTGACAACACCTTGTCAATCGAATGCCGCAAGAGCGCCACACACCCTCTCGTCAGAGGACGCGCGTCTTGAAGCACGCATA ATCACAGCAGAACGCACCTTCAAGAGGAAGAATGACAACTGCTGGAAATAACTACNTACACAACCAAGTAGCCATGCTNACCTG CAGATCCCAAAAGCCCACCACCATANTTCTTG	242 283 R
	Gm	ITSA	TCCATAACTTCATGTAGAACTACCTAGAGTGAGGTTCTGTGCAGAAGTTGCACCTCGGTGCCGCTGGGCAGAAGCTCGAGGGCA GCAAAAGGTTGGGGTGACAGGTGCGTGCGTCTGATCTTGCTGCCGGTTCTGTAAAGGAATTT	146
		ITSB	GCTGCGGAAG CAGAGAGGCNCAGACAGACAACACAGTTCAGCAGAGTGAGGCTTAGACCCGATACTGAGCTGAAAGCATTGC TGC GGTTGTCGATCAAGTGCGTCTAAAGTGCTGCAGAGCGCACATTCAAATGCACAGGTCTCACTCCTCCTAGCCCGA CAGG CAAAGGGTCATGGAAA	180
	Gy	ITSA	na		
		ITSB	na		

na – no amplification; R – re-sequencing.