BIODIVERSITY IN ENCLOSED SEAS

Recurrent high-biomass blooms of *Alexandrium taylorii* (Dinophyceae), a HAB species expanding in the Mediterranean

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Abstract Summer outbreaks of the dinoflagellate *Alexandrium taylorii* Balech are recurrent events in nearshore waters of Sicily (Italy)—a central region in the Mediterranean Sea—producing dense yellowish–green patches. Beyond the local phenomenon, the problem covers a broader geographic scale, involving also other European localities, mostly in Spain.

Biological, environmental, and molecular data are reported here from a semi-closed bay of Sicily (Vulcano Island, Tyrrhenian Sea, 2000–2003), showing in summer the recurrence of high-biomass blooms and events of water discolouration. With-out underestimating the setbacks to the tourism industry, the ecological impact of *A. taylorii* blooms may be important considering the high levels of biomass produced (West Bay, Vulcano: up to a magnitude order of 10^7 cells l^{-1}, 50–180 μg-Chl$a$ l^{-1}, June 2002 and 2003) and coincident conditions of oxygen supersaturation of the waters (130–170%). Trophic trends in the Tyrrhenian site indicate high amounts of nutrients linked to the increased anthropogenic activity in summer, although recently there has been an apparent shift of the marked eutrophic conditions towards a slighter eutrophy.

Genetic data on isolates of *A. taylorii* from the Mediterranean Sea are also discussed. Molecular analyses implied the sequencing of target rDNA regions (5.8S rDNA and ITS regions) of several isolates from different Mediterranean localities, as well as the application of species-specific PCR assays for rapid species identification in preserved field samples. The confirmation of the specific identity provided new insights into the biogeography of this species and further evidence of the occurrence of *A. taylorii* in a number of Mediterranean localities, both in the western side (the Catalan coast of Spain) and the eastern area (Greece). Analyses of the molecular diversity of geographically distinct isolates of *A. taylorii* from Italy, Spain, and Greece based on the 5.8S rDNA-ITS region sequences showed a high level of similarity, indicating the existence of an unique Mediterranean population.

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Introduction

Blooms of phytoplankton organisms are recurrent events in European coastal waters and worldwide (Anderson, 1989), being potentially harmful to human health, marine ecosystems and resources such as tourism, fisheries and aquaculture. The socio-economic impact of HABs (Harmful Algal Blooms) highlights the need for ecological studies addressed to the understanding of HAB dynamics and processes of bloom formation and dispersal. Summer outbreaks of the dinoflagellate *Alexandrium taylorii* Balech produce dense yellowish–green patches of limited extension in coastal waters of Sicily, Italy (Giacobbe & Yang, 1999; Penna et al., 2002), a central island in the Mediterranean Sea. Beyond the local phenomenon, similar events are also observed in beach areas along the Costa Brava and Balearic Islands (Spain), causing in some localities water discolouration lasting two months (Garcés et al., 1999). Dense aggregates of cells are also found near the sediment, this representing a possible way of avoiding advection from the area and prevent population losses. As a whole, the observations reported in literature suggest that the magnitude of the event is increasing in some places and that this species is in a phase of expansion, probably due to ballast water release and other causes.

Some authors have shown the morphological variability of *A. taylori*, the high production of temporary cysts having a significant role in the bloom dynamics (Garcés et al., 2002), and the existence of a sexual resting stage as a part of its life cycle (Delgado et al., 1997, Giacobbe & Yang, 1999). Thus, the specific identity may be easily masked when dealing with encysted stages—vegetative or sexual resting cysts.

In this study, we examined a set of biological and environmental data obtained in the last four years (2000–2003, EU Project Strategy, EVK3-CT-2001–00046) from a Tyrrhenian locality of Sicily subject to bloom events. Sequence analyses of different strains of *A. taylorii* isolated from various Mediterranean areas, as well as results of the application of molecular probes (by PCR assays) to preserved field samples, are also provided.

Study area

The West Bay of Vulcano (Sicily), a tourist locality of the Aeolian Islands (Tyrrhenian Sea), is a shallow, semicircular area extending approximately 300×100 m. The site is located at 38°25′04″–10″ Lat N and 14°57′14″–23″ Long E and covers as a whole about 7 ha. Recurrent summer blooms of *A. taylorii* take place in some nearshore points of the bay with evident water discolouration due to the high-biomass produced by this dinoflagellate (Penna et al., 2002). Before 2000, there had been no studies on HABs in this Tyrrhenian locality, although popular references on events of red tide date back to 1995.

Materials and methods

Sample collection and analyses

Three nearshore stations (1 m depth) located in the West Bay of Vulcano (Stn 1: 38°25′4″ N, 14°57′14″ E; Stn 2: 38°25′6″ N, 14°57′19″ E; Stn 3: 38°25′10″ N, 14°57′23″ E) were sampled in spring-summer 2000–2003, on 7–19 occasions each year. 100–250 ml of surface seawater samples were collected and fixed with Lugol's solution (0.4% final concentration). Samples were taken at the same time of day (between 12 and 15 h). The bloom density was determined by cell counts of target species and other phytoplankton groups. The procedure for identification and quantification of phytoplantkon cells involved: sedimentation of a subsample in 10 or 50 ml settling chambers and counts of cells in an appropriate area (Throndsen, 1995) using an inverted Zeiss Axiovert 200 microscope equipped with epifluorescence. The identity of *A. taylorii* was confirmed by staining the cells with Calcofluor White M2R (Fritz & Triemer, 1985) and using a Zeiss UV filter set Fs 01.

Measures of environmental parameters such as water temperature, salinity and oxygen were taken by a mobile Multiprobe (YSI 556 MPS). Nutrient samples were filtered and frozen immediately after water collection. Concentrations of nitrate, nitrite and phosphate were determined following the Strickland and Parsons (1972) method, and ammonia according to Aminot & Chaussepied (1983).

Clonal cultures of *Alexandrium taylorii* were established at the Consiglio Nazionale delle Ricerche (CNR, Messina), Institut de Ciències del Mar (CSIC, Barcelona), and Instituto Español de Oceanografia (IEO, Vigo) from unfixed seawater samples taken at various sites of the Mediterranean. All marine cultures were maintained in F/2 or F/20 medium (see http://ccmp. bigelow.org/), at $18 \pm 1°C$ and 12:12 h (light:dark) photoperiod. Illumination was provided by a photon irradiance of 100 µmol m^{-2} s^{-1}.

Molecular assays

Total DNA extractions of different *A. taylorii* isolates and mixed phytoplankton populations from seawater samples collected in different localities of the Mediterranean basin—Vulcano (Italy), St. Carles, Catalan Sea (Spain), and Saronikos Gulf (Greece)—were performed by using DNeasy Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. PCR amplification conditions of the 5.8S gene and ITS regions were performed as described in Penna & Magnani (1999). PCR amplified products of all *A. taylorii* isolates used in this study were directly sequenced. The sequences were deposited at EMBL Bank and given in Table 1.

Sequence alignment was done with Clustal X software and subsequently improved by eye. The rDNA-ITS regions were aligned with other ITS sequences of *Alexandrium* species, listed in GenBank. Sequence alignment analyses were also carried out by including other sequence data of *Alexandrium* species from GenBank (Table 2). Phylogenetic relationships, based on the 5.8S rDNA-ITS regions, were inferred using the Neighbor-Joining (NJ) (Saitou & Nei, 1987). The sequence of *Gymnodinium sanguineum* Hirasaka

[=*Akashiwo sanguinea* (Hirasaka) Hansen & Moestrup] (GenBank AF131075) was used as outgroup. Robustness of the phylogenetic tree generated by NJ was tested by using the non-parametric bootstrap (Felsenstein, 1985) with 10,000 pseudoreplicates. The above analysis was performed with the software package MEGA ver. 3.0.

A. taylorii specific PCR assays were carried out on the genomic DNA of mixed phytoplankton population samples by using two species-specific designed primers (Penna et al., 2004). The PCR products were visualized on 2.0% agarose gel.

Results

Spring-summer *A. taylorii* densities in the last four years (2000–2003) showed exceptionally high values (Fig. 1), compared with the concentrations of phytoplankton usually found in Sicilian coastal waters. On various occasions (2001–2003), the bloom density reached a 10^7 order of magnitude, with maxima of 2–3×10^7 cells l^{-1} and amounts of Chl*a* as high as 50–180 µg l^{-1} in June 2002 and 2003. During these blooms, the water temperature was 27.5–32°C, salinity 37.1–37.2, dissolved inorganic nitrogen 2.42–2.88 µM DIN, phosphates 2.32–5.48 µM, with a corresponding condition of oxygen supersaturation of the waters (up to 170%). The highest concentrations of DIN were due to nitrates, or alternatively to ammonia, and phosphates in some cases reached values of ≈10 µM (2001, Fig. 1) with a slight decreasing trend through the years.

The water discolouration lasted 1–2 months, as confirmed again in 2004 and 2005 (data not shown), gradually disappearing in July or August–September (2001). The bloom persistence and discolouration was apparently favoured by optimal, summer weather conditions. The horizontal distribution of *A. taylorii* cells within the West Bay (Vulcano) was characterized in all cases by the assembling of cells nearshore, in the most sheltered part of the bay, as shown in Fig. 2, although in some cases a wind-driven advection of cells to NE of the bay was observed.

The percentage of temporary cysts versus motile cells of *A. taylorii* (Fig. 3), as determined

Table 1 List of *A. taylorii* strains, sample locations, origin, source and EMBL accession numbers from this study

Species	Strain	Sampling location and year	Origin cellular phase	Source	EMBL accession no.
A. taylorii	[a]CNR-AT4	Mediterranean, Ionian Sea, Siracusa, Italy, 1997	vegetative	M.G. Giacobbe	AJ251653
A. taylorii	[a]CNR-ATAYB2	Mediterranean, Tyrrhenian Sea, Vulcano, Italy, 2000	vegetative	M.G. Giacobbe	AJ300451
A. taylorii	Field sample	Mediterranean, Tyrrhenian Sea, Vulcano, Italy, 2000	temporary cysts	M.G. Giacobbe/A. Penna	AJ291785
A. taylorii	[b]CBA-1	Mediterranean, Aegean Sea, Kavala, Italy, 2001	vegetative	K. Koukavas	AJ416856
A. taylorii	[c]CSIC-AV8	Mediterranean, Catalan Sea, La Fosca, Spain, 1995	vegetative	E. Garcés/S. Fraga	AJ251654

[a] CNR = Consiglio Nazionale delle Ricerche, Messina, Italy

[b] CBA = Centro di Biologia Ambientale, Urbino, Italy

[c] CSIC-ICM = Institut de Cienciès del Mar, Barcelona, Spain

Table 2 List of *Alexandrium* species 5.8S rDNA and ITS region sequences used in this study

| Species	Strain	Location	Source[a]	Accession No.	Toxicity
A. affine	H1	Harima Nada, Japan	Adachi et al. 1996	AB006995	No-PSP
A. catenella	M17	Harima Nada, Japan	Adachi et al. 1996	AB006990	Yes-PSP
A. catenella	ACC01	Chile	Marin et al. 2001	AJ272120	Yes-PSP
A. fraterculus	AF-1	Pusan, Korea	Cho 1999	AF208242	No-PSP
A. insuetum	S1	Shoudoshima, Japan	Adachi et al. 1996	AB006996	No-PSP
Alexandrium sp.	AS-1	Pusan, Korea	Cho 1999	AF208243	No-PSP
A. margalefii	[b]CNR-AM1	Oliveri, Italy	Penna et al. 1999	AJ251208	No-PSP
A. minutum	LAC 27	Trieste, Italy	Penna et al. 2000	AJ005050	Yes-PSP
A. minutum	[b]CNR-AMIA1	Siracusa, Italy	Penna et al. 2003	Vila et al. 2005	Yes-PSP
A. minutum	[b]CNR-AMIA4	Siracusa, Italy	Penna et al. 2003	Vila et al. 2005	Yes-PSP
A. minutum	[b]CNR-AMIA5	Siracusa, Italy	Penna et al. 2003	Vila et al. 2005	Yes-PSP
A. minutum	[c]IEO-AL8C	Arenys, Spain	Penna et al. 2003	Vila et al. 2005	Yes-PSP
A. minutum	[c]IEO-AL9C	Arenys, Spain	Penna et al. 2003	Vila et al. 2005	Yes-PSP
A. minutum	[d]CSIC-D1	Arenys, Spain	Penna et al. 2003	Vila et al. 2005	Yes-PSP
A. pseudogonