UNIVERSITAT DE BARCELONA

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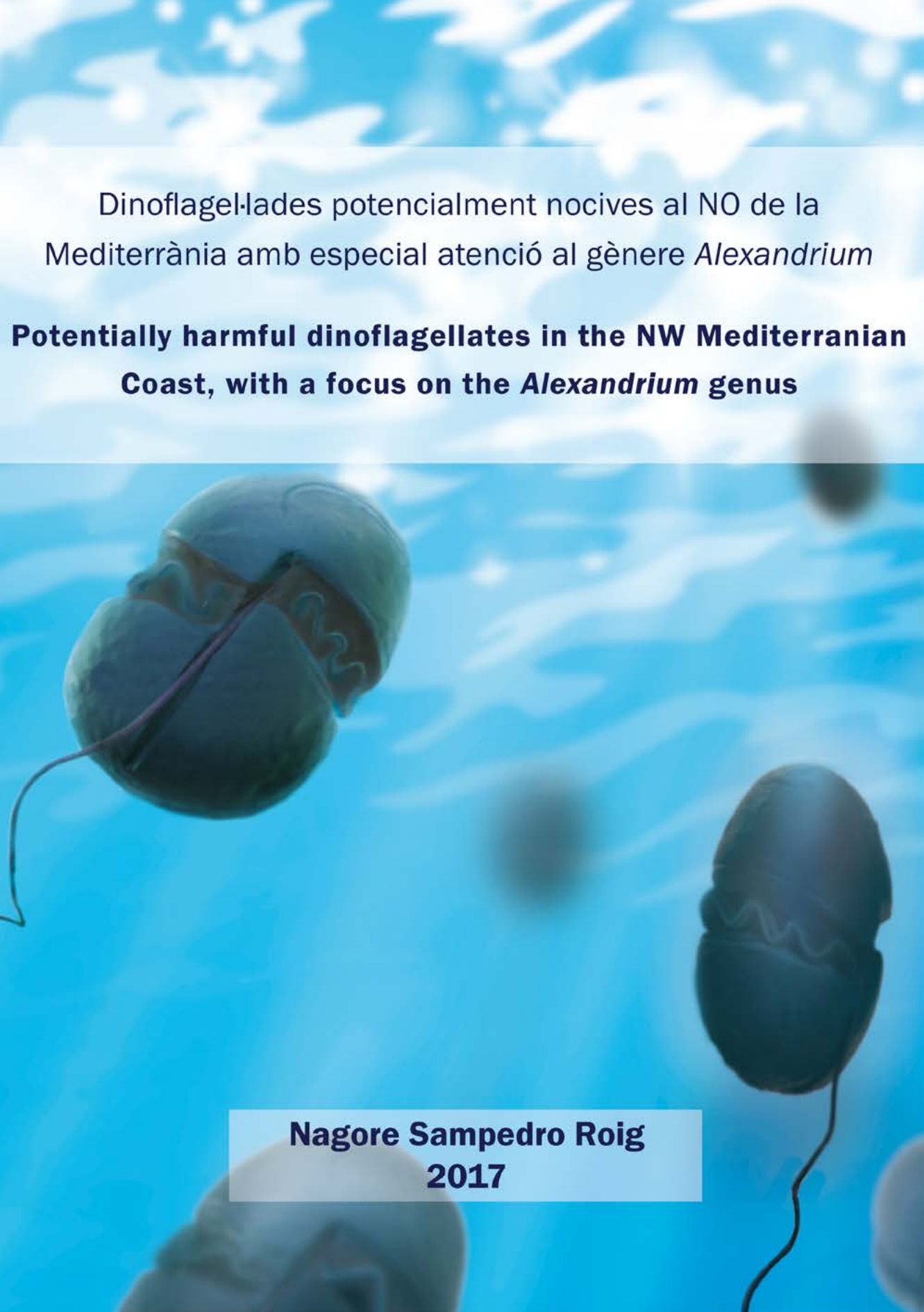

Nagore Sampedro Roig

2017

DINOFLAGEL·LADES POTENCIALMENT NOCIVES AL NO DE LA MEDITERRÀNIA AMB ESPECIAL ATENCIÓ AL GÈNERE *ALEXANDRIUM*

POTENTIALLY HARMFUL DINOFLAGELLATES IN THE NW MEDITERRANEAN COAST, WITH A FOCUS ON THE ALEXANDRIUM GENUS

Nagore Sampedro i Roig

Memòria presentada per optar al grau de Doctor per la Universitat de Barcelona

Programa de Doctorat en Ecologia, Ciències Ambientals i Fisiologia Vegetal

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Una tesi és com una PAN, depèn de molts factors, i per a que tingui èxit han d'haver-hi tot un conjunt de condicions favorables. Gràcies a tots per ser les meves condicions favorables!! Espero no haver estat gaire tòxica ;)

CONTINGUTS

Introducció general

El fitoplàncton és el conjunt d'organismes microscòpics i majoritàriament fotosintètics que viuen suspesos en els ecosistemes aquàtics. Comprèn diversos tipus d'organismes unicel·lulars, tant procariotes (bacteris) com eucariotes (protistes). Els grups més importants que inclou serien les diatomees, les dinoflagel·lades, les haptofícies i els cianobacteris. El fitoplàncton és la base de la xarxa tròfica marina, donat que és els responsable de la major part de la producció primària dels oceans. Utilitza l'energia solar i aigua amb nutrients per fixar el diòxid de carboni (CO_2) en matèria orgànica i alliberar oxigen (O_2). D'aquesta manera proveeixen d'aliment a nivells tròfics superiors, contribueixen a la captació de CO_2 de l'atmosfera i a la producció d'oxigen, fet pel qual la seva funció és vital no només pels ecosistemes marins sinó per tot el planeta.

1. Proliferacions Algals Nocives

Una proliferació és qualsevol augment de la concentració basal d'una espècie. Hi ha diversos grups microalgals que poden produir aquestes proliferacions: diatomees, dinoflagel·lades, silicoflagel·lades, primnesofícies i rafidofícies. Però només una minoria de les espècies d'aquests grups, de l'ordre d'un 7%, són capaces de donar lloc a proliferacions massives capaces de produir fenòmens perceptibles. Les proliferacions poden causar diversos efectes no desitjats tant a l'ecosistema marí com a l'espècie humana (**Figura 1**). Els principals problemes serien:

- La discoloració o canvi de color de l'aigua. L'elevada concentració d'aquests organismes que contenen pigments modifica la coloració de l'aigua de mar. La discoloració tot i que popularment és anomenada "marea roja" pot ser de diferents colors: verdosa, marronosa vermellosa i fins i tot blanquinosa. El sol fet de que una proliferació produeixi una discoloració repercuteix en l'activitat turística de les platges que la pateixen donat a la pèrdua de transparència de l'aigua però també pot afectar per exemple a les macroalgues del bentos degut a la disminució de la penetració de la llum.

- Depleció d'oxigen a la columna d'aigua. L'elevada biomassa que es concentra en aquestes proliferacions es va degradant a mesura que es sedimenta cap

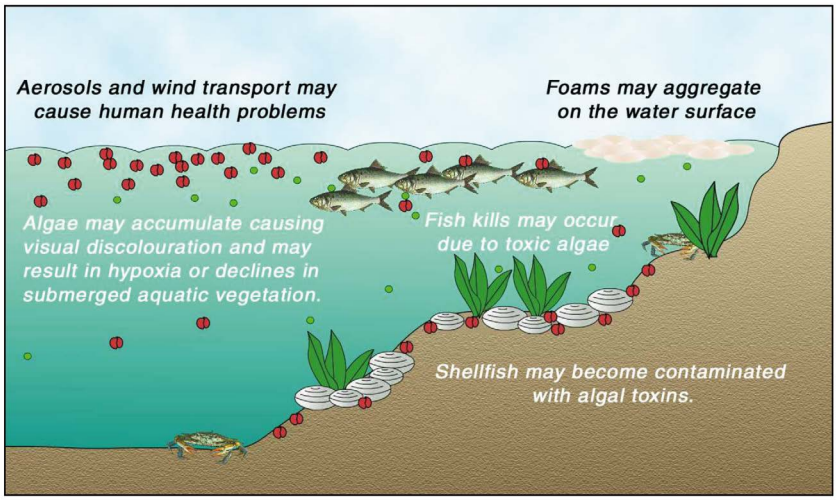


Figura 1. Esquema resum dels impactes de les Proliferacions Algals Nocives. Extreta del primer informe del GEOHAB (Science Plan, 2001).

al sòl marí. Aquest procés de degradació consumeix l'oxigen de la columna d'aigua que va disminuint fins que de vegades crea anòxies prop del fons que produeixen la mort sobretot als organismes sèssils de la fauna bentònica.

- Mortalitat de peixos per efecte mecànic. Algunes algues fitoplanctòniques amb morfologies punxents poden produir la mort als peixos per l'oclusió de les ganyes (ex: *Dictyocha fibula*).

- Producció d'escumes i mucílags. Alguns d'aquests organismes poden produir escumes i mucílags. Això pot generar diversos problemes: per un costat la gran quantitat de matèria orgànica acumulada pot produir depleció d'oxigen a la columna d'aigua i les conseqüències anteriorment comentades. Per altra banda pot produir la mortaldat de peixos per l'oclusió de les ganyes i, per últim, sobretot els mucílags, poden produir pèrdues econòmiques en el sector pesquer degut a l'obturació que produeixen a les xarxes de pesca.

- Producció de biotoxines. Un petita part de les espècies fitoplanctòniques (2%) poden generar potents toxines. Aquestes toxines poden acumulant-se i concentrant-se a través de la xarxa tròfica i produir intoxicacions i fins i tot la mort, tant a depredadors del medi marí (peixos, manatís, ocells marins etc..) com a l'home.

La via d'intoxicació per l'home acostuma a ser a través de mol·lusc bivalves com poden ser: musclos, escopinyes, cloïsses, vieires, ostres, que són organismes filtradors que acumulen ràpidament les toxines en els seus teixits. I tot i que generalment aquestes toxines no acostumen a afectar a la viabilitat dels bivalves (almenys en adults), quan aquests mol·luscs contaminats són ingerits per l'home, aquest pot patir diferents síndromes depenent de la toxina: paralitzant, diarreic, amnèsic, neurotòxic i azaspiràcid (PSP, DSP, ASP, NSP i AZP) (**Quadre 1**). L'home i altres consumidors finals també poden patir aquestes síndromes amb el consum d'organismes depredadors que s'alimenten de mol·luscs bivalves intoxicats com els cargols de mar, els crancs i les llagostes (Shumway, 1995). En zones tropicals i subtropicals, els humans també poden resultar enverinats per ciguatera pel consum de peixos (CZP) o per palitoxines i anàlegs pel consum del diferents tipus de marisc.

Una altra via a través de la qual algunes toxines (com és el cas de les brevitoxines) poden afectar als humans són els aerosols que es poden generar per acció coordinada del vent i l'hidrodinamisme en aigües on es dóna una proliferació de certs organismes que les produeixen (ex: *Karenia brevis*). L'exposició directa a aquest aerosols poden ocasionar problemes respiratoris similars a l'asma, i irritació de les membranes nasals i oculars.

Hi ha casos d'organismes fitoplanctònics que creen toxines que poden afectar als peixos però no a l'home, són el que anomenem organismes ictiotòxics (ex: *Karlodinium* spp). Aquests organismes causen grans pèrdues econòmiques en l'aqüicultura.

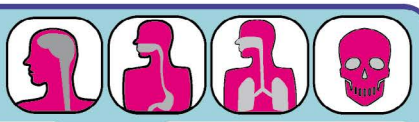
Quan la proliferació està produïda per espècies que són potencialment tòxiques o nocives parlarem de PAN (proliferació algal nociva) o HAB (de l'anglès *harmful algal blooms*).

2. Condicions adequades per a les PANs

Margalef (1978) va proposar un model conceptual, l'actualment anomenat **Mandala de Margalef** (**Fig. 2**), en què considerava la disponibilitat dels nutrients i l'energia externa (en forma de convecció de l'aigua i turbulència) els principals factors de selecció dels diferents grups fitoplanctònics. Segons aquest model, les diatomees creixien ràpidament en condicions d'aigües riques en nutrients i turbulentes. En

Quadre 1. Síndromes causats per toxines produïdes per algunes espècies fitoplanctòniques.

PSP — enverinament paralitzant pel consum de mol·luscs bivalves



Alexandrium spp., *Gymnodinium catenatum*, *Pyrodinium bahamense* var. *compressum*

Saxitoxines i derivats

Entumiment de la boca que s'estén cap a la cara, coll i dits de mans i peus. Mal de cap i símptomes gastrointestinals. Parestèsia que progressa per extremitats, rigidesa muscular, sensació de flotació, atàxia (pèrdua de control dels moviments) i vertigen. En casos més greus: parèsia (paràlisi muscular), atàxia severa, alteracions de l'estat mental, insuficiència respiratòria i mort dins les primeres 24 hores. La tassa de letalitat és particularment alta en nens. El pronòstic es favorable quan es sobreviu les primeres 24 hores.

Anderson et al., 1989; Harada et al., 1982; Prakash and Taylor, 1966

NSP — enverinament neurotòxic pel consum de mol·luscs

Karenia brevis

Brevitoxines

Vòmits, nàusees, símptomes neurològics lleus (trastorns de la parla, parestèsia i entumiment, dolors musculars i atàxia). Quan l'intoxicació és a través del consum de mol·luscs bivalves, el quadre és més banal, sense paràlisi i autolimitat en 48 hores.
Les brevitoxines, poden arribar per via respiratòria per mitjà d'aerosols marins, fins i tot en nedar en una proliferació tòxica. En aquest cas, cursa amb broncoconstricció, irritació de mucoses nasals i oculars i rinorrea.

Fleming et al., 2009; Fleming et al., 2007; Fleming et al., 2011

AZP — enverinament per azaspiràcid pel consum de mol·luscs bivalves

Azadidium spp.

Azaspiràcids

Els agents causals d'aquest síndrome que s'ha donat a certs països d'Europa del nord almenys des de 1995 han estat recentment descoberts. Es tracta d'espècies de dinoflagel·lades del gènere *Azadidium*. Els símptomes són similars als del DSP, nàusees, vòmits, diarrea severa i rampes a l'estómac.

McMahon and Marine, 1996; Tillmann et al., 2009

Quadre 1 (cont.). Síndromes causats per toxines produïdes per algunes espècies fitoplanctòniques.

DSP

enverinament diarreic pel consum de mol·luscs bivalves

Dinophysis spp. *Prorocentrum* spp.

Àcid ocadaic i derivats

Diarrees, nàusees, vòmits i dolors abdominals. Els símptomes solen desaparèixer tres dies després de la intoxicació. Una exposició crònica a la toxina pot produir la formació de tumors en el sistema digestiu.

Fujiki et al., 1988; Murakami et al., 1982; Reguera and Pizarro, 2008; Yasumoto et al., 1980

ASP

enverinament amnèsic pel consum de mariscs bivalves

Pseudo-nitzschia spp. and *Nitzschia* spp.

Àcid domoic i isòmers

Símptomes gastrointestinals en les primeres hores i neurològics (vertigen, mareig, perdua d'equilibri, visió borrosa, al·lucinacions, desorientació, confusió, amnèsia) passades les 24 hores del consum de bivalves. Especialment en edat avançada pot evolucionar a confusió, coma i mort. La letalitat d'aquest quadre és del 2%. Diversos mesos després de l'intoxicació, les víctimes segueixen mostrant déficit crònic de memoria residual i neuropatía motora o axenopatia.

Bates et al., 1989; Kotaki et al., 2000; Smida et al., 2014

CFP

enverinament per ciguatera pel consum de peixos

Gambierdiscus spp., *Fukuyo* spp.

Ciguatoxina

Símptomes molt variables. Generalment gastrointestinals a l'inici, que poden anar seguits de símptomes neurològics com entumiment de la boca i de les parts distals de les extremitats, alteració en la sensació tèrmica, dolor muscular i articular. També es poden donar símptomes cardiovasculars. La mortaldat és molt baixa, però els símptomes neurològics es poden prolongar setmanes, mesos i fins i tot anys, presentant-se remissions i aguditzacions.

Friedman et al., 2008; Holmes, 1998; Kohli, 2015

Palitoxicosi

Ostreopsis spp.

Palitoxina, Ostreocin, Ovatoxin, scarenotoxin

Provocada pel consum de marisc o la inhalació d'aerosols marins o el contacte directa amb l'aigua. Cursa amb símptomes gastrointestinals, letàrgia, entumiment, rabdomiòlisi i, en cas d'inhalació, símptomes respiratoris . La intoxicació alimentària és típica de tròpics i subtròpics mentre les irritacions cutànies i respiratòries, de les platges mediterrànies.

Ciminiello et al., 2014; Deeds i Schwartz, 2010; Ramos i Vasconcelos, 2010; Tubaro et al., 2011; Usami et al., 1995

canvi, les grans dinoflagel·lades, amb expansions i amb flagells que els hi permeten migrar en la columna d'aigua, serien més abundants en aigües estratificades i pobres en nutrients. Mentre que les coccolitoforals tendirien a ocupar una posició intermèdia respecte a les condicions tròfiques. Dins d'aquest esquema Margalef va distingir un subgroup de dinoflagel·lades formadores de PANs (de mida petita i formes esfèriques), les quals serien afavorides per una disponibilitat elevada de nutrients i situacions d'estabilitat (baixa turbulència). Aquestes situacions són cada cop més comuns en les zones costaneres, atesa la modificació que ha patit la costa per l'activitat humana. L'home per una banda ha creat ports i espigons que alberguen aigües confinades amb poca turbulència i per altra banda ha augmentat l'eutrofització de la zona costanera.

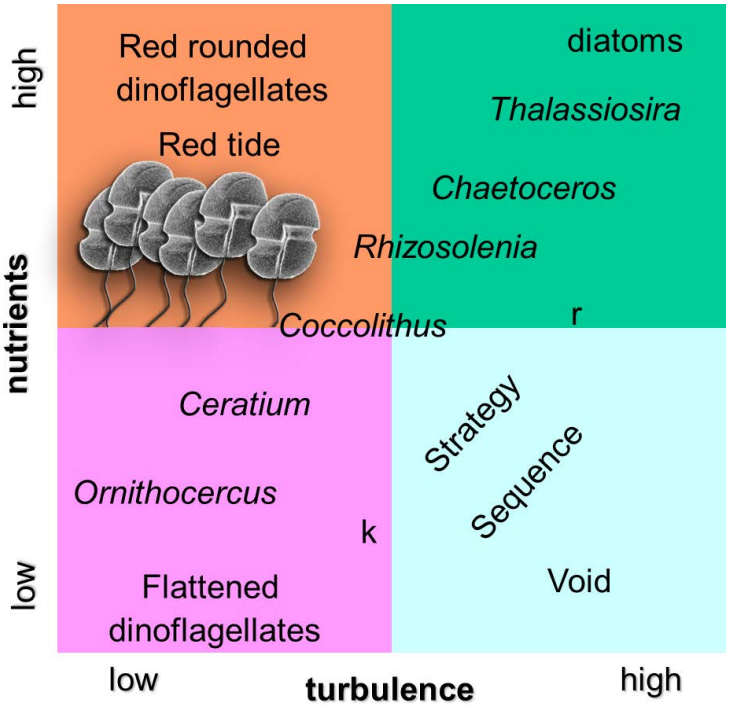


Figura 2. "Mandala" de Margalef. Els diferents grups del fitoplàncton estan graficats en funció de les concentracions de nutrients i de la turbulència de l'aigua.

3. Expansió de les PANs

En les últimes dècades s'ha observat un augment dels registres de PANs a nivell mundial (Hallegraeff, 1993). Abans dels anys 70 relativament pocs països semblaven afectats per proliferacions algals nocives mentre que ara es té registre que afecten a la majoria de països costaners (**Fig. 3**) i es donen en major freqüència.
Aquest increment observat pot ser degut a diverses raons: unes a causa de la millora dels mètodes de detecció, un millor coneixement sobre les espècies i el nombre d'observadors i observacions; i les altres, a l'increment de factors afavoridors de les proliferacions (Anderson, 1989; Hallegraeff, 1993; Hallegraeff, 2010). Dins aquest segon grup es debaten causes relacionades amb mecanismes naturals i causes que serien produïdes per l'activitat humana. S'acostumen a citar com a possibles causes:

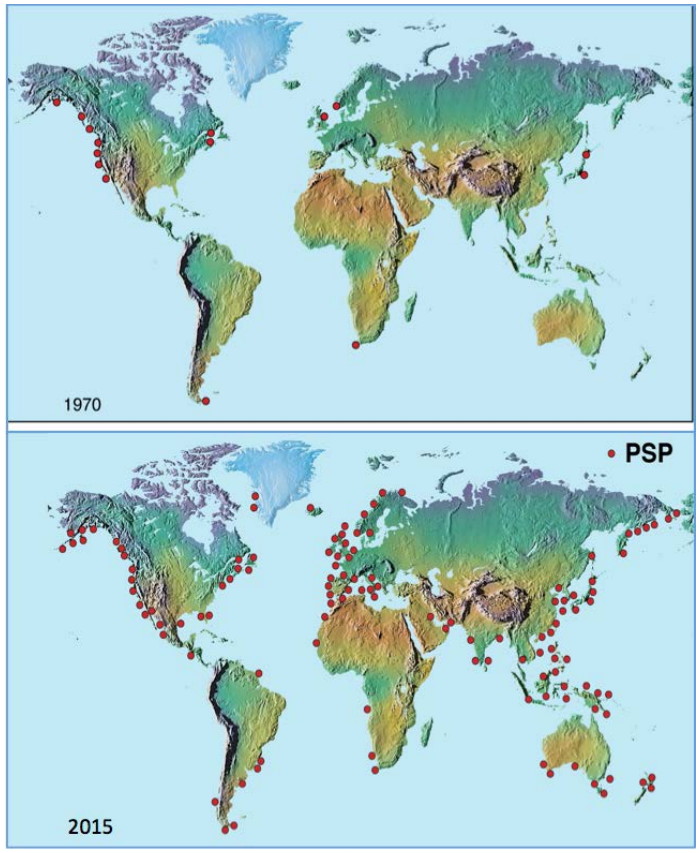


Figura 3. Observacions de PSP a diferents indrets del món al 1970 (dalt) i al 2015 (baix). Extret de https://www.whoi.edu/redtide/regions/world-distribution

- L'increment de l'eutrofització (Anderson 1989, Hallegraeff 1993) de les aigües costaneres per part de l'home. La disponibilitat de nutrients modula fortament molts aspectes de l'ecologia de les proliferacions. Però no només l'eutrofització específicament pot contribuir a l'augment de PANs sinó també els canvis de la proporció del nutrients i de les formes predominants dels nutrients que es donen a la costa deguts, per exemple, a l´ús de certs tipus de fertilitzants per part de l'home. L'arribada a la costa d'aigües amb proporcions de nutrients que difereixen dels que es produeixen de forma natural alteraria la composició de les espècies d'algues podent afavorir algunes espècies d'algues nocives.
- L'increment de la utilització d'aigües costaneres per l'aqüicultura, que augmenta l'eutrofització i el transport d'espècies mitjançant la translocació dels estocs de mol·luscs.
- L'augment del transport marítim que facilitaria la dispersió d'espècies per l'aigua de llast dels vaixells (Hallegraeff, 1998)
- Condicions meteorològiques inusuals com el "canvi climàtic".
- La creació de zones costeres d'aigües confinades amb baixa turbulència com per exemple: ports i platges amb espigons (Garcés i Camp, 2012; Vila et al., 2001a; Vila et al., 2001b).

Actualment, algunes d'aquestes causes i la importància relativa de cadascuna d'elles en cada lloc i situació concrets són objecte de debat.

4. Plans de Vigilància de PANs

El creixent interès per les PANs durant els anys 80 va incrementar el nombre de deteccions arreu del món, i en raó dels riscos per la salut humana va dur a molts països a establir regulacions per tal de garantir la seguretat alimentaria als consumidors de productes marins. Es van establir límits per a cada toxina per sobre dels quals es considera que el producte és perillós i no segur per al consum humà i es van establir també les metodologies oficials per tal de mesurar-ho.

Els reglaments també estableixen l'obligació de realitzar programes de vigilància d'especies fitoplanctòniques potencialment productores de toxines en zones de producció de mol·luscs bivalves (EU Directive 91/492/EEC). Els tancaments de les zones de producció es realitzen per les autoritats competents quan es detecten nivells de toxines excessius en els mol·luscs però també es poden donar quan

Taula 1. Tancaments administratius de les zones de producció de bivalves de Catalunya durant el període 2000-2012.
Tipus de toxicitat: PSP (en vermell) = tancaments produïts per toxicitat paralitzant, DSP (en groc)= tancaments produïts per toxicitat diarreica, ASP (en verd) = tancaments produïts per toxicitat amnèsica.

Zones de producció	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Llançà- Port de la Selva													
L'Escala-Roses-Cadaqués												16 d	16+18 d
Badia de l'Estartit													
Palamós- Cap Negre													
Sant Feliu de Guíxols													
Tossa de Mar													
Calella de la Costa-Lloret de Mar								18 d					
Mataró- Arenys de Mar- Sant Pol													
El Masnou-Sant Joan de Vilassar													
Port Olímpic- Barcelona													
Port de Barcelona-Port Olímpic													
La Murtra- Port de Barcelona													
Torre Barona- La Murtra													
Vilanova i la Geltrú- Torre Barona													39 d
Cap Gros-Vilanova i la Geltrú				25d*		25 d		36 d*	16 d	50 d*	22 d	9 d	22 d
Cap de Salou- Cap Gros			16d[1]					40 d[1]					
Vandellós i L'H.-Cap de Salou													
L'Ametlla- Vandellós i l' H.													
Badia del Fangar i Golf de l'Ampolla		Fals-7d 30d[1]	prev.-6d	12d	32 d		7d	64 d		16 d	15 d		13 d
Costa Nord del Delta de l'Ebre													
Costa Sud del Delta de l'Ebre						9 d							
Badia dels Alfacs	40 d	Fals-3 d	93d	33d		98 d	76 d	111 d	32 d	18 d	16 d		86
Alcanar- St. Carles de la Ràpita						83 d	64 d	32 d	49 d	43 d			

* *Alexandrium minutum*
[1] *Alexandrium pacificum*

d=dies
Prev.=preventiu

Fals = tancament degut a un fals positiu de toxicitat

s'observen canvis en les poblacions fitoplanctòniques tòxiques que poguessin donar lloc a una acumulació de toxines (Reglament (CE) 854/2004).

Catalunya consta de 23 zones de recol·lecció de mol·luscs bivalves al llarg de tota la costa. Les zones de producció inclouen bancs naturals de marisc en aigües obertes i zones de cultiu de marisc localitzades a l'interior de badies costeres semi-tancades del Delta de l'Ebre, aquestes darreres representen el 90-95 % del total de la producció de bivalves i és on es va establir el primer programa de vigilància de fitoplàncton tòxic a finals dels anys 80 que es va materialitzar a partir del RD 345/1993 amb un pla de vigilància.

A principis dels anys 90 es mostrejaven també les altres zones de marisqueig al llarg de la costa, els punts de mostreig es localitzaven a la superfície d'on es trobaven els bancs naturals de bivalves. Cap al 1995, en substitució, es va establir un nou programa de vigilància on es mostrejaven diversos ports amb la idea que en aquests mostrejos es detectaven de manera anticipada les proliferacions fitoplanctòniques nocives o tòxiques. En molts casos, abans que es donessin en aigües exteriors.

Per tal de posar de manifest la rellevància que té per l'aqüicultura el fitoplàncton tòxic a la costa catalana es presenta a la **taula 1** els tancaments de l'activitat marisquera, produïts per la detecció de toxines fitoplanctòniques, que es van dur a terme a les diferents zones de producció durant el període del 2000 al 2012.

Ja en el segle XXI a Europa es comencen a establir normatives ambientals i normatives d'aigües de bany que també necessiten el seguiment del fitoplàncton. La Directiva Marc de l'Aigua (DMA) 2000/60/CE (EC, 2000) és una normativa ambiental, que busca el bon estat de les aigües litorals i exigeix l'ús d'un indicador de qualitat biològic (BQE) basat en el fitoplàncton per avaluar l'estat ecològic de les zones costaneres i les masses d'aigua de transició. Per tal de dur a terme la implementació i compliment dels requeriments d'aquesta directiva, a Catalunya, s'estableixen estacions de mostreig en diferents punts de la costa, que s'aniran modificant per tal d'adequar-se a les decisions preses a les diverses reunions europees. A l'any 2006 es va publicar la Directiva 2006/7/CE del Parlament europeu i del Consell de 15 de febrer de 2006, relativa a la gestió de la qualitat de les aigües de bany que es transposada per l'Estat espanyol mitjançant el RD 1341/2007, d'11 d'octubre. Aquesta normativa estableix entre d'altres les mesures de control de les zones d'aigües de bany, i té com a objectiu la protecció de la

salut pública i la protecció i la millora de la qualitat del medi ambient. Respecte al fitoplàncton, aquesta normativa estableix que quan el perfil de les aigües de bany mostri propensió a la proliferació de fitoplàncton marí, es duran a terme les investigacions i els control necessaris per determinar l'acceptabilitat de l'aigua i es comunicaran a la autoritat sanitària que avaluarà els riscos per la salut. En aquest aspecte a Catalunya s'ha dut a terme el seguiment d'algunes platges considerades de risc de patir PANs i per tal de estudiar els fenòmens relacionats amb el fitoplàncton que puguin perjudicar o influir sobre els banyistes.

Els plans de vigilància són una font d'informació valuosa molt útil per l'estudi de la dinàmica de poblacions i el coneixement de les espècies i la seva ecologia en general.

De manera general els plans de vigilància de les zones de marisqueig han estat la principal font de coneixement de les espècies fitoplanctòniques tòxiques al món. En canvi, en altres zones on generalment no hi mira ningú hi ha un buit de coneixement d'aquests organismes.

*En aquesta tesi, s'estudia la distribució espai-temporal de dinoflagel·lades potencialment nocives en aquestes zones arran de costa on hi ha aquest buit de coneixement i on poden sorgir possibles problemàtiques que afectin, la salut de les persones, la qualitat de les zones de bany o que provoquin danys ambientals (**Capítol 3**).*
Bona part de la informació d'aquesta tesi s'extreu del programa de vigilància realitzat entre el 2000 i el 2012 entre l'ICM i l'ACA (l'Agència Catalana de l'Aigua) que inclou mostrejos per garantir la seguretat alimentaria als consumidors de productes marins, mostrejos realitzats pel compliment de la DMA i mostrejos per avaluar la qualitat de les aigües de bany.

5. Dinoflagel·lades

Un 75% de les espècies que produeixen PANs pertanyen al grup de les dinoflagel·lades (Smayda, 1997), grup en el qual es centra la present tesi. Les dinoflagel·lades és un grup de protistes unicel·lulars que poden formar colònies. Comprèn aproximadament unes 2000 espècies vives, de les quals la majoria són marines però també viuen en aigua dolça i salobre. Poden ser planctòniques, bentòniques, epibionts i també paràsites o simbionts. També són un grup divers

quant a la seva forma d'alimentar-se, puix que hi ha especies autòtrofes, heteròtrofes i també mixòtrofes.

Les dinoflagel·lades presenten una capa simple de vesícules aplanades anomenades vesícules de l'amfiesma o alvèols (Morrill i Loeblich, 1983; Netzel i Dürr, 1984) situades sota de la membrana cel·lular. En funció del contingut d'aquestes vesícules les cèl·lules poden ser atecades (nues) o tecades (amb teca). En les espècies atecades, les vesícules estan buides o contenen material amorf. A les tecades, les vesícules contenen cel·lulosa, formant plaques de cel·lulosa que conformen la teca. Els patrons formats per les plaques de la teca són claus per a la taxonomia d'aquests organismes.

Les dinoflagel·lades poden alternar reproducció asexual i sexual. La reproducció asexual és la més ràpida i serveix per expandir el genotip millor adaptat, essent la reproducció predominant en condicions òptimes i la que permet el creixement de la població i les proliferacions. La reproducció sexual, contribueix a la variabilitat genètica de la població.

Hi ha espècies de dinoflagel·lades holoplanctòniques que viuen sempre a la columna d'aigua i espècies meroplanctòniques que presenten a més un estadi quiescent que habita al bentos (el cist). De manera que aquests darrers alternen una fase planctònica amb una fase bentònica. Es poden formar diferents tipus de cistos. Els cistos anomenats temporals, d'ècdisi o pel·liculars poden germinar en poc temps (d'hores a dies) i els cistos de resistència o hipnozigots germinaran després d'una fase de maduració (període de latència) obligatòria que pot durar dies o alguns mesos. En els cas dels cistos sexuals, la germinació donarà un planomeiozigot diploide que es dividirà per formar dues cèl·lules vegetatives haploides (Walker, 1984).

La proliferació d'aquest organismes es desenvolupa en les següents tres fases:

- L'inici de la proliferació requereix un inòcul de cèl·lules vegetatives que pot provenir de cèl·lules que ja es trobaven a l'aigua en baixes concentracions, o de la germinació de cistos de resistència que es trobarien al sediment; així com de l'advecció de la població d'altres zones. Els factors que desencadenen l'inici de la proliferació són encara poc coneguts per la majoria d'espècies. La població apareixerà d'una manera més o menys irregular i recurrent en funció de la fisiologia de l'estat resistent, que germinarà un cop completada

la fase de maduració i quan les condicions li siguin adequades o en funció de les taxes de creixement de les cèl·lules vegetatives presents en la columna de l'aigua. Per una banda, els cistos representen una gran avantatge per a la supervivència de l'espècie donat que poden sobreviure fins i tot 100 anys en el sediment (Ellegaard et al., 2008), per l'altra, la supervivència de les cèl·lules en la columna d'aigua dependrà de si les condicions són prou favorables durant tot l'any.

- El creixement exponencial i manteniment. Un cop iniciada la proliferació la població creixerà exponencialment responent a les condicions ambientals (com llum, fotoperíode, temperatura, disponibilitat de nutrients, estabilitat de la columna d'aigua) fins a la fase de manteniment, on el creixement sempre estarà compensat per les pèrdues. Aquestes pèrdues poden ser biològiques com a depredació, parasitisme, encistament i dispersió de la població per pròpia motilitat o poden ser físiques, on les variables que influeixen en el temps de renovació de l'aigua seran importants.

- La finalització de la proliferació arriba quan les pèrdues superen els increments de la població. Generalment es sol atribuir a condicions adverses pel creixement vegetatiu, però en molts casos les causes de la finalització de les proliferacions no s'han aclarit, probablement perquè es deu a una combinació de factors biològics i físics.

6. Dinàmica poblacional i patrons de variabilitat

La dinàmica de poblacions estudia els canvis en la mida i l'estructura de la població així com els factors i els mecanismes que els produeixen. Les variacions poblacionals depenen de factors endògens de l'espècie, així com de les característiques topogràfiques de l'ambient on es troben i de les condicions fisico-químiques i interaccions biològiques. Les variabilitats de les poblacions del fitoplàncton ocorre en diversos patrons: cicles (fluctuació en un període conegut), tendències, fluctuacions, esdeveniments inusuals, pulsos irregulars; i a diverses escales: horària o inferior, diària, estacional, anual, decadal etc..(Smayda, 1998).

Les sèries temporals són eines imprescindibles per esbrinar aquests patrons poblacionals. Per poder conèixer i entendre la dinàmica de les espècies es necessari una observació detallada de la població en un període llarg de temps

i de les variables que poden influir en aquesta dinàmica. Això ens permetrà per exemple detectar patrons de la població persistents o ocasionals, canvis en l'abundància de les espècies degut a canvis ambientals, i determinar quins factors governen el desenvolupament de les proliferacions (Trainer et al., 2002). El coneixement de la seva dinàmica poblacional i els factors que la regulen pot ajudar a prevenir, minimitzar o resoldre situacions de risc.

Les sèries temporals més llargues de l'abundància d'organismes fitoplanctònics s'han obtingut en estudis paleoecològics analitzant els frústuls de les diatomees o els cistos de dinoflagel·lades preservats en els sediments (ex: Lundholm et al., 2010; Mousing et al., 2013; Ribeiro et al., 2012) i/o l'ADN ancià en el sediment juntament amb la datació d'aquests sediments (ex: Klouch et al., 2016). Aquestes sèries resulten de molta utilitat donat que permet estudiar les comunitats fitoplanctòniques en sediments en períodes de temps relativament llargs (majors a 50 anys) però, per contra, depenen de la preservació dels organismes durant tots aquests anys i dels límits de les anàlisis de l'ADN ancià i de la datació dels sediments. Aquest tipus de sèries permet l'estudi de sèries llargues des del punt de vista, per exemple, de la caracterització de les tendències, mentre que d'altres aspectes no es poden abordar degut al seu nivell de detall i als límits de la datació, com l'estudi dels patrons estacionals.

D'altra banda, ara comencen a haver-hi sèries multianuals de més de 10 anys, de recomptes fitoplanctònics. En la majoria de casos, aquestes sèries són fruit dels programes de seguiment de fitoplàncton establerts en àrees costeres a partir dels anys vuitanta. Els treballs realitzats sobre l'estudi d'aquestes sèries multianuals es basen en gran part dels casos en clorofil·les (ex: Winder i Cloern, 2010) o en grans grups fitoplanctònics (ex: Suikkanen et al., 2007), tot i que també s'ha treballat a nivell de gènere (ex: Shuler et al., 2012) i més ocasionalment a nivell d'espècies (ex: Bode et al., 2015).

Si es vol estudiar la dinàmica poblacional de certes espècies, l'estudi a nivell de gènere no és suficient. La tendència, i la resta de patrons temporals del gènere no ha de ser la mateixa de l'espècie en concret, per exemple Lundholm et al. (2010) va documentar un canvi en la composició específica del gènere *Pseudo-nitzschia* al llarg de gairebé 100 anys a Sill-fjord (Dinamarca) mentre que l'abundància relativa del total de *Pseudo-nitzschia* romania més o menys igual. Quan es tracta de l'estudi de gèneres que inclouen espècies potencialment tòxiques, la identificació a nivell d'espècie encara és més important. La dificultat

per a una correcta identificació taxonòmica en microscòpia òptica de moltes espècies fitoplanctòniques, junt amb el baix nombre d'experts capaços de reconèixer certs organismes a nivell d'espècie i el poc valor que sovint es dona a aquesta tasca, ha conduit, però, a una gran mancança de sèries temporals llargues i fiables inclús d'espècies de gèneres importants per l'espècie humana i els ecosistemes com és el gènere de dinoflagel·lades tòxiques *Alexandrium*. Moltes d'altres causes afecten la qualitat de les sèries, que estan influïdes pel canvi de persones que fan els recomptes al llarg de la sèrie, el que fàcilment produeix un biaix en aquesta, o per la freqüència de mostreig entre d'altres. Altres problemàtiques, com la dificultat per mantenir econòmicament sèries llargues en un món de prioritats canviants també han contribuït al reduït nombre de sèries multianuals relativament llargues.

Patrons de variabilitat

El fitoplàncton, com tots els components tòxics de l'ecosistema marí, exhibeix variacions anuals i canvis a llarg termini. Les variacions, però, no són iguals a tot arreu. A la costa es dóna un major rang de heterogeneïtat de patrons estacionals de biomassa fitoplanctònica entre i dintre dels ecosistemes que contrasta amb les fluctuacions estacionals recurrents i sincròniques dels productors primaris en amplies zones terrestres i oceàniques (Cloern i Jassby, 2008; Winder i Cloern, 2010). A què és deguda aquesta alta variabilitat de patrons a la zona costanera? Mentre que a mar obert la majoria de patrons venen regulats pels cicles anuals de radiació solar i entrada de calor atmosfèric (Cushing, 1959; Sverdrup, 1953), prop de la costa es donen processos addicionals degut a la interacció entre la terra, l'oceà, l'atmosfera i els fons subjacents (Cloern 1996). Per exemple, l'entrada de pulsos d'aigua dolça del continent, la resuspensió dels sediments pels vents en zones poc profundes, o el fluxe de cèl·lules vegetatives des del sediments somers que poden actuar com un banc de cistos d'espècies fitoplanctòniques. A més, la gran pressió que l'home exerceix sobre aquest zona pot modificar molts d'aquests processos (ex. Regulació del cabals d'aigua dels rius o de la seva concentració de nutrients) i introduir-ne de nous (ex: transformació de l'hàbitat, per exemple amb la construcció de ports i dics). A la costa doncs, intervenen molts factors i molts d'ells locals que explicarien el gran espectre de patrons trobats a la costa i específics de cada lloc i que poden emmascarar respostes a fenòmens més globals com el canvi climàtic. Estem encara en una etapa descriptiva de la recerca

fitoplanctònica, lluny de poder explicar la seva variabilitat (Smetacek i Cloern, 2008). Però, els patrons ens poden donar pistes dels processos de variabilitat (Cloern i Jassby, 2010), per això l'estudi d'aquests patrons és determinant per a conèixer les variables que governen la dinàmica del fitoplàncton i poder-la predir.

Les variacions fitoplanctòniques a més llarg termini, com les variacions interanuals i les tendències, poden venir determinades per cicles naturals (per exemple hidrogràfics o meteorològics) de períodes de diversos anys o bé de rellotge intern, que li confereixi a la població recurrències en períodes de temps majors que un any. Les tendències en fitoplàncton també poden anar associades a tendències de nutrients, ex. Henriksen, 2009. En general les tendències dels organismes fitoplanctònics no són linears i són espècie-específiques de manera que no totes les espècies d'un grup de fitoplanctònic (com dinoflagel·lades o diatomees) segueixen la mateixa tendència (Bode et al., 2015). Bode et al. (2015) van trobar que, per a un mateix període de temps en el mateix lloc, unes especies no mostraven cap tendència, altres decreixien, altres creixien durant un temps concret i altres ho feien durant tot el període. Es coneix també espècies que han desaparegut d'una zona on portava molts anys, com és el cas d' *Alexandrium minutum* al port d' Alexandria (Egipte) que formava proliferacions des del 1958 (Halim, 1960) fins l'any 1994 (Ismael i Khadr, 2003; Labib i Halim, 1995).

Per altra banda, es poden donar fluctuacions no recurrents per pertorbacions hidrogràfiques, climàtiques, químiques i biològiques. Canvis bruscs en el sistema climàtic de gran escala i també canvis ambientals locals, en moltes ocasions induïts per l'home, poden produir canvis freqüentment únics i permanents, per exemple en l' estacionalitat del fitoplàncton (Cloern i Jassby, 2008), o en el nivell mig de l'abundància d'una espècie com és el cas de *Skeletonema* spp. a Narragansett Bay durant els 80 (Borkman i Smayda, 2009). Per altra banda, gran part de la variabilitat fitoplanctònica és impredictible, de duració variable però temporal i freqüentment respon a disrupcions físiques de l'habitat (Smayda, 1998).

Els patrons anuals o estacionals regulars del fitoplàncton impliquen un control per un procés periòdic com la temperatura, la radiació solar, el fotoperíode, el cabal d'un riu o d'altres factors directament relacionats amb aquests com pugui ser un fort control herbívor que oscil·la sobre el cicle anual de temperatura. Prop de la costa però la biomassa fitoplanctònica pot arribar al seu punt màxim durant qualsevol estació de l'any, el que evidència que l'estacionalitat del fitoplàncton a

la interfase terra-mar és impulsada per més d'uns pocs factors climàtics (Cloern i Jassby, 2008). Pel que fa a la periodicitat, la major part de les sèries temporals de clorofil·la de zones temperades i subtropicals tenen períodes dominants de 12 mesos, altres de 6 mesos, i altres de períodes curts de 2-4 mesos. En alguns casos, aquest períodes no persisteixen al llarg de tota la sèrie i els períodes dominants canvien. Alguns casos no presenten cap període cíclic (Winder i Cloern, 2010).

De la mateixa manera que ho fa el màxim de clorofil·la, cada una de les espècies poden presentar diferents periodicitats. De manera que hi ha espècies que proliferen regularment un cop a l'any mentre hi ha d'altres que ho poden fer dues vegades. La recurrència anual de les proliferacions de dinoflagel·lades està molt lligada als cistos de resistència. La presència d'aquests cistos en els sediments, que representa un reservori de llavors, s'ha relacionat amb la ocurrència recurrent de les proliferacions (ex. Anderson i Wall, 1978). El període de maduració dels cistos de resistència així com els patrons de germinació, ambdues coses específiques de cada espècie tot i que poden estar afectades per les condicions ambientals, poden explicar en part els patrons de les proliferacions. Ishikawa i Taniguchi (1996, 1997) va identificar tres patrons de germinació en espècies productores de cists que poden ajudar a explicar l'estacionalitat de les espècies.

La dinàmica de les proliferacions està lluny de ser entesa atès el gran nombre de factors que hi intervenen i el baix nombre de sèries llargues de qualitat que amb què es compta.

*En aquesta tesi, en els capítols 1, 2 i 3 s'estudia la dinàmica de la fase planctònica de diverses espècies d'*Alexandrium *al llarg d'un període de 13 anys en diferents ambients costers del NO de la Mediterrània que inclouen ports, platges i una llacuna. S'estudien les fluctuacions temporals, tant dins un mateix any com al llarg dels anys, per tal de diferenciar els seus patrons i identificar els factors que contribueixen en aquestes variacions. S'avalua també en aquests capítols la diversitat i la distribució a una escala regional de les espècies d'*Alexandrium *i el seu canvi al llarg dels anys*.

La major part de dades que s'analitzen en aquest tres capítols són fruit del programa de vigilància de fitoplàncton tòxic de la Costa Catalana establert entre l'ICM i l'ACA esmentat anteriorment. Tot i que aquest sistema de vigilància és anterior a l'inici de la sèrie analitzada en aquesta tesi (del 2000 al 2012) s'ha escollit l'anàlisi d'aquest període per la homogeneïtat de les seves dades. En totes les sèries, al llarg d'aquest període s'ha utilitzat el mateix procediment de mostreig, el mateix mètode de preservació, preparació i recompte de la mostra, utilitzant calcofluor de manera rutinària per a la identificació d'espècies del gènere Alexandrium*, i han estat analitzades per les mateixes persones expertes per a la identificació del gènere* Alexandrium*. A més, de manera puntual quan ha estat convenient s'ha utilitzat dades d'anys anteriors que s'han considerat fiables i comparables o d'algun altre sistema de programa de vigilància on la determinació taxonòmica del gènere* Alexandrium *ha estat revisada.*

7. Taxonomia de dinoflagel·lades

Històricament les dinoflagel·lades s'han classificat per caràcters morfològics identificables en microscòpia òptica. L'arribada de la microscòpia electrònica va permetre l'observació d'altres detalls de la superfície de la cèl·lula i dels seus orgànuls. L'ús de les tècniques moleculars a partir dels 90 han ajudat sobretot a establir la diversitat d'espècies en aquells grups amb menys caràcters morfològics distingibles (com són els atecats, dins les dinoflagel·lades o els petits flagel·ladets), per poder distingir espècies críptiques i per poder establir la relació filogenètica entre els organismes. La majoria d'estudis moleculars en eucariotes utilitzen els gens del ADN ribosomal (ADNr) com a marcadors moleculars donat que són fragments relativament llargs que es troben en tots els eucariotes. Els gens ADNr dels eucariotes consten de 3 fragments de diferent mida: la 18S (la subunitat petita, SSU), 5.8S i 28S (la subunitat gran, LSU). Aquests fragments estan separats per 2 espaiadors transcrits interns (ITS1 i ITS2). Els diferents fragments tenen diferents taxes d'evolució. De manera que la SSU és emprada per establir alts nivells taxonòmics puix que és una regió altament conservada, mentre que la LSU, que és menys conservada, s'utilitza per estudis a nivell de gènere o espècie. La ITS, encara més variable, és utilitzada normalment per comparacions intraespecífiques.

La identificació morfològica de les dinoflagel·lades és diferent segons es tracti de dinoflagel·lades tecades o atecades.

La taxonomia de cèl·lules atecades ha estat sempre més complicada que la de les tecades, donat que les cèl·lules no posseeixen cap estructura rígida que la sustenti i la seva fixació produeix la deformació d'aquests fràgils organismes. A més la morfologia externa de la cèl·lula no presenta molts caràcters taxonòmics i menys encara visibles fàcilment mitjançant tècniques de microscopia òptica. Després de molts anys d'utilitzar criteris morfològics no fiables (com el desplaçament cingular) la classificació de les dinoflagel·lades atecades va patir un punt d'inflexió a partit del treball de Daugbjerg i coautors al 2000. En base a aquest treball que va combinar les observacions morfològiques, d'ultraestructura i filogenètica, la taxonomia morfològica actual basa la classificació dels seus gèneres principalment en la morfologia d'un solc que es dona a la part apical de l'epiconus (l'episoma de les atecades) anomenat acrobase o solc apical**.** Per exemple el gènere *Takayama* es defineix per tenir una acrobase en forma sigmoïdal i el gènere *Gymnodinium* per tenir una acrobase en forma de ferradura que gira en sentit contrari de les agulles del rellotge (**Fig. 4**). En els darrers anys nombrosos gèneres i espècies han estat erigits i altres han esta reclassificats. (veure Reñé , 2014 i les referències que conté).

Dins de cada gènere d'atecats la classificació de les espècies ve donada per diferents caràcters com la mida, la morfologia del contorn, la posició del nucli i el nombre i la posició dels cloroplastos entre d'altres.

La classificació de les tecades té en compte les plaques de la teca. Per a l'estudi de les plaques, Kofoid (1909) va crear un sistema de nomenclatura de plaques que es basa en la disposició latitudinal de diverses sèries de plaques (**Fig. 5**). Començant per l'àpex cap a l'antàpex, les plaques de les sèries s'anomenen: apicals (′), precingulars (″), singulars (c), postcingulars (‴) i antiapicals (⁗). Les plaques de cada sèrie latitudinals es numeren en direcció contrària a les agulles del rellotge, començant per la més ventral. Les plaques que puguin haver-hi situades entre les apicals i les precingulars s'anomenen intercalars anteriors i es denominen amb un número seguit de la lletra (a). Mentre les que puguin haver entre les postcingulars i les antiapicals s'anomenen intercalars inferiors i es denominen amb un número seguit de la lletra (p). Les plaques que conformen el sulcus s'anomenen sulcals. La placa més anterior serà la sulcal anterior (S. a.) i la més posterior la sulcal posterior (S. p.). A partir d'aquesta nomenclatura per a la

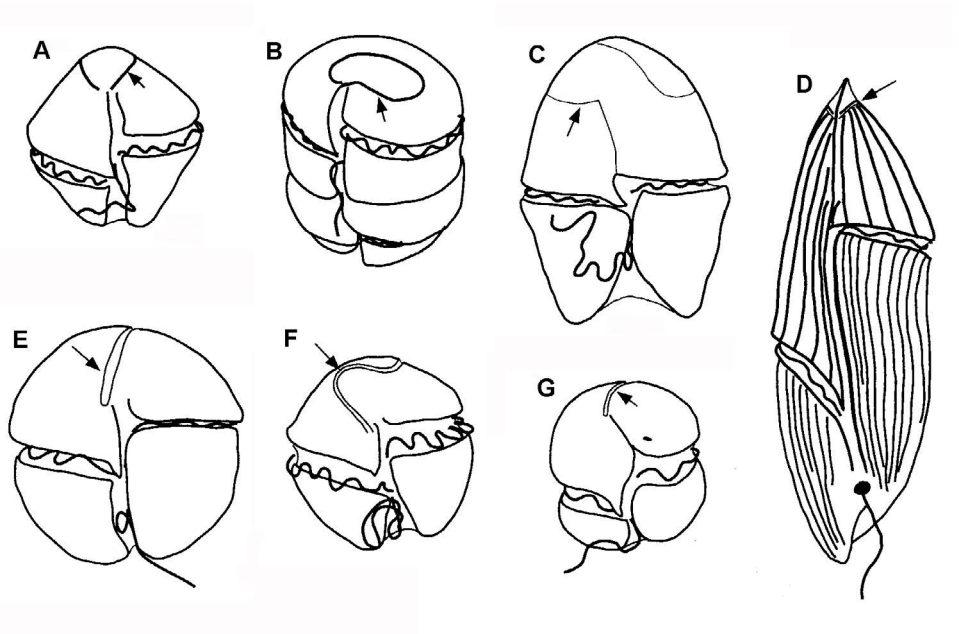


Figura 4. Morfologies d'acrobases diferents (fletxes) que identifiquen / defineixen diferents gèneres de dinoflagel·lades atacades. A) *Gymnodinium* B) *Polykrikos* C) *Akashiwo* D) *Gyrodinium* E) *Karenia* F) *Takayama* G) *Karlodinium*. Extret de Reñé, 2014 (modificat de Daugbjerg et al. 2000)

classificació dels gèneres de dinoflagel·lades tecades s'utilitza la fórmula tecal de Kofoid (1909). La fórmula es basa en anomenar la quantitat de plaques de cada sèrie de plaques i la presència o no dels diferents tipus de plaques de la teca.

Dins de cada gènere, la identificació de les diferents espècies es pot basar en característiques com: la mida i la morfologia del contorn cel·lular, la presència d'extensions, espines i reticulacions, la capacitat o no de formar cadenes, la morfologia de certes plaques i/o en petits detalls d'aquestes.

Per l'observació de les plaques i dels seus detalls en microscòpia òptica es necessària la tinció de la teca de cel·lulosa. Aquesta tinció s'acostuma a fer amb calcofluor (Fritz i Triemer, 1985), que tenyeix la cel·lulosa i permet veure les plaques sota llum ultraviolada.

La identificació taxonòmica ha estat una part important de la realització d'aquesta tesi.

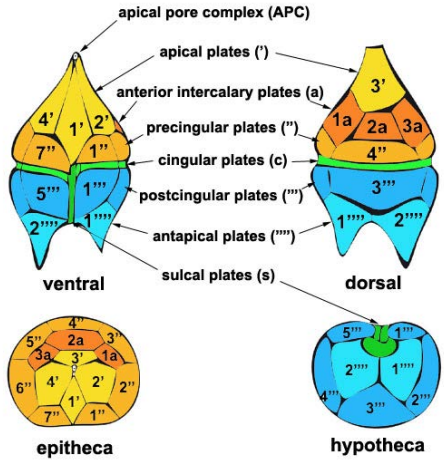


Figura 5. Nomenclatura de plaques. Extret de Hoppenrath and Saldarriaga (2008) ToLweb page.

Per tal de poder estudiar les espècies, les seves característiques, els seus patrons i les seves dinàmiques és necessària la seva identificació.

La identificació a nivell d'espècie o inclús de gènere però no és sempre possible, ja sigui per la limitació del mètode emprat que no permeti, per exemple, veure els caràcters taxonòmics; ja sigui perquè aquests caràcters siguin malmesos en el procés de fixació de la mostra o ja sigui perquè l' espècie a classificar no ha estat descrita prèviament.

*En el **capítol 4** es descriu un nou gènere de dinoflagel·lada atecada i la seva espècie tipus. Aquesta espècie era una de les espècies no identificades en el capítol 3 inclosa dintre dels "Gymnodinioids" que desenvolupava proliferacions d'elevada biomassa a la platja de la Fosca junt amb* Alexandrium taylori *causant discoloracions.*

8. Gènere *Alexandrium*

Alexandrium, en què es centra part d'aquesta tesi, és un gènere de dinoflagel·lada tecada planctònica que pertany a la família de les gonyaucals (ex. Saldarriaga et al., 2004). Es troba àmpliament distribuït arreu del món, en diferents ambients tant de regions subàrtiques, temperades i tropicals de ambdós hemisferis (Taylor

et al., 1995). Pot créixer tant en aigües relativament prístines com repletes de nutrients pel que és difícil generalitzar sobre un nínxol nutricional d'*Alexandrium* (Anderson et al., 2012). I és que les estratègies nutricionals d'aquest gènere són molt diverses: inclou des de l'ús de nutrients inorgànics (autotròfia), el consum de components orgànics (mixotròfia) com la urea, la guanina o la guanosina (Proctor et al., 1975), com la ingesta d'altres organismes (fagotròfia) com bacteris, flagel·lats, petites diatomees i inclús d'altres dinoflagel·lades (Jeong et al., 2010).

La taxonomia d'aquest gènere no ha estat mai fàcil, i en aquesta tesis s'ha fet un esforç especial per a la identificació de les seves espècies. El gènere *Alexandrium* consta actualment de 33 espècies moltes de les quals no van ser descrites en un primer moment com pertanyent a aquest gènere. Halim al 1960 va descriure el gènere *Alexandrium* amb *Alexandrium minutum* com a espècie tipus (Halim, 1960), però ja abans algunes espècies que ara pertanyen al gènere s'haurien descrit originalment dins d'altres gèneres, la majoria dins del gènere *Gonyaulax*. La causa d'aquest fet es deu a que el sistema de Kofoid pot ser interpretat per diferents autors de diferent manera i no té en compte la homologia de plaques. Així per exemple la 1', que en moltes espècies d' *Alexandrium* toca tant a la part apical com al cíngul, va ser interpretada per alguns autors com primera apical (1′) i per altres com primera precingular (1″) i per tant com a diferents gèneres. Balech i altres coautors al 1985 (Balech, 1985; Balech i Tangen, 1985) van transferir un grup d'espècies molt similars del gènere *Gonyaulax*, les anomenades "*Gonyaulax* del grup *tamarensis* o *catenella*" a *Alexandrium*. Balech alhora també va redefinir el gènere assignant-li la fórmula tecal Po, 4'(la 1'amb contacte o no amb Po), 6", 6C, 5"', 2"", 9-10S (Balech, 1985) que vol dir que presenta 4 plaques apicals, 6 precingulars, 6 cingulars, 5 postcingulars, 2 antiapicals i 9 o 10 sulcals (**Fig. 6**). Altres característiques del gènere serien el cíngol, generalment ben excavat i descendent, normalment la mateixa amplada del cíngol sense solapar-se. No presenten extensions ni espines i tenen el nucli transversalment elongat (Balech, 1995).

Per a la classificació de les espècies del gènere *Alexandrium*, a part de la connexió o no de la 1' amb la Po que separava el gènere en dos subgeneres, Balech li va donar molta importància a altres caràcters com són: la mida, la forma, la capacitat o no de formar cadenes, la presència de reticulació, la morfologia de certes plaques com la 1', 6'', S.p., S.a. i S.s.a. (sulcal anterior esquerra), la presència o no de porus ventral a la 1', la seva ubicació en la placa i la seva mida (**Fig.7**).

Amb l'arribada de la informació genètica, la majoria de les espècies descrites per Balech al 1995 han coincidit amb anàlisis filogenètiques i el gènere *Alexandrium* es considera monofilètic. Els dos subgèneres, però, no tenen consistència genètica i alguns complexes han trigat a ser resolts. El més estudiat ha estat el complexe d'espècies *A. tamarense*. Basant-se en les anàlisis genètiques de l'ADNr aquest complexe havia estat separat en 5 grups, que Lilly et al. 2007 van numerar del I al V. En la revisió realitzada posteriorment d'aquest complexe, John et al. (2014) van assignar un nom d'espècie a cadascun dels grups ja que els seus resultats eren consistents en que cada grup representava una espècie críptica. Un altre complex resolt recentment seria el complex d'espècies *A. ostenfeldii*. L'espècie *A. peruvianum*, inclosa en aquest complex, ha estat proposada com un sinònim heterotípic d' *A. ostenfeldii* per Kremp et al (2014) basant-se en anàlisis genètics, morfomètrics i dels perfils de toxines.

Des de la monografia de Balech (1995) altres espècies del gènere *Alexandrium* s'han anat descrivint: *A. camurascutulum* (MacKenzie et Todd, 2002), *A. gaarderae* i *A. globosum* (Nguyen-Ngoc et Larsen, 2004), *A. tamutum* (Montresor, 2004), *A.*

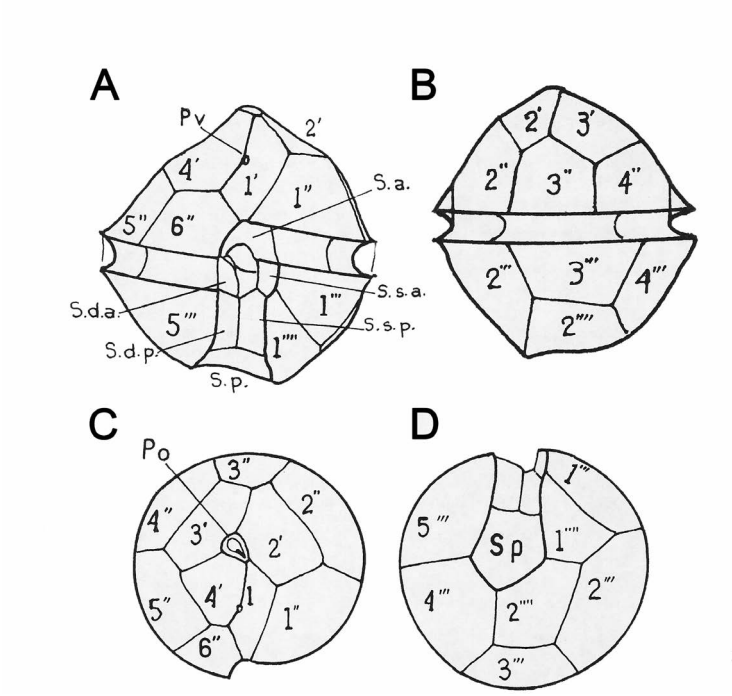


Figura 6. Plaques de la teca d'un *Alexandrium*. Vista ventral (1), vista dorsal (1B), vista apical, vista antiapical. Pv = porus ventral; Po = placa del porus apical. Adaptat de Balech 1995.

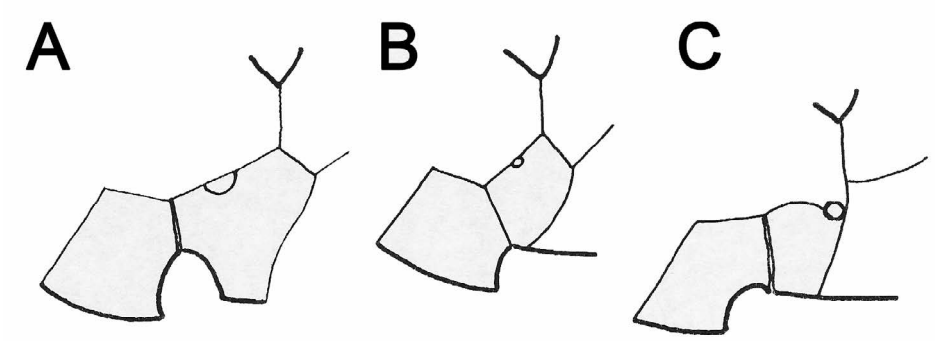


Figura 7. Diferents mides i posició del Pv en *A. pseudogonyalax* (A) *A. insuetum* (B) *A. margalefi* (C). Adaptat de Balech 1995.

pacificum, *A. australiensis* i *A. mediterraneus* (John et al., 2014), *A. diversaporum* (Murray et al., 2014) i *A. pohangense* (Lim et al., 2015). És per això pel que aquest gènere continua essent un desafiament taxonòmic.

A més de noves espècies, la caracterització genètica ens ha permès descobrir la variabilitat dins d'una mateixa espècie d'*Alexandrium* (Ex: Lilly et al., 2005; McCauley et al., 2009). L'anàlisi genètica basat en el ADNr de soques d' *A. minutum* d'arreu del món per exemple va permetre diferenciar dos grups diferents dintre la mateixa espècie (Lilly et al., 2005). Generalment però, són altres marcadors d'escala més fina com els microsatèl·lits els que han permès distingir poblacions dintre d'una espècie (ex. McCauley et al., 2009).

Alexandrium és un gènere emblemàtic molt estudiat arreu del món, la seva importància ve donada principalment perquè algunes espècies d'aquest gènere són productores de toxicitat PSP. De les 33 espècies actuals d'*Alexandrium*, unes 15 han estat considerades potencialment tòxiques. Només 10 d'aquestes es coneixen com a productores de PSP (Moestrup, 2009 en endavant), ja que altres han estat considerades nocives per altres motius com per produir mortalitat de peixos, substàncies antifúngiques o ser hemolítiques (**Taula 2**).

La toxicitat PSP és produïda per diferents toxines del grup de les saxitoxines (SXT) que actuen bloquejant els canals de sodi. La composició de toxines PSP típicament inclou diversos membres d'un o més dels següents subgrups: (1) toxines carbamoïl que inclou saxitoxines (STX), neosaxitoxines (NEO) i Gonyautoxines GTX (GXT1-4) (2) N-sulfocarbamoil (GTX5, GTX6, C1-C4) (Anderson et al., 2012).

Taula 2. Efecte nociu de les diferents espècies d*'Alexandrium*

Espècies tòxiques	Efecte danyí	Referència
A. acatenella	PSP	Prakash i Taylor 1966
A. andersonii	PSP	Ciminiello et al., 2000
A. australiensis	PSP	John et al., 2014
A. balechii	Mortalitat de peixos	Steidinger 1971
A. catenella	PSP	Burke et al., 1960
A. fundyense	PSP	Anderson et al., 1990
A. hiranoi	Antifúngic	Murakami et al., 1998
A. leei	Ictiotòxic	Tang et al., 2007
A. minutum	PSP i mortalitat de peixos	Hallegraef et al., 1988
A. monilatum	Ictiotòxic. Efecte hemolític	Howell 1953; Clemons et al. 1980
A. ostenfeldii	PSP i espiròlids	Cembella et al., 2000; Franco et al., 2006; Hansen et al., 1992
A. pacificum	PSP	John et al., 2014
A.pseudogonyaulax	Ictiotòxic	Triki et al., 2016
A. tamiyavanichii	PSP	Kodama et al., 1988
A. taylori	PSP i hemolític	Emura et al., 2004; Lim et al., 2005

La relació entre la presència de dinoflagel·lades gonyaulacals (ordre al que pertany el gènere *Alexandrium*) a l'aigua de mar i la presència de toxines PSP en mol·luscs filtradors es va començar a evidenciar cap als anys 30 (Sommer i Meyer, 1937; Sommer et al., 1937). No va ser però fins molts anys després que una d'aquestes neurotoxines, la saxitoxina (STX), es va aïllar d'una cloïssa groga (*Saxidomus giganteus*) d'Alaska i de musclos de California (*Mytilus californianus*) (Schantz et al., 1966). Amb l'objectiu de determinar si la toxina del musclos de Califòrnia provenia *d' Alexandrium catenella*, Burke et al. (1960) va estudiar les propietats físiques i químiques d'un extracte purificat de toxines extretes de cèl·lules d' *A. catenella*. Els resultats van indicar que els components eren idèntics, i que per tant la toxina trobada als musclos de Califòrnia tenien el seu origen en *A. catenella*. Va ser per tant *A. catenella* la primera espècie de dinoflagel·lada en ser relacionada amb la producció de toxines PSP.

Des d'aleshores s'ha anat demostrant la producció de toxines PSP per altres espècies del gènere *Alexandrium* (**Taula 2**), però la toxicitat d'algunes espècies encara no és ben coneguda. Les poques soques aïllades d'algunes espècies junt

amb la gran variabilitat intraespecífica que s'ha vist en el contingut cel·lular de toxines i els patrons de toxines d'espècies tòxiques (ex. Kremp et al., 2014), fa necessari l'estudi curós de la toxicitat d'un major nombre de soques.

Un cas que il·lustra la incertesa a l'hora d'assignar toxicitat a les espècies és el d'*A. andersonii*. Aquesta espècie, va ser descrita per Balech (1990) a partir de material recol·lectat a Cape Cod (NO Atlàntic). El mateix any, Anderson et al. (1990) van reportar la soca com a no tòxica. Uns anys més tard, al 1998 l'espècie va ser trobada per primera vegada a la Mediterrània (Golf de Nàpols) (Montresor et al., 1998). Un soca establerta a partir d'un cist va ser reportada com a tòxica per Ciminiello et al. (2000).

La detecció d'*A. andersonii* a la Costa Catalana per primera vegada durant el període d'estudi d'aquesta tesi, i el fet que la única referència de toxicitat es basava en una única soca aïllada a la Mediterrània (Ciminiello, et al., 2000; Montresor et al., 1998), ens van fer preguntar quines repercussions podria tenir la seva presència a les nostres costes.

*En el **capítol 5** d'aquesta tesi s'ha estudiat la toxicitat i la variabilitat intraespecífica d'*Alexandrium andersonii *amb diferents metodologies per tal de clarificar la seva toxicitat a la Mediterrània i en general tenir un major coneixement sobre la seva fisiologia i d'altres característiques d'aquesta espècie tan poc coneguda.*

9. Àrea d'estudi

9.1. La zona costanera Mediterrània

La Mediterrània és un mar semi-tancat i relativament petit (2.5 X 10^6 Km^2) sense marees importants (màxima amplitud d'uns 20 cm a l'Adriàtic) amb entrada d'aigües Atlàntiques superficials menys salades i amb menys nutrients per l'estret de Gibraltar, i amb un insignificant intercanvi d'aigües amb el Mar Negre per l'orient. El Mediterrani actua com una conca de concentració puix que l'entrada d'aigües fluvials i de pluja no compensen la seva pèrdua per evaporació.

En contra de l'opinió generalitzada de fa tant sols unes dècades, el Mediterrani és un lloc d'alt risc per a les PANs (Garcés i Camp, 2012). Les aigües de la plataforma continental són marcadament diferents a les aigües de les zones oceàniques considerades oligotròfiques. L'entrada d'aigua continental menys salada i carregada de nutrients a traves de rius, rieres, estacions de depuració d'aigües residuals i sobreeixidors confereix a aquesta zona més costanera aigües més dessalades i amb més nutrients. De manera que, l'entrada de nutrients no es limita a l'hivern, quan es produeixen els fenòmens de barreja, i de manera més gradual a la tardor, quan s'enfonsa la termoclina. Això comporta que els nutrients no siguin el factor limitant de la producció primària en certes èpoques com a l'estiu, com succeeix mar endins, i per tant les poblacions fitoplanctòniques no segueixen el típic cicle que es dóna a la Mediterrània. Aquestes propietats de les aigües de la plataforma s'aguditzen com més a prop de la línia de costa i encara es modifiquen més quan les aigües es troben incloses dins una estructura com són les estructures portuàries o les badies. La forta càrrega de nutrients afecta a tots els processos que es donen a la costa, tant és així que aquestes aportacions de nutrients des del continent han provocat per exemple problemes de depleció d'oxigen en la zona Nord de l'Adriàtic (Justić, 1991) produïts per l'increment de biomassa algal. L'augment d'aigües confinades a la zona costera és també, com l'eutrofització d'aquestes zones, una conseqüència de l'antropització del litoral. L'important transformació de la línia de costa modificant platges, construint passeigs costaners, espigons i ports ha fet augmentar els espais costaners amb aigües confinades o semi-confinades, on l'eutrofització és encara major i les característiques físiques es particularitzen.

Les àrees d'estudi d'aquesta tesi es situen a la costa Mediterrània NO. La major part de la tesi es centra a la costa catalana (NE d'Espanya), que s'estén al llarg d'aproximadament 580 km des del sud de França (3º19'59.94 "E, 42º29'0.09" N) al sud del Delta de l'Ebre (0º 9'41.69 "E, 40 ° 31'27.56" N), mentre que en el ***capítol 2*** *una de les àrees d'estudi es localitza a la costa Mediterrània francesa. Les àrees d'estudi de la tesi corresponen a diferents ambients costaners.*

10. Ambients estudiats a la tesi

10.1. Ambients costaners tancats, els ports, badies i les llacunes.

*Aquests ambients conformen les àrees d'estudi dels primers dos capítols de la tesi (**Capítol 1 i 2**).*

Un port és una massa d'aigua semi-tancada de poca fondària que rep influència tant marina com fluvial (si es troba situat a prop d'un riu) per la bocana, com continental per mitjà d'aigua d'escorrentia pluvial o residual que pugui anar a desembocar-hi. El confinament i l'eutrofització, es veuen més acusats degut al reduït contacte amb el mar a través de bocana. Aquest reduït contacte, provoca també que la renovació de l'aigua sigui menor que en platges o badies i per tant que la dilució dels nutrients i els organismes sigui més lenta. Per bé que les aigües portuàries difereixen de les de mar obert, també difereixen entre elles depenent entre d'altres de l'estructura i mida portuària, les aportacions d'aigua continental que li arribin, les activitats que hi realitzi l'home, l'època de l'any, la meteorologia i el microclima concret de la zona, la biologia de les espècies que es troben en aquest port i la de les espècies que hi arriben (per mitjà d'aigües de llast, plàstics, aigües continentals etc..). A Catalunya, el número de ports ha incrementat de 12 al 1950 a 48 al 2015. En la tesi com a mostra d'aquest ambient un total de 16 ports formen part de les àrees d'estudi (**Capítol 1 i 2**).

Una altre tipus de massa d'aigua semi-tancada que es pot trobar a la costa catalana són les badies. Les badies del Fangar i d'Alfacs estan ubicades a la zona Nord i Sud del delta de l'Ebre respectivament (Sud de Catalunya) i són les majors àrees de cultiu i recol·lecció de marisc de tot el litoral català. Les badies són més grans que els ports, són naturals i tenen un major contacte amb el mar. En aquesta tesi s'han realitzat alguns mostrejos a la badia dels Alfacs (**Capítol 1**).

Les llacunes d'aigua de mar són també masses d'aigua de poca fondària amb poc contacte amb el mar. En aquesta tesi s'ha utilitzat dades provinents de la Llacuna de Thau (França) per la presència recurrent de proliferacions d'*Alexadrium pacificum* (**Capítol 2**). Aquesta llacuna es troba al NO de la Mediterrània, a uns 120 km de la Costa Catalana pel que comparteix el seu mateix marc Mediterrani coster.

10.2. Ambients costaners oberts o semi-oberts: Platges

Les platges, a diferencia dels ports i les badies, presenten masses d'aigua obertes o semi-obertes amb una alta renovació de l'aigua. La construcció d'espigons, però, en algunes platges també fa augmentar el confinament d'aquestes aigües, a més d'afectar, de la mateixa manera que els ports, al transport de sorres. Donada la heterogeneïtat de la costa catalana, les platges que hi trobem són molt diverses, hi ha platges naturals i artificials, de sorra fina o de còdols, llargues obertes o petites i tancades, abruptes o baixes, algunes més influenciades per rius i d'altres amb poca influencia d'aigua dolça..etc. Al llarg del litoral català hi ha un total de 566 platges de les quals una part important (258 platges) s 'ha mostrejat alguna vegada dins el marc del pla de vigilància de fitoplàncton tòxic de la costa catalana i conformen les àrees d'estudi del **capítol 3**.

Figura 8. Diferents ambients costaners. Fotografies de Jordi Camp.

Així doncs tot i el petit tram de costa que representa la costa catalana en relació a la costa Mediterrània, la quantitat d'ambients costaners, tant tancats, semi-tancats o oberts, diferents que hi ha a la costa catalana dóna lloc a multitud d'hàbitats litorals cadascun amb les seves característiques i la seva dinàmica particular. En aquesta tesis s'ha treballat en tots aquests ambients en els **capítols 1, 2 i 3** el que ens permet comparar el que succeeix en els diferents ambients en relació a la dinàmica poblacional de les dinoflagel·lades nocives i amb especial atenció a la dinàmica de diferents espècies del gènere *Alexandrium*.

11. Coneixements previs de fitoplàncton a la costa catalana

El fitoplàncton del Mar Català ha estat tradicionalment (a partir dels anys 40) estudiat en campanyes oceanogràfiques, tant a nivell de tota la comunitat (ex. Estrada, 1979; Estrada, 1980; Margalef, 1948; Margalef, 1957; Margalef, 1965; Margalef i Estrada 1987) com de grups concrets d'organismes fitoplanctònics (ex. Cros, 2001). A partir dels 80, van començar a realitzar-se també estudis més costaners, com els duts a terme a les badies del Delta de l'Ebre per Delgado (1986). Però va ser a partir de la troballa de la primera proliferació fitoplanctònica tòxica a la costa catalana (al port de Sant Carles de la Ràpita) (Delgado et al., 1990), que es va començar a posar èmfasi en l'estudi de les espècies potencialment tòxiques i nocives en les aigües més costaneres. Primerament en algunes àrees concretes de la costa catalana (ex: Garcés et al., 1999a; Garcés et al., 1999b) i més tard Vila (Vila et al., 2001a; Vila et al., 2001b) amb dades recollides durant quatre anys va donar una visió de la diversitat i abundància de les espècies tòxiques i nocives de les zones confinades (ports) al llarg de la costa catalana i d'algunes platges puntuals. Durant els darrers anys s'han realitzat estudis sobre grups o gèneres potencialment tòxics o nocius com el gènere *Ostreopsis* (ex. Vila et al., 2016; Vila et al., 2008) el gènere *Pseudo-nitschia* (Andree et al., 2011; Quijano-Scheggia, 2008), el gènere *Karlodinium* (Garcés et al., 2006), les Gymnodinials (Reñé, 2014). Alguns aspecte del gènere *Alexandrium* han estat també estudiats de manera paral·lela al transcurs d'aquesta tesi com la distribució (Anglès et al., 2010; Bravo et al., 2008; Garcés et al., 2004), diversitat (Bravo et al., 2006) i paper dels cistos (Anglès et al., 2012; Estrada et al., 2010; Garcés et al., 2004), el paper de les variables meteorològiques en els blooms de *A. minutum* (Estrada et al., 2008; Van Lenning et al., 2007) i les condicions fisico-químiques que es donen en les proliferacions d'algunes espècies d'*Alexandrium* (Bravo et al., 2008; Vila et al., 2005).

Objectius

El principal objectiu d'aquesta tesi ha estat ampliar el coneixement local i global de les dinoflagel·lades potencialment nocives al NO de la Mediterrània i amb especial atenció sobre el gènere *Alexandrium*, pel que fa a la seva presència i abundància, característiques de les espècies i de les seves proliferacions, patrons de distribució espai-temporals, dinàmica poblacional, tendències i risc associat en aigües costaneres del NO de la Mediterrània.

D'acord amb les principals línies de recerca exposades anteriorment, els objectius específics serien:

1. Identificació, quantificació i distribució espai-temporal de les espècies de dinoflagel·lades potencialment nocives.

   Identificar quines espècies de dinoflagel·lades potencialment nocives trobem a les platges de la costa catalana (**Capítols 3** i **4**).

   Identificar quines espècies del gènere *Alexandrium* són presents i en quines quantitats tant en zones costeres confinades com en zones costeres obertes. (**Capítols 1** i **3**).

   Determinar la distribució espai-temporal de les espècies trobades **(Capítols 1 i 3)**.

2. Descripció de la dinàmica poblacional i factors que determinen la seva dinàmica.

   Avaluar la recurrència de les proliferacions d'algunes espècies d'*Alexandrium* problemàtiques i investigar els factors que les provoquen (**Capítols 1, 2, i 3**).

   Analitzar les tendències d'algunes espècies d'*Alexandrium* problemàtiques per respondre a la pregunta si estan en augment a la zona costanera i investigar si aquest augment respon a factors antropogènics (**Capítols 1, 2 i 3**).

3. Avaluació de riscos associats.

   Determinar la presència o absència de toxicitat en algunes espècies en les que no es coneix o es posa en dubte i que es troben presents a la costa catalana (**Capítols 4 i 5**).

4. Caracterització d'algunes espècies poc conegudes.

   Caracteritzar la morfologia externa la ultraestructura, la composició de pigments i d'àcids grassos, i determinar la posició filogenètica dintre de les dinoflagel·lades de la nova espècie descrita en aquesta tesi *Barrufeta bravensis* (**Capítol 4)**.

   Caracteritzar la variabilitat intraespecífica d'*Alexandrium andersonii* quant la seva morfologia, genètica, composició de pigments i taxes de creixement (**Capítol 5)**.

La recerca desenvolupada durant aquesta tesi per aconseguir aquests objectius es presenta en cinc capítols. Seguit d'aquests articles es discuteixen tots els resultats en una **Discussió general** i s'extreuen les conclusions en l'apartat de **Conclusions generals**.

Informe del director de tesi

El Dr. Jordi Camp i Sancho, director de la tesi titulada: "**Dinoflagel·lades potencialment nocives al NO de la Mediterrània amb especial atenció al gènere *Alexandrium***"- "**Potentially harmful dinoflagellates in the NW Mediterranean coast, with a focus on the *Alexandrium* genus**" realitzada per Nagore Sampedro Roig,

Informa de la implicació de doctoranda en cada article científic desenvolupat per la present tesi i que cap dels articles, ni les dades aquí presentades, han estat usades per a la memòria d'una altra tesi. I també s'informa del Factor d'Impacte dels treballs ja publicats.

ARTICLE 1: "Diversity and distribution of the dinoflagellate genus Alexandrium (Halim) in confined waters of the Catalan coast (NW Mediterranean Sea)". Autors: Nagore Sampedro, Sílvia Anglès, Laura Arin, Magda Vila, Esther Garcés, Albert Reñé, and Jordi Camp.

Article no publicat, la versió que es presenta a la memòria de la tesi és la versió revisada i consensuada pels diversos coautors que haurà de ser reduïda per enviar a una revista especialitzada. El disseny del mostreig correspon al que es va considerar adequat per la vigilància de fitoplàncton tòxic de la costa catalana. La doctoranda va intervenir en disseny del mostreig del pla de vigilància i en el recompte de les mostres, en el tractament de dades, l'anàlisi i la interpretació dels resultats i va redactar l'article amb l'assessorament dels coautors.

ARTICLE 2: "*Alexandrium pacificum* bloom dynamics over 11-years in two anthropic Mediterranean coastal environaments" Autors: Nagore Sampedro, Magda Vila, Silvia Anglès, Jordi Solé, Eric Abadie, Yves Collos, Jordi Camp, Esther Garcés.

Article no publicat, la versió que es presenta a la memòria de la tesi és la versió revisada i consensuada pels diversos coautors i en preparació per enviar a una revista especialitzada. El disseny del mostreig correspon al que es va considerar adequat per la vigilància de fitoplàncton tòxic de la costa catalana en el cas de Tarragona i de la Badia de Thau. La doctoranda va intervenir en el disseny de mostreig del pla de vigilància de la costa catalana i el recompte de les mostres de Tarragona a més va reexaminar les mostres de Thau per tal de comptabilitzat el gènere *Alexandrium* a nivell d'espècie, va realitzar el tractament de dades, la major part de l'anàlisi, la interpretació dels resultats i va redactar l'article amb l'assessorament dels coautors.

ARTICLE 3: The spatio-temporal distribution of the potentially harmful dinoflagellates at Catalan beaches (NW Mediterranean) Autors: Nagore Sampedro, Albert Reñé, Sílvia Anglés, Laura Arin, Magda Vila, Esther Garcés, Jordi Camp.

Article no publicat, la versió que es presenta a la memòria de la tesi és la versió revisada i consensuada pels diversos coautors i en preparació per enviar a una revista especialitzada. El disseny del mostreig correspon al que es va considerar adequat per la vigilància de fitoplàncton tòxic de la costa catalana. La doctoranda va intervenir en el disseny de mostreig i el recompte de les mostres, el tractament de dades, l'anàlisi i la interpretació dels resultats i va redactar l'article amb l'assessorament dels coautors.

ARTICLE 4: "*Barrufeta bravensis* gen. Nov. Sp. Nov. (dinophyceae): a new Bloom-forming species from the northwest Mediterranean sea". Autors: Nagore Sampedro, Santiago Fraga, Antonella Penna, Silvia Casabianca, Manuel Zapata, Claudio Fuentes Grünewald, Pilar Riobó, Jordi Camp.

Article publicat a la revista "Journal of Phycology", amb índex d'impacte de 2.071 a l'any 2011 i situada al Q2 quartil (categoria "Marine & freshwater biology"). La doctoranda va estudiar els cultius amb diferents tipus de microscòpia LM, SEM, TEM i analitzar les seves imatges. Va cultivar la soca i preparar les mostres per a les anàlisis de pigments, toxines, lípids i genètica, va interpretar els resultats i va redactar l'article amb l'assessorament dels coautors.

ARTICLE 5: The toxicity and intraspecific variability of *Alexandrium andersonii* Balech Autors: Nagore Sampedro; José M. Franco; Manuel Zapata; Pilar Riobó; Esther Garcés; Antonella Penna; Amandine Cailleaud, Jorge Diogène; Emiliano Cacho; Jordi Camp.

Article publicat a la revista "Harmful Algae", amb índex d'impacte de 3.339 a l'any 2013 i situada al Q1 quartil (categoria "Marine & freshwater biology"). La doctoranda va examinar morfològicament les diferents soques per LM i va realitzar les corbes de creixement. Va cultivar les diferents soques i preparar les mostres per a les anàlisis de pigments, toxines, i genètica, va interpretar els resultats i va redactar l'article amb l'assessorament dels coautors.

Dr. Jordi Camp i Sancho
Director

Chapter 1

Diversity and distribution of the dinoflagellate genus *Alexandrium* (Halim) in confined waters of the Catalan coast (NW Mediterranean Sea)

Scientia Marina, in preparation

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Abstract

The diversity and spatio-temporal distribution of dinoflagellates belonging to the *Alexandrium* (Halim) genus were studied using traditional microscopy techniques during a 13-year period (2000–2012). The investigation was carried out at 16 confined coastal stations along the Catalan coast (NW Mediterranean Sea) and included their physico-chemical conditions. The 11 *Alexandrium* species detected in the NW Mediterranean Sea (*Alexandrium andersonii, A. insuetum, A. cf. leei, A. margalefi, A. minutum, A. ostenfeldii, A. pacificum, A. pseudogonyaulax, A. tamarense, A. tamutum*, and *A. taylori*) represent one-third of those described worldwide thus far. Most of these species produced blooms (abundances ≥ $1X10^4$ cells L^{-1}) in the enclosed environments of the study area. According to the spatio-temporal distributions observed, *Alexandrium* species in confined waters can be separated in i) settled species in a high number of different harbors (e.g., *A. minutum*) ii) settled species in few or one specific harbors (e.g., *A. pacificum* and *A. taylori)* iii) occasional species that appeared synchronized along much of the coast (e.g., *A. insuetum* or *A. margalefi*) iv) occasional species that appeared in a particular place (e.g., *A. pseudogonyaulax* or *A. tamutum*). While the settled species produces recurrent annual blooms, the occasional species appear irregularly at high or low abundances. Diverse temporal trends were observed among annually recurring species. The most frequent and widely distributed species, *A. minutum*, maintained or increased its presence over time. Sampling sites with increasing trends of *A. minutum* abundances showed a reduction of inputs of nitrate-loaded freshwater over the same period. By contrast, *A. pacificum*, which expanded its geographical distribution in the late 1990s, was, at the end of this study, detected in only one sampling site. The results show that toxin-producing species along the Catalan coast exhibit independent responses to the same climatic variability. Accordingly, the prediction of PSP bloom events in the NW Mediterranean Sea and other susceptible areas must consider not only local as well as more wide-ranging environmental changes but also the individual responses of PSP-causing species.

1. Introduction

The dinoflagellate genus *Alexandrium* has been extensively studied worldwide because of its harmful effects. Approximately 25% of *Alexandrium* species produce saxitoxins or their derivatives, all of which cause paralytic shellfish poisoning (PSP). PSP results in considerable morbidity, such as numbness of the mouth and limbs, loss of motor control, respiratory distress, and paralysis, and even death in humans following the consumption of contaminated shellfish (Hallegraeff, 1993). These species also exert negative effects on marine organisms through the food web (e.g., Geraci et al., 1989). Other toxins produced by some *Alexandrium* species include spirolides and gymnodimines (Cembella et al., 2000; Salgado et al., 2015; Van Wagoner et al., 2011). However, the formation of high-biomass blooms by non-PSP producer species has also caused fish mortality (Howell, 1953; Steidinger, 1971) and, due to the discoloration of coastal waters, economic losses in the tourism sector (Garcés et al., 1999; Satta et al., 2010). The number of harmful algal blooms (HABs), including those produced by *Alexandrium* species, has increased worldwide during recent decades (Hallegraeff, 1993). While this can in part be explained by increased scientific awareness and improved detection techniques (Anderson et al., 1989), factors favoring the proliferation and dispersion of *Alexandrium* species must be considered as well (Smayda, 2007). For example, global and local environmental changes, such as the eutrophication of coastal waters and global warming, could promote their proliferation. One explanation scarcely explored is the increase in confined waters, e.g., in the form of harbors, in many places around the world, including the Mediterranean Sea (Garcés and Camp, 2012; Vila et al., 2001a).

According to Margalef's "mandala of phytoplankton," red tide dinoflagellates, including the genus *Alexandrium*, are favored by high water column stability and nutrient availability (Margalef, 1978). These optimal conditions for proliferation are found along the coastal shorelines of bays and in man-made areas such as harbors, where confinement and eutrophication are more pronounced due to reduced exchange of the enclosed with open waters. The low rate of water renewal leads to a low dilution of nutrients and microorganisms in these enclosed areas. In addition to their differences with the open sea,

confined waters differ among themselves with respect to their size, structure, the contribution of continental waters, human activities (e.g., ship traffic), seasonality, meteorology, and microclimate. Harbors are relatively new environments; along the Catalan coast (NW Mediterranean Sea, NE Spain) most were built in the second half of the twentieth century and the number has increased during the current one. Thus, while there were 12 harbors along the Catalan coast in 1950, as of 2015 there were 48.

Early studies in coastal as well as open waters reported the occasional presence of *Alexandrium* species in very low abundances along the Catalan coast [Margalef and Estrada, 1987 (as *Gonyaulax catenell*a)]. But in 1989, the first toxic PSP outbreak (caused by *A. minutum*) was detected in Sant Carles de la Ràpita Harbor, located inside Alfacs Bay (Delgado et al., 1990). Since then, regular monitoring of the Catalan coast (Vila et al., 2001a) has identified other *Alexandrium*-related toxic events in the confined coastal waters of this region. Vila et al. (2001a) demonstrated the utility of a systematic sampling program for the early detection of HA species in Catalan coastal harbors. During monitoring carried out from 1995 to 1999, three *Alexandrium* species, (*A. pacificum* (formerly *A. catenella*), *A. minutum*, and *A. taylori*, were regularly detected at high abundances in the planktonic form, while one species (*A. tamarense*) occurred only rarely and at low abundance. The presence of *A. pacificum* in an increasing number of Catalan coastal stations during those 5 years suggested the expansion of this species in the NW Mediterranean Sea (Vila et al., 2001b). Parallel monitoring efforts at the Ebre Delta detected the *Alexandrium* species *A. pseudogonyaulax* (Delgado et al., 1999).

Additional parallel studies on the diversity of dinoflagellate cysts in sediments collected from harbors and other confined waters along the Catalan coast identified the presence of several other *Alexandrium* species in these environments: *A. kutnerae, A. ostendeldii* (as *A. peruvianum*), *A. margalefi, A. pseudogonyaulax*, and *A. tamarense* (Bravo et al., 2006; Satta et al., 2013). Both planktonic and benthonic studies provided the background of this genus in this geographical area.

Alexandrium species are difficult to distinguish morphologically by conventional light microscopy and require special staining techniques for this purpose. Correct species identification relies on the recognition of small details in the thecal plates of these dinoflagellates: the presence or absence of the ventral

pore, the size and shape of the plates, and the connection between the pore plate and first apical plate (Balech, 1995). These details can be observed by staining the cells with Calcofluor and observing the fluorescence under epifluorescence microscopy. In addition, because species co-occur in nature, the identification and quantification of a target species are often challenging. All of this has led to non-species-specific identifications and a lack of reliable long-term field studies on the presence and abundance of *Alexandrium* species worldwide. As knowledge of the distribution of a species over time is the basis for recognizing its spatial and temporal expansion as well as the factors influencing its abundance and persistence, unequivocal identification is needed.

The present study examined the diversity and spatio-temporal distribution of *Alexandrium* species in the confined waters of the Catalan coast over a

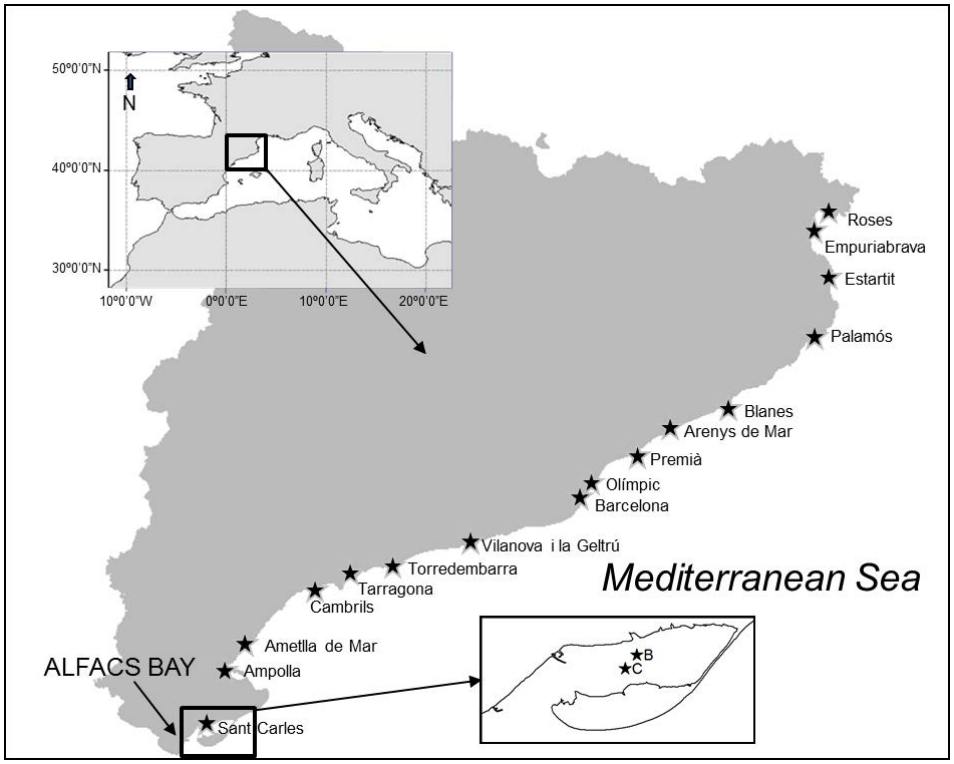

Figure 1. Study area showing the sampling locations along the Catalan coast.

13-year period, from 2000 to 2012. *Alexandrium* species were systematically identified by epifluorescence microscopy of Calcofluor-stained samples. Molecular analyses corroborating our morphological identification of most of the species has been published in other works (e.g. Penna et al., 2008; Sampedro et al., 2013). Using the time-series data produced, the following topics were addressed in this long-term investigation: 1) the identification of the *Alexandrium* species present in Catalan confined waters and a determination of their spatial and temporal patterns along the coast; 2) the expansion of potential PSP producer species such as *A. minutum* and *A. pacificum*, and the implications with respect to bloom frequency; 3) the identification of the annual and long-term patterns of *A. minutum*, the most frequently occurring *Alexandrium* species in Catalan confined waters; 4) investigation of the environmental conditions linked to the long-term abundances of *A. minutum* in confined and eutrophic environments; and 5) assessment of the risk posed by the genus *Alexandrium*, both for public health and the coastal ecosystem.

2. Materials and methods

2.1. Study area

The Catalan coast is located in the NW Mediterranean Sea (NE Spain). It extends along approximately 580 km, from the south of France to south of the Ebre Delta, following a NE–SW orientation (**Fig. 1**). The Mediterranean Sea is microtidal, with a maximum range of 0.2 m at the Catalan coast. The most important current is the Catalan current, which occurs close to the coast of the Catalan-Balearic Sea and is a continuation of the southwards-moving (roughly NE to SW) Ligurian-Provençal current (Font et al., 1988).

The offshore waters of the Mediterranean Sea are oligotrophic. The annual maximum inorganic nutrient concentration in the upper layer occurs in winter, due to intense vertical mixing. However, inshore waters are very different from the water in the open ocean because they are subject to varying inputs of continental waters with low salinity and high nutrient concentrations. The irregular entry of continental water affecting coastal waters depends greatly on the Mediterranean climate of the region. The Catalan coast has a

Mediterranean climate characterized by mild winters, hot and dry summers, and irregular precipitation (total annual precipitation in Catalonia: 400–1200 mm). Rivers and streams contribute markedly less water in summer and many of them remain dry during the respective months. Autumn is the rainiest season and torrential rains are not unusual.

Sampling stations were located in 16 harbors along the Catalan coast (**Fig. 1**), including the two largest harbors (Barcelona and Tarragona) and Alfacs Bay, the major shellfish cultivation and harvesting area along the coast. Alfacs Bay has a surface area of 49 km², an average depth of ~3 m, and a mouth that is 2 km wide. Inputs of freshwater from irrigation canals providing water to rice-paddies in the Ebre Delta enter the bay from April to December (Serra et al., 2007) and unload considerable amounts of organic and inorganic nutrient into the bay. As a stratified type B estuary (Pritchard, 1955), Alfacs Bay has a salinity-dominated stratification, with an outwardly moving superficial layer (0 to 2–3 m depth) of low salinity (30–35 psu) and an inwardly moving deep salty layer (36–38 psu). Complete mixing of the two layers occurs during strong wind events.

2.2. Samplings at harbor stations

The 16 harbor stations were sampled one to four times per month from March to September and once or twice per month for the rest of the year depending on the station and the year (**Table 1**). Nine stations were sampled from 2000 to 2012 and the remaining seven stations from 2000 to 2005. Sampling was carried out in the most confined area of each harbor. Sampling points have been maintained during the studied period except in Barcelona Harbor where it was moved 200 m away from the original site in September 2003. Temperature and salinity were measured directly using a microprocessor conductivity meter (WTW model LF197). Surface water samples were collected for phytoplankton enumeration, Chlorophyll-*a* determination, and inorganic nutrient analysis. From each sample, a volume of 150 mL was preserved with Lugol's iodine solution for microscopic analysis; an additional 60 mL were filtered on Whatman GF/F glass-fiber filters and frozen at –20°C for chlorophyll analysis, and another 60 mL were frozen immediately (–20°C) for nutrient analyses.

2.3. Samplings in Alfacs Bay

Alfacs Bay (Ebre Delta, southern Catalonia) is an aquaculture site and has been monitored weekly for the presence of harmful phytoplankton by the Institut de Recerca Tecnologia Agroalimentaries (IRTA) since 1989 in several locations (see Furones et al., 2004). From May to July of 2003 and from January to April of 2007, samples from this monitoring program were kindly provided by the IRTA staff. Samples from Alfacs central station (C) (**Fig. 1**) were quantified and examined under epifluorescence microscopy at the Institut de Ciències del Mar (ICM) to characterize outbreaks of *A. pseudogonyaulax* and *A. andersonii*, respectively. Water samples were collected at the surface and bottom and from the entire water column (integrated sample) at the central station and were fixed with formalin. Additional samplings were carried out during the *A. pseudogonyaulax* bloom event to determine its vertical distribution. Figure 1 shows station B, where the vertical prolife was determined on June 16, 2003.

2.4. Chl-*a* and nutrient analyses

Chl-*a* was extracted in 8 mL of 90% acetone for 48 h at 4°C and its concentration was measured with a Turner Designs fluorometer (Yentsh and Menzel, 1963). Dissolved inorganic nutrient (including NO_3, NO_2, NH_4, PO_4, and SiO_4) concentrations were analyzed as described by Grasshoff et al. (1983), using an Alliance Instruments Evolution II autoanalyzer (samples collected from 2000 to 2007) or an analyzer AA3 (Bran+Luebbe; samples collected from 2007 to 2012). Stoichiometric nutrient limitations were calculated as described in Justic et al. (1995). Potential limitations were determined for P ($P < 0.1$ µM; DIN: $PO_4 > 22$; Si: $PO_4 > 22$), N (DIN < 1 µM; DIN: $PO_4 < 10$; Si: DIN >1), and Si (Si < 2 µM; Si: $PO_4 < 10$; Si: DIN < 1).

2.5. Phytoplankton enumeration by light and epifluorescence microscopy

From the fixed sample, a 50-mL aliquot was settled during the same week of its collection for 1 day in a counting chamber and following the Utermöhl method (Utermöhl, 1958). For phytoplankton enumeration, half of the chamber was scanned at 200× using a light microscope. The minimum concentration detected by this method was 40 cells L^{-1}. The appropriate area of the chamber

Table 1. Sampling frequency in the different harbors during the study period.

Station	Harbor	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
1	Roses	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY							
2	Empuriabrava	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY							
3	Estartit	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	BWS-MY
4	Palamós	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY
5	Blanes	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY							
6	Arenys de Mar	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	BWS-MY
7	Premià	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY							
8	Olimpic	BWY	BWY	BWY	BWY	BWY	BWY	BWY	BWY	BWY	BWY	BWY	BWY	MY
9	Barcelona	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	BWS-MY
10	Vilanova	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	BWS-MY
11	Torredemb.	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY							
12	Tarragona	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	BWS-MY
13	Cambrils	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY
14	L'Ametlla	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	WS-MY	BWS-MY
15	L'Ampolla	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY							
16	St. Carles	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY	WS-BWY							

BWS-MY Biweekly in summer months, and monthly the rest of the year

WS-BWY Weekly in summer months, and biweekly the rest of the year

WS-MY Weekly in summer months, and monthly the rest of the year

MY Monthly all year

BWY Biweekly all year

was then scanned at 200–400× magnification, depending on the cell density of each species, using a Leica-Leitz DM-II inverted microscope (Throndsen, 1995) and counting a minimum of 30 cells for each species. After enumeration, when *Alexandrium* cells were detected in the sedimentation chamber the thecal plates were stained with the fluorescent dye Calcofluor white M2R (final concentration 10–20 mg mL^{-1}; Fritz and Triemer, 1985). The chambers were then examined at 400× magnification under bright-field and epifluorescence (lamp 50W) microscopy using a Leica DM IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted microscope connected to a ProgRes C10 digital camera. *Alexandrium* species were confirmed by the tabulation of their thecal plates, based on Balech's criteria (1995) together with more recent descriptions (Montresor et al., 2004). If the number of cells or their position (e.g., dorsal) did not allow taxonomic characterization, they were examined individually by turning them with a needle to provide further details aiding in species determination. Cells that could not be unequivocally identified at the species level were identified at the genus level as *Alexandrium* spp. Special case are the species comprising the *A. tamarense* complex that were recently re-described by John et al. (2014). The morphologically based determination of the species of this complex is presented in Chapter 2 of this thesis.

2.6. Statistical analyses and graphs

The spatio-temporal distributions of the species and physical variables were plotted using Surfer 8 (Golden Software Inc., Golden, USA). Both the abundances of the most frequent and abundant species and the physical variables were estimated using the kriging interpolation method. Box plots were used to compare sampling sites. To partly avoid the bias arising from the different monthly sampling frequencies in the different harbors, the boxes were calculated with fortnightly averages of all sampled years. However, because some harbors were sampled less often than others (6 instead of 13 years) not all harbors were fully comparable. In the figure, these less frequently sampled harbors are indicated with an asterisk.

For some graphical representations and for statistical analyses, the cell abundances of *Alexandrium* species were log-transformed according to $v' = \log_{10}(v + 1)$.

The phytoplankton time series used in this study covered the period 2000–2012. Moreover, when data from 1996–1999 (Vila et al., 2001a) were available from the monitoring program and deemed to be of interest, they were included in the temporal graphs. Descriptive figures were performed using Kaleidagraph Software.

In this study, the term "bloom" refers to cell abundances ≥ $1X10^4$ cells L^{-1}. To analyze the trend of PSP blooms over the studied period, in figure 10 the annual percentages of samples with blooms of PSP producer species were calculated based on data of the nine harbors sampled over the entire study period. Percentages were calculated to avoid the bias arising from the different number of samplings in 2012.

The mean annual cycles of *Alexandrium minutum* abundances, and the environmental variables were determined by calculating the fortnightly averages of data collected during the study period.

The Mann-Kendall (MK) trend test (Kendall, 1975; Mann, 1945) was performed on target species and environmental series to detect statistically significant trends. The MK trend test is a non-parametric test for monotonic trends that is typically used to detect whether there is a significant increasing or decreasing trend in natural time series (e. g. hydrological or climatological) as this test is not affected by the actual distribution of the data and is less sensitive to outliers than parametric trend tests, such as the regression coefficient. We applied the MK test since most of the data series were not normally distributed according to the Shapiro-Wilk's test, calculated using STATISTICA 10 Software (StatSoft, 2011). In the MK test, the initial assumption is the null hypothesis (H0), which assumes that there is no trend in the data series over time. The alternative hypothesis (H1) is that there is a significant trend (increasing or decreasing) over time. The significance level of the test was set at $\alpha = 0.05$. If the P-value < 0.05 the H0 is rejected, meaning that there is a significant trend in the time series. Kendall's tau (τ) is a value between −1 and 1 that indicates the direction of the trend over time. An increasing trend is indicated by $\tau > 0$ and a decreasing one by $\tau < 0$. The MK test was applied using the Kendall package in R software version 3.0.3 (Team, 2015) to deseasonalized monthly mean series data obtained by subtracting the monthly global means from monthly means (hereafter monthly anomalies), in order to better discern the trend in the variable over time. The change per unit time in a time series

having a linear trend was estimated by applying a simple non-parametric procedure, namely Sen's estimator of slope (Sen, 1968). We considered as significant those trends of monthly anomalies in which both Kendall's tau τ was significant and the 95% confidence interval of the Sen's estimator of slope did not include 0.

In order to depict trends in the abundance of *A. minutum*, and some environmental variables of selected harbors, the Lowess smoothing was calculated for each data series. To compare the magnitude of the 3 components of *A. minutum* variability in the Catalan harbors, we first calculated the average data series of the abundances of *A. minutum* with the nine-data series obtained from the more sampled harbors to summarize all the variability of the species in a series. Next, we decomposed it into seasonal, trend and irregular components using Seasonal Decomposition of Time Series procedure based on Loess (STL) with R software.

To study in detail the influence of temperature in some harbors, anomalies were plotted and data were smoothed using a moving average filter with a span of 12 months based on the fortnightly temperature anomalies.

For the analysis of the relationship between the log of *A. minutum* abundances and environmental variables we established a linear mixed-effects model that takes into account the repeated measures along time. A linear mixed model (LMM) is a generalization of the standard linear model, allowing fit data where a response variable has multiple measurements on the same experimental unit. Thus, these models consider the correlation among observations and the heterogeneous variance (Littell et al., 1996). In contrast to simple linear models, a linear mixed model contains both fixed and random effects, with parameters which are allowed to vary over individuals.

The model established to analyze our data, included as fixed-effects: water temperature, salinity, Chl-*a*, nitrate, nitrite, ammonium, phosphate and silicate. Variable time was introduced as both fixed and random effect. As a random effect, a structure of correlations among the 12 months of the year was assumed. To structure the variability, we used a covariance structure based on ARMA (autoregressive moving averages).

All results of the fitted models were obtained using the MIXED procedure of SAS v9.4, SAS Institute Inc., Cary, NC, USA. The significance level was fixed at 0.05.

3. Results

3.1. Environmental conditions

The environmental parameters and Chl-*a* levels measured at the 16 stations during the 13-year time series are shown in **Fig. 2** and **3** and **Table S1**. The marked seasonality of temperature was evidenced by maximum and minimum mean values in summer and winter, respectively. A spatial gradient, with colder waters in the north than in the south, was apparent as well. Salinity levels and inorganic nutrient concentrations were highly different among the harbors and varied widely in some of them (**Fig. 3**). Salinities were remarkably lower at harbors influenced by the Ebre River and regional irrigation canals, and in Arenys de Mar and Premià harbors that are subjected to freshwater inputs.

3.2. Diversity and spatio-temporal distribution of the genus *Alexandrium* in enclosed coastal areas

Eleven morphospecies belonging to the genus *Alexandrium* were detected in the coastal superficial waters sampled during the study period, including three potential PSP producers: *A. minutum, A. pacificum*, and *A. ostenfeldii* (**Fig. 4**, **Table 2**).

The identification of most of the detected species was confirmed repeatedly by molecular sequencing of the cultured strain, and published in other works (**Table 2**).

The maximum cell abundances and the percentage of blooms comprising each of the detected species during the 13-year study are shown in **Table 2**. The spatio-temporal distributions are plotted only for the more frequently detected *Alexandrium* species.

Alexandrium andersonii Balech

In the winter of 2007 a proliferation of *A. andersonii* was detected in Alfacs Bay that coincided with the time of year when the irrigation canals reaching the bay are closed. This was the first detection of this species in the Mediterranean

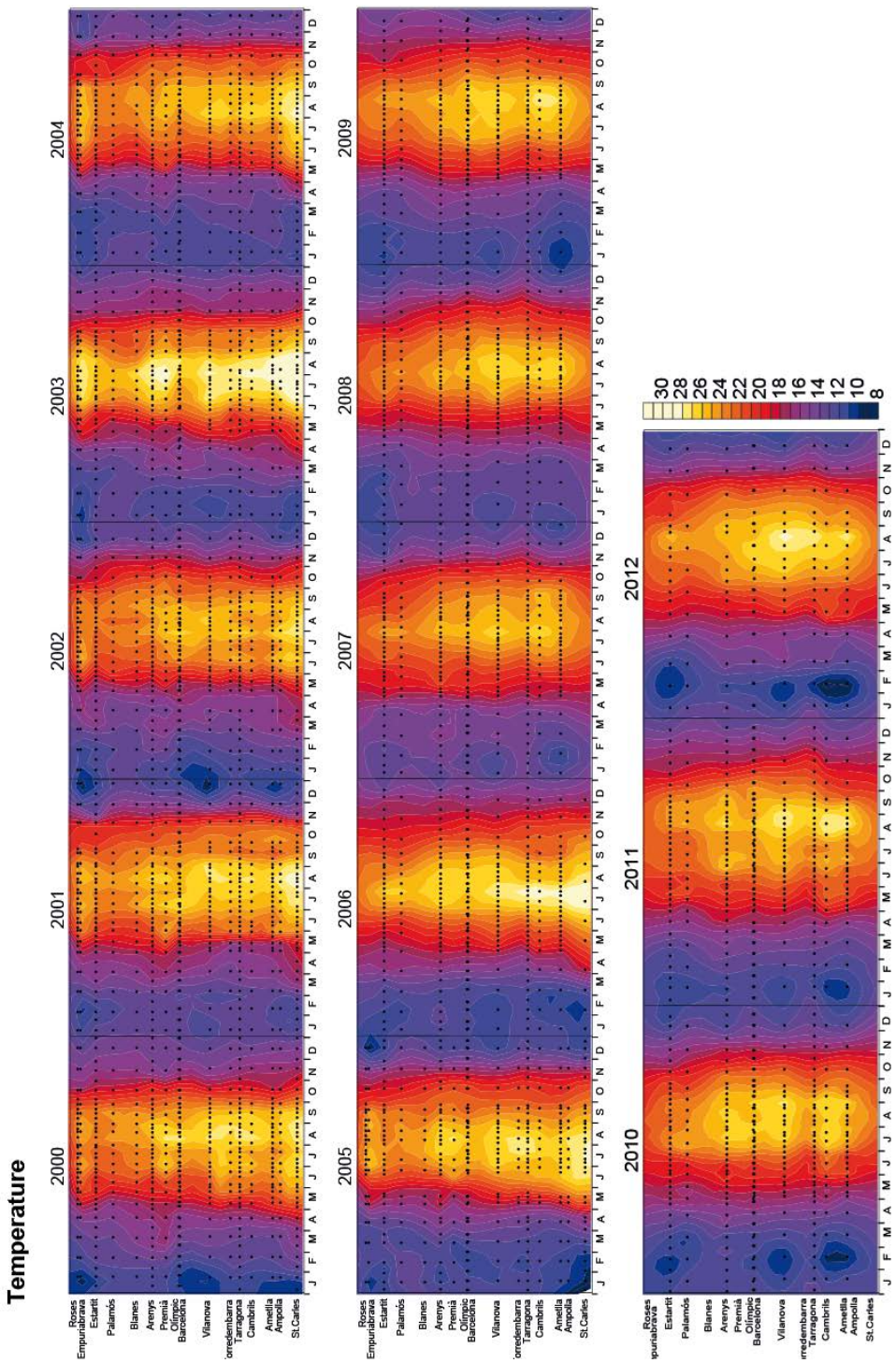

Figure 2(A). Spatio-temporal distribution of temperature in Catalan coastal waters at the harbors included in this study.

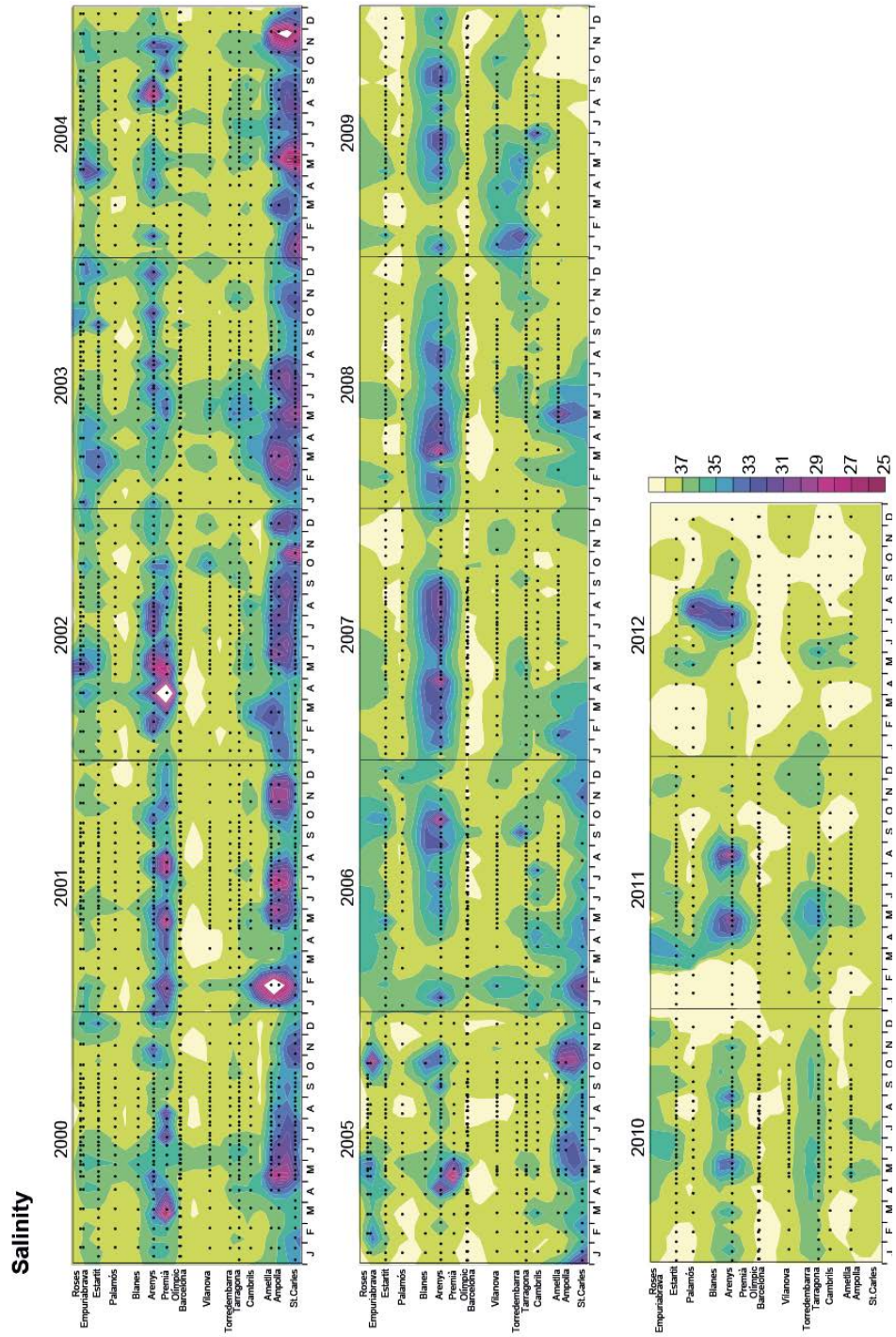

Figure 2(B). Spatio-temporal distribution of salinity in Catalan coastal waters at the harbors included in this study.

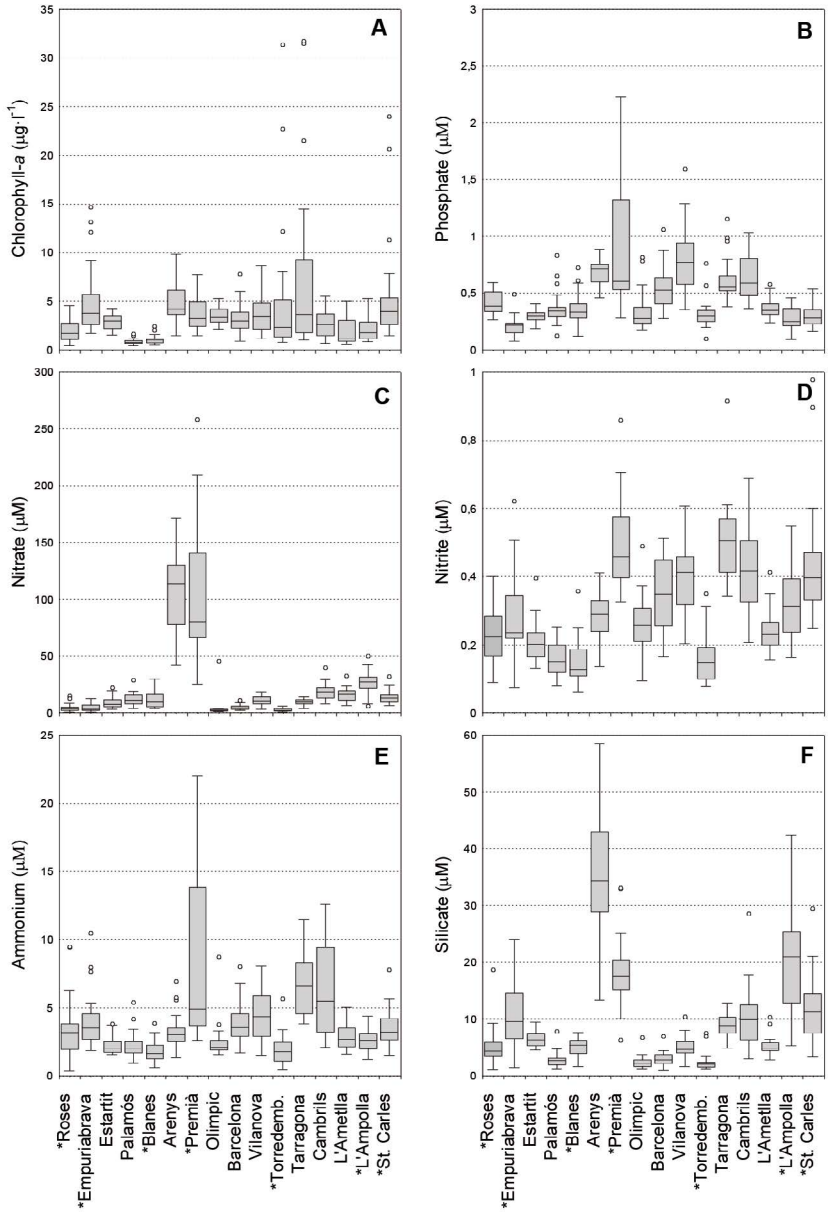

Figure 3. Box plots of environmental variables concentrations calculated with the fortnightly averages in each harbor during all sampled years. Box limits: 25th and 75th percentile of the variable; Horizontal line: median value; Whisker: minimum and maximum values withouh outliers; Empty circles: oultiers. Outliers of ammonium, nitrite, and phosphate at Premià Harbor and phosphate at Palamós Harbor were not plotted, for illustrative purposes. Note the different scales of the different graphs. Asterisks show the harbors sampled until 2005.

Table 2. *Alexandrium* species detected in the Mediterranean Sea and their presence or absence along the Catalan coast. Maxim cell abundances, percentages of blooms, and toxicity in Catalan coastal waters (from 2000 to 2012). n.d. not detected. References for the presence in the Mediterranean waters are also reported for comparison porpoises.

	Max cell abundances	Blooms (%) N=4279	Toxicity in Catalan coastal waters	Reference for toxicity	Catalan cultures sequenced	Reported presence in the Mediterranean
A. affine	n.d.					Penna et al., 2008
A. andersonii	4700	0.00	no	Sampedro et al., 2013	Sampedro et al., 2013	Montresor et al., 1998
A. balechii	n.d.					Montresor et al., 1990
A. compressum	n.d.					Caroppo (2000)
A. concavum	n.d.					Daly Yahia-Kefi et al., 2001
A. foedum	n.d.					Balech (1990)
A. insuetum	164787	0.02	?	-	Penna et al., 2008	Daly Yahia-Kefi et al. (2001)
A. cf. leei	2600	0.00	?	-		Daly Yahia-Kefi et al. (2001)
A. margalefi	2080	0.00	?	-	Penna et al., 2008	Daly Yahia-Kefi et al. (2001
A. minutum	7236834	5.56	PSP toxins	Van Lening et al., 2007	Penna et al., 2008	Halim (1960)
A. mediterraneum	n.d.					John et al., 2003
A. ostenfeldii	2800	0.00	spirolides	Franco et al., 2006	Penna et al., 2008,	Lecal, J. (1954), Mikhail, K. 2001
A. pacificum	26447339	0.89	PSP toxins	unpublished	Penna et al., 2008	Vila et al., 2001b
A. pseudogonyaulax	217200	0.02	?		Penna et al., 2008	Daly Yahia-Kefi et al. (2001); John et al., 2003
A. kutnerae	n. d.					Daly Yahia-Kefi et al. (2001)
A. cf. tamarense	19505	0.02	no	Riobó P., pers. comm.	Satta et al., 2013	Penna et al., 2008
A. tamutum	33670	0.02	no	Vila et al 2004	Penna et al., 2008	Montresor et al., 2004
A. taylori	107070	0.30	no	unpublished	Penna et al., 2008	Delgado et al.,1997 Garcés et al., 1999

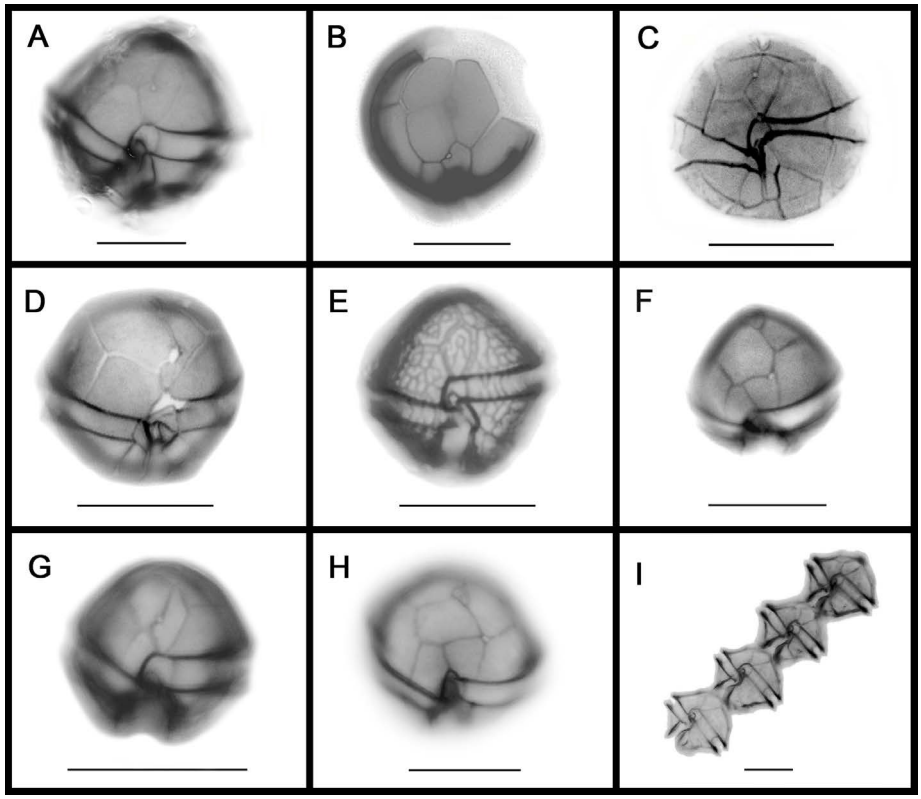

Figure 4. Micrographs of wild *Alexandrium* cells stained with Calcofluor and observed using epifluorescence microscopy. The cells are seen in ventral view. (A) *A.* cf. *leei*, (B) *A. pseudogonyaulax*, (C) *A. tamutum*, (D) *A. ostenfeldii* (E), *A. insuetum*, (F) *A. margalefi*, (G) *A. minutum*, (H) *A. taylori*, (I) *A. pacificum*. Scale bars: 20 µm.

coastal waters of Spain. *A. andersonii* was first detected in mid-January, after which its abundance progressively increased until a maximum (7.2 $X10^3$ cells L^{-1} in integrated sample) was reached on February 19, with cell numbers decreasing thereafter (**Fig. 5A**). The outbreak occurred in water that was relatively cold (8.9–15.4ºC) for this area and over a wide range of salinities (33.9–37.4). During this time, the water column was mixed or of moderate stratification, after a period of strong stratification that lasted most of January (**Fig. 5A**). The end of the bloom event, in early April, coincided with the presence of strong winds from the northwest (not shown). At the central station and throughout the study period, *A. andersonii* abundances were higher in samples from the water column (integrated) than in those obtained

from surface and bottom waters. A very large difference was recorded on February 19, when slight stratification was evident (**Fig. 5A, B**).

These observations suggested that *A. andersonii* had accumulated in a thin layer within the water column but not in the surface or bottom water. During the bloom event, the presence of *Alexandrium* species other than *A. andersonii* was noted, including *A. minutum*, the second most abundant *Alexandrium* species in the bloom, as well as *A. margalefi*, *A. insuetum*, *A.* cf. *leei*, *A. tamutum*, and *A. tamarense*. Thereafter, *A. andersonii* was detected only in Barcelona Harbor, where in May of the same year (2007) it occurred at very low abundances.

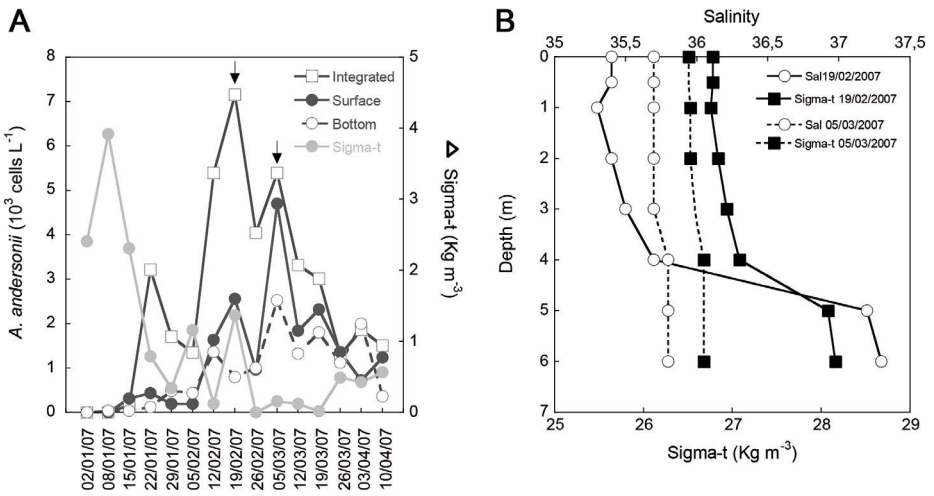

Figure 5. (A) Temporal variation of *A. andersonii* cell abundances as measured in surface, bottom, and integrated water column samples from the central station (St. C) of Alfacs Bay, and temporal variation of the sigma-t difference between surface and bottom waters. (B) Vertical profiles of salinity and density determined on two different occasions during the proliferation (marked with arrows in plot A).

A. insuetum Balech

This species was first detected in 2004 and at almost all the stations sampled between May and July. It abundances did not exceed of 10^4cells L^{-1}, except in

Cambrils Harbor, where 1.65 X10^5 cells L^{-1} were recorded during June 2004. In the following years, *A. insuetum* was detected on fewer occasions but always during the same 3 months of the year (**Fig. 6A**).

***A.* cf. *leei* Balech**

Alexandrium cells with a morphology consistent with that of *A. leei* (see **Fig. 4**) were found for the first time in August 2006, in Arenys de Mar Harbor. The following year the species was also present in additional samples taken in Alfacs Bay during the winter proliferation of *A. andersonii*, and again in Arenys de Mar Harbor, in the summer, but always in very low abundances. The maximum abundance of *A. leei* was recorded in Tarragona Harbor samples in the summer of 2009 (**Table 2**).

***A. margalefi* Balech**

Cells of this species have been found occasionally and at low abundances (max. 2080 cells L^{-1} in Arenys de Mar) since 2007, always in summer (from June to August) and most frequently in the two southern-most harbors (Vilanova and Tarragona). In August 2010, the species was detected simultaneously in several harbors along the Catalan coast (**Fig. 6B**). It was also observed in Alfacs Bay, albeit at very low abundances (max. 120 cells L^{-1}), in the winter of 2007 during the proliferation of *A. andersonii*.

***A. minutum* Halim**

A. minutum produced numerous blooms at the sampling stations and accounted for the highest percentage of blooms among the studied species (**Table 2**). It was also the most widely distributed *Alexandrium* species, both along the Catalan coast, where it was present in all the studied harbors, and over time, as it was detected throughout the year, with blooms occurring mostly between January and August (**Fig. 6C**). Except for the Empuriabrava, Tarragona, and Ampolla stations, A. minutum bloom abundances (> 1 X10^4 cells L^{-1}) were observed in all harbors. Blooms of this species occurred every year in Vilanova, Arenys de Mar, and Estartit harbors and were recurrent most years in Olímpic and Cambrils harbors. Bloom abundances were rarely reached in autumn, and were never observed in October. Annual maximum

cell abundances occurred mostly in the spring, always > 1 $X10^5$ cells L^{-1} and even > 1 $X10^6$ cells L^{-1} in most of the studied years. These higher abundances were detected at Arenys de Mar, Cambrils, Estartit, Olímpic, and Vilanova harbors.

***A. ostenfeldii* (Paulsen) Balech & Tangen**

The first detection of *A. ostenfeldii* was in 2005 in Estartit Harbor. Thereafter, it was also detected occasionally in different harbors along the Catalan coast during different months of the year, usually in spring (**Fig. 6D**), and always at low abundances (max 2.8 $X10^3$ cells L^{-1}, **Table 2**).

***A. pacificum* Litaker**

During the study period, bloom-level abundances of *A. pacificum* were found occasionally in Olímpic and Vilanova and routinely in Tarragona harbors (**Fig. 6E**). The blooms in Tarragona usually occurred in summer and some reached very high abundances (> 1 $X10^6$ cells L^{-1}). The distribution of *A. pacificum* narrowed over time. While initially found at 10 stations spread across the central and southern Catalan coast, its distribution later declined to include fewer harbors and lower abundances. After September 2008, it was detected only in Tarragona Harbor, where it continued to reach bloom-level abundances.

***A. pseudogonyaulax* (Biecheler) Horiguchi ex Kita & Fukuyo**

A. pseudogonyaulax was first detected in 2003 and then occasionally in harbors located in the central and southern portions of the study area, usually from April to June. Its abundances were typically low (<10^3 cells L^{-1}) except in Sant Carles Harbor, where bloom-level abundances (> 1.0 $X10^4$ cells L^{-1}) were reached in June 2003 (**Fig. 6F**). This harbor is located inside Alfacs Bay and the bloom affected the entire bay from June 2 to July 21 of that year (**Fig. 7**). Cell abundance inside Alfacs Bay reached a maximum on June 16. The bloom started during a period of surface water renovation driven mainly by freshwater inputs. Vertical profiles obtained during the event revealed a stratified water column with a thermocline and a halocline, both clearly distinguishable at 3–4 m depth, and higher (1–2 orders of magnitude) *A. pseudogonyaulax* cell abundances in the layer just below the pycnocline (**Fig. 8**).

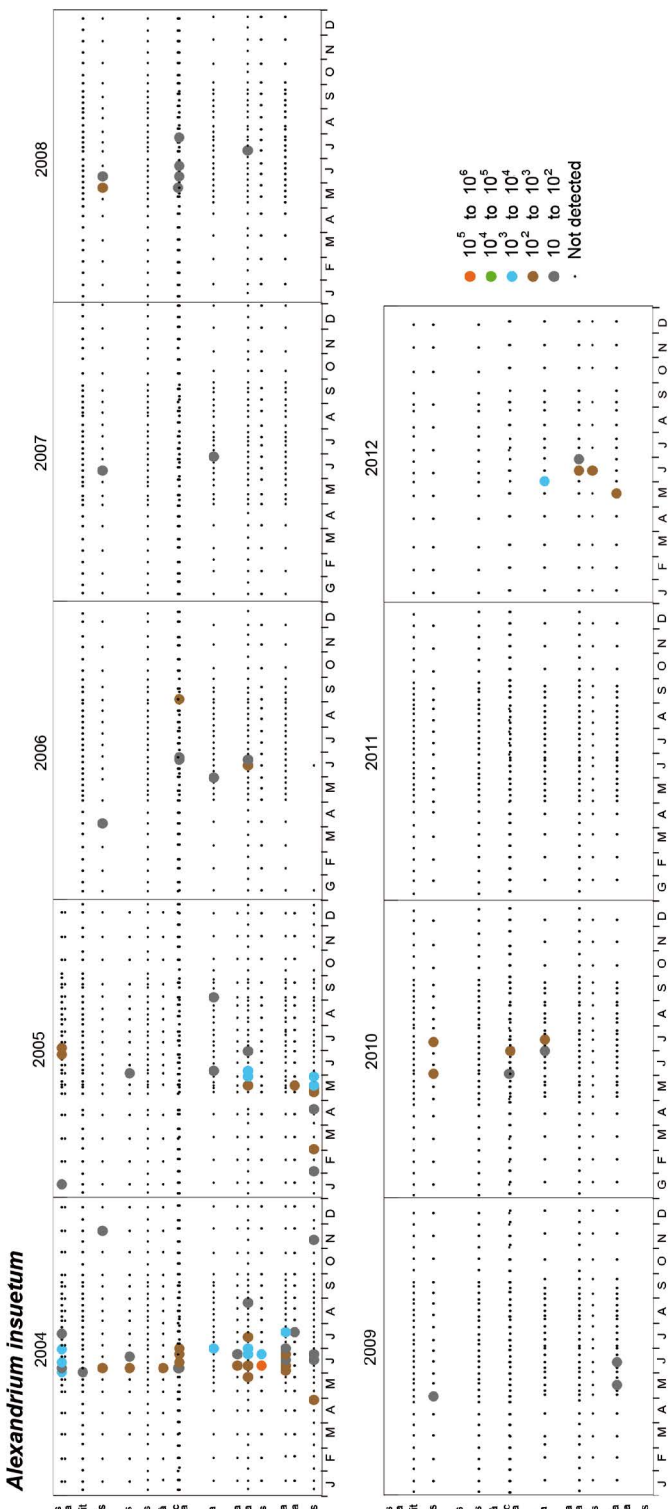

Figure 6(A). Spatio-temporal distribution of *Alexandrium insuetum* in Catalan coastal waters at the studied harbors.

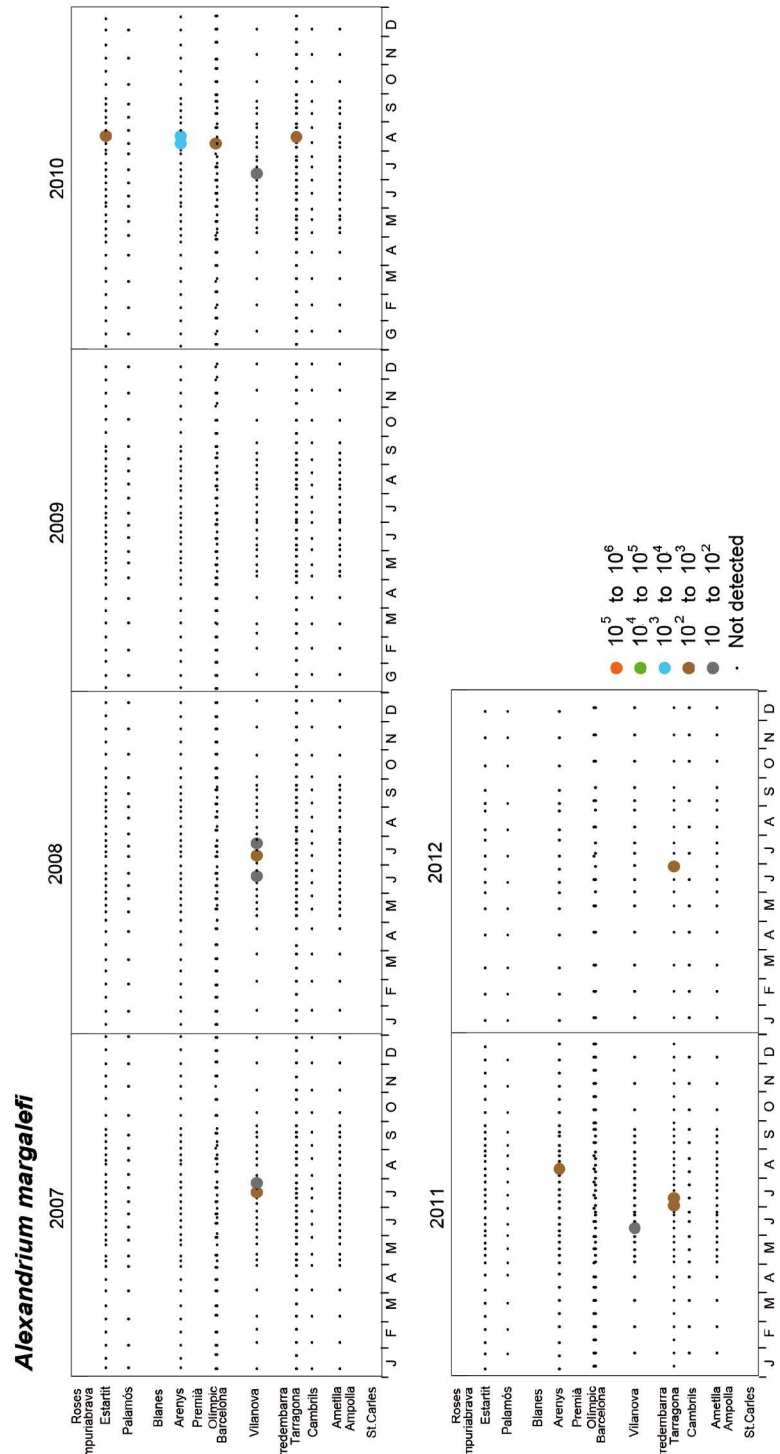

Figure 6(B). Spatio-temporal distribution of *Alexandrium margalefi* in Catalan coastal waters at the studied harbors.

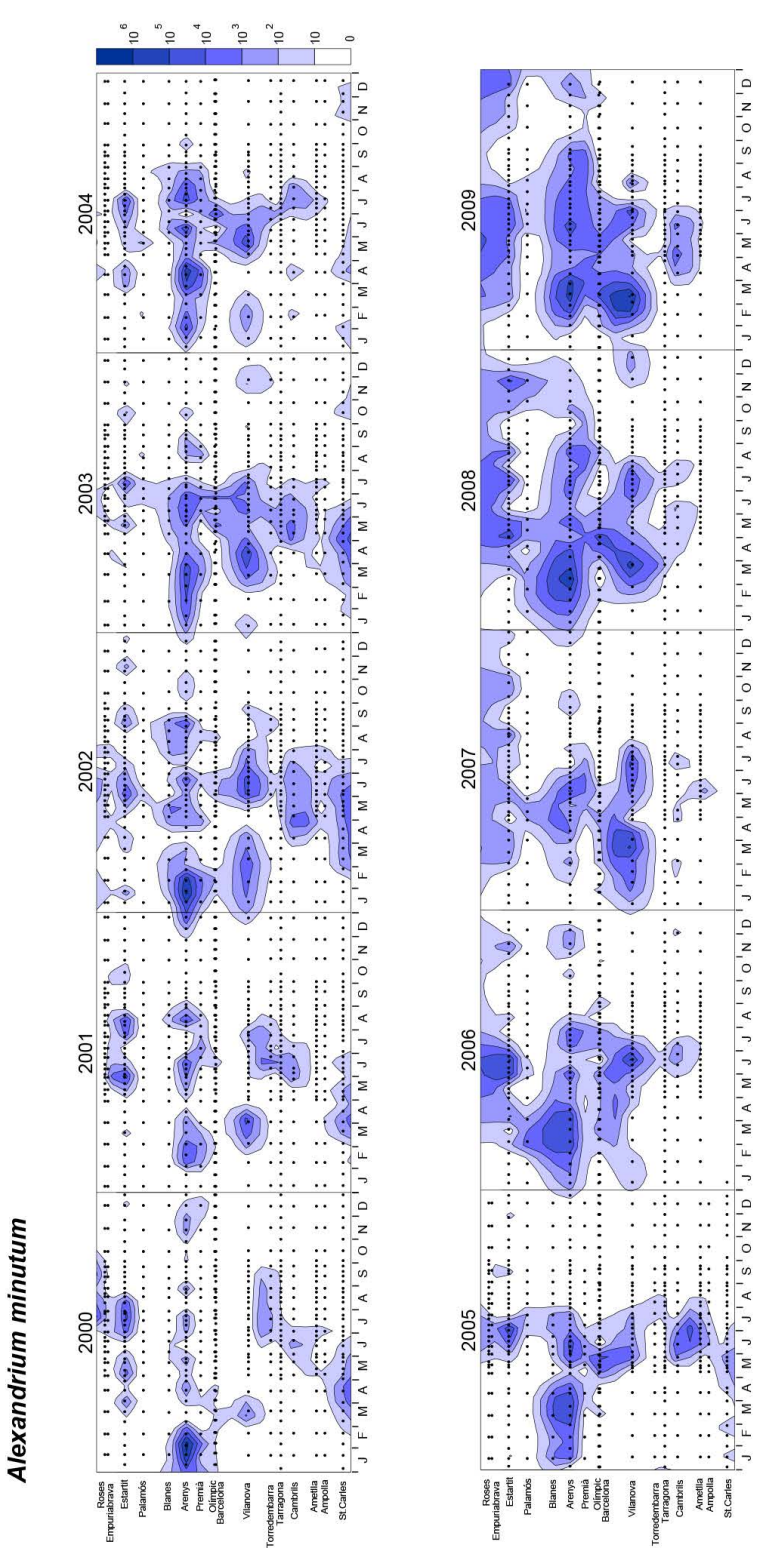

Figure 6(C). Spatio-temporal distribution of *Alexandrium minutum* in Catalan coastal waters at the studied harbors.

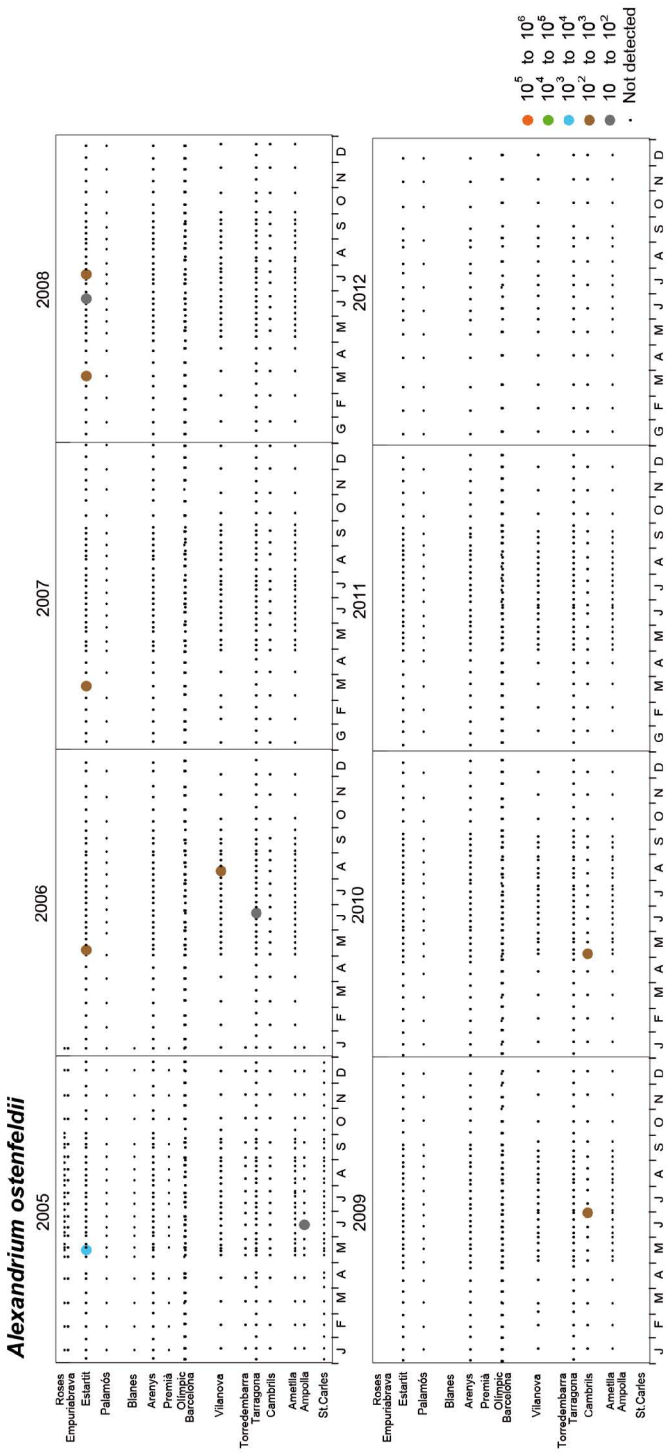

Figure 6(D). Spatio-temporal distribution of *Alexandrium ostenfeldii* in Catalan coastal waters at the studied harbors.

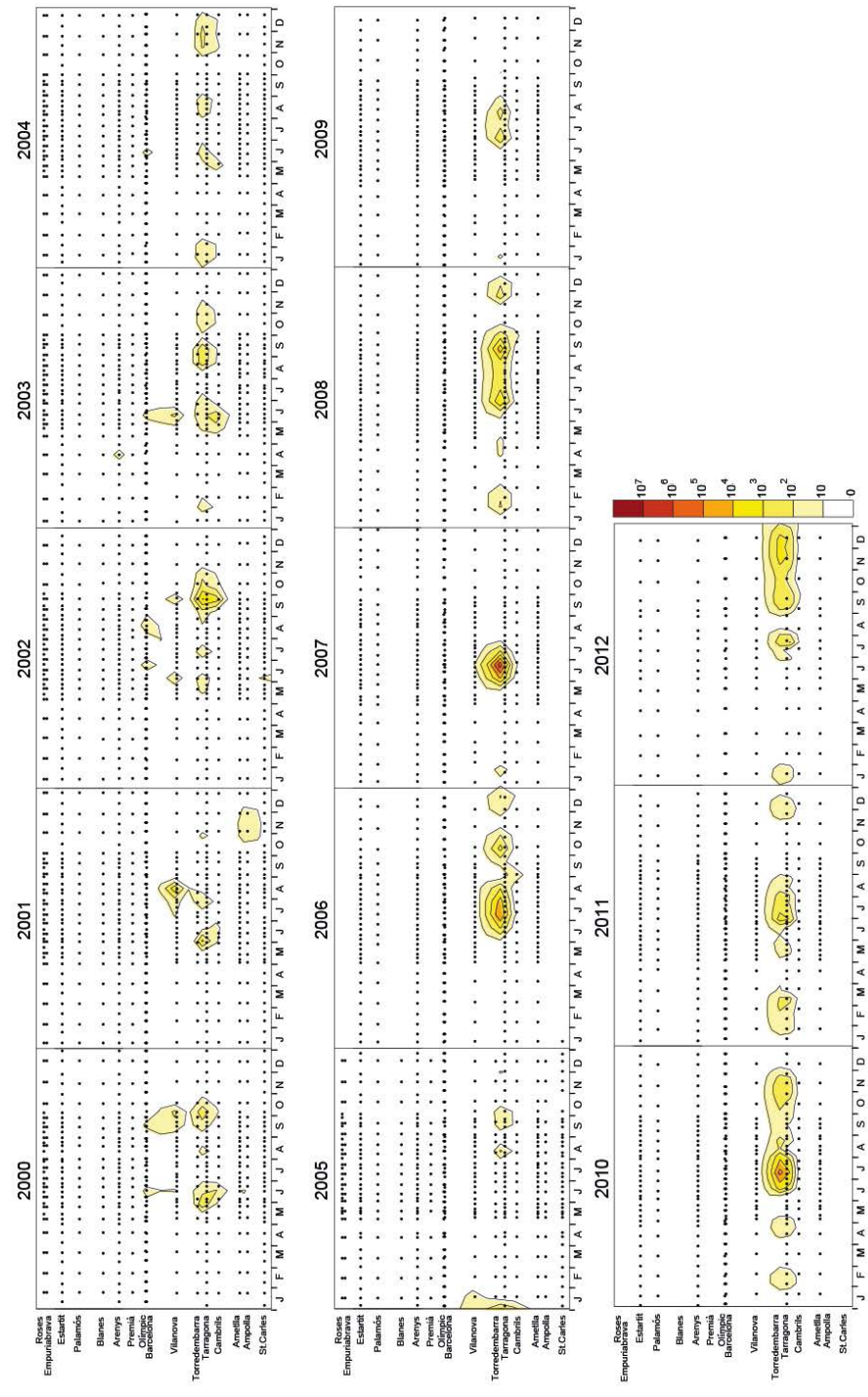

Figure 6(E). Spatio-temporal distribution of *Alexandrium pacificum* in Catalan coastal waters at the studied harbors.

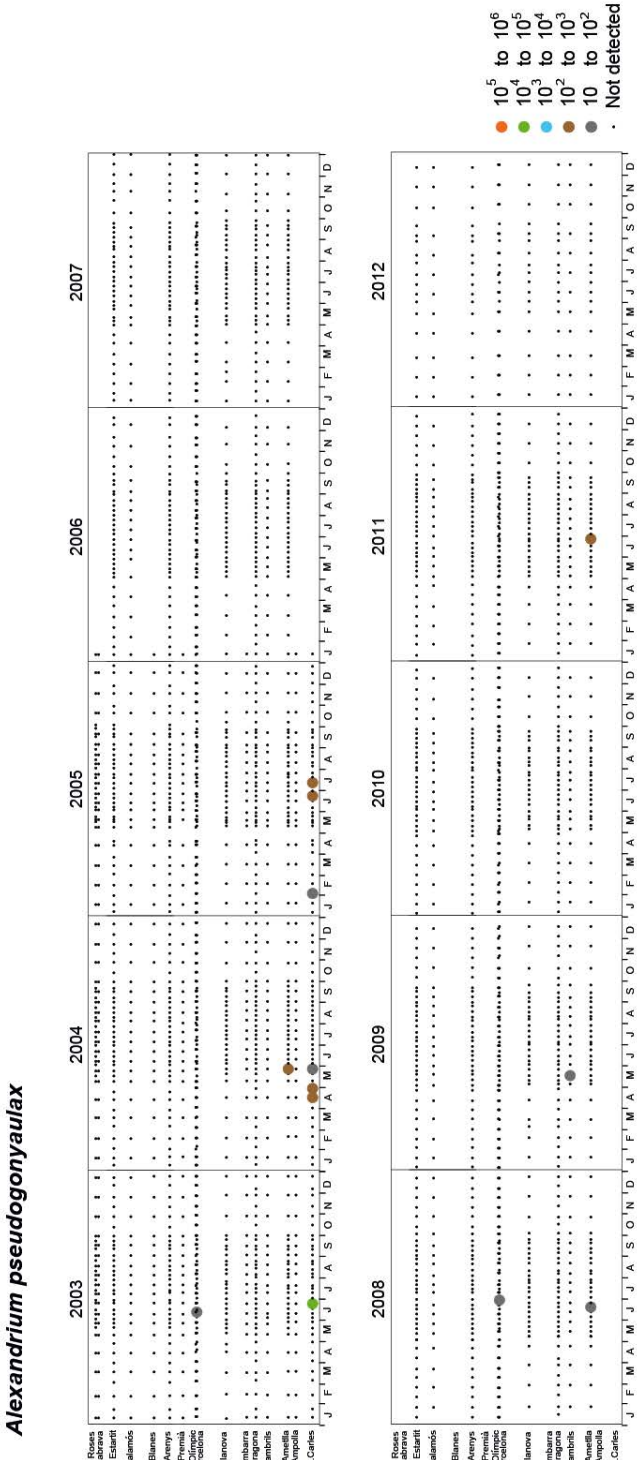

Figure 6(F). Spatio-temporal distribution of *Alexandrium pseudogonyaulax* in Catalan coastal waters at the studied harbors.

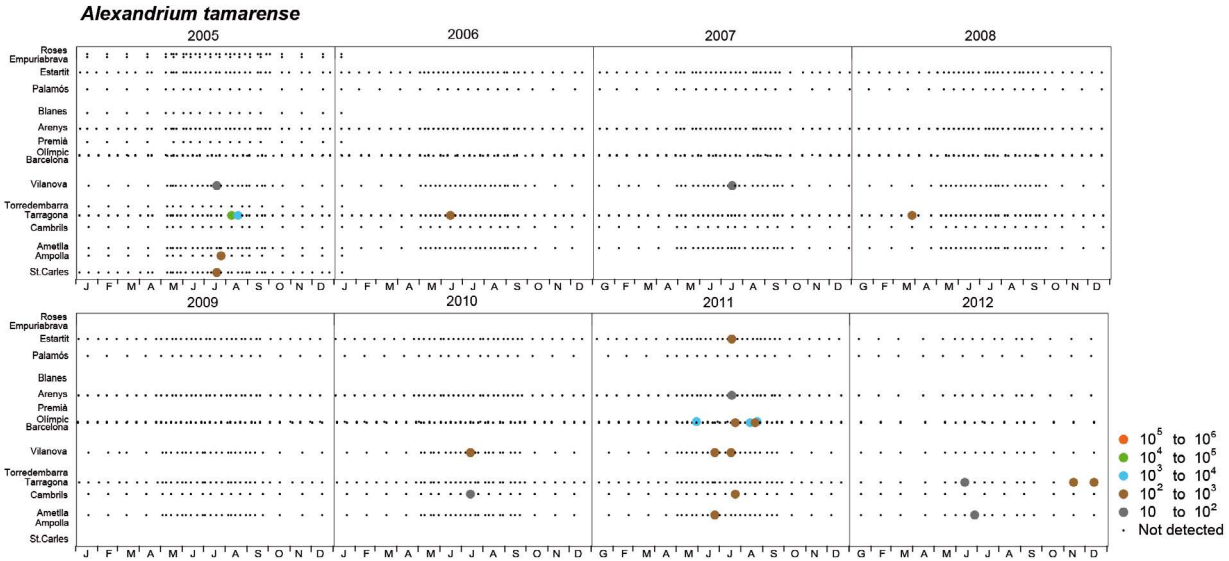

Figure 6(G). Spatio-temporal distribution of *Alexandrium tamarense* in Catalan coastal waters at the studied harbors.

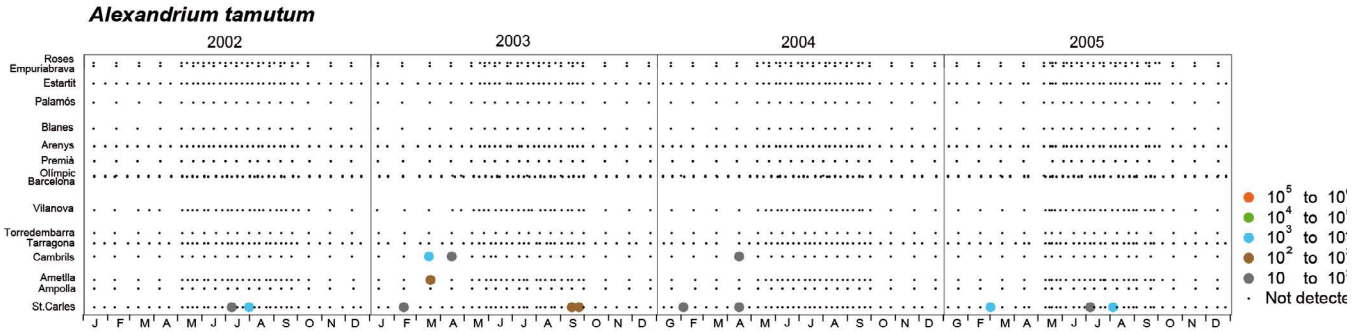

Figure 6(H). Spatio-temporal distribution of *Alexandrium tamutum* in Catalan coastal waters at the studied harbors.

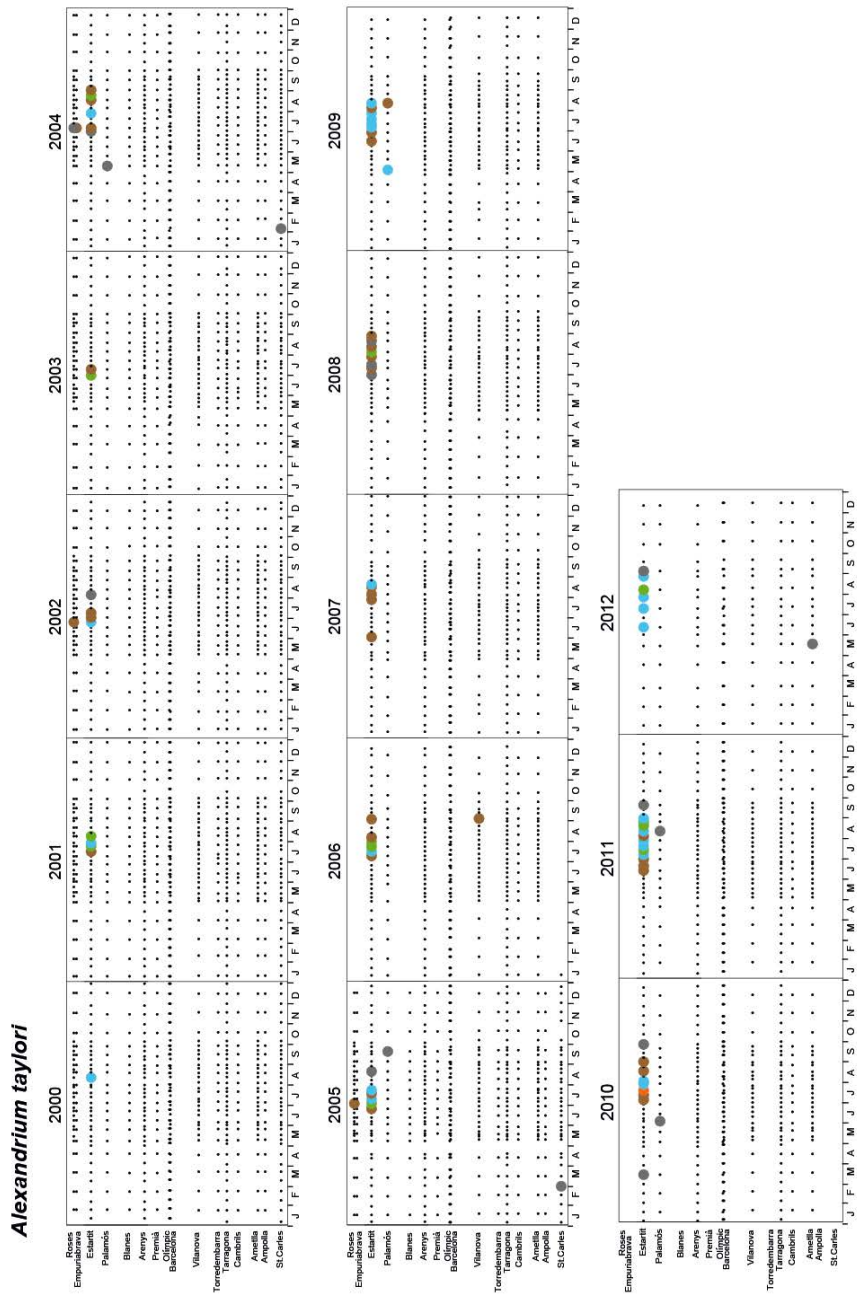

Figure 6(I). Spatio-temporal distribution of *Alexandrium taylori* in Catalan coastal waters at the studied harbors.

***A.* cf. *tamarense* (Lebour) Balech emended John**

A. cf. *tamarense* was detected beginning in 2005, generally in the summer months, in Vilanova, Tarragona, Ampolla and St. Carles harbors. In 2011, this species was detected all along the coast. The abundances of *A*. cf. *tamarense* were usually < 10^3 cells L^{-1} except in 2005 when bloom-level abundances were reached in Tarragona Harbor (**Table 2**, **Fig. 6G**).

***A. tamutum* Montresor, Beran & John**

After its first detection in 2002, this species was occasionally observed in three harbors located along the southern part of the coast, usually at cell abundances < 3 X10^3 cells L^{-1} and during different seasons of the year (**Fig. 6H**). *A. tamutum* was more frequently detected in St Carles de la Ràpita Harbor, inside Alfacs Bay, where in July 2002 it reached abundances up to 3.4 X10^4 cells L^{-1}. After 2006, this harbor and six others were no longer sampled leaving the monitoring to 9 sites. In these monitored sites, *A. tamutum* was not longer

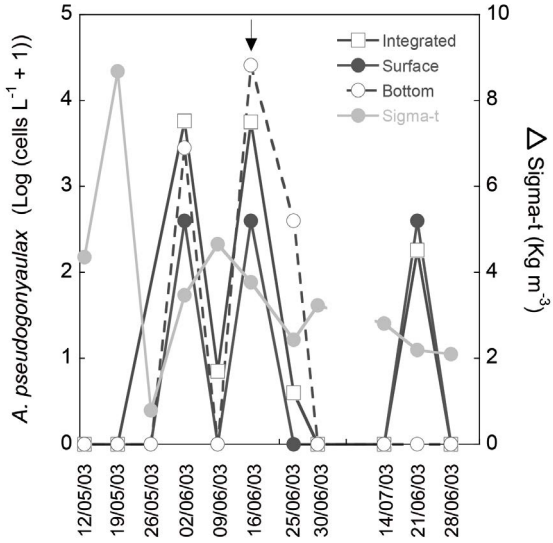

Figure 7. Temporal variation of *A. pseudogonyaulax* cell abundances as measured in surface, bottom, and integrated water column samples from the central station (station C) of Alfacs Bay, and temporal variation of the sigma-t difference between surface and bottom waters. Arrow indicates the date when the vertical profile shown in Fig. 8 was obtained.

detected. Prior to the description of *A. tamutum* in 2004 by Montresor et al. (2004), specimens that differed morphologically from all described species were found along the Catalan coast. A subsequent re-examination of the images, samples, and established cultures led to the identification of these specimens as *A. tamutum*.

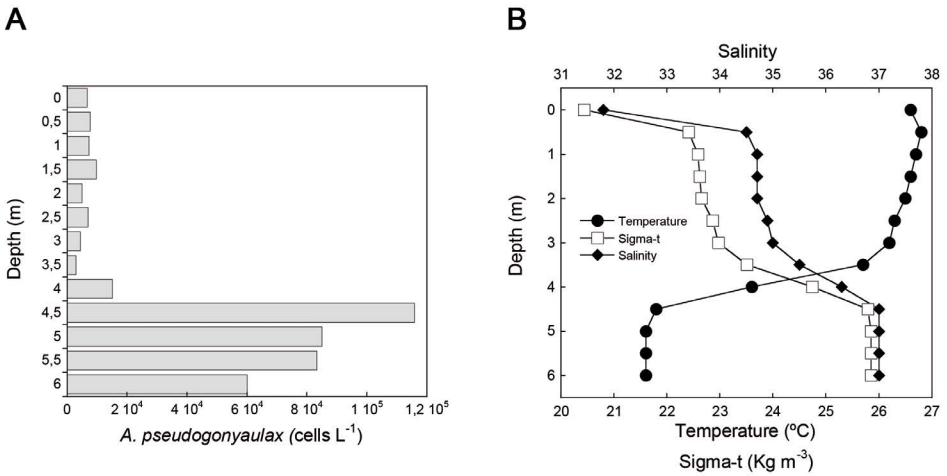

Figure 8. (A) Vertical profile of *Alexandrium pseudogonyaulax* and (B) vertical profiles of salinity, density (sigma-t), and temperature determined on June 16, 2003 at station B.

A. taylori Balech

Over the study period, *A. taylori* was detected in several harbors, but only in Estartit Harbor (located on the Costa Brava) did it reach bloom-level abundances, recurrent every summer. The presence of this species in the other harbors was occasional and at low abundances (**Fig. 6l**).

3.3. Population dynamics of *Alexandrium* PSP producer species

Figure 9 shows the long-term (based on the study period and previous studies for which data are available) variation of *A minutum* and *A. pacificum* (PSP producer species) in five harbors in which both species were present. *A.*

minutum had been detected in these harbors since the start of the monitoring program, with different, fluctuating abundances in each one over the course of the study. After the emergence of *A. pacificum,* its abundance at Olímpic, Barcelona, Vilanova, and Cambrils declined until by the end of the study this species was no longer detected in any of them. By contrast, the presence of *A. pacificum* was maintained at Tarragona Harbor, where it caused recurrent blooms (maximum cell abundances of 26.5 $X10^6$ cells L^{-1}; summer of 2003). However, cell abundances were lower than the bloom level in 2004, 2005, and 2009; in 2004 and 2005 the maximum was only 8.3 $X10^3$ cells L^{-1}, which was much lower than in either the previous or subsequent years. These two years the abundances of *A. pacificum* in the other harbors also were lower or disappeared (**Fig. 9**).

Despite the coexistence of *A. minutum* and *A. pacificum* in the studied harbors, when one species reached bloom-level abundances, the other was present at very low cell numbers, if detectable at all. Moreover, the sum of the abundances of the two PSP species never reached bloom densities if the abundance of each one was below the bloom level. Over the 13-year study, blooms attributed to *Alexandrium* PSP producer species in the nine studied harbors, for which data from 2000 to 2012 were available, increased significantly ($p < 0.05$, slope: 0.343, $r^2 = 0.445$; **Fig. 10**). This was largely due to the higher bloom percentages of *A. minutum* ($p < 0.05$, slope: 0.362, $r^2 = 0.508$) whereas those of *A. pacificum* decreased slightly, but not significantly ($p > 0.05$, slope: −0.016, $r^2 = 0.007$).

3.4. Temporal variability of *A. minutum* and its relation to environmental factors

Alexandrium minutum was the most abundant and widely distributed species in the confined waters of the Catalan coast and its bloom percentages were the largest. Thus, we focused on this species studying its temporal variability and their relationships to the environmental factors. The temporal variability of *A. minutum* was decomposed in 3 components: the seasonality, the trend and the remainder component not caused by the seasonality or the tendency. Here, we first determined the relative weight of each component in the variability of *A. minutum*. Then we studied the long-term patterns of *A. minutum* and of the environmental variables in the nine harbors that were sampled over the

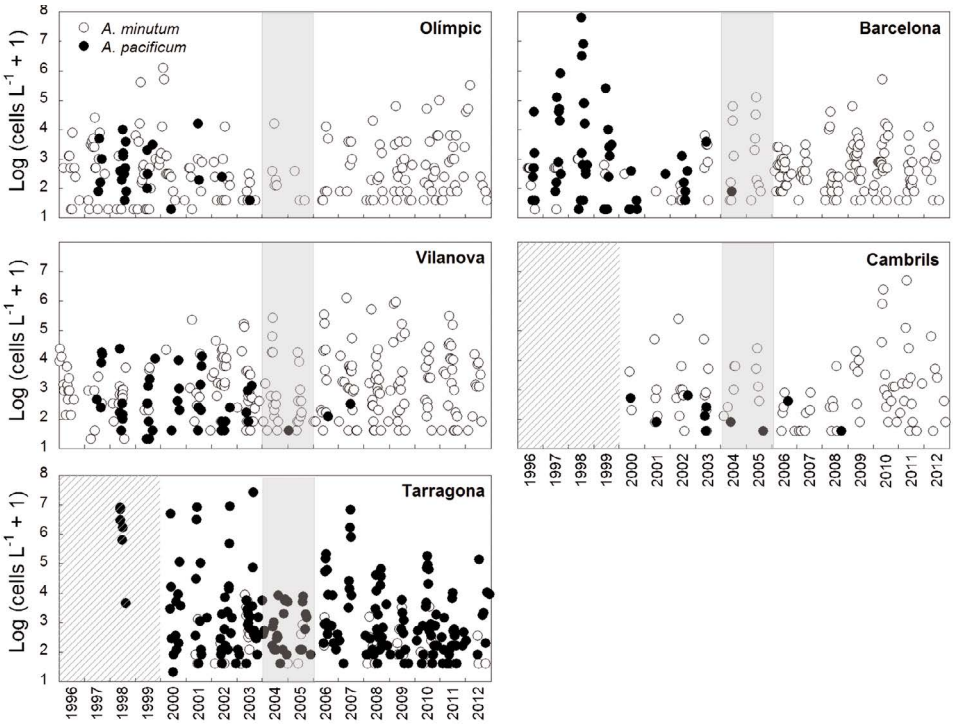

Figure 9. Temporal variation of *A. pacificum* and *A. minutum* cell abundances at five different harbors. Cambrils Harbor was not sampled before 2000 (striped area). Tarragona Harbor was sampled only occasionally before 2000 (striped area). Gray areas indicate the 2004 and 2005 period.

whole studied period. More detailed information about the seasonal patterns of *A. minutum* abundances and the environmental variables was obtained by analyzing the three harbors where this species bloomed every year. Finally, the results of LMM to establish significant relationships between *A. minutum* abundances and the environmental variables analyzed are shown.

Although the proportion of the *A. minutum* variability due to the seasonality and the trend is different according to the harbor (not shown), in general the magnitude of the seasonal variability was higher than the magnitude of the trend variability (**Fig. 11**). However, the remainder component, mostly explained by random processes, is the largest of the 3 components of *A. minutum* abundances variability in general (**Fig. 11**) and in each harbor (not showed).

Table 3. Results of the Mann-Kendall (MK) Trend Test and Sen's slope of the monthly anomalies of environmental and biological series. Significant P-values (α=0.05) resulted of MK test for the detection of trends and Sen's estimator of slopes that not include 0 in the 95% interval of confidence are showed in bold.

Environmental variable	A. minutum abundances					Temp. (ºC)	Salinity	Chlorophyll a (µg/L)	NO_3 (µM)	NO_2 (µM)	NH_4 (µM)	DIN (µM)	PO_4 (µM)	SiO_4 (µM)
	MK Trend Test		Sen's slope											
Harbors	Tau (τ)	P-Value	Slope	Confidence limits										
Estartit	0.102	0.061	0.003	0.007	0	↓	↑	↓	–	–	↓	–	–	↓
Palamós	0.068	0.225	0	0.001	0	–	↑	–	–	–	–	–	–	↓
Arenys de mar	0.217	**6.0E-05**	**0.008**	0.012	0.004	–	↑	–	↓	–	–	↓	–	↓
Olímpic	0.137	**0.012**	**0.0046**	0.009	3,00E-04	↑	↑	↓	↓	↓	↓	↓	↓	↓
Barcelona	0.331	**2,22E-16**	**0.0076**	0.011	0.005	↑	↑	–	↓	↓	↓	↓	↓	↓
Vilanova	0.106	0.052	0.004	0.008	0	↑	–	–	↑	↑	–	–	↑	–
Tarragona	0.051	0.366	0	7,00E-04	0	–	–	–	–	–	–	–	–	↑
Cambrils	0.189	**5,70E-04**	**0.003**	0.007	0.001	–	↑	–	↓	–	–	↓	↑	↓
L'Ametlla	-8,26E-04	0.990	0	0	0	–	↑	–	↓	↓	–	↓	↑	↓

– Not significant trend. Arrows: significant trends, ↑ (positive) ↓ (negative).

Table 4. Results of the Linear Mixed-Effects model for the logarithm of *A. minutum* abundance in Catalan harbors. Boldface indicates a statistical significant coefficient. December was fixed as the reference month.

Effect	Estimate	Standard Error	P-value (Pr>/t/)
Month	.	.	**<.0001**
January	0.2319	0.1070	**0.0316**
February	0.4875	0.1156	**<.0001**
March	0.8020	0.1321	**<.0001**
April	0.6516	0.1262	**<.0001**
May	0.8652	0.1289	**<.0001**
June	1.0437	0.1642	**<.0001**
July	0.6292	0.1877	**0.0010**
August	-0.04485	0.1841	0.8078
September	-0.3350	0.1597	**0.0375**
October	-0.2680	0.1180	**0.0244**
November	-0.1160	0.08348	0.1664
Temperature (°C)	0.02737	0.01358	**0.0455**
Salinity	0.002222	0.01106	0.8410
Chlorophyll a (µg/L)	0.001127	0.003452	0.7444
NO_3 (µM)	0.000998	0.000656	0.1300
NO_2 (µM)	-0.2751	0.09950	**0.0063**
NH_4 (µM)	0.002303	0.001766	0.1940
PO_4 (µM)	-0.04761	0.02868	0.0987
SiO_4 (µM)	-0.00077	0.002808	0.7851

The tendency, which in general seems to increase (**Fig. 10** and **11**), was analyzed with the MK trend test and Sen's Slope applied to the log abundances of *A. minutum* in the nine different harbors from 2000 to 2012 (**Table 3**). Results show that the log abundances of *A. minutum* increased significantly in four harbors (Arenys de Mar, Olímpic, Barcelona and Cambrils), increased but not significantly in two harbors (Estartit and Vilanova), and did not show any trend in three harbors (Palamós, Tarragona and Ametlla). Trends in the environmental variables at the nine harbors, shown in **Table 3**, revealed a positive tendency of salinity levels and a negative tendency of silicate at most

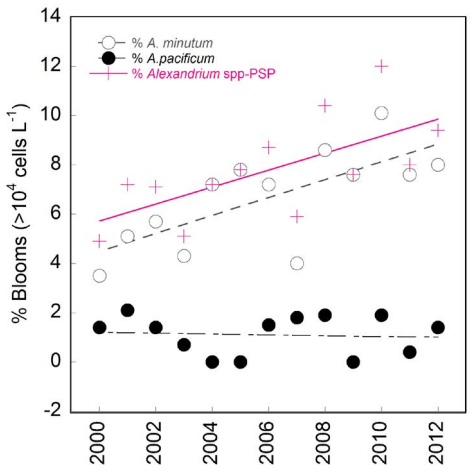

Figure 10. Temporal variation of the annual percentages of blooms of PSP producer species calculated for the nine harbors sampled over the entire study period. Linear trends are also shown. *Alexandrium* spp-PSP= *A. minutum* + *A. pacificum.*

of the harbors. These tendencies, in addition to the decrease of nitrate, were shared by the four harbors that showed a significant increase of *A. minutum* between 2000 and 2012 (**Table 3**, **Fig. 12**).

A. minutum reached bloom abundances during each of the 13 years of the study in three widely separated (with the nearest being ~100 km away) harbors: Estartit, Arenys de Mar, and Vilanova. The bloom period in these harbors differed (**Fig. 13**), but bloom-level abundances were generally not detected during the last 4 months of the year (in autumn) in any one of them. In Estartit Harbor, *A. minutum* usually bloomed between May and August; blooms were never detected at the beginning of the year. By contrast, in Arenys de Mar Harbor *A. minutum* usually reached its highest (bloom-level) abundances during the early months of the year. In Vilanova Harbor, high-density blooms of *A. minutum* were also detected between February and April but less regularly; however, the sampling frequency at this site was lower. Although *A. minutum* formed blooms in the same harbor every year during the same months and thus showed a slight seasonality, the blooms were not characterized by a well-defined temporal pattern (**Fig. 13**).

The three harbors differed greatly in their nutrient concentrations and salinity (**Figs. 2** and **3**), with remarkably higher concentrations of silicate and nitrate and a lower salinity at Arenys de Mar Harbor, and higher median nitrite and ammonium concentrations at Vilanova Harbor. The salinity level measured at Arenys de Mar Harbor was highly anomalous compared to the other two harbors. The annual patterns of physico-chemical variables and Chl-*a* of the three harbors are plotted in **Fig. S1**. Concerning potential nutrient limitation, DIN and phosphate were stoichiometrically limiting for strictly autotrophic phytoplankton in a low percentage of the samples from the three harbors

(maximum 2% and 8%, respectively, both in Estartit Harbor). The stoichiometric limitation of silicate was detected in a 16% of the samples from Vilanova Harbor but in only 1% of those from Arenys de Mar Harbor. In some cases, *A. minutum* blooms coincided with the limitation of some of the nutrients.

To study the relationship between the log of *A. minutum* abundances and environmental variables, we established a LMM. The model estimates that the effects that were significantly related to *A. minutum* abundances in harbors were temperature, nitrite and the month of the year, although not all months show the same weight (**Table 4**). Temperature affected *A. minutum* abundances positively but with a low estimate, while nitrite showed a negative relationship with *A. minutum* with a higher estimate. Concerning the influence of the month, August and November did not show significant influence, September and October affected negatively, and the months from January to July affected positively on the abundances of *A. minutum*, with June showing the highest estimate.

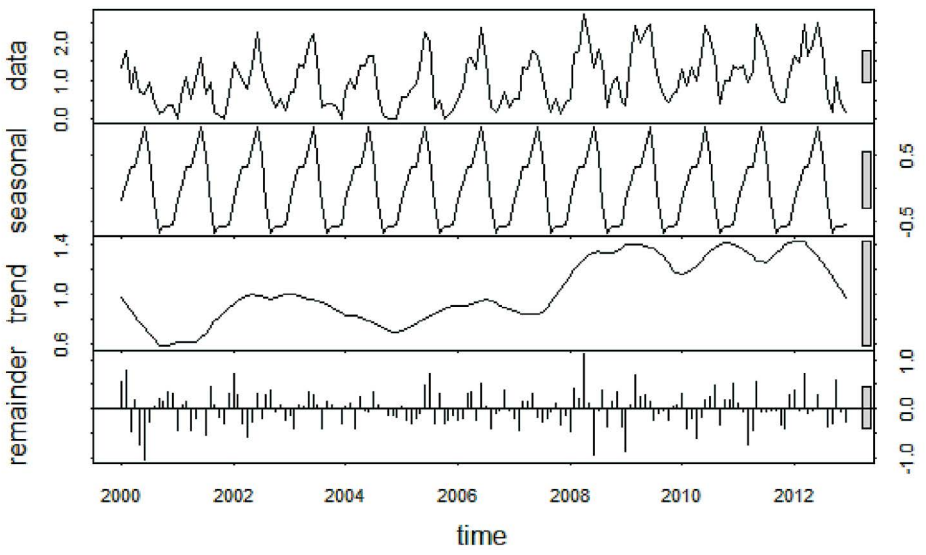

Figure 11. The average data series of *A. minutum* in habors (top) resulted from the average of nine-time series (from the 9 harbors that were sampled over the whole studied period) and its three patterns of variability (seasonal, trend and remainder) obtained from a STL decomposition.

4. Discussion

4.1. Identification and diversity of *Alexandrium*

A full understanding of the population ecology of most *Alexandrium* species has been limited by difficulties in their taxonomic identification. This study is the first to provide detailed, species-level records of *Alexandrium* detected along the Catalan coast, to assess the relationship of these species with environmental conditions and to identify temporal trends based on a 13-year data series.

A third of the globally described *Alexandrium* species have been found in confined Catalan coastal waters, and the abundances of 7 of the 11-detected species reached bloom levels (> 10^4 cells L^{-1}) in these coastal environments. All 11 species have been reported elsewhere in the Mediterranean Sea, although some species very scarce as in the case of *A. leei*. This species has been generally reported in North West Pacific (Balech, 1995; Fukuyo et al., 1988; Kim et al., 2005; Kodama et al., 1982; Liang et al., 2000; Nguyen-Ngoc and Larsen, 2004; Tang et al., 2007; Usup et al., 2002). In the Mediterranean Sea, *A. leei* is a rare species, reported in Tunisian waters (Daly Yahia-Kefi et al., 2001) and once in a Corsican lagoon sample (Nézan personal communication; Gómez, 2003), but neither strains nor cells from these Mediterranean Sea isolates have been sequenced. Cultures of *A.* cf. *leei* isolates from the Catalan coast have also not been established, nor has this species been studied genetically. However, our observations supported the presence of *A. leei* in the Mediterranean Sea.

Seven species detected in other areas of the Mediterranean Sea (*A. balechii, A. compressum, A. concavum, A. foedum, A. kutnerae, A. affine*, and *A. mediterraneum*) (Gómez, 2003; Penna et al., 2008) were not detected in this study. Some of these species were reported along the Catalan coast in other studies. This is the case of *A. kutnerae* and *A. mediterraneum*. A strain of *A.* cf. *kutnerae* was germinated from a cyst isolated at Vilanova Harbor in May of 2003 by Bravo et al. (2006); but after several months of culture it lost one of its identifying characteristics and phylogenetic analyses placed it among the ME clade of the '*A. tamarense*' species complex (now *A. mediterraneum*) (see Penna et al., 2008). Given this unusual occurrence and the fact that

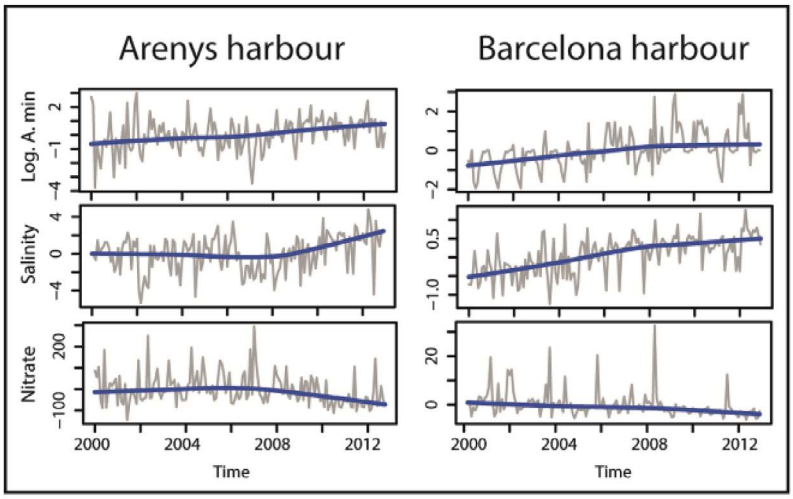

Figure 12. Anomaly (grey) of Log *A. minutum* abundances (top), salinity (center) and nitrate (bottom) in Arenys Harbor (left) and Barcelona Harbor (right) and the Lowess smoothing showing the trend (blue).

vegetative cells of the *A. kutnerae* morphotype have never been observed in samples from the monitoring program, we cannot be sure of the presence of this species along the Catalan coast. Given the worldwide absence of *A. kutnerae* sequences, Penna et al. (2008) suggested that the ME clade (now *A. mediterraneum*) constitutes *A. kutnerae*, although contamination of the strain cannot be ruled out. On the other hand, Busch et al. (2016) detected *A. mediterraneum* and *A. catenella* (the latter following Fraga et al., 2015 but referred to as *A. fundyense* by Bush et al., 2016) in Fangar Bay (northern Ebre Delta) by 454 pyrosequencing of LSU rDNA from environmental DNA. In the case of *A. catenella* (Group I), this would represent the first detection of the species in the Mediterranean Sea. However, given that the taxonomic resolution of short reads is limited at the species level and 454 pyrosequencing technology is relatively error prone (Luo et al., 2012), there are not reliable evidences of its presence and detection of the species should be confirmed by other techniques.

The number of *Alexandrium* species in a specific harbor over the course of the study varied from two to seven, depending largely on the number of samples collected at each one (data not shown). Thus, we are aware that neither the number nor the distribution of *Alexandrium* species in Catalan coastal waters

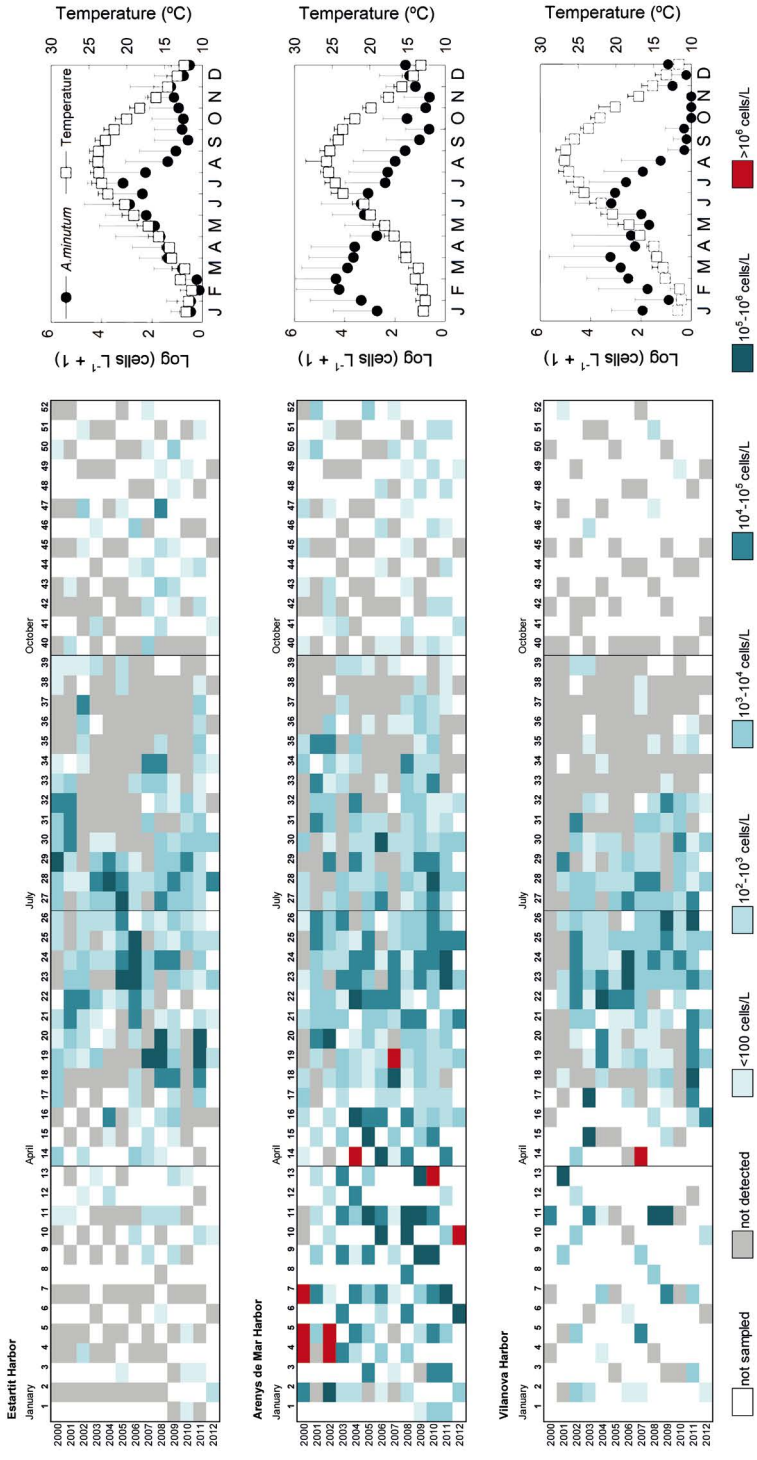

Figure13 . Graphical summary of the occurrences of *A. minutum* during the 13-year study period (left) and annual cycle of *A. minutum* abundances and Temperature (right) in Estartit (A), Arenys de Mar (B), and Vilanova (C) harbors.

could be fully determined, and a larger number of samples may yield more definitive results.

4.2. Spatial and temporal distribution

Most of the species reached bloom densities in the confined waters sampled, which confirmed that harbors and bays are prone for the development of *Alexandrium*.

According to the spatio-temporal distributions observed, *Alexandrium* species in confined waters can be separated in settled or occasional species. The settled species are frequently found at high abundances producing recurrent blooms, some of the species in a high number of different harbors (such as

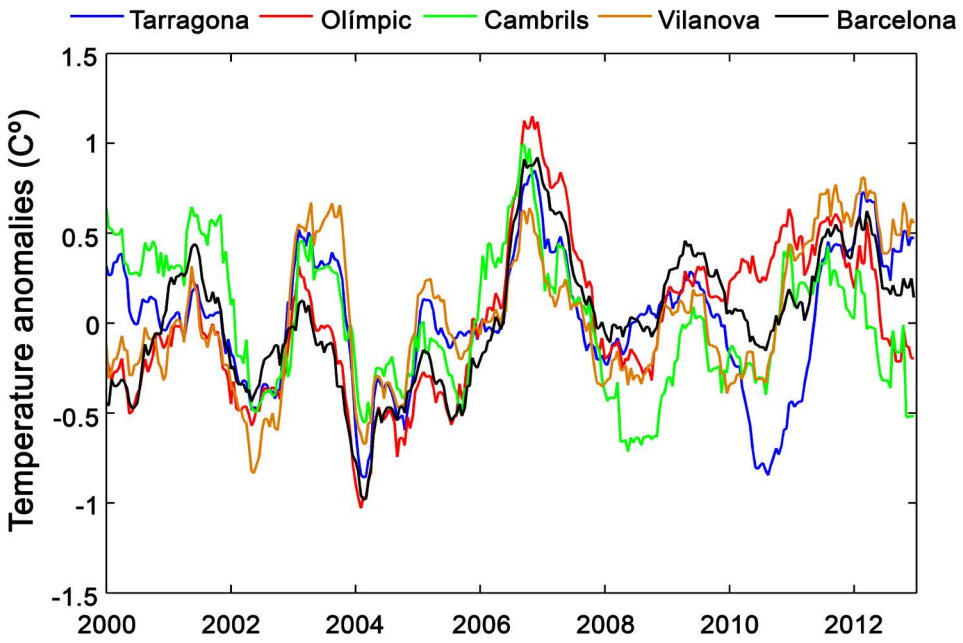

Figure 14. Water temperature anomalies in Barcelona (red), Tarragona (dark blue), Olímpic (green), Cambrils (light blue), and Vilanova (orange) harbors. Data were fitted to a smoothed curve.

A. minutum) and other species just in one or few specific harbors (such as *A. pacificum* and *A. taylori*). Probably, while the former species are well adapted in general to the conditions of this habitat, the latter are adapted to some particularity or particularities of a specific harbor. The meroplanktonic life cycle of most *Alexandrium* species includes resting cysts apart from pellicle cysts. The benthic resting stages enable survival during unfavourable conditions and provide an inoculum for bloom initiation. The recurrent annual blooms of *A. minutum* and *A. taylori* along the Catalan coast have been associated with cyst germination (Anglès et al., 2012; Garcés et al., 1999). Cysts can be produced in large quantities during blooms, resulting in a cyst bed in the sediments of the affected area that provides a long-term source of cell inoculum favouring the establishment and recurrence of the species in the area.

The occasional species appear at a certain moment, sometimes in bloom abundances (irregular blooms), and the following years they often appear in the same place with less intensity until they disappear. This appearance can be synchronized along much of the coast (such as *A. insuetum* or *A. margalefi*) or can be located in a particular place (e. g. *A. pseudogonyaulax* or *A. tamutum*). These irregular pulses are probably induced events, often occurring as responses to short-term climatic events or other abiotic factors that produce a temporary modification of the ecosystem (Winder and Cloern, 2010). In the case of synchronized events, the inducing factors would be regional scale factors, while in the case of local events would be local factors.

Spatially, most of the species identified in this study occurred in different harbors along the entire coast, although species such as *A. pacificum* and *A. taylori* were found in more restricted adjacent areas (south and north, respectively). Whether each species has been introduced in each locality simultaneously due to the entry of an extensive proliferation from offshore or there is a contagious distribution due to the relatively short distances (mean of ~10 km) between the harbors that may have facilitated species dispersal is a hypothesis to be tested.

In the following, we discuss the spatio-temporal distributions of the two most important *Alexandrium* species present in confined waters. We also explore this distribution in relation to environmental conditions.

4.2.1 Distribution and abundances of *Alexandrium* over time

A. pacificum

One of the main questions explored in our long-term investigation was whether *Alexandrium* species had expanded along the Catalan coast over the 13-year period and whether the hypothesis proposed by Vila et al., (2001b), regarding the expansion of. *A. pacificum*, is still valid with the new contributed data. After the first bloom report of *A. pacificum* (previously reported as *A. catenella*) in coastal Mediterranean waters in 1994, in Valencia Harbor (southernmost Catalonia), this species was detected in diverse localities in other Mediterranean coastal localities (Abadie et al., 1999 (as *A. tamarense*); Gomis et al., 1996; Luglie et al., 2003; Mikhail, 2001; Vila et al., 2001b). Vila et al. (2001b) described the rapid expansion of this toxic dinoflagellate along the Catalan coast between 1995 (when *A. pacificum* was not detected) and 1999, when it was detected in nine sampling stations, including three where it reached bloom abundances (> 10^4 cells L^{-1}). In Barcelona Harbor, these blooms were recurrent over the 4-year period from 1996 to 1999, during which those authors detected *A. pacificum*. Even after taking into account the reduction in the number of sampling stations since 2005, our 13-year data series covering 2000 to 2012 clearly showed that, after an initial period of expansion along the Catalan coast, the trend in the distribution of *A. pacificum* reversed, with the exception of Tarragona Harbor (which was not monitored regularly before 2000) where recurrent blooms were documented. In Barcelona Harbor, *A. pacificum* cell abundances > 10^4 cells L^{-1} were not detected after 1999, and after 2004 were at least below the detection limit of our methods. Vila et al. (2005) suggested that the change in phytoplankton community composition determined in Barcelona Harbor from 1996 to 2003 was in part due to the environmental restoration carried out at this site from 2000 to 2003 (reduction of sewage inputs and the addition of a second harbor mouth). However, this would contrast with the conclusions reached by the authors of other studies in which the first records and increasing of *A. pacificum* (as *A. catenella* or/and *A. tamarense*) coincided with a reduction of nutrient concentration (Hadjadji et al., 2014; Lugliè et al., 2003). Despite the very large decrease in the cell abundances of *A. pacificum* coinciding with the changes in Barcelona Harbor in 2000 and the low abundances of *A. pacificum* in the following years, a

weak tendency of increased abundances was observed from 2001 until 2003 (**Figura 9**). However, very low abundances were observed in 2004 and beginning in 2005 there were no further observations of this species. In 2004 and 2005, reduced abundances were also noted at the harbors in Vilanova, Tarragona, and Cambrils, while at Olímpic Harbor the last observation was in 2003. This suggests that a global climatic factor negatively affected the dynamics of *A. pacificum* in 2004-2005. Unusual climatological conditions across the entire region were reflected by the anomalies in the water temperature of these harbors (**Fig. 14**)—where a colder period occurred from roughly 2004 to 2006 at all five of them, followed by a warmer period—and may have affected *A. pacificum*. In the winter of 2004 and 2005, the unusually cold, dry, windy weather in the NW Mediterranean (Font et al., 2007) resulted in hydrological changes, giving rise to the Western Mediterranean Transition (WMT) (Schroeder et al., 2013). The spring of 2004 was also unusually cold in Catalonia (http://www.meteo.cat/wpweb/climatologia). Nonetheless, these unusual climatological conditions in 2004 and 2005 do not explain the earlier disappearance of *A. pacificum* from Olímpic Harbor. Therefore, other factors, including an as yet undescribed internal cycle of the species, must be explored to account for its decline in Catalan harbors.

A. minutum

Our long-term time series revealed trends suggesting an expansion of *A. minutum* in Catalan confined coastal waters. The presence of *A. minutum* in these waters was first detected in 1989, when a bloom occurred in Sant Carles de la Ràpita Harbor (Delgado et al., 1990). Thereafter, from 1995 to 1999, *A. minutum* was detected by Vila et al. (2001a) in all 11 harbors also sampled in the present study, including 7 in which bloom abundances (≥ 10^4 cells L^{-1}) were reached. In the other four harbors without documented blooms and included both in our study and in that of Vila et al. (2001a), *A. minutum* was detected as late as 2012, but only in Empuriabrava Harbor did it fail to reach bloom abundances. This observation, the increasing percentage of blooms recorded in this study, and the positive trend of *A. minutum* abundances observed during the study period in six harbors (four of them significantly) evidenced, at least until 2012, the increased abundance of this dinoflagellate in part of the harbors. Our results also showed that this species remains

widely distributed along Catalan confined waters demonstrating that it is well adapted to this environment.

What could explain the increase of *A. minutum* in some harbors?

The effect of climate change on phytoplankton in general and in specific species is still uncertain. In this work, not all harbors where abundances of *A. minutum* increased significantly showed an increase of temperature. Chl-*a* did not show an increasing trend in the harbors, and it even showed a decreasing trend in Olímpic Harbor where water temperature showed an increasing trend. Therefore, it does not seem that an increase in temperature is the cause of the increase of *A. minutum* nor is related to an increase in chlorophyll.

Changes in nutrient loads in coastal ecosystems have been related to changes in phytoplankton biomass or community structure (e. g. change of dominant species, Nishikawa et al., 2010). The increase of HABs has been linked to the eutrophication of the coast (e. g. Lancelot, 1995; Trainer et al., 2007). Contrary to this idea, in this work we found an increasing trend of *A. minutum* abundances in harbors that showed a process of oligotrophication as a result of a decrease of nitrate-loaded freshwater (and other nutrients in the more urban harbors) inputs. This decrease of nitrate is not reflected in a reduction of chlorophyll (except for Olímpic Harbor).

Nevertheless, none of the abiotic factors discussed above provide a clear explanation of the increase of *A. minutum* in some Catalan harbors. An alternative determinant could be the presence of a large cyst bed in these harbors. Cysts have been shown to have an important role in the bloom dynamics of *A. minutum* (Anglès et al., 2012; Estrada et al., 2010). This large cyst bed would enable the presence of cells in the water column throughout the germination period, that in the case of *A. minutum* is all year. The continuous presence of cells would increase the likelihood of bloom development under appropriate conditions.

In addition to trends, other long-term changes such as sudden shifts in phytoplankton community structure and abundance provoked by abrupt climatological changes (eg. Borkman and Smayda, 2009) could be occurring. Unlike *A. pacificum*, whose populations may have decreased or even disappeared partly presumably in response to the unusual climatological conditions in 2004–2005, *A. minutum* seems to have been impervious to these climatological conditions (**Fig. 9**).

The two species show independent responses to the same environmental variability. Accordingly, predictions of PSP events in an area must consider not only environmental changes, both local and on a larger scale, but also the species-specific responses of the PSP producers.

4.2.2. General considerations on species expansion

Before 2000, five *Alexandrium* species [*A. minutum*, *A. pacificum* (as *A. catenella*), *A. taylori*, *A. pseudogonyaulax*, and *A. tamarense*] had been detected in Catalan coastal waters (Delgado et al., 1999; Garcés et al., 1999; Vila et al., 2001a). At the end of our study, in 2012, 11 *Alexandrium* species had been detected. Of these, only *A. minutum*, *A. pacificum*, and *A. taylori* continued to cause recurrent, high-abundance blooms at the investigated stations. Although the Catalan monitoring program has included harbors since 1996 and Ebre bays since 1989, the presence of other *Alexandrium* species before 2000 cannot be ruled out. Whether species such as *A. andersonii* and *A. insuetum* have expanded into these waters or are newly introduced could not be determined because of the lack of verifiable information about their earlier presence or absence in these waters.

It is also important to note that, despite the general assumption of a worldwide expansion of HABs, this is not uniformly true. For example, *A. pacificum* populations along the Catalan coast have apparently regressed, even though confined and nutrient-replete areas such as harbors would seem to be highly favourable for the further establishment of this and other cyst-forming species. This observation highlights the importance of long-term data series, since shorter records may suggest that, for example, a species is expanding, whereas longer-term observations, while also incomplete, may indicate the opposite, as was the case of our data with *A. pacificum*.

Therefore, the increase of PSP blooms is not fulfilled at all scales of space and time and for all the species. It depends on the length of the time series, the localities sampled, not only in the adequate environment where the species can increase more easily, but also to sample with a good spatial resolution, and the species.

4.2.3 Annual pattern of *Alexandrium minutum*

The low abundances of some species hindered recognition of their annual patterns, but most species (except *A. minutum* and *A. andersonii*) showed a clear trend in the warmer months (May to August or September), when conditions are generally suitable for dinoflagellate growth. The annual cycle of *A. pacificum* in Tarragona Harbor is described in detail in Chapter 2.

The annual pattern of *A. minutum*, the most abundant species of *Alexandrium* in the confined waters of the Catalan coast, differed in each of the three harbors studied in detail. However, all three shared the low-level detection of *A. minutum* in autumn (as observed in the other harbors included in this study) and the high-level detection of this species from April to July. In their study of Arenys de Mar Harbor, Anglès et al. (2012), suggested that *A. minutum* undergoes continuous excystment, with more active excystment occurring during periods of increasing irradiance and water temperature (from the end of December to July). Blooms of *A. minutum* in this harbor occurred during these conditions, as described previously (Van Lening et al., 2007) and as demonstrated for this and Estartit and Vilanova harbors in this study. Moreover, the results of LMM also corroborate the significant relationship of these months with *A. minutum* abudances. However, blooms were frequent in Arenys de Mar, Estartit, and Vilanova harbors from April to July–August, but during the first 3 months of the year only in some of them. These different patterns may have been the result of local factors, such as wind regime, precipitation, or freshwater inputs that regulate the growth of vegetative cells during periods of intense excystment. Van Lening et al. (2007), in their study of *A. minutum* in Arenys de Mar Harbor, and Estrada et al. (2008), who studied this species in the Arenys de Mar and Olímpic harbors, noted the importance of a prolonged period of good weather for the blooming of *A. minutum*. Interestingly, in December 2015 and January 2016, blooms of *A. minutum* were detected in Vilanova Harbor [information provided by Departament d'Agricultura, Ramaderia i Pesca (DARP)] during an unusual winter of calm, dry conditions and warm temperatures provoked by a strong El Niño–Southern Oscillation. Additional meteorological studies at Vilanova and Estartit harbors are necessary to understand the relative contributions of climate conditions in the annual patterns of *A. minutum* at these sites.

The varying levels of sufficient or excessive nutrients according to seasons as a result of factors such as the flow of freshwater inputs could also influence the seasonality of the phytoplankton species. Hence, due to the negative relationship found between nitrite levels and *A. minutum* cell abundances, varying nitrite concentrations may explain the different annual patterns of *A. minutum* determined in the three harbors. For example, high concentrations of nitrite could inhibit the growth of *A. minutum*. Because nitrite is not normally included in growth experiments as an N-source, little information is available about the nitrite affinity and tolerance of *Alexandrium* species. Lu et al. (2011) tested nitrite concentrations ≥ 0.15 mM and showed that *A. minutum* failed to thrive when nitrite was the sole N source. Growth inhibition of dinoflagellates due to a high concentration of N substrates has been described largely for ammonium (e.g., Chang and McClean, 1997; Leong and Taguchi, 2004). In Catalan harbors without bloom events, nitrite concentrations (mean= 0.31 µM, max= 6.71 µM) are probably not high enough to inhibit the growth of *A. minutum*. The negative relation between *A. minutum* and nitrite need not necessarily be direct. Another possibility to explain this relation is that low nitrite conditions are favorable or high nitrite conditions are unfavorable for *A. minutum* development. In this scenario, high nitrite concentrations arising due to mixing in the water column would repress the proliferation of *A. minutum*, which needs calm waters for its growth. Further laboratory studies and more frequent field observations are necessary to understand the clear negative temporal correlation between *A. minutum* and nitrite dynamics.

In an effort to explain the temporal variability of phytoplankton species abundances, especially of toxic species in the coastal zone, variables such as concentrations of inorganic nutrients are typically measured along with the abundances of species. Usually non-causal correlations are established, which depend largely on the length of the series, but in some cases either the possible explanation for the correlations is unclear (for example the negative relationship between nitrite and *A. minutum*) or the correlations provide little information. Our results show that a significant portion of the abundance variability of *A. minutum* is explained by the seasonality. However, the seasonality of *A. minutum* is different in each harbor, which suggests that local factors not considered in this study would explain most of the predictable variability of this organism in confined areas.

4.3. Public health and environmental implications

During the 13-year study period, *A. minutum* and *A. pacificum* blooms led to the closure of several shellfish harvest zones, for a total of 111 and 86 days, respectively (information provided by DARP). These species had been detected at high abundances along this coast before 2000. But, while there was not further increase in proliferation of *A. pacificum* and the species is no longer found in many harbors, the frequency of *A. minutum* blooms has doubled. Moreover, because this species is very well adapted to anthropogenic habitats, the frequency of bloom events in these environments can be expected to increase further.

Neither *A. andersonii* nor *A. ostenfeldii* were reported at the investigated stations of the Catalan coast prior to this study. *A. andersonii* was initially considered to be a toxic species in the Mediterranean Sea (Ciminiello et al., 2000) but recent studies suggest that it does not pose a danger for human health (Sampedro et al., 2013; Stüken et al., 2011) (Table 2). However, strains of *A. ostenfeldii* (as *A. peruvianum*) isolated in the Catalan coast produce spirolides (Franco et al., 2006), although PSP toxins were not detected (Kremp et al., 2014). The latter have been described in other phylogenetic subgroups of *A. ostenfeldii* present in other areas of the world (Kremp et al., 2014). This species was first detected along the Catalan coast in 2003, in cyst stages isolated from sediments of Palamós Harbor (Bravo et al., 2006), but vegetative cells were not observed in the water column until 2005, in Estartit Harbor, and have never been observed in Palamós. The maximum cell abundances of *A. ostenfeldii* detected in the confined waters of the study locations during our 13-year investigation were low and were reached infrequently; but whether this species poses a future threat remains to be seen.

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7. Supplementary material

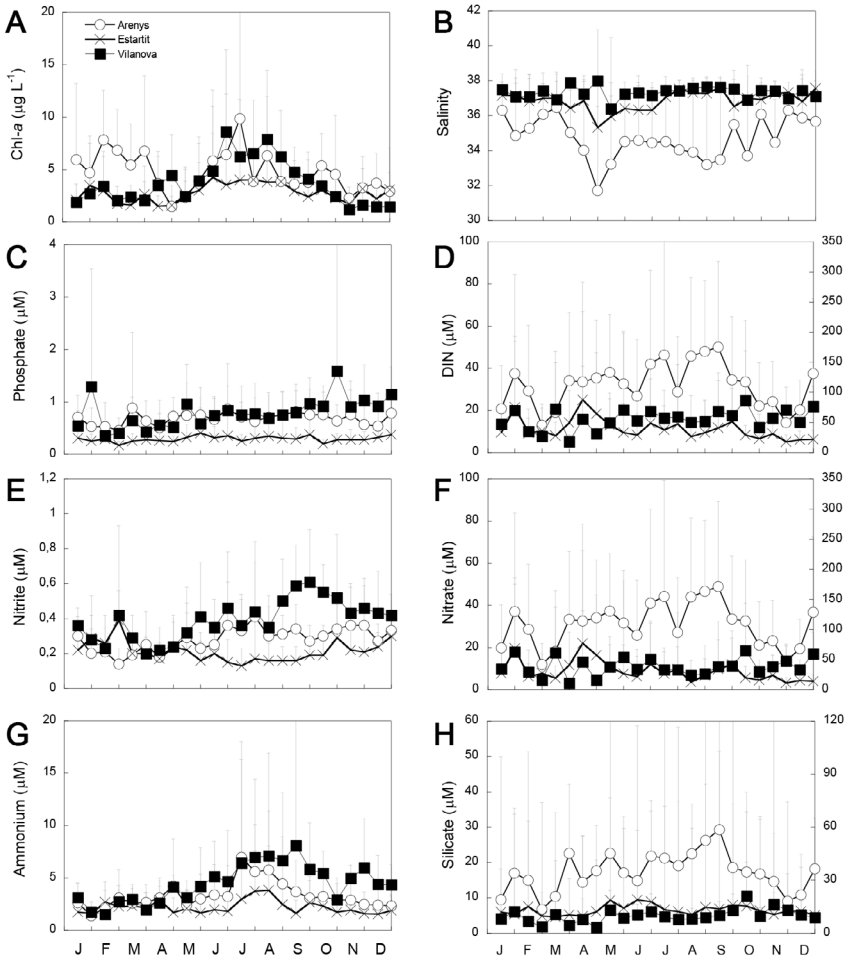

Figure S1. Annual patterns of biological and physico-chemical parameters in Estartit, Arenys de Mar and Vilanova harbors with recurrent blooms of *A. minutum*.

Table S1. Descriptive analysis of environmental and biological data from harbors.

	Mean	Std	Min	Q1	Median	Q3	Max	N
Temperature (ºC)	19,97	5,07	6,5	15,2	20,9	24,3	32	4219
Salinity	36,46	2,63	10,4	36,2	37,3	37,8	38,4	3929
Chl-*a* (µg/l)	4,23	10,87	0,04	1,2	2,34	4,36	383,57	3742
Nitrate (µM)	23,92	60,2	0,01	2,61	5,83	16,02	904,47	3745
Nitrite (µM)	0,31	0,27	0,01	0,14	0,24	0,39	6,71	3745
Ammonium (µM)	4,32	16,41	0,01	1,42	2,57	4,43	896,61	3745
DIN	28,55	64,84	0,15	5,41	10,14	21,99	1392,83	3745
Phosphate (µM)	0,51	0,84	0	0,21	0,36	0,6	31,46	3745
Silicate (µM)	10,09	16,06	0,01	2,28	4,55	9,97	174,57	3745

Chapter 2

Alexandrium pacificum bloom dynamics over 11 years in two anthropic Mediterranean coastal environments

Harmful Algae, in preparation

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Abstract

The toxic dinoflagellate *Alexandrium pacificum* (group IV) reaches bloom abundances every year in two enclosed coastal environments in the NW Mediterranean Sea: Tarragona harbor (Spanish coast) and Thau lagoon (French coast). Using an 11-year data series (2000–2010) from these localities, we studied the annual pattern, seasonal variability and trends of *A. pacificum*. We also studied the different patterns of variability of this organism along with environmental variables. While no trend was detected in both series for the period studied, the interannual and seasonal variability were different in both locations, highlighting the importance of the local factors for the bloom development. In addition, bloom conditions of *A. pacificum* in the study areas were defined and the environmental requirements suggested in the literature were discussed. Abundances higher than 10^4 cells L^{-1} of this species were found in a wide range of temperature (11.8 – 29.9ºC) and inorganic nutrients such us ammonium (0.19 – 92.72µM), and nitrate (0.02 – 44.03 µM).

1. Introduction

Toxic phytoplankton blooms are widely spread phenomena occurring in the coastal waters of the world's oceans. Several microalgae produce toxins that can affect human health through different pathogenic routes (Moestrup, (2009 onwards); Van Dolah, 2000). Beginning in the 1990s, most developed countries implemented monitoring programs as part of their efforts to control the occurrence of harmful algal blooms (HABs). Several dinoflagellate genera are involved in human intoxications. Among them, the genus *Alexandrium,* which includes some neurotoxins producer species*,* is one of the most common causes of paralytic shellfish poisoning (PSP) events. *A. pacificum* (Litaker) is one of this toxic species. Its name was recently assigned by John et al. (2014) to the group IV ribotype of the *Alexandrium tamarense/catenella/fundyense* species complex (Lilly et al., 2007 nomenclature). Since it was first detected (as *Gonyaulax catenella*) in Japan in 1975 (Hashimoto et al 1976), this chain-forming species has become common in the temperate waters of Japan, Korea, China, Australia, and New Zealand, with PSP shellfish contamination consistently reported (Farrell et al., 2013; Gu et al., 2013; Lilly et al., 2007; MacKenzie et al., 2004; Scholin et al., 1995).

In the late 1990s, blooms of the *Alexandrium tamarense/catenella/fundyense* species complex were recorded for the first time in the northwest Mediterranean Sea (Abadie et al., 1999; Gomis et al., 1996; Lugliè et al., 2003; Vila et al., 2001). Since then, several events characterized by PSP toxicity in shellfish of the Mediterranean coast have been attributed to *A. catenella* and they resulted in the closure of bivalve aquaculture areas for several weeks (Lugliè et al., 2011; Masselin et al., 2001; Sampedro et al., 2008).

Genetic analyses of *A. catenella* morphotype strains isolated from two harbors of the Catalan coast (NE Spain), both from vegetative cells (blooms in 1998, 2002 and 2003) and from cysts (collected in 2001 and 2002), identified this recurrent species as "*A. catenella*" (group IV) (Penna et al., 2008; Penna et al., 2005), now referred to as *A. pacificum* following the nomenclature of John et al. (2014). This species has been detected at very high abundances in several harbors along the Catalan coast, including those of Barcelona and

Tarragona (Vila et al., 2001), but only in the latter has this species continued to form recurrent blooms, based on data from regular monitoring of this harbor, which started in 2000 (Chapter 1).

A. pacificum has bloomed in Tarragona Harbor at least since the summer of 1998, as determined in sporadic samplings carried out during a water discoloration episode caused by this species that coincided with widespread blooms of *A. pacificum* in non-confined coastal waters along 100 km of coastline (Vila et al., 2001).

Along the French coast, recurrent blooms of *Alexandrium* belonging to the "*tamarense/catenella/funyense* species complex" have been observed in Thau Lagoon since 1995 (Abadie et al., 1999). *A. tamarense* (Lebour) Balech was identified as the causative organism of the bloom that occurred in 1998. Thereafter, the existence of *A. catenella* belonging to the Temperate Asian ribotype reported by Scholin et al. (1995) (included in group IV of Lilly et al., 2007) in Thau Lagoon was demonstrated by the analysis (RFLP and sequence analysis of LSU rDNA) of two clonal cultures established from the 1998 bloom (Lilly et al., 2002). The latter authors pointed out that both species were present in the 1995 and 1998 blooms. In 2008, co-occurrence of the non-toxic species *A. tamarense* (belonging to group III) and the toxic species *A. catenella* (group IV; now *A. pacificum*) in Thau Lagoon were demonstrated by sequencing the ITS1-5.8SrDNA-ITS2 nuclear ribosomal DNA region of monoclonal strains isolated from bloom events that occurred in the spring and autumn of 2007 in Angle Creek (northeast part of Thau Lagoon) (Genovesi et al., 2008; Genovesi et al., 2011). But the relevance of each species of the *tamarense/catenella/fundyense* species complex over the Thau Lagoon time series produced by the monitoring program was not resolved.

The emergence of problems related to *A. pacificum* blooms in the Mediterranean Sea and other areas in the world led scientists to study the ecology and physiology of this species in order to elucidate its population dynamics and the conditions promoting its blooms. From the results of these studies, the different physico-chemical and meteorological parameters favoring the development of *A. pacificum* blooms in the Mediterranean Sea were proposed. For instance, in Thau Lagoon it was suggested an initial wind stress (>4 m s^{-1}) is needed to resuspend *A. pacificum* cysts buried in the sediment, followed by a calm period and water temperatures close to 20°C to promote blooms of this organism

(Laanaia et al., 2013). In previous studies of *A. pacificum* in Catalan harbors, Vila et al. (2001) related bloom development to water temperatures >20°C. Studies based on 4-year data of the occurrences of blooms in the same area showed an association with high inorganic nutrient levels, specifically nitrate and ammonium (~50 and 20 µM, respectively) (Bravo et al., 2008). In addition, in their study conducted at Annaba Bay (Algeria) and Thau Lagoon, covering a year before an *Alexandrium* bloom (1992) and two years of bloom formation, 2002 (the first bloom to occur at Annaba) and 2010 (a major bloom at Annaba), Hadjadji et al. (2014) associated the emergence of *Alexandrium tamarense/ catenella* at both sites to a decrease in the concentration of soluble reactive phosphorus (SRP) to <1 µM.

However, field studies are often based on short time series and/or a time series from a single location. Therefore, their results are restricted to a short temporal window and the place studied. Comparative studies of the population dynamics of a single species but at different habitats and of the environmental variables at each one are needed to better understand the mechanisms and factors that ultimately lead to bloom development. With the implementation of monitoring programs and thus the availability of relatively long (>10 years), multiannual time series of the abundances of HAB species, these types of comparative studies can be conducted at different time scales (annual, inter-annual, and long-term variations) and the factors responsible for the identified patterns explored.

Here we analyze an 11-year time series of *A. pacificum* abundances and the physico-chemical, biological, and climatic variables of the two confined habitats in the northwest Mediterranean Sea where this species has been recurrently detected for several years: a harbor located on the Spanish coast and a lagoon on the French coast (Chapter 1). The objectives of the study were: (1) to describe and compare the different variability patterns of *A. pacificum* at the two sites to elucidate the mechanisms and factors driving the dynamic of the species and (2) to define the conditions supporting blooms of *A. pacificum* in two different Mediterranean habitats and thereby obtain a broader view of the requirements of this species to form blooms. Finally, we discuss the factors that may contribute to variations in *A. pacificum* abundance, including those suggested in the literature.

2. Methods

2.1. Study area

A. pacificum blooms were monitored over an 11-year period (2000–2010) at two target sites, Tarragona Harbor and Thau Lagoon, both located on the northwest Mediterranean coast. In this study, we define as "bloom" as cell abundances >1 $\times$ 10^4 L^{-1}. The two sites are confined and affected by high human pressure. Tarragona Harbor is situated on the Catalan coast (NE Spain) at 41º 51'N and 1º 13' E (**Fig. 1**). It is one of the largest (4.5 km long; 600 m to 2 km wide; extension ~5 km^2= 500 ha; maximum depth: 26 m) and most active Mediterranean harbors, with considerable commercial (mainly petroleum-related products) and fishing activities. Its longitudinal axis is oriented in a northeast direction with a southern entrance protecting the inside waters from the dominant northeastern and eastern wave storms. The water depth inside the harbor is in the range of ~10–20 m. South of the harbor is a 3-km long stretch of sandy beaches that ends at the prominent Cape Salou. The harbor receives the direct input of high levels of dissolved inorganic nutrients from the Francolí River. This relatively short river (85 km) ends near the harbor mouth (inner side) (Fig. 1) and is characterized by large variations in its flow rate throughout the year, with the maximum flows occurring in autumn and winter. However, the amount of river flow is usually low (average: 0.9 m^3 s^{-1}, median: 0.5 m^3 s^{-1}) and often either absent or characterized by occasional discharges from late spring to late summer. Thau Lagoon is a shallow marine lagoon located on the French Mediterranean coast (43º 22' N, 3º 35' E) and covering 75 km^2 (**Fig. 1**). It has a mean depth of 4 m and a maximum depth of 10 m. The lagoon is connected to the sea by three narrow channels and receives freshwater from several streams and two channels, the Canal du Midi and the Canal du Rhône a Seté, which connects the Rhône River with the lagoon. Three oyster farming zones are located along the northwestern shore and account for 10% of French oyster production. In fact, these zones comprise the main oyster production center in the Mediterranean. However, since 1998, recurrent blooms of the *A. tamarense/catenella/fundyense* complex

have periodically threatened economic activities at the lagoon. The blooms consistently originate in Angle Creek, a shallow embayment located in the northeastern part of the lagoon.

2.2. Sampling in Tarragona Harbor and Thau Lagoon

The sampling site in Tarragona Harbor was situated in the innermost part of the harbor (**Fig. 1**). Samples were obtained routinely during the 11-year period, either weekly (from May to October) or fortnightly (from November to April). Bucket samples were taken from the pier for phytoplankton, chlorophyll (Chl), and dissolved inorganic nutrient analyses. Associated temperature and salinity were measured at the surface using a microprocessor conductivity meter WTW Model LF 197.

The sampling point in Thau Lagoon (station A5) was located in Angle Creek (**Fig. 1**). Phytoplankton, sea surface temperature, salinity, and inorganic nutrients (2002–2003) were routinely sampled weekly. Due to the absence of Chl samples in station A5, Chl data sampled fortnightly from station Bouzigues (Bouz) were used instead.

Chlorophyll: Water samples of 60 mL (Tarragona Harbor) or 750 mL (Thau Lagoon) were filtered through glass-fiber filters (GF/F) and frozen at –20°C until the analysis. Tarragona Harbor samples were extracted in 8 mL of 90% acetone for 48h at 4°C. Chl-*a* was measured using a Turner Designs fluorometer (Yentsh and Menzel, 1963). For Thau Lagoon samples, Chl-*a* was measured within a few weeks by spectrofluorometry (Neveux and Lantoine, 1993).

Nutrients: The nutrient sampling methodology differed for the two sites. At Thau Lagoon, 60-mL water samples were filtered water through GF/F. At Tarragona Harbor, the 60-mL water samples were immediately frozen (–20°C) until nutrient quantification. The dissolved inorganic nutrient (NO_3, NO_2, NH_4, PO_4 and SiO_4) concentrations were measured as described by Grassohoff et al. (1983), using an Alliance Instruments Evolution II autoanalyzer (samples collected from 2000 to 2007) or a continuous flow analyzer AA3 (Bran+Luebbe; samples collected from 2007 to 2010). For Thau Lagoon, the nutrients in samples from 2002–2003 were analyzed. Samples for ammonium (NH_4) determination were fixed onboard and measured using the method of

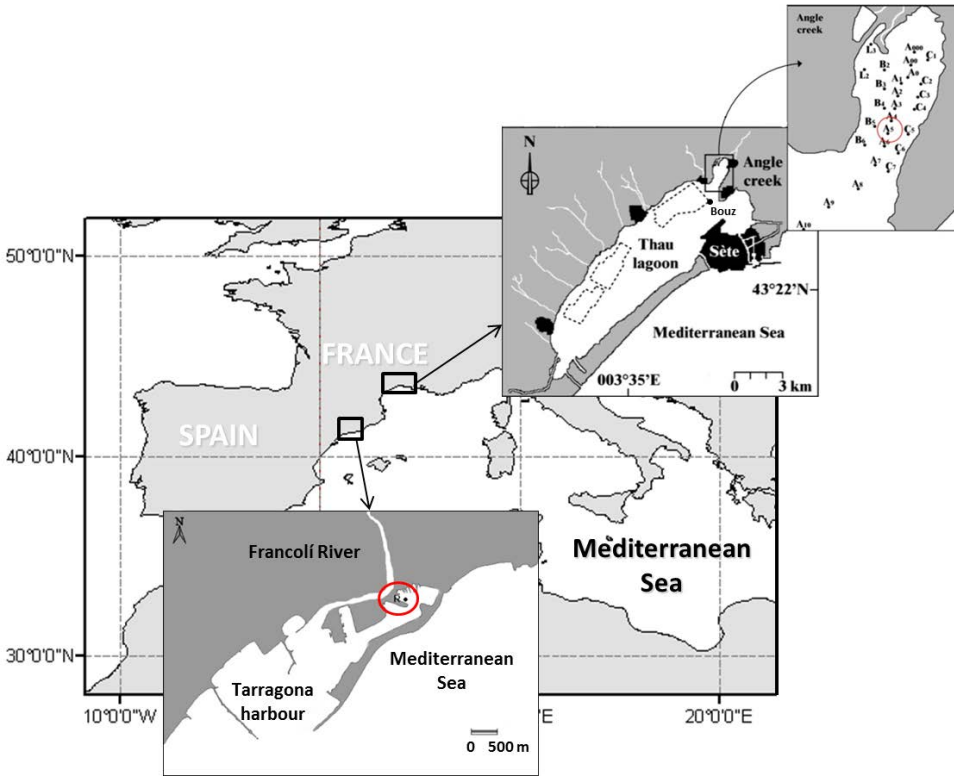

Figure 1. Study areas and sampling locations (circles).

Koroleff (1976). The precision of the measurement was 5% and the detection limit 0.05 µM. All samples were filtered and stored within 1 h of sampling. The contents of the sampling bottles were pre-filtered through a 200-µm screen to remove large zooplankton, stored in acid pre-cleaned polycarbonate bottles, and then transported to the laboratory for filtration in an all-glass filtering system with a vacuum under 10 cmHg. The samples were filtered through a pre-combusted (450°C for 6 h) Whatman GF/F and the filtrates frozen immediately at –20°C in pre-combusted Pyrex flasks. The analyses were performed within 2 months (Macdonald and McLaughin, 1982). After thawing the samples gently at ambient temperature, SRP, nitrate (NO_3), and nitrite (NO_2) were measured. For the determination of reactive silicate (Si), filtrates stored at 4°C in polycarbonate bottles were analyzed within a few weeks. SRP, NO_3, NO_2, and Si were measured in triplicate using a Technicon AutoAnalyzer II (Tréguer and Le Corre, 1975) with a precision of 1%.

Potential nutrient limitations for autotrophic phytoplankton were estimated following the criteria of Justic et al. (1995). The criteria of probable limitation were as follows: P limitation [P <0.1 μM; dissolved inorganic nitrogen (DIN): P >22; Si: P >22]; N limitation (DIN <1 μM; DIN: P <10; Si: DIN >1) and Si limitation (Si <2 μM; Si: PO_4 <10; Si: DIN <1).

Phytoplankton: Phytoplankton samples (150 mL) from Tarragona Harbor were preserved in Lugol's iodine solution. For observation and enumeration, a 50-mL aliquot was settled in a counting chamber for 1 day. For phytoplankton enumeration, the contents of at least half the chamber were examined, scanning the appropriate area at 63–400× magnification using a Leica-Leitz DM-IL inverted microscope (Throndsen, 1995), depending on the cell density of each sample. A minimum of 30 cells were counted, when possible. The minimum concentration detectable by this method was 20 cells L^{-1}.

Thau Lagoon phytoplankton samples (20 mL) were preserved in 2 mL of 40% formaldehyde and kept in the dark at 4°C until their identification using an optical Olympus IMT 2 light inverted microscope according to the method of Utermöhl (1958). The samples were allowed to sediment in 10-mL counting sedimentation chambers for a minimum of 8 h. For phytoplankton enumeration, the contents of the whole chamber were observed. Depending on the phytoplankton concentration, a 10× or 20× lens was used for enumeration. The detection limit was 100 cells L^{-1}.

The phytoplankton time series from Tarragona Harbor was generated as part of the Catalan Harmful Phytoplankton Monitoring Program and the time series from Thau Lagoon within the Ifremer REPHY monitoring program. A problem in the comparative exploration of the two series was that *Alexandrium* species from Thau Lagoon were determined by light microscopy assessments of cell size and morphology rather than on the morphological characteristics of the thecal plates. Light microscopy alone does not allow species with similar morphological features to be distinguished. At the time this study was planned, we were concerned about the identity of *Alexandrium* species in the Thau Lagoon time series because of the co-existence in the lagoon of two similar species: *A. tamarense* and *A. pacificum* (reported as *A. catenella*) (Genovesi et al. 2011). These two species belong to the *Alexandrium tamarense/catenella/fundyense* species complex and are difficult to separate using only bright-field microscopy. Additional observations using epifluorescence microscopy

together with Calcofluor staining reveals the characteristics of the thecal plates that are important for discriminating these two species: the observation of the first apical plate and the presence or absence of the ventral pore (Vp).

To specifically determine *A. pacificum* abundances in Thau Lagoon and compare them with those in Tarragona Harbor, 55 samples from the Thau Lagoon time series, most of them obtained at bloom abundances, were re-examined using the Calcofluor technique. The majority of the re-examined samples were from the routine A5 sampling site, but some of them were from other Angle Creek stations close to A5 (**Fig. 1**). Thus, all blooms detected during the 11-year period (2000–2010) were included, with the exception of the bloom event in August 2007, for which there were no samples. The morphologies of 50 cells per sample were checked for their assignment to one or the other *Alexandrium* species. If <50 cells were present in the sample, all of them were checked. If necessary, a needle was used to turn the cells to a position that allowed observation of their taxonomic characters.

2.3 ***Alexandrium*** **identification**

Alexandrium species were identified based on cell shape and size, chain formation, and the characteristics of the thecal plates (Balech, 1995) after staining the samples by the addition into the chamber of a few drops of the fluorescent dye Calcofluor White M2R (final concentration 10–20 mg mL^{-1}; Fritz and Triemer, 1985). The stained cells were examined at 400× or 630× magnification using an inverted microscope under UV excitation fluorescent illumination.

A. catenella and *A. tamarense* have long been differentiated based on their ability to form chains and the absence or presence of a Vp along the margin between plate 1' and 4' (observable after Calcofluor staining) (Balech, 1995). *A. catenella*, in contrast to *A. tamarense* was described as a chain-forming species without a Vp. However, the validity of these characters has been questioned in recent years, and advances in genetics have shown that strains belonging to the same phylogenetic group (following John et al., 2014, each group is assigned to a different species) may vary with respect to these characters. As recently described by John et al. (2014), the Vp is generally not present in *A. pacificum* and many of the strains belonging to group IV

lack a Vp. In the emended description of *A. tamarense* (group III), the authors reported that a Vp is generally present. Therefore, the absence of a Vp in *A. pacificum* and its presence in *A. tamarense* are not always reliable features, but the opposite is seen only rarely. The same authors noted that *A. tamarense* cells are almost always single and rarely found in two-cell chains in culture, whereas in field samples *A. pacificum* cells can be arranged in chains or as single cells.

The variable morphological characters, such as the presence/absence of a Vp within the same species in some species belonging to the *Alexandrium* genus, is geographically specific (Gu et al., 2013) and may thus be useful to distinguish different species in a particular area. Indeed, in their analysis of 52 strains established from water and sediment samples collected from Thau Lagoon in 2004 and 2007, Genovesi et al. (2011) showed a correspondence between the ribotypes of *A. tamarense* (group III) and *A. catenella* (group IV) and morphospecies designations based on Vp presence /absence and chain formation. This relationship reinforces the utility of the presence/absence of the Vp as a morphological character to distinguish *A. tamarense* from *A. pacificum*, respectively, in samples from the northwest Mediterranean Sea. In the present study, these characters were used to distinguish the two species.

2.4. Meteorological parameters

Wind is one of the factors that most commonly and immediately impact phytoplankton dynamics in coastal ecosystems, as it relates to mixing and circulation (Zingone et al., 2010). Moreover, it has also already been associated with *A. pacificum* bloom dynamics in the Mediterranean (Laanaia et al., 2013). Accordingly, in this study wind speed were determined at the two study sites.

Daily mean measurements (at 10 m high) were made from 2003 to 2010 by Servei Meterologic de Catalunya from a permanent station located in Torredembarra, 14 km north of Tarragona Harbor. The same variables from Sète for the period 2000–2010 were provided by MeteoFrance. For graphical representation with *A. pacificum* cell abundances, the daily mean wind speed data were pre-filtered by assuming that daily means wind speeds of <4 m/s (considered as a calm) were equal to 0.

2.5. Statistical analyses and graph trends

To explore the different variability patterns of *A. pacificum*, the periodic components of the variability in abundance were extracted from each time series using Fourier spectral analysis and wavelet analysis. The former allowed the identification of characteristic periods in the time series of *A. pacificum* cell abundances at both locations, and the latter a determination of how the periodic components of the time series changed over time. Time series data were pre-processed before their use in the Fourier decomposition algorithm (Fast Fourier Transform code in Matlab R2009a). The pre-process comprised two steps: first, a linear interpolation was applied to obtain a constant time sampling interval in the two time series. Second, the series were normalized (dividing by the standard deviation) and detrended (subtracting the trend). Wavelet analysis was used to visualize periodicities in *A. pacificum* cell abundances and the timing of those periodicities in the two locations. Matlab functions developed by Torrence and Compo (1998) and Grinsted et al. (2004) were applied. Morlet wavelet was used as the wavelet base function. Prior to the wavelet analysis, the data were log-transformed, the monthly averages calculated to obtain a constant time step, and the results detrended.

To study the different components of the variability in greater detail, we determined the seasonality, trends, and inter-annual variability of both the *A. pacificum* abundances and the environmental variables as follows:

To reveal the seasonality of the time series, the mean annual cycles of the biological (*A. pacificum* abundances, Chl-*a*) and environmental (temperature, salinity, inorganic nutrients) variables were determined by calculating the fortnightly averages of data collected during the study period.

The Mann-Kendall (MK) trend test (Mann, 1945; Kendall, 1975) was performed on the target species and the environmental series to detect statistically significant trends. This non-parametric test for monotonic trends is typically used to detect a significantly increasing or decreasing trend in natural time series (e.g., hydrological or climatological), as the test is not affected by the actual distribution of the data and is less sensitive to outliers than parametric trend tests, such as the regression coefficient. We applied this test because only one of the data series anomalies was normally distributed according

to the Shapiro-Wilk's test. The initial assumption in the MK test is the null hypothesis (H0), which assumes that there is no trend in the data series over time. The alternative hypothesis (H1) is that there is a significant trend (increasing or decreasing) over time. The significance level of the test was set at $\alpha = 0.05$. Thus, at $P < 0.05$, H0 is rejected and there is a significant trend in the time series. Kendall's tau (τ) is a value between −1 and 1 that indicates the direction of the trend over time. An increasing trend is indicated by $\tau > 0$ and a decreasing trend by $\tau < 0$. The MK test (Kendall package in the R software, version 3.0.3; Team, 2015) was applied to the deseasonalized monthly mean series data obtained by subtracting the monthly global means from the monthly means, to better discern the trend in Y over time. The change per unit time in a time series having a linear trend was estimated by applying Sen's estimator of slope (Sen, 1968), a simple non-parametric procedure. Trends in the monthly anomalies in which both Kendall's τ was significant and the 95% confidence interval of Sen's estimator of slope did not include 0 were considered significant.

While the inter-annual variability of *A. pacificum* was assessed using the raw data, box plots were used to determine the inter-annual variability in the environmental variables. The variations in temperature and salinity over time were studied in greater detail by plotting the anomalies, while smoothing the data using a moving average filter with a span of 6 months based on the fortnightly temperature and salinity anomalies.

Finally, to analyze the relationship between the log of *A. pacificum* abundances and environmental variables, we established a linear mixed model (LMM) that took into account the repeated measures over time. A LMM is a generalization of the standard linear model; it allows the fitting of data in which a response variable has multiple measurements within the same experimental unit. Thus, these models take into account correlations among observations and the heterogeneous variance (Littell et al., 1996). In contrast to simple linear models, LMMs include both fixed and random effects, with parameters that are allowed to vary over individuals. The model established to analyze our data included, as fixed-effects, water temperature, salinity, Chl-*a*, and wind speed. The variable time was introduced as both a fixed and a random effect. For the latter, a structure of correlations among the 12 months of the year was assumed. To structure the variability, we used a covariance structure based

on ARMA (autoregressive moving averages). Inorganic nutrients were not included in the LMM because of the scarce data for Thau Lagoon. All results of the fitted models were obtained using the MIXED procedure of SAS v9.4, SAS Institute Inc., Cary, NC, USA. The significance level was fixed at 0.05. Since nutrients were not included in the LMM, Spearman's rank correlation was computed between *A. pacificum* abundances and inorganic nutrients using STATISTICA 10 software (StatSoft, 2011). Prior to the correlation analyses, all variables were log-transformed according to $v' = \log_{10} (v + 1)$.

3. Results

3.1. Morphological identification

Samples collected over the study period from Thau Lagoon contained both the *A. catenella*-morphotype, hereafter *A. pacificum,* and the *A. tamarense*-morphotype, hereafter *A. tamarense* (**Fig. 2**), together with other *Alexandrium* species (e.g., *A. minutum* and *A. pseudogonyaulax*), as determined by Calcofluor staining; however, almost all the blooms at this study site were caused by *A. pacificum*. Only in two cases were the proliferations not dominated by this species: during the bloom of September 2007, in which the abundances of *A. pacificum* and *A. tamarense* were similar, and in early June 2009, when *Alexandrium* abundances were still below bloom concentrations. The abundance of *A. tamarense* in the latter case was higher than that of *A. pacificum* (83% of *Alexandrium* cells belonging to *A. tamarense* vs. 16% to *A. pacificum*). By mid-June 2009, 96% of the proliferation was due to *A. pacificum* and only 2% to *A. tamarense*. On other occasions, the percentage of *A. tamarense* was low (≤ 12% of *Alexandrium* cells). The abundances of the other *Alexandrium* species (*A. minutum* and *A. pseudogonyaulax*) were always < 3% of the total abundance of *Alexandrium* cells. Based on all of this information, we recalculated the *A. pacificum* abundances for the Thau Lagoon time series.

In Tarragona Harbor, over the 11-year period other *Alexandrium* species, specifically, *A. tamarense*, *A. minutum*, and *Alexandrium insuetum*, were also detected together with *A. pacificum*. Bloom abundances of *A. tamarense*

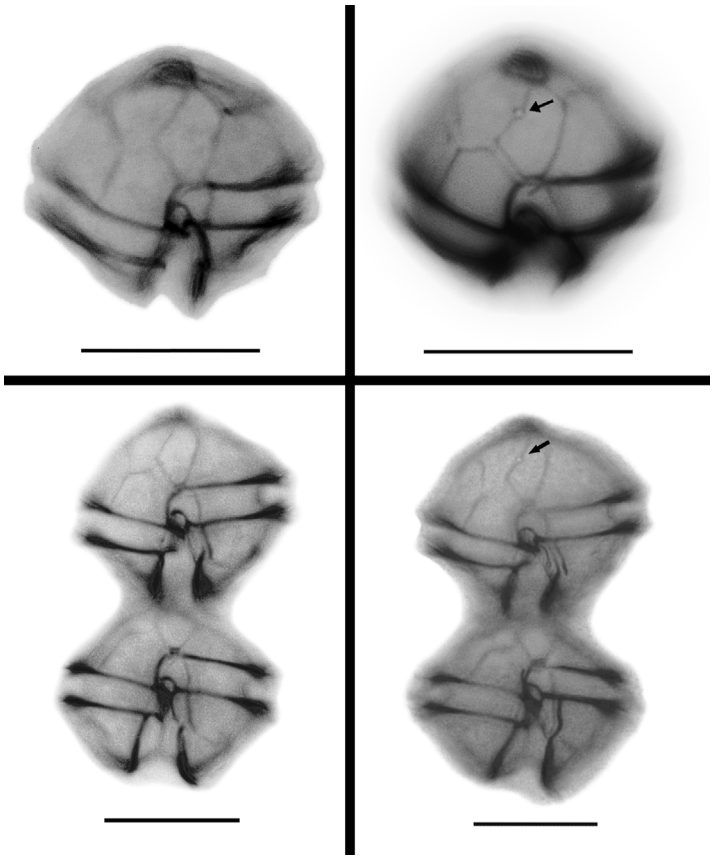

Figure 2. Cells of *A. catenella* (left) and *A. tamarense* (right) morphotypes from Thau lagoon stained with calcofluor. Arrows show the ventral pore. Scale bars: 20 µm.

(2×10^4 cells L^{-1}) were recorded in August 2005, coinciding with one of the highest *A. pacificum* abundances (~5×10^3 cells L^{-1}) recorded that year. The abundances of *A. minutum* and *A. insuetum* over the 11-year period were low (≤ 8.6×10^3 cells L^{-1}).

3.2. Temporal variability of *A. pacificum*

After regular monitoring started in Tarragona Harbor, in 2000, *A. pacificum* cells were detected every year, reaching bloom concentrations (>1×10^4 cells L^{-1}) in all years except 2004, 2005, and 2009 (**Fig 3A**). Cell abundances >1×10^6 cells L^{-1} were recorded during bloom years, whereas in non-bloom years they were no higher than 8.3×10^3 cells L^{-1}. Proliferations of *A. pacificum* in

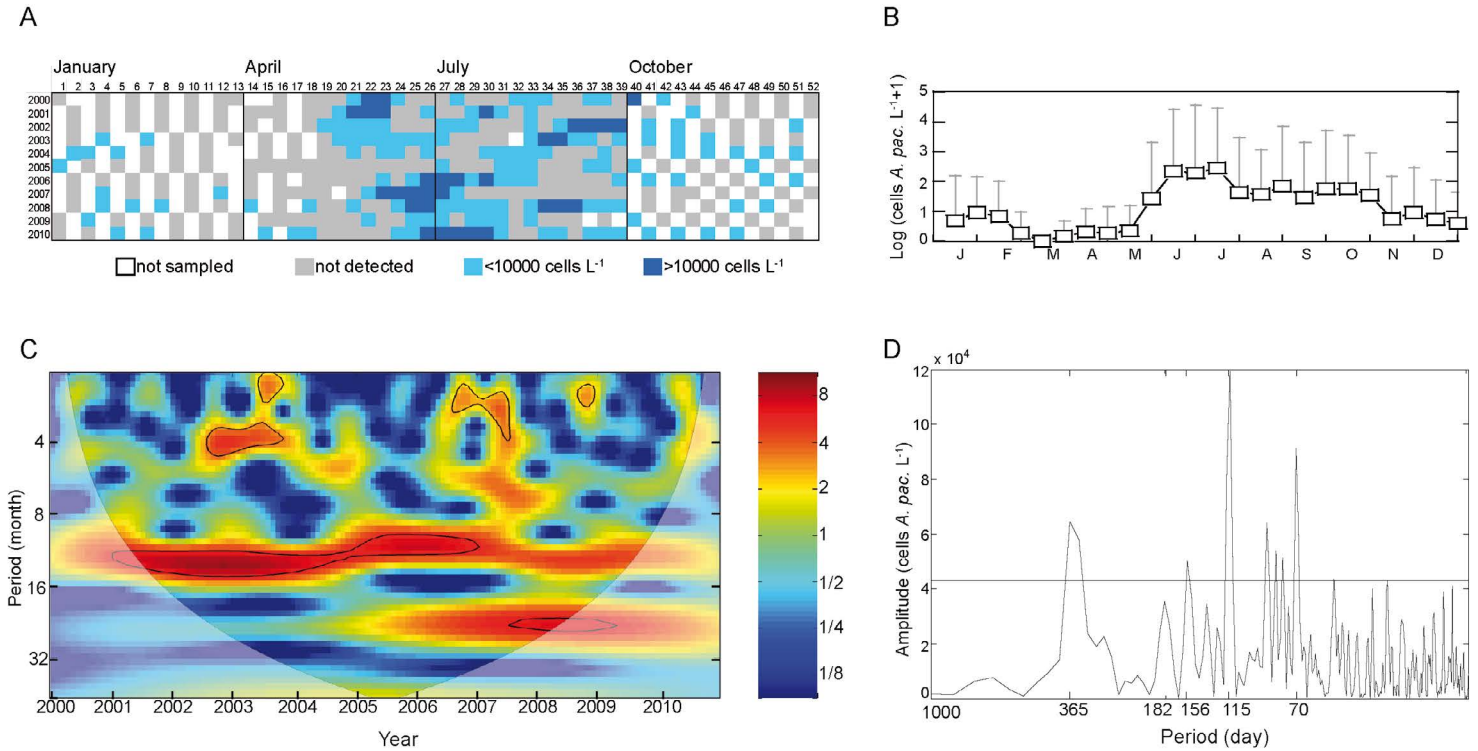

Figure 3 . Temporal variability and characterization of periodic frequencies of Tarragona Harbor *A. pacificum* abundances time series from 2000 to 2010 (A) Seasonal and inter-annual variability of *A. pacificum* data showing the bloom occurrences and the presences at low densities (< 10^4 cellsL^{-1}) (B) fortnightly averages (+ s.d.) over the sampling period showing the global seasonality (C) continuous wavelet power spectrum showing how the periodicities changed over time. Colour bar indicates the wavelet power (red indicates high intensity). Areas surrounded by black contour lines delineate the areas where the power is significant at the 95% confidence level. Shaded areas represent the cone of influence (where edge effects become important and might distort the signal) (D) Fourier spectra of the series showing characteristic periods of the time series. Horizontal line shows the 95% significance level.

Tarragona Harbor occurred once or more between mid-May and mid-October (**Fig. 3A**, **B**). Exactly when *A. pacificum* reached bloom abundances varied from one year to another, but it was never during the first 3 weeks of August. After 2005, the proliferations seemed to start later and blooms occurred generally in June–July (**Fig. 3A**). The Fourier spectra resulting from Tarragona Harbor time series showed significant periods of ~ 4 months, 1 year, and several periods of 2–3 months, while the continuous wavelet power spectrum revealed a persistent 1-year periodicity over the sampling period (**Fig. 3C**, **D**). The wavelet spectrum also revealed a period of 4 months or shorter some years, and a 2-year period in 2008, in an area of the graph where the edge effect was important.

A. pacificum bloomed every year in Thau Lagoon, except 2006 and 2009 (**Fig. 4A**). In contrast to Tarragona Harbor, cell abundances exceeded 1×10^6 cells L^{-1} only in 2001 (data not shown). The first of the two yearly blooming periods was from May until June, and the second from September until November (**Fig. 4B**). Significant periods of 6 months, ~ 4 months, and 1 year were seen on the Fourier spectra. The continuous wavelet spectrum revealed different annual patterns of *A. pacificum* abundances over time, with fluctuations at periods of 6 months or/and 12 months depending on the year (**Fig. 4C**, **D**). Periodicities of 4 months were also present in some years.

There was no temporal trend in the cell abundance anomalies of *A. pacificum* in Tarragona Harbor over the study period. For the Thau Lagoon data, the MK trend test showed a significant negative trend but Sen's estimator slope included 0 in the 95% confidence interval. Accordingly, it was assumed that during the study period there no linear trend for *A. pacificum* (T**able 1**).

3.3. Environmental conditions at Tarragona Harbor and Thau Lagoon

To characterize the environmental conditions that accompanied the bloom periods, basic statistics (mean, ranges, median) were calculated together with the environmental characteristics of each site (**Table 2**). The mean seasonal cycle, inter-annual variability, and trends in the environmental variables are shown in Figs. 5, 6, and 7, and Table 1, respectively. Detailed information is presented below.

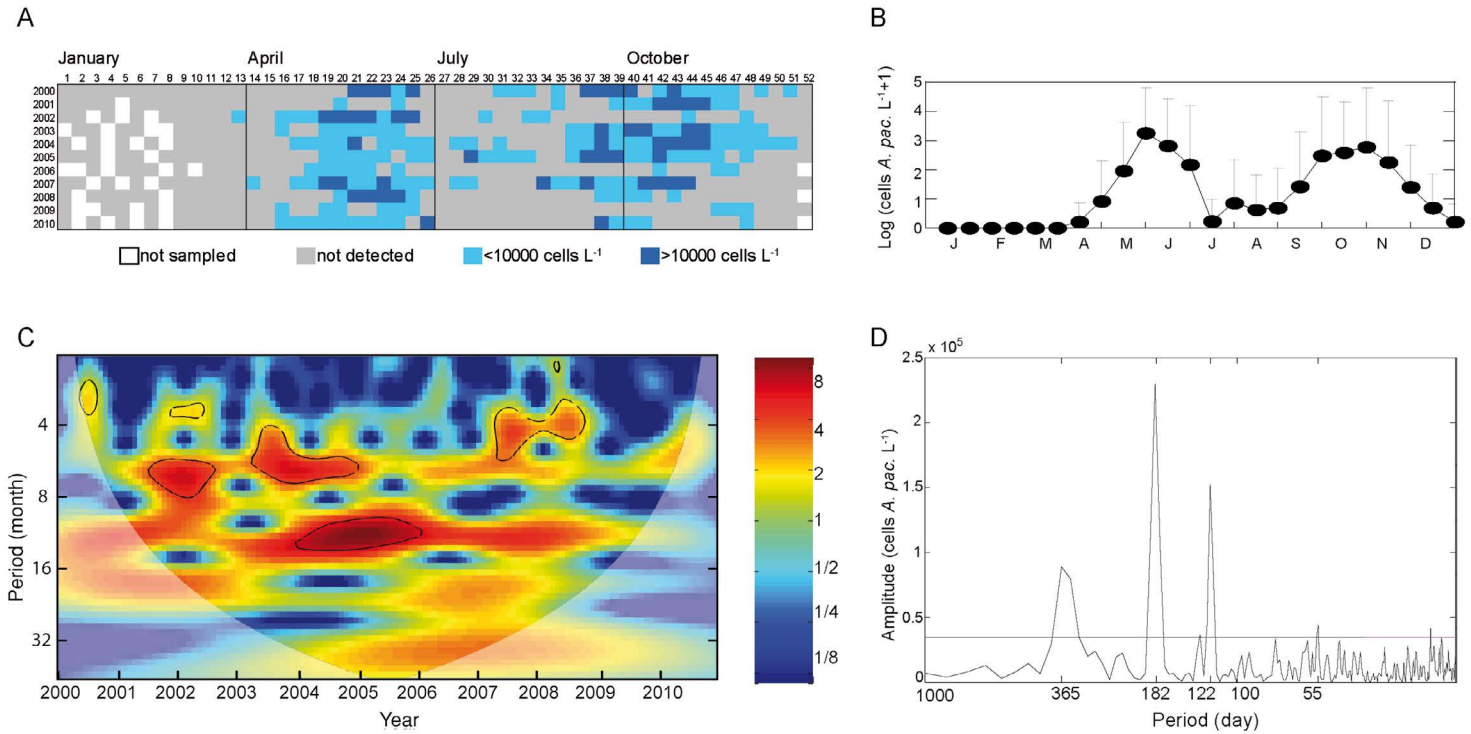

Figure 4 . Temporal variability and characterization of periodic frequencies of Thau Lagoon *A. pacificum* abundances time series from 2000 to 2010 (A) Seasonal and inter-annual variability of *A. pacificum* data showing the bloom occurrences and the presences at low densities (< 10^4 cellsL^{-1}) (B) fortnightly averages (+ s.d.) over the sampling period showing the global seasonality (C) continuous wavelet power spectrum showing how the periodicities changed over time. Colour bar indicates the wavelet power (red indicates high intensity). Areas surrounded by black contour lines delineate the areas where the power is significant at the 95% confidence level. Shaded areas represent the cone of influence (where edge effects become important and might distort the signal) (D) Fourier spectra of the series showing characteristic periods of the time series. Horizontal line shows the 95% significance level.

3.3.1 Water temperature

Water temperature followed the same general pattern in Tarragona Harbor as in Thau Lagoon (**Fig 5A**). The lowest temperatures were detected from the end of December to January, and the highest in summer; from the first fortnight of July to the first fortnight of August in Thau Lagoon (up to 29.3ºC) and from the second fortnight of July to the second fortnight of August in Tarragona Harbor (up to 29.9ºC). Despite the similar maximum water temperatures in the two localities, there were large differences in the low temperatures. Thus, the winter temperatures were lower in Thau Lagoon than in Tarragona Harbor,

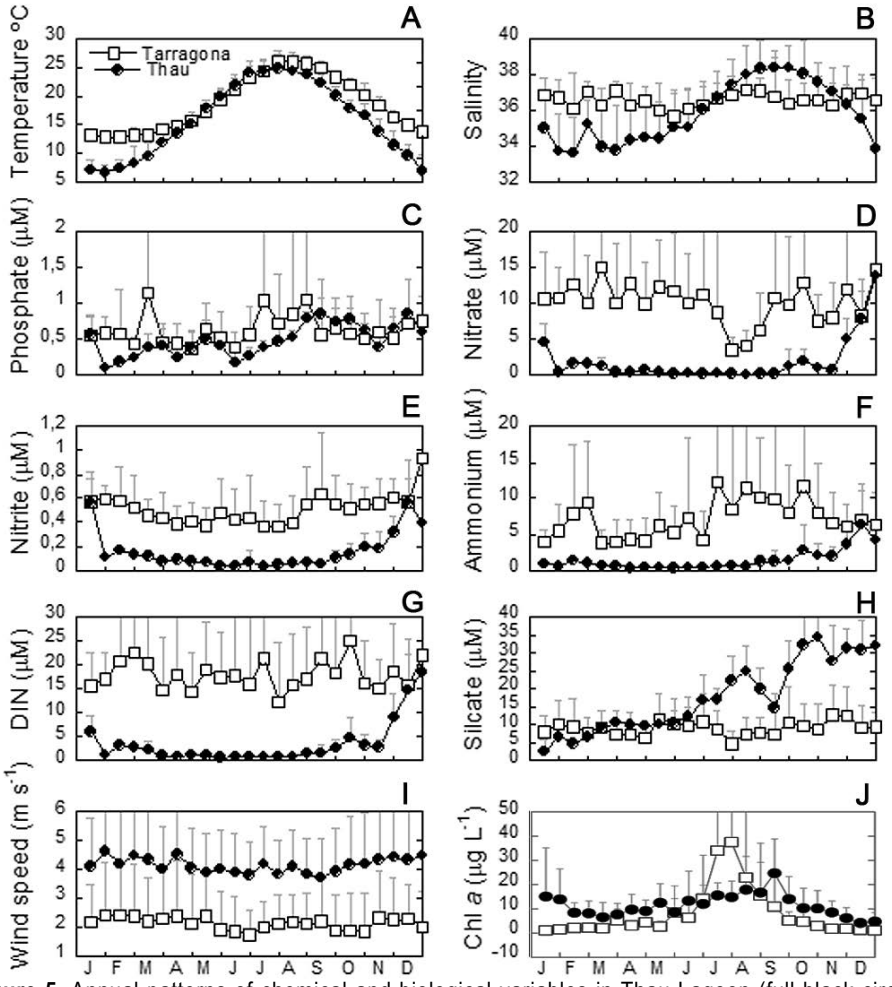

Figure 5. Annual patterns of chemical and biological variables in Thau Lagoon (full black circle) and Tarragona Harbor (open grey square). Whisker: standard deviation.

Table 1. Results of the Mann-Kendall Trend Test and Sen´s slope of the monthly anomalies of environmental and biological series. Significant (at 95% level of confidence) P-values resulted of MK test for the detection of trends and Sen's estimator of slopes that not include 0 in the 95% interval of confidence are showed in bold.

Environmental variable	Tarragona harbor					Thau lagoon				
	MK Trend Test		Sen's slope			MK Trend Test		Sen's slope		
	Tau (τ)	P-Value	Slope	Confidence limits		Tau (τ)	P-Value	Slope	Confidence limits	
A.pacificum **(log cells L^{-1} +1)**	0.0557	0.3488	0.0013	0.0063	-0.0015	-0.148	**0.0142**	-0.0028	0.00E+00	-0.007
Temperature (°C)	-0.0541	0.3583	-0.002	0.0019	-0.0059	-0.0909	0.1225	-0.0047	0.0014	-0.0109
Salinity	-0.121	**0.0404**	**-0.0034**	-0.0002	-0.0067	0.21	**0.0004**	**0.0134**	0.021	0.0061
Chlorophyll *a* (µg/L)	0.045	0.4492	0.0061	0.0213	-0.0085	-0.281	**2.32E-06**	**-0.0137**	-0.0085	-0.0192
NO_3 (µM)	0.0621	0.2953	0.0102	0.0304	-0.009					
NO_2 (µM)	0.203	**0.0006**	**0.0014**	0.0021	0.0006					
NH_4 (µM)	0.188	**0.0015**	**0.0279**	0.0454	0.011					
DIN (µM)	0.159	**0.0073**	**0.0399**	0.0711	0.0113					
PO_4 (µM)	0.0458	0.4408	6.00E-04	0.002	-9.00E-04					
SiO_4 (µM)	0.264	**8.46E-06**	**0.0462**	0.0665	0.026					
Wind speed (m/s)	-0.449	**9.02E-11**	**-0.0122**	-0.0087	-0.0163	0.106	0.0716	0.0017	0.0035	-2.00E-04

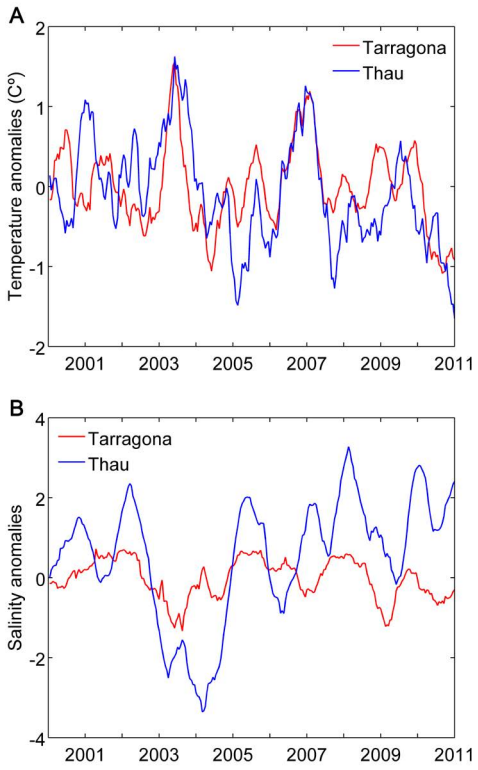

Figure 6. Moving averages of the fortnightly anomalies of the temperature (A) and salinity (B).

with a difference in their minimum values of 9°C.

During the study period there were no significant trends in the water temperatures of Tarragona Harbor and Thau Lagoon (**Table 1**) and a similar inter-annual pattern characterized the two localities. In 2004–2005 and the second half of 2010, the water temperature at both sites was anomalously cold compared to that in the remainder of the study period, whereas positive anomalies were observed in the summer of 2003 and during the second half of 2006–winter of 2007. These anomalies were more pronounced in Thau Lagoon than in Tarragona Harbor (**Fig 6A**).

3.3.2 Salinity

Salinity in Tarragona Harbor remained fairly constant throughout the year, with a mean of 36.6 and a range between 26.9 and 38.0; the seasonal minimum was in May. By contrast, there was a clear seasonal pattern at Thau Lagoon, with minimum values from December to June (lower than those in Tarragona Harbor). At both localities, the highest salinity values were detected in summer (**Fig 5B**), but the salinity of Thau Lagoon reached higher values. It also showed a high inter-annual variability and a significant tendency to increase over the study period (**Table 1**). In 2003 and 2004, the salinity at the lagoon was markedly lower than in the other years (**Fig 6B**), with a minimum salinity in the winter of 2003–2004, and peaks of maximum anomalies in the winters of 2007–2008 and 2009–2010 and at the end of 2010. Salinity at the harbor showed a significant decreasing trend (**Table 1**) and the anomalies were less pronounced. Lower salinities were recorded in 2003 and at the beginning of 2009.

3.3.3 Dissolved inorganic nutrients concentration and limitations

Mean and median DIN values were an order or magnitude higher in Tarragona Harbor than in Thau Lagoon (means of 17.21 vs. 3.01 μM during the bloom period, and medians of 9.62 vs. 1.29 μM, respectively). PO_4 levels were also slightly higher in Tarragona Harbor than in Thau Lagoon (means of 1.00 vs. 0.53 μM and medians of 0.55 vs. 033 μM, respectively). By contrast, SiO_4 was higher in Thau Lagoon than in in Tarragona Harbor (means of 8.54 vs. 28.5 μM, and medians of 7.25 vs. 33.86 μM, respectively).

Inorganic nutrients differed in their seasonal patterns at each locality (**Fig. 5C–H**). Despite the difference in nitrate concentrations at Tarragona Harbor vs. Thau Lagoon, a nitrate minimum was detected during the last fortnight of July and the first fortnight of August (**Fig 5D**) at both sites. In Tarragona Harbor, this minimum coincided with a decrease and at Thau Lagoon an increase in silicate levels (**Fig 5H**).

In Thau Lagoon, DIN was limiting for strict autotrophic phytoplankton in a third of the samples (for the period 2002–2003, when conventional nutrient data were available). There was no limitation of phosphate, and silicate was limited only once in this locality. Nutrient limitation occurred on a few occasions at Tarragona Harbor: ~ 5% of samples were limited by silicate, ~ 4% by phosphate (never coinciding with a bloom), and < 1% by DIN.

The time series of NO_2, NH_4, DIN, and SiO_4 from Tarragona Harbor showed statistically significant positive trends ($p < 0.05$) (**Table 1**). Nutrients, with the exception of nitrite, varied largely between years (**Fig. 7A–D**). Thus, higher levels of silicate were recorded in 2009, while in 2007 the ammonium concentration was higher. Trends in Thau Lagoon nutrients were not analyzed due to the few years they were measured.

3.3.4 Meteorological conditions: Wind speed

Wind speed was globally higher in Thau Lagoon than in Tarragona Harbor. The mean daily wind speed was roughly double at Thau Lagoon than at Tarragona Harbor (**Fig. 5I**). On average, wind intensity at Tarragona Harbor was lower at the end of spring and the beginning of autumn, and higher in winter, with a maximum daily value of 10.9 m s^{-1}. This seasonality was similar

in Thau Lagoon, where the wind intensity was lower at the end of spring and the end of summer and higher in colder months, with a daily maximum of 12.7 m s^{-1}. Dominant winds blew in from the west. At Tarragona Harbor, the fastest winds were usually from the northeast and northwest, while at Thau Lagoon the dominant and fastest winds blew in from the northwest. Daily wind speeds of >4 m s^{-1} occurred during 10% of the total study days at Tarragona Harbor and during 47% at Thau Lagoon. Over the study period (2003–2010), the wind speed at Tarragona Harbor had a significant decreasing trend (**Fig 7G**, **Table 1**), whereas there was no trend in the wind speed at Thau Lagoon. The years characterized by a lower wind speed were the latest of the study period in Tarragona Harbor but occurred in 2006 in Thau Lagoon.

3.3.5 Chlorophyll *a* and abundant phytoplankton species

Chl-*a* concentrations in Tarragona Harbor ranged from 0.3 to 386.6 µg L^{-1}, with a median of 3.6 µg L^{-1}. Maximum values were reached in July and minimum values in winter (**Fig. 5J**). The inter-annual variability was large (**Fig 5J**) and no trend over this period was detected (**Table1**).

Blooms of *A. pacificum* in Tarragona Harbor were frequently accompanied by high abundances of other species, including *Prorocentrum triestinum*, *Gymnodinium impudicum*, *Dinophysis sacculus*, *Prorocentrum micans*, *Skeletonema costatum*, *Thalassionema nitzschioides*, *Pseudo-nitzschia* spp., and *Lingulodinium poliedrum*. Despite the absence of *A. pacificum* blooms during the first half of August, the Chl-*a* concentration was very high because other species bloomed during this period (**Fig. 5J**). Blooms of *Gymnodinium impudicum*, another chain-forming dinoflagellate, were the most common, followed by those of the cocolithophorid *Holococcolithophora sphaeroidea* (syn. *Calyptrosphaera sphaeroidea*) or *Emiliania huxleyi*. Blooms of cocolithophorids were common in summer, from June to August, although these proliferations did not normally co-occur with *A. pacificum* blooms. Other dinoflagellates (*Prorocentrum triestinum*, *Gonyaulax spinifera*, *Lingulodinium poliedrum*), or even diatoms such as *Leptocylindrus danicus* or small unidentified nanoflagellates, bloomed as well. High Chl-*a* concentrations (**Fig 7E**) were also recorded during *A. pacificum* non-bloom years (2004, 2005, and 2009), which were instead characterized by blooms of *G. impudicum* (up to 4.5×10^6 cells L^{-1}) and *H. sphaeroidea* (up to 26.5×10^6 cells L^{-1}) in 2004

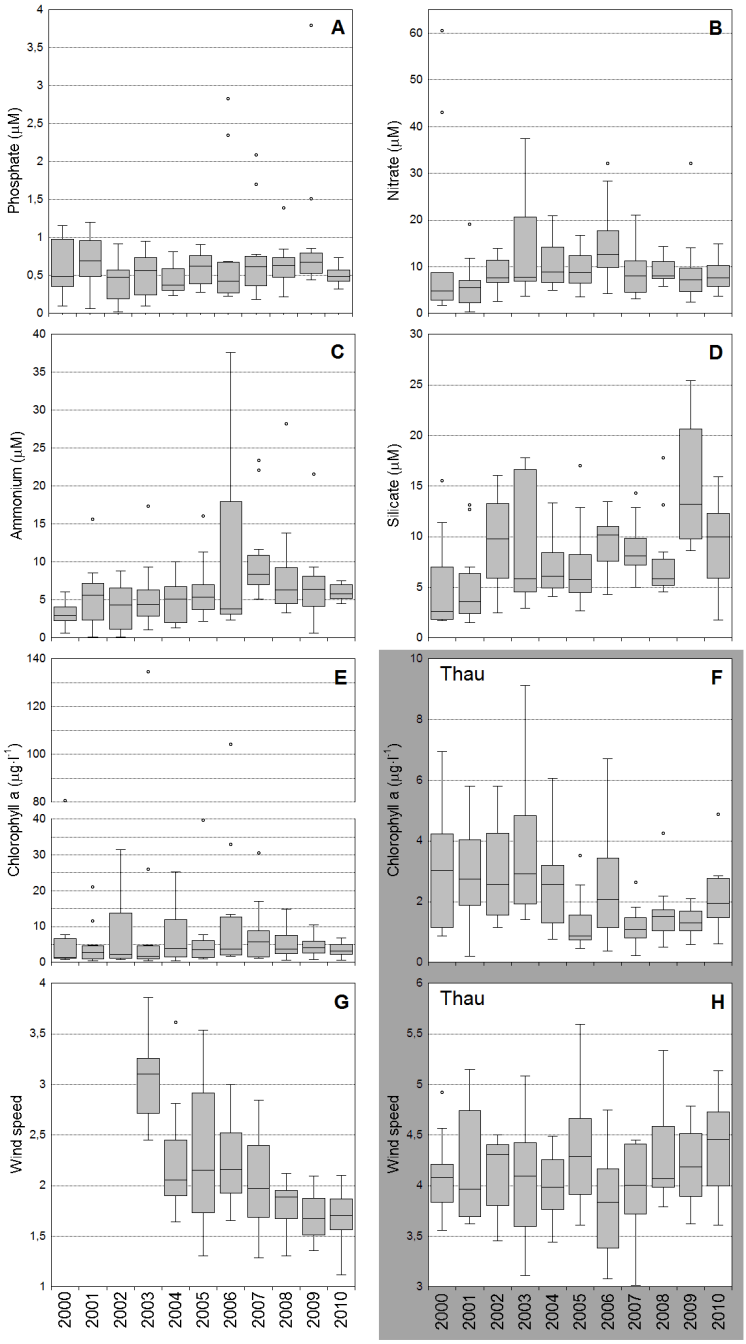

Figure 7. Inter-annual variation of the environmental variables of Tarragona Harbor and Thau Lagoon (shaded panel). Box limits: 25th and 75th percentile of the variable; Horizontal line: median value; Whisker: minimum and maximum values withouh outliers; Empty circles: oultiers. Note the different scales of the different graphs.

and 2005, respectively. In 2009, a succession of the diatoms *Thalassionema nitzschioides, L. danicus, Cylindrotheca closterium*, and *Skeletonema costatum* dominated the harbor waters from June to mid-September, reaching species abundances of 1–5 $\times$ 10^6 cells L^{-1}.

G. impudicum is thus a species whose blooms coincide or not with those of *A. pacificum*, but only from June to September, with maximum abundances attained in July–August (not shown).

In Thau Lagoon, the Chl-*a* concentration ranged from 0.1 to 13.7 µg L^{-1} with a median of 1.7 µg L^{-1}. There was a significant decreasing tendency over the study period (Fig. 7H, Table 1), although Chl-*a* concentrations were lowest in 2005. Maximum values were reached in September and minimum values in December (Fig. 5J). During bloom events, *A. pacificum* was accompanied by *P. triestinum*, *Mesodinium rubrum, Skeletonema* sp*., Asterionella* sp., and *Pseudo-nitzschia* sp*.* During summer, in the absence of *A. pacificum* blooms, *Chaetoceros* sp*, Ceratoneis closterium, L. minimus, Rhizosolenia* sp., and *Synechococcus* sp. were detected. In 2006, the most abundant species were *Chaetoceros* sp*., Cylindrotheca closterium, Gymnodinium* sp*., Nitzschia longissima*, and *Pseudo-nitzschia* sp*.* In 2009, diatoms dominated the phytoplankton communities, with the most abundant species being *Chaetoceros* sp*., C. closterium, N. longissima, Pseudo-nitzschia* sp., and *Rhizosolenia setigera.*

3.4. Relationship between *A. pacificum* patterns and environmental conditions

The relationship between *A. pacificum* abundances and environmental variables was assessed using a LMM. The results of the model showed that month was significantly related to *A. pacificum* abundances, although not all months (**Table 3**) as only June and November had a significant positive influence.

Since inorganic nutrients could not be included in the LMM due to the few years measured in Thau Lagoon, the Spearman correlations of the environmental variables and *A. pacificum* cell abundances were calculated (**Table 4**). The results showed that the correlations with nutrients were not the same at the two sampling points. At Tarragona Harbor, using all raw data, slightly negative correlations with *A. pacificum* abundances were determined only for nitrate and nitrite.

Table 2 . T Basic statistics of environmental data and the *A. pacificum* cell densities in both localities over the 2000-2011 period for all cases and for bloom (>1 X10^4 cells *A. pacificum* L^{-1}).

		Tarragona harbour						Thau lagoon					
		N	Min	Max	Mean	Std. Desv.	Median	N	Min	Max	Mean	Std. Desv.	Median
A. pacificum	Bloom>10^4	32	-	26,5·10^6	2,0·10^6	5,1·10^6	7,4·10^4	51	-	1,3·10^6	1,2·10^5	2,1·10^5	5,2·10^4
(Cells/l)	All data	400	0	26,5·10^6	1,6·10^6	1,5·10^6	0	541	0	1,3·10^6	1,2·10^4	7,4·10^4	0
Temp (ºC)	Bloom>10^4	31	20,1	29,9	24,0	2,4	23,8	48	11,8	26,6	19,1	3,4	18,9
	All data	394	11,1	29,9	20,1	4,9	20,7	491	2,1	29,3	16,4	6,5	16,8
Salinity	Bloom>10^4	29	32,7	37,9	36,8	1,0	37,1	36	31,6	39,7	37,0	1,7	36,8
	All data	368	26,9	38,0	36,6	1,2	36,9	301	27,6	40,8	35,9	2,5	36,5
Chl.-*a* (µg/l)	Bloom>10^4	26	2,2	234,7	32,2	52,0	10,8						
	All data	344	0,3	383,6	10,4	29,4	3,6	269	0,1	13,7	2,3	2,0	1,7
NO_3 (µM)	Bloom>10^4	26	0,10	44,03	7,64	11,80	3,71	12	0,02	6,48	1,42	2,01	0,63
	All data	343	0,10	72,30	9,90	9,60	6,85	105	0,01	14,83	1,52	2,93	0,40
NO_2 (µM)	Bloom>10^4	26	0,02	0,93	0,35	0,23	0,31	12	0,03	0,39	0,14	0,11	0,10
	All data	343	0,02	2,53	0,49	0,29	0,43	105	0,01	0,97	0,14	0,17	0,08
NH_4 (µM)	Bloom>10^4	26	0,19	92,72	9,22	17,97	4,23	11	0,26	9,60	1,92	2,74	0,55
	All data	343	0,04	92,72	7,44	9,15	5,10	103	0,17	11,39	1,44	2,07	0,63
DIN (µM)	Bloom>10^4	26	0,67	93,06	17,21	22,05	9,62	11	0,32	11,52	3,01	3,57	1,29
	All data	343	0,67	106,24	17,82	14,09	13,97	103	0,32	24,16	3,09	4,81	1,19
PO_4 (µM)	Bloom>10^4	26	0,13	7,55	1,00	1,48	0,55	12	0,21	1,10	0,53	0,32	0,33
	All data	343	0,02	10,49	0,66	0,92	0,49	105	0,05	1,74	0,52	0,31	0,47
SiO_4 (µM)	Bloom>10^4	26	1,28	37,40	8,54	7,44	7,25	12	10,6	44,08	28,05	13,12	33,86
	All data	343	0,01	37,40	8,94	6,36	7,32	99	0,00	44,08	18,65	10,95	15,25

3.5. Bloom conditions

A. pacificum blooms occurred in Thau Lagoon at water temperatures that were colder than those of Tarragona Harbor (**Fig 8A**, **Table 2**). While in the latter all blooms occurred at temperature >20°C, in Thau Lagoon more than half occurred between 11.8 and 20ºC. In relation to salinity, blooms occurred at salinities between 31.6 and 39.7 (**Fig. 8B**, **Table 2**). At both localities, the mean temperature and mean salinity were higher during bloom events (**Table 2**). While on some occasions *A. pacificum* blooms at Tarragona Harbor formed at very high ammonium and nitrate concentrations (up to ~93 µM and ~44 µM, respectively) (**Table 2**), the vast majority occurred at concentrations of <13 µM and 8 µM, respectively (**Fig. 8C**). Total DIN was normally lower than 16 µM, while phosphate levels were < 2 µM (**Fig. 8D**).

Cell abundances of *A. pacificum* and the daily mean of the pre-filtered wind speed were plotted together to determine the relationship between the two variables both at Tarragona Harbor and at Thau Lagoon. In Tarragona Harbor, *A. pacificum* consistently bloomed during a calm period (**Fig. 9**, see 2008 and 2010) whereas bloom abundances in Thau Lagoon coincided on several occasions with wind speeds > 4 m s^{-1} (**Fig. 9**, 2002 and 2003).

During non-bloom years, calm periods at both sites would have been long enough to allow bloom development during the appropriate annual periods (**Fig. 9**, see 2004 and 2009 in Tarragona Harbor, and 2006 in Thau Lagoon). Strong winds during the days or weeks before the bloom event did not seem to be a prerequisite for bloom development, at least at Tarragona Harbor. As shown in **Fig 9**, in 2010 a bloom occurred in July but the last strong wind before the bloom was in April.

On some occasions, the decline of *A. pacificum* abundances at both locations coincided with high wind speeds (**Fig. 9**, Tarragona 2004 and Thau 2006).

Table 3. Results of the Linear Mixed model for the logarithm of *A. pacificum* in Tarragona and Thau. Boldface indicates a statistical significant coefficient. December has fixed as a reference month.

Effect	Estimate	Standard Error	P-value (Pr>/t/)
Month	.	.	**<.0001**
January	-0.05556	0.4994	0.9115
February	-0.3876	0.4988	0.4381
March	-0.9230	0.4919	0.0621
April	-0.3270	0.5380	0.5440
May	1.1276	0.6581	0.0883
June	1.6289	0.8243	**0.0496**
July	0.06054	0.9763	0.9506
August	0.2693	0.9600	0.7794
September	0.6287	0.8347	0.4523
October	1.1107	0.6587	0.0934
November	1.1256	0.4703	**0.0177**
Temperature (°C)	0.07423	0.06292	0.2396
Salinity	-0.02935	0.07278	0.6872
Chlorophyll *a* (µg/L)	0.01030	0.008432	0.2232
Wind speed (m/s)	0.09988	0.1357	0.4625

4. Discussion

Studies of algal blooms during several years at a particular place can provide insights that allow identification of the factors controlling bloom onset and maintenance, distinguishing them from those that play no relevant role. Studies of more than one locality also allow features significant only for a specific place to be discarded as the main drivers of a specific bloom. The aim of this study was to identify the factors that promote *A. pacificum* blooms, by comparing the similarities and differences of these events at two confined stations in the northwest Mediterranean Sea based on two 11-year phytoplankton time series (2000–2010). **Table S1** summarizes the characteristics of Tarragona Harbor and Thau Lagoon and the similarities and differences between *A. pacificum* blooms at each site and the related parameters.

4.1 Taxonomy

The results of the morphological analyses of *Alexandrium* species performed in this study are in agreement with those based on molecular tools (Genovesi et al., 2008; Lilly et al., 2002; Penna et al., 2005. They evidenced that the *Alexandrium* species that blooms regularly in Tarragona Harbor and in Thau Lagoon is *A. pacificum* (*A. catenella* morphotype belonging to group IV). Furthermore, to the best of our knowledge, reliable evidence of the presence in the Mediterranean Sea of *A. catenella* (group I), the species morphologically most similar to *A. pacificum*, has not been obtained (see Discussion in Chapter 1). The *A. tamarense* morphotype observed in the Thau Lagoon and Tarragona Harbor samples almost certainly belongs to *A. tamarense* (Group III). Sequences previously obtained from organisms collected in Thau Lagoon

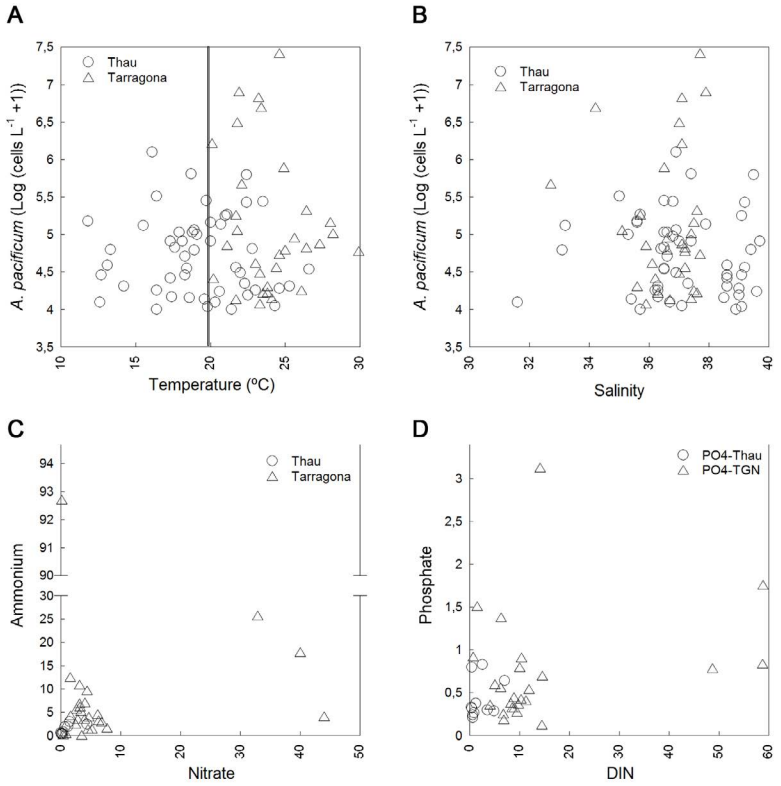

Figure 8. Bloom conditions are represented by plotting only the cases with *A. pacificum* (>1X10^4 cells L^{-1}. Note the break in Y-axis of plot C and the different scales before and after the break. An outlier from Tarragona Harbor data corresponding to DIN= 93.1 and PO_4= 7.5 were not plotted for illustrative purposes. Only blooms of Thau Lagoon belonging to years 2002 and 2003 are represented in the graphs below.

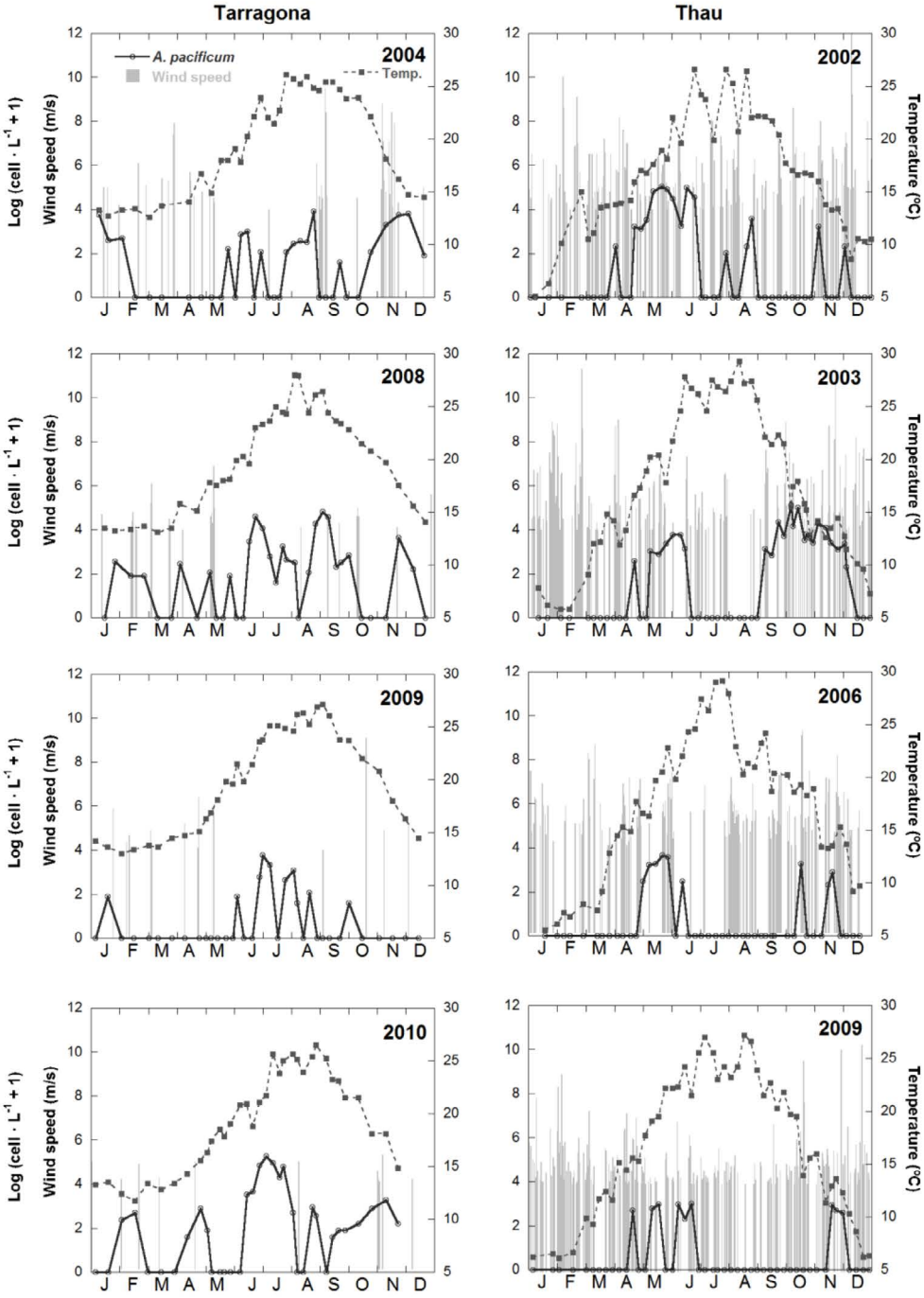

Figure 9. Winds pre-filtered (bars), temperature (squares) and cell abundances of *A. pacificum* (circles) in different years in Tarragona Harbor (left) and Thau Lagoon (right).

included those representing this species (Genovesi et al., 2008). Although the *A. tamarense* morphotype has never been isolated and sequenced from Tarragona Harbor, a cyst belonging to group III was isolated from Fangar Bay (65 km south of Tarragona) (Satta et al., 2013).

In addition, most of our findings of the presence of *A. pacificum* or *A. tamarense* in the reexamined samples from Thau Lagoon agreed with those of Genovesi et al. (2011), who determined the relative abundance of each species in the water column during 2007 and in the sediment in 2004 identifying 558 clonal strains by molecular methods. For example, these authors did not detect the *A. tamarense* ribotype during the bloom of November 2004 coinciding with our results for the same date, where the 99% of the *Alexandrium* cells lacked Vp (thus corresponding to *A. pacificum*). Similarly, the prevalence of the *A. tamarense* ribotype detected in September 2007 by Genovesi and coauthors coincided with one of our two samples in which a high percentage of cells with Vp was found (50%).

4.2 Variability in *A. pacificum* cell abundances

4.2.1 Annual patterns

The different annual patterns observed for *A. pacificum* and the different bloom periods in the two localities studied are consistent with the continuous cyst germination pattern documented for this species both in culture (Figueroa et al., 2005) and in situ (Ishikawa et al., 2014). Continuous germination ensures the presence of cells that can increase in abundance and develop blooms when conditions become favorable for growth (Anglès et al. 2012; Ishikawa et al., 2014). However, at both study sites no blooms were observed between December and April; whether this period is characterized by less active excystment or conditions inappropriate for growth is not known. At Thau Lagoon, where no cells were detected during the coldest months, the water temperature may have been the cause (see below); however, at Tarragona Harbor, the water is warmer and cells were present during these 4–5 months. At each locality, wind speeds were higher between December and April (**Fig. 5l**), but the substantial difference in the wind speed of the harbor vs. the lagoon and the non-significance of the wind speed in the LMM

Table 4. Spearman rank correlations between environmental and biological factors, and *A. pacificum* cell abundances calculated based on data collected from both sampling localities between 2000 and 2010. Data from the two localities with recurrent *A. pacificum* blooms, climatology (mean annual cycle) data and anomalies. Bold indicated significant correlations ($p<0.05$).

Environmetal variables	Both points	Tarragona	Thau
	All data	**All data**	**All data**
	N=408	N=343	N=65
NO_3 (µM)	**-0,179**	**-0,198**	-0,150
NO_2 (µM)	**-0,160**	**-0,172**	-0,143
NH_4 (µM)	-0,089	-0,082	-0,095
DIN (µM)	**-0,148**	**-0,162**	-0,114
PO_4 (µM)	-0,023	-0,019	-0,065
SiO_4 (µM)	-0,028	-0,066	0,102

led us to discard this variable as the sole factor explaining the absence of blooms during this time. In support of this conclusion, a wavelet analysis of the wind speed of Thau Lagoon did not reveal the same periodicities observed for *A. pacificum*, including the 6-month periodicity (data not shown). Thus, the absence of *A. pacificum* blooms between December and April could have been due to a general factor not measured in this work or to a mixture of factors that occurred during these months.

Despite the coincidence of the absence of bloom between December and April and the annual recurrence of *A. pacificum* in both zones, the seasonality of *A. pacificum* differed at the two sites and was more marked at Thau Lagoon than at Tarragona Harbor. The significant positive relationship of abundance in June and November indicated by the LMM showed that seasonality was significant. A bimodal distribution, with two peaks per year, in spring and in autumn, similar to that observed in Thau Lagoon has also been reported for other phytoplanktonic species (e.g., Borkman and Smayda, 2009; Li and Smayda, 2000) and for *A. pacificum* (as *A. catenella*) in other localities, such as Ago Bay, Japan (Ishikawa et al., 2014). These two annual peaks were generally associated with seasonal changes in mixing intensity, nutrient availability, grazing, and shifts in phytoplankton community structure (Winder and Cloern, 2010). According to our data, despite the DIN limitation in Thau Lagoon, nutrients do not explain the seasonality of *A. pacificum.* In the case of Tarragona Harbor, the 12-month periodicity dominated during the study period.

However, the duration, intensity, and time of onset of the annual bloom, while occurring within the bloom period described, varied from year to year. This observation suggests a mixture of processes that include biological factors, such as predation, competition, and parasitism, as well as other external factors. In any case, the seasonality at both sites did not seem to be marked by a common general or regional factor but rather by local factors.

In addition to the annual peak, the time point of ~4 months was significant in Tarragona Harbor (115 days, Fig. 3C–D) and Thau Lagoon (122 days, Fig. 4C–D), but other, shorter periodicities identified in some years in Tarragona (by the wavelet spectrum were also significant Fig 3C–D). However, as this variability in the 2–4 months band is not a well-established feature of phytoplankton variability, its detection in other studies has been interpreted by the respective authors as either a true pattern dominated by irregular, short-term bloom events, but also as a sampling error (e.g., too few measurements) or attributable to artifacts produced by the analytical methodology (Winder et Cloern, 2010).

4.2.2 Tendency-interannual variability

Seasonality dominated the variability in *A. pacificum* abundance more than the variability in the tendency because there was no statistically significant temporal trend in *A. pacificum* cell abundances at either Tarragona Harbor or Thau Lagoon. This information adds to the insights gained after the first detection of *A. pacificum*, in Barcelona Harbor (Catalonia, Spain), when from 1996 to 1999 this species expanded into several neighboring harbors along the Catalan coast (Vila et al. 2001). However, at beginning of the 21st century, the abundance of *A. pacificum* was lower in most harbors, and since 2008 this species has been detected only in Tarragona Harbor (see also Chapter 1). Nonetheless, based on our analysis of 11 years (from 2000 to 2010) of data we found neither an increasing nor a decreasing temporal trend in the cell abundances of *A. pacificum* in Tarragona Harbor, despite the increasing tendency of some nutrients (ammonium, nitrite and silicate) and the decreasing wind speed in this locality. The same was observed in Thau Lagoon, where tendencies of an increasing salinity and a decreasing Chl-*a* concentration were instead determined.

The absence of bloom occurrence at the two localities in the same year, as was the case in 2009, suggested a role for a global or regional factor in bloom regulation. However, temperature of this year did not show any unusual pattern and it was not an especially rainy year at either location (data not shown). In addition, the wind speed was very different at Thau Lagoon, where it was relatively high, than at Tarragona Harbor, where it was very low (Fig. 9, Thau and Tarragona 2009); thus, meteorological variables do not explain the absence of blooms at both sites. Common features were the presence of blooms of various diatom species and a decrease in salinity, which was more marked at Tarragona. There, the year 2009 was characterized by several noteworthy features: the silicate concentration was the highest and the flow of the Francolí River (which flows into the harbor) the second largest during the study period (daily flow: 42 m^3 s^{-1}, data obtained from the Catalan Water Agency (ACA) website). The high flow occurred a few days after a rainfall of 36.8 mm in Tarragona in April. Among the biological variables, the Chl-*a* concentrations of 2009 were not different from those of other years, but *G. impudicum* (a dinoflagellate species that produces recurrent high-biomass blooms during the summer months in Tarragona Harbor) detections were the lowest of the study period; only once, in September, did the abundances of this species exceed 10^3 cells L^{-1}. These findings together suggest that no proliferation of *A. pacificum* took place, due to competition with diatoms, whose growth was/could have been favored by the high concentration of silicates, at least in Tarragona; for Thau Lagoon nutrient data were not available.

In contrast to 2009, when no climatic changes were recorded, the climate conditions in 2004–2005 were unusual—with negative anomalies in water temperatures in both regions—and may have influenced bloom dynamics. Specifically, the unusual climate conditions at Tarragona Harbor may have contributed to the lower cell abundances and the absence of blooms (see Chapter 1). However, as a regional phenomenon, the unusual climate conditions should have had the same consequences at Thau Lagoon, but bloom events were documented there during those years. Nevertheless, the wavelet analysis of *A. pacificum* abundances in Thau Lagoon, showed a shift in the periodicity during 2004–2005. Shifts in the annual pattern of phytoplankton have been reported (Winder and Cloern, 2010), including as a result of abrupt changes in climate (e.g., Borkman and Smayda, 2009). Our results suggest differential effects of climate changes on the population

dynamics of *A. pacificum* at the two study sites. However, a different local factor each year in Tarragona could also be the cause of the low abundances of *A. pacificum* these two years. For example, the winds in 2004 could have prevented *A. pacificum* from reaching bloom abundances (**Fig. 9**). These hypotheses are not mutually exclusive and together may explain the data.

Similar to Tarragona Harbor in 2004, bloom development at Thau Lagoon in 2006 might also have been disrupted by winds (**Fig. 9**).

4.3. Bloom conditions of *A. pacificum*

Previous studies have associated certain conditions with bloom events of *A. pacificum* in Mediterranean coastal waters. In this work, we studied several physico-chemical and meteorological factors at two sites to determine whether they were common and thus perhaps necessary for bloom development.

Is wind strength a determinant of bloom initiations?

In their study of Thau Lagoon, Laanaia et al. (2013) suggested the necessity of a strong wind (>4 m s^{-1}) followed by a calm period and stable sea surface temperature (20± 2ºC) to allow the resuspension and germination of buried cysts and vegetative cell growth. However, our results for Tarragona Harbor did not suggest a need for wind stress prior to calm weather for bloom formation. Rather, cyst resuspension in both studied environments may have been due to other natural forces, such as seiches (a natural sea-level oscillation of the order of minutes that can cause cyst resuspension in enclosed coastal systems; Anglès et al., 2010; Jordi et al., 2008; Niedda et Greppi, 2007), and/or to anthropogenic activities (e.g., dredging, ship traffic).

Is a calm period, with wind speeds <4 m s^{-1}, necessary for bloom development?

As proposed by Laanaia et al. (2013) for Thau Lagoon based on data from 2000 to 2007, our results for Tarragona Harbor showed that blooms coincided with periods in which the wind speed was <4 m s^{-1}. By contrast, data from Thau Lagoon from 2000 to 2010 did not support a role for wind speed, since

mean daily winds >4 m s^{-1} were recorded either during or a few days before a bloom event. Since wind can be considered a proxy of water instability, it may be that a specific number of days (>1 day) in which the mean daily winds speed is ≥ 4 m s^{-1} and blows in the appropriate direction are needed to produce water instability and thus impede bloom formation.

Do temperatures of ~20°C mark the bloom period?

While Laanaia et al. (2013) suggested the necessity of a stable surface temperature of 20±2°C for bloom development in Thau Lagoon, our results show that this is not the case for the proliferation of *A. pacificum* in Mediterranean waters, since 33% of *A. pacificum* blooms in Thau Lagoon occurred at temperatures between 11.8°C and 18°C. Thus, the relationship between a surface temperature of 20±2°C and *A. pacificum* blooms is probably not a direct one but most likely indicative of a role for conditions usually associated with temperature, such as water stability, in the growth of *A. pacificum*. However, low temperatures (<11.8°C) may be unfavorable for the growth of *A. pacificum* despite the stable waters of Thau Lagoon, where the temperatures in winter were far lower (minimum of 2.1ºC) than in Tarragona Harbor (minimum of 11.1°C, **Fig. 5**). Indeed, in winter *A. pacificum* cells were below the detection limit in Thau Lagoon, whereas planktonic cells were detected year-round in Tarragona Harbor. The laboratory results reported by Laabir et al. (2011) support this possibility. These authors did not observe the laboratory growth of strain ACT03 from Thau Lagoon at temperatures <12°C; moreover, the decrease in cell abundances after inoculation suggested cell mortality. Thus, the influence of environmental conditions detrimental to the survival of *A. pacificum* in the water column during winter in Thau Lagoon cannot be ruled out.

Are bloom abundances associated with high ammonium and high nitrate levels?

An association of *A. pacificum* blooms at the Catalan coast with high levels of both ammonium (20 µM) and nitrate (50 µM) in coastal waters was previously suggested (Bravo et al. 2008). However, our results do not support this conclusion (Fig 8C), as the mean and median concentrations of ammonium at Tarragona Harbor measured during blooms that occurred within the study period were 9.22 µM and 4.23 µM, and the concentrations for nitrate 7.64 µM

and 3.71 µM, respectively (**Table 2**). The former study considered different sites along the Catalan coast whereas ours focused only on Tarragona Harbor, where the largest blooms of this species occur (Bravo et al. 2008, Chapter 1) and which account for almost all *A. pacificum* blooms detected in the area. This allowed us to compare our results with those of Bravo et al. (2008). Data from Thau Lagoon similarly refuted the association of high ammonium and nitrate levels with *A. pacificum* blooms, since the concentrations of these inorganic nutrients were lower than in Tarragona (**Table 2**), also in the presence of blooms. In addition, Thau Lagoon is a nitrogen-limited system, according to the criteria of Justic et al. (1995). Furthermore, blooms of *A. pacificum* were also shown to occur without any significant inputs of nitrate; thus dissolved organic N must play a role in their dynamics (Collos et al., 2014).

Are phosphate levels < 1 µM a prerequisite for bloom development?

The emergence of *A. tamarense/catenella* blooms in Annaba Bay (Algeria) and in Thau lagoon was associated with a decrease in SRP to a concentration of <1 µM (Hadjadji et al., 2014). Although the mean phosphate concentration in bloom situations in Tarragona was 1 µM, the mean values were also <1 µM (**Table 2**). However, the relationship between a decrease in SRP and bloom emergence is far from universal, as demonstrated by the case of Barcelona Harbor (Vila et al., 2005). There, blooms of *A. pacificum* were recurrent from 1996 to 1999, until *A. pacificum* densities decreased drastically, coinciding with the beginning of the environmental restoration carried out at this site from 2000 to 2003. This restoration affected the concentrations of phosphate and other nutrients, with the annual mean concentration of phosphate decreasing from > 2 µM before 2000 to < 1 µM thereafter.

The relevance of dissolved inorganic nutrients such as nitrate or phosphate to the growth of *A. pacificum* is unclear because this species is mixotrophic (Anderson et al., 2012). This species can use either dissolved organic forms of N (Carlsson et al., 1998; Loureiro et al., 2009) or P (Jauzein et al., 2010; Matsuda et al., 1999), or particulate organic forms by phagotrophy (Jeong et al., 2010). Thus, at the study sites, *A. pacificum* probably compensates for occasional deficits in conventional nutrients by the uptake of dissolved and particulate N and P. While dissolved inorganic nutrients can support bloom development, they are not a necessary presence. The mechanisms involving

nutrients in the blooms of this species remain elusive, but a diversity of nutrient sources is probably an important element in bloom formation. As we found no evidence of a relationship between dissolved inorganic nutrients and *A. pacificum* cell numbers, our results are in agreement with those of Heisler et al. (2008), who concluded that future models of such blooms should "move away from simplistic inorganic nutrient-dose-yield models."

5. Conclusions

The two sites that were the focus of this study are located in the northwest Mediterranean Sea, separated by a distance of 350 km, with a common Mediterranean weather regime, and a non-relevant tidal range. The recurrent blooms of *A. pacificum* at the two sites differed in their seasonality (occurring at different periods of the year), suggesting that the annual cycle of this species is not driven by a global factor such as irradiation (or temperature). Rather, bloom-modulating factors probably do not follow the same patterns at the two localities. Among the environmental variables analyzed in the present work, the water temperature range for bloom development was wider than reported in previous studies and its direct role in the seasonality of the species is unclear. On the other hand, uncommon climatological conditions on a regional scale, reflected as a negative thermal temperature anomaly, could have changed the annual maximum abundances severely and / or caused a change in the annual pattern of *A. pacificum*. Inorganic nutrients also do not seem to explain the seasonality of *A. pacificum*, and the concentrations during the documented blooms were highly variable. Seasonality also does not seem to be modulated by the wind speed, which, nonetheless, in some years may have prevented bloom development.

This study showed that conclusions drawn from an analysis of a single location and short time series may not coincide with those more accurate deduced with more places and more years data. Instead comparative studies of more than one place and using a long-term series are needed. While our own results must be validated by more data, they suggest that some of those published in the literature about bloom dynamic or blooms requirements are not general rules. Furthermore, other additional variables are probably necessary to understand the bloom dynamic of *A. pacificum*.

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8. Supplementary material

Table S1. Characteristics of Tarragona Harbor and Thau Lagoon and the similarities and differences between *A. pacificum* blooms at each site and the related parameters.

	Tarragona harbor	**Thau Lagoon**
General *A. pacificum* information		
First record	1998	1995
Highest abundances (normal maximal anual abundances)	2.6 X10^7 cells/L (>10^6 cells/L)	1.2 X 10^6 cells/L (>10^5 cells/L)
Presence if vegetative cells	All year	Spring, autumn
Major cysts banks identified	Inside the harbor, near the Francolí river (Bravo et al., 2008)	Inside the lagoon, in Cirque-de-l'Angle, and Etang-des-Eaux-Blanches (Genovesi et al., 2013)
Bloom periods	Mid May - mid October	May-June; September-November
Years without blooms (<10^4 cells/L)	2004, 2005, 2009	2006, 2009
Other biological factors		
Chl-*a* - range (mean) μg·L^{-1}	0.3 - 386 (3.6)	0.1 to 13.7 (station B NOAA database)
Period of maximal Chl-*a* values	July and August	Generally in August
Main species producing blooms of high abundances	*G. impudicum* and *H. sphaeroidea*	*Skeletonema* sp., *Thalassiosira* sp.
Aquaculture	None	Oysters, mussels
Physical factors		
Freshwater influence	Urban	Continental
Freshwater inputs	Francolí river, sewage collectors	Water from the Canal du Midi, the Canal du Rhône a Seté and several streams

Table S1 (cont.).

	Tarragona harbor	**Thau Lagoon**
Period of maximal freshwater inputs	Usually in autumn and winter (almost negligible from spring to late summer)	Autumn and winter
Effect of freshwater in basin circulation	No significant effects of the Francolí river outflow on harbor circulation (Mestres et al., 2007)	Very limited
Water temperature- range (mean)	11.1 - 29.9 (20.1)	2.1 - 29.3 (16.4)
Salinity- range (mean)	26.9 - 38.0 (36.6)	31.6 - 39.7 (37.0)
Main forcing of the water circulation in the basin	Wind stress (Mestres et al., 2007)	Wind (Millet, 1989)
Chemical factors		
Inorganic nutrients*		
DIN - range (mean) µM	One order magnitude higher; 0.67 - 106.24 (17.82)	0.32 - 24.16 (3.09)
PO_4 - range (mean) µM	0.02 - 10.49 (0.66)	0.05 - 1.74 (0.52)
SiO_4 - range (mean) µM	0.01 - 37.40 (8.94), constant during the year cycle	0.00 - 44.08 (18.65); increase during the year
NO_3 - range (mean) µM	0.10 - 72.30 (9.90)	0.01 - 14.83 (1.52)
NH_4 - range (mean) µM	0.04 - 92.72 (7.44)	0.17 - 11.39 (1.44)//minimum without the two bloom periods
DIN and PO_4 limitations	Not limiting	1/3 cases limited by DIN
Topography	Shallow, semi-confined	Large and shallow, confined
Extension (surface area)	5 km²	75 km²

Table S1 (cont.).

Maximum and mean depth	26 m; 10-20	10; 4.5 m
Depth in the sampling point	4.5 m	2 m
capacity//volume (m^3)	75 million cubic meters	337 million cubic meters
Time of residence	2-11 days (Sánchez-Arcilla et al., 2005; Mestres et al., 2007)	1-5 months (Vaulot, D. &G.F. Frisoni, 1986)
Connections to the sea	1 mouth (450 m wide, 26 m depth)	3 narrow channels
Orientation of the connections to the sea	SW	SE
Orientation of the basin (longitudinal axis)	NE-SW	NE-SW
Meteorological conditions		
Maximum daily wind intensity	10.9 m s^{-1}	12.7 m s^{-1}
Wind dominant regime	W	NW
Faster winds	NE - NW	NW
Windy period	Winter	Winter
Initial wind stress needed for bloom formation	No	Yes. NW winds (Laanaia et al., 2013)
Bloom occur in calm period (<4 m/s)	Yes	Yes
Possible interruption of bloom due to strong winds	Yes	Yes. NW winds (Laanaia et al., 2013)
Anthropogenic influence		
Anthropogenic activities	Commercial (petroleum-related products, agricultural products and cereals) and fishing activities	Aquaculture activities and tourists on small barges
Dredgings over the studied period	In 2003, pier fishermen adjacent area (46800 m^3) In 2004, Marina Tarraco adjacent area	No in Angle creek

* nutrient info from Tarragona (11 years data), Thau (2 years data)

Chapter 3

The spatio-temporal distribution of the potentially harmful dinoflagellates at Catalan beaches (NW Mediterranean)

Harmful Algae, in preparation

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Abstract

Most Harmful Algal Bloom (HAB) monitoring programs target shellfish culturing and shellfishing areas, but ignore other nearshore waters vulnerable to HAB outbreaks. As a result, the toxic and bloom-forming algal species in these areas remain largely unknown. In this study, we investigated the distribution at high-scale resolution of potentially harmful dinoflagellates along several Catalan beaches (NW Mediterranean Sea). Based on analyses of samples collected from ~258 beaches from 2000 to 2012, we found species causing PSP, DSP, species producing yessotoxin and palytoxin, ichthyotoxic species, and species that cause a deterioration of water quality due to high-biomass blooms or mucilage production. Seventeen of the identified taxa reached bloom abundances, with *A. taylori*, Gymnodinioids, and *Ostreopsis* spp. producing the largest number of blooms. Most of the taxa were widely distributed along the coast, although a few of them showed a clearly limited distribution. The beaches of the Costa Brava (north coast) are the most severely affected by dinoflagellate blooms. Nearly all blooms of dinoflagellates occurred between April and September. In addition, we examined the spatio-temporal dynamics of several *Alexandrium* species, focusing on the dynamics of *A. taylori*, which bloomed in ~33 % of the Costa Brava beaches. Our results evidenced that this species has expanded forming blooms in an additional area. Moreover, our findings demonstrate that the different degrees and forms of anthropogenically related eutrophication and the quantity of freshwater inputs do not explain the distribution of *A. taylori* blooms.

1. Introduction

The open Mediterranean Sea is an oligotrophic (and, in its eastern basin, ultra-oligotrophic) system with low levels of chlorophyll in its superficial waters that periodically increase either in response to spatial structures (such as fronts, cyclonic gyres), or seasonally, during late winter and early spring, when mixing within the water column induces nutrient inputs from bottom to superficial waters. In this ecosystem, the probability of high-biomass bloom formation by phytoplankton and the persistence of these blooms are low (Garcés et Camp, 2012). By contrast, coastal areas receive continental inflows, mainly in the form of freshwater and nutrients, and are thus characterized by very strong physico-chemical gradients, and the impact of biogeochemical processes is intensified. Moreover, as the human population density along coastal Mediterranean areas has increased over the last several decades, so have the socio-economic activities of people living in these areas (including commercial and recreational activities), which has resulted in an over-enrichment of nutrients in coastal waters and an increase in the number of confined water bodies (such as harbors). Both are thought to be the main factors accounting for the increase in harmful algal bloom (HAB) development in the NW Mediterranean Sea (Flo et al., 2011a; Garcés et Camp, 2012; Vila et al 2001a; Vila et al., 2001b). Furthermore, some human activities contribute to increasing the translocation of harmful phytoplankton species from near or distant localities, for example, with the movement of shellfish stocks for aquaculture, drifting plastic debris (Masó et al., 2003), or ballast water. Together, these factors explain why Mediterranean coastal waters, in contrast to the sea's open waters, constitute a eutrophic system where chlorophyll levels are high and the formation of HABs is frequent (e.g., Garcés et al., 1999; Vila et al., 2001a; Vila et al., 2001b).

HABs adversely affect human and marine wildlife health, marine ecosystems, and human resources such as tourism industry, fisheries, and aquaculture. They can produce toxic effects in human health and other organisms (i.e., through the consumption of contaminated shellfish). In addition, the high biomasses of harmful algal species cause a deterioration of the water quality (discoloration, foul odor, oxygen depletion).

HAB monitoring programs have mostly focused on shellfish culture and fishing areas, whereas other nearshore waters vulnerable to HABs have for the most part been ignored. As a result, little is known about the presence and dynamics of the toxin-producing and bloom-forming species that inhabit these areas. With the implementation of EU Directives, such as the European Water Framework Directive (Directive 2000/60/EC), the aim of which is to achieve good-quality coastal waters, and Directive 2006/7/EC, which addresses the quality of recreational (swimming) waters, the monitoring of phytoplankton in nearshore waters is now mandatory.

In Catalonia (NW Mediterranean Sea), shellfish culturing areas are mostly located in the two bays of the Ebre Delta (Alfacs and Fangar), which have been monitored for harmful phytoplankton species since 1989. In that year, the first toxic event in the area was detected, when a bloom of the dinoflagellate *Alexandrium minutum* caused Paralytic Shellfish Poisoning (PSP) in mussels (Delgado et al., 1990). Shellfish, however, could be collected both in the delta bays where they are cultured and in the natural settlements along the coast, where they grow in the wild. Since 1995, the monitoring program has expanded to include several harbors spaced along the Catalan coast. The extensive monitoring of HABs in this region has contributed to an early warning system for HAB development all along the coast.

Catalonia is a popular tourism area, with a coastline of approx. 580 km (linearly) that includes 562 beaches used for swimming and other recreational purposes. Nonetheless, before 2000, only a few beaches were monitored, and only occasionally. These few samplings revealed toxic and environmental problems at the beaches due to HABs, many of them caused by the genus *Alexandrium*, which includes PSP-producing species and other species that produce high-biomass blooms. For example, in the spring of 1998 there was an important outbreak of the PSP producer *Alexandrium pacificum* (previously called *A. catenella*) along the southern Catalan coast (Vila et al., 2001b). High-biomass blooms of *Alexandrium taylori* Balech, which produces brownish-green discolorations of the affected water, have been documented at La Fosca beach (Costa Brava) since the late 1990s (Garcés et al., 1999), and the unappealing water quality has resulted in economic losses in the tourism sector.

The study described herein was carried out to provide much-needed information on the toxic and harmful phytoplankton species present at beaches along the Catalan coast. As the taxonomic group that includes many especially harmful species, we focused on dinoflagellates. Our specific aims were to characterize the diversity of harmful dinoflagellates in Catalan beaches, to investigate their spatial distribution at high-spatial resolution, and to determine their maximal abundances at this environment. This knowledge yielded insights into the health-related, economic, and environmental threats posed by dinoflagellates at Catalan beaches and allowed us to identify the most problematic beaches. In addition, we examined the spatio-temporal dynamics of several *Alexandrium* species (including non-HAB species), focusing on the dynamics of prominent bloom-forming species (*A. taylori*), and the environmental factors at three beaches where blooms of this species are recurrent. Based on the data acquired from samplings of up to 258 beaches from 2000 to 2012, together with environmental data obtained during some of the samplings, our study includes a discussion of the influence of specific environmental factors as promoters of HAB formation along Catalan beaches.

2. Material and methods

2.1 Study area

The Catalan coast is located in the NW Mediterranean Sea (NE Spain) and extends in a NE–SW orientation from southern France (3º19'59.94"E, 42º29'0.09"N) to the southern tip of the Ebre Delta (0º 9'41.69"E, 40º 31'27.56"N) (**Fig. 1**). Differences in coastal morphology, geological characteristics, land use, population, and freshwater entries account for the different physico-chemical and biological properties of Catalan coastal waters. The coastline can be broadly divided into three areas. The northern coast (Costa Brava) is mainly composed of metamorphic and granite rocks and has a high abundance of natural pocket beaches, but also alluvial coastal plains reflecting the deposition of recent sediments supplied by the Fluvià, Muga, and Ter rivers. These three small rivers account for most of the continental inflows in this northeast region such that it is subject to less pressure than other Catalan

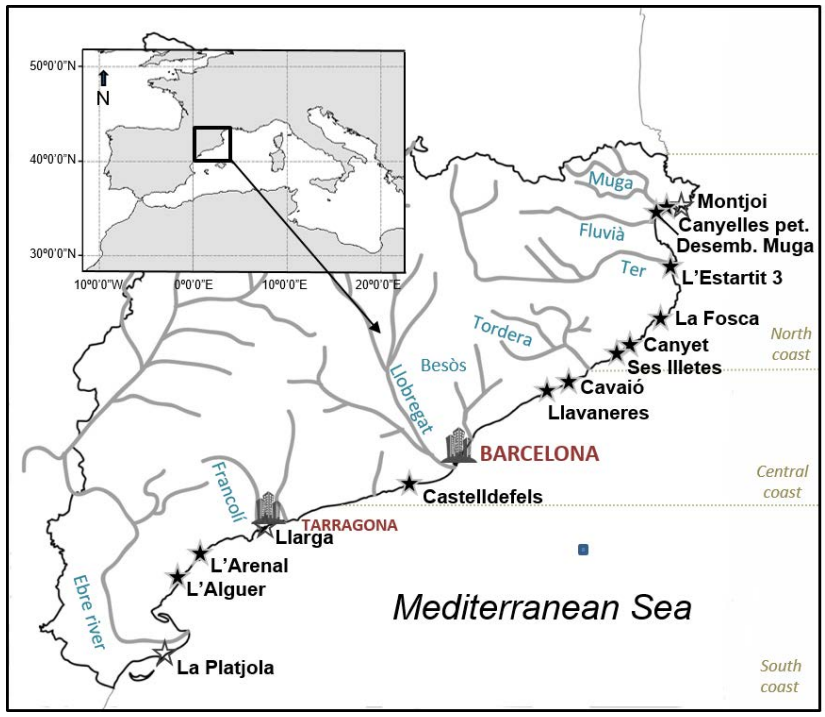

Figure 1. Study area showing the more frequently sampled locations (stars) along the Catalan coast. Black stars show the beaches sampled more regularly beginning in 2005.

regions and its coastal waters have intermediate values of salinity and silicates (Flo et al., 2011a). The human population is also of moderate size in this area. By contrast, the mostly low, sandy central coast is the most populated area of the Catalan coastline as it includes the city of Barcelona and its metropolitan area (3.3 million inhabitants). The coast north of Barcelona is a 40-km-long continuous beach interrupted only by a series of harbors developed over granite rocks. The border with the northern Costa Brava area is formed by the Tordera River, which supplies the area with its sandy sediments. The city area of Barcelona includes artificial pocket beaches and a large harbor that extends for more than 15 km. The border to the south includes the delta plain of the Llobregat River, whose sediments form the 18-km-long sandy coastline and, further south, the abrupt Garraf coastline, whose low calcareous cliffs form small natural pocket beaches. Because of the intense urban influences, the concentrations of inorganic nutrients (ammonium, phosphate, and nitrite) measured along the coast of metropolitan Barcelona are very high (Flo et al.,

2011a). The low-lying southern Catalan coast (Costa Daurada) lacks steep slopes and is thus characterized by open sandy beaches, while the harbors and piers in this region have given rise to artificial, enclosed beaches. The southernmost segment is the Ebre Delta, whose sandy delta plain of 320 km^2 includes two bays: Fangar Bay to the north and Alfacs Bay to the south. Both are the major areas of shellfish cultivation and harvesting along the Catalan coast. A large part of the Ebre Delta is used for agricultural purposes. As the most important Catalan river, the Ebre drains a watershed of 84,230 km^2 and has a mean water discharge at its mouth of 416 $m^3\ s^{-1}$ (Ludwig et al., 2009). The population density along the Costa Daurada is moderate to low, with Tarragona as the most important urban area (140,000 inhabitants). The fluvial and agricultural influences account for the high levels of silicates and nitrate as well as the large freshwater content that characterizes the coastal waters of this region (Flo et al., 2011a).

The Catalan coast is microtidal, with maximum tidal range of 0.2 m. The most important current close to the coast is the Catalan current, a continuation of the south-moving Ligurian-Provençal current (Font et al., 1988), which travels approximately along the coast (NE to SW) with an average speed of 20cm s^{-1}.

2.2 Sampling and measurements of physical variables

Most of the samplings were performed as part of the consecutive contracts between the Catalan Water Agency (ACA) and the Institute of Marine Sciences (ICM–CSIC), which mandated the monitoring of potentially toxic or harmful phytoplankton species in the harbors and beaches of the Catalan coast. Other samplings were carried out during research projects conducted at the ICM–CSIC. In total, samples were collected at up to 258 (45% of the total number of Catalan beaches) beaches between 2000 and 2012.

Extensive sampling

Extensive samplings, covering the entire Catalan coast, were carried out from 2001 to 2010 and included 114–243 (depending on the year) beaches sampled once per year (twice in 2001). The coastal surveys were conducted

nearly simultaneously, with all samples for a given year obtained within three consecutive days. Generally, sampling was conducted during the peak of summer, at the end of July or the beginning of August, during calm weather (never on rainy days), as these conditions are the most favorable for dinoflagellates and tourism at the beaches is at its highest level. The extensive water samplings (150 mL each) for phytoplankton analyses were collected exclusively from surface waters and were preserved with Lugol's iodine solution.

Continuous sampling

From 2000 to 2012, several beaches along the Catalan coast (2 to 17 depending on the year) were sampled annually at different frequencies (**Table S1**). **Figure 1** shows the location of the 14 more frequently sampled beaches. From 2005 to 2011 or 2012 (depending on the beach), 11 of these 14 beaches (indicated in **Figure 1**) were sampled on a more regular basis than in other years, with a minimum monthly sampling frequency (**Table S1**). The samples collected during these continuous samplings were also taken from surface waters and used in analyses of phytoplankton, chlorophyll *a*, and inorganic nutrients. Specifically, from each sample 150 mL were preserved with Lugol's iodine solution for microscopic analysis of phytoplankton; 60 mL were filtered onto Whatman GF/F glass-fiber filters and frozen at –20°C for Chl-*a* analysis, and another 60 mL were frozen immediately (–20°C) for nutrient analyses. Temperature and salinity were measured *in situ* using a conductivity meter (WTW Model LF 197). Samples used in the nutrients and Chl-*a* determinations were frozen until analyzed. Since the cell abundance of certain dinoflagellates, especially *Alexandrium taylori*, in surface waters differs depending on the sampling time, due to the daily surface-to-bottom migration of the organisms, sampling was performed routinely between 11:00 and 16:00 h.

2.3 Chlorophyll *a* and nutrient analyses

The samples were extracted in 8 mL of 90% acetone for 48 h at 4°C, and the fluorescence of the extract then measured with a Turner Designs fluorometer following the method of Yentsch and Menzel (1963). Dissolved inorganic nutrients (including NO_3, NO_2, NH_4, PO_4, and SiO_4) were analyzed as described by Grassohoff et al. (1983), using an Alliance Instruments

Evolution II autoanalyzer (samples collected from 2000 to 2007) and with a Bran+Luebbe analyzer AA3 (samples collected from 2007 to 2012). Probable inorganic dissolved nutrient limitations were calculated as in Justic et al. (1995) according to the following criteria: P limitation [P <0.1 μM; dissolved inorganic nitrogen (DIN): P >22; Si: P >22], N limitation (DIN <1 μM; DIN: P <10; Si: DIN >1) and Si limitation (Si <2 μM; Si: PO4 <10; Si: DIN <1).

2.4 Phytoplankton identification and enumeration

To observe and enumerate known harmful dinoflagellates, including *Alexandrium* species and other dinoflagellate species, present in bloom abundances (>10^4 cells L^{-1}), a 50-mL water sample was left to settle in a counting chamber for 1 day. For species enumeration, the appropriate area of the chamber was observed at 200–400× magnification, depending on the cell density of each sample, using a Leica-Leitz DM-IL inverted microscope (Throndsen, 1995). Usually, at least half the chamber was examined; thus, the minimum cell concentration that can be detected by this method is 40 cells L^{-1}. *Alexandrium* and other species (as appropriate) were identified by thecal plate tabulation after the addition of several drops of the fluorescent dye Calcofluor White M2R (final concentration 10–20 mg mL^{-1}; Fritz and Triemer, 1985) to the chamber. The organisms were examined at 400× or 630× magnification under UV excitation fluorescent illumination using an inverted microscope. Calcofluor staining of the thecal plates reveals several taxonomic characters of thecated dinoflagellates, including the absence or the presence of the ventral pore and the size and shape of specific plates. Taxonomical identification followed in general Tomas (1997) for dinoflagellates and that of Balech (1995) for the genus *Alexandrium*, aided by subsequent new descriptions and re-descriptions. Particular cases are the species comprising the *Alexandrium tamarense* complex that were recently re-described by John et al. (2014). The morphologically based determination of the species of this complex is presented in Chapter 2 of this thesis. Most of these taxa were identified to the species level, six at genus level, and one at a group morphology level. None of them included all species known for the respective genus. Thus, for example, the *Ostreopsis* spp. identified in this study included *O. ovata* and *O. siamensis* (Penna et al., 2005a); *Karlodinium* spp. consisted of *K. armiger* and *K. veneficum* (see Garcés et al., 2006). Dinoflagellates described by the group morphology term “Gymnodinioid” included *Barrufeta bravensis*, *Gymnodinium*

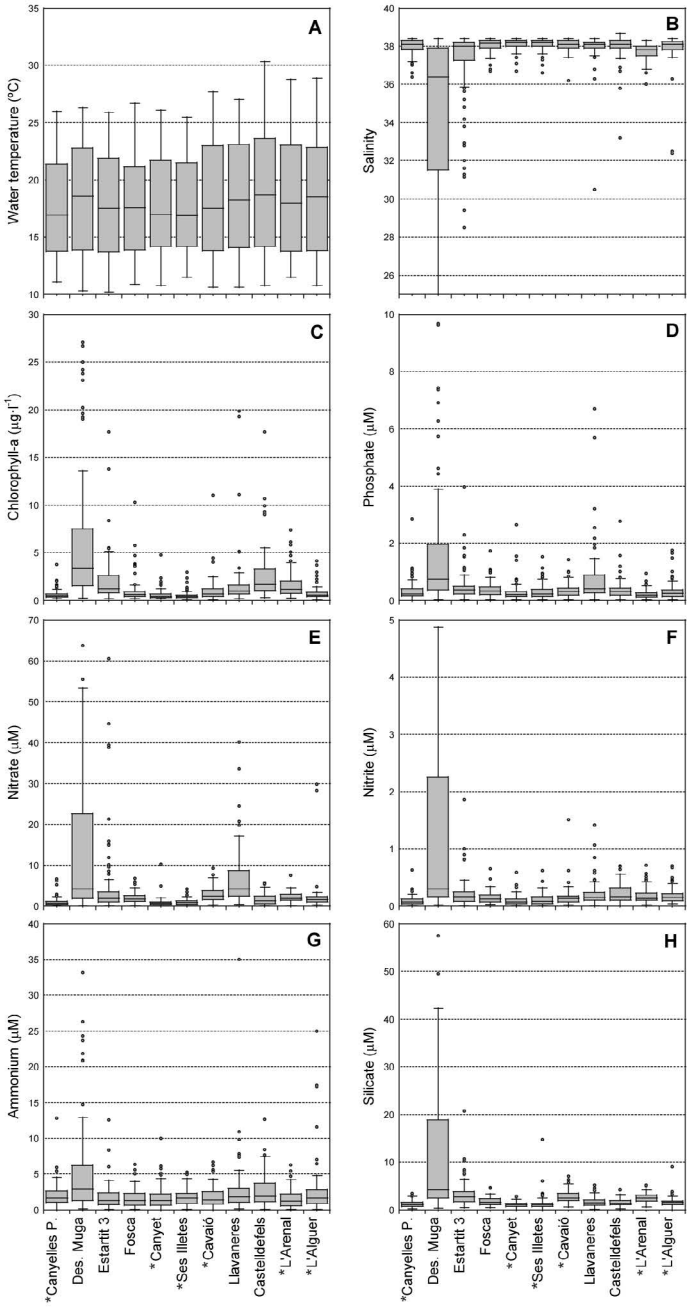

Figure 2. Box plots of the concentrations of environmental variables at each beach calculated with the monthly means using data from 2005 to 2011 or 2012. Box limits: 25th and 75th percentiles of the variable; horizontal line: median value; whisker: minimum and maximum values without outliers; empty

litoralis, and *Levanderina fissa (=Gymnodinium instriatum)* (see Reñé et al., 2014 and Sampedro et al., 2011). *G. impudicum* was counted separately. The *Amphidinium* spp. did not include *Amphidinium carterae* but could include the potentially toxic species *Amphidinium* cf. *operculatum* (Reñé et al., 2015). The *Dinophysis acuminata* complex included *D. sacculus* and *D. acuminata morphotypes* but not *D. ovum*.

2.5 Statistical analyses

The environmental variables of the more regularly sampled sites were plotted in box plots using data collected from 2005 to 2011 or 2012. To partly avoid bias due to differences in the monthly sampling frequencies at the different beaches, the boxes were calculated using the mean of every month. Descriptive analyses were performed using Kaleidagraph.

The mean annual cycles of the biological [*A. taylori* abundance (log cells L^{-1} +1), Chl-*a*] and environmental (temperature, salinity, inorganic nutrients) variables were determined by calculating the fortnightly averages of data collected during the study period.

The Mann-Kendall (MK) trend test (Mann, 1945; Kendall, 1975) was used to detect statistically significant trends in target species and environmental series, since most of the respective data were not normally distributed according to the Shapiro-Wilk's test [calculated using STATISTICA 10 Software (StatSoft, 2011)]. Thus, the MK test was applied using the Kendall package in the R software version 3.0.3 (R Core Team 2015). The data series used consisted of the deseasonalized monthly mean, obtained by subtracting the monthly global mean from the monthly mean (hereafter, the monthly anomalies), which revealed the trends in the variables over time. The significance level of the test was set at $\alpha = 0.05$. The change per unit time in a time series having a linear trend was estimated by applying Sen's estimator of slope (Sen, 1968), a simple non-parametric procedure. Trends in monthly anomalies were significant when Kendall's tau τ was significant and the 95% confidence interval of Sen's slope estimator did not include 0. To depict trends in the abundance of *A. taylori* at selected beaches, Lowess smoothing was calculated using R software.

Prior to the correlation analyses, all variables were log-transformed according to $v' = \log10 (v + 1)$. Since not all variables were normally distributed, only

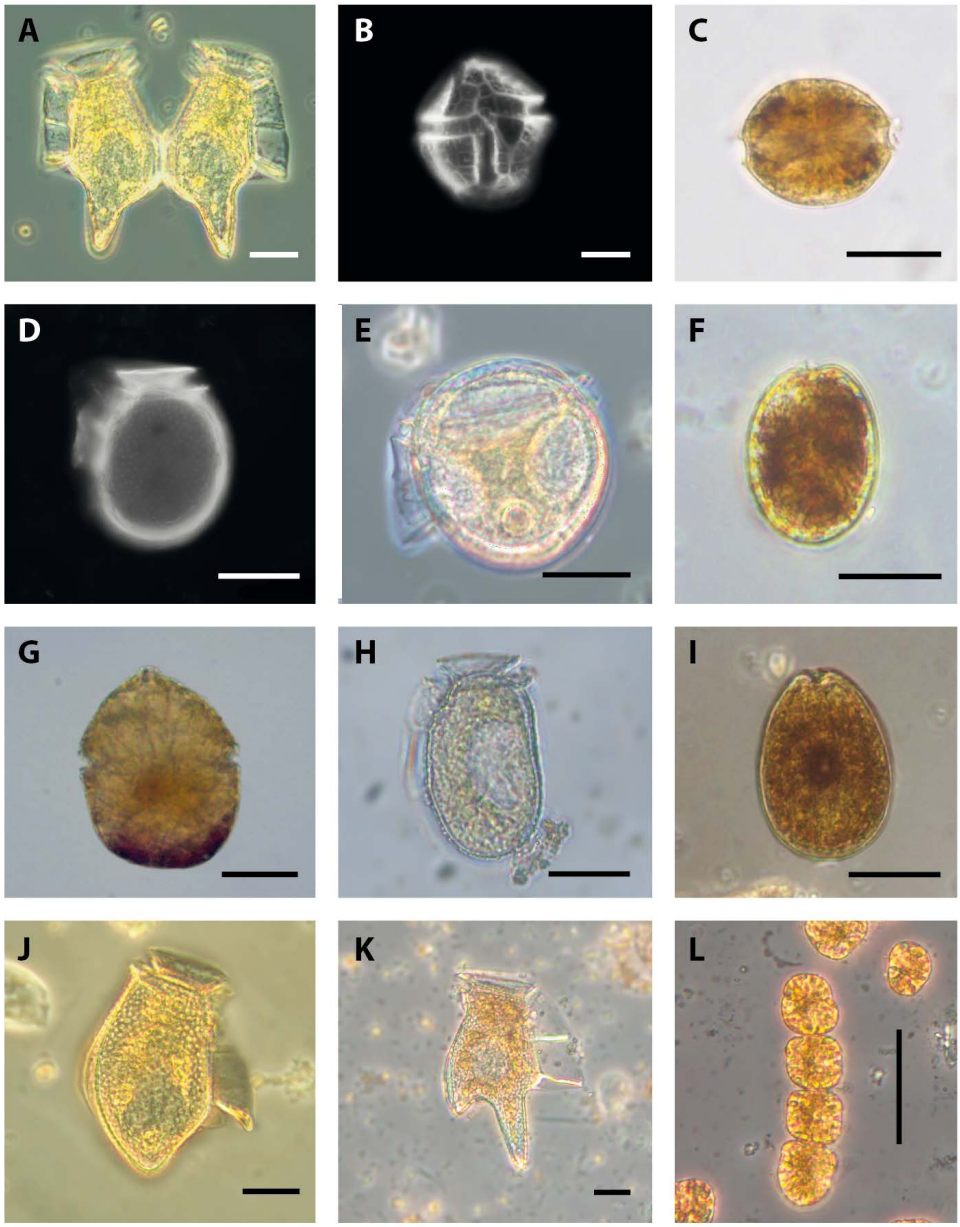

Figure 3. Light micrographs of: A) *Dinophysis caudata*, B) *Protoceratium reticulatum*, C) *Alexandrium taylori*, D) *Dinophysis ovum*, E) *Phalachroma rotundatum*, F) *Prorocentrum rhathymum*, G) *Gonyaulax fragilis*, H) *Dinophysis sacculus morphotype*, I) *Prorocentrum lima*, J) *Dinophysis acuta*, K) *Dinophysis tripos*, and L) *Gymnodinium impudicum*. Scale bars= 20 µm.

non-parametric statistical analyses were applied. Spearman's rank correlation between *A. taylori* abundances and abiotic variables was computed using 'STATISTICA 10 Software'.

3. Results

3.1 Environmental conditions

Figure 2 and **Table S2** describe the very wide ranges of inorganic nutrient and Chl-*a* concentrations as well as the salinity levels in open and semi-open sampled Mediterranean coastal waters. The ranges and medians varied greatly depending on the beach (**Fig. 2**). For example, the salinity level at Desembocadura de la Muga (Desemb. Muga) was much lower than at the other beaches included in the study but it also was wide-ranging since the beach is located at the end of the Muga River. The riverine waters influence of other rivers is also apparent in the salinity of the two southernmost stations (influenced by Ebre River) and Estartit 3 beach (influenced by Ter River) although ranges are narrower than in Desembocadura de la Muga beach. The inorganic nutrients and Chl-*a* concentrations at Desemb. Muga were very high, whereas high levels of phosphate and nitrate were measured at Llavaneres beach, and high levels of nitrite and ammonium at Castelldefels beach. As expected, the water temperature was colder at northern than at southern stations.

3.2 Distribution, abundances, and seasonality of harmful dinoflagellates

Both potentially toxic dinoflagellates (as listed in the IOC Taxonomic Reference List of Toxic Plankton Algae, Moestrup et al., 2009 onwards) and species that reached bloom abundances (>10^4 cells L^{-1}) detected along the Catalan coast over the studied period are listed in **Table 1**, which, for purposes of general interest, also includes all *Alexandrium* species detected.

The potentially harmful taxa identified as a result of the extensive and continuous samplings of beaches from 2000 to 2012 included species causing PSP, DSP (diarrheic shellfish poisoning), species producing yessotoxin and

palytoxin, ichthyotoxic species, and species that cause a deterioration of water quality due to their high-biomass blooms or mucilage production. Micrographs of some of the species are shown in **Fig. 3**.

The harmful taxa (including those that are non-toxic but were found at bloom abundances) most frequently detected were *A. minutum*, *Coolia monotis*, *Dinophysis caudata*, the *D. acuminata* complex, *Phalacroma rotundatum, Ostreopsis* spp, *Prorocemtrum lima, Pr. rhathymum, Pr. cordatum,* Gymnodinioids *(not G. impudicum),* and *Pr. micans* (**Table 1**). Seventeen of the identified taxa reached bloom densities, with *A. taylori*, Gymnodinioids, and *Ostreopsis* spp. producing the largest number of blooms. The maximum abundance detected was 65.1 X 10^6 cell L^{-1}, determined for Gymnodinioids at Estartit 3 beach, where this taxon exceeded 1 X 10^6 cell L^{-1} for several years. However, the abundances of some species, such as *Lingulodinium polyedrum* and *Protoceratium reticulatum* were very low. This was the case for the eight-detected toxic dinophysiales species, the abundances of which never surpassed 2000 cell L^{-1} (**Table 1**).

Most of the taxa were widely distributed along the coast (see **Fig. 4B**), although the distribution of others, such as *A. pacificum*, *A. taylori*, and *G. impudicum*, was clearly limited (**Fig. 4**, **Table 1**). *A. pacificum* commonly occurs together with *G. impudicum* and their spatial distributions match. Coinciding blooms of these species have been observed in harbors (chapter 2; Vila et al., 2001a). Similarly, we observed blooms of *A. taylori* along the beaches of the northern coast co-occur with those of Gymnodinioids, although the predominant species of gymnodinioid differs depending on the beach, the year and the time during the bloom. Because in this work phytoplankton were analyzed by light microscopy, the abundances of the various Gymnodinioids could not be determined because of the impossibility of identifying these organisms using only this methodology. Furthermore, two of the gymnodinioid species that co-occurred with *A. taylori* during the study period were identified as new species (Reñé et al., 2011; Sampedro et al., 2011). Since the Gymnodinioids are the sum of several species with similar morphologies, their distribution is wider than that of *A. taylori* but their blooms always coincide with the distribution of *A. taylori*. In consequence, the beaches of the Costa Brava (north coast) are those most severely affected by dinoflagellate blooms, largely due to the blooms produced by *A. taylori* and Gymnodinioids, but also to *Ostreopsis* spp. The latter were detected at 188 beaches (73% of the beaches sampled) and

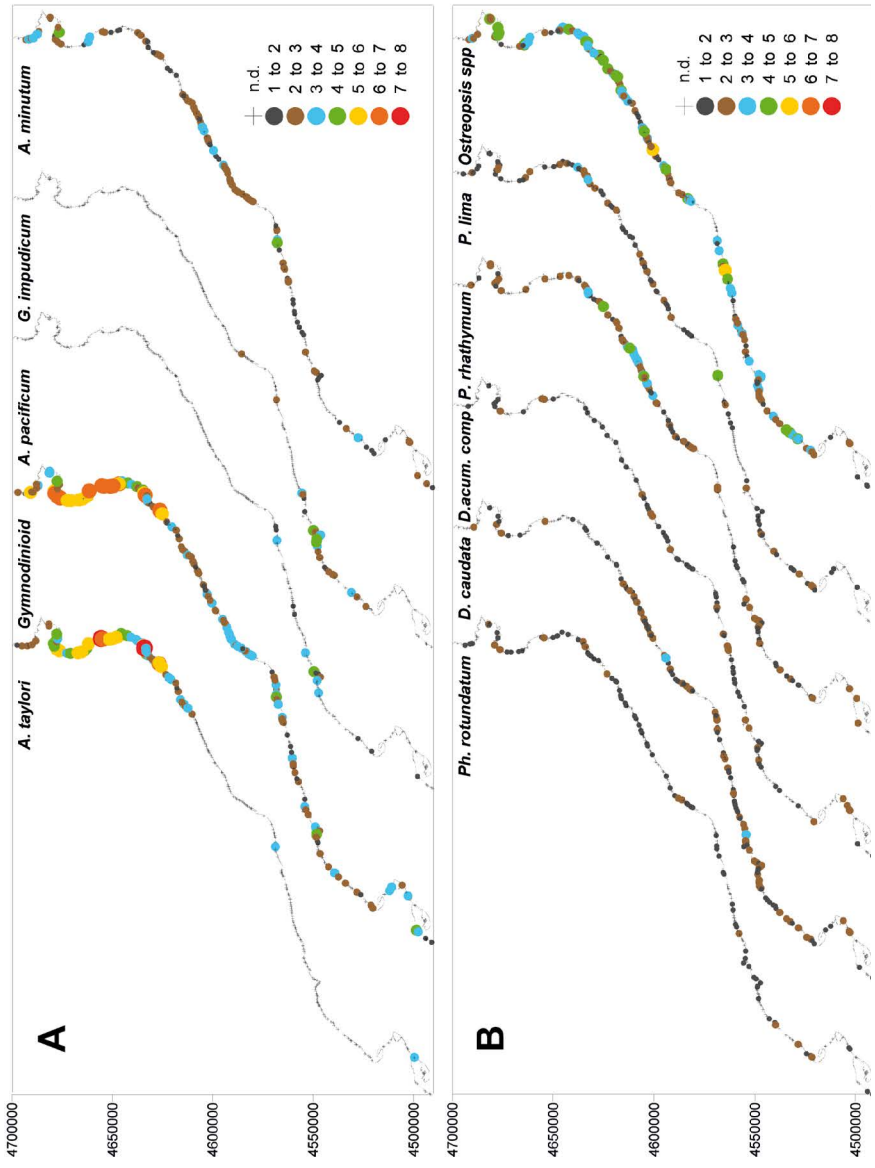

Figure 4. Spatial distribution of the most frequently detected harmful species along the Catalan coast. Dots of different colors show the maximum abundances (expressed as log cell number L^{-1}) determined in all samplings from each sampling point. Crosses indicate the sampling points where harmful dinoflagellates were not detected.

Table 1. Potentially harmful dinoflagellate species or taxa observed along Catalan beaches, their maximum abundances, harmful impact (DSP: diarrheic shellfish poisoning; HB: high-biomass blooms; ITX: ichthyotoxic, "ITX": possibly ichthyotoxic; NSP: neurotoxic shellfish poisoning; PSP: paralytic shellfish poisoning). Toxins with potentially harmful, not-well-known effects are indicated in parentheses (YTXs: yessotoxins). The species distribution was classified as: wide (all along the coast), narrow (along a part of the coast); localized (in specific places), or unknown (detections were too few to deduce the distribution of the species). The number of months with detections, months of bloom densities, number of detections, and number of blooms during all the studied period are also shown. Most of the non-toxic bloom forming species were counted only when they achieved bloom abundances, thus the number of detections and the seasonality are not shown for these species.

Genus/Species	Maximum abundance	Harmful impact	Distribution	Seasonality (months with detections)	Seasonality of blooms (months)	Number of detections	Num of blooms
Alexandrium minutum Halim	30448	PSP	wide	All	3, 7	316	2
Alexandrium ostenfeldii (Paulsen) Balech & Tangen	40	PSP, (spirolides)	unknown	1, 2, 9	-	5	0
Amphidinium carterae Hulburt	159156	ITX	unknown	6–9	7,8,9	8	4
Alexandrium pacificum Litaker	11205	PSP	narrow	6, 7 8, 12	8	19	1
Coolia monotis	12802	HB	wide	All	7	324	1
Dinophysis acuminata complex	560	DSP	wide	2–12	-	203	0
Dinophysis acuta Ehrenberg	120	DSP	wide	4,5,6,7,8	-	10	0
Dinophysis caudata Saville-Kent	1960	DSP	wide	3, 5–12	-	97	0
Dinophysis fortii Pavillard	40	DSP	unknown	9	-	1	0
Dinophysis ovum (F.Schütt) T.H.Abé	920	DSP	wide	All	-	71	0
Dinophysis tripos Gourret	40	DSP	unknown	5,6	-	2	0
Phalacroma mitra F.Schütt	40	DSP	unknown	8	-	1	0

Table 1 (cont.).

Phalacroma rotundatum (Claparéde & Lachmann) Kofoid & Michener	520	DSP	wide	All	-	175	0
Lingulodinium polyedrum (F.Stein) J.D.Dodge	200	"DSP" (YTXs)	wide	1, 5 to 8	-	24	0
Ostreopsis spp. (O. ovata Fukuyo + O. siamensis Johs.Schmidt)	205632	Palytoxicosis, respiratory and skin irritations	wide	All	6 to 9, 11	903	52
Prorocentrum lima (Ehrenberg) F.Stein	17290	DSP	wide	2,3,5–12	8	249	1
Prorocentrum rhathymum A.R.Loeblich III, Sherley & Schmidt	29064	DSP	wide	4, 6–12	7, 8	373	3
Protoceratium reticulatum (Claparède & Lachmann) Bütschli	200	"DSP" (YTXs)	wide	1, 3–10	-	41	0
Karlodinium spp.	9640	ITX, Mortality invertebrates	wide	4, 6 to 8, 10 to 1	-	16	0
Amphidinium spp	440	ITX	wide	1, 3 to 11	-	77	0
Prorocentrum cordatum (Ostenfeld) J.D.Dodge,	43160	ITX	wide	All	7,8	181	4
Karenia spp.	880	NSP, (gymnodimine)	wide	1, 4–12	-	24	0
Prorocentrum emarginatum Y.Fukuyo,	138	Hemolytic activity	wide	3–10, 11	-	35	0

Table 1 (cont.).

Prorocentrum concavum Y.Fukuyo,	120	DSP	unknown	7–10	-	10	0
Alexandrium taylori Balech	16727924	HB	narrow	3–10, 12	5 to 9	856	301
Gymnodinium impudicum (Fraga et Bravo) Hansen et Moestrup	98355	HB	narrow	6–9	7,8	43	5
Gymnodinioid (no *G. impudicum*)	65131159	HB	wide	All	5 to 11	997	336
Gonyaulax fragilis (Schütt) Kofoid	2160	Mucilage producer	narrow	7, 9	-	49*	0
Heterocapsa sp.	1494304	Mortality shellfish and fish	-	-	4 to 8	-	6
Prorocentrum micans Ehrenberg	38083	HB	wide	4–9, 11	5, 8	175	4
Prorocentrum triestinum J.Schiller	246705	HB	wide	3–8	3, 5 to 8	76	11
Protoperidinium quinquecorne (Abé, 1927) Balech	38083	HB	-	-	8	-	3
Scrippsiella spp.	155655	HB	wide	-	6 to 8	-	29
Kryptoperidinium foliaceum (Stein) Lindemann	320396	HB	localized	1–7, 9–12	4 to 6, 10, 12 to 2	41*	9
Alexandrium cf. *tamarense* (Lebour) Balech emended John	2320	PSP	unknown	6, 7, 8	-	10	0
Alexandrium cf. *leei* Balech	120	Other species of interest	unknown	6, 7	-	5	0
Alexandrium margalefi Balech	480	Other species of interest	unknown	5, 6	-	2	0

Table 1 (cont.).

Alexandrium tamutum Montresor, Beran & John	40	Other species of interest	unknown	8	-	1	0
Alexandrium pseudogonyaulax (Biecheler) Horiguchi ex Kita & Fukuyo	120	"ITX"	unknown	5, 6, 7, 9	-	4	0
Alexandrium insuetum Balech	200	Other species of interest	wide	4–8	-	20	0

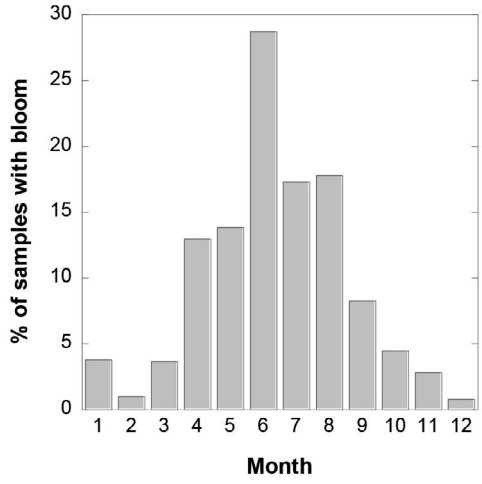

Figure 5. Monthly percentages of samples containing bloom cell abundances of at least one species of dinoflagellate.

reached bloom abundances at 28 different beaches (11%) along the entire Catalan coast, but especially along beaches of the north coast. However, the beaches with the highest *Ostreopsis* abundances (> 10^5 cell·L^{-1} were Llavaneres and Terramar beach, all of which are located on the central coast. Other species occurred at only a few beaches; for example, *Kryptoperidinium foliaceum* (**Table 1**) was detected at only three beaches (Desemb. Muga, Estartit 3, and La Platjola) and reached bloom always at the same beach (Desemb. Muga).

An analysis of the seasonality of the species showed that although many species were detected during every season, nearly all blooms of dinoflagellates occurred between April and September. The highest percentages of samples with cell abundances indicating bloom formation were obtained in June, July, and August, whereas blooms were infrequent in the colder months (**Table 1**, **Fig. 5**). Half of the blooms recorded from October to March were those of non-identified dinoflagellate and they occurred at different beaches. *Kryptoperidinium foliaceum*, Gymnodinioids, *Ostreopsis* spp., and *A. minutum* formed blooms in colder but also warmer months. For example, *Ostreopsis* spp. blooms were most often detected in July and August.

In addition to the dinoflagellates listed in Table 1, 116 blooms of non-identified dinoflagellates were recorded over the study period.

3.3 Spatio-temporal distribution of the *Alexandrium* genus

The ten species of *Alexandrium* detected in this study were *A.insuetum*, *A.* cf. *leei, A. margalefi, A. minutum, A. ostenfeldii, A. pacificum, A. pseudogonyaulax, A. tamarense, A. tamutum,* and *A. taylori*. Most were recorded only occasionally in few locations and in low abundances.

From those recorded ocasionally, *A. tamutum* was detected only once, and at the detection limit (40 cells·L^{-1}), at a beach located in the southern area (La Punta del Riu) in August 2005. *A. margalefi* was detected once at two different beaches, one located in the north and the other in the south, with a maximum cell abundance of 480 cells·L^{-1}. The first detection was in 2007 and the second in 2011 (**Table 2**), both during the spring season. *A. ostenfeldii* was detected during three different years (**Table 2**) and in January, February, or at the end of September, but always at the minimum of 40 cells·L^{-1}. The detections occurred at four different beaches, two in the north and two in the south. Three were at stations located close to the mouth of a nearby river. *A. pseudogonyaulax* was also detected occasionally and at very low cell abundances during May to September of three different years (**Table 2**). Three of the four detections were at La Platjola, located in the southern Ebre Delta (**Fig. 1**). The fourth was at another beach near the delta.

Some others were recorded also ocasionally but the appearance was synchronized at different places along the coast. *A.* cf. *leei* was detected in June–July 2007 at five beaches scattered along the coast. The cell abundances were very low (maximum of 120 cells·L^{-1} at Desemb. Muga). *A. insuetum* was first detected in 2004, at several beaches along the coast, and then during most of the following years albeit always at low cell abundances (**Table 2**). While the majority of the detections were in June samples, they also occurred in samples from the end of April to the beginning of August.

A. cf. *tamarense* and *A. pacificum* were detected during several years, usually at low cell abundances (<1000 cells L^{-1}), but at abundances up to 2320 cells L^{-1} for *A.* cf. *tamarense* in 2008 and up to 11.2 X 10^3 cells L^{-1} for *A. pacificum* in 2005. Beginning in 2005, *A.* cf. *tamarense* was detected in June, July, and August at ten beaches spread along the Catalan coast. The highest cell abundance (see above) was at La Gola beach (north coast) in August 2008. The species was also detected on the same day at the two near beaches but at lower cell abundances. *A. pacificum* was detected at several central and southern beaches between Viladecans and Cambrils (Prat d'en Forés beach) (**Fig. 6**) in 2001, and later at its southern boundary, in Parc de Garbí beach (**Fig. 6**), in 2004. The maximum cell abundances were in the August 2005 samples from Els Prats beach, at the southern end of Tarragona Harbor, where recurrent blooms of this species occurred (see Chapter 2 and **Fig. 1**). The species was also detected in June, July, and August during other years

and on one occasion in December, although at an abundance at the limit of detection.

A. minutum and *A. taylori* were detected every year at many Catalan beaches (**Fig. 7**and **8**), with *A. minutum* the more widely distributed although usually at low abundances. It was detected all along the coast (**Fig. 4A**), affecting 131 of the 258 (51.2%) sampled beaches, mostly in the central and northern areas. The cell abundances at those locations were typically < 4000 cells L^{-1} and twice they were >10^4 cells L^{-1}: at Canyelles Petites beach (northern coast, **Fig. 1**) in March 2008 (30.5 X 10^3 cells L^{-1}) and at Castelldefels beach (central coast, **Fig. 1**) in July 2010 (10.7X 10^3 cells L^{-1}). At most of the more frequently sampled beaches *A. minutum* was detected on a few occasions but at very low abundances. Among the beaches that were monitored throughout the year over several years and with at least 60 samples (range: 60–174), *A. minutum* was not detected in some of them (l'Arenal beach). Rather, in other beaches this species was present in up to 18.5% of the samples and especially in those from Llavaneres beach, located south of Arenys Harbor, where it produced recurrent blooms every year. At Llavaneres beach, together with the beaches of Castelldefels, La Platjola, Desemb. Muga, and Estartit 3, *A. minutum* was detected in > 12% of the samples from these beaches and throughout the year but mostly from May to July–August. During the study period, *A. minutum* was most widespread in 2006, along the central coast (**Fig 7**). There was no trend in the cell abundances at Llavaneres beach (where the species was most frequently detected) from 2005 to 2012 (p-value=0.02 but Sen's slope=0).

The most abundant species at the sampled beaches was *A. taylori*, which produced high-biomass blooms in which cell densities reached 16.7 X 10^6 cells L^{-1} (la Fosca beach, August 2000). This species was mostly delimited to the northern Catalan coast (**Fig. 4A**). It was present in samples from 81 sampling stations of the Costa Brava (including Malgrat), representing ~90% of the beaches in this area. At 30 of them (~33%), *A. taylori* was detected at bloom abundances (>10^4 cells L^{-1}) at least once between June and September. At 18 of these beaches, abundances reached 10^5 cells L^{-1}. At 11 sampling stations, *A. taylori* was settled producing recurrent blooms (blooms in more than half of the years of the study period). These 11 sampling stations were as follows: la Muga, Cala Montgó, Estartit (3 sampling stations), La Gola, del Grau, Castell, La Fosca, and Sant Pol (2 sampling stations) (see **Fig. 8**). Only

Table 2. Maximum abundances of *Alexandrium* species detected along Catalan beaches during 12-years of the studied period.

Years	N	*A.minutum*	*A.* cf. *tamarense*	*A. pacificum*	*A. ostenfeldii*	*A. pseudogonyaulax*	*A. taylori*	*A.* cf. *leei*	*A. margalefi*	*A. tamutum*	*A. insuetum*
2001	512	160	0	2884	0	0	$11,9 X10^6$	0	0	0	0
2002	223	360	0	0	0	0	$2,4 X10^6$	0	0	0	0
2003	399	1720	0	0	0	0	$8,1 X 10^6$	0	0	0	0
2004	296	1211	0	80	0	40	$3,3 X 10^6$	0	0	0	120
2005	450	640	40	11205	0	0	$1,4 X10^6$	0	0	40	200
2006	444	2280	0	0	40	0	$7,1 X 10^6$	0	0	0	120
2007	342	2763	830	40	0	0	$1 X10^6$	120	40	0	0
2008	342	30448	2320	0	40	0	$8,8 X10^5$	0	0	0	40
2009	353	3335	0	0	0	80	$2,2 X10^6$	0	0	0	0
2010	440	10726	622	40	0	0	$3,7 X 10^6$	0	0	0	80
2011	190	320	240	0	40	120	$1 X 10^6$	0	480	0	40
2012	60	120	0	0	0	0	$5,1 X10^5$	0	0	0	120
Max.	4142	30448	2320	11205	40	120	$11,9 X 10^6$	120	480	40	200

at Estartit 3, Sant Pol, and La Fosca beaches did the abundances exceed 10^6 cells L^{-1}. Isolated detections of *A. taylori* were also recorded at Castelldefels beach (central coast) and Trabucador beach (southern coast). There were also occasional detections in March, April, October, and December (**Table 1**), although at low abundances (maximum of 7470 cells L^{-1}, at Canyelles Petites beach).

3.4 Temporal variability of *A. taylori* and its relation to environmental factors

As the species that produces the largest number of blooms along Catalan beaches, *A. taylori* was investigated in detail in this study together with the environmental factors in the affected areas. We therefore examined the long-term variability of this species at three of the beaches in the Costa Brava (north coast) where it was recurrently detected (Desemb. Muga, Estartit 3, and La Fosca beaches), using samples collected over the longest period of the study (2005–2012) from sites that were more regularly sampled. As *A. taylori* blooms recurrently at yearly intervals, its annual bloom patterns at these three beaches were investigated as well.

Based on the results of the MK trend test and Sen's slope (**Table 3**), applied to the log abundances of *A. taylori*, there was no significant trend for this species at any of the three beaches during the period 2005 to 2012 (**Fig. 9**). However, analyses of the environmental data obtained over the same period showed significant increasing trends in both temperature and the ammonium concentration at La Fosca and Estartit 3 beaches.

As La Fosca beach was sampled every summer between 1995 and 2012, an additional MK trend test was performed using the mean abundance in July during those same years, to identify changes occurring over a longer period. However, there was no trend in the July abundances of *A. taylori* ($p=0.969$).

The three studied beaches, spread along the Costa Brava, differed markedly in their freshwater inputs and therefore in their physicochemical characteristics (**Fig. 2**), including inorganic nutrient concentrations and salinity. At Desemb. Muga beach, characterized by a strong riverine influence, inorganic nutrient concentrations were high and the salinity was low. At Estartit 3 beach, the riverine influence is moderate and the salinity is usually > 37, but it can be very low. Nutrient concentrations were high, especially in the case of nitrates,

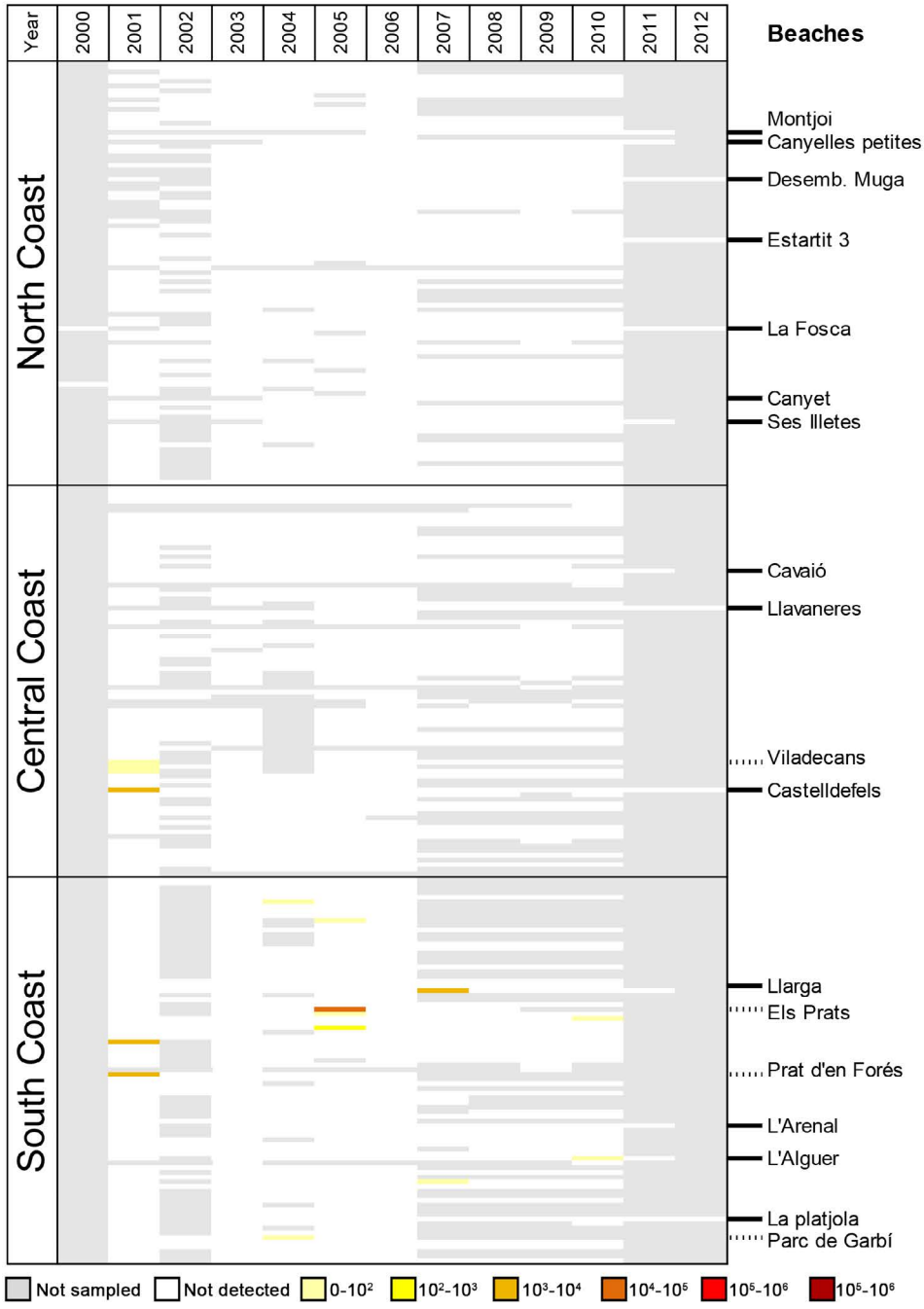

Figure 6. Maximum anual abundances (expressed as cells L^{-1}) of *A. pacificum* detected in samples from 258 Catalan coast beaches during the study period. Black lines indicate the locations of the more frequently sampled beaches, and dotted lines the locations of beaches referred to in the text.

but they were lower than at Desemb. Muga. At La Fosca beach, where there is no riverine influence, the salinity was relatively stable (**Fig. 2B**) year-round and inorganic nutrient concentrations were low. These differences in the freshwater inputs were also reflected in the silicate limitations at each beach; thus, potential stoichiometric limitations of silicate occurred in 37.8% of the samples at La Fosca, 16.8% of those at Estartit 3, and 6.3% of those at Desemb. Muga. For the other nutrients of interest, phosphate was seldom stoichiometrically limiting for strict autotrophic phytoplankton, with a maximum of 4.1% of the samples at La Fosca beach. The stoichiometric limitation of DIN was also low at La Fosca and Desemb. Muga beaches (max 2.7% of the samples) but much higher at Estartit 3 beach (11%).

Despite the differences in their environmental variables, La Fosca Estartit 3, and Desemb. Muga were subject to annually recurrent blooms of *A. taylori* (**Fig. 10**), with clear unimodal patterns that were synchronous for the three beaches (**Fig. 11A**). Vegetative cells first appeared in mid-May, after which the cell densities increased until maximum abundances were reached, usually between July and early August. Thereafter, the abundances declined until September, when *A. taylori* was no longer detectable.

The annual patterns in the biological and physico-chemical parameters are plotted in Figure 11. Chl-*a* levels at the three beaches were higher in summer although in Desemb. Muga the chlorophyll peaks did not always coincide with the maximum abundances of *A. taylori* (**Fig. 11A, B**). The annual salinity pattern was similar at the three beaches, with high salinities at the beginning and end of the year and from the end of July until the beginning of September, and low salinities at the end of spring and at the beginning of summer (**Fig. 11 D**). DIN was clearly dominated by nitrate and thus had the same annual pattern as this nutrient but also of nitrite (**Fig. 11F, G, I**), the lowest values of which were detected during June and July. Concentrations of ammonium were more stable in the second half of the year whereas peaks occurred in the first half of the year (**Fig. 11H**). The annual pattern of phosphate was different at the three beaches (**Fig. E**).

Table 4 shows the Spearman correlation between *A. taylori* cell abundances and different variables. A clear correlation of *A. taylori* cell abundances with water temperature and with Chl-*a* levels was determined for all three beaches. But, a (negative) correlation between *A. taylori* cell density and salinity was

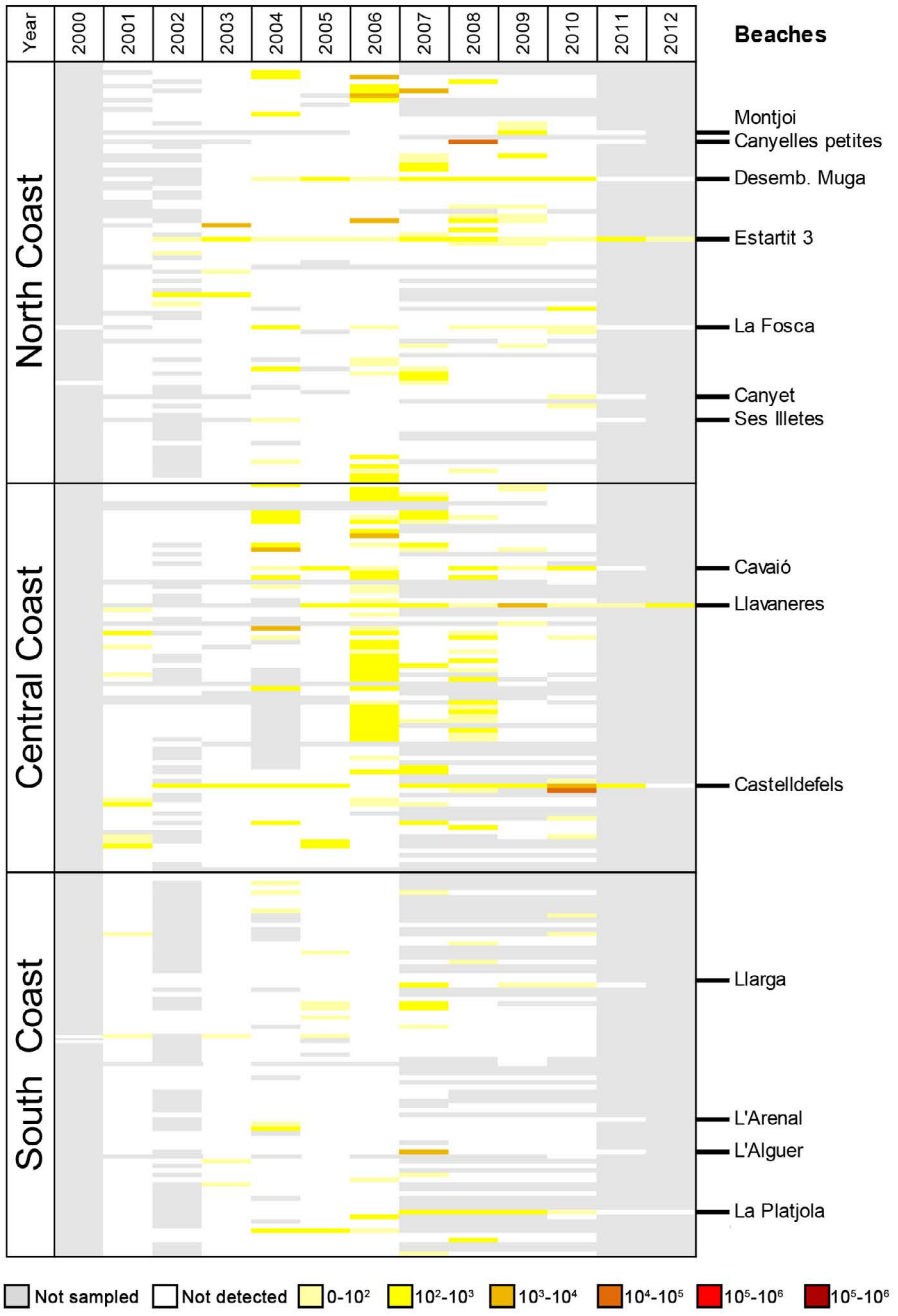

Figure 7. Maximum anual abundances (expressed as cells·L^{-1}) of *A. minutum* detected at 258 Catalan coast beaches during the study period.

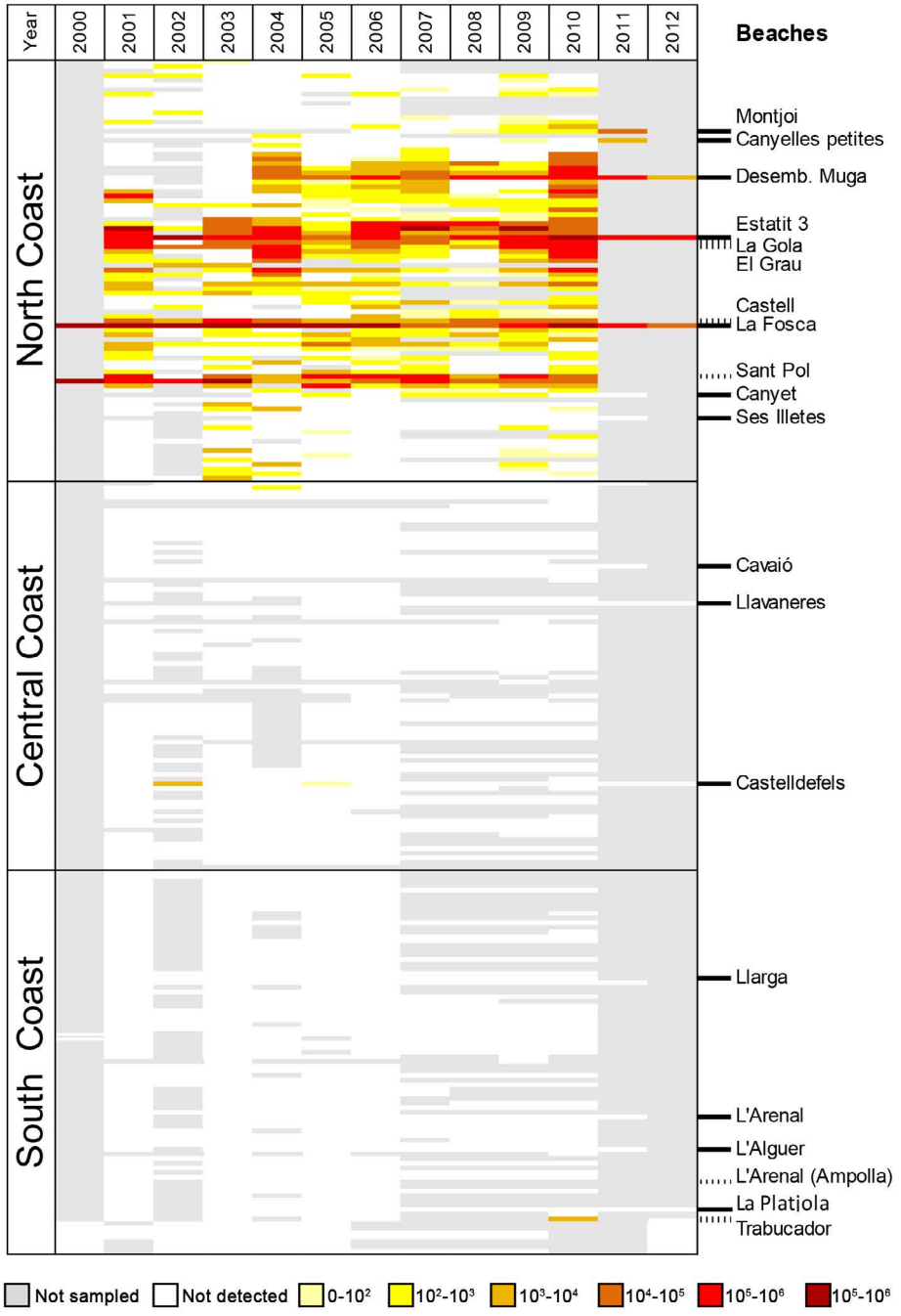

Figure 8. Maximum anual abundances (expressed as cells L^{-1}) of *A. taylori* detected at 258 Catalan coast beaches during the study period. Black lines show the location of the more frequently sampled beaches, and dotted lines the location of beaches referred to in the text.

detected only at La Fosca and Estartit 3 beaches. Correlations between the cell abundances of *A. taylori* and inorganic nutrients varied and depended on the beach.

4. Discussion

4.1. Harmful dinoflagellates and associated problems

At Catalan beaches *A. taylori* was the main bloom-forming species causing water discolorations. In cultures of *A. taylor*i isolated from the Malaysian coast, Lim et al. (2005) detected PSP toxins by HPLC. In their study of Japanese strains, Emura et al. (2004) suggested that this species could produce a hemolytic exotoxin. By contrast, PSP toxins have never been detected in Mediterranean strains (Delgado et al., 1997; Penna et al., 2005b) and the species in the Mediterranean Sea is not considered to be toxic. Nonetheless, the proliferation of this species, often accompanied by high abundances of Gymnodinioids, causes a deterioration of the water quality at beaches used for swimming. In Sardinia, blooms of *A. taylori* have co-occurred with those of *Barrufeta bravensis* and *Levanderina fissa (=Gymnodinium instriatum*) (Satta et al., 2014). In the Balearic Islands, *A. taylori* was also found with *Barrufeta bravensis* (Illoul, 2014). The abundances of these Gymnodinioids often surpassed those of *A. taylori.* Recently (21/07/2015), a monospecific bloom of *Barrufeta bravensis* with abundances of 6.7 X 10^6 cells L^{-1} and causing water discoloration was detected at a Barcelona beach, on the central coast (personal observation). Previously, this organism had only been found on the north coast (Reñé et al., 2015; Sampedro et al., 2011). The data analyzed in the present study did not indicate any blooms of Gymnodinioids along the central coast. Therefore, the range of this species may be expanding, which, due to the formation of blooms, could become problematic.

Other problematic dinoflagellates along the Catalan coast include members of the genus *Ostreopsis*, which is thought to induce respiratory difficulties and other symptoms in people exposed to seawater or to marine aerosols. These problematic events have been detected in many Mediterranean coastal localities since 1998 (see a review of references in Vila et al., 2016,

Table 1). Moreover, blooms of this genus have also been related to massive macrofaunal mortalities (e.g., Shears and Ross, 2009). *Ostreopsis* species are epiphytic, which may explain they are observed habitually at beaches where the rock substratum is rich in macroalgae, although they have also been found at high abundances in the water column of these areas. Along the Catalonia coast, respiratory symptoms attributable to *Ostreopsis* outbreaks have been detected only at Llavaneres Beach (Vila et al., 2016; Vila et al., 2008). However, our data indicate the presence of this species at three-quarters of the beaches sampled, more than previously reported (Vila et al., 2008), including a considerable number of beaches where bloom abundances were reached. Thus, if the causality between *Ostreopsis* and symptoms in humans are confirmed, then the users of many other beaches may be affected by these respiratory symptoms. On the other hand, unique features of Llavaneres beach may explain the intoxications that occurred there. For example, because the beach is a large rocky platform totally covered with macroalgae, it is one of the

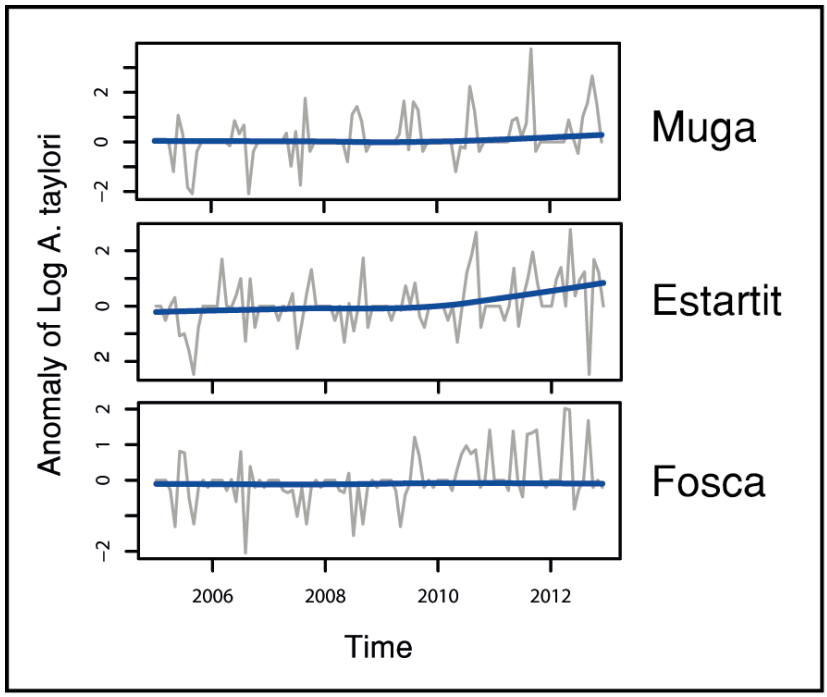

Figure 9. Anomalies in *A. taylori* abundances (log cells L^{-1} +1) at Desembocadura de la Muga, Estartit, and La Fosca beach (gray lines), and the Lowess smoothing showing the trend (blue lines).

few beaches where *Ostreopsis* abundances reach 10^5 cells L^{-1} and blooms are annually recurrent. This would increase the probability of a toxic event if other environmental conditions are also needed. Another consideration is that in addition to respiratory symptoms, along an extensive part of the coast there is a potential risk of poisoning by the consumption of palytoxin through the trophic chain, as reported in other areas of the world (Alcala et al., 1988; Randall, 2005) but thus far not for the Catalan or any European coast.

Kryptoperidinium foliaceum is a bloom-forming species that in some cases has been associated with fish mortalities (Lewitus et al., 2003; Wolny et al., 2004) and damage to oyster cultures (Kempton et al., 2002). As it tolerates a wide range of salinities (Saburova et al., 2012), blooms of this species in brackish habitats (e.g., Figueroa et al., 2009) but also in hypersaline conditions (Saburova et al., 2012) have been reported. In 2003, a bloom of *K. foliaceum* was detected in a coastal lagoon in front of Estartit 3 beach but not at the beach itself (López-Flores et al., 2005). High abundances also occurred in 2015, in a lagoon near the previously mentioned one (Anglès et al., 2017). South of the Catalan coast, cysts of *K. foliaceum* were identified in sediments collected in March 2009 from Fangar Bay (Ebre River Delta). Ours is the first report of vegetative cells of this species at Catalan coast beaches, and specifically, at three beaches, which are characterized by inputs of freshwater. However, only at Desem. Muga, where the salinity is much lower than at the other beaches included in this study and the concentrations of inorganic nutrients vary widely, was *K. foliaceum* found at bloom abundances. Blooms were detected during the last 3 years of the study and during different seasons, except in summer. The dynamics differed from those reported for this species, for example, at the estuary of the Miñor River (northwest Spain), where recurrent blooms have been recorded in summer (Figueroa et al., 2009).

The accumulation of mucilage in coastal waters adversely impacts tourism at the affected beaches as well as local fishing. *Gonyaulax fragilis* has been linked to mucilage events in the Mediterranean Sea (Pompei *et al*., 2003) and, in the summer of 2006, at the Catalan coast (Sampedro et al., 2007). We detected this species in many samples collected in the summer of 2006 from beaches of the central and southern coasts, where aggregates of mucilage were also observed (Sampedro et al., 2007). However, the cell abundances in these samples were much lower than those found in the mucilage (Sampedro

et al., 2007). Thus, it may be that high abundances of *Gonyaulax fragilis* are restricted to mucilage, whereas in nearby water samples abundances are low, such that blooms of this species will not be detected if the mucilage is not sampled.

Among the PSP-producing species, *A. minutum*, with its potential for high abundances and its widespread distribution, is the one that raises the most concern. Its abundances at the studied beaches were high enough to intoxicate shellfish species (although abundances >5000 cells L^{-1} were infrequent), especially at beaches close to the southern end of harbors, where this species usually blooms (see below). Despite the scarce detection of *A. pacificum* and its low abundances, outbreaks of this species, such as occurred in 1998–1999 along the southern half of the Catalan coast, cannot be ruled out. Whether such blooms are a recurrent phenomenon, with a recurrence interval longer than the length of our study period, is unclear.

Among the DPS-producing species, only *Prorocentrum lima* and *Pr. rhathymum* were found in bloom abundances. However, low abundances (a few hundred cells per liter) of dinophysiales, found in many of the beaches, are enough to produce DSP episodes (Blanco et al., 1998; Reguera et al., 1993) and thus to contaminate seafood.

4.2. Spatial and temporal distribution of *Alexandrium*

Many of the *Alexandrium* species recorded in this study were detected very occasionally and for the first time in water samples from Catalan beaches. These detections were in some cases in the same year as their first detections in Catalan harbors while in others they occurred in later years (chapter 1), thus confirming the utility of sampling in confined areas to improve the early detection of dinoflagellates in coastal waters, in agreement with Vila et al. (2001a). In the following, we discuss the spatial and temporal evolution of the most problematic *Alexandrium* species along the Catalan coast.

4.2.1 *A. minutum* and *A. pacificum* (PSP producers)

A. minutum was detected all along the Catalan coast. Its abundances were highest in March and July, which coincided with the distribution and annual patterns observed in confined waters, where this species frequently reaches

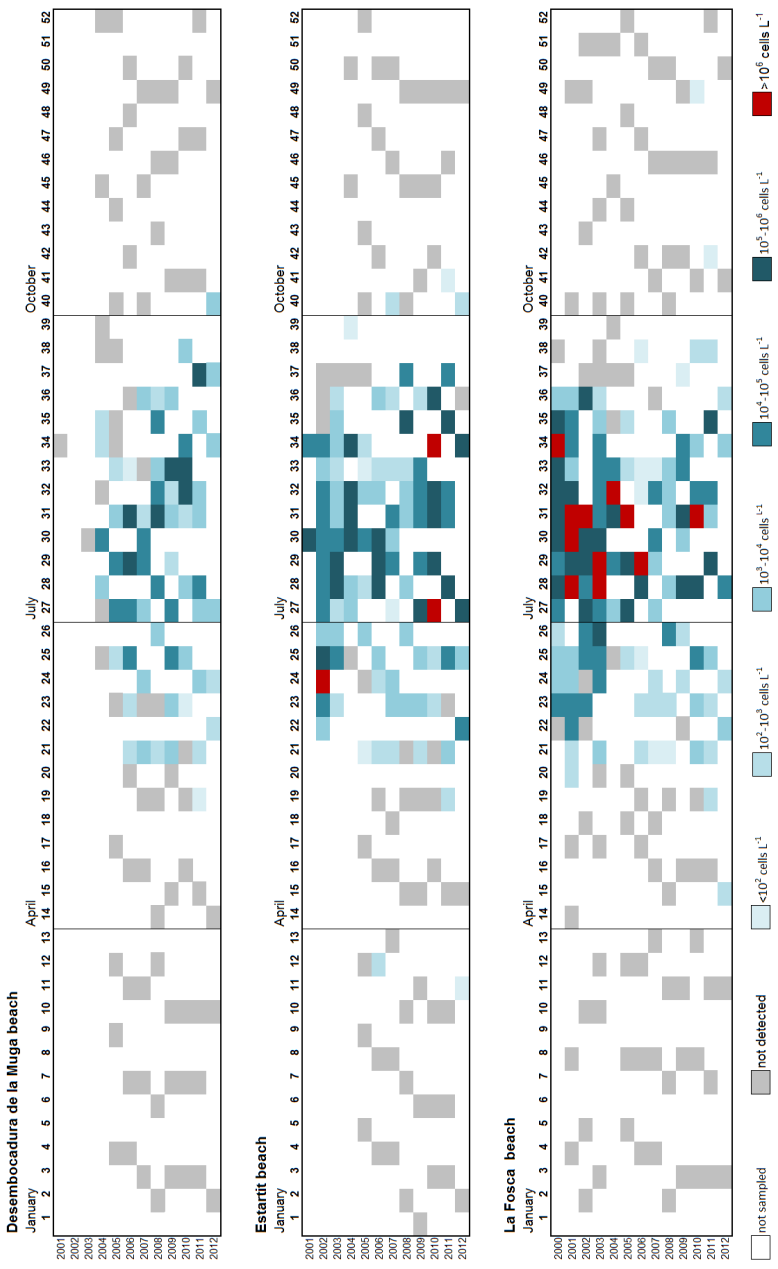

Figure 10. Graphical summary of the occurrences of *A. taylori* at Desembocadura de la Muga (A), Estartit (B), and La Fosca (C) beaches during the 13-year study period.

bloom abundances (see Chapter 1). However, most of the samplings were carried out in July and August, whereas this species is able to reach high abundances, and therefore produce blooms, in winter and spring (Chapter 1) and the maximum abundances achieved by this species at beaches may be accordingly skewed.

Changes in the distribution of *A. minutum* during the study period could not be accurately determined due to the different number of beaches sampled every year and the low sampling frequencies at most of them. However, the number of beaches where the species was detected was much lower in 2001 than in the following summers, suggesting the expansion of this species. Indeed, *A. minutum* was observed consistently at Castelldefels beach beginning in 2002, but not at this location previously (see below).

In the case of *A. pacificum*, no other expansive bloom of this species occurred during the study period that was comparable to the outbreaks reported in 1998 and 1999 (Vila et al., 2001b, reported as *A. catenella*). Our detections of this species were very rare and included only one at bloom abundance. Half of the detections were in 2001 whereas the detections in the final years of the study were sporadic. Castelldefels beach was monitored from 1996 to 1999 by Vila et al. (2001b), who detected *A. pacificum* (as *A. catenella*) during the final 2 years at abundances of up to 1.2×10^4 cells L^{-1}, coinciding with expansive blooms of this species. During our regular monitoring of this beach from 2001 to 2012, this species was detected only three times, all of them in 2001, at a maximum abundance of 2.3×10^3 cells L^{-1} (**Fig. 6**). This species has not been detected at Castelldefels since then, in contrast to the detections of *A. minutum* at this beach beginning in 2002 (**Fig. 5**). A similar change on the dominance of these two species was observed in Barcelona Harbor (Chapter 1). Thus, the water at Castelldefels beach could have been influenced by that of the nearby harbor.

Interestingly, a large number of the detections occurred in beaches located south of the two large harbors (those of Barcelona and Tarragona) where *A. pacificum* blooms recurrently (Chapter 2 and Vila et al., 2005). Similarly, the beach where *A. minutum* was frequently observed is located south of Arenys Harbor, where high-abundance blooms of this species occur every year.

The distribution of *A. minutum* and *A. pacificum* along Catalan beaches

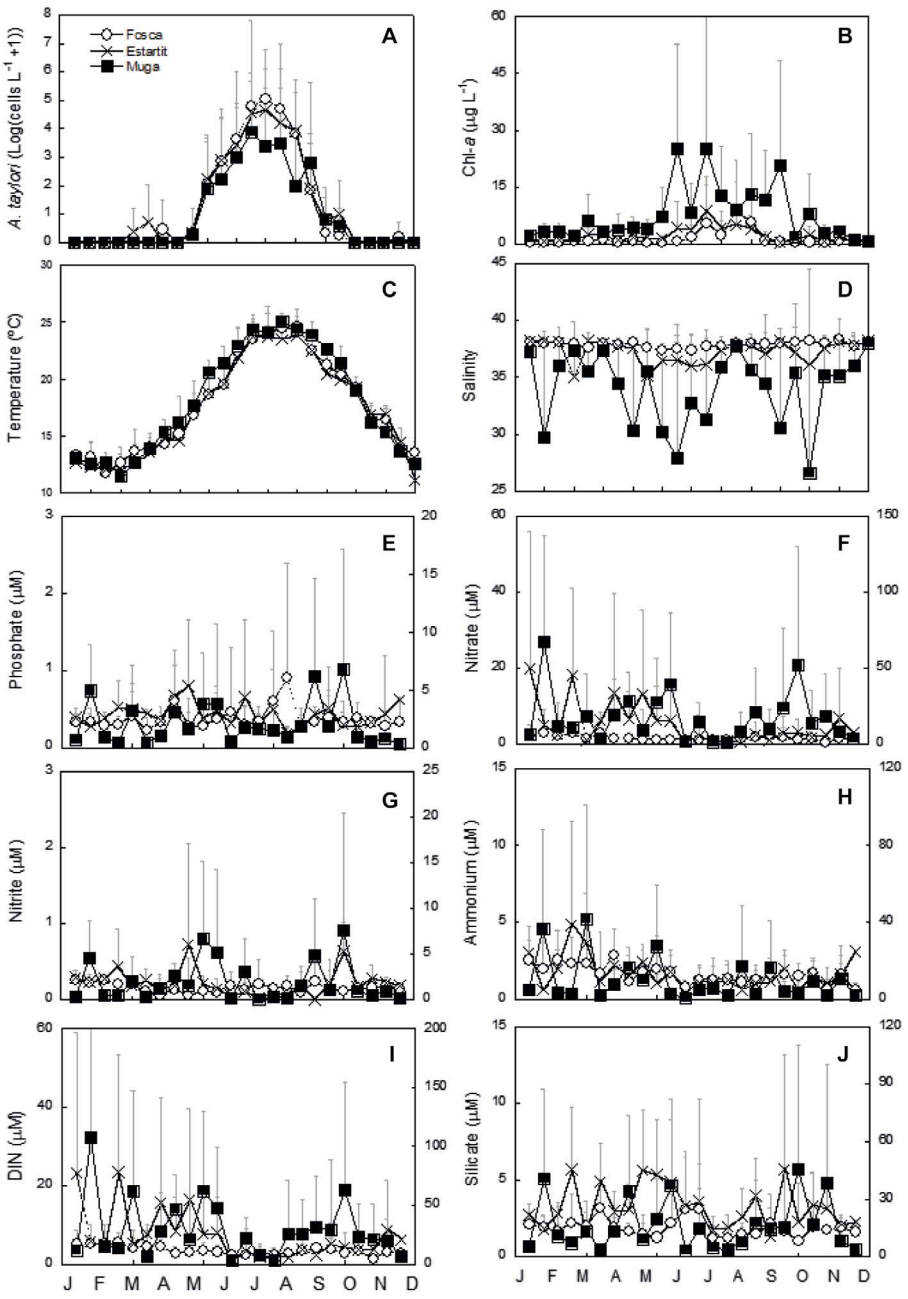

Figure 11. Annual patterns of the biological and physico-chemical parameters at beaches with recurrent blooms of *A. taylori*: Desembocadura de la Muga (righ y axes), Estartit, and la Fosca (left y axes).

Table 3. Results of the Mann-Kendall trend test and Sen's slope of the monthly anomalies of environmental and biological series. Significant P-values (α=0.05) in the MK test for the detection of trends and the values of Sen's estimator of slopes that did not include 0 in the 95% confidence interval are shown in bold.

Environmental variable	Desem. Muga beach					Estartit 3 beach					Fosca beach				
	MK trend test		Sen's slope			MK trend test		Sen's slope			MK trend test		Sen's slope		
	Tau (τ)	P-value	Slope	Confidence limits		Tau (τ)	P-value	Slope	Confidence limit		Tau (τ)	P-value	Slope	Confidence limit	
L. *A.taylori* (log cells L^{-1} +1)	0.184	**0.014**	0.000	0.004	0.000	0.234	**0.001**	0.004	0.013	0.000	0.193	**0.008**	0.003	0.006	0
Temperature (°C)	-0.011	0.877	-3.00E-04	0.009	-0.010	0.215	**0.002**	**0.013**	0.021	0.004	0.144	**0.038**	**0.009**	0.016	5.00E-04
Salinity	-0.046	0.512	-0.011	0.021	-0.044	0.000	1.000	0.000	0.006	-0.007	-0.018	0.795	-2.00E-04	0.002	-0.002
Chlorophyll *a* ($\mu g \cdot L^{-1}$)	-0.075	0.283	-0.016	0.014	-0.050	0.055	0.431	0.003	0.014	-0.004	-0.066	0.344	-0.001	0.002	-0.004
NO_3 (μM)	-0.006	0.937	-0.005	0.169	-0.132	-0.076	0.272	-0.019	0.015	-0.055	-0.024	0.730	-0.002	0.006	-0.009
NO_2 (μM)	-0.029	0.674	-0.003	0.013	-0.020	0.027	0.695	2.00E-04	0.001	-7.00E-04	-0.063	0.369	-2.00E-04	2.00E-04	-7.00E-04
NH_4 (μM)	-0.110	0.114	-0.044	0.008	-0.105	0.165	**0.018**	**0.010**	0.018	0.002	0.191	**0.006**	**0.012**	0.0198	0.003
DIN (μM)	-0.017	0.812	-0.029	0.219	-0.302	-0.028	0.688	-0.008	0.028	-0.048	0.129	0.063	0.012	0.024	-7.00E-04
PO_4 (μM)	-0.019	0.783	-0.001	0.010	-0.015	0.137	**0.049**	0.002	0.004	0.000	-0.047	0.500	-5.00E-04	0.001	-0.002
SiO_4 (μM)	0.021	0.768	0.017	0.010	-0.015	0.114	0.102	0.009	0.020	-0.002	0.053	0.449	0.002	0.009	-0.004

Table 4. Spearman rank correlations between environmental and biological factors and *A. taylori* cell abundances calculated using the data from all beaches and from the three beaches with recurrent blooms of *A. taylori*. Correlations significant at p<0.01 are indicated in bold.

Environmental and biological variables	All beaches All data N=1193	Muga All data N=106	Estartit3 All data N=129	Fosca All data N=186
Temperature (ºC)	**0.342**	**0.780**	**0.760**	**0.745**
Salinity	**−0.154**	−0.127	**−0.241**	**−0.241**
Chl-*a* (µg L^{-1})	**0.180**	**0.374**	**0.476**	**0.428**
NO_3 (µM)	**−0.137**	−0.149	**−0.453**	**−0.320**
NO_2 (µM)	**−0.132**	−0.129	**−0.457**	−0.008
NH_4 (µM)	**−0.199**	−0.231	**−0.344**	−0.054
DIN (µM)	**−0.198**	−0.117	**−0.462**	**−0.246**
PO_4 (µM)	0.056	0.076	0.050	0.124
SiO_4 (µM)	0.026	−0.077	0.002	**−0.213**

corroborates the wide and limited distribution, respectively, reported in confined areas (Chapter 1). In addition, the pattern of *A. pacificum* detection during the study period is consistent with the decline of this species in harbors. As for *A. minutum*, although it may have been detected at some other beaches after the first years of the study, an increase in its blooms was not observed.

4.2.2 *A. taylori*

Among *Alexandrium* species, *A. taylori* produces the largest number of blooms along the Catalan coast. Bloom formations at the beaches of other Mediterranean areas were reported already at the beginning of the present century (Basterretxea et al., 2005; Giacobbe et al., 2007; Masó et al., 2002; Satta et al., 2014; Satta et al., 2010). Giacobbe et al. (2007) obtained evidences for the spread of *A. taylori* from the western to the eastern Mediterranean Sea. The first detection in the Mediterranean was at La Fosca beach, where recurrent summer discolorations had been reported since 1982, although *A. taylori* was not identified as the causative species until 1994 (Delgado et al., 1997). Garcés et al. (1999) described the presence of *A. taylori* and its blooms at several beaches along the northern Catalan coast in 1996, although the

highest abundances, reaching 10^6 cells L^{-1}, were detected only at La Fosca beach.

Our data provide a picture, at high spatial resolution, of the true biogeographic distribution of *A. taylori*, which allows an assessment of potential outbreaks along the entire Catalan coast as well as in other Mediterranean regions with more recent introductions of this species.

During our study, the distribution of *A. taylori* was largely restricted to the northern coast, with a few exceptions along the central and southern coasts. For the detection along the northern coast, our results showed non-temporally related trends from 2005 to 2012 (**Table 3**) in Desemb. Muga, Estartit, and La Fosca beaches. However, in samples from Desemb. Muga collected in August 2001 and July 2003 this species was not detected, which suggests the relatively new involvement of this area by bloom formation. This change between 2001–2003 and 2004 was observed in all areas surrounding Desemb. Muga (**Fig. 8**) and corroborates the emergence of this area during the study period as a site conducive to bloom formation, perhaps due to its contamination from another nearby affected beach. Blooms of *A. taylori* at Estartit 3 and La Fosca beaches were reported previously (Garcés et al., 1999). In 1996, Garcés et al. (1999) monitored the abundances of *A. taylori* at 18 beaches along the northern coast, all of them south of Desemb. Muga (Gulf of Roses), over the course of the year. Our results showed bloom abundances of *A. taylori* at beaches where they were also detected by Garcés et al. (1999). While at beaches where these authors did not detect blooms, we observed the same results in seven of them and we detected blooms in the other two. In general, following the detection of a bloom by *A. taylori*, blooms recurred in subsequent years. Thus, at the northern coast, at the three areas where Garcés et al. (1999) measured high abundances of *A. taylori* (Estartit, La Fosca, and St Pol), the same dynamics have been maintained. Also, as evidenced by our data, since then the species has expanded forming blooms in an additional area (Desemb. Muga).

We detected *A. taylori* at an abundance of 7080 cells L^{-1} in the most southern part of the Catalan coast, at Trabucador beach (near the Ebre Delta), although as a unique event. A more recent observation (11/07/2016) was at l'Arenal (Ampolla) beach, located in the northern Ebre Delta and different from the l'Arenal beach indicated in Fig. 1 (see **Fig. 8**). The high cell abundance

recorded at this site, 5 X 10^5 cells L^{-1}, suggests that this area is vulnerable to recurrent blooms of *A. taylori*.

4.2.3 An evaluation of the seasonality of *A. taylori*

Our data documenting a large number of annual blooms of *A. taylori* at different beaches, including those where blooms had not been described before, agree with previous observations by Garcés et al. (1999), who described the nearly simultaneous appearances of *A. taylori* at different beaches of the Costa Brava. However, whereas vegetative cells were detected during every year of the study during the last fortnight in May, according to Garcés et al. (1999) they first appeared in early July. We found that bloom abundances were reached, as previously described, in July and August (Garcés et al., 1999), but in some years in June. Differences in bloom initiation between years may reflect variations in the environmental parameters that promote bloom formation. High cell abundances were typically seen in July at La Fosca and other beaches, but in 2003 cells densities > 10^5 cells·L^{-1} were detected in La Fosca already in the last week of June and coincided with a strong positive temperature anomaly (+4.6) around that date. Similarly, densities of *A. taylori* of 7.4 X 10^5 cells L^{-1} were recently detected in Estartit 3 beach, on May 31, 2017, coinciding with a water temperature anomaly in the area (vs. the climatic mean between 1973 and 2012) of +2.5ºC; thus, the water temperature was the same as that typically occurring during the third week of June (data from http://meteolestartit.cat). This indicates that, although blooms may appear every year within a well-marked period, early warming of the water can accelerate their development. It also provides further evidence that water temperature drives *A. taylori* to reach bloom densities. Temperature can promote *A. taylori* blooms in different ways. Based on cell growth estimations, Basterretxea et al. (2007) concluded that an increase in the specific growth rate of this species during bloom formation was associated with the seasonal increase in the water temperature. The warming of beach water together with calm weather can result in a nearshore niche with low water renewal, adequate for the accumulation and bloom development of *A. taylori* (Basterretxea et al., 2007; Garcés., 1999). Basterretxea et al. (2005) also pointed out the importance of the daily summer breeze cycle — a consequence of the contrasting thermal response of the land and sea that occurs more intensely

during warmer periods— in determining the occurrence and persistence of nearshore massive blooms.

However, factors others than temperature are necessary to explain the early bloom that occurred at Estartit 3 beach in June 2002 (**Fig. 10**) despite the negative anomalies of temperature there and at other beaches.

The marked seasonality that characterizes proliferations of *A. taylori* suggests that this organism has a synchronous germination pattern, in which germination occurs at a specific time. However, the isolated detections outside the warmer months (such as December and March, **Fig. 10**), not previously observed by Garcés et al. (1999), leave open the possibility that this species undergoes incessant germination, with peaks at certain times of the year. This pattern has been demonstrated for *A. minutum* (Anglès et al., 2012). In addition, the abundance of vegetative cells in the water is not determined by the flow of germinating cysts (Estrada et al., 2010; Ishikawa et al., 2014; Ishikawa and Taniguchi, 1997). Thus, vegetative abundances seldom parallel germinating cyst flow. *A. taylori* could excyst all year long but with population growth occurring only in the summer months, when supported by environmental conditions. In situ studies on excystment are needed to investigate this hypothesis.

4.3 Factors promoting harmful dinoflagellate bloom formations along Catalan coast beaches

A comparison of our data with the data in Chapter 1 and those published in Vila et al. (2001a) clearly shows that most of the dinoflagellates reported in the latter sources reached higher abundances inside harbors and other confined waters than in the beaches studied in the present work. This can be explained by the preference of dinoflagellates for environments with a low level of turbulence (Margalef, 1978). However, species such as *A. taylori*, *Ostreopsis* spp., and *Kryptoperidinium foliaceum* typically produce blooms at beaches, where turbulence is characteristically higher than in harbors.

Biological, physical, chemical, and geomorphological factors have been proposed to explain HAB formation at Mediterranean beaches (eg: Basterretxea et al., 2007; Garcés et al., 1999; Giacobbe et al., 2006; Satta et al., 2014).

Anthropogenic activities affecting the coast have often been associated as well, as different forms of land use determine the concentrations and types of nutrients that flow into nearshore waters and may affect the phytoplankton community (Heisler et al., 2008). For Catalan coastal waters, anthropogenic pressures on the coastal zone have been calculated using the Land Uses Simplified Index (LUSI) (Flo et al., in prep.). LUSI is a tool to assess continental pressures on coastal waters based on land use type (urban, industrial, and agricultural pressures), as estimated from land cover maps and salinity data (riverine pressure) obtained during coastal monitoring (see Flo et al., 2011b for more information). This index has been validated against dissolved inorganic nutrients in coastal waters (Flo et al., in prep.). Our data show that blooms of *A. taylori* are common at beaches where the continental impact on coastal waters is large, such as the area around Desemb. Muga and Estartit 3 (LUSI = 6.25), but also at beaches where the continental pressure is very low, such as the area of La Fosca (LUSI=1). We also found large differences in nutrient concentrations, forms, and the presence or absence of nutrient limitations, such as in the case of silicate, as well as differences in the salinity at beaches where *A. taylori* blooms. Our findings demonstrate that the different degrees and forms of anthropogenically related eutrophication and the quantity of freshwater inputs do not explain the distribution of *A. taylori* blooms. This is probably due to the fact that the minimum quantities of nutrients required for bloom development were present at all of the studied beaches. However, we also determined that blooms of other species, such as *Kryptoperidinium foliaceum*, develop in coastal waters with large freshwater inputs, as previously reported (eg. Figueroa et al., 2009)

Garcés et al. (1999) reported a strong positive thermal anomaly in the waters of La Fosca beach (where the abundances of *A. taylori* are usually higher), determined by comparison with the water temperatures of other surrounding beaches during the studied bloom period. The positive temperature anomaly indicated water confinement and thus little renewal. Basterretxea et al. (2007) examined water renewal at two beaches in Mallorca (Catalan Sea) during the development of a bloom of *A. taylori* and also determined a low level of water renewal at both. Thus, the beaches where dinoflagellates produce high-biomass blooms may have similar hydrologies in summer, in which a nearshore niche with low water renewal is formed that is conducive to bloom development. In addition, beach morphology may influence water renewal and

therefore the accumulation and growth of phytoplankton. For example, blooms of *A. taylori* are preferentially associated with pocket beaches (Basterretxea et al., 2005; Garcés et al., 1999; Penna et al., 2002), although Satta et al. (2014) recently reported blooms of *A. taylori* in an extended and open beach in Sardinia, although the events lasted only a few days. In our study, the beaches where blooms of *A. taylori* comprised cell densities > 10^5 cells L^{-1} included enclosed, semi-enclosed, and open beaches, such as Sant Pere Pescador, del Grau (where blooms are recurrent) and Gran de Pals beach (beach morphology data from "Llibre verd de l'estat de la zona costanera a Catalunya", Gerenalitat de Catalunya 2010; http://territori.gencat.cat/ca/01_departament/documentacio/territori-i-urbanisme/ordenacio_territorial/llibre_verd_estat_de_la_zona_costanera/). However, other factors can affect water renewal, such as predominantly low wind intensity, the direction of the beach, and the predominant wind direction, and thus bloom development (Basterretxea et al., 2007).

The ability of a species to reach bloom abundances may be related not only to the planktonic phase but also to the benthic phase (cysts) and its relationship with the geomorphology and sedimentology of the site. Satta et al. (2014) found that, in Sardinia, the distribution of harmful algal species (especially *A. taylori*, as the most widely distributed) correlated negatively and significantly with a gravel (>2 mm diameter) substratum of the foreshore and positively, although not significantly, with a medium-fine sand (<0.5 mm) one. The beaches where we detected blooms of dinoflagellates may be those with a sediment deposition zone inside or near the beach, since cysts behave as passive particles of sediments governed by hydrodynamic and sedimentary processes.

Finally, some of the bloom-forming species present in the water samples collected at the studied beaches are epiphytic, and their abundances in the water column therefore also depend on the beach substrate and the extent of macroalgal growth. Thus, in the case of *Ostreopsis*, members of this genus mostly bloomed at the typically rocky beaches of the north coast. In this study, we have observed the importance of these epiphytic organisms all along the coast but an adequate sampling of beaches, not performed in this study, is needed to assess more properly its distribution and potentially problematic areas.

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7. Supplementary material

Table S1. Graphical summary of the occurrences of *A. taylori* at Desembocadura de la Muga (A), Estartit (B), and La Fosca (C) beaches during the 13-year study period.

Beaches	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Gran de Cadaqués		(8) S											
Montjoi							(2) Y	MY	MY	MY	MY	MY	
Canyelles Petites					(6) Y	BWS-MY	MY	MY	MY	MY	MY	MY	
Desembocadura de la Muga					(11) S	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY-FN
de cala Montgó				(16) S									
Estartit 3			(16) S	(14) S	(11) S	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY-FN
del Grau			(8) S	(27) Y									
de sa Riera			(15) S	(16) S									
Llafranc			(14) S	(15) S									
Castell			(6) S	(15) S									
La Fosca	(40) S	(31) Y	(34) Y	(43) Y	(11) S	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY-FN
Sant Pol	(23) S	(16) S	(8) S	(14) S									
Canyet					(1) Y	BWS-MY	MY	MY	MY	MY	MY	MY	
ses Illetes					(5) Y	BWS-MY	MY	MY	MY	MY	MY	MY	

Table S1 (cont.).

Cavaió	(7) S			(5) S	BWS-MY	MY	MY	MY	MY	MY	MY	
Llavaneres					BWS	BWS-MY	MY	MY	BWS-MY	BWS-MY	BWS-MY	MY-FN
del Parc del Litoral				(7) S	BWS-MY	BWS-MY						
Castelldefels	(10) S	(14) S	(14) S	(9) S	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY-FN
de la Ribera	(9) S											
de la Tèrmica	(10) S											
Llarga						(2) Y	MY	MY	MY	MY	MY	
Llevant	(11) S			(10) Y	BWS-MY	BWS-MY						
Prat d'en Forés	(12) S											
l'Arenal (Vandellós)				(1) Y	BWS-MY	MY	MY	MY	MY	MY	MY	
L'Alguer				(6) Y	BWS-MY	MY	MY	MY	MY	MY	MY	
La Platjola							BWS-MY	BWS-MY	BWS-MY	BWS-MY	BWS-MY	MY-FN
l'Eucaliptus						BWS-MY						
Parc de Garbí				(9) Y	BWS-MY	BWS-MY						

From 2000 to 2004, whether sampling was conducted throughout the year (Y) or only during the summer months (S) is indicated, as is the number of samples taken in that year.

(x)S	X=number of samples obtained in summer	(x)Y	X=number of samples obtained during the year	MY-FN	Monthly all year, but lags in February and November	BWS-MY	Biweekly in the summer months, and monthly the rest of the year	MY	Monthly all year

Table S2. Graphical summary of the occurrences of *A. taylori* at Desembocadura de la Muga (A), Estartit (B), and La Fosca (C) beaches during the 13-year study period.

	Mean	Std	Min	Q1	Median	Q3	Max	N
Temperature (ºC)	19.8	4.8	7.6	15.3	20.7	24.0	31.4	1903
Salinity	37.1	3.0	5.9	37.4	37.9	38.2	39.6	1862
Chlorophyll a (µg/l)	2.6	6.8	0.0	0.5	0.9	2.1	136.0	1775
Nitrate (µM)	4.29	10.60	0.02	0.82	1.63	3.27	143.09	1679
Nitrite (µM)	0.36	1.33	0.00	0.07	0.13	0.23	25.41	1679
Ammonium (µM)	2.78	7.18	0.01	0.73	1.39	2.63	124.28	1680
DIN	7.44	16.06	0.13	2.22	3.63	6.08	247.15	1675
Phosphate (µM)	0.51	1.19	0.01	0.16	0.27	0.45	20.79	1680
Silicate (µM)	3.72	8.60	0.04	1.18	1.87	3.03	120.73	1676

Chapter 4

Barrufeta bravensis gen. nov. sp. nov. (Dinophyceae): a new bloom-forming species from the Northwest Mediterranean sea.

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Abstract

The present study describes a new dinoflagellate genus, *Barrufeta N.* Sampedro et S. Fraga gen. nov., with one new species, *B. bravensis* Sampedro et S. Fraga sp. nov., isolated from the Costa Brava (NW Mediterranean Sea). The dinoflagellate was characterized at the genus and species levels by LM and EM; LSU and internal transcribed spacer (ITS) rDNA sequences; and HPLC analyses of the pigments, fatty acids, and possible presence of toxins of several cultured strains. The new *Barrufeta* species is oval shaped (22–35 μm long and 16–25 μm wide) and dorsoventrally flattened. It possesses numerous small chloroplasts that radiate from two large equatorially located pyrenoids and is a typical peridinin-containing dinoflagellate. The nucleus is in the anterior part of the epicone. The apical groove has a characteristic ‘‘Smurf-cap’’ shape that runs counterclockwise on the epicone and terminates on its right posterior part.

B. bravensis is similar to the previously described species *Gyrodinium resplendens* Hulburt in its external morphology, but the original report of the latter lacked a description of the complete shape of the apical groove. It is therefore likely that some of the *G. resplendens* species reported in the literature are *Barrufeta* since they possess a *Barrufeta*-type apicalgroove. Fatty acids of *Barrufeta* were more similar to those of *Karenia brevis* than those obtained from other unarmored analyzed species including three species of *Gymnodinium* and *Akashiwo sanguinea*.

1. Introduction

Unarmored dinoflagellates have always been difficult to classify. Indeed, early species descriptions were based only on basic morphological characteristics visible by LM, such as cell shape and size; girdle displacement; number, position, and color of the chromatophores; position of the nucleus; and the nature of the cell surface (smooth or striated). One of the problems with these first descriptions was that in some cases, they referred to fixed samples or to organisms in poor condition and thus in neither case representative of living cells. Inaccurate descriptions can also result from the extreme sensitivity of the living organisms to confinement between the slide and the coverglass and from observation only of nonmoving cells. Furthermore, the taxonomic criteria previously used to distinguish between two genera of unarmored dinoflagellates were frequently not reliable. Since these criteria were drawn from continuous characteristics (e.g., the degree of cingular displacement; Kofoid and Swezy, 1921), such as used to distinguish the genera *Gymnodinium* and *Gyrodinium*, the cutoff that determined one genus versus the other could not be precisely defined, with particular difficulty arising in many borderline cases.

The taxonomy of unarmored dinoflagellates progressed following the information obtained from ultrastructural studies (Dodge, 1974; Steidinger et al., 1978) as well as from analyses of pigment composition (Jeffrey et al., 1975 and references therein; Bjørnland and Tangen, 1979) and the morphology of the dinoflagellate apical groove (Takayama, 1981, 1985, Takayama and Adachi ,1984). More recently, the morphological and ultrastructural investigations have been combined with phylogenetic determinations (Saunders et al., 1997;Hansen et al., 2000a), which, in turn, provide a new focus to the classification of genera comprising the unarmored dinoflagellates. Daugbjerg et al. (2000) used this approach to redescribe the most important genera of unarmored dinoflagellates (*Gymnodinium* and *Gyrodinium*), in addition to describing three new genera and proposing a total of 17 new genus-species combinations. The features of the new genera were based on the phylogenetic analysis, specifically, of the LSU of rDNA, in combination with morphological evaluation of the apical groove, several ultrastructural details (e.g., the presence

or absence of nuclear chambers in the nuclear envelope), and the identities of the major accessory pigments. Since the shape of the apical groove was determined to differ in each genus, it provided a reliable generic characteristic, one that was usually observable by SEM. This new classification of unarmored dinoflagellates likewise yielded new genus-species combinations in addition to a new genus, *Takayama* (de Salas et al., 2003; Murray et al., 2007). However, Daugbjerg et al. (2000) also made a few exceptions: for example, the apical groove of *Lepidodinium viride* M. Watanabe, S. Suda, I. Inouye, Sawaguchi et Chihara is similar to that of *Gymnodinium*, and the analysis of SSU rDNA sequences places this species within or close to *Gymnodinium* sensu stricto (Saunders et al., 1997); nonetheless, the *Lepidodinium* genus was retained based on the presence of both an outer layer of body scales and pigments of Chlorophyta origin (Watanabe et al., 1990). Hansen et al. (2007) compared *L. viride* with *Gymnodinium chlorophorum* Elbr. et Schnepf. Although the latter species lacks body scales, similarities between the two species at the ultrastructural, pigment, and genetic levels led the authors to propose the new combination *L. chlorophorum* (Elbr. et Schnepf) Gert Hansen, Botes et de Salas. The use of fatty acids as a chemotaxonomic tool in marine microalgae has been suggested by some authors. Fatty acids of unarmored dinoflagellates have also been studied in this way. Mooney et al. (2007), for example, examined eight species of marine gymnodinioids and proposed the ratio of 28:7n6／28:8n3 as a chemotaxonomic marker at the species level. There are, to the authors' knowledge, no studies on chemotaxonomic tools based on fatty acids to distinguish between unarmored genera.

Since 1982, blooms of *Alexandrium taylori* Balech causing intensely brown/green-colored waters have been observed along some of the beaches of the Costa Brava (NW Mediterranean Sea) in the summer months (from June to September), when the influx of tourists is the greatest (Garcés et al., 1999).

Since 1995, routine monitoring of HABs at La Fosca beach has led to the additional detection of an unarmored dinoflagellate during the same period that the *A. taylori* bloom occurs, but the preservation of this gymnodinioid species with Lugol's solution prevented its identification. Therefore, to properly identify this species, several strains from the blooms that occurred in 2002 and 2005 were isolated. Our efforts led to the identification of *B. bravensis* gen. et sp. nov, described herein, which was based on LM and SEM examinations of its

external morphology; phylogenetic analysis of its LSU and ITS rDNA; and analyses of its ultrastructure, pigments, and fatty acids composition.

2. Materials and methods

Strain isolation and cultures. Strains isolated from La Fosca beach, Costa Brava, during September 2002 and August 2005 were grown in L1 medium without silicate (Guillard and Hargraves, 1993) and prepared with coastal seawater. The cultures were incubated at 20ºC with a 12:12 light: dark (L:D) photoperiod and an irradiance of ~100 µmol photons x m^{-2} x s^{-1}. The salinity was adjusted to 32 (psu) for LM and analyses of pigment, fatty acid, and toxin content and 37.8 (psu) for SEM and TEM. The culture used for TEM analysis, belonging to the strain VGO864, was accompanied by a small, unknown photosynthetic oval, almost spherical, stramenopile measuring ~3 µm long and ~2.5 µm wide. The cultures were deposited at the Culture Collection of Microalgae of the Centro Oceanográfico de Vigo (CCVIEO; Vigo, Spain) and in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; West Boothbay Harbor, ME, USA). Other unarmored dinoflagellates [*Akashiwo sanguinea* (Hirasaka) Gert Hansen et Moestrup 2000 (VGO626), *Gymnodinium microreticulatum* Bolch (VGO328), *Gymnodinium catenatum* L. E. Graham (Est14H5), *Gymnodinium impudicum* (S. Fraga et I. Bravo) Gert Hansen et Moestrup (VGO665), and *Karenia brevis* (C. C. Davis) Gert Hansen et Moestrup (CCMP2281)] were cultivated under the same conditions to allow comparison of their fatty acid contents with those of *B. bravensis* (VGO864).

2.1. Microscopy

LM. Live cells were examined and photographed under bright-field and epifluorescence (lamp 50 W) microscopy using a Leica DM IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted microscope connected to a ProgRes C10 (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) digital camera. Nuclei were stained with 4',6-diamidino-2- phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2 µg X mL^{-1}. A stained nucleus was photographed through a UV filter, and immediately afterward, another image was taken with a blue filter in order to capture

the chloroplast's autofluorescence in the same cell. The two images were overlapped using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA). Cellular dimensions and the degree of girdle displacement were determined in 50 cells using ProgRes capturePro v 2.1 software (JENOPTIK Optical Systems GmbH). Nomarski microphotographs were taken with a Canon EOS D60 (Canon Inc., Tokyo, Japan) digital camera connected to a Leica DMLA light microscope (Leica Microsystems GmbH).

SEM. Cells of *B. bravensis* were fixed for 15 min at room temperature with an adequate volume of osmium tetroxide 4% (dissolved in seawater) to reach a final concentration of 1%; pH was not adjusted. The cells fixed were then filtered through a 13 mm diameter Nucleopore (Pleasanton, CA, USA) polycarbonate filter with a pore size of 2 or 5 µm. The filtered cells were then washed in distilled water; dehydrated in 25, 50, 75, 95, and 100% ethanol, 10 min each; and critical-point-dried. The filters were mounted on stubs, sputter-coated with gold, and examined with a JEOL JSM-6500F scanning electron microscope (JEOL-USA Inc., Peabody, MA, USA). To compare the structure of the apical groove of *B. bravensis* with the same structure in a related species of *Gymnodinium*, two SEM preparations used 17 years ago for the description of the *G. impudicum* (see Fraga et al., 1995 for more information on the methodology of SEM) were reexamined using a variable-pressure scanning electron microscope Hitachi S-3500N (Hitachi High Technologies Corp., Japan).

TEM. *B. bravensis* VGO864 was fixed in an equal volume of a mixture of 4% glutaraldehyde and 0.4% osmium tetroxide (previously dissolved in distilled water to 4% concentration, and from 4% to 0.4% in culture medium) for 30 min at 4ºC, pelleted by centrifugation, washed in culture medium, and finally covered in 2.5% warm agar. After the agar had cooled and solidified, it was peeled off the filter (Hernández, 1992), divided into smaller pieces, and postfixed for 1 h in 0.8% FeCNK and 1% osmium tetroxide prepared in 0.1 M Na-cacodylate buffer (pH 7.4). After washing in buffer, the material was dehydrated in an acetone series (50, 70, 80, 2 · 90, 3 · 96, 3 · 100%), embedded in Spurr's resin, and then sectioned on an Reichert Jung Ultracut E (Capovani Brothers Inc., Scotia, NY, USA) ultramicrotome using a diamond knife (Diatome, Hatfield, PA, USA). The sections were collected on a 200-mesh grid and placed on a Formvar film and then stained in 2% uranyl acetate and lead citrate following the method of Reynolds (1963). Sections were examined

in a JEOL JEM-1010 electron microscope (JEOLUSA Inc.) operated at 80 kV. Micrographs were taken using a Gatan, BioScan model 792 (Gatan Inc., Pleasanton, CA, USA) digital camera.

2.2 Pigment analyses

Sample preparation: Prior to HPLC pigment analysis, the health and characteristic morphology of the cells were confirmed by LM. Three hours into the light cycle, cells from cultures in the exponential phase of growth were harvested, and a 14 mL aliquot of the culture was filtered onto Whatman GF⁄F filters (Whatman plc, Maidstone, Kent, UK) under reduced pressure. The filters were frozen immediately at)25ºC and analyzed within 12 h.

Pigment extraction: The frozen filters were placed in Teflonlined screw-capped tubes, and the pigments were subsequently extracted with 5 mL of 90% acetone, using a stainless steel spatula to grind the filter. The tubes were chilled in a beaker of ice, and the contents sonicated for 5 min in an ultrasonic bath. The extracts were then filtered through syringe filters (MFS HP020, 25mm, 0.20 µm pore size, hydrophilic PTFE, Advantec, MFS Inc. Dublin, CA, USA) to remove cell and filter debris. An aliquot (0.5 mL) of acetone extract was mixed with 0.2 mL of water, followed by immediate injection of a 200 µL sample into the HPLC column. This procedure avoids distortion of the early eluting peaks (Zapata and Garrido, 1991) and prevents the loss of nonpolar pigments prior to column injection.

HPLC analysis. Pigments were separated by HPLC in a system consisting of a Waters (Waters Corporation, Milford, MA, USA) Alliance 2695 separation module and a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector by means of a Sat ⁄ In analog interface. The chromatographic system was controlled using Millenium 32 software (Waters). Pigments were separated according to the HPLC method of Zapata et al. (2000), with a reformulated mobile phase A and using a C_8 monomeric Waters Symmetry column (150 X 4.6 mm, 3.5 lm particle size, 10 nm pore size). Eluent A consisted of methanol: acetonitrile:0.025 M aqueous pyridine (50:25:25 v ⁄ v ⁄ v), and eluent B of methanol:acetonitrile:acetone (20:60:20 v⁄v⁄v). The elution gradient was as follows (time, %B): t_0, 0%; t_{22}, 40%; t_{28}, 95%; t_{37}, 95%; t_{40}, 0%. The flow rate was 1.0 mL X min^{-1}, and the column temperature was 25ºC. Solvents were HPLC

grade (Romil-*SpS*™ Ltd., The Source Convent Waterbeach, Cambridge, UK); pyridine was reagent grade (Merck, Darmstadt, Germany).

Pigments were identified either by cochromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (see Zapata et al., 2000). The purity of the peaks was confirmed, and the spectral information then compared with a library of Chl and carotenoid spectra of pigments extracted from standard phytoplankton cultures (Zapata et al., 2000) or supplied by DHI (DHI Laboratory Products, Hørsholm, Denmark). The molar extinction coefficients (Ɛ; l X mol^{-1} X cm^{-1}) provided by Jeffrey (1997) were used for pigment quantification.

2.3. Toxin analyses

Samples of *B. bravensis* cultures were filtered onto 47 mm glass fiber filters (Whatman GF/C). One of the filters was transferred to a 15 mL centrifuge tube containing PBS (Emura et al., 2004) for protein extraction. The other filters were treated with 0.1 M HCl to extract dinoflagellate toxins. These acidic extracts were used for the mouse bioassay (MBA), chemical analyses, and the hemolytic assay; the PBS extract was used only for the hemolytic assay.

The presence or absence of hemolytic compounds in both the PBS and the acidic extracts was determined following the method published by Riobó et al. (2008) with slight modifications. This assay was carried out using sheep blood diluted in Alsever's solution, kindly provided by Cz Veterinaria, S.A. (Porriño, Pontevedra, Spain).

The presence or absence of paralytic shellfish poisoning (PSP) toxins in cultures was determined according to the Association of Official Analytical Chemist's (AOAC) MBA method (AOAC 1990) and the HPLC method, the latter including postcolumn oxidation and fluorometric detection (Franco and Fernandez, 1993).

2.4. Fatty acid analyses

Fatty acid analyses were carried out on the strain of *B. bravensis* (VGO864) and other strains of unarmored dinoflagellates (*A. sanguinea* [VGO626], *G. microreticulatum* [VGO328], *G. catenatum* [Est14H5], *G. impudicum*

[VGO665], and *K. brevis* [CCMP2281]). Duplicate or triplicate (500mL) exponentially growing cultures were filtered through Whatman GF/F (25 mm) precombusted glass-fiber filters, immediately frozen in liquid N_2, freeze-dried for 12h, and stored at -20ºC until analysis. Cellular lipids were extracted with 3:1 DCM:MeOH (dichloromethane:methanol), according to the method of Ruiz et al. (2004). The samples were redissolved in 0.5 mL of chloroform and eluted through a 500 mg aminopropyl minicolumn (Waters Sep-Pak@ Cartridges) previously activated with 4 mL of n-hexane according to the method of Fuentes-Grünewald et al. (2009). The extracts were stored at -20C until gas chromatography (GC) analysis in a Thermo Finnigan Trace GC ultra instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a flame ionization detector and splitless injector and fitted with a DB-5 Agilent column (30 m length, 0.25 mm internal diameter, and 0.25 µm phase thickness) (Agilent Technologies Inc., Santa Clara, CA, USA). Helium was used as the carrier gas at a flow rate of 33 cm X s^{-1}. The oven temperature was programmed to increase from 50ºC to 320ºC at 10ºC X min^{-1}. Injector and detector temperatures were 300ºC and 320ºC, respectively. Fatty acid methyl esters (FAME) were identified by comparing their retention times to those of standard fatty acids (37 FAME compounds, Supelco@ Mix C4-C24; Sigma-Aldrich, St. Louis, MO. USA). Fatty acids were quantified by integrating the areas under the peaks in the GC traces (Chromquest 4.1 software; Thermo Fisher Scientific Inc.), with calibrations derived from internal standards (2-octyldodecanoic acid and 5b-cholanic acid).

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

Cultures of *B. bravensis* were collected during the exponential growth phase by filtration on 3 µm pore-size Isopore membrane filters (Millipore, Billerica, MA, USA). DNA was extracted and purified as described in Penna et al. (2005). Nuclear-encoded 5.8S rDNA and ITS regions were PCRamplified as described in Penna et al. (2008). The nuclearencoded LSU (D1/D2 regions) was amplified by using the primers D1R and D2C (Scholin and Anderson, 1994) and the amplification protocol of Penna et al. (2008). Genomic DNA (1ng) was amplified in a 50 µL reaction mix containing 50 µM each of dATP, dTTP, dCTP, and dGTP; 0.4 µM of each primer; 4 mM $MgCl_2$; 1 X reaction buffer (Diatheva, Fano, Italy); and 1.0 U Hot Rescue DNA Polymerase (Diatheva).

Thermocycling was as follows: 10 min initial denaturation at 95ºC; 35 cycles of 1 min at 95ºC, 1 min at 50ºC, and 2.5 min at 72ºC; and a final elongation step of 7 min at 72ºC. Three PCR-amplified products corresponding to the D1XD2 regions of the LSU gene and the 5.8S rDNA and ITS regions were pooled, purified, and then directly sequenced using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer Corp., Applied Biosystems, Foster City, CA, USA) and the dye terminator method provided in the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit, Perkin Elmer Corp.). Sequences obtained from this study were aligned with those from GenBank using the CLUSTAL X2 program (Larkin et al. 2007) with default settings. Alignments were rechecked visually and edited manually; nonalignable regions were excluded prior to the phylogenetic analyses. The strains used in the molecular analyses are listed in Tables S1 and S2 (see the supplementary material), together with the GenBank accession numbers of their LSU and 5.8S-ITS rDNA sequences. Phylogenetic relationships, based on the D1/D2 LSU and 5.8S-ITS rDNA data, were inferred using the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. Sequences of *Alexandrium affine* (H. Inoue et Y. Fukuyo) Balech (AY294612) and *Oxyrrhis marina* Dujard. CCMP604 (AY566415) were used as outgroups in the LSU and ITS-5.8S rDNA phylogeny, respectively. The best-fit model of nucleotide substitution for the phylogenetic analyses was the Akaike information criterion implemented in Modeltest 3.06 (Posada and Crandall 1998). For the LSU rDNA, the TVM+G model with gamma distribution for among-site variation was selected, with the alpha value of the gamma distribution equal to 0.497. For the 5.8S rDNA-ITS, the TrN+I+G model with gamma distribution for among-site variation was adopted, with alpha value of the gamma distribution equal to 0.924. This evolutionary model was used in the NJ distance matrix. MP analysis was performed using heuristic searches with treebisection-reconnection branch swapping. Branches were collapsed if their minimum length was 0; ambiguities and gaps were considered as missing data. The robustness of the NJ and MP trees was determined by bootstrapping with 1,000 pseudoreplicates. Phylogenetic analyses were carried out using the software packages PAUP* ver. 4.0b10 (Swofford, 2002). ML analyses were run with RaxML (randomized axelerated maximum likelihood) software ver. 7.0.4 (Stamatakis et al., 2005), which adopts a general time reversible (GTR) substitution model and allows estimation of several parameters, such as the proportion of invariant sites and the alpha values of the gamma distribution

for among-site rate variation. The LSU and 5.8-ITS rDNA sequences were subjected to ML analyses. Bootstrap values were calculated with 1,000 pseudoreplicates. Standard molecular indices were determined with Arlequin v. 3.0 (Excoffier et al., 2005).

3. Results

Barrufeta N. Sampedro et S. Fraga ***gen. nov.*** Dinoflagellati nudi cum peridinina ut principali pigmento lucis captore. Sulcus apicalis incipit in anteriore parte intersectionis surci et cinguli, currit diagonali modo in partem anteriorem ad dexteram, postea redit, sensu contrario motus horologii, in gyrum apicalis partis cellulae perlongum nodum transversalem componendum et finit prope dexteram partem ejus originis.

Unarmored dinoflagellates occur with peridinin as the major light-harvesting accessory pigment. An apical groove that starts in the anterior part of the intersection between the sulcus and the cingulum, runs diagonally to the anterior right side of the cell, and then turns counterclockwise around the apical part of the cell in a very elongated transversal loop before ending near the right side of its origin.

Etymology: Named after the shape of the epicone, which is due to the shape of the apical groove. It is similar to the cap of a ‘‘Smurf’’ (originally a ‘‘Schtroumpf’’), a comic strip character invented by Peyo in 1958 and adapted later to television. In Catalan, *barrufet* means ‘‘Smurf,’’ with *barrufeta* as the feminine form.

Type species: *Barrufeta bravensis* N. Sampedro et S. Fraga sp. nov. ***Barrufeta bravensis*** N. Sampedro et S. Fraga **sp. nov.**

Cellulae ovoideae dorsiventraliter complanatae, 22–35 μm longae 16–25 μm latae. Et epiconus et hypoconus eamdem longuitudinem habent. Epiconus orbiculatus et frequenter cum parva protuberantia. Sulcus apicalis incipit in anteriore parte intersectionis surci et cinguli, currit diagonali modo in partem anteriorem ad dexteram, postea redit, sensu contrario motus horologii, in gyrum apicalis partis cellulae, perlongum nodum transversalem componendum et finit prope dexteram partem ejus originis. Hypoconus suaviter truncatus et leviter bilobulatus, extensionis sulci antapicem attingentis causa. Cingulum

descendens 1.5-2 quantum habet latitudinis retractum. Permulti parvi cloroplasti pallidi obscuri, ex quibus multi exeunt e duobus magnis pyrenoidis sub cingulo positis in utroque latere sulci. Peridinina ut principale pigmentum lucis captore. Nucleus plus minusve in centro epiconi locatus.

Cells oval in outline, dorsoventrally flattened, 22– 35 µm long and 16–25 µm wide. Epicone and hypocone are similar in length. Epicone is rounded and frequently found showing a slight protuberance. An apical groove that starts in the upper part of the intersection between the sulcus and the cingulum, runs diagonally to the anterior right side, and then it turns counterclockwise around the apex, forming a wide transversal loop and ending near the right side of its origin. Hypocone is smoothly truncate and slightly bilobate due to the sulcus extension until the antapex. Cingulum is descending and displaced by 1.5–2 cingulum widths. Numerous small yellow-brownish chloroplasts, with many of them radiating from two large pyrenoids, are equatorially located with one on each side of the sulcus. Peridinin occurs as the major light-harvesting accessory pigment. Nucleus is more or less centered in the epicone of the cell.

Holotype: **Figure 1** of culture VGO864, isolated from La Fosca beach, located on the Mediterranean coast of Catalonia, Spain. The culture was deposited in two culture collections (CCVIEO and CCMP3277). Sequences were deposited to GenBank under the accession numbers FN647670 (ITS) and FN647673 (LSU).

Isotype: **Figure 2F**.

Type locality: La Fosca beach, Catalonia, Spain (**Fig. 3**) (41º51'29'' N, 3º08'40'' E).

Etymology: Named after Costa Brava, the coast where this species produces blooms.

Distribution: NW Mediterranean Sea.

Habitat and ecology: Marine plankton. High cell densities (>10^6 cells L^{-1}) of *B. bravensis* have been detected in coastal waters during the summer months (June–September) at water temperatures of 20ºC–27ºC and salinities of 36.5-38.4. This species has been found accompanying blooms of *A. taylori* at beaches located inside semi-enclosed bays. In laboratory cultures, *B. bravensis* produces mucus.

External cell morphology. The cells are slightly elongated and dorsoventrally flattened (**Fig. 2C**), 26.9 ± 3 µm (22–35.4 µm, *n* = 50) long and 20.1 ± 2.3 µm (16–25.3 µm, *n* = 50) wide. The length/width ratio ranges from 1.2 to 1.5 (*n* = 50). The epicone and hypocone are similar in length (**Fig. 2, A, D,** and **E**). The sulcus extends to the antapex sometimes giving the hypocone a slightly bilobate shape (**Fig. 2, A, D,** and **E**). The apex is generally rounded and frequently has a slight protuberance that is limited by the apical groove (**Figs. 2A** and **B**; **4B** and **E**). The descending cingulum is deep and wide and displaced by 1.5-2 cingulum widths, which represent 16% to 26% of the body length (**Fig. 4A** and **B**). The sulcus has a small intrusion in the epicone and runs deep and straight to the antapex. The epicone has an apical groove, Smurf-cap shaped, running counterclockwise around the apex. The proximal part of the groove starts at the intrusion of the sulcus in the epicone and then ascends diagonally for a short distance toward the right anterior side of the cell. The groove then turns toward the left and crosses a large part of the epicone across the ventral part of the cell, surrounds the apex of the cell, and returns parallel to the cingulum across the dorsal side of the epicone. Lastly,

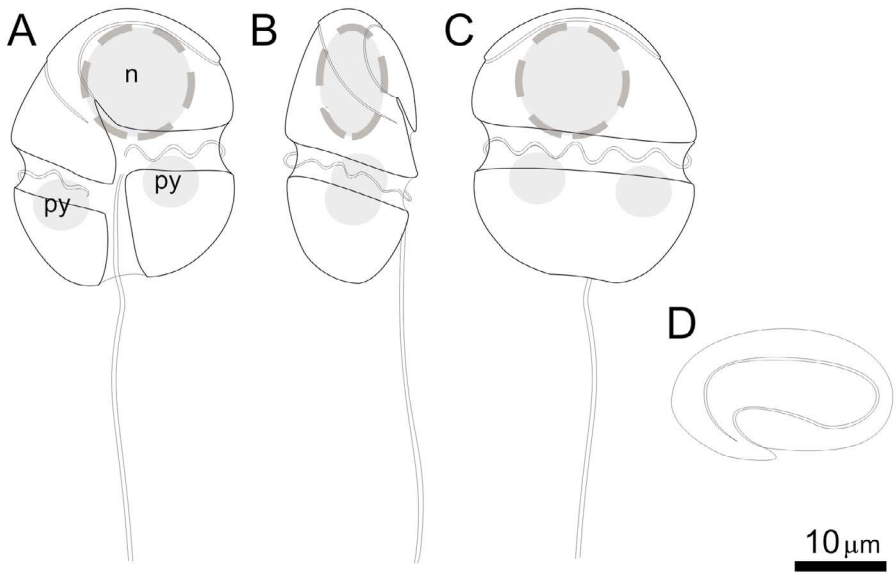

Figure 1. Schematic representation of *Barrufeta bravensis* showing the position of the (n) nucleus and the (py) pyrenoids: (A) ventral, (B) lateral, (C) dorsal, and (D) apical views.

it turns toward the ventral part, finishing near its origin, without reaching to the sulcus of the cell (**Figs. 1A-D**; **2A, B** and **D**; **4A-E**). The apical groove consists of three elongated vesicles, the middle one ornamented with small knobs (**Fig. 5A**). The cell surface is covered with polygonal amphiesmal vesicles (usually quadrangular or pentagonal) that do not show a regular pattern, whereas the border of the girdle presents an array of well-aligned rectangular vesicles (**Fig. 4F**). The longitudinal flagellum varies from 1 to 1.5 times the body length.

For the purpose of comparison, the structure of the apical groove of *G. impudicum* is shown in Figure 5, B and C. Its apical groove consists of three elongated vesicles; the lateral ones are ornamented with a line of small knobs. This morphological detail was not included in the original description of this species.

Resting cyst morphology. Cysts of *B. bravensis* were observed in cultures. The resting cysts are flattened and range in shape from circular (21.8–29.6 µm diameter; *n* = 10) to oval (25.4–34.9 µm length, 22.3–30 µm width; n = 12) and occasionally irregular, as seen in the frontal view (**Fig. 6A** and **B**). The cyst

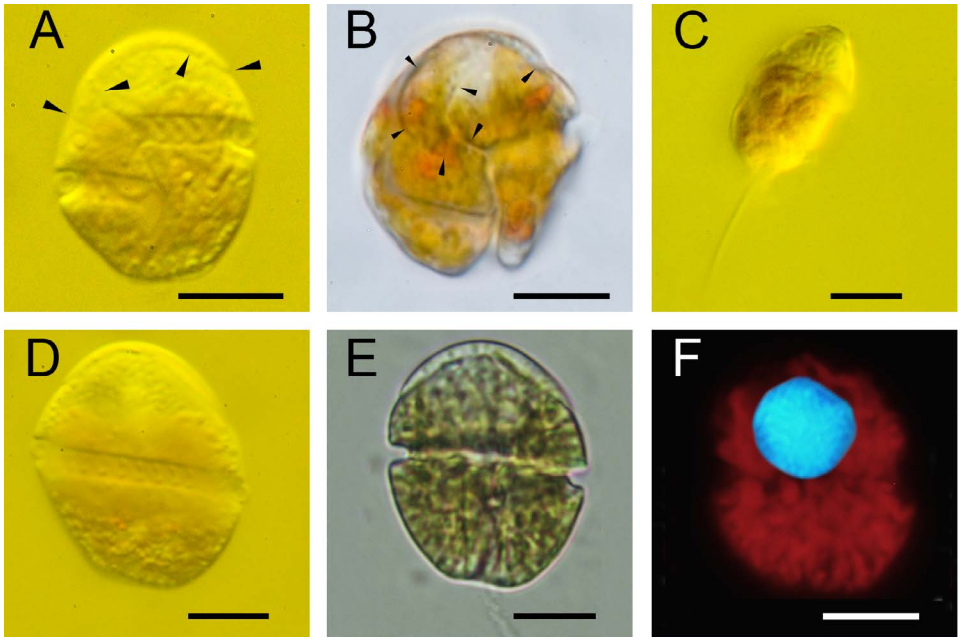

Figure 2. Light micrographs of vegetative cells. (A–B) Ventral views showing the apical groove (arrowheads); (C) lateral view; (D–E) dorsal views; (F) epifluorescence image showing DAPI-stained nucleus and autofluorescent chloroplasts. Scale bars, 10 µm. DAPI, 4',6-diamidino-2-phenylindole.

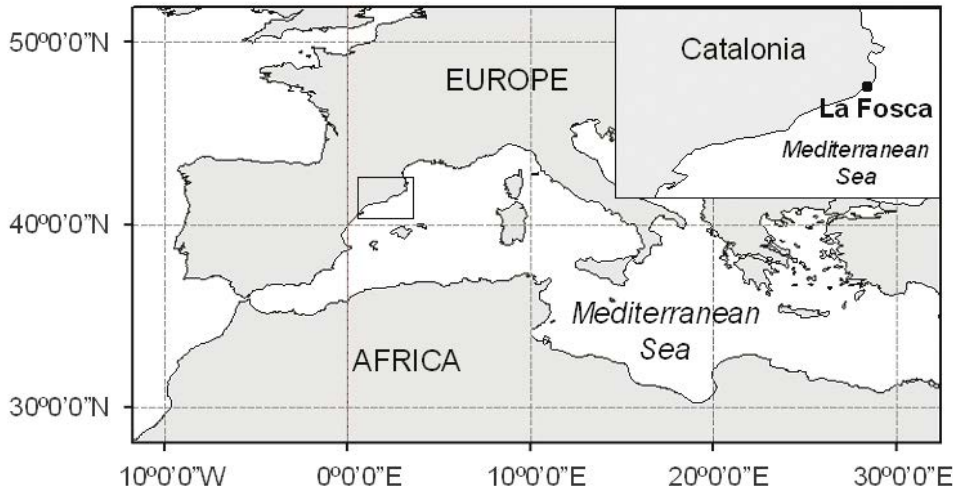

Figure 3. Location of the sampling area, La Fosca beach.

is double-walled, smooth, and thick, with white-grayish granular contents and one or more orange spots. The cysts are covered by a transparent substance that takes irregular forms. These mucoid cysts are usually strongly stuck to the bottom of the wells in culture conditions.

Cell ultrastructure. The almost spherical nucleus (~10.5 µm in diameter) is situated in the epicone and, as seen in lateral and ventral view of the cell, centered (**Figs. 2F**; **7A** and **B**). It is a typical dinokaryon with permanently condensed chromosomes and with the presence of a nucleolus (**Fig. 8G**). Golgi apparatus was observed adjacent to the nucleus (**Fig. 8I**). A structure resembling vesicular chambers of the nuclear envelope was observed in some cells (**Fig. 8H**), although nuclear pores were not observed in it. Two large and almost spherical pyrenoids are equatorially located, one on each side of the sulcus (**Fig. 7, A–D**). Chloroplasts are numerous, small, elongated, and radially arranged (**Figs. 2F**, **7D**, and **8A**) giving the cell a yellow-brownish appearance. Many of the chloroplasts radiate from the pyrenoids to the cell periphery (**Fig. 7A**, **C**, and **D**). Thylakoids are stacked generally in fours to form lamellae (**Fig. 8B**). Many lengthened vacuoles with electron-transparent contents are radially distributed and inserted in between the chloroplasts; they occupy a large part of the distal area of the cell (**Fig. 7D**). The vermiform vesicles situated beneath the amphiesma have a fibrillar content that is densely packed in the middle; these vacuoles are probably mucocysts (**Fig.**

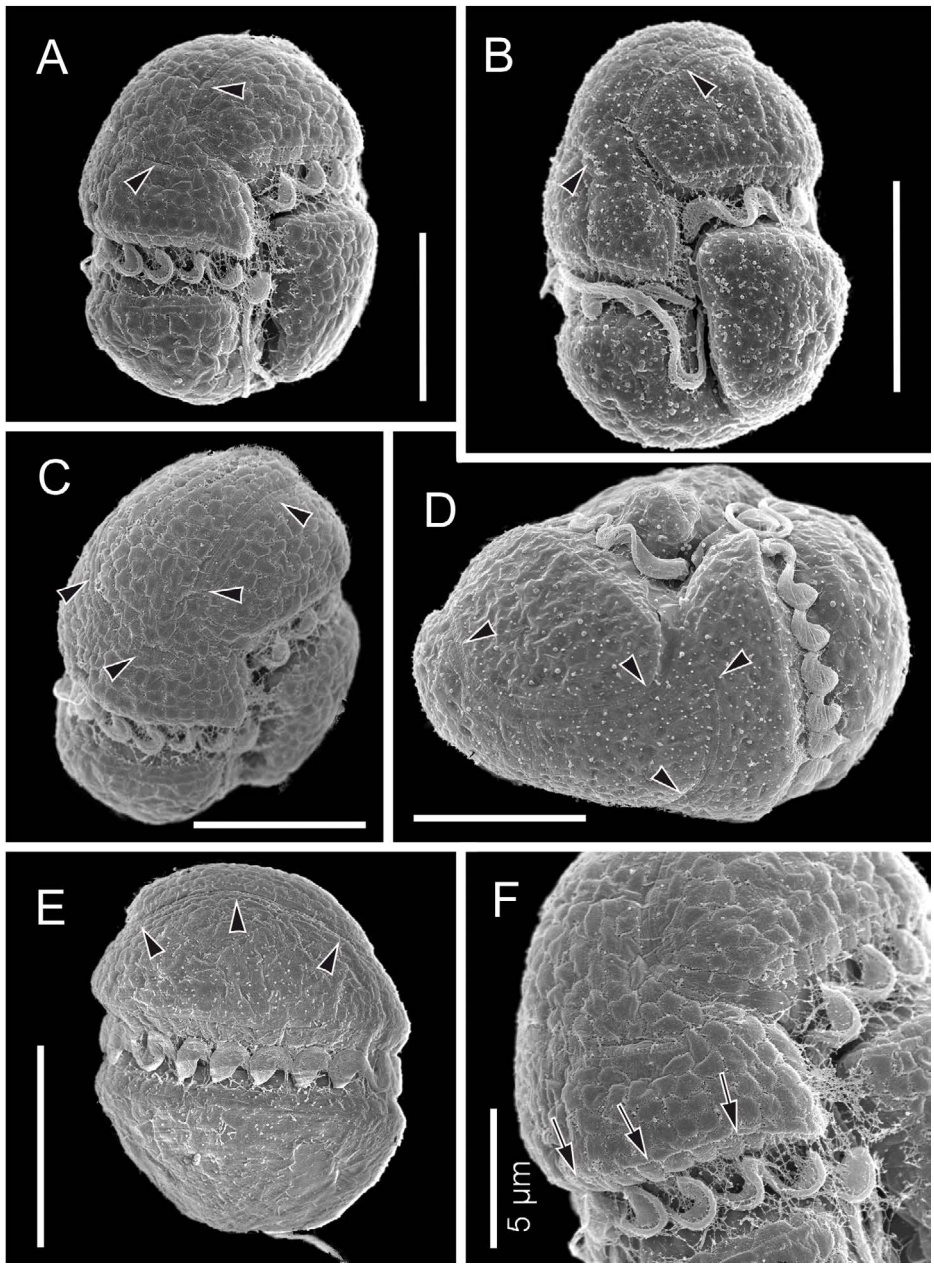

Figure 4. Scanning electron micrographs of strain of *Barrufeta bravensis*. Arrowheads point to the apical groove. (A) Ventral view of a cell and (B) of a cell with a slight protuberance. (C) Epicone and (D) epicone showing the nearly complete apical groove (arrowheads). (E) Dorsal view of a cell. (F) The detail of the cell surface shows the polygonal amphiesmal vesicles. Arrows point to the border of the cingulum, made up of a line of rectangular vesicles Scale bars, 10 lm (unless otherwise indicated).

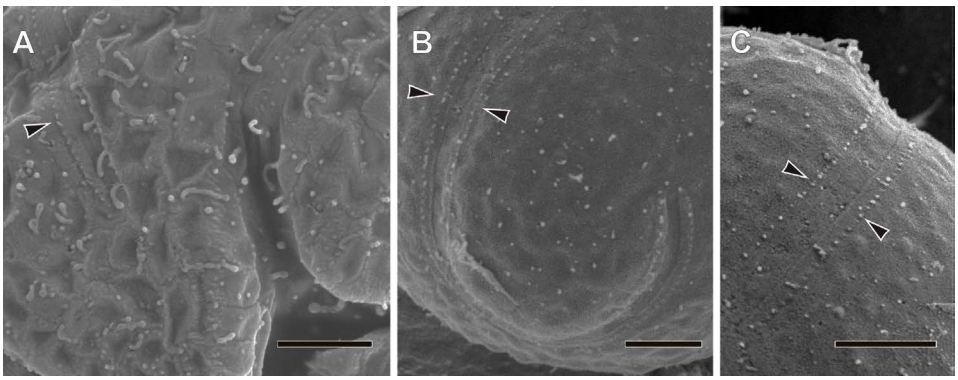

Figure 5. Scanning electron micrographs show (A) a detailed view of the apical furrow in *Barrufeta bravensis*, including three elongated vesicles. The middle one is ornamented with small knobs (arrowhead). (B, C) Details of the apical furrow in *Gymnodinium impudicum* strain GY1VA (used for the species description). The furrow consists of three elongated vesicles; the lateral ones are ornamented with a line of small knobs (arrowheads). Scale bars, 2 µm.

7A and **8E**). Another kind of mucocyst, wider than the first type and oval in shape, was observed under amphiesma (**Fig. 8F**). Trichocysts are abundant and distributed throughout the cell. These extrusomes are composed of an apical cap, a neck, and a dense body that is quadrangular shaped in cross-section (**Fig. 8C**). Both the neck and the body possess an external striated cover (**Fig. 8E**). In addition, the cells contain lipid droplets and starch grains scattered throughout the cytoplasm, as well as mitochondria with tubular christae (**Fig. 9D**).

Spherical food-vacuole-like structures measuring ~2 µm in diameter were observed in some cells (**Fig. 7B** and **9B**); their contents were similar in size to an unknown photosynthetic stramenopile present in the culture VGO864 used for TEM analysis (**Fig. 9C**). These structures were mainly located in the central area of the dinoflagellate cell.

The amphiesma is composed of flattened vesicles that contain a thin layer of electro-opaque material. Cortical microtubules are present under the amphiesmal vesicles in groups of two or three but sometimes more (**Fig. 8D**).

The pusular system is not very complex. This kind of pusular system was described by Dodge (1972, p. 288) as a ‘‘pusule with collecting chamber which branches from the flagellar canal.’’ It consists of a prolonged collecting chamber that is surrounded by numerous pusular vesicles, which are joined

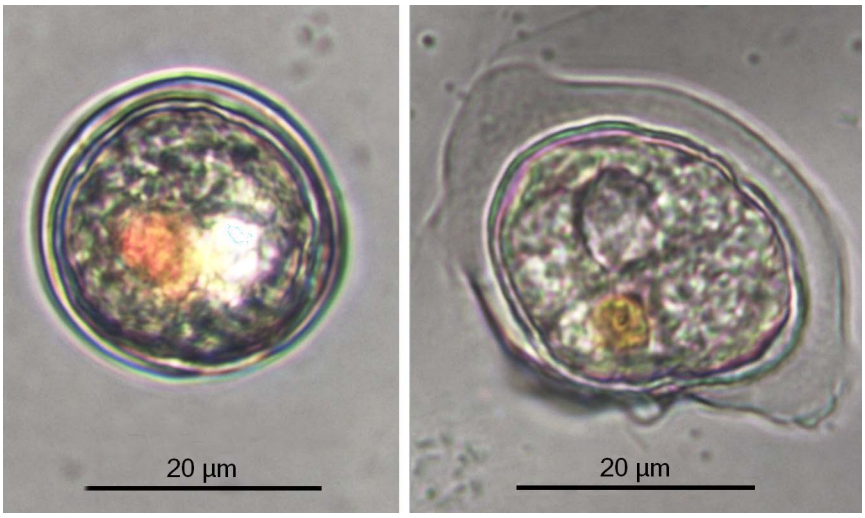

Figure 6. Light micrographs of the (A) circular-shaped and (B) oval-shaped resting cysts of cultured *Barrufeta bravensis*, as seen in a frontal view. Scale bars, 20 µm.

to it. This collecting chamber branches from the flagellar canal of longitudinal flagellum (**Fig. 9A**).

Pigment composition. As seen in the HPLC chromatogram, the pigment profile of *B. bravensis* strain VGO864 (**Fig. 10**) was characteristic of peridinin-containing dinoflagellates (Jeffrey et al. 1975), with Chl-c_2 as the major accessory Chl (Chl-c_2:Chl-*a* = 0.20), traces of Mg-2,4-divinyl pheporphyrin (MgDVP:Chl-*a* = 0.01), and no Chl-c_1. Diadinoxanthin was the major carotenoid (Diadino:Chl-*a* = 0.58), followed in relative importance by peridinin (Per:Chl-*a* = 0.40), dinoxanthin (Dino:Chl-*a* = 0.23), and diatoxanthin (0.03). Two unknown carotenoids (t_R = 26.00 and 29.00 min) sharing similar spectra (λmax:[424], 453, 477 nm) were also detected in minor amounts (pigment to Chl-*a* ratios of 0.05 and 0.02, respectively).

Toxin analyses. No PSP toxins were detected by HPLC with fluorescence detection or by MBA. The PBS and HCl extracts did not show any hemolytic activity. Lipid-soluble toxins were not tested.

Fatty acid composition. B. bravensis shows the major concentration in saturated fatty acids as a stearic acid C18:0 and palmitic acid C16:0 (4.53% and 2.30%, respectively) (**Table 1**). The second most important lipids, monounsaturated C16:1*n*7 and C18:1*n*9, were present at low concentrations

(1.22% and 1.78%, respectively). All polyunsaturated fatty acids (PUFAs) were detected in concentrations of <1%. The results of fatty acids analyses of other unarmored species cultured under the same culture conditions (*A. sanguinea, G. microreticulatum, G. catenatum, G. impudicum, K. brevis*) are shown for comparison purposes in Table 1. Differences in the fatty acid profile, in addition to their percentage, have been observed between *Barrufeta* and other related genera such as *Gymnodinium*. The fatty acid profile of *K. brevis* was similar to that of *B. bravensis* in terms of percentages and lipid content. For example, C12:0 was detected only in *B. bravensis* and *K. brevis* but not in any of the three *Gymnodinium* species or in the *A. sanguinea*, despite identical culture conditions. For C14:1, the opposite case was true; this fatty

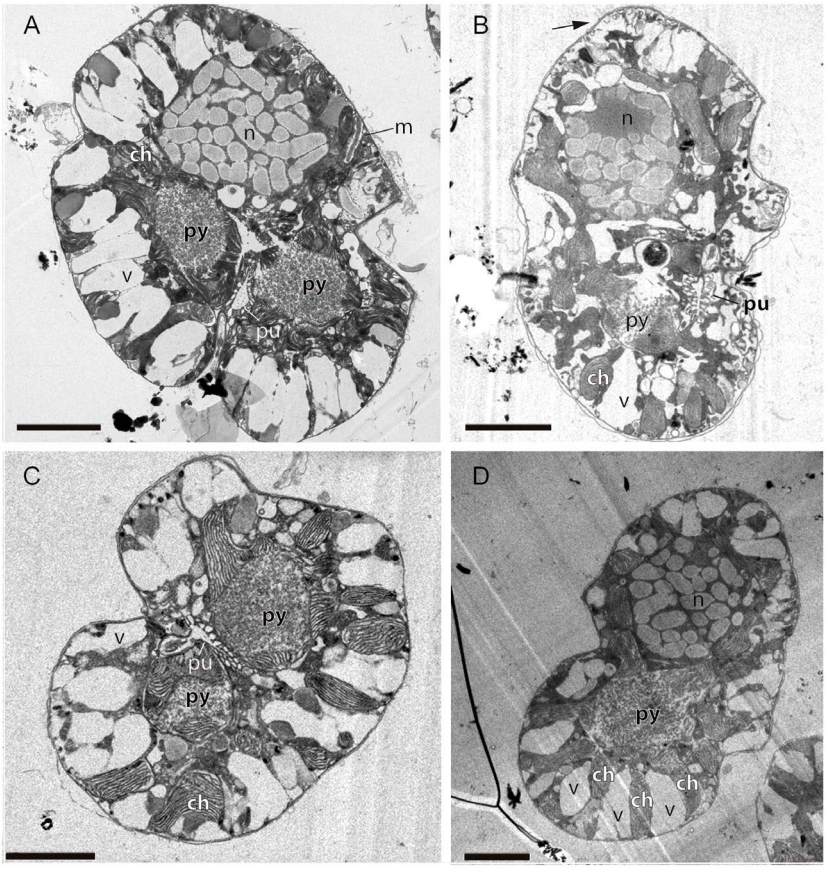

Figure 7. General ultrastructure of *Barrufeta bravensis*. (A) Longitudinal section of a cell shows the arrangement of the main organelles: (ch) chloroplast, (m) mucocyst, (n) nucleus, (pu) pusule, (py) pyrenoid, and (v) vesicle. (B) Lateral view of the cell. (C) Transverse section of the hypocone shows the two pyrenoids and the pusular system. (D) Slanting longitudinal section of a cell shows the chloroplasts radiating from the pyrenoids to the cell periphery. Scale bars, 5 µm.

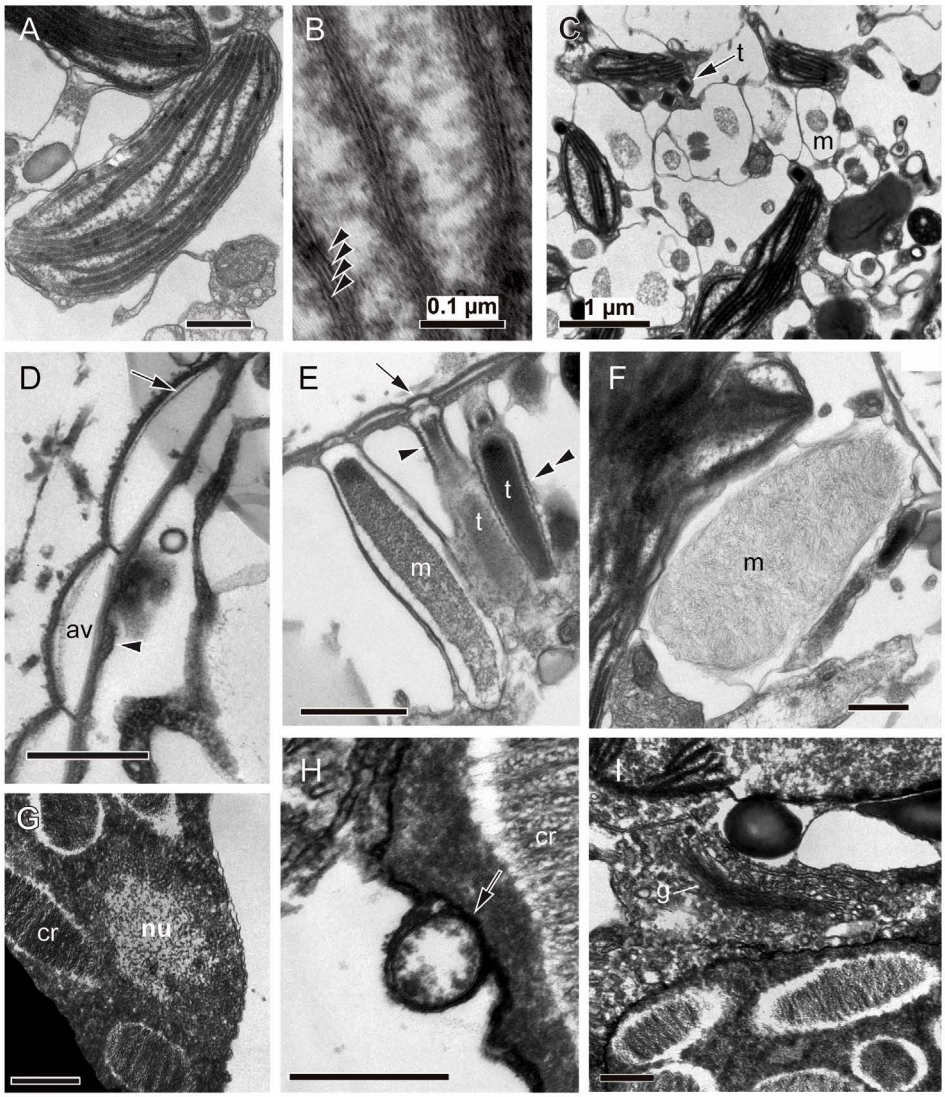

Figure 8. Transmission electron micrographs of *Barrufeta bravensis*. (A) Whole chloroplast. (B) Detail of a chloroplast showing thylakoids grouped in fours (arrowheads) to form lamellae. (C) Section of a subsuperficial area of the cell, showing chloroplasts, trichocysts (t), and mucocyst-like vesicles (m) in a transverse section. (D) A transverse section of amphiesma shows the amphiesmal vesicle (av), which contains platelike material (arrow); the arrowhead indicates cortical microtubules in groups of two or three. (E) Longitudinal section of two trichocysts (t), showing the cap (arrow) and the striation in both the neck (arrowhead) and body (double arrowhead); a vermiform mucocyst (m) with dense material in its core also has a cap. (F) Oval mucocyst and (G) detail of the nucleus, including the nucleolus (nu) and chromosome (cr). (H) In a detailed view of the nucleus, a chromosome and a structure resembling a vesicular chamber (arrow). (I) Part of the Golgi (g) adjacent to the nucleus. Scale bars, 0.5 µm (unless otherwise indicated).

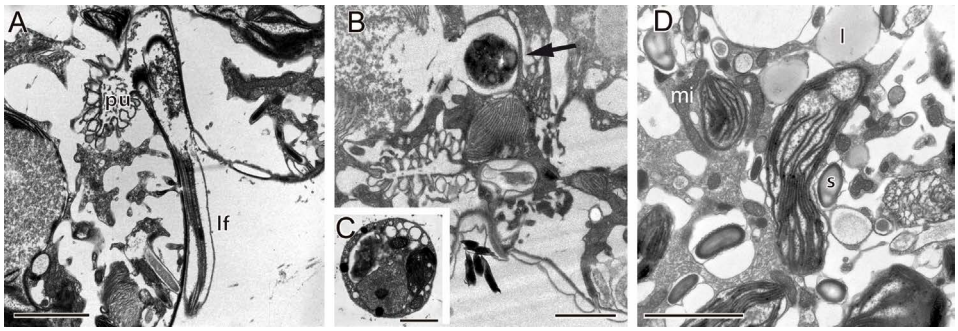

Figure 9. Transmission electron micrographs of *Barrufeta bravensis*. (A) The pusular system (pu) connects with the longitudinal flagellar canal; longitudinal flagella (lf). (B) Food-vacuole-like structure (arrow). (C) Unknown photosynthetic stramenopile cultured with *Barrufeta*. The size of the unknown photosynthetic stramenopile is similar to that of the content of the food-vacuole-like structure. Scale bar, 1 µm. (D) Micrograph of the cytoplasm, showing droplets of lipids (l), starch grains (s), and mitochondrion (mi). Scale bars (A, B, D), 2 µm.

acid was not detected in *Barrufeta* but was present in the other three genera. The percentage of palmitoleic acid (C16:1n7) was higher in *Gymnodinium* and *Akashiwo* than in *Barrufeta* and *Karenia*. The percentages of PUFA C18:2 and saturated C18:0 measured in *Barrufeta* were also different from those in *Gymnodinium*, *Karenia*, and *Akashiwo*. In addition, *Gymnodinium* had an important concentration of saturated lipids, with percentages ranging between 37.4% and 42.1% depending on the particular strain. This amount was almost 3-fold higher than the amount in *B. bravensis*, as shown in Table 1.

Molecular and phylogenetic analysis. The final alignment of *B. bravensis* with *O. marina* CCMP604 as outgroup was 647 bp in length (C, 24.60%; T, 29.06%; A, 19.69%; G, 26.65%) with 607 polymorphic sites and a transition/transversion ratio of 1.6 for the 5.8S rDNA-ITS gene sequences. With *A. affine* as the outgroup, the aligned sequence was 722 bp in length (C, 20.46%; T, 26.34%; A, 24.31%; G, 28.89%), with a transition/transversion ratio of 1.6 and 601 polymorphic sites for the LSU rDNA gene sequences.

As substantial identity was determined across NJ, MP, and ML analyses, with only minor differences, only the ML phylogenetic tree is shown (**Fig. 11** and **12**). According to the ITS-5.8S rDNA phylogeny (**Fig. 11**), the first lineage delineated by the outgroup comprised two species, *A. affine* and *Coolia monotis* Meunier. The ITS-5.8S rDNA phylogeny provided evidence of a major cluster within the one that included the new genus, *Barrufeta*. All *B. bravensis*

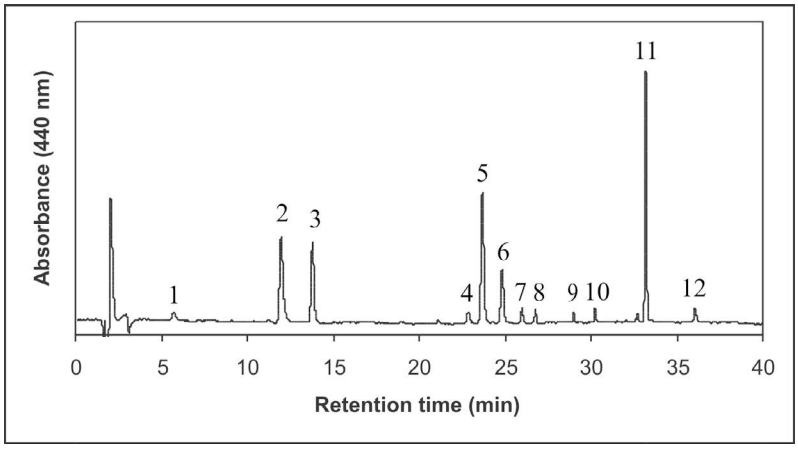

Figure 10. HPLC chromatogram of pigments from *Barrufeta bravensis* strain VGO864. Peak identification: (1) peridininol, (2) Chl-*c*2, (3) peridinin, (4) diadinochrome, (5) diadinoxanthin, (6) dinoxanthin, (7) unknown carotenoid, (8) diatoxanthin, (9, 10) unknown carotenoids, (11) Chl-*a*, (12) β, β-carotene. Detection by absorbance at 440 nm.

isolates constituted a distinct and clearly separated group, which was a sister clade of two other genera represented by *L. viride* and *G. impudicum*. This grouping of two sister clades was supported by high bootstrap values. *B. bravensis* was included in the clade called by some authors as ‘‘*Gymnodinium* sensu stricto clade’’ together with *Lepidodinium* and *Gymnodinium* species. The tree inferred from the data and from model setting showed that different species of the Gymnodiniales were separated into several clades.

Phylogenetic analysis of the sequence generated from the LSU rDNA (**Fig. 12**) showed that *B. bravensis* formed a homogeneous group as a sister clade of *Gymnodinium dorsalisulcum* (Hulburt, J. A. McLaughlin et Zahl) S. L. Murray, de Salas et Hallegr.); this grouping was supported by high bootstrap values in the NJ and ML analyses. *B. bravensis* was included in the ‘‘*Gymnodinium* sensu stricto clade’’, which was partially resolved. In the LSU phylogeny based on *A. affine* as outgroup, two major groups diverged: one group including different taxa segregated at different branching orders and a second one, ‘‘*Gymnodinium* sensu stricto clade’’, which seemed better resolved. Within the first main grouping, the first cluster included *Tovellia*, *Amphidinium*, and *Jadwigia*. The second cluster consisted of two further groups: one group that comprised *Akashiwo*, *Biecheleria*, *Gyrodinium falcatum*, and *Ceratium fusus* together with the sister taxon of *Cochlodinium fulvescens*; the other

group included three genera belonging to the family *Kareniaceae* (*Karenia*, *Takayama*, *Karlodinium*) as a sister branch of one that included *Gymnodinium instriatum* and *Gyrodinium spirale*. In the LSU phylogeny, the second main grouping ‘‘*Gymnodinium* sensu stricto clade’’ included two different clusters. One cluster consisted of two sister clades, one of them composed of different species of *Gymnodinium* including the type species *G. fuscum* (Ehrenb.) F. Stein, and the other one composed of the genus *Lepidodinium*, *Barrufeta*, and two species of *Gymnodinium*; the other cluster also included genera such as *Polykrikos*, *Gymnodinium*, and *Pheopolykrikos* with the exception of *Dissodinium* (Blastodiniales).

Table 1. Relative abundance (%) of fatty acids in different marine unarmored dinoflagellates cultured under the same conditions and harvested in the same growth phase.

Fatty acids	*Barrufeta bravensis* (VGO864)	*Karenia brevis* (CCMP2281)	*Gymnodiniu microreticulatum* (VGO328)	*G. catenatum* (Est14H5)	*G.impudicum* (VGO665)	*Akashiwo sanguinea* (VGO626)
C12:0	0.28	0.1	0.0	0.0	0.0	0.0
C14:1	0.0	0.5	1.0	1.1	1.0	1.3
C14:0	0.24	1.0	1.0	1.3	1.7	2.0
C15:0	0.12	0.2	0.8	1.1	2.3	0.6
C16:1*n*7	1.22	1.3	6.4	5.9	4.9	8.0
C16:0	2.30	2.8	14.7	14.0	13.2	18.0
C17:0	0.17	0.1	0.7	0.5	1.0	0.5
C18:5*n*3	0.24	0.5	2.9	1.0	0.5	1.6
C18:3	0.29	1.1	2.6	1.4	0.9	3.9
C18:2	0.95	0.0	0.0	0.0	0.0	0.0
C18:1*n*9	1.78	1.3	6.6	7.0	7.0	4.3
C18:0	4.53	3.6	19.9	23.4	23.9	13.1
C20:5*n*3	0.21	0.0	2.5	0.0	0.0	3.4
C20:4	0.0	0.0	7.8	1.3	0.0	12.2
C20:0	0.10	0.10	0.3	0.0	0.0	0.6
C22:0	0.0	0.0	0.0	0.0	0.0	0.3
C24:0	0.0	0.0	0.0	0.0	0.0	0.4
SFA	**7.7**	**7.9**	**37.4**	**40.3**	**42.1**	**35.5**
MUFA	**3.0**	**3.1**	**14.0**	**14.0**	**12.9**	**13.6**
PUFA	**1.7**	**1.6**	**15.8**	**3.7**	**1.4**	**21.1**

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

4. Discussion

Daugbjerg et al. (2000) identified both the shape of the apical groove and pigment composition as critical features allowing the differentiation of unarmored dinoflagellate genera, as they were consistent with phylogenetic trees based on rDNA sequencing. This approach resulted in the redescribed genera *Gymnodinium* and *Gyrodinium* as well as new genera, such as Akashiwo, distinguished by differences in the shape of the apical groove (Daugbjerg et al., 2000). Another genus, *Takayama*, with yet a different apical groove shape was described later (de Salas et al., 2003). The shape of the apical groove of new genus *Barrufeta* also differs from that of other gymnodinioids. Furthermore, referring to the different kinds of apical-furrow apparatuses described in the literature (Hansen and Daugbjerg, 2009; Moestrup et al., 2009a, b), we made a detailed structural comparison between the apical groove of *Barrufeta* and that of a species belonging to the genus *Gymnodinium*, *G. impudicum*. Although both contained three elongated vesicles, small knobs ornamented the central vesicle in *B. bravensis*, whereas in *G. impudicum* the lateral vesicles were ornamented. This is the first time that this difference has been noted. However, more species belonging to the two genera need to be examined in future to determine whether this difference is maintained within each genus and to resolve if it can be used as a taxonomic character at the genus level.

The species closest to *B. bravensis*, both phylogenetically and morphologically, are *G. dorsalisulcum*, *L. viride*, and *Lepidodinium chlorophorum* (see Table 2). *G. dorsalisulcum* is a benthic species with a *Gymnodinium*-type apical groove according to Murray et al. (2007) and is thus different from *Barrufeta*. Also, unlike in *Barrufeta*, the epicone of *G. dorsalisulcum* is longer than its hypocone, whereas the two species share the presence of an anteriorly positioned nucleus, two large equatorial pyrenoids, and numerous radiating plastids. In our opinion, the path of the apical groove of *G. dorsalisulcum* shown in Murray et al. (2007) does not seem to be quite the same as a typical horseshoe-shaped *Gymnodinium* apical groove; it forms a shape around the cell vaguely resembling *Barrufeta* overall in the dorsal view. Further studies involving other genes, ultrastructure, and a more accurate examination of the apical groove should determine if the position of *G. dorsalisulcum* within the *Gymnodinium* genus is still appropriate.

Both *Lepidodinium* species have an apical groove of the *Gymnodinium* type, and their pigments derive from Chlorophyta, as deduced from plastid-encoded gene phylogeny (Takishita et al., 2008). Although several other dinoflagellate genera included within Gymnodiniales, such as *Karenia*, *Karlodinium*, and *Takayama*, acquired their chloroplasts through tertiary endosymbiosis from haptophytes (Tengs et al., 2000; de Salas et al., 2003; Garcés et al., 2006), *Barrufeta* is a typical peridinin-containing dinoflagellate and is thus similar to *Gymnodinium* but different from *Lepidodinium*. In addition to the *B. bravensis* strain VGO864, the other strains that were analyzed (VGO859 and VGO860) were observed to have similar pigment patterns and ratios, with diadinoxanthin as the dominant carotenoid, followed by peridinin. This result could be explained by a culture light environment prone to eliciting a photoprotection mechanism that results in a reduced proportion of the light-harvesting pigment peridinin and an increased pool of the photoprotective carotenoid diadinoxanthin.

Since Daugbjerg et al. (2000) described the *Gymnodinium* sensu stricto clade, several genera have been included in this clade in addition to *Gymnodinium*, such as *Lepidodinium* (Hansen et al., 2007), *Polykrikos* (Hoppenrath and Leander, 2007), *Dissodinium* (Gómez et al., 2009b), *Warnowia* (Gómez et al., 2009a), *Nematodinium* (Hoppenrath et al., 2009), *Erythrosidinium* (Gómez et al., 2009a), *Chytriodinium* (Gómez et al., 2009b), and *Paragymnodinium* (Kang et al., 2010); consequently, the name of this group does not seem to be at the moment the most appropriate. The new phylogenetic data on gymnodinioids together with data from the literature (e.g., Gómez et al., 2009b) show that species considered *Gymnodinium* are located in different branches of this clade. This fact supports the need to establish many new genera in order to include species currently considered as belonging to *Gymnodinium*. Species such as *G. catenatum*, *G. nolleri*, and *G. microreticulatum* are monophyletic and have reticulate cysts, and although their horseshoe-shaped apical groove is of the *Gymnodinium* type, it is slightly different from the groove of other *Gymnodinium* species (i.e., *G. impudicum*, *G. dorsalisulcum*, and *G. fuscum*). Accordingly, they should be distinguished within a new genus.

The presence of nuclear chambers and nuclear fibrous connective (NFC) have been considered characteristics of the genus *Gymnodinium* (Daugbjerg et al., 2000), although it has not been demonstrated in all species that

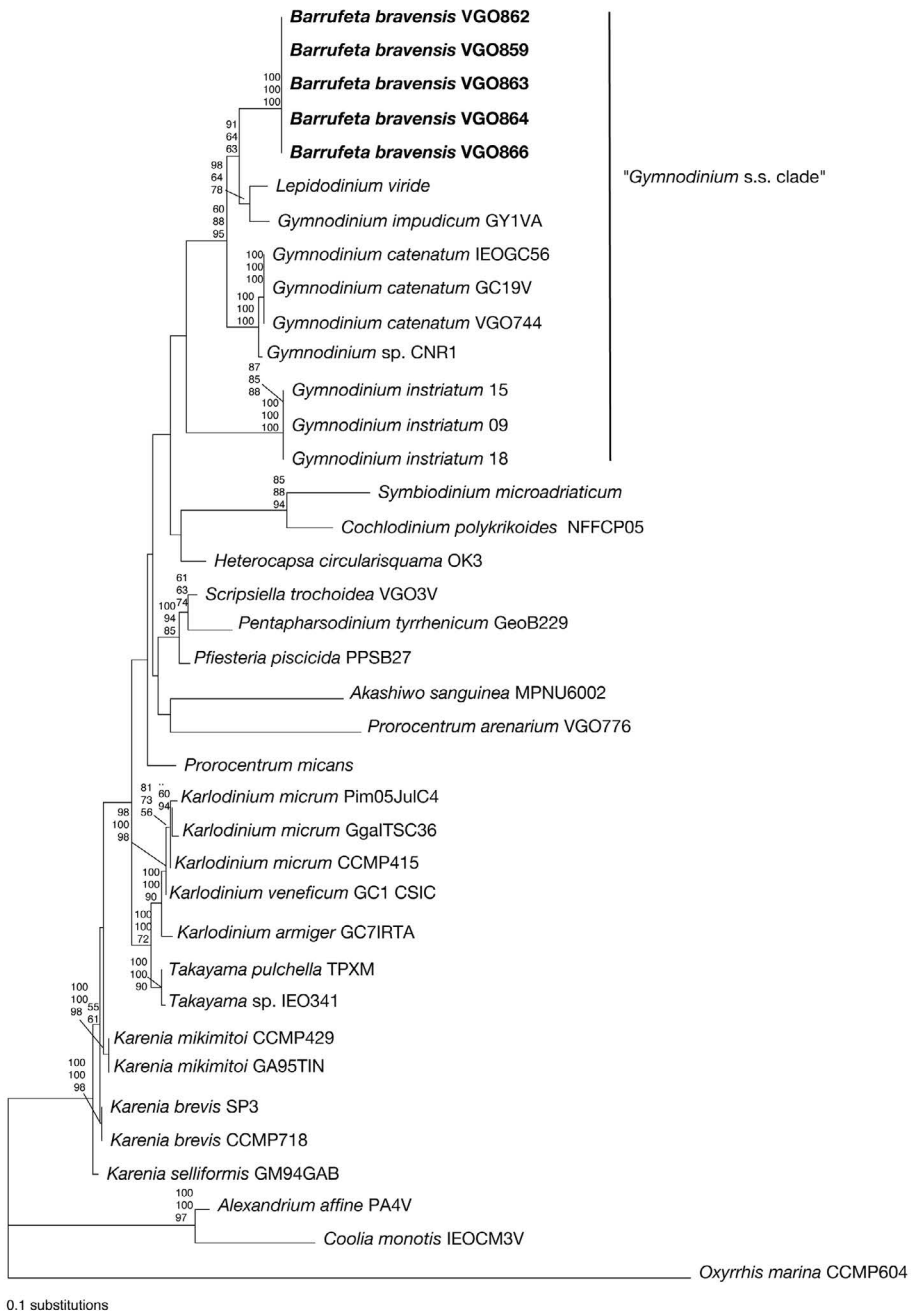

Figure 11. Maximum-likelihood (ML) tree inferred from the alignment of ITS-5.8S rDNA sequences. Numbers on the major nodes represent, from top to bottom, neighbor-joining (1,000 pseudoreplicates), maximum-parsimony (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Oxyrrhis marina* (AY566415) as outgroup. ITS, internal transcribed spacer.

Daugbjerg transferred to *Gymnodinium* genus. Nevertheless, both structures have also been observed in *L. chlorophorum* and *L. viride* (Hansen et al., 2007), although the NFC component is not as large. The NFC has also been detected in other dinoflagellate genera, such as *Polykrikos* (Bradbury et al., 1983), *Actiniscus* (Hansen, 1993), *Nematodinium* (Roberts and Taylor, 1995), and *Biecheleriopsis* (Moestrup et al., 2009b). While nuclear chambers were observed in *Barrufeta*, they are not as numerous as in *Gymnodinium fuscum* (Hansen et al., 2000b) or *L. viride* (Hansen et al., 2007), and nuclear pores were not observed. NFC was not seen in *Barrufeta*, but the presence of this structure cannot yet be ruled out entirely because unfortunately serial sections of samples were not examined with a transmission electron microscope. The large number of the vacuoles with electron-transparent contents and arranged along the periphery alternating with the chloroplast is unusual. Other highly vacuolated species are, for example, *Polykrikos lebourae* Herdman (Hoppenrath and Leander, 2007) and *Amphidinium cryophilum* G. J. Wedem., L. W. Wilcox et L. E. Graham (Wilcox et al., 1982). In *B. bravensis*, the vacuoles with fibrillar content are probably mucocysts, similar to those observed in *P. lebourae* and *Prorocentrum tsawwassenense* Hoppenrath et B. S. Leander and consistent with the observation that *B. bravensis* is a mucus producer. The strain of *Barrufeta* only used in this study for TEM was accompanied by a small, unknown photosynthetic stramenopile. The observation of food-vacuole-like structures with a size similar to that of the unknown photosynthetic stramenopile and the fact that the mixed cultures grew better than the nonmixed ones led us to conclude that *B. bravensis* is a mixotrophic species.

There exists a major discussion about the utility of fatty acids as a chemotaxonomic tool, since these lipids show a variable profile during the growth curve and under different growth conditions (Thompson et al., 1992; Xu and Beardall,1997; Zhu et al.,1997; Xu et al., 2008). This fact, added to the different methodologies used for the analysis of fatty acids in the literature, makes most of the data from the literature uncomparable. In this work, we wanted to compare the profile of fatty acids of *B. bravensis* with those of other unarmored dinoflagellates. To avoid growth-, methodology-, and culture-related differences in fatty acid profiles, all of the strains examined in this study were cultured under the same conditions, examined during the same growth phase and under the same methodology. In this way, the results obtained in this

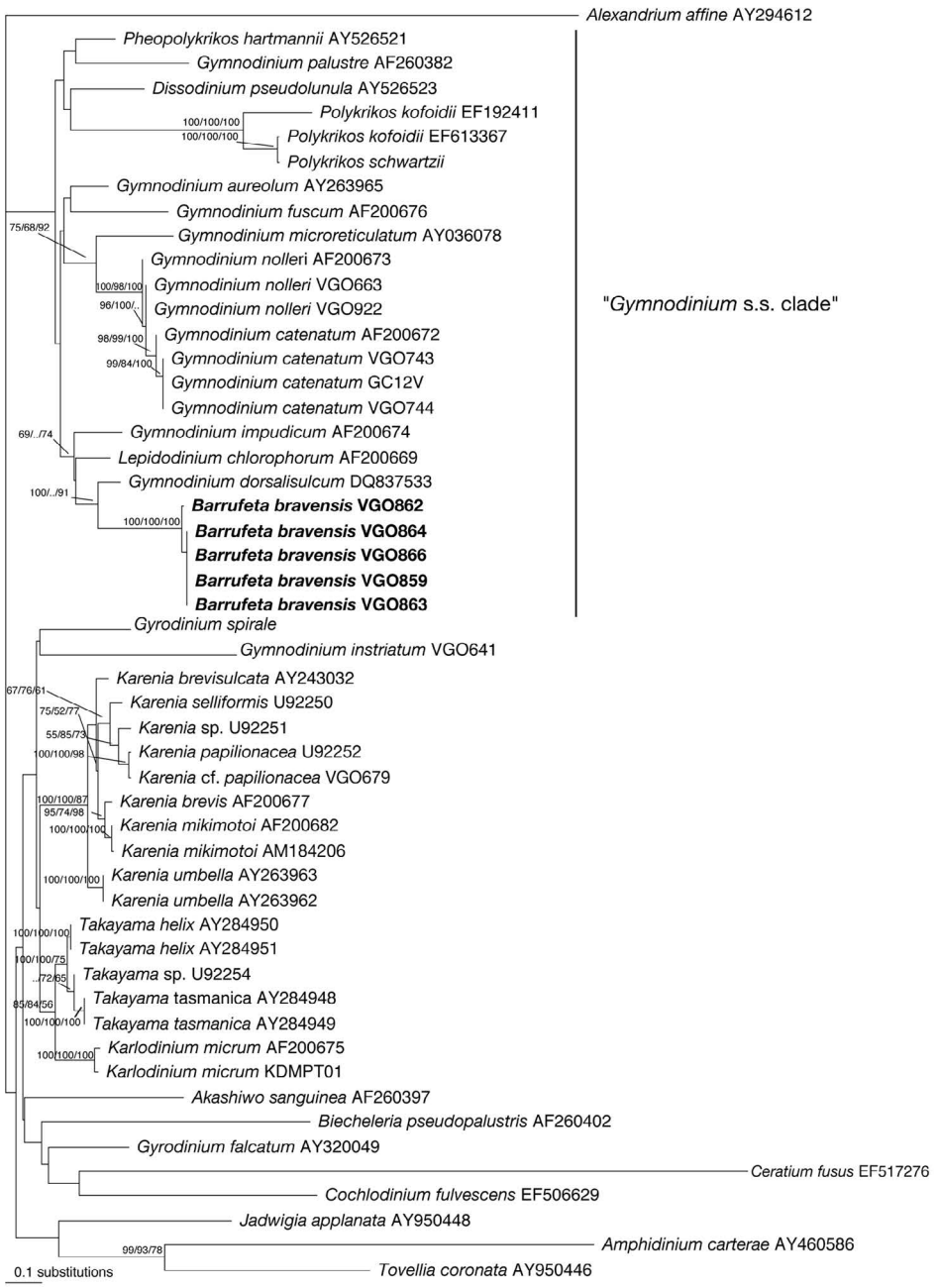

Figure 12. Maximum-likelihood (ML) tree inferred from alignment of the D1 / D2 domains of the LSU rDNA sequences. Numbers on the major nodes represent, from top to bottom, neighbor-joining (1,000 pseudoreplicates), maximum-parsimony (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Alexandrium affine* (AY294612) as outgroup.

Table 2. A comparison of the major morphological features of similar and closest species to *Barrufeta brevensis*; based on Hulburt (1957)[1], Watanabe et al. (1990)[6], Fraga et al. (1995)[5], Takayama (1998)[3], Skovgaard (2000)[2], Murray et al. (2007)[4]

	B. bravensis **(NW Mediterranean Catalonia)**	***Gyrodinium resplendens*** [1] **(NW Atlantic, Woods Hole)**	***G. resplendens*** [2] **(NW Atlantic, Maryland)**	***G. resplendens*** [3] **(Seto Inland Sea)**	***Gymnodinium dorsalisulcum*** [4]	***Gymnodinium impudicum*** [5]	***Lepidodinium viride*** [6]
Shape	Oval-shaped, truncate or slightly bilobate antapex, dorsoventrally flattened	Broadly fusoid, truncate apex and antapex, moderately dorsoventrally flattened	Close to spheroid but moderately dorsoventrally flattened. Some individuals more elongate.	Oval-shaped, truncate antapex	Cells are oval to elongate oval from the ventral side, and dorsoventrally flattened	Chain-forming. Longer and shorter cells can be observed.	Cell subglobular, dorsiventrally flattened. Cell surface with box-shaped scales
Cellular size (µm)	22–35L, 16–25W	36–62L, 32–48W	25–46L, 20–46W	45L, 34W	25–40L, 15–28W	14–37L, 16–32W	22–52L, 19–38W
Apical groove Shape	Smurf cap-shaped	Sulcus extending onto epicone as very narrow superficial groove, diverging to right	Smurf cap-shaped	Smurf cap-shaped	Horseshoe-shaped resembling to Barrufeta in dorsal view	Horseshoe-shaped	Horseshoe-shaped
Chloroplast	Numerous small radiating chloroplasts	Oval, rich brown, radiating, many- tiered	Numerous small, oblong, or disc-shaped chloroplasts lined the inner periphery of the cell		Numerous, yellow-brown, and appear to radiate from the center of the cell	Numerous small elongated chloroplasts	Usually single and is seen as a peripherically situated reticulum
Position of the nucleus	Anterior part of the epicone	Somewhat anterior of center, wider than long	In the epicone	In the epicone	In the epicone	Central, slightly displaced towards the hypocone or the epicone, depending on the position of the cell in the chain	Centrally to anteriorly located
Presence of food vacuoles	+	+	+				

L, long; W, wide.

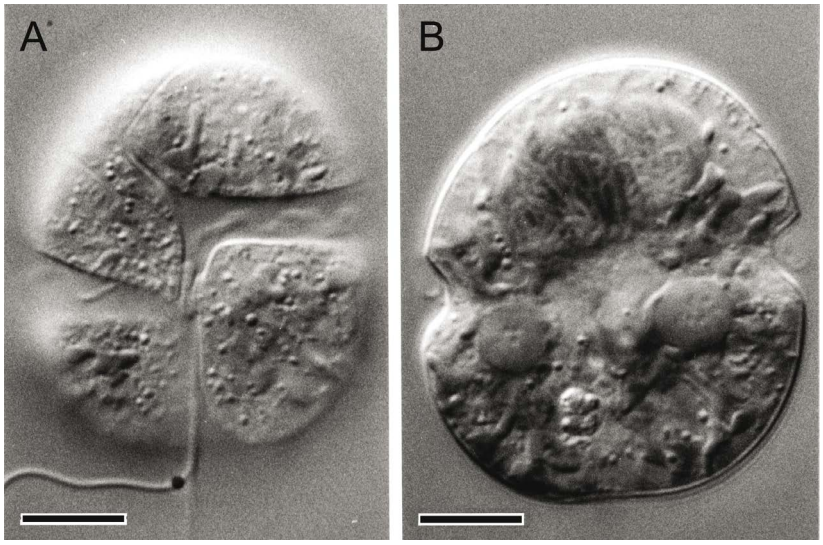

Figure 13. (A, B) Light micrographs of *Gyrodinium resplendens*. Reproduced from Takayama (1998), figures 3 and 4, respectively, with permission. Scale bars, 10 μm

work demonstrate that the fatty acids of *B. bravensis*, in terms of percentages and composition, are more similar to those of *K. brevis* than to those of other species more related, such as the three species of *Gymnodinium* analyzed.

The *Barrufeta* genus could include some other species previously described in the literature. This may be the case for *G. resplendens* (Hulburt, 1957, p. 210), which shares several characteristics with *Barrufeta*, such as the presence of numerous radiating chloroplasts (see **Table 2**). Hulburt (1957) described the apical groove of this species as ‘‘the sulcus extending onto the epicone as a very narrow superficial groove, diverging to the right.’’ This is similar to the proximal part of the apical groove of *Barrufeta*, but the end of the apical groove of *G. resplendens* was not described, or its apical groove is short and only located on its ventral part. In addition, the presence of the pyrenoids was not described, and the size of the latter species is larger than that of *Barrufeta* (36–62 μm length, 32–48 μm width), although smaller cells were identified as in *G. resplendens* by Campbell (1973). Daugbjerg et al. (2000), based on the apical groove of *G. resplendens*, suggested that this species could belong to *Akashiwo*, but some organisms not belonging to the latter genus have been identified as *G. resplendens* by other authors. A strain isolated

from a brackish water (salinity 11) fish pond in Maryland, on the East Coast of the USA, and identified as *G. resplendens* was used by Skovgaard (2000) in a study on mixotrophy, which provided new morphological and behavioral details regarding this plastidic species. Even though Skovgaard (2000) realized that in Hulburt's drawing the apical groove did not encircle the apex of the cell, he assumed that Hulburt had overlooked this feature; based on other similarities with the original *G. resplendens* material, he therefore assigned his organism to this species. Our sample material was similar to that used in Skovgaard's study with respect to the shape of the apical groove, cingulum displacement, the positions and shapes of the chloroplast and nucleus, the presence of food vacuoles, the shape of the smooth-walled cysts, and the presence and position of two pyrenoids, which can be observed in a differential interference contrast (DIC) photograph (Fig. 7 in Skovgaard, 2000). However, Skovgaard described cells that were generally rounded and only moderately dorsoventrally flattened, whereas *B. bravensis* cells are more elongated and more dorsoventrally flattened. Moreover, Skovgaard observed two types of cysts, the most frequent of which had a smooth surface with either a few irregular spines or without spines, while the other, a more uncommon one had a spiny wall. The surfaces of *Barrufeta* cysts are exclusively completely smooth, and most of them are surrounded by mucus. Other aspects of the two species, such as the salinity of the water where they were found, are also different. In another study of *G. resplendens* (Loeblich and Smith, 1968), the reported pigment composition was consistent with our results, although, in contrast to *B. bravensis*, peridinin was found to be the major carotenoid (40%) in *G. resplendens*, followed by diadinoxanthin (33%). Takayama (1998) studied *G. resplendens* from material collected from the Seto Inland Sea and showed the complete apical groove, which had the same shape as we have described in *Barrufeta*. Furthermore, DIC images showed similar positions of the nucleus and of the two large pyrenoids (**Fig. 13**). Mainly due to the probability of the incompletely described apical groove in the original report of *G. resplendens*, we cannot be sure that the *G. resplendens* described by Hulburt is a *Barrufeta*; however, the species studied as *G. resplendens* by Takayama and by Skovgaard, which are not necessarily the same one described by Hulburt, can most certainly be assigned to the genus *Barrufeta*.

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7. Supplementary material

Table S1. European Molecular Biology Laboratory (EMBL) accession numbers and sample locations of the different strains used in internal transcribed spacer (ITS) sequence studies. EMBL accession numbers in bold were generated in this study.

Species	Strain	EMBL accession no.	Sampling location
Akashiwo sanguinea	MPNU6002	AF131075	South Korea
Alexandrium affine	PA4V	AJ632094	Ria de Vigo, Europe Atlantic, Spain
Barrufeta bravensis	VGO859	**FN645813**	La Fosca, Cataluña
Barrufeta bravensis	VGO862	**FN647668**	La Fosca, Cataluña
Barrufeta bravensis	VGO863	**FN647669**	La Fosca, Cataluña
Barrufeta bravensis	VGO864	**FN647670**	La Fosca, Cataluña
Barrufeta bravensis	VGO866	**FN647671**	La Fosca, Cataluña
Coolia monotis	IEO-CM3V	AJ491337	East Atlantic, Vigo, Spain, 1985
Cochlodinium polykrikoides	NFFCP05	EU418935	South Korea: Jangheung
Gymnodinium catenatum	VGO744	**AM998536**	Algier, Algeria
Gymnodinium catenatum	GC19V	AF208247	Ría de Vigo, Spain
Gymnodinium catenatum	CCVIEO GC56AM	AY506592	Gibraltar Strait, Spain
Gymnodinium impudicum	GY1VA	EU244463	Ría de Vigo, Spain
Gymnodinium instriatum	09	AJ534386	Shenzhen Bay, China
Gymnodinium instriatum	15	AJ534387	Shenzhen Bay, China
Gymnodinium instriatum	18	AJ534388	Shenzhen Bay, China
Gymnodinium sp.	CNR-1	AM184202	Sicily, Italy
Heterocapsa circularisquama	OK3	AB084091	Fukui, Obama Bay, Japan

Table S1. (cont.)

Species	Strain	EMBL accession no.	Sampling location
Karenia brevis	CCMP718	AF352827	Gulf of Mexico, USA
Karenia brevis	SP3	AF352825	Gulf of Mexico, USA
Karenia mikimotoi	GA95TIN	AF318224	Atlantic coast, France
Karenia mikimotoi	CCMP429	AM184206	Plymouth, UK
Karenia selliformis	GM94GAB	AF318225	Atlantic coast, France
Karlodinium micrum	GgaITSC36	AF352367	North Carolina, USA
Karlodinium micrum	Pim05JulC4	AY245692	Florida, USA
Karlodinium micrum	CCMP415	AJ557026	North Sea, Norway
Karlodinium veneficum	GC1 CSIC	AJ534656	Alfacs Bay, Ebro Delta, Spain
Karlodinium armiger	GC7IRTA	AJ557024	Alfacs Bay, Ebro Delta, Spain
Lepidodinium viride		DQ499645	South Africa
Oxyrrhis marina	CCMP604	AY566415	UK
Pentapharsodinium thyrrenicum	GeoB 229	AY499512	
Pfiesteria piscicida	PPSB27	DQ991382	Indonesia: Surabaya
Prorocentrum arenarium	VGO776	EU244470	Tenerife
Prorocentrum micans		DQ485145	China
Scrippsiella trochoidea	VGO-3V	**AM998537**	East Atlantic, Vigo, Spain
Symbiodinium microadriaticum	#61	AF333505	
Takayama sp.	IEO341	AM183261	Ría de Vigo, Spain
Takayama pulchellum	TPXM	AY764179	Xiamen, China

Table S2. European Molecular Biology Laboratory (EMBL) accession numbers and sample locations of the different strains used in LSU sequence studies. EMBL accession numbers in bold were generated in this study.

Species	Strain	EMBL Accession no.	Sampling Location
Akashiwo sanguinea	NEPCC354	AF260397	
Alexandrium affine		AY294612	Vietnam
Amphidinium carterae	CCMP1748	AY460586	Belize, Caribbean Sea
Barrufeta bravensis	VGO859	**FN647674**	Spain
Barrufeta bravensis	VGO862	**FN647675**	Spain
Barrufeta bravensis	VGO863	**FN647672**	Spain
Barrufeta bravensis	VGO864	**FN647673**	Spain
Barrufeta bravensis	VGO866	**FN647676**	Spain
Biecheleria pseudopalustris	AJC12cl-915	AF260402	
Ceratium fusus	UAMI-14A	EF517276	Acapulco, Mexico
Cochlodinium fulvescens	CS01	EF506629	Canada
Dissodinium pseudolunula	JHW0205-1	AY526523	Korea
Gymnodinium catenatum	VGO743	**FN647677**	Algeria
Gymnodinium catenatum	VGO744	**FN647678**	Algeria
Gymnodinium catenatum	GC12V	**FN647679**	Spain
Gymnodinium catenatum		AF200672	Spain
Gymnodinium instriatum	VGO641	**FN649410**	Spain
Gymnodinium nolleri	VGO663	**FN649408**	Spain
Gymnodinium nolleri	VGO922	**FN649409**	Spain
Gymnodinium nolleri	DK4	AF200673	

Table S2. (cont.)

Species	Strain	EMBL Accession no.	Sampling Location
Gymnodinium aureolum	GAAD0	AY263965	Australia
Gymnodinium dorsalisulcum	KDAAD	DQ837533	Australia
Gymnodinium impudicum	JL30	AF200674	
Gymnodinium. fuscum	CCMP1677	AF200676	
Gymnodinium palustre	AJC14-732	AF260382	
Gyrodinium falcatum	GFPL01	AY320049	Australia
Gymnodinium. microreticulatum	GMNC01	AY036078	Tasmania
Gyrodinium spirale		AY571371	
Jadwigia applanata	FW 145	AY950448	
Karenia mikimotoi	K-0579	AF200682	
Karenia mikimotoi	CCMP429	AM184206	Plymouth, UK
Karenia. brevis	JL32	AF200677	
Karenia brevisulcata	-	AY243032	New Zealand
Karenia selliformis	G01FVXNZCAWD37	U92250	New Zealand
Karenia sp.	G02HAWNZ IS17	U92251	Australia
Karenia. umbella	KUTN05	AY263963	Australia: Tasmania
Karenia umbella	KULV01	AY263962	Australia: Spring Bay, Tasmania
Karenia papilionacea	G01HAWNZ *IS16*	U92252	Hawke's Bay, North Island, New Zealand,
Karenia. cf. papilionacea	VGO679	**FN649411**	
Karlodinium micrum	KDMPT01	AY263964	New Zealand
Karlodinium micrum	K-0522	AF200675	New Zealand

Table S2. (cont.)

Species	Strain	EMBL Accession no.	Sampling Location
Lepidodinium chlorophorum	K-0539	AF200669	
Pheopolykrikos hartmannii		AY526521	
Polykrikos kofoidii	PKHK00	EF192411	
Polykrikos kofoidii	JHC0412a	EF613367	
Polykrikos schwartzii	PSSH00	EF192409	
Takayama tasmanica	TTDE01	AY284948	Tasmania
Takayama tasmanica	TTTL02	AY284949	Australia
Takayama helix	TTNWB01	AY284950	Tasmania
Takayama helix	TTPA01	AY284951	Tasmania
Takayama sp.	GPKAWNZ CAWD02	U92254	
Tovellia coronata	F1	AY950446	

Chapter 5

The toxicity and intraspecific variability of *Alexandrium andersonii* Balech

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Abstract

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

1. Introduction

In a recent issue of the journal *Harmful Algae* (Pitcher, 2012), the need for species-specific information to predict the occurrence of harmful algal blooms (HABs) was emphasized, especially given the wide-ranging differences in the morphology, phylogeny, lifecycles, growth requirements, etc., of HAB species. In fact, even within the same species there is extensive genotypic and phenotypic variability that challenges our traditional notion of morphospecies (e.g., Alpermann et al., 2010). Therefore, it is clear that to appreciate the plasticity of a species and thus its adaptive potential, more than one strain must be studied.

Phylogenetic studies on diverse phytoplankton species have revealed the geographic differentiation of several traditional morphospecies at global (McCauley et al., 2009; Penna et al., 2010), regional (Casabianca et al., 2012), and local (Godhe and Härnström, 2010) scales. Other studies have found phenotypic differences among strains from different localities or even within the same bloom population (e.g., Alpermann et al., 2010; Calbet et al., 2011; Tillmann et al., 2009). Within a given species there is a high degree of plasticity with respect to toxin content (Alpermann et al., 2010; Thessen et al., 2009; Tillmann et al., 2009), growth rates (Calbet et al., 2011; Hadjadji et al., 2012; Thessen et al., 2009; Tillmann et al., 2009), size (Calbet et al., 2011), lipid composition (Calbet et al., 2011), feeding behavior (Calbet et al., 2011), and allelochemical activity (Alpermann et al., 2010; Tillmann et al., 2009). These and other variations could reflect geographic adaptations of the population but they may also be due to environmental factors. In some cases populations within the same geographic area have been shown to differ in their toxin profiles (i.e., Alpermann et al., 2010; Oh et al., 2010) whereas in others these

differences have been used to distinguish distant populations (i.e., Kim et al., 1993; Mackenzie et al., 1996; Oshima et al., 1993). Similarly, associations of toxic and non-toxic populations of *Alexandrium minutum* in different areas of the coastal waters of Ireland have been described (Touzet et al., 2007). Moreover, there are several reports of temperature-related differences in the toxin content of *Alexandrium* species (Etheridge and Roesler, 2005; Lim et al., 2006; Navarro et al., 2006; Ogata et al., 1987; Wang et al., 2006).

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

Alexandrium andersonii was described by Balech in 1990 based on a clonal culture derived from a cell collected on Cape Cod (NW Atlantic). The same year, Anderson et al. (1990) reported that the strain was non-toxic. In 1998, the first reports documenting the detection of this species in the Mediterranean Sea (Gulf of Naples) were published (Montresor et al., 1998). Ciminiello et al. (2000) determined the PSP toxicity of *A. andersonii* by means of a mouse bioassay and in nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) studies, preceded by the isolation and purification of a high-biomass culture. The toxins obtained in the greatest abundances were saxitoxin (STX) and neosaxitoxin (neoSTX), purified from strain SZN12. This strain was originally obtained from a cyst found in the sediment of the Gulf of Naples. The cellular toxin content of strain SZN12 when grown in the presence

of different nitrogen and phosphorus supplies was analyzed by Frangopulos et al. (2004), who used high-performance liquid chromatography (HPLC). These authors found a very low amount of toxin per cell, mostly gonyautoxin-2 (GTX2), whereas neither STX nor neoSTX was detected. In strains from the Irish coast, Touzet et al. (2008) were unable to detect PSP toxins. Therefore, the nature of the toxicity of strain SZN12 and whether it is indeed toxic at all remain unclear.

In the winter of 2007, *Alexandrium andersonii* was detected for the first time along the Catalan coast (NW Mediterranean Sea), in Alfacs Bay (Ebre Delta, Catalonia), the major shellfish harvesting area in this region (Sampedro et al., 2007). A mouse bioassay of mussel samples collected from the bay identified the low-level presence of PSP toxins (44 µg STX eq./100 g) (Fernández-Tejedor et al., 2007). However, they could not be directly ascribed to the *A.*

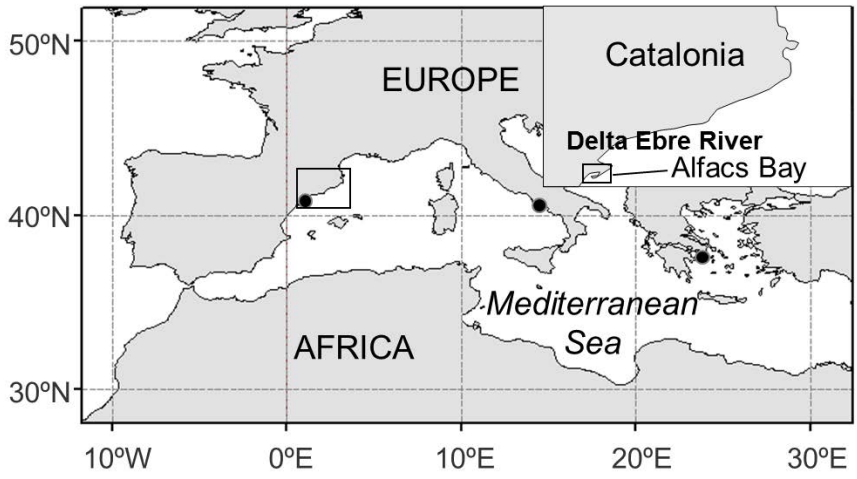

Fig. 1. Map showing the location of Alfacs Bay. Dots show the sites where the Mediterranean strains were isolated.

andersonii population, since other *Alexandrium* PSP-producing species were simultaneously present in the water column.

The recent detection of *Alexandrium andersonii* in different areas of the Mediterranean has brought to light the deficits in our knowledge of this species. This applies not only to its toxin-producing ability but also to its physiology, ecology, and distribution as well as its intraspecific variability, none of which has been studied.

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

2. Materials and methods

2.1. *Alexandrium andersonii* isolation and culture

Alexandrium andersonii culture ICMB222 was isolated in March 2007 from Alfacs Bay (**Fig. 1**), the southern bay of the Ebre River Delta (NW Mediterranean Sea) and the major shellfish harvesting area along the Catalan coast. The other cultures used in this study were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP) of Bigelow Laboratory (USA) and from the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo (Spain). All cultures were adapted for one year to a salinity of 36 and to growth in L1 medium (Guillard and Hargraves, 1993).

2.2. Phenological characterization of *A. andersonii* strains

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

Speculating that variations in temperature influenced the toxin content of *Alexandrium* species, we carried out an additional growth experiment at 14 ºC (the water temperature during the bloom of *Alexandrium andersonii* in Alfacs Bay). Duplicate cultures of strains VGO664, SZN012, ICMB222, and CCMP1718 were grown at 14 ºC under the same conditions used in the first growth experiment, with 2-mL subsamples taken every 2 or 3 days for cell quantification. For HPLC analyses of toxin profiles, 20–40 mL of each culture was removed (depending on the concentration) during the exponential and stationary phases of growth and filtered on Whatman GF/C glass-fiber filters.

2.3. Growth rate calculation and statistical analyses

The cell abundances obtained in the growth experiments were used to determine the exponential growth rates according to Guillard (1973). The growth rate for each flask culture was then estimated using data from different days depending on the start and duration of the respective exponential phase.

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

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

The growth rates and maximum cell abundances at the two temperatures (14 and 20 ºC) were compared. Since the normality of the data could not be assured, due to the small data sets, the paired sign test was used in this analysis. Kruskal–Wallis tests were used to determine significant differences in growth rates and in maximum cell abundances between clones (data from different temperatures were treated separately). All statistical analyses were conducted using Statistica'99 software (StatSoft Inc., USA).

2.4. Light microscopy

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

Species identification was based on the criteria of Balech (1995) together with more recent descriptions (Mackenzie and Todd, 2002; Montresor et al., 2004; Nguyen-Ngoc and Larsen, 2004). The plate tabulation of the cells was examined in detail, following the calcofluor method of Fritz and Triemer (1985).

The cells were stained with calcofluor white M2R (Sigma–Aldrich Co., St. Louis, MO, USA) and examined under a Leica DM IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted microscope with epifluorescence (lamp 50 W).

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

2.5. Measures and statistical analyses

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

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

Cultures of *Alexandrium andersonii* were collected during the exponential growth phase by filtration on 3-µm pore size Isopore membrane filters (Millipore). DNA was extracted and purified as described in Penna et al. (2005). Nuclear-encoded 5.8S rDNA and ITS regions were PCR-amplified as described in Penna et al. (2008). Genomic DNA (1 ng) was amplified in a 50-µL reaction mix containing 50 µM each of dATP, dTTP, dCTP, and dGTP, 0.4 µM of each primer, 4 mM MgCl2, 1 reaction buffer (Diatheva, Fano, Italy), and 1.0 U of Hot Rescue DNA polymerase (Diatheva, Fano, Italy). PCR of the LSU rDNA regions (D1/D2) was carried out in a 50-µL reaction mix containing 1 µL of genomic DNA, 0.25 µM of each primer (D1R and D2C by Scholin et al., 1994), 600 µM of each dNTP, 3 µL MgCl2 (25 mM), 1 reaction buffer

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

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

(Qiagen, USA, including 1.5 mM MgCl2), and 2.5 U of Taq DNA polymerase (Qiagen, USA). Thermocycling conditions for the 5.8S rDNA and ITS regions were as follows: a 10-min initial denaturation at 95 ºC and then 35 cycles

of 1 min at 95 ºC, 1 min at 50 ºC, and 2.5 min at 72 ºC; a final elongation step consisted of 7 min at 72 ºC. For LSU rDNA, an initial denaturation at 95 ºC for 5 min was followed by 40 cycles of 20 s at 95 ºC, 30 s at 55 ºC, and 1 min at 72 ºC, with a final elongation step of 10 min at 72 ºC. The three PCR-amplified products corresponding to the 5.8S rDNA and ITS regions were pooled, purified, and then directly sequenced using the ABI PRISM 310 genetic analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) and the dye terminator method as described in the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction kit, Perkin Elmer Corp., Foster City, CA). LSU rDNA sequencing was carried out by an external service (Macrogen Inc., Korea). Sequences obtained from this study were aligned with those from GenBank using the CLUSTAL X2 program (Larkin et al., 2007) with default settings. Alignments were rechecked visually and edited manually; non-alignable regions were excluded prior to the phylogenetic analyses. The strains used in the molecular determinations are listed in **Table 2** and in supplementary material (**Table 1**) together with the GenBank accession numbers of their 5.8S ITS and LSU rDNA sequences. Phylogenetic relationships, based on the 5.8S ITS rDNA data, were inferred using the neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods. Sequences of *Gonyaulax spinifera* (Claparède et Lachmann) Diesing (EMBL-EBI ENA: AF051832) served as the outgroup in the ITS 5.8S rDNA phylogeny. NJ and MP analyses were performed using euristic searches with tree-bisection-reconnection branch swapping. Branches were collapsed if their minimum length was 0; ambiguities and gaps were considered as missing data. The robustness of the NJ and MP trees was determined by bootstrapping with 1000 pseudoreplicates. Phylogenetic analyses were carried out using the software packages PAUP* ver. 4.0b10 (Swofford, 2002). ML analyses were run with RaxML (Randomized Axelerated Maximum Likelihood) software ver. 7.0.4 (Stamatakis et al., 2005), which adopts a general time reversible (GTR) substitution model and allows for the estimation of several parameters, including the proportion of invariant sites

and the alpha values of the gamma distribution for among-site rate variation. Bootstrap values were calculated with 1000 pseudoreplicates.

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

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

2.7. Pigment analyses

Cultures were examined by light microscopy prior to HPLC pigment analysis to ensure the health and intact morphology of the cells. Three hours into the 12-h light cycle, the cells were harvested from exponentially growing cultures by filtering variable volumes (10–36 mL) of the cultures onto Whatman GF/F filters (Whatman International Ltd., UK) under reduced pressure. The filters were frozen immediately at 25 ºC and processed within 12 h. Cells trapped on the frozen filters were extracted under low light in Teflon-lined screw-capped tubes with 5 mL of 90% acetone, using a stainless steel spatula to grind the filters. The contents of the tubes were chilled in a beaker of ice, sonicated for 5 min in an ultrasonic bath, and then filtered through syringe filters with a 25-mm diameter (MFS HP020, 0.20-µm pore size, hydrophilic PTFE) to remove cell and filter debris. A 0.5-mL aliquot of the acetone extract was mixed with 0.2 mL of water, and 200 µL were injected immediately. This protocol avoids peak distortion by early-eluting peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to injection of the extract in an HPLC system (Latasa et al., 2001).

Pigments were separated following a previously described method (Zapata et al., 2000) on a C8 Waters Symmetry column (150 mm 4.6 mm, 3.5-µm particle-size, 10-nm pore size) using a Waters Alliance HPLC system (Waters Corp., Milford, MA) consisting of a 2695 separation module, a Waters 996 diode-array detector, and a Waters 474 scanning fluorescence detector (excitation: 440 nm, emission: 650 nm). Eluent A consisted of methanol:acetonitrile:0.025 M aqueous pyridine (50:25:25, v/v/v), and eluent B of methanol:acetonitrile:acetone (20:60:20, v/v/v). The elution gradient (time: %B) was as follows: t_0 = 0%, t_{22} = 40%, t_{28} = 95%, t_{37} = 95%, and t_{40} = 0%. The flow rate was 1.0 mL min^{-1} and the column temperature was 25 ºC. The solvents were HPLC grade (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). After the confirming the purity of the peaks, we compared the spectral information with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see Jeffrey and Wright, 1997).

2.8. Toxicity and toxin analyses

Toxin analyses by HPLC as well as the toxicity assays (mouse bioassay, hemolytic and cytotoxicity tests) were carried out using extracts prepared from the five strains, following their culture, without replicates, in 5.5-L Pyrex bottle with aeration. The cultures were maintained in the same medium and at the same salinity, temperature, and photoperiod as described for the first growth experiment but with a slightly higher light intensity (110 µmol photons m^{-2} s^{-1}). Two-mL aliquots were sampled every 1–4 days and fixed with Lugol's for quantification in order to determine the growth phase of the cultures. During late exponential phase (on different days, depending on the culture), approximately 4 L of every culture was filtered under reduced pressure onto Whatman GF/C glass-fiber filters that had been previously heated in an oven

at 450 ºC for a minimum of 4 h. The filters were frozen immediately at -20 ºC until needed. The extraction for PSP toxin determination was performed with 0.1 M HCl following the official method (AOAC, 2000). The same methodology, but substituting phosphatebuffered saline (PBS) for 0.1 M HCl, was used to obtain extracts of possible hemolytic proteins.

2.8.1. HPLC-FLD analysis of PSP toxins

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

HPLC-FLD with post-column derivatization was performed according to Franco and Fernandez (1993). The HPLC system consisted of a Waters 474 fluorescence detector (excitation: 330 nm, emission: 390 nm), a Waters 717 automatic injector, a Waters 600 HPLC pump to deliver the mobile phases, and two Waters 510 pumps to deliver the post-column reagents. Separation was achieved with an Agilent 5µ Lichrosphere 100 RP18 cartridge (125 mm 4.6 mm). The column temperature was 30 ºC and the flow rate was 0.8 mL min^{-1}. The mobile phases consisted of 1.5 mM octansulfonic in 10 mM ammonium phosphate (pH 7) for GTX separations and 95% of this eluent solution plus 5% acetonitrile for the separation of neoSTX, dcSTX, and STX.

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

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

GTX5, and GTX6.

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

2.8.2. Cytotoxicity assay

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

To prevent interferences in the HCl extract, prior to the analysis, an aliquot of the acidic *Alexandrium andersonii* extract (see Section 2.8.3) was further purified using solid-phase extraction (SPE) cartridges (C18 AccuBond II, 500 mg, 3 mL). The SPE cartridges were first conditioned using 6 mL of absolute methanol followed by 6 mL of MilliQ water. One mL of acidic extract was loaded and eluted twice with 2 mL of MilliQ water. The volume of the eluate was further adjusted to 4 mL using MilliQ water.

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

2.8.3. Hemolytic activity

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

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

Triplicate samples made up of 5 mL of the blood-cell suspensions and 5 mL of the toxin-containing solution were combined in centrifuge tubes, incubated at 37 ºC for 6 h, and then maintained at room temperature for an additional 18 h. At intervals of 1, 5, 6, and 24 h, 1-mL aliquots of the mixture were transferred to Eppendorf vials, which were centrifuged for 10 min at 1500 rpm at a temperature of 10 ºC. Two hundred µL aliquots of each supernatant were then added to the wells of one microwell plate. Absorption of these samples was measured at 405 nm with a BioRad microplate reader model 550.

2.8.4. Mouse bioassay

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

For each sample, two mice were i.p. injected with 1 mL of the HCl extract and then observed continuously for 60 min. Symptom occurrence and time of death

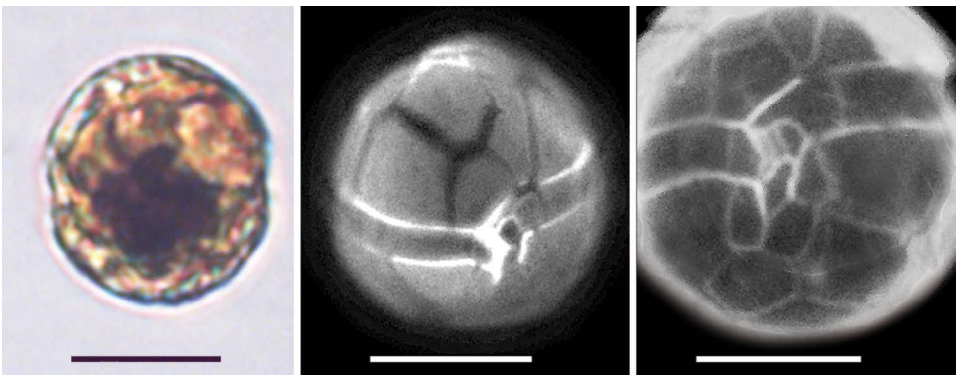

Fig. 2. Micrographs of an *Alexandrium andersonii* cell observed using light (A) and epifluorescence (B), (C) microscopy. Scale bars, 10 µm.

were recorded. Mice still alive after this time were observed intermittently for a total of 48 h.

3. Results

3.1. Morphology and measurements

The morphology of *Alexandrium andersonii* from Alfacs Bay of the Ebre Delta (**Fig. 2**) generally well matched the description of Balech (1990), except that the wild cells were smaller (length: 21.7 ± 3.1 µm, range: 17–28.3 µm, n = 30; width: 21.1±2.7 µm, range: 15.5–26.4 µm, n = 30) than those of the original description (length: 21–35 µm, width: 18–33 µm).

Differences in the cell sizes of the strains compared to the Alfacs Bay field samples are shown in **Fig. 3**. Cells of the Catalan strain ICMB222 were the smallest while those of one of the two American strains, CCMP1718, were the largest, although wild cells were larger than cells from any of the strains studied. ANOSIM analysis, which included strains and field cells, showed that these differences in cell size were significant ($p = 0.00001$). According to the pairwise tests, each of the strains was significantly different from the others

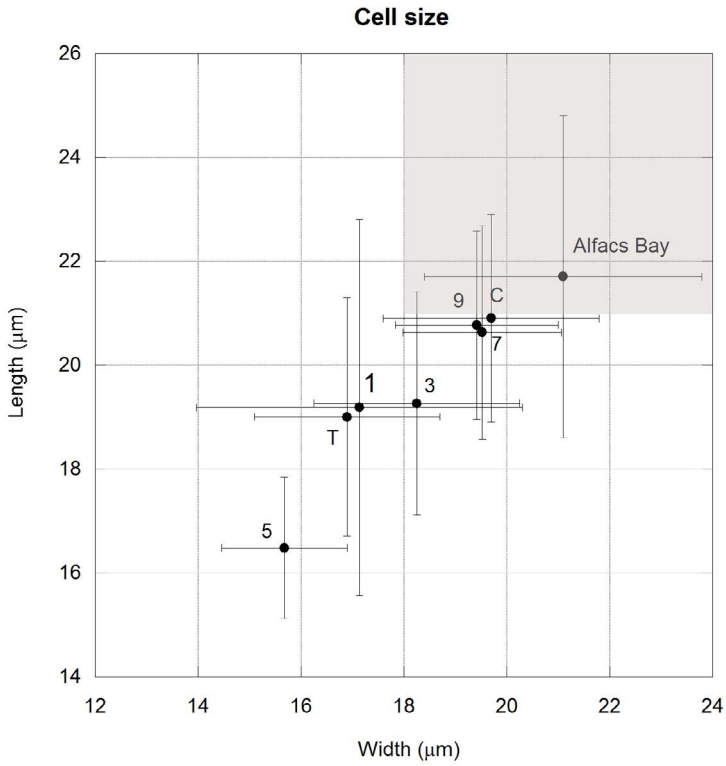

Fig. 3. Mean and standard deviation of the measurements of 30 cells from each of the five strains of *A. andersonii* (1, VGO664; 3, SZN012; 5, ICMB222; 7, CCMP1718; 9, CCMP1597) and from cells in natural samples from Alfacs Bay. Measurements from other authors have been included (C, Ciminiello et al.,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

and from the field samples ($p < 0.05$), with the exception of the two American strains (CCMP1718 and CCMP1597).

3.2. Phylogenetic analyses

Based on the 5.8S rDNA, ITS regions, and LSU regions of the *Alexandrium* isolates, almost identical topologies were obtained by NJ, MP, and ML and by NJ, UPGMA, and ML analyses, respectively; therefore, only ML phylogenetic trees are shown in **Fig. 4** (also see the supplementary material). *Alexandrium andersonii* constituted a homogeneous clade supported by high bootstrap

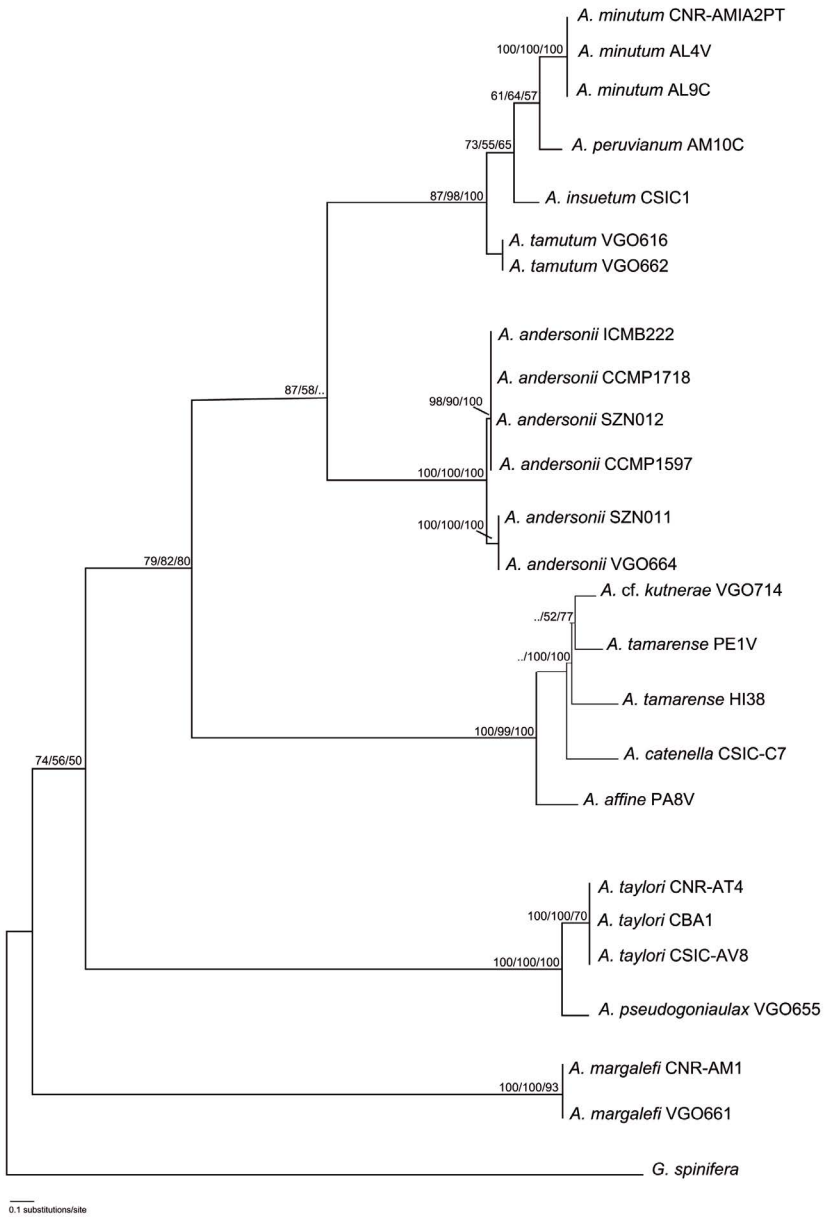

Fig. 4. Maximum-likelihood (ML) tree inferred from the alignment of ITS 5.8S rDNA sequences. Numbers on the major nodes represent, from right to left, neighbor-joining (1000 pseudoreplicates), maximum-parsimony (1000 pseudoreplicates), and ML (1000 pseudoreplicates) bootstrap values. Only bootstrap values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

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

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

values in both phylogenetic trees, consistent with a lack of substantial variability among the isolates analyzed. The *A. andersonii* group constituted a sister clade of the *Alexandrium minutum*/*Alexandrium tamutum* group within the genus *Alexandrium*.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2013.02.003.3.2.

3.3. Growth rates

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

The maximum cell densities reached by each culture differed between strains but not significantly, with a maximum of $3.9X10^7$ cells L^{-1} achieved by strain CCMP1718 at 20 ºC. Significantly higher maximum densities were obtained at 20 ºC than at 14 ºC (non-parametric paired sign tests). At 14 ºC, only strain SZN012 grew to reach densities $>10^7$ cells L^{-1}.

Different patterns of growth were observed for the five strains. Thus, strains ICMB222 and CCMP1597 (for strain CCMP1597 replicates were not available) had lower growth rates and longer exponential phases (**Fig. 5C** and **D**), while strains SZN012 and CCMP1718 grew faster, with shorter exponential phases (**Fig. 5B** and **D**). For strain VGO664, temperature-specific differences were

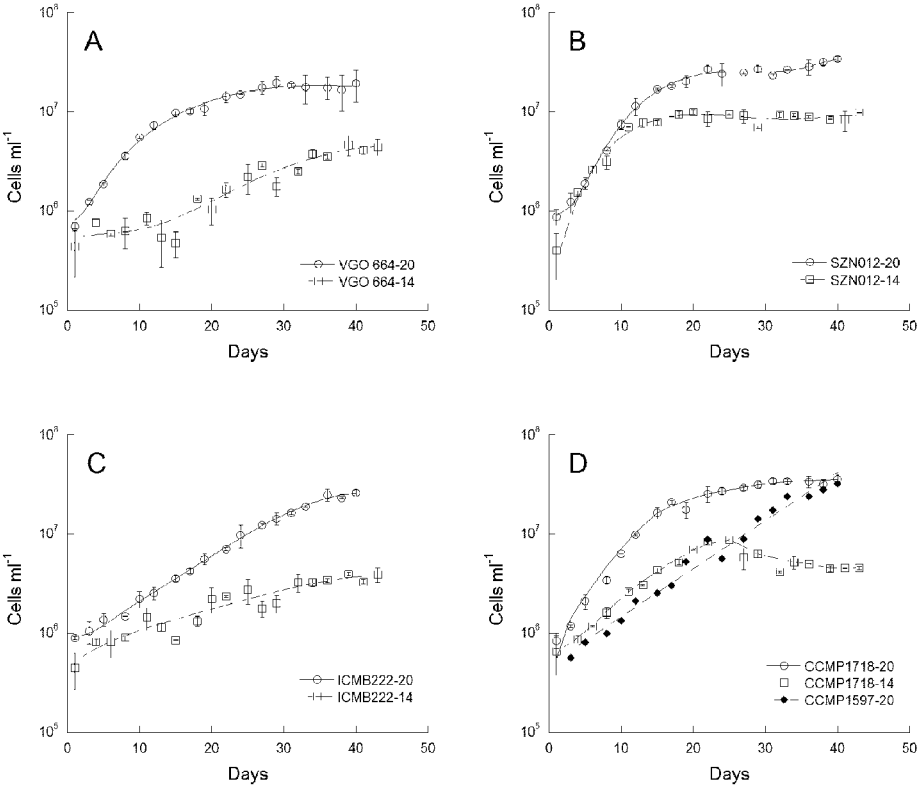

Fig 5. Growth curves of the different strains of *A. andersonii* cultured at 20 ºC (circles or rhombus) and at 14 ºC (squares) in L1 medium. For illustrative purposes, the data were fitted to a curve. Bars represent the standard deviation of the duplicates.

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

Strain	Per/chl-*a* ± SD	Per/c_2 ± SD	chl-*c*2/chl-*a* ± SD	Diadino/ chl-*a* ± SD	Dino/chl-*a* ± SD
VGO664	1.04 ± 0.01	4.93 ± 0.10	0.21 ± 0	0.23 ± 0	0.09 ± 0.01
SZN012	0.90 ± 0.01	4.43 ± 0	0.20 ± 0	0.23 ± 0	0.08 ± 0
ICMB222	0.83 ± 0.01	4.14±0.57	0.20 ± 0.03	0.21 ± 0.02	0.07 ± 0.01
CCMP1718	0.76 ± 0.06	3.03 ± 0.11	0.25 ± 0.02	0.23 ± 0.01	0.10 ± 0.01
CCMP1597	0.46	1.91	0.24	0.21	0.08

noted. At 14 ºC, this strain grew slowly, after a long lag phase, whereas at 20 ºC growth was more rapid (**Fig. 5A**).

3.4. Pigment analyses

Alexandrium andersonii is a typical peridinin-containing dinoflagellate and the pigment composition of all five strains was similar, with peridinin as the major carotenoid. However, there were slight differences in the pigment ratios with respect to chlorophyll (Chl) *a* (**Table 4**). Specifically, the ratio of peridinin to either Chl-*a* or Chl-c_2 was highest (1.04±0.01 and 4.93±0.10, respectively) in the Greek strain VGO644 and lowest (0.46 and 1.91, respectively) in strain CCMP1597 (for strain CCMP1597 replicates were not available), isolated from the west coast of the North Atlantic. Other pigment ratios (Chl-c_2/Chl-*a*, diadino/Chl-*a*, dino/Chl-*a*) were more consistent irrespective of the strain analyzed.

3.5. Toxicity and toxin analyses

3.5.1. HPLC

PSP toxins were not detected in samples of *Alexandrium andersonii* cultured at either 20 ºC or 14 ºC. For some of the samples, a small peak in the

chromatogram with a retention time somewhat earlier than that of the GTX4 or GTX1 standard was noted. Since these peaks were also observed in the absence of oxidant they were considered to represent interfering fluorescent compounds (see the example in **Fig. 6**).

3.5.2. Cytotoxicity assay

All of the *Alexandrium andersonii* strains studied were toxic to neuro-2a cells, with a response characteristic of the presence of VGSC-inhibiting toxins (data not shown). A semi-quantitative estimation of the STX-like compounds produced by *A. andersonii* strains is provided in **Table 5**. The values ranged between 1.4 and 14.7 µg STX equivalents $X10^{-5}$ $cells^{-1}$.

3.5.3. Hemolytic activity

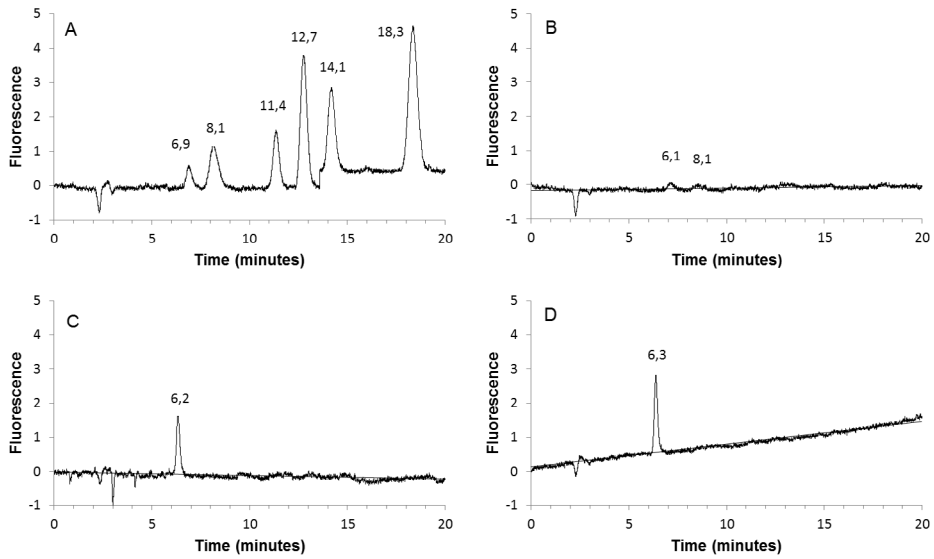

Fig. 6. HPLC-FLD chromatograms of stationary phase cells of strain SZN012 cultured at 14 ºC. (A) SZN012 cells + GTXs standard with oxidant; (B) SZN012 cells + GTXs standard without oxidant; (C) SZN012 cells with oxidant; (D) SZN012 cells without oxidant. Retention times (min) of GTXs: GTX4: 6.9; GTX1: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

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

Strain	Cells (mL culture)$^{-1}$	µg STX eq.(mL culture)$^{-1}$	pg STX eq. X cells^{-1}
VGO664	34692	1.99	57.3
SZN012	27000	3.971	147
ICMB222	42870	0.858	20
CCPM1718	35320	0.478	13.5
CCPM1597	20857	2.178	104

All of the tested strains showed hemolytic activity (**Table 6**). After 1 h of incubation, the absorbances of the PBS-extracted samples were indicative of hemolysis, which reached 100% after 5 h. While the HCl extracts were also positive, the response was slightly different: intact erythrocytes were detected after 1 h, ruptured red cells after 5 h, and 100% hemolysis after 24 h of incubation. Therefore, hemolytic activity was characteristic of all the strains. The positive result obtained with the PBS extracts suggests the involvement of proteinaceous compounds, while that obtained with the HCl extracts is consistent with the presence of non-protein compounds, the nature of which is not yet known.

3.5.4. Mouse bioassay

None of the strains appeared to express PSP toxins since none of the bioassayed mice exhibited the appropriate symptoms. Mice injected with extracts from strains SZN012 and CCMP1597 died at various times, as detailed in **Table 7**. Following injection with the HCl extracts from these strains, the mice became weak and lethargic, with a progressive decline in activity until they finally died. However, these symptoms are not typical of those induced by toxins of the PSP complex. Nonetheless, to increase the possible toxic content

and thereby better distinguish toxin-related symptoms, samples from strains VGO664 and SZN012 were concentrated five- and six-fold, respectively, prior to their injection, in which case the mice died approximately 2 h later but still did not exhibit PSP-like symptoms.

4. Discussion

4.1. Morphology and measurements

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

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

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

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

Strain	Time until death (h)		Comments
VGO664	—	—	Alive (48h)
SZN012	30	48	
ICMB222	—	—	Alive (48h)
CCPM1718	—	—	Alive (48h)
CCPM1597	10	48	
VGO664X5	2	2	Death probably
ICMB222X6	1.5	2	due to excess salt

A. andersonii strains used in this study were smaller than those obtained by Balech. Moreover, the sizes of the field cells were within the lowest end of the range reported in that original description.

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

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

4.2. Phylogenetic analyses

The phylogenetic homogeneity of *Alexandrium andersonii* strains based on the 5.8 ITS and LSU regions did not allow us to distinguish among individual populations from different Mediterranean areas, unlike in other *Alexandrium* species (Lilly et al., 2005; McCauley et al., 2009). However, genetic diversity within *A. andersonii* cannot be ruled out and its detection may instead require finer-scale markers (such as microsatellites), as was the case for *Alexandrium*

minutum in studies carried out at global and Mediterranean scales (Casabianca et al., 2012; McCauley et al., 2009).

4.3. Growth rates

The net growth rates of the studied strains under standard conditions did not differ significantly. This was also reported for *Karlodinium veneficum* (Bachvaroff et al., 2009) but not for *Karenia brevis* (Loret et al., 2002). In our study, differences in the growth patterns of the five strains and even between a subclonal strain and its parent strain (CCMP1718 and CCMP1597) were observed. Along the Catalan coast (NW Mediterranean, Spain), *Alexandrium* species have been observed in detail for over 17 years. During this time, there have been no blooms of *Alexandrium andersonii* in response to warm temperatures; rather, the cells have only rarely been detected and only at very low densities (<100 cells L^{-1}). In contrast, a bloom of *A. andersonii* occurred in the winter (from January to April) of 2007 in Alfacs Bay, coinciding with water temperatures between 10 and 15 ºC. During that event, cell densities reached a maximum of 7159 cells L^{-1}, recorded on February 19th.

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

4.4. Pigment analyses

As all members of the genus *Alexandrium* identified thus far contain the pigment peridinin, in this study only differences in the accessory pigment ratios were expected. In dinoflagellates, pigments occur in stoichiometric proportions in the extrinsic water-soluble peridinin-Chl *a*-protein (PCP) and intrinsic PCP

(membrane-bound) light-harvesting antennae. Since the proportions of the two antenna types are determined by genetic as well as environmental factors, these in turn will affect the ratios of accessory pigments.

In the specific case of *Alexandrium andersonii*, our data point to the role of intrinsic and thus probably genetic factors in the quantitative differences in pigment ratios, since the five strains were cultured under the same environmental conditions. Zapata et al. (2012) studied three common strains of *A. andersonii* (CCMP1718, VGO664, and SZN012) and found similar differences in pigment ratios. However, the absolute molar pigment ratios in that study were slightly different than those determined in the present work, most likely due to variable culture conditions (e.g., light and salinity).

4.5. Toxicity and toxin analyses

Under the experimental conditions of this study, PSP toxins were not detected by either HPLC or mouse bioassay, in contrast to the results of Ciminiello et al. (2000) and Frangopulos et al. (2004). However, all the studied strains exhibited both a response characteristic of the expression of VGSC-inhibiting toxins, as shown in the neuroblastoma neuro-2a cell assay, and hemolytic activity. These results can be alternatively explained as follows: The neuro-2a cytotoxicity assay is able to detect not only PSP-type toxicity, similar to the HPLC-FLD method, but also the toxicity of other biomolecules that interact with VGSCs, such as tetrodotoxin (TTX) (Kogure et al., 1988), lipopeptide (Edwards et al., 2004; Wu et al., 2000), and polypeptide (Catterall et al., 2007; Jacob and McDougal, 2010). Consequently, this assay may overestimate toxin concentrations. Thus, an interpretation of our results must include the consideration that the low toxicity detected was not due to PSP toxins. Similarly, the hemolytic activity of the different strains could have been due not only to STX and its derivatives but also to an excess of salt or/and to a hemolytic toxin, as reported for other *Alexandrium* species (Emura et al., 2004; Simonsen et al., 1995). As an alternative explanation of our findings,

the amount of toxicity may have been so low that it was not detected either by the mouse bioassay or by the HPLC method, which have limits of detection of 0.3 µg STX eq. mL^{-1} and 0.01–0.15 µg STX eq. mL^{-1}, respectively, whereas it was detectable in the neuro-2a assay (detection limit of 0.91–58.41 nM, or 0.27–17.5X10^{-3} µg STX eq. mL^{-1}) (Cañete and Diogène, 2008). However, the first explanation is the more likely one since the toxicity of the strains as determined in the cytotoxicity assay was clearly higher than the detection limit of the HPLC method (0.01–0.15 µg STX eq. mL^{-1}), which therefore should have yielded a positive result.

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

Frangopulos et al. (2004), are unusual. Indeed, significant shifts tend to occur only in batch and semi-continuous cultures in response to extreme changes in the growth regime (Anderson et al., 2012).

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

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

A few studies reported in the literature used *Alexandrium andersonii* as the toxic species in ingestion experiments but only in some of them were the toxin profiles analyzed. The *A. andersonii* strains used by Shaber and Sulkin (2007) and by Perez and Sulkin (2005) were obtained as toxic strains of *A. andersonii* from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The latter study used strain CCMP1718, one of the five strains of *A. andersonii* that we examined in this work. In describing its toxicity, the authors cited the CCMP website. However, the CCMP, to the best of our knowledge, has not evaluated their *A. andersonii* cultures for toxicity (J. Sexton, personal communication). Garcia et al. (2011) also used strain CCMP1718 for ingestion experiments. Its toxicity, as verified by CyanoHAB Services, is based on the results of an ELISA, which determined a toxin concentration of 5.1 μg STX eq. L^{-1}. Both Perez and Sulkin (2005) and Garcia et al. (2011) reported the accelerated mortality of larval *Hemigrapsus oregonensis* when fed *A. andersonii* cells, but according to the latter authors the results could not be ascribed with absolute certainty to toxin effects.

Stüken et al. (2011) recently proposed that in dinoflagellates nuclear genes are required for STX synthesis but they were unable to detect these genes (specifically *sxt*A1 and *sxt*A4) in *Alexandrium andersonii* strains CCMP1597 and CCMP2222 (the latter synonymous with SZN012 of this study). Although we cannot draw any conclusions regarding the ability of *A. andersonii* to synthesize toxins, neither of the two official methodologies for the detection of PSP toxins used in our study, i.e., HPLC and mouse bioassay, yielded evidence of their production, thus supporting the results of those authors.

Our findings obtained with the less specific methodologies (cell bioassays) are inconclusive since, as discussed above, it is unclear whether the response of neuroblastoma 2a cells was genuinely due to STX (or to its derivatives). In any case, under the study conditions only trace amounts of toxins were produced by the tested strains of *Alexandrium andersonii*. Therefore, based

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

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

on our current knowledge, we do not consider *A. andersonii* as a species that is toxic to humans.

4.6. Aquaculture implications

In this study, the non-toxic behaviors of both the Neapolitan strain of *Alexandrium andersonii* and the other four Mediterranean and North Atlantic Ocean strains, all tested under the same conditions, suggest that the toxicity detected in field mussels from the Alfacs Bay in the winter of 2007 (Fernández-Tejedor et al., 2007), concurrent with a bloom of *A. andersonii*, was probably due to the presence of other, toxic species. In fact, *Alexandrium minutum*, which is very common along the Catalan coast and whose toxicity is well established (Delgado et al., 1990; Van Lenning et al., 2007), was also present during that bloom period (Sampedro et al., 2007).

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

While in the present work *Alexandrium andersonii* strains exhibited hemolytic

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

5. Conclusions

Our experiments show that *Alexandrium andersonii* strains originating from different locations differ markedly in their size, several of their pigment ratios, and in their patterns of growth, despite phylogenetic homogeneity in their 5.8 ITS and LSU regions.

This intraspecific variability was also noted between a subclonal strain and the parent strain of this species (CCMP1718 and CCMP1597, respectively). Moreover, strains cultured under the same laboratory conditions did not express PSP toxins, as determined by HPLC and in a mouse bioassay.

6. Acknowledgements

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8. Supplementary material

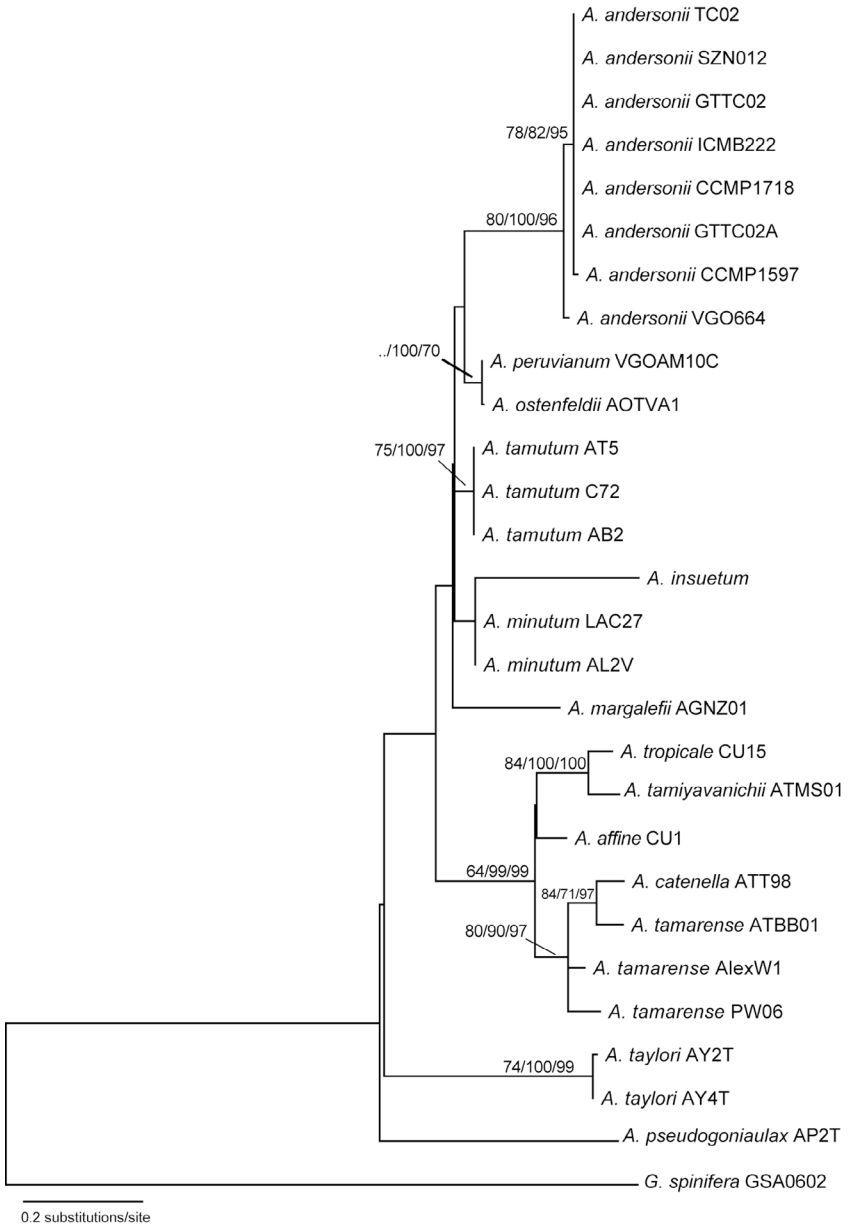

Figure S1. Maximum-likelihood (ML) tree inferred from the alignment of LSU rDNA sequences. Numbers on the major nodes represent, from right to left, neighbor-joining (1,000 pseudoreplicates), UPGMA (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using Goniaulax spinifera (EU805591) as outgroup.

Table S1. EMBL-EBI ENA accession numbers and sampling locations of the different strains used in LSU sequence studies.

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

Discussió global

1. La diversitat del gènere *Alexandrium*

La costa catalana presenta una gran diversitat d'espècies del gènere *Alexandrium*. Altres zones del món amb treballs específics sobre la biodiversitat del gènere *Alexandrium*, mostren una diversitat menor amb 6 o 7 espècies (Mackenzie et al., 2004; Farrell et al., 2013; Touzet et al., 2008). La major diversitat d'espècies d'*Alexandrium* trobada a la costa catalana (11 espècies) i en general a la Mediterrània (18) pot ser degut entre d'altres factors al gran tràfic marítim que s'hi dona. Els ports reben vaixells i presenten condicions adequades pel creixement d'espècies de dinoflagel·lades el que pot provocar l'introducció d'aquests organismes al litoral. Són vies d'entrada, i d'ampliació que a més están interconectats podent propiciar a més la dispersió d'espècies en el territori.

La llista d'espècies a la costa catalana i a la Mediterrània és possiblement major a la descrita fins ara. Investigacions recents presentades a la 17a Conferència Internacional sobre algues nocives, van descriure una nova espècie d' *Alexandrium, A. fragae* Branco, Oliveira, Salgueiro & Menezes (Branco et al., 2016), anteriorment observada a les aigües costaneres catalanes entre d'altres àrees i confosa amb una morfologia singular d' *A. minutum* (amb una hipoteca reticulada), p. ex., Fig 1.1 a Garcés, 1998 i Fig 3b a Montresor et al., 1990. Durant el nostre període estudiat, aquesta nova espècie es va observar anecdòticament.

2. Contribució a la taxonomia de dinoflagel·lades

Es calcula que hi ha moltes espècies de dinoflagel·lades sense descriure i d'altres es troben mal descrites donades les limitacions metodològiques de l'època de la descripció original. En els darrers anys amb l'arribada dels mètodes moleculars una gran quantitat de gèneres i espècies de dinoflagel·lades s'han descrit o redescrit. En aquesta tesi es descriu un nou gènere (capítol 4) amb una sola espècie *Barrufeta bravensis* i es proposa que

una altre organisme descrit com *Gyrodinium resplendens* podria pertànyer a aquest nou gènere. Per alguns taxònoms la descripció d'un gènere amb una sola espècies pot resultar arriscada. La recent redescripció i nova combinació de *Barrufeta resplendens* per Gu et al. (2015) reafirma però la descripció del gènere *Barrufeta*.

3. La dinàmica poblacional i els factors que la governen

L'estudi de la dinàmica poblacional d'espècies fitoplanctòniques i dels factors que la regulen ha estat limitada per les curtes series temporals de què es disposaven. A més, en el cas d'espècies de difícil identificació com les del gènere *Alexandrium* la sèrie acostuma a quedar identificada a nivell de gènere. El que no permet estudiar realment la dinàmica temporal d'una espècie que pot ser molt diferent a la d'una altra espècies del mateix gènere (ex. Lundholm et al., 2010).

Ara es comencen a tenir series multianuals de més de 10 anys fruits de plans de vigilància de fitoplàncton que ens permeten estudiar més adequadament els patrons espai-temporals d'aquestes espècies. La manera en que les espècies es comporten en diferents hàbitats costers és important pel que fa a la gestió de la zona costera, sobretot en gèneres amb especies tòxiques i nocives.

En aquesta tesi s'ha fet un gran esforç per identificar acuradament a nivell d'espècie, i es descriuen els seus patrons a diferents escales tant espacials com temporals en diferents ambients costers. Els nostres resultats mostren com de diferents poden ser els patrons de les diverses espècies d'un mateix gènere, i com ells poden també ser diferents en dos ambients costaners diferents. Cal tenir en compte però, que els patrons observats poden estar esbiaixats per la diferent freqüència de mostreig en els dos ambients i pel fet que els patrons s'han extret de mostres superficials. Com s'ha vist en el cas de la proliferació d' *A. pseudogonyaulax* a la Badia dels Alfacs (Capítol 1, Fig. 8) les abundàncies d'aquestes espècies poden variar molt verticalment depenent de l'estructura de la columna d'aigua.

En aquesta memòria s'estudia més en detall la dinàmica temporal i espacial de tres espècies d' *Alexandrium* problemàtiques, dues potencialment productores de PSP (*A. minutum* i *A. pacificum*) i l' altre causant de proliferacions d'alta

biomassa a platges (*A. taylori*). Les tres espècies s'han mostrat molt diferents en quant la seva dinàmica poblacional tant pel que fa a la seva tendència general en les diferents localitats estudiades com pel seu cicle estacional.

Els patrons són determinats per diferents factors a diferents nivells espai-temporal, per exemple es creu que l'escalfament global i altres factors antropogènics poden canviar la freqüència, la intensitat i la extensió geogràfica de les PANs al llarg del temps.

En els tres primers capítols d'aquesta memòria s'analitza els nutrients inorgànics dissolts, i determinats factors climàtics (temperatura, vent) per tal de veure si determinen l'estacionalitat de les proliferacions d' algunes espècies objectiu, les localitats on es donen aquestes proliferacions i la tendència d'aquestes espècies. Les dades s'analitzen en un marc espacial local i regional i en un marc temporal estacional i interanual (13 anys).

A escala global, el fitoplàncton respon a l'eutrofització significativa i estadísticament positiva (Smith, 2006) i, en alguns casos a escala local (ex. Verity and Borkman, 2010). Dins de l'àmbit de les PANs, l'eutrofització ha estat relacionada amb el desenvolupament, persistència i l'expansió d'aquest fenomen (Heisler et al., 2008). Les nostres dades però, a nivell d'espècie d'*Alexandrium* i a nivell de clorofil·la no mostren aquesta tendència interanual a escala local o regional dins el NO de la Mediterrània. Li et al., 2010 va demostrar que en ecosistemes costaners a nivell local la relació entre els nutrients i el fitoplàncton no sempre és evident a les diferents escales de temps. Els resultats d'aquesta tesi tampoc mostren que la dinàmica estacional d'aquestes espècies segueixin la dinàmica dels nutrients ni que les localitats on aquestes espècies produeixen proliferacions siguin les que tenen unes majors concentracions de nutrients.

Si bé algunes de les sèries temporals analitzades a la tesi mostraven una tendència significativa a augmentar la temperatura durant el període estudiat, aquest augment no es correspon en la majoria de casos a un augment de l'espècie estudiada, ni tampoc de la clorofil·la. La temperatura doncs tampoc sembla determinar la tendència de les espècies d'*Alexandrium* ni de la clorofil·la dintre de l'àmbit espai-temporal estudiat, i es relaciona directament amb les variacions anuals només en el cas d'*A. taylori*.

Expansió espai-temporal

En les darreres dècades del segle passat ja es va observar que les PANs incrementaven a nivell global (Hallegraeff, 1993). Aquest augment dels registres de proliferacions de PSP observat a nivell mundial però podria ser explicat principalment per dos factors. Per un increment en els llocs observats i en el nombre d'observacions o per un increment real de les proliferacions. Una de les preguntes que es va voler respondre en aquesta tesi és si les proliferacions d'espècies productores de PSP augmenten realment a la costa catalana. Els resultats obtinguts permeten concloure que les proliferacions d'organimes productors de PSP han augmentat a la costa catalana en concret en les zones confinades i degut a *A. minutum*. Curiosament però, les proliferacions d'aquesta espècie han estat anecdòtiques a les platges on no s'ha notat cap augment. L'augment real de les proliferacions es pot deure a l'increment de factors afavoridors de les proliferacions que inclouria causes relacionades amb mecanismes naturals o causes que serien produïdes per l'activitat humana (Anderson, 1989; Hallegraeff, 1993; Hallegraeff, 2010) com l'eutrofització cultural o el canvi climàtic ja comentat anteriorment, o la modificació de la línia de costa.

La diferència observada en la dinàmica de l'espècie entre dintre i fora dels ports en la mateixa costa ens evidencia que en el cas de la costa catalana el factor clau per a que es produeixi un augment de proliferacions PSP són els ports. Una de les diferències més notables entre les aigües dels ports i les aigües de les platges és la concentració de nutrients. A la tesi s'ha observat que *A. minutum* pot proliferar recurrentment en ports amb concentracions de nutrients molt diferents (Capítol 1). Tot i així, veient les diferències de la concentració de DIN entre els ports i les platges (veure **Taula S1, capítol 1** i **Taula S2, capítol 3**), hom pot tendir a pensar que són les diferències d'aquests nutrients o del seus coeficients (ex: DIN/P) el que pot portar a *A. minutum* a fer proliferacions als ports i no a les platges. Les dades obtingudes però, no mostren cap relació entre l'abundància màxima d'*A. minutum* i les concentracions de DIN promig o de nitrat o els coeficients DIN/P i NO3/NH4 en cada port i platja (**Fig. 1**). L'efecte del confinament de les aigües portuàries d'acord amb el Mandala d'en Margalef (Margalef, 1978), i la facilitat de l'establiment de llits de cistos en ports sembla una explicació més probable per explicar la diferent dinàmica d'aquesta espècie en els dos ambients. L'augment de proliferacions de dinoflagel·lades

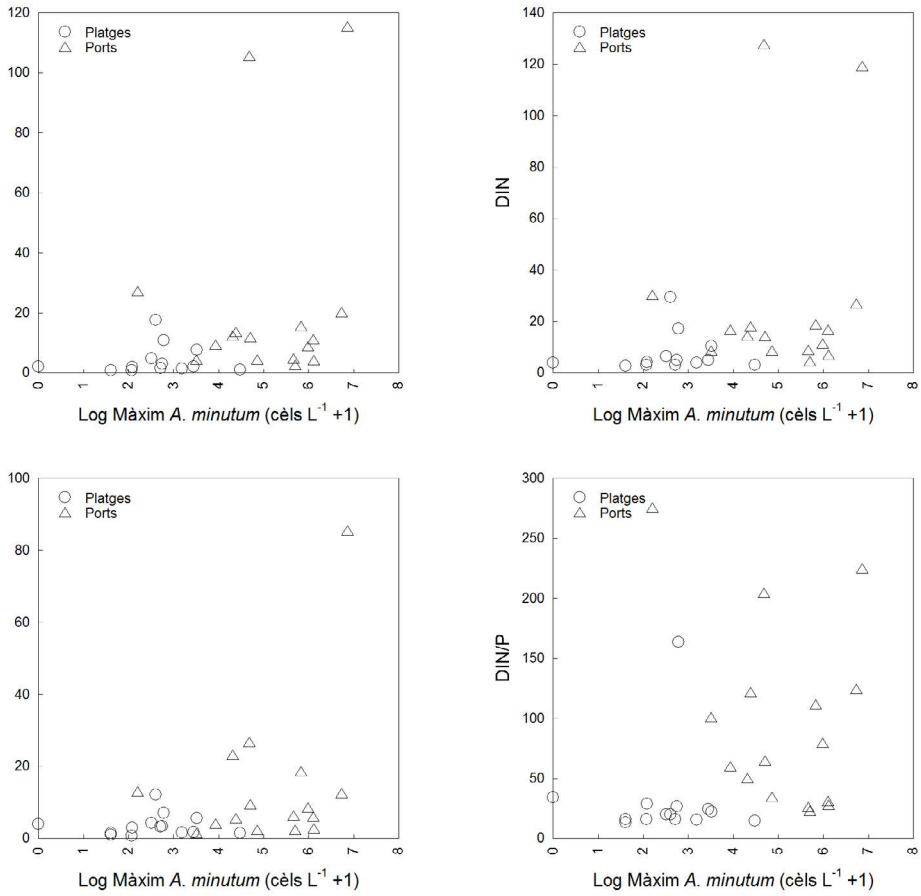

Figura 1. Abundàncies màximes d'*A. minutum* detectada a cada ports i platja mostrejada regularment respecte a les concentracions de Nitrat (µM), DIN (µM), Nitrat/Amoni i DIN/P promig de cada estació de mostreig.

per la construcció de ports al Mediterrani ja havia estat proposat per Vila et al., 2001a. Les nostres dades confirmarien l'augment de proliferacions de PSP a la costa catalana per aquesta causa.

Tot i l'augment de les proliferacions de PSP degut a l'augment d' *A. minutum*, la dinàmica d' *A. pacificum* va ser completament diferent. Aquesta espècie es va començar a detectar als 90 en diverses localitats del NO de la Mediterrània (Abadie et al., 1999; Gomis et al., 1996; Luglie et al., 2003; Vila et al., 2001). A la costa catalana aquesta espècie es va detectar entre el 1996 i el 1999 en un nombre d'estacions cada cop major el que va suggerir una ràpida expansió

d'aquesta espècie (Vila et al., 2001b) per aquesta regió. Amb dades aportades en aquesta tesi però, s'ha vist un clar retrocés de l'espècie en molts ports i en platges i només ha quedat formant proliferacions recurrents en llocs concrets. Observant com s'havia donat aquest retrocés i la diferencia interanual de les abundàncies d' *A. pacificum* al port de Tarragona ens va sorgir la hipòtesis de que les condicions climatològiques inusuals que es van donar al 2004 – 2005 podrien haver afectat la dinàmica d'aquest organisme negativament. Aquesta hipòtesi podria ser compatible també amb el que va ocorre en altres llocs de la Mediterrània com a dues estacions de Sardenya (Itàlia) situades al Golf de Olbia (**Fig. 2**) on *A. pacificum* havia estat detectada per primera vegada al 1999 i on també aquest organisme va acabar desapareixent abans del 2008 i després d' una forta minva de la seva població al 2004-2005.

Les observacions a la llacuna de Thau presentades en aquesta tesi però posa en dubte la hipòtesis. En aquesta badia, que també va ser afectada per les inusuals condicions climàtiques del 2004-2005, les abundàncies d'aqueta espècie no van desaparèixer ni disminuir durant aquests anys. Tot i que sí que es va observar un canvi en el patró anual d'*A. pacificum* en aquest anys.

Mentre que amb totes les dades que tenim la hipòtesi de la regressió d' *A. pacificum* a causa d'unes condicions climatològiques inusuals al 2004-2005 no és concloent, un estudi només centrat en la costa catalana podria haver-ho conclòs. Més enllà de saber la causa de la minva d'aquesta espècie en el NO de la Mediterrània això ens mostra la necessitat de tenir una visió ample del que succeeix en molts llocs per arribar a conclusions definitives.

Alternativament, el patró de desaparició d'*A. pacificum* podria ser explicat per altres causes, com que formés part d'un cicle intern de l'espècie o que

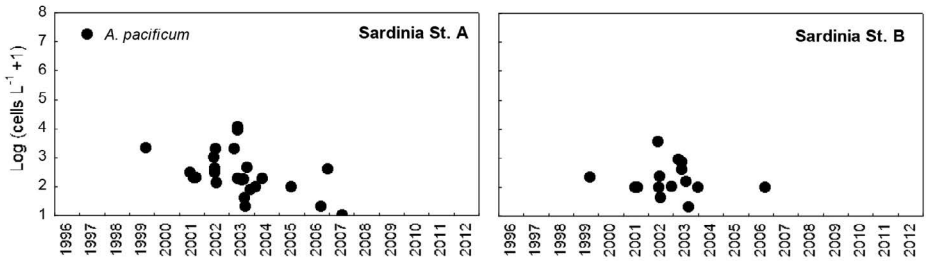

Figura 2. Abundàncies d'*Alexadrium pacificum* en dos punts del Golf de Olbia (Sardenya). Extret de Sampedro et al., 2016.

es tractés d'una introducció o colonització fallida, degut per exemple que no es complissin els requisits del medi físic o a causa de factors biològics com la depredació o la competència. Això hauria acabat fent recular l'espècie a la majoria de llocs excepte en alguns, com el port de Tarragona i la llacuna de Thau, on les condicions que si donen haurien estat les apropiades perquè la introducció o colonització tingués èxit i s'hauria quedat fent proliferacions recurrents. En aquesta tesi hem estudiat aquests llocs per tal d'intentar trobar les condicions comuns que ens expliquessin els factors que li són favorables a *A. paficium*. Les variables estudiades han resultat per això ser molt diferents en les dues localitats i no ens expliquen la dinàmica d'aquesta espècie.

Pel que fa a *A. taylori* aquesta espècie tot i la seva expansió en una àrea nova de la Costa Brava, continua estant bastant ben delimitada en aquesta costa. Aquesta espècie a diferència d' *A. minutum* fa blooms a platges i no n'acostuma a fer a ports amb l'excepció del port de l'Estartit, on aquesta espècie fa blooms recurrents. Es podria pensar que la distribució singular d'una espècie en un àrea específica de la costa s'ha de regir per una condició ambiental particular exclusiva d'aquest àmbit. No obstant això, les evidències en la literatura són rares, i altres factors, inclosos els que són aleatoris, solen intervenir. Per exemple, el fet que *A. taylori* es trobés pràcticament només al port d'Estartit podria no ser només degut a una condició mediambiental exclusiva d'aquest port, sinó també al fet que aquesta espècie floreix cada estiu en abundàncies molt elevades a la platja situada just al sud de la bocana del port. L'aigua del mar que conté elevades densitats d'*A. taylori* es podria introduir a l'interior del port per la bocana. En suport d'aquesta hipòtesi, les fotografies aèries

Figura 3. Fotografies aèries de la platja i el port de l'Estartit on es pot observar la discoloració de l'aigua deguda a la presència d'*A. taylori* i gymnodinioids.

preses d'aquesta zona mostren aigües discolorides que arriben al port (**Fig. 3**). D'altra banda, no es pot descartar l'inici de la proliferació a l'interior del port, ja que és un dels pocs ports que inclouen una platja a l'interior.

Estacionalitat

Els cicles anuals de les tres espècies han resultat ser molt diferents, *d'Alexandrium minutum* i *A. pacificum* varien depenent de la zona confinada concreta, el que ens remarca el paper dels factors locals en aquestes espècies i dins d'aquest habitat. En canvi *A. taylori* presenta un patró anual sincrònic a les diferents platges, així com també en el port de l'Estartit (**Fig. 4**), que està determinat per la temperatura.

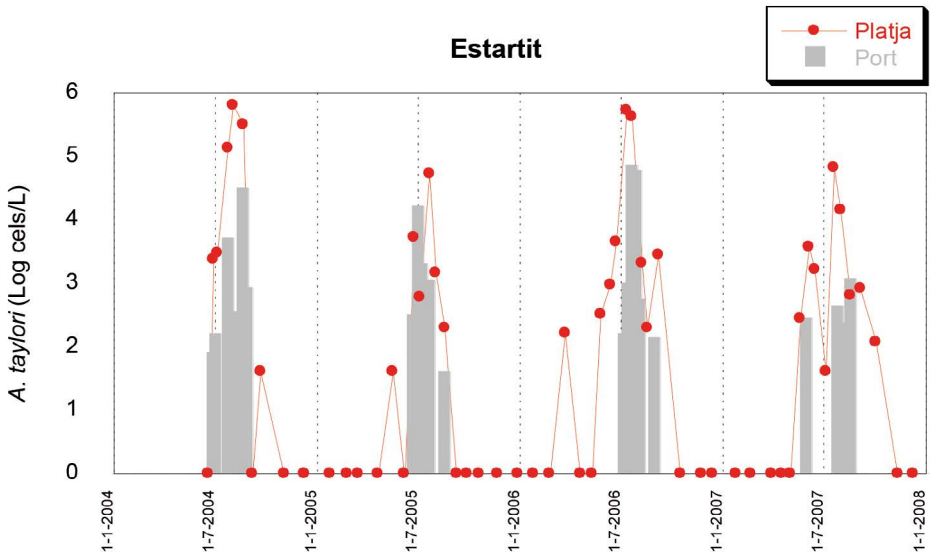

Figura 4. Abundàncies d'*A. taylori* durant 4 anys en el port de l'Estartit i la platja de l'Estartit.

És destacable que tot i la recurrència anual des de fa anys d'*A. minutum* al port d'Arenys i *A. taylori* a la platja de la Fosca, les tendències d'aquests organismes en aquestes localitats és d'augmentar en el primer cas mentre que en el segon cas no hi ha cap tendència. Això podria ser degut a que la finestra de condicions òptimes durant l'any és més reduïda en el cas del *A. taylori* que ve marcada pel mesos amb elevades temperatures, mentre que *A. minutum* que s'ha vist que pot proliferar a totes les estacions de l'any excepte

a la tardor aquesta finestra és més ample i permet aquesta espècie pugui anar expandint-se dins aquest període de temps, i gràcies al llit de cistos del port que el proveeix d'inòcul.

4. Avaluació de riscos associats

La informació recollida en aquesta tesi ens serveix entre d'altres coses per avaluar els possibles riscos associats a les dinoflagel·lades potencialment tòxiques a la costa catalana. Els anàlisi de toxines ens han permés per exemple descartar problemàtiques de PSP associades a *A. andersonii* i *Barrufeta bravensis*. Tot i això, les anàlisi de les tendències de les proliferacions de PSP ens han indicat un augment d'aquestes proliferacions a les àrees confinades. Aquest fet no afectaria directament la salut pública donat que en els ports no està permesa la recol·lecció de mol·luscs bivalves. Indirectament però, aquest organismes poden sortir cap a les platges i afectar a les diferents zones de producció de marisc amb bancs naturals repartides al llarg de la costa. Les observacions de les mostres de platges ens mostren en canvi elevades abundàncies d'*A. minutum* només en comptades ocasions, si bé és cert, que aquestes poques observacions coincideixen en platges situades al sud de ports on s'acostumen a donar proliferacions d' *A. minutum* de manera recurrent. El risc més important de PSP es troba doncs en les àrees situades al sud dels ports on s'han trobat proliferacions recurrents de l'espècie *A. minutum*.

5. Observacions finals

Aquesta tesi mostra la utilitat de les sèries temporals generades pels plans de vigilància del fitoplàncton tòxic. Mentre que molt cops les limitacions dels projectes científics no permeten observar en un espai i temps amplis, els plans de vigilància de fitoplàncton permeten obtenir dades en escales espacials i temporals majors. Així disposem d' informació més general en comptes dels freqüents estudis de fets aïllats que poden portar a conclusions parcials o errònies. La tesi és una foto d' un temps i espai mitjos, però tot i així és una visió parcial, mai un treball acabat donat els continus canvis que es donen en la dinàmica poblacional. Tot i així, revisitar les dades amb certa perspectiva en un espai-temps més ampli ens permet veure coses no vistes o descartar d'altres que ens semblava haver vist.

Conclusions

1. Les dinoflagel·lades que produeixen més proliferacions a les platges de la costa catalana serien *A. taylori,* diferents espècies de Gymnodinials i *Ostreopsis* spp, essent les platges de la Costa Brava les més afectades per proliferacions de dinoflagel·lades. En quant a l'estacionalitat la gran majoria de proliferacions es dona a les platges entre els mesos d'Abril i Setembre.

2. La diversitat del gènere *Alexandrium* a la Costa Catalana és gran; s'han trobat 11 espècies diferents, el que representa un terç de les espècies existents d'aquest gènere. La diversitat trobada ha estat semblant en zones confinades i en platges però la majoria de les espècies es troben en major freqüència i en majors abundàncies a les aigües confinades on arriben freqüentment a formar proliferacions.

3. Els tipus de patrons espai-temporals del gènere *Alexandrium* poden ser diferents depenent de l'espècie però també de l'hàbitat coster, tot i que la seva presència es trobi en ambdós hàbitats.

4. Les proliferacions de PSP van incrementar a les zones confinades de la costa catalana durant el període estudiat, tot i que les dues espècies d'*Alexandrium* responsables d'aquestes proliferacions van presentar evolucions molt diferents. Mentre que *A. minutum* durant els 13 anys estudiats ha doblat el nombre de proliferacions a ports, el nombre de proliferacions d'*A. pacificum* han disminuït o almenys s'ha mantingut en aquests hàbitats.

5. En contrast amb la dinàmica observada en àrees confinades, *A. minutum* no ha mostrat cap augment de proliferacions en platges, fet que senyala la construcció de ports com el principal factor determinant per a l'increment de proliferacions de PSP a la costa catalana.

6. El seguiment d' *A. pacificum,* tant en zones confinades com obertes, durant els anys d'estudi (2000-2012) ens indica que aquesta espècie, que havia estat en expansió a la costa catalana entre el 1996 i el 1999, ha anat retrocedint tant en ports com en platges i només es continua

detectant al port de Tarragona on al final d'aquest estudi continuava formant proliferacions recurrents.

7. Durant el període d'estudi *A. taylori* s'ha expandit dins la Costa Brava formant blooms recurrents en una nova area (Desemb. Muga). Tot i que aquesta espècie continua estant pràcticament delimitada a la Costa Brava algunes platges del Delta de l'Ebre són vulnerables a proliferacions recurrents.

8. Els cicles anuals *d'Alexandrium minutum* i *A. pacificum* varien depenent de la zona confinada, el que ens remarca el paper dels factors locals en aquestes espècies i dins d'aquest habitat. En canvi, *A. taylori* presenta un patró anual sincrònic a les diferents platges, que sembla determinat per factors estacionals o regionals de més gran escala com la temperatura.

9. L'espècie que forma proliferacions recurrents a la llacuna de Thau és *A. pacificum*, mentre que *A. tamarense*, la primera citada, s'ha trobat en poques ocasions com a l'espècie dominant.

10. La dinàmica estacional d' *A. pacificum* no sembla modulada per la temperatura, la velocitat del vent o la concentració de nutrients inorgànics.

11. L'espècie no descrita que acompanya *Alexandrium taylori* a les platges de la Costa Brava, produint també proliferacions d'elevada densitat, cel·lular ha estat identificada com un gènere nou de dinoflagel·lada i s'ha descrit amb el nom de *Barrufeta bravensis*. Aquesta espècie es situa filogenèticament a prop dels gèneres *Lepidodinium* i *Gymnodinium*, té peridinina com a principal pigment accessori, és mixotròfica i no produeix toxines PSP.

12. En contrast a altres treballs sobre la toxicitat d' *A. andersonii* del NO del Mediterrani, els anàlisi realitzats indiquen que aquesta espècie no produeix toxines PSP. Malgrat això, els resultats amb mètodes no oficials indiquen la presència d'una petita quantitat de toxina inhibidora dels canals de sodi depenents de voltatge.

13. *A. andersonii* no presenta diferències significatives en la seva taxa de creixement a les diferents temperatures testades en aquesta tesi, però obté abundàncies màximes més elevades a 20ºC que a 14ºC.

14. Les soques de diferents orígens d'*A. andersoni* estudiades no presenten variabilitat intraspecífica en la genètica (regions ITS i LSU) ni en les taxes de creixement però sí en la seva mida, la relació de pigments i el seu patró de creixement.

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Annex
Publicacions

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BARRUFETA BRAVENSIS GEN. NOV. SP. NOV. (DINOPHYCEAE): A NEW BLOOM-FORMING SPECIES FROM THE NORTHWEST MEDITERRANEAN SEA[1]

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The present study describes a new dinoflagellate genus, *Barrufeta* N. Sampedro et S. Fraga gen. nov., with one new species, *B. bravensis* Sampedro et S. Fraga sp. nov., isolated from the Costa Brava (NW Mediterranean Sea). The dinoflagellate was characterized at the genus and species levels by LM and EM; LSU and internal transcribed spacer (ITS) rDNA sequences; and HPLC analyses of the pigments, fatty acids, and possible presence of toxins of several cultured strains. The new *Barrufeta* species is oval shaped (22–35 μm long and 16–25 μm wide) and dorsoventrally flattened. It possesses numerous small chloroplasts that radiate from two large equatorially located pyrenoids and is a typical peridinin-containing dinoflagellate. The nucleus is in the anterior part of the epicone. The apical groove has a characteristic "Smurf-cap" shape that runs counterclockwise on the epicone and terminates on its right posterior part. *B. bravensis* is similar to the previously described species *Gyrodinium resplendens* Hulburt in its external morphology, but the original report of the latter lacked a description of the complete shape of the apical groove. It is therefore likely that some of the *G. resplendens* species reported in the literature are *Barrufeta* since they possess a *Barrufeta*-type apical groove. Fatty acids of *Barrufeta* were more similar to those of *Karenia brevis* than those obtained from other unarmored analyzed species including three species of *Gymnodinium* and *Akashiwo sanguinea.*

***Key index words: Barrufeta bravensis;* dinoflagellates; *Gymnodinium; Gyrodinium;* molecular phylogeny; ultrastructure**

***Abbreviations:* av, amphiesmal vesicles; CCVIEO, Culture Collection of Microalgae of the Centro Oceanográfico de Vigo; ch, chloroplasts; cr, chromosomes; CTP, cytidine triphosphate; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast microscopy; g, golgi; GC, gas chromatography; ITS, internal transcribed spacer; l, lipid droplet; lf, longitudinal flagella; m, mucocyst-like vesicles; MgDVP, Mg-2,4-divinyl pheoporphyrin; ML, maximum likelihood; MP, maximum parsimony; MUFA, monounsaturated fatty acid; n, nucleus; NFC, nuclear fibrous connective; NJ, neighbor joining; nu, nucleolus; pu, pusule; PUFA, polyunsaturated fatty acid; py, pyrenoid; s, starch grain; SFA, saturated fatty acid; t, trichocysts; TTP, thymidine triphosphate; v, vacuoles**

Unarmored dinoflagellates have always been difficult to classify. Indeed, early species descriptions were based only on basic morphological characteristics

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visible by LM, such as cell shape and size; girdle displacement; number, position, and color of the chromatophores; position of the nucleus; and the nature of the cell surface (smooth or striated). One of the problems with these first descriptions was that in some cases, they referred to fixed samples or to organisms in poor condition and thus in neither case representative of living cells. Inaccurate descriptions can also result from the extreme sensitivity of the living organisms to confinement between the slide and the coverglass and from observation only of nonmoving cells. Furthermore, the taxonomic criteria previously used to distinguish between two genera of unarmored dinoflagellates were frequently not reliable. Since these criteria were drawn from continuous characteristics (e.g., the degree of cingular displacement; Kofoid and Swezy 1921), such as used to distinguish the genera *Gymnodinium* and *Gyrodinium*, the cutoff that determined one genus versus the other could not be precisely defined, with particular difficulty arising in many borderline cases.

The taxonomy of unarmored dinoflagellates progressed following the information obtained from ultrastructural studies (Dodge 1974, Steidinger et al. 1978) as well as from analyses of pigment composition (Jeffrey et al. 1975 and references therein, Bjørnland and Tangen 1979) and the morphology of the dinoflagellate apical groove (Takayama 1981, 1985, Takayama and Adachi 1984). More recently, the morphological and ultrastructural investigations have been combined with phylogenetic determinations (Saunders et al. 1997, Hansen et al. 2000a), which, in turn, provide a new focus to the classification of genera comprising the unarmored dinoflagellates. Daugbjerg et al. (2000) used this approach to redescribe the most important genera of unarmored dinoflagellates (*Gymnodinium* and *Gyrodinium*), in addition to describing three new genera and proposing a total of 17 new genus-species combinations. The features of the new genera were based on the phylogenetic analysis, specifically, of the LSU of rDNA, in combination with morphological evaluation of the apical groove, several ultrastructural details (e.g., the presence or absence of nuclear chambers in the nuclear envelope), and the identities of the major accessory pigments. Since the shape of the apical groove was determined to differ in each genus, it provided a reliable generic characteristic, one that was usually observable by SEM. This new classification of unarmored dinoflagellates likewise yielded new genus-species combinations in addition to a new genus, *Takayama* (de Salas et al. 2003, Murray et al. 2007). However, Daugbjerg et al. (2000) also made a few exceptions: for example, the apical groove of *Lepidodinium viride* M. Watanabe, S. Suda, I. Inouye, Sawaguchi et Chihara is similar to that of *Gymnodinium*, and the analysis of SSU rDNA sequences places this species within or close to *Gymnodinium* sensu stricto (Saunders et al. 1997); nonetheless, the *Lepidodinium* genus was retained based on the presence of both an outer layer of body scales and pigments of Chlorophyta origin (Watanabe et al. 1990). Hansen et al. (2007) compared *L. viride* with *Gymnodinium chlorophorum* Elbr. et Schnepf. Although the latter species lacks body scales, similarities between the two species at the ultrastructural, pigment, and genetic levels led the authors to propose the new combination *L. chlorophorum* (Elbr. et Schnepf) Gert Hansen, Botes et de Salas. The use of fatty acids as a chemotaxonomic tool in marine microalgae has been suggested by some authors. Fatty acids of unarmored dinoflagellates have also been studied in this way. Mooney et al. (2007), for example, examined eight species of marine gymnodinioids and proposed the ratio of 28:7n6/28:8n3 as a chemotaxonomic marker at the species level. There are, to the authors' knowledge, no studies on chemotaxonomic tools based on fatty acids to distinguish between unarmored genera.

Since 1982, blooms of *Alexandrium taylori* Balech causing intensely brown/green-colored waters have been observed along some of the beaches of the Costa Brava (NW Mediterranean Sea) in the summer months (from June to September), when the influx of tourists is the greatest (Garcés et al. 1999). Since 1995, routine monitoring of HABs at La Fosca beach has led to the additional detection of an unarmored dinoflagellate during the same period that the *A. taylori* bloom occurs, but the preservation of this gymnodinioid species with Lugol's solution prevented its identification. Therefore, to properly identify this species, several strains from the blooms that occurred in 2002 and 2005 were isolated. Our efforts led to the identification of *B. bravensis* gen. et sp. nov, described herein, which was based on LM and SEM examinations of its external morphology; phylogenetic analysis of its LSU and ITS rDNA; and analyses of its ultrastructure, pigments, and fatty acids composition.

MATERIALS AND METHODS

Strain isolation and cultures. Strains isolated from La Fosca beach, Costa Brava, during September 2002 and August 2005 were grown in L1 medium without silicate (Guillard and Hargraves 1993) and prepared with coastal seawater. The cultures were incubated at 20°C with a 12:12 light: dark (L:D) photoperiod and an irradiance of ~100 µmol photons $\cdot m^{-2} \cdot s^{-1}$. The salinity was adjusted to 32 (psu) for LM and analyses of pigment, fatty acid, and toxin content and 37.8 (psu) for SEM and TEM. The culture used for TEM analysis, belonging to the strain VGO864, was accompanied by a small, unknown photosynthetic oval, almost spherical, stramenopile measuring ~ 3 µm long and ~ 2.5 µm wide. The cultures were deposited at the Culture Collection of Microalgae of the Centro Oceanográfico de Vigo (CCVIEO; Vigo, Spain) and in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; West Boothbay Harbor, ME, USA). Other unarmored dinoflagellates [*Akashiwo sanguinea* (Hirasaka) Gert Hansen et Moestrup 2000 (VGO626), *Gymnodinium microreticulatum* Bolch (VGO328), *Gymnodinium catenatum* L. E. Graham (Est14H5), *Gymnodinium impudicum* (S. Fraga et I. Bravo) Gert Hansen et Moestrup (VGO665), and *Karenia*

brevis (C. C. Davis) Gert Hansen et Moestrup (CCMP2281)] were cultivated under the same conditions to allow comparison of their fatty acid contents with those of *B. bravensis* (VGO864).

LM. Live cells were examined and photographed under bright-field and epifluorescence (lamp 50 W) microscopy using a Leica DM IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted microscope connected to a ProgRes C10 (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) digital camera. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2 $\mu g \cdot mL^{-1}$. A stained nucleus was photographed through a UV filter, and immediately afterward, another image was taken with a blue filter in order to capture the chloroplast's autofluorescence in the same cell. The two images were overlapped using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA). Cellular dimensions and the degree of girdle displacement were determined in 50 cells using ProgRes capturePro v 2.1 software (JENOPTIK Optical Systems GmbH). Nomarski microphotographs were taken with a Canon EOS D60 (Canon Inc., Tokyo, Japan) digital camera connected to a Leica DMLA light microscope (Leica Microsystems GmbH).

SEM. Cells of *B. bravensis* were fixed for 15 min at room temperature with an adequate volume of osmium tetroxide 4% (dissolved in seawater) to reach a final concentration of 1%; pH was not adjusted. The cells fixed were then filtered through a 13 mm diameter Nucleopore (Pleasanton, CA, USA) polycarbonate filter with a pore size of 2 or 5 μm. The filtered cells were then washed in distilled water; dehydrated in 25, 50, 75, 95, and 100% ethanol, 10 min each; and critical-point-dried. The filters were mounted on stubs, sputter-coated with gold, and examined with a JEOL JSM-6500F scanning electron microscope (JEOL-USA Inc., Peabody, MA, USA). To compare the structure of the apical groove of *B. bravensis* with the same structure in a related species of *Gymnodinium*, two SEM preparations used 17 years ago for the description of the *G. impudicum* (see Fraga et al. 1995 for more information on the methodology of SEM) were reexamined using a variable-pressure scanning electron microscope Hitachi S-3500N (Hitachi High Technologies Corp., Japan).

TEM. B. bravensis VGO864 was fixed in an equal volume of a mixture of 4% glutaraldehyde and 0.4% osmium tetroxide (previously dissolved in distilled water to 4% concentration, and from 4% to 0.4% in culture medium) for 30 min at 4°C, pelleted by centrifugation, washed in culture medium, and finally covered in 2.5% warm agar. After the agar had cooled and solidified, it was peeled off the filter (Hernández 1992), divided into smaller pieces, and postfixed for 1 h in 0.8% FeCNK and 1% osmium tetroxide prepared in 0.1 M Na-cacodylate buffer (pH 7.4). After washing in buffer, the material was dehydrated in an acetone series (50, 70, 80, 2×90, 3×96, $3 \times 100\%$), embedded in Spurr's resin, and then sectioned on an Reichert Jung Ultracut E (Capovani Brothers Inc., Scotia, NY, USA) ultramicrotome using a diamond knife (Diatome, Hatfield, PA, USA). The sections were collected on a 200-mesh grid and placed on a Formvar film and then stained in 2% uranyl acetate and lead citrate following the method of Reynolds (1963). Sections were examined in a JEOL JEM-1010 electron microscope (JEOL-USA Inc.) operated at 80 kV. Micrographs were taken using a Gatan, BioScan model 792 (Gatan Inc., Pleasanton, CA, USA) digital camera.

Pigment analyses. Sample preparation: Prior to HPLC pigment analysis, the health and characteristic morphology of the cells were confirmed by LM. Three hours into the light cycle, cells from cultures in the exponential phase of growth were harvested, and a 14 mL aliquot of the culture was filtered onto Whatman GF/F filters (Whatman plc, Maidstone, Kent, UK) under reduced pressure. The filters were frozen immediately at −25°C and analyzed within 12 h.

Pigment extraction: The frozen filters were placed in Teflon-lined screw-capped tubes, and the pigments were subsequently extracted with 5 mL of 90% acetone, using a stainless steel spatula to grind the filter. The tubes were chilled in a beaker of ice, and the contents sonicated for 5 min in an ultrasonic bath. The extracts were then filtered through syringe filters (MFS HP020, 25 mm, 0.20 μm pore size, hydrophilic PTFE, Advantec, MFS Inc. Dublin, CA, USA) to remove cell and filter debris. An aliquot (0.5 mL) of acetone extract was mixed with 0.2 mL of water, followed by immediate injection of a 200 μL sample into the HPLC column. This procedure avoids distortion of the early eluting peaks (Zapata and Garrido 1991) and prevents the loss of nonpolar pigments prior to column injection.

HPLC analysis. Pigments were separated by HPLC in a system consisting of a Waters (Waters Corporation, Milford, MA, USA) Alliance 2695 separation module and a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface. The chromatographic system was controlled using Millenium 32 software (Waters). Pigments were separated according to the HPLC method of Zapata et al. (2000), with a reformulated mobile phase A and using a C_8 monomeric Waters Symmetry column (150×4.6 mm, 3.5 μm particle size, 10 nm pore size). Eluent A consisted of methanol:acetonitrile:0.025 M aqueous pyridine (50:25:25 v/v/v), and eluent B of methanol:acetonitrile:acetone (20:60:20 v/v/v). The elution gradient was as follows (time, %B): t_0, 0%; t_{22}, 40%; t_{28}, 95%; t_{37}, 95%; t_{40}, 0%. The flow rate was 1.0 $mL \cdot min^{-1}$, and the column temperature was 25°C. Solvents were HPLC grade (Romil-*SpS*™ Ltd., The Source Convent Waterbeach, Cambridge, UK); pyridine was reagent grade (Merck, Darmstadt, Germany).

Pigments were identified either by cochromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (see Zapata et al. 2000). The purity of the peaks was confirmed, and the spectral information then compared with a library of chl and carotenoid spectra of pigments extracted from standard phytoplankton cultures (Zapata et al. 2000) or supplied by DHI (DHI Laboratory Products, Hørsholm, Denmark). The molar extinction coefficients (ε; $l \cdot mol^{-1} \cdot cm^{-1}$) provided by Jeffrey (1997) were used for pigment quantification.

Toxin analyses. Samples of *B. bravensis* cultures were filtered onto 47 mm glass fiber filters (Whatman GF/C). One of the filters was transferred to a 15 mL centrifuge tube containing PBS (Emura et al. 2004) for protein extraction. The other filters were treated with 0.1 M HCl to extract dinoflagellate toxins. These acidic extracts were used for the mouse bioassay (MBA), chemical analyses, and the hemolytic assay; the PBS extract was used only for the hemolytic assay.

The presence or absence of hemolytic compounds in both the PBS and the acidic extracts was determined following the method published by Riobó et al. (2008) with slight modifications. This assay was carried out using sheep blood diluted in Alsever's solution, kindly provided by Cz Veterinaria, S.A. (Porriño, Pontevedra, Spain).

The presence or absence of paralytic shellfish poisoning (PSP) toxins in cultures was determined according to the Association of Official Analytical Chemist's (AOAC) MBA method (AOAC 1990) and the HPLC method, the latter including postcolumn oxidation and fluorometric detection (Franco and Fernandez 1993).

Fatty acid analyses. Fatty acid analyses were carried out on the strain of *B. bravensis* (VGO864) and other strains of unarmored dinoflagellates (*A. sanguinea* [VGO626], *G. microreticulatum* [VGO328], *G. catenatum* [Est14H5], *G. impudicum* [VGO665], and *K. brevis* [CCMP2281]). Duplicate or triplicate

(500 mL) exponentially growing cultures were filtered through Whatman GF/F (25 mm) precombusted glass-fiber filters, immediately frozen in liquid N_2, freeze-dried for 12 h, and stored at −20°C until analysis. Cellular lipids were extracted with 3:1 DCM:MeOH (dichloromethane:methanol), according to the method of Ruiz et al. (2004). The samples were redissolved in 0.5 mL of chloroform and eluted through a 500 mg aminopropyl minicolumn (Waters Sep-Pak® Cartridges) previously activated with 4 mL of *n*-hexane according to the method of Fuentes-Grünewald et al. (2009). The extracts were stored at −20°C until gas chromatography (GC) analysis in a Thermo Finnigan Trace GC ultra instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a flame ionization detector and splitless injector and fitted with a DB-5 Agilent column (30 m length, 0.25 mm internal diameter, and 0.25 μm phase thickness) (Agilent Technologies Inc., Santa Clara, CA, USA). Helium was used as the carrier gas at a flow rate of 33 $cm \cdot s^{-1}$. The oven temperature was programmed to increase from 50°C to 320°C at 10°C · min^{-1}. Injector and detector temperatures were 300°C and 320°C, respectively. Fatty acid methyl esters (FAME) were identified by comparing their retention times to those of standard fatty acids (37 FAME compounds, Supelco® Mix C4-C24; Sigma-Aldrich, St. Louis, MO. USA). Fatty acids were quantified by integrating the areas under the peaks in the GC traces (Chromquest 4.1 software; Thermo Fisher Scientific Inc.), with calibrations derived from internal standards (2-octyldodecanoic acid and 5β-cholanic acid).

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses. Cultures of *B. bravensis* were collected during the exponential growth phase by filtration on 3 μm pore-size Isopore membrane filters (Millipore, Billerica, MA, USA). DNA was extracted and purified as described in Penna et al. (2005). Nuclear-encoded 5.8S rDNA and ITS regions were PCR-amplified as described in Penna et al. (2008). The nuclear-encoded LSU (D1/D2 regions) was amplified by using the primers D1R and D2C (Scholin and Anderson 1994) and the amplification protocol of Penna et al. (2008). Genomic DNA (1 ng) was amplified in a 50 μL reaction mix containing 50 μM each of dATP, dTTP, dCTP, and dGTP; 0.4 μM of each primer; 4 mM $MgCl_2$; 1 × reaction buffer (Diatheva, Fano, Italy); and 1.0 U Hot Rescue DNA Polymerase (Diatheva). Thermocycling was as follows: 10 min initial denaturation at 95°C; 35 cycles of 1 min at 95°C, 1 min at 50°C, and 2.5 min at 72°C; and a final elongation step of 7 min at 72°C. Three PCR-amplified products corresponding to the D1/D2 regions of the LSU gene and the 5.8S rDNA and ITS regions were pooled, purified, and then directly sequenced using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer Corp., Applied Biosystems, Foster City, CA, USA) and the dye terminator method provided in the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit, Perkin Elmer Corp.). Sequences obtained from this study were aligned with those from GenBank using the CLUSTAL X2 program (Larkin et al. 2007) with default settings. Alignments were rechecked visually and edited manually; nonalignable regions were excluded prior to the phylogenetic analyses. The strains used in the molecular analyses are listed in Tables S1 and S2 (see the supplementary material), together with the GenBank accession numbers of their LSU and 5.8S-ITS rDNA sequences. Phylogenetic relationships, based on the D1/D2 LSU and 5.8S-ITS rDNA data, were inferred using the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. Sequences of *Alexandrium affine* (H. Inoue et Y. Fukuyo) Balech (AY294612) and *Oxyrrhis marina* Dujard. CCMP604 (AY566415) were used as outgroups in the LSU and ITS-5.8S rDNA phylogeny, respectively. The best-fit model of nucleotide substitution for the phylogenetic analyses was the Akaike information criterion implemented in Modeltest 3.06 (Posada and Crandall 1998). For the LSU rDNA, the TVM+G model with gamma distribution for among-site variation was selected, with the alpha value of the gamma distribution equal to 0.497. For the 5.8S rDNA-ITS, the TrN+I+G model with gamma distribution for among-site variation was adopted, with alpha value of the gamma distribution equal to 0.924. This evolutionary model was used in the NJ distance matrix. MP analysis was performed using heuristic searches with tree-bisection-reconnection branch swapping. Branches were collapsed if their minimum length was 0; ambiguities and gaps were considered as missing data. The robustness of the NJ and MP trees was determined by bootstrapping with 1,000 pseudoreplicates. Phylogenetic analyses were carried out using the software packages PAUP* ver. 4.0b10 (Swofford 2002). ML analyses were run with RaxML (randomized axelerated maximum likelihood) software ver. 7.0.4 (Stamatakis et al. 2005), which adopts a general time reversible (GTR) substitution model and allows estimation of several parameters, such as the proportion of invariant sites and the alpha values of the gamma distribution for among-site rate variation. The LSU and 5.8-ITS rDNA sequences were subjected to ML analyses. Bootstrap values were calculated with 1,000 pseudoreplicates. Standard molecular indices were determined with Arlequin v. 3.0 (Excoffier et al. 2005).

RESULTS

Barrufeta N. Sampedro et S. Fraga **gen. nov.**

Dinoflagellati nudi cum peridinina ut principali pigmento lucis captore. Sulcus apicalis incipit in anteriore parte intersectionis surci et cinguli, currit diagonali modo in partem anteriorem ad dexteram, postea redit, sensu contrario motus horologii, in gyrum apicalis partis cellulae perlongum nodum transversalem componendum et finit prope dexteram partem ejus originis.

Unarmored dinoflagellates occur with peridinin as the major light-harvesting accessory pigment. An apical groove that starts in the anterior part of the intersection between the sulcus and the cingulum, runs diagonally to the anterior right side of the cell, and then turns counterclockwise around the apical part of the cell in a very elongated transversal loop before ending near the right side of its origin.

Etymology: Named after the shape of the epicone, which is due to the shape of the apical groove. It is similar to the cap of a ''Smurf'' (originally a ''Schtroumpf''), a comic strip character invented by Peyo in 1958 and adapted later to television. In Catalan, *barrufet* means ''Smurf,'' with *barrufeta* as the feminine form.

Type species: Barrufeta bravensis N. Sampedro et S. Fraga sp. nov.

Barrufeta bravensis N. Sampedro et S. Fraga **sp. nov**.

Cellulae ovoideae dorsiventraliter complanatae, 22–35 μm longae 16–25 μm latae. Et epiconus et hypoconus eamdem longuitudinem habent. Epiconus orbiculatus et frequenter cum parva protuberantia. Sulcus apicalis incipit in anteriore parte intersectionis surci et cinguli, currit diagonali modo in partem anteriorem ad dexteram, postea redit, sensu contrario motus horologii, in gyrum apicalis

partis cellulae, perlongum nodum transversalem componendum et finit prope dexteram partem ejus originis. Hypoconus suaviter truncatus et leviter bilobulatus, extensionis sulci antapicem attingentis causa. Cingulum descendens 1.5–2 quantum habet latitudinis retractum. Permulti parvi cloroplasti pallidi obscuri, ex quibus multi exeunt e duobus magnis pyrenoidis sub cíngulo positis in utroque latere sulci. Peridinina ut principale pigmentum lucis captore. Nucleus plus minusve in centro epiconi locatus.

Cells oval in outline, dorsoventrally flattened, 22–35 μm long and 16–25 μm wide. Epicone and hypocone are similar in length. Epicone is rounded and frequently found showing a slight protuberance. An apical groove that starts in the upper part of the intersection between the sulcus and the cingulum, runs diagonally to the anterior right side, and then it turns counterclockwise around the apex, forming a wide transversal loop and ending near the right side of its origin. Hypocone is smoothly truncate and slightly bilobate due to the sulcus extension until the antapex. Cingulum is descending and displaced by 1.5–2 cingulum widths. Numerous small yellow-brownish chloroplasts, with many of them radiating from two large pyrenoids, are equatorially located with one on each side of the sulcus. Peridinin occurs as the major light-harvesting accessory pigment. Nucleus is more or less centered in the epicone of the cell.

Holotype: Figure 1 of culture VGO864, isolated from La Fosca beach, located on the Mediterranean coast of Catalonia, Spain. The culture was deposited in two culture collections (CCVIEO and CCMP3277). Sequences were deposited to GenBank under the accession numbers FN647670 (ITS) and FN647673 (LSU).

Isotype: Figure 2F.

Type locality: La Fosca beach, Catalonia, Spain (Fig. 3) (41°51′29″ N, 3°08′40″ E).

Etymology: Named after Costa Brava, the coast where this species produces blooms.

Distribution: NW Mediterranean Sea.

Habitat and ecology: Marine plankton. High cell densities ($>10^6$ cell $\cdot$ L^{-1}) of *B. bravensis* have been detected in coastal waters during the summer months (June–September) at water temperatures of 20°C–27°C and salinities of 36.5–38.4. This species has been found accompanying blooms of *A. taylori* at beaches located inside semi-enclosed bays. In laboratory cultures, *B. bravensis* produces mucus.

External cell morphology. The cells are slightly elongated and dorsoventrally flattened (Fig. 2C), 26.9 ± 3 μm (22–35.4 μm, $n = 50$) long and 20.1 ± 2.3 μm (16–25.3 μm, $n = 50$) wide. The length/width ratio ranges from 1.2 to 1.5 ($n = 50$). The epicone and hypocone are similar in length (Fig. 2, A, D, and E). The sulcus extends to the antapex sometimes giving the hypocone a slightly bilobate shape (Fig. 2, A, D, and E). The apex is generally rounded and frequently has a slight protuberance that is limited by the apical groove (Figs. 2, A and B; 4, B and E). The descending cingulum is deep and wide and displaced by 1.5–2 cingulum widths, which represent 16% to 26% of the body length (Fig. 4, A and B). The sulcus has a small intrusion in the epicone and runs deep and straight to the antapex.

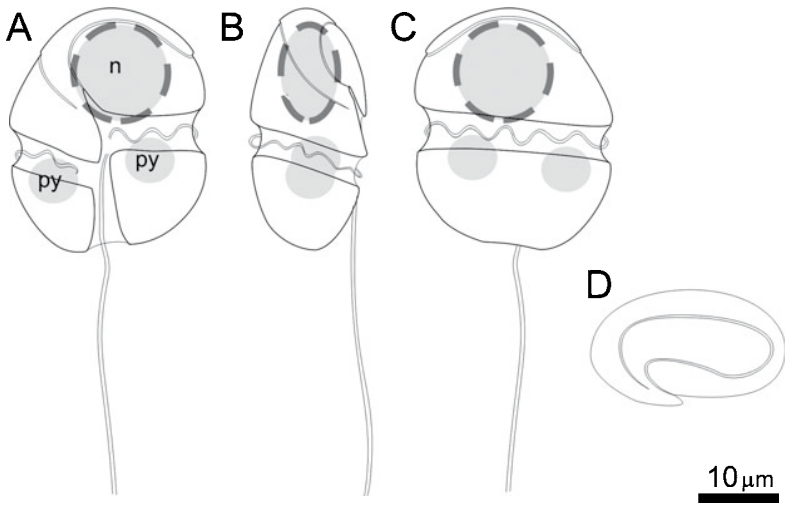

FIG. 1. Schematic representation of *Barrufeta bravensis* showing the position of the (n) nucleus and the (py) pyrenoids: (A) ventral, (B) lateral, (C) dorsal, and (D) apical views.

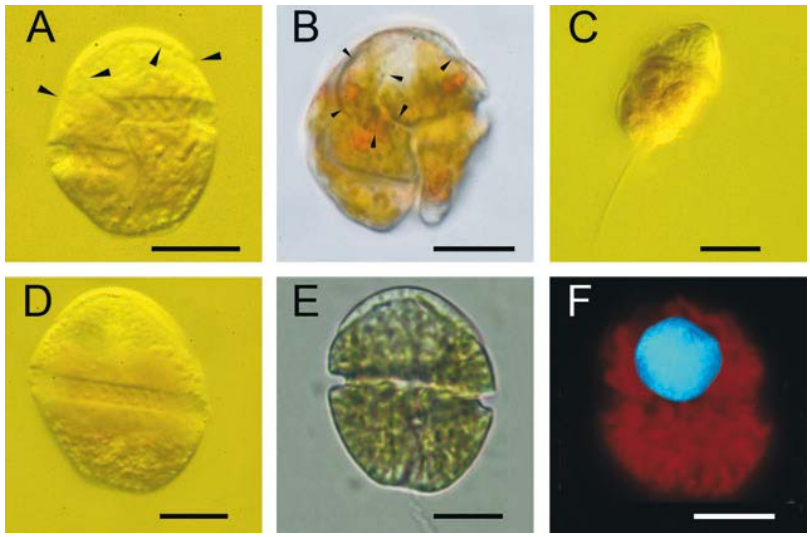

FIG. 2. Light micrographs of vegetative cells. (A–B) Ventral views showing the apical groove (arrowheads); (C) lateral view; (D–E) dorsal views; (F) epifluorescence image showing DAPI-stained nucleus and autofluorescent chloroplasts. Scale bars, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole.

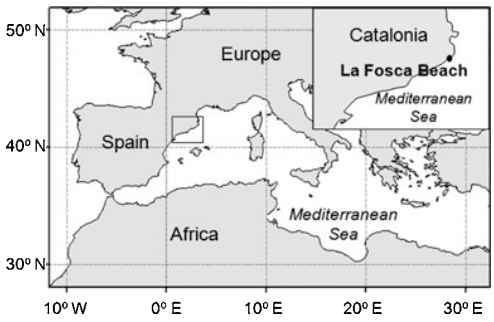

FIG. 3. Location of the sampling area, La Fosca beach.

The epicone has an apical groove, Smurf-cap shaped, running counterclockwise around the apex. The proximal part of the groove starts at the intrusion of the sulcus in the epicone and then ascends diagonally for a short distance toward the right anterior side of the cell. The groove then turns toward the left and crosses a large part of the epicone across the ventral part of the cell, surrounds the apex of the cell, and returns parallel to the cingulum across the dorsal side of the epicone. Lastly, it turns toward the ventral part, finishing near its origin, without reaching to the sulcus of the cell (Figs. 1, A–D; 2, A, B and D; 4, A–E). The apical groove consists of three elongated vesicles, the middle one ornamented with small knobs (Fig. 5A). The cell surface is covered with polygonal amphiesmal vesicles (usually quadrangular or pentagonal) that do not show a regular pattern, whereas the border of the girdle presents an array of well-aligned rectangular vesicles (Fig. 4F). The longitudinal flagellum varies from 1 to 1.5 times the body length.

For the purpose of comparison, the structure of the apical groove of *G. impudicum* is shown in Figure 5, B and C. Its apical groove consists of three elongated vesicles; the lateral ones are ornamented with a line of small knobs. This morphological detail was not included in the original description of this species.

Resting cyst morphology. Cysts of *B. bravensis* were observed in cultures. The resting cysts are flattened and range in shape from circular (21.8–29.6 μm diameter; $n = 10$) to oval (25.4–34.9 μm length, 22.3–30 μm width; $n = 12$) and occasionally irregular, as seen in the frontal view (Fig. 6, A and B). The cyst is double-walled, smooth, and thick, with white-grayish granular contents and one or more orange spots. The cysts are covered by a transparent substance that takes irregular forms. These mucoid cysts are usually strongly stuck to the bottom of the wells in culture conditions.

Cell ultrastructure. The almost spherical nucleus (~10.5 μm in diameter) is situated in the epicone and, as seen in lateral and ventral view of the cell, centered (Figs. 2F; 7, A and B). It is a typical dinokaryon with permanently condensed chromosomes

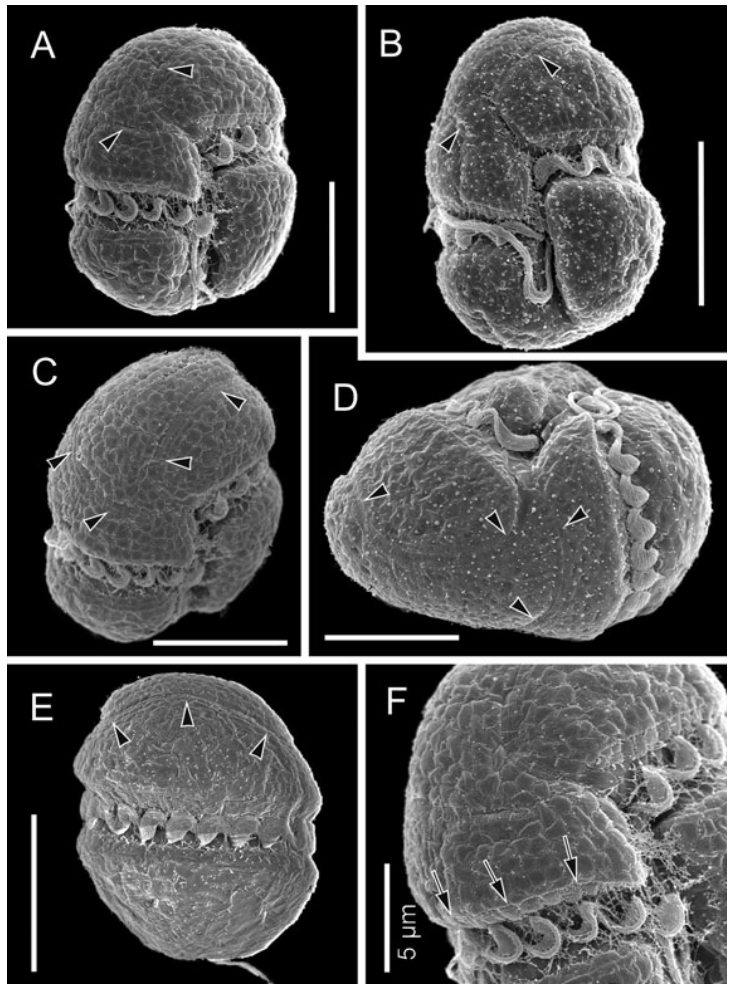

FIG. 4. Scanning electron micrographs of strain of *Barrufeta bravensis*. Arrowheads point to the apical groove. (A) Ventral view of a cell and (B) of a cell with a slight protuberance. (C) Epicone and (D) epicone showing the nearly complete apical groove (arrowheads). (E) Dorsal view of a cell. (F) The detail of the cell surface shows the polygonal amphiesmal vesicles. Arrows point to the border of the cingulum, made up of a line of rectangular vesicles Scale bars, 10 µm (unless otherwise indicated).

and with the presence of a nucleolus (Fig. 8G). Golgi apparatus was observed adjacent to the nucleus (Fig. 8I). A structure resembling vesicular chambers of the nuclear envelope was observed in some cells (Fig. 8H), although nuclear pores were not observed in it. Two large and almost spherical pyrenoids are equatorially located, one on each side of the sulcus (Fig. 7, A–D). Chloroplasts are numerous, small, elongated, and radially arranged (Figs. 2F, 7D, and 8A) giving the cell a yellow-brownish appearance. Many of the chloroplasts radiate from the pyrenoids to the cell periphery (Fig. 7, A, C, and D). Thylakoids are stacked generally in fours to form lamellae (Fig. 8B). Many lengthened vacuoles with electron-transparent contents are radially distributed and inserted in between the chloroplasts; they occupy a large part of the distal area of the cell (Fig. 7D). The vermiform vesicles situated beneath the amphiesma have a fibrillar content that is densely packed in the middle; these vacuoles are probably mucocysts (Figs. 7A and 8E). Another kind of mucocyst, wider than the first type and oval in shape, was observed under amphiesma (Fig. 8F). Trichocysts are abundant and distributed throughout the cell. These extrusomes are composed of an apical cap, a neck, and a dense body that is quadrangular shaped in cross-section (Fig. 8C). Both the neck and the body possess an external striated cover (Fig. 8E). In addition, the cells contain lipid droplets and starch grains scattered throughout the

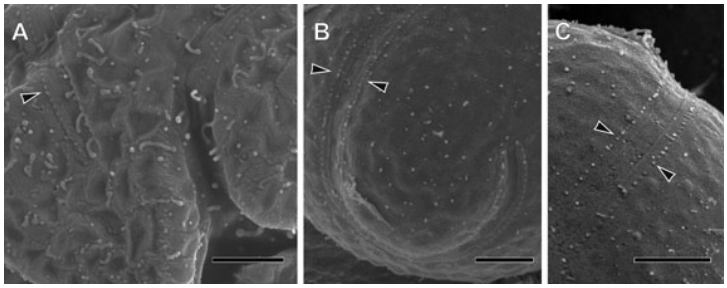

FIG. 5. Scanning electron micrographs show (A) a detailed view of the apical furrow in *Barrufeta bravensis*, including three elongated vesicles. The middle one is ornamented with small knobs (arrowhead). (B, C) Details of the apical furrow in *Gymnodinium impudicum* strain GY1VA (used for the species description). The furrow consists of three elongated vesicles; the lateral ones are ornamented with a line of small knobs (arrowheads). Scale bars, 2 μm.

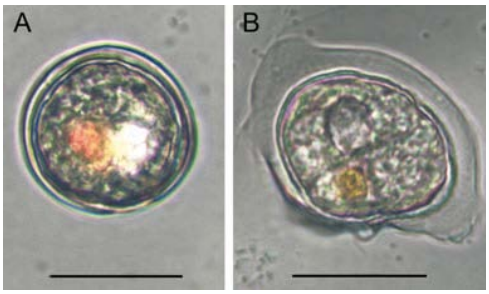

FIG. 6. Light micrographs of the (A) circular-shaped and (B) oval-shaped resting cysts of cultured *Barrufeta bravensis*, as seen in a frontal view. Scale bars, 20 μm.

cytoplasm, as well as mitochondria with tubular christae (Fig. 9D).

Spherical food-vacuole-like structures measuring ~ 2 μm in diameter were observed in some cells (Figs. 7B and 9B); their contents were similar in size to an unknown photosynthetic stramenopile present in the culture VGO864 used for TEM analysis (Fig. 9C). These structures were mainly located in the central area of the dinoflagellate cell.

The amphiesma is composed of flattened vesicles that contain a thin layer of electro-opaque material. Cortical microtubules are present under the amphiesmal vesicles in groups of two or three but sometimes more (Fig. 8D).

The pusular system is not very complex. This kind of pusular system was described by Dodge (1972, p. 288) as a ''pusule with collecting chamber which branches from the flagellar canal.'' It consists of a prolonged collecting chamber that is surrounded by numerous pusular vesicles, which are joined to it. This collecting chamber branches from the flagellar canal of longitudinal flagellum (Fig. 9A).

Pigment composition. As seen in the HPLC chromatogram, the pigment profile of *B. bravensis* strain VGO864 (Fig. 10) was characteristic of peridinin-containing dinoflagellates (Jeffrey et al. 1975), with chl c_2 as the major accessory chl (chl c_2:chl a = 0.20), traces of Mg-2,4-divinyl pheporphyrin (MgDVP:chl a = 0.01), and no chl c_1. Diadinoxanthin was the major carotenoid (Diadino:chl a = 0.58), followed in relative importance by peridinin (Per:chl a = 0.40), dinoxanthin (Dino:chl a = 0.23), and diatoxanthin (0.03). Two unknown carotenoids (t_R = 26.00 and 29.00 min) sharing similar spectra (λ_{max}:[424], 453, 477 nm) were also detected in minor amounts (pigment to chl a ratios of 0.05 and 0.02, respectively).

Toxin analyses. No PSP toxins were detected by HPLC with fluorescence detection or by MBA. The PBS and HCl extracts did not show any hemolytic activity. Lipid-soluble toxins were not tested.

Fatty acid composition. *B. bravensis* shows the major concentration in saturated fatty acids as a stearic acid C18:0 and palmitic acid C16:0 (4.53% and 2.30%, respectively) (Table 1). The second most important lipids, monounsaturated C16:1*n*7 and C18:1*n*9, were present at low concentrations (1.22% and 1.78%, respectively). All polyunsaturated fatty acids (PUFAs) were detected in concentrations of <1%. The results of fatty acids analyses of other unarmored species cultured under the same culture conditions (*A. sanguinea, G. microreticulatum, G. catenatum, G. impudicum, K. brevis*) are shown for comparison purposes in Table 1. Differences in the fatty acid profile, in addition to their percentage, have been observed between *Barrufeta* and other related genera such as *Gymnodinium*. The fatty acid profile of *K. brevis* was similar to that of *B. bravensis* in terms of percentages and lipid content. For example, C12:0 was detected only in *B. bravensis* and *K. brevis* but not in any of the three *Gymnodinium* species or in the *A. sanguinea*, despite identical culture conditions. For C14:1, the opposite case was

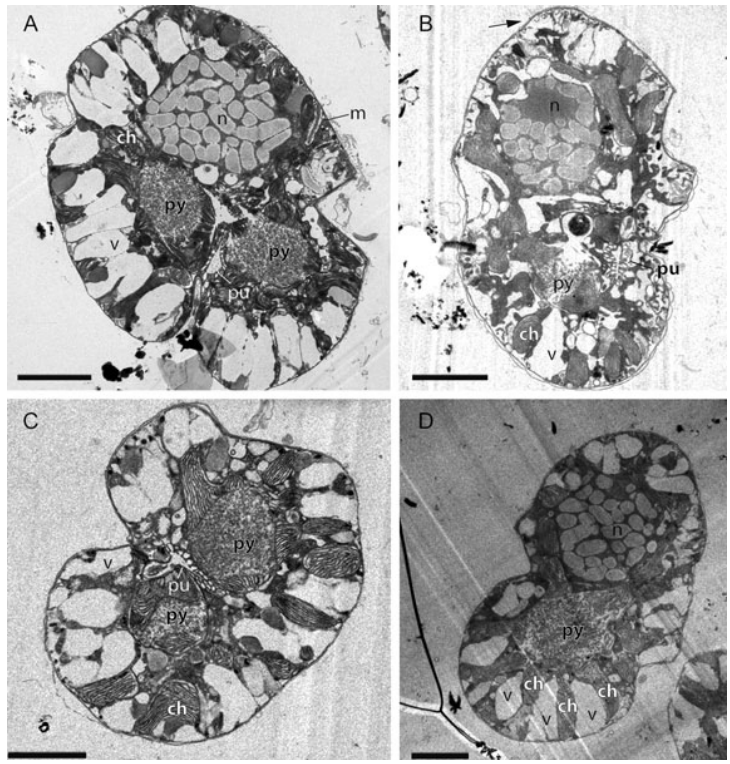

FIG. 7. General ultrastructure of *Barrufeta bravensis*. (A) Longitudinal section of a cell shows the arrangement of the main organelles: (ch) chloroplast, (m) mucocyst, (n) nucleus, (pu) pusule, (py) pyrenoid, and (v) vesicle. (B) Lateral view of the cell. (C) Transverse section of the hypocone shows the two pyrenoids and the pusular system. (D) Slanting longitudinal section of a cell shows the chloroplasts radiating from the pyrenoids to the cell periphery. Scale bars, 5 μm.

true; this fatty acid was not detected in *Barrufeta* but was present in the other three genera. The percentage of palmitoleic acid (C16:1*n*7) was higher in *Gymnodinium* and *Akashiwo* than in *Barrufeta* and *Karenia*. The percentages of PUFA C18:2 and saturated C18:0 measured in *Barrufeta* were also different from those in *Gymnodinium*, *Karenia,* and *Akashiwo*. In addition, *Gymnodinium* had an important concentration of saturated lipids, with percentages ranging between 37.4% and 42.1% depending on the particular strain. This amount was almost 3-fold higher than the amount in *B. bravensis*, as shown in Table 1.

Molecular and phylogenetic analysis. The final alignment of *B. bravensis* with *O. marina* CCMP604 as outgroup was 647 bp in length (C, 24.60%; T, 29.06%; A, 19.69%; G, 26.65%) with 607 polymorphic sites and a transition/transversion ratio of 1.6 for the 5.8S rDNA-ITS gene sequences. With *A. affine* as the outgroup, the aligned sequence was 722 bp in length (C, 20.46%; T, 26.34%; A, 24.31%; G, 28.89%), with a transition/transversion ratio of 1.6 and 601 polymorphic sites for the LSU rDNA gene sequences.

As substantial identity was determined across NJ, MP, and ML analyses, with only minor differences, only the ML phylogenetic tree is shown (Figs. 11 and 12). According to the ITS-5.8S rDNA phylogeny (Fig. 11), the first lineage delineated by the outgroup comprised two species, *A. affine* and *Coolia monotis* Meunier. The ITS-5.8S rDNA phylogeny provided evidence of a major cluster within the one that included the new genus, *Barrufeta*. All *B. bravensis* isolates constituted a distinct and clearly separated group, which was a sister clade of two other genera represented by *L. viride* and *G. impudicum*. This grouping of two sister clades was supported by high bootstrap values. *B. bravensis* was included in the clade called by some authors as ''*Gymnodinium* sensu stricto clade'' together with *Lepidodinium* and *Gymnodinium* species. The tree inferred from the data and from model setting showed that different species of the Gymnodiniales were separated into several clades.

Phylogenetic analysis of the sequence generated from the LSU rDNA (Fig. 12) showed that *B. bravensis* formed a homogeneous group as a sister clade of *Gymnodinium dorsalisulcum* (Hulburt, J. A. McLaughlin et Zahl) S. L. Murray, de Salas et Hallegr.); this

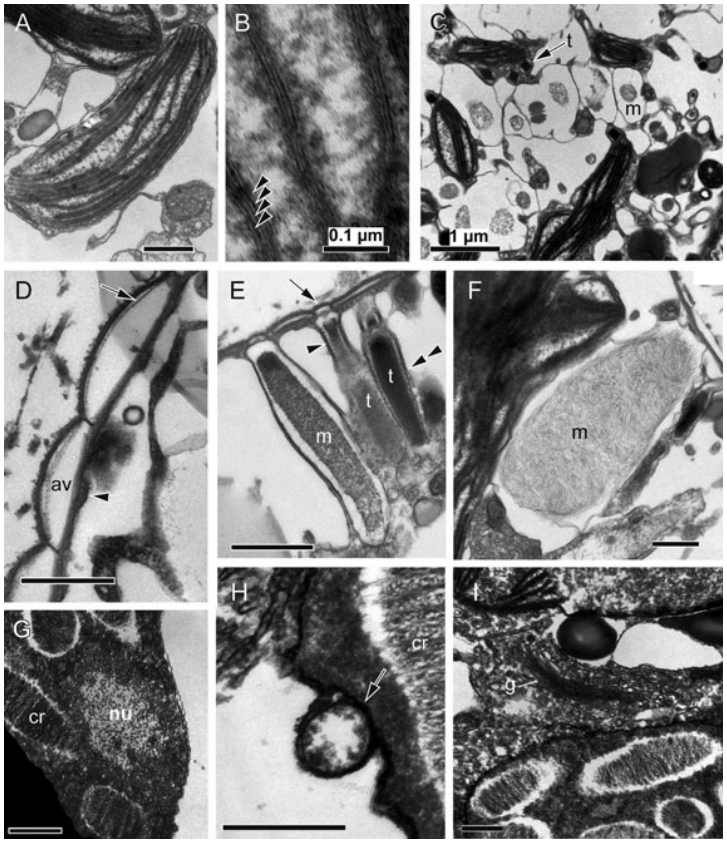

FIG. 8. Transmission electron micrographs of *Barrufeta bravensis*. (A) Whole chloroplast. (B) Detail of a chloroplast showing thylakoids grouped in fours (arrowheads) to form lamellae. (C) Section of a subsuperficial area of the cell, showing chloroplasts, trichocysts (t), and mucocyst-like vesicles (m) in a transverse section. (D) A transverse section of amphiesma shows the amphiesmal vesicle (av), which contains platelike material (arrow); the arrowhead indicates cortical microtubules in groups of two or three. (E) Longitudinal section of two trichocysts (t), showing the cap (arrow) and the striation in both the neck (arrowhead) and body (double arrowhead); a vermiform mucocyst (m) with dense material in its core also has a cap. (F) Oval mucocyst and (G) detail of the nucleus, including the nucleolus (nu) and chromosome (cr). (H) In a detailed view of the nucleus, a chromosome and a structure resembling a vesicular chamber (arrow). (I) Part of the Golgi (g) adjacent to the nucleus. Scale bars, 0.5 μm (unless otherwise indicated).

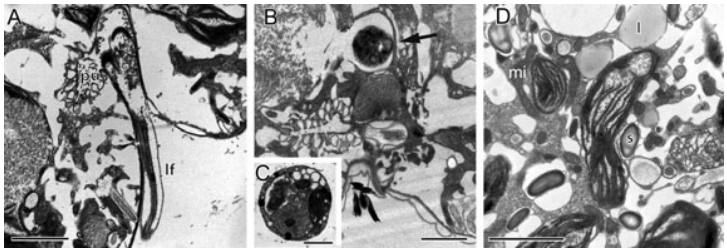

FIG. 9. Transmission electron micrographs of *Barrufeta bravensis*. (A) The pusular system (pu) connects with the longitudinal flagellar canal; longitudinal flagella (lf). (B) Food-vacuole-like structure (arrow). (C) Unknown photosynthetic stramenopile cultured with *Barrufeta*. The size of the unknown photosynthetic stramenopile is similar to that of the content of the food-vacuole-like structure. Scale bar, 1 μm. (D) Micrograph of the cytoplasm, showing droplets of lipids (l), starch grains (s), and mitochondrion (mi). Scale bars (A, B, D), 2 μm.

grouping was supported by high bootstrap values in the NJ and ML analyses. *B. bravensis* was included in the ''*Gymnodinium* sensu stricto clade,'' which was partially resolved. In the LSU phylogeny based on *A. affine* as outgroup, two major groups diverged: one group including different taxa segregated at different branching orders and a second one, ''*Gymnodinium* sensu stricto clade,'' which seemed better

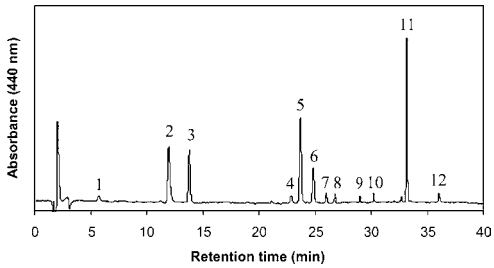

FIG. 10. HPLC chromatogram of pigments from *Barrufeta bravensis* strain VGO864. Peak identification: (1) peridininol, (2) chl *c2*, (3) peridinin, (4) diadinochrome, (5) diadinoxanthin, (6) dinoxanthin, (7) unknown carotenoid, (8) diatoxanthin, (9, 10) unknown carotenoids, (11) chl *a*, (12) β, β-carotene. Detection by absorbance at 440 nm.

resolved. Within the first main grouping, the first cluster included *Tovellia*, *Amphidinium*, and *Jadwigia*. The second cluster consisted of two further groups: one group that comprised *Akashiwo*, *Biecheleria*, *Gyrodinium falcatum,* and *Ceratium fusus* together with the sister taxon of *Cochlodinium fulvescens*; the other group included three genera belonging to the family Kareniaceae (*Karenia*, *Takayama*, *Karlodinium*) as a sister branch of one that included *Gymnodinium instriatum* and *Gyrodinium spirale*. In the LSU phylogeny, the second main grouping ''*Gymnodinium* sensu stricto clade'' included two different clusters. One cluster consisted of two sister clades, one of them composed of different species of *Gymnodinium* including the type species *G. fuscum* (Ehrenb.) F. Stein, and the other one composed of the genus *Lepidodinium*, *Barrufeta,* and two species of *Gymnodinium*; the other cluster also included genera such as *Polykrikos*, *Gymnodinium*, and *Pheopolykrikos* with the exception of *Dissodinium* (Blastodiniales).

DISCUSSION

Daugbjerg et al. (2000) identified both the shape of the apical groove and pigment composition as critical features allowing the differentiation of unarmored dinoflagellate genera, as they were consistent with phylogenetic trees based on rDNA sequencing. This approach resulted in the redescribed genera *Gymnodinium* and *Gyrodinium* as well as new genera, such as *Akashiwo*, distinguished by differences in the shape of the apical groove (Daugbjerg et al. 2000). Another genus, *Takayama*, with yet a different apical groove shape was described later (de Salas et al. 2003). The shape of the apical groove of new genus *Barrufeta* also differs from that of other gymnodinioids. Furthermore, referring to the different kinds of apical-furrow apparatuses described in the literature (Hansen and Daugbjerg 2009, Moestrup et al. 2009a, b), we made a detailed structural comparison between the apical groove of *Barrufeta* and that of a species belonging to the genus *Gymnodinium*, *G. impudicum*. Although both contained three elongated vesicles, small knobs ornamented the central vesicle in *B. bravensis,* whereas in *G. impudicum* the lateral vesicles were ornamented. This is the first time that this difference has been noted. However, more species belonging to the two genera need to

TABLE 1. Relative abundance (%) of fatty acids in different marine unarmored dinoflagellates cultured under the same conditions and harvested in the same growth phase.

Fatty acids	*Barrufeta bravensis* (VGO864)	*Karenia brevis* (CCMP2281)	*Gymnodinium microreticulatum* (VGO328)	*Gymnodinium catenatum* (Est14H5)	*Gymnodinium impudicum* (VGO665)	*Akashiwo sanguinea* (VGO626)
C12:0	0.28	0.1	0.0	0.0	0.0	0.0
C14:1	0.0	0.5	1.0	1.1	1.0	1.3
C14:0	0.24	1.0	1.0	1.3	1.7	2.0
C15:0	0.12	0.2	0.8	1.1	2.3	0.6
C16:1*n7*	1.22	1.3	6.4	5.9	4.9	8.0
C16:0	2.30	2.8	14.7	14.0	13.2	18.0
C17:0	0.17	0.1	0.7	0.5	1.0	0.5
C18:5*n3*	0.24	0.5	2.9	1.0	0.5	1.6
C18:3	0.29	1.1	2.6	1.4	0.9	3.9
C18:2	0.95	0.0	0.0	0.0	0.0	0.0
C18:1*n9*	1.78	1.3	6.6	7.0	7.0	4.3
C18:0	4.53	3.6	19.9	23.4	23.9	13.1
C20:5*n3*	0.21	0.0	2.5	0.0	0.0	3.4
C20:4	0.0	0.0	7.8	1.3	0.0	12.2
C20:0	0.10	0.10	0.3	0.0	0.0	0.6
C22:0	0.0	0.0	0.0	0.0	0.0	0.3
C24:0	0.0	0.0	0.0	0.0	0.0	0.4
SFA	7.7	7.9	37.4	40.3	42.1	35.5
MUFA	3.0	3.1	14.0	14.0	12.9	13.6
PUFA	1.7	1.6	15.8	3.7	1.4	21.1

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

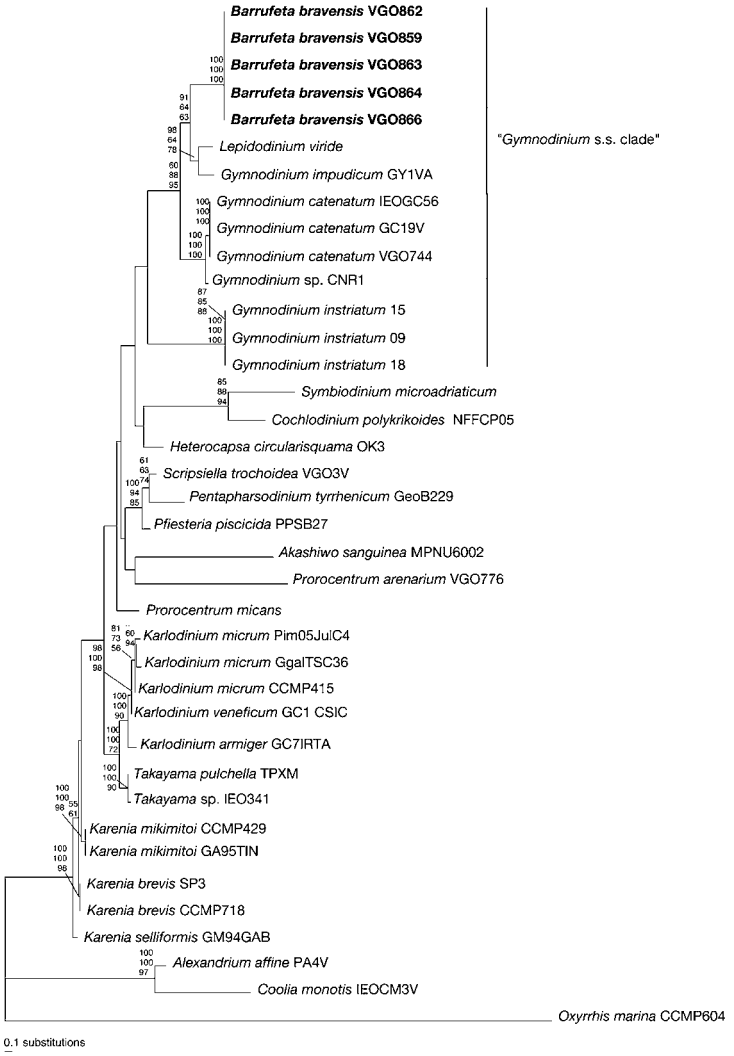

FIG. 11. Maximum-likelihood (ML) tree inferred from the alignment of ITS-5.8S rDNA sequences. Numbers on the major nodes represent, from top to bottom, neighbor-joining (1,000 pseudoreplicates), maximum-parsimony (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Oxyrrhis marina* (AY566415) as outgroup. ITS, internal transcribed spacer.

be examined in future to determine whether this difference is maintained within each genus and to resolve if it can be used as a taxonomic character at the genus level.

The species closest to *B. bravensis*, both phylogenetically and morphologically, are *G. dorsalisulcum*, *L. viride,* and *Lepidodinium chlorophorum* (see Table 2). *G. dorsalisulcum* is a benthic species with a *Gymnodinium*-type apical groove according to Murray et al. (2007) and is thus different from *Barrufeta*. Also, unlike in *Barrufeta*, the epicone of *G. dorsalisulcum* is longer than its hypocone, whereas the two species share the presence of an anteriorly positioned nucleus, two large equatorial pyrenoids, and numerous radiating plastids. In our opinion, the path of the apical groove of *G. dorsalisulcum* shown in Murray et al. (2007) does not seem to be quite the same as a typical horseshoe-shaped *Gymnodinium* apical groove; it forms a shape around the cell vaguely resembling *Barrufeta* overall in the dorsal view. Further studies involving other genes, ultrastructure, and a more accurate examination of the apical groove should determine if the position of G. *dorsalisulcum* within the *Gymnodinium* genus is still appropriate.

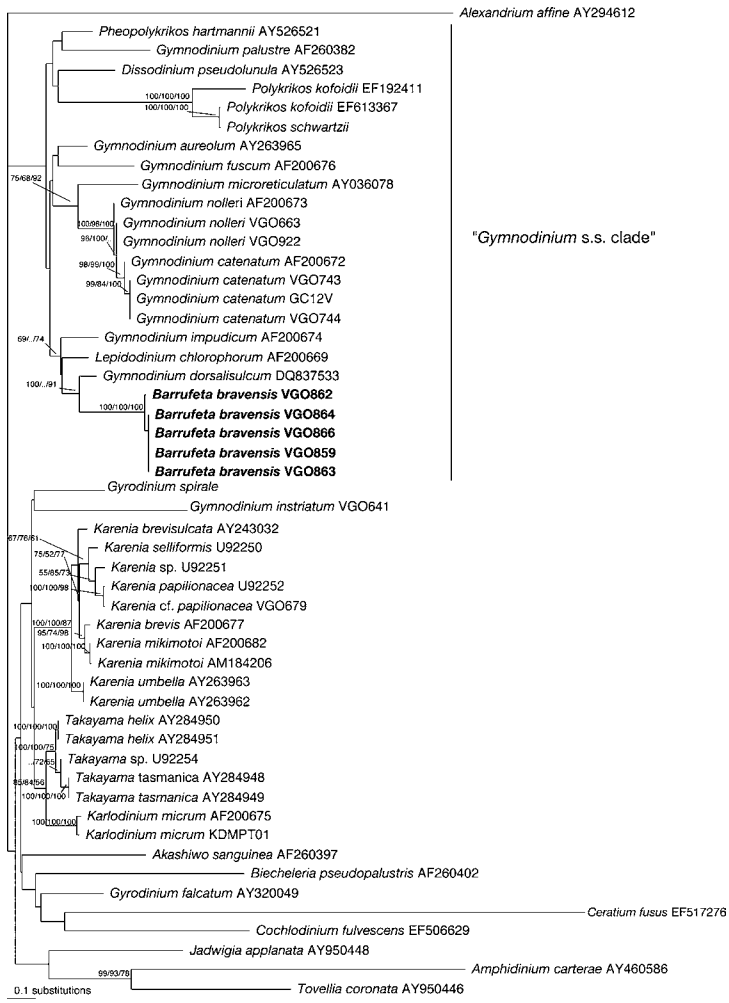

FIG. 12. Maximum-likelihood (ML) tree inferred from alignment of the D1/D2 domains of the LSU rDNA sequences. Numbers on the major nodes represent, from top to bottom, neighbor-joining (1,000 pseudoreplicates), maximum-parsimony (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Alexandrium affine* (AY294612) as outgroup.

Both *Lepidodinium* species have an apical groove of the *Gymnodinium* type, and their pigments derive from Chlorophyta, as deduced from plastid-encoded gene phylogeny (Takishita et al. 2008). Although several other dinoflagellate genera included within Gymnodiniales, such as *Karenia*, *Karlodinium*, and *Takayama,* acquired their chloroplasts through tertiary endosymbiosis from haptophytes (Tengs et al. 2000, de Salas et al. 2003, Garcés et al. 2006), *Barrufeta* is a typical peridinin-containing dinoflagellate and is thus similar to *Gymnodinium* but different from *Lepidodinium.* In addition to the *B. bravensis* strain VGO864, the other strains that were analyzed (VGO859 and VGO860) were observed to have similar pigment patterns and ratios, with diadinoxanthin as the dominant carotenoid, followed by peridinin. This result could be explained by a culture light environment prone to eliciting a photoprotection mechanism that results in a reduced proportion of the light-harvesting pigment peridinin and an increased pool of the photoprotective carotenoid diadinoxanthin.

Since Daugbjerg et al. (2000) described the *Gymnodinium* sensu stricto clade, several genera have been included in this clade in addition to *Gymnodinium*, such as *Lepidodinium* (Hansen et al. 2007), *Polykrikos* (Hoppenrath and Leander 2007), *Dissodinium* (Gómez et al. 2009b), *Warnowia* (Gómez et al.

TABLE 2. A comparison of the major morphological features of similar and closest species to *Barrufeta brevensis;* based on Hulburt (1957)[1], Watanabe et al. (1990)[6], Fraga et al. (1995)[5], Takayama (1998)[3], Skovgaard (2000)[2], Murray et al. (2007)[4].

	B. bravensis (NW Mediterranean Catalonia)	*Gyrodinium resplendens*[1] (NW Atlantic, Woods Hole)	*G. resplendens*[2] (NW Atlantic, Maryland)	*G. resplendens*[3] (Seto Inland Sea)	*Gymnodinium dorsalisulcum*[4]	*Gymnodinium impudicum*[5]	*Lepidodinium viride*[6]
Shape	Oval shaped, truncate or slightly bilobate antapex, dorsoventrally flattened	Broadly fusoid, truncate apex and antapex, moderately dorsoventrally flattened	Close to spheroid but moderately dorsoventrally flattened. Some individuals more elongated.	Oval shaped, truncate antapex	Cells are oval to elongate oval from the ventral side and dorsoventrally flattened	Chain forming. Longer and shorter cells can be observed.	Cell subglobular, dorsiventrally flattened. Cell surface with box-shaped scales.
Cellular size (μm)	22–35L, 16–25W	36–62L, 32–48W	25–46L, 20–46W	45L, 34W	25–40L, 15–28W	14–37L, 16–32W	22–52L, 19–38W
Apical groove shape	Smurf-cap shaped	Sulcus extending onto epicone as very narrow, superficial groove, diverging to right	Smurf-cap shaped	Smurf-cap shaped	Horseshoe shaped, resembling *Barrufeta* in dorsal view	Horseshoe shaped	Horseshoe shaped
Chloroplast	Numerous small radiating chloroplasts	Oval, rich brown, radiating, many tiered	Numerous small oblong or disk-shaped chloroplasts lined the inner periphery of the cell		Numerous, yellow brown, and appear to radiate from the center of the cell	Numerous small elongated chloroplasts	Usually single and is seen as a peripherally situated reticulum
Position of the nucleus	Anterior part of the epicone	Somewhat anterior of center, wider than long	In the epicone	In the epicone	In the epicone	Central, slightly displaced toward the hypocone or the epicone, depending on the position of the cell in the chain	Centrally to anteriorly located
Presence of food vacuoles	+	+	+				

L, long; W, Wide.

2009a), *Nematodinium* (Hoppenrath et al. 2009), *Erythrosidinium* (Gomez et al. 2009a), *Chytriodinium* (Gomez et al. 2009b), and *Paragymnodinium* (Kang et al. 2010); consequently, the name of this group does not seem to be at the moment the most appropriate. The new phylogenetic data on gymnodinioids together with data from the literature (e.g., Gomez et al. 2009b) show that species considered *Gymnodinium* are located in different branches of this clade. This fact supports the need to establish many new genera in order to include species currently considered as belonging to *Gymnodinium.* Species such as *G. catenatum*, *G. nolleri*, and *G. microreticulatum* are monophyletic and have reticulate cysts, and although their horseshoe-shaped apical groove is of the *Gymnodinium* type, it is slightly different from the groove of other *Gymnodinium* species (i.e., *G. impudicum*, *G. dorsalisulcum,* and *G. fuscum*). Accordingly, they should be distinguished within a new genus.

The presence of nuclear chambers and nuclear fibrous connective (NFC) have been considered characteristics of the genus *Gymnodinium* (Daugbjerg et al. 2000), although it has not been demonstrated in all species that Daugbjerg transferred to *Gymnodinium* genus. Nevertheless, both structures have also been observed in *L. chlorophorum* and *L. viride* (Hansen et al. 2007), although the NFC component is not as large. The NFC has also been detected in other dinoflagellate genera, such as *Polykrikos* (Bradbury et al. 1983), *Actiniscus* (Hansen 1993), *Nematodinium* (Roberts and Taylor 1995), and *Biecheleriopsis* (Moestrup et al. 2009b). While nuclear chambers were observed in *Barrufeta*, they are not as numerous as in *Gymnodinium fuscum* (Hansen et al. 2000b) or *L. viride* (Hansen et al. 2007), and nuclear pores were not observed. NFC was not seen in *Barrufeta,* but the presence of this structure cannot yet be ruled out entirely because unfortunately serial sections of samples were not examined with a transmission electron microscope. The large number of the vacuoles with electron-transparent contents and arranged along the periphery alternating with the chloroplast is unusual. Other highly vacuolated species are, for example, *Polykrikos lebourae* Herdman (Hoppenrath and Leander 2007) and *Amphidinium cryophilum* G. J. Wedem., L. W. Wilcox et L. E. Graham (Wilcox et al. 1982). In *B. bravensis*, the vacuoles with fibrillar content are probably mucocysts, similar to those observed in *P. lebourae* and *Prorocentrum tsawwassenense* Hoppenrath et B. S. Leander and consistent with the observation that *B. bravensis* is a mucus producer. The strain of *Barrufeta* only used in this study for TEM was accompanied by a small, unknown photosynthetic stramenopile. The observation of food-vacuole-like structures with a size similar to that of the unknown photosynthetic stramenopile and the fact that the mixed cultures grew better than the nonmixed ones led us to conclude that *B. bravensis* is a mixotrophic species.

There exists a major discussion about the utility of fatty acids as a chemotaxonomic tool, since these lipids show a variable profile during the growth curve and under different growth conditions (Thompson et al. 1992, Xu and Beardall 1997, Zhu et al. 1997, Xu et al. 2008). This fact, added to the different methodologies used for the analysis of fatty acids in the literature, makes most of the data from the literature uncomparable. In this work, we wanted to compare the profile of fatty acids of *B. bravensis* with those of other unarmored dinoflagellates. To avoid growth-, methodology-, and culture-related differences in fatty acid profiles, all of the strains examined in this study were cultured under the same conditions, examined during the same growth phase and under the same methodology. In this way, the results obtained in this work demonstrate that the fatty acids of *B. bravensis*, in terms of percentages and composition, are more similar to those of *K. brevis* than to those of other species more related, such as the three species of *Gymnodinium* analyzed.

The *Barrufeta* genus could include some other species previously described in the literature. This may be the case for *G. resplendens* (Hulburt 1957, p. 210), which shares several characteristics with *Barrufeta,* such as the presence of numerous radiating chloroplasts (see Table 2). Hulburt (1957) described the apical groove of this species as ''the sulcus extending onto the epicone as a very narrow superficial groove, diverging to the right.'' This is similar to the proximal part of the apical groove of *Barrufeta,* but the end of the apical groove of *G. resplendens* was not described, or its apical groove is short and only located on its ventral part. In addition, the presence of the pyrenoids was not described, and the size of the latter species is larger than that of *Barrufeta* (36–62 μm length, 32–48 μm width), although smaller cells were identified as in *G. resplendens* by Campbell (1973). Daugbjerg et al. (2000), based on the apical groove of *G. resplendens*, suggested that this species could belong to *Akashiwo,* but some organisms not belonging to the latter genus have been identified as *G. resplendens* by other authors. A strain isolated from a brackish water (salinity 11) fish pond in Maryland, on the East Coast of the USA, and identified as *G. resplendens* was used by Skovgaard (2000) in a study on mixotrophy, which provided new morphological and behavioral details regarding this plastidic species. Even though Skovgaard (2000) realized that in Hulburt's drawing the apical groove did not encircle the apex of the cell, he assumed that Hulburt had overlooked this feature; based on other similarities with the original *G. resplendens* material, he therefore assigned his organism to this species. Our sample material was similar to that used in Skovgaard's study with respect to the shape of the apical groove, cingulum displacement, the positions and shapes of the chloroplast and nucleus, the presence of food

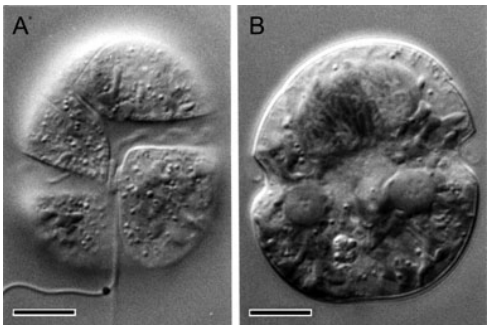

FIG. 13. (A, B) Light micrographs of *Gyrodinium resplendens*. Reproduced from Takayama (1998), figures 3 and 4, respectively, with permission. Scale bars, 10 μm.

vacuoles, the shape of the smooth-walled cysts, and the presence and position of two pyrenoids, which can be observed in a differential interference contrast (DIC) photograph (fig. 7 in Skovgaard 2000). However, Skovgaard described cells that were generally rounded and only moderately dorsoventrally flattened, whereas *B. bravensis* cells are more elongated and more dorsoventrally flattened. Moreover, Skovgaard observed two types of cysts, the most frequent of which had a smooth surface with either a few irregular spines or without spines, while the other, a more uncommon one had a spiny wall. The surfaces of *Barrufeta* cysts are exclusively completely smooth, and most of them are surrounded by mucus. Other aspects of the two species, such as the salinity of the water where they were found, are also different. In another study of *G. resplendens* (Loeblich and Smith 1968), the reported pigment composition was consistent with our results, although, in contrast to *B. bravensis*, peridinin was found to be the major carotenoid (40%) in *G. resplendens*, followed by diadinoxanthin (33%). Takayama (1998) studied *G. resplendens* from material collected from the Seto Inland Sea and showed the complete apical groove, which had the same shape as we have described in *Barrufeta*. Furthermore, DIC images showed similar positions of the nucleus and of the two large pyrenoids (Fig. 13). Mainly due to the probability of the incompletely described apical groove in the original report of *G. resplendens*, we cannot be sure that the *G. resplendens* described by Hulburt is a *Barrufeta*; however, the species studied as *G. resplendens* by Takayama and by Skovgaard, which are not necessarily the same one described by Hulburt, can most certainly be assigned to the genus *Barrufeta*.

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The toxicity and intraspecific variability of *Alexandrium andersonii* Balech

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ABSTRACT

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

1. Introduction

In a recent issue of the journal *Harmful Algae* (Pitcher, 2012), the need for species-specific information to predict the occurrence of harmful algal blooms (HABs) was emphasized, especially given the wide-ranging differences in the morphology, phylogeny, life-cycles, growth requirements, etc., of HAB species. In fact, even within the same species there is extensive genotypic and phenotypic variability that challenges our traditional notion of morphospecies (e.g., Alpermann et al., 2010). Therefore, it is clear that to appreciate the plasticity of a species and thus its adaptive potential, more than one strain must be studied.

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

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

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

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

The recent detection of *Alexandrium andersonii* in different areas of the Mediterranean has brought to light the deficits in our knowledge of this species. This applies not only to its toxin-producing ability but also to its physiology, ecology, and distribution as well as its intraspecific variability, none of which has been studied.

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

2. Materials and methods

2.1. Alexandrium andersonii *isolation and culture*

Alexandrium andersonii culture ICMB222 was isolated in March 2007 from Alfacs Bay (Fig. 1), the southern bay of the Ebre River Delta (NW Mediterranean Sea) and the major shellfish harvesting area along the Catalan coast. The other cultures used in this study were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP) of Bigelow Laboratory (USA) and from the Culture Collection of

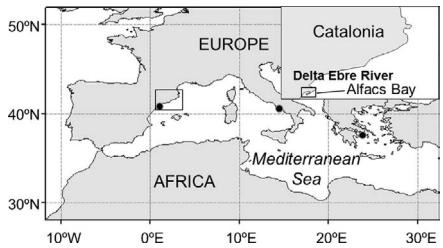

Fig. 1. Map showing the location of Alfacs Bay. Dots show the sites where the Mediterranean strains were isolated.

Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo (Spain). All cultures were adapted for one year to a salinity of 36 and to growth in L1 medium (Guillard and Hargraves, 1993).

2.2. Phenological characterization of A. andersonii *strains*

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

Speculating that variations in temperature influenced the toxin content of *Alexandrium* species, we carried out an additional growth experiment at 14 °C (the water temperature during the bloom of *Alexandrium andersonii* in Alfacs Bay). Duplicate cultures of strains VGO664, SZN012, ICMB222, and CCMP1718 were grown at 14 °C under the same conditions used in the first growth experiment, with 2-mL subsamples taken every 2 or 3 days for cell quantification. For HPLC analyses of toxin profiles, 20–40 mL of each culture was removed (depending on the concentration) during the exponential and stationary phases of growth and filtered on Whatman GF/C glass-fiber filters.

2.3. Growth rate calculation and statistical analyses

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

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

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

2.4. Light microscopy

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

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

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

2.5. Measures and statistical analyses

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

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

Cultures of *Alexandrium andersonii* were collected during the exponential growth phase by filtration on 3-μm pore size Isopore membrane filters (Millipore). DNA was extracted and purified as described in Penna et al. (2005). Nuclear-encoded 5.8S rDNA and ITS regions were PCR-amplified as described in Penna et al. (2008). Genomic DNA (1 ng) was amplified in a 50-μL reaction mix containing 50 μM each of dATP, dTTP, dCTP, and dGTP, 0.4 μM of each primer, 4 mM $MgCl_2$, 1× reaction buffer (Diatheva, Fano, Italy), and 1.0 U of Hot Rescue DNA polymerase (Diatheva, Fano, Italy). PCR of the LSU rDNA regions (D1/D2) was carried out in a 50-μL reaction mix containing 1 μL of genomic DNA, 0.25 μM of each primer (D1R and D2C by Scholin et al., 1994), 600 μM of each dNTP, 3 μL $MgCl_2$ (25 mM), 1× reaction buffer (Qiagen, USA, including 1.5 mM $MgCl_2$), and 2.5 U of Taq DNA polymerase (Qiagen, USA). Thermocycling conditions for the 5.8S rDNA and ITS regions were as follows: a 10-min initial denaturation at 95 °C and then 35 cycles of 1 min at 95 °C, 1 min at 50 °C, and 2.5 min at 72 °C; a final elongation step consisted of 7 min at 72 °C. For LSU rDNA, an initial denaturation at 95 °C for 5 min was followed by 40 cycles of 20 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final elongation step of 10 min at 72 °C. The three PCR-amplified products corresponding to the 5.8S rDNA and ITS regions were pooled, purified, and then directly sequenced using the ABI PRISM 310 genetic analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) and the dye terminator method as described in the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction kit, Perkin Elmer Corp., Foster City, CA). LSU rDNA sequencing was carried out by an external service (Macrogen Inc., Korea). Sequences obtained from this study were aligned with those from GenBank using the CLUSTAL X2 program (Larkin et al., 2007) with default settings. Alignments were re-checked visually and edited manually; non-alignable regions were excluded prior to the phylogenetic analyses. The strains used in the molecular determinations are listed in Table 2 and in supplementary material (Table 1) together with the GenBank accession numbers of their 5.8S ITS and LSU rDNA sequences. Phylogenetic relationships, based on the 5.8S ITS rDNA data, were inferred using the neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods. Sequences of *Gonyaulax spinifera* (Claparède et Lachmann) Diesing (EMBL-EBI ENA: AF051832) served as the outgroup in the ITS 5.8S rDNA phylogeny. NJ and MP analyses were performed using heuristic searches with tree-bisection-reconnection branch swapping. Branches were collapsed if their minimum length was 0; ambiguities and gaps were considered as missing data. The

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

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

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

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

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

2.7. Pigment analyses

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

2.8. Toxicity and toxin analyses

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

2.8.1. HPLC-FLD analysis of PSP toxins

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

HPLC-FLD with post-column derivatization was performed according to Franco and Fernandez (1993). The HPLC system consisted of a Waters 474 fluorescence detector (excitation: 330 nm, emission: 390 nm), a Waters 717 automatic injector, a Waters 600 HPLC pump to deliver the mobile phases, and two Waters 510 pumps to deliver the post-column reagents. Separation was achieved with an Agilent 5μ Lichrosphere 100 RP18 cartridge (125 mm × 4.6 mm). The column temperature was 30 °C and the flow rate was 0.8 mL min^{-1}. The mobile phases consisted of 1.5 mM octansulfonic in 10 mM ammonium phosphate (pH 7) for GTX separations and 95% of this eluent solution plus 5% acetonitrile for the separation of neoSTX, dcSTX, and STX.

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

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

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

2.8.2. Cytotoxicity assay

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

To prevent interferences in the HCl extract, prior to the analysis, an aliquot of the acidic *Alexandrium andersonii* extract (see Section

2.8.3) was further purified using solid-phase extraction (SPE) cartridges (C18 AccuBond II, 500 mg, 3 mL). The SPE cartridges were first conditioned using 6 mL of absolute methanol followed by 6 mL of MilliQ water. One mL of acidic extract was loaded and eluted twice with 2 mL of MilliQ water. The volume of the eluate was further adjusted to 4 mL using MilliQ water.

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

2.8.3. Hemolytic activity

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

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

Triplicate samples made up of 5 mL of the blood-cell suspensions and 5 mL of the toxin-containing solution were combined in centrifuge tubes, incubated at 37 °C for 6 h, and then maintained at room temperature for an additional 18 h. At intervals of 1, 5, 6, and 24 h, 1-mL aliquots of the mixture were transferred to Eppendorf vials, which were centrifuged for 10 min at 1500 rpm at a temperature of 10 °C. Two hundred μL aliquots of each supernatant were then added to the wells of one microwell plate. Absorption of these samples was measured at 405 nm with a BioRad microplate reader model 550.

2.8.4. Mouse bioassay

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

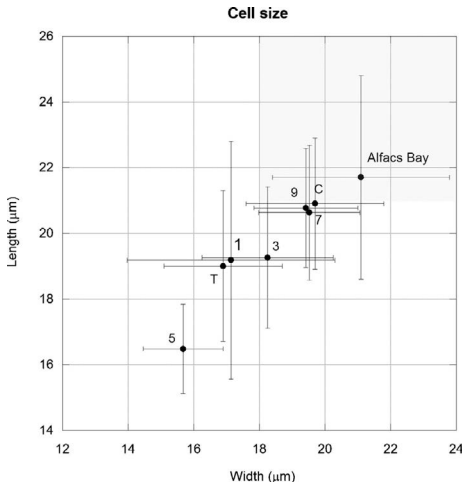

Fig. 3. Mean and standard deviation of the measurements of 30 cells from each of the five strains of *A. andersonii* (1, VGO664; 3, SZN012; 5, ICMB222; 7, CCMP1718; 9, CCMP1597) and from cells in natural samples from Alfacs Bay. Measurements from other authors have been included (C, Ciminiello et al.,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

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

3. Results

3.1. Morphology and measurements

The morphology of *Alexandrium andersonii* from Alfacs Bay of the Ebre Delta (Fig. 2) generally well matched the description of Balech (1990), except that the wild cells were smaller (length: 21.7 ± 3.1 μm, range: 17–28.3 μm, $n = 30$; width: 21.1 ± 2.7 μm, range: 15.5–26.4 μm, $n = 30$) than those of the original description (length: 21–35 μm, width: 18–33 μm).

Differences in the cell sizes of the strains compared to the Alfacs Bay field samples are shown in Fig. 3. Cells of the Catalan strain ICMB222 were the smallest while those of one of the two American strains, CCMP1718, were the largest, although wild cells were

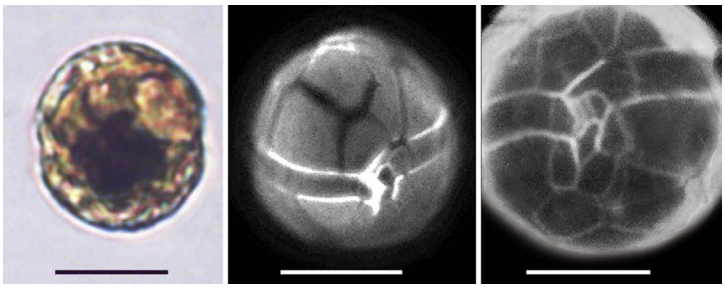

Fig. 2. Micrographs of an *Alexandrium andersonii* cell observed using light (A) and epifluorescence (B), (C) microscopy. Scale bars, 10 μm.

larger than cells from any of the strains studied. ANOSIM analysis, which included strains and field cells, showed that these differences in cell size were significant ($p = 0.00001$). According to the pairwise tests, each of the strains was significantly different from the others and from the field samples ($p < 0.05$), with the exception of the two American strains (CCMP1718 and CCMP1597).

3.2. Phylogenetic analyses

Based on the 5.8S rDNA, ITS regions, and LSU regions of the *Alexandrium* isolates, almost identical topologies were obtained by NJ, MP, and ML and by NJ, UPGMA, and ML analyses, respectively; therefore, only ML phylogenetic trees are shown in Fig. 4 (also see the supplementary material). *Alexandrium*

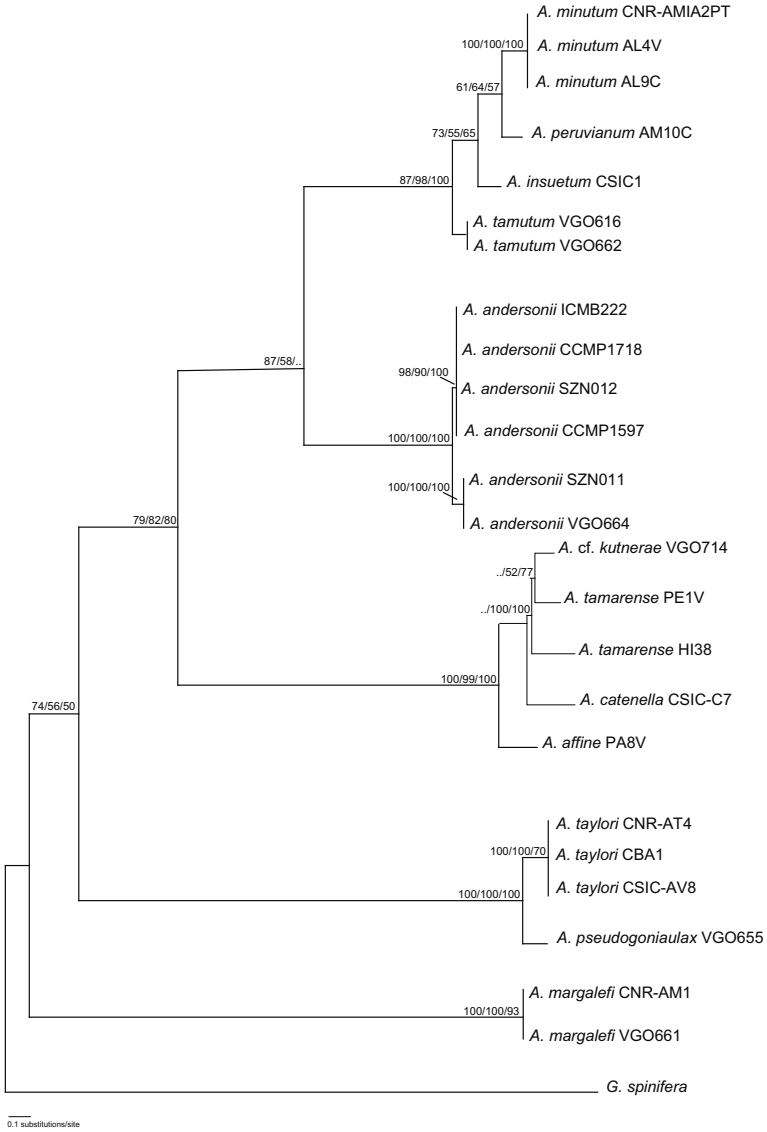

Fig. 4. Maximum-likelihood (ML) tree inferred from the alignment of ITS 5.8S rDNA sequences. Numbers on the major nodes represent, from right to left, neighbor-joining (1000 pseudoreplicates), maximum-parsimony (1000 pseudoreplicates), and ML (1000 pseudoreplicates) bootstrap values. Only bootstrap values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

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

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

andersonii constituted a homogeneous clade supported by high bootstrap values in both phylogenetic trees, consistent with a lack of substantial variability among the isolates analyzed. The *A. andersonii* group constituted a sister clade of the *Alexandrium minutum*/*Alexandrium tamutum* group within the genus *Alexandrium*.

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

3.3. Growth rates

The growth rates and maximum cell densities reached by the five *Alexandrium andersonii* strains at 20 °C and 14 °C are summarized in Table 3. Estimated growth rates ranged from 0.05 to 0.31 d^{-1} and were not significantly different among the strains either at 20 °C or at 14 °C (Kruskal–Wallis test, $p = 0.1212$ and $p = 0.0916$, respectively). The growth rates were more

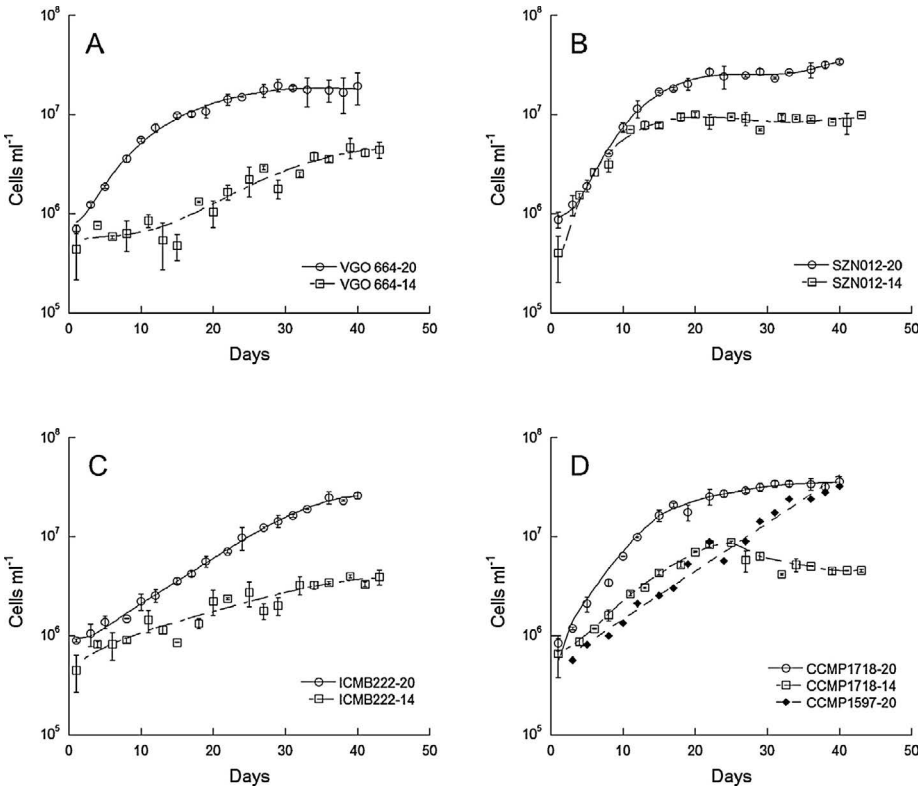

Fig. 5. Growth curves of the different strains of *A. andersonii* cultured at 20 °C (circles or rhombus) and at 14 °C (squares) in L1 medium. For illustrative purposes, the data were fitted to a curve. Bars represent the standard deviation of the duplicates.

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

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

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

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

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

3.4. Pigment analyses

Alexandrium andersonii is a typical peridinin-containing dinoflagellate and the pigment composition of all five strains was similar, with peridinin as the major carotenoid. However, there were slight differences in the pigment ratios with respect to chlorophyll (chl) *a* (Table 4). Specifically, the ratio of peridinin to either chl *a* or chl c_2 was highest (1.04 ± 0.01 and 4.93 ± 0.10, respectively) in the Greek strain VGO644 and lowest (0.46 and 1.91, respectively) in strain CCMP1597 (for strain CCMP1597 replicates were not available), isolated from the west coast of the North Atlantic. Other pigment ratios (chl c_2/chl *a*, diadino/chl *a*, dino/chl *a*) were more consistent irrespective of the strain analyzed.

3.5. Toxicity and toxin analyses

3.5.1. HPLC

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

3.5.2. Cytotoxicity assay

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

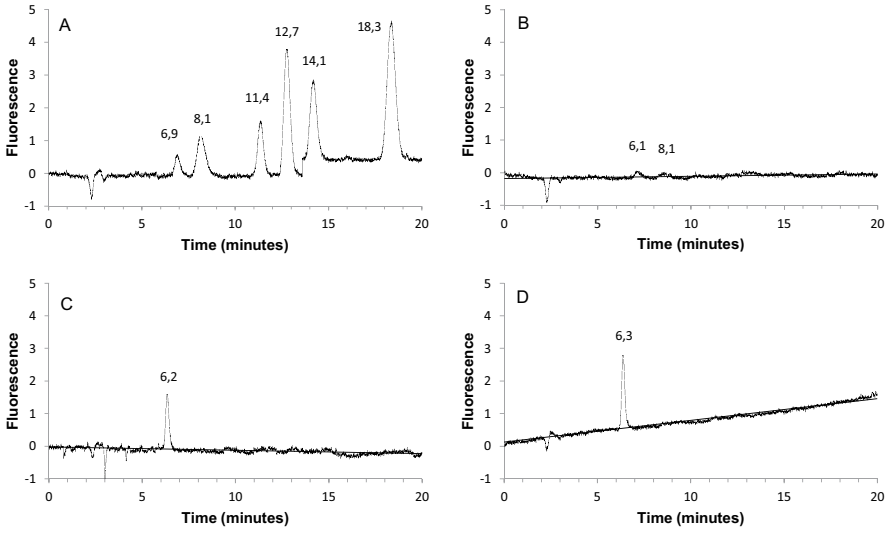

Fig. 6. HPLC-FLD chromatograms of stationary phase cells of strain SZN012 cultured at 14 °C. (A) SZN012 cells + GTXs standard with oxidant; (B) SZN012 cells + GTXs standard without oxidant; (C) SZN012 cells with oxidant; (D) SZN012 cells without oxidant. Retention times (min) of GTXs: GTX4: 6.9; GTX1: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

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

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

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

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

3.5.3. Hemolytic activity

All of the tested strains showed hemolytic activity (Table 6). After 1 h of incubation, the absorbances of the PBS-extracted samples were indicative of hemolysis, which reached 100% after 5 h. While the HCl extracts were also positive, the response was slightly different: intact erythrocytes were detected after 1 h, ruptured red cells after 5 h, and 100% hemolysis after 24 h of incubation. Therefore, hemolytic activity was characteristic of all the strains. The positive result obtained with the PBS extracts suggests the involvement of proteinaceous compounds, while that obtained with the HCl extracts is consistent with the presence of non-protein compounds, the nature of which is not yet known.

3.5.4. Mouse bioassay

None of the strains appeared to express PSP toxins since none of the bioassayed mice exhibited the appropriate symptoms. Mice injected with extracts from strains SZN012 and CCMP1597 died at various times, as detailed in Table 7. Following injection with the HCl extracts from these strains, the mice became weak and lethargic, with a progressive decline in activity until they finally died. However, these symptoms are not typical of those induced by toxins of the PSP complex. Nonetheless, to increase the possible toxic content and thereby better distinguish toxin-related symptoms, samples from strains VGO664 and SZN012 were concentrated five- and six-fold, respectively, prior to their injection, in which case the mice died approximately 2 h later but still did not exhibit PSP-like symptoms.

4. Discussion

4.1. Morphology and measurements

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

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

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

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

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

4.2. Phylogenetic analyses

The phylogenetic homogeneity of *Alexandrium andersonii* strains based on the 5.8 ITS and LSU regions did not allow us to distinguish among individual populations from different Mediterranean areas, unlike in other *Alexandrium* species (Lilly et al., 2005; McCauley et al., 2009). However, genetic diversity within *A. andersonii* cannot be ruled out and its detection may instead require finer-scale markers (such as microsatellites), as was the case for *Alexandrium minutum* in studies carried out at global and Mediterranean scales (Casabianca et al., 2012; McCauley et al., 2009).

4.3. Growth rates

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

Along the Catalan coast (NW Mediterranean, Spain), *Alexandrium* species have been observed in detail for over 17 years. During this time, there have been no blooms of *Alexandrium andersonii* in response to warm temperatures; rather, the cells have only rarely been detected and only at very low densities (<100 cells L^{-1}). In contrast, a bloom of *A. andersonii* occurred in

Table 8
Mouse bioassay of different concentrations of acetic acid.

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

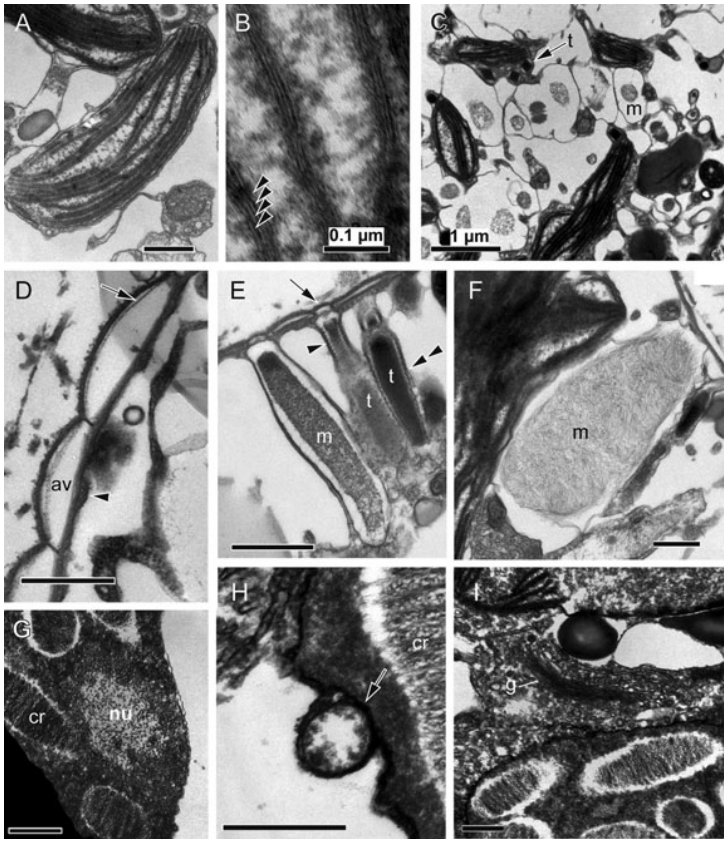

FIG. 8. Transmission electron micrographs of *Barrufeta bravensis*. (A) Whole chloroplast. (B) Detail of a chloroplast showing thylakoids grouped in fours (arrowheads) to form lamellae. (C) Section of a subsuperficial area of the cell, showing chloroplasts, trichocysts (t), and mucocyst-like vesicles (m) in a transverse section. (D) A transverse section of amphiesma shows the amphiesmal vesicle (av), which contains platelike material (arrow); the arrowhead indicates cortical microtubules in groups of two or three. (E) Longitudinal section of two trichocysts (t), showing the cap (arrow) and the striation in both the neck (arrowhead) and body (double arrowhead); a vermiform mucocyst (m) with dense material in its core also has a cap. (F) Oval mucocyst and (G) detail of the nucleus, including the nucleolus (nu) and chromosome (cr). (H) In a detailed view of the nucleus, a chromosome and a structure resembling a vesicular chamber (arrow). (I) Part of the Golgi (g) adjacent to the nucleus. Scale bars, 0.5 µm (unless otherwise indicated).

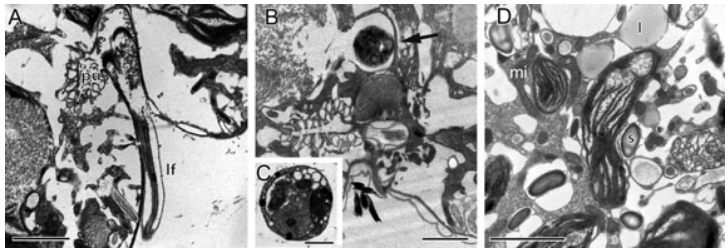

FIG. 9. Transmission electron micrographs of *Barrufeta bravensis*. (A) The pusular system (pu) connects with the longitudinal flagellar canal; longitudinal flagella (lf). (B) Food-vacuole-like structure (arrow). (C) Unknown photosynthetic stramenopile cultured with *Barrufeta*. The size of the unknown photosynthetic stramenopile is similar to that of the content of the food-vacuole-like structure. Scale bar, 1 µm. (D) Micrograph of the cytoplasm, showing droplets of lipids (l), starch grains (s), and mitochondrion (mi). Scale bars (A, B, D), 2 µm.

grouping was supported by high bootstrap values in the NJ and ML analyses. *B. bravensis* was included in the ''*Gymnodinium* sensu stricto clade,'' which was partially resolved. In the LSU phylogeny based on *A. affine* as outgroup, two major groups diverged: one group including different taxa segregated at different branching orders and a second one, ''*Gymnodinium* sensu stricto clade,'' which seemed better

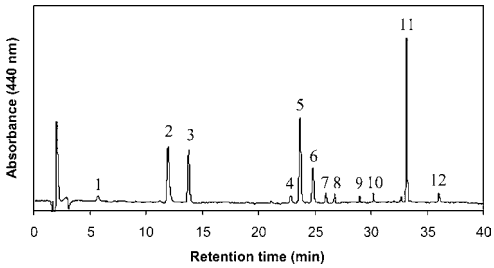

FIG. 10. HPLC chromatogram of pigments from *Barrufeta bravensis* strain VGO864. Peak identification: (1) peridininol, (2) chl *c*2, (3) peridinin, (4) diadinochrome, (5) diadinoxanthin, (6) dinoxanthin, (7) unknown carotenoid, (8) diatoxanthin, (9, 10) unknown carotenoids, (11) chl *a*, (12) β, β-carotene. Detection by absorbance at 440 nm.

resolved. Within the first main grouping, the first cluster included *Tovellia*, *Amphidinium*, and *Jadwigia*. The second cluster consisted of two further groups: one group that comprised *Akashiwo*, *Biecheleria*, *Gyrodinium falcatum*, and *Ceratium fusus* together with the sister taxon of *Cochlodinium fulvescens*; the other group included three genera belonging to the family Kareniaceae (*Karenia*, *Takayama*, *Karlodinium*) as a sister branch of one that included *Gymnodinium instriatum* and *Gyrodinium spirale*. In the LSU phylogeny, the second main grouping ''*Gymnodinium* sensu stricto clade'' included two different clusters. One cluster consisted of two sister clades, one of them composed of different species of *Gymnodinium* including the type species *G. fuscum* (Ehrenb.) F. Stein, and the other one composed of the genus *Lepidodinium*, *Barrufeta*, and two species of *Gymnodinium*; the other cluster also included genera such as *Polykrikos*, *Gymnodinium*, and *Pheopolykrikos* with the exception of *Dissodinium* (Blastodiniales).

DISCUSSION

Daugbjerg et al. (2000) identified both the shape of the apical groove and pigment composition as critical features allowing the differentiation of unarmored dinoflagellate genera, as they were consistent with phylogenetic trees based on rDNA sequencing. This approach resulted in the redescribed genera *Gymnodinium* and *Gyrodinium* as well as new genera, such as *Akashiwo*, distinguished by differences in the shape of the apical groove (Daugbjerg et al. 2000). Another genus, *Takayama*, with yet a different apical groove shape was described later (de Salas et al. 2003). The shape of the apical groove of new genus *Barrufeta* also differs from that of other gymnodinioids. Furthermore, referring to the different kinds of apical-furrow apparatuses described in the literature (Hansen and Daugbjerg 2009, Moestrup et al. 2009a, b), we made a detailed structural comparison between the apical groove of *Barrufeta* and that of a species belonging to the genus *Gymnodinium*, *G. impudicum*. Although both contained three elongated vesicles, small knobs ornamented the central vesicle in *B. bravensis*, whereas in *G. impudicum* the lateral vesicles were ornamented. This is the first time that this difference has been noted. However, more species belonging to the two genera need to

TABLE 1. Relative abundance (%) of fatty acids in different marine unarmored dinoflagellates cultured under the same conditions and harvested in the same growth phase.

Fatty acids	*Barrufeta bravensis* (VGO864)	*Karenia brevis* (CCMP2281)	*Gymnodinium microreticulatum* (VGO328)	*Gymnodinium catenatum* (Est14H5)	*Gymnodinium impudicum* (VGO665)	*Akashiwo sanguinea* (VGO626)
C12:0	0.28	0.1	0.0	0.0	0.0	0.0
C14:1	0.0	0.5	1.0	1.1	1.0	1.3
C14:0	0.24	1.0	1.0	1.3	1.7	2.0
C15:0	0.12	0.2	0.8	1.1	2.3	0.6
C16:1*n7*	1.22	1.3	6.4	5.9	4.9	8.0
C16:0	2.30	2.8	14.7	14.0	13.2	18.0
C17:0	0.17	0.1	0.7	0.5	1.0	0.5
C18:5*n3*	0.24	0.5	2.9	1.0	0.5	1.6
C18:3	0.29	1.1	2.6	1.4	0.9	3.9
C18:2	0.95	0.0	0.0	0.0	0.0	0.0
C18:1*n9*	1.78	1.3	6.6	7.0	7.0	4.3
C18:0	4.53	3.6	19.9	23.4	23.9	13.1
C20:5*n3*	0.21	0.0	2.5	0.0	0.0	3.4
C20:4	0.0	0.0	7.8	1.3	0.0	12.2
C20:0	0.10	0.10	0.3	0.0	0.0	0.6
C22:0	0.0	0.0	0.0	0.0	0.0	0.3
C24:0	0.0	0.0	0.0	0.0	0.0	0.4
SFA	7.7	7.9	37.4	40.3	42.1	35.5
MUFA	3.0	3.1	14.0	14.0	12.9	13.6
PUFA	1.7	1.6	15.8	3.7	1.4	21.1

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

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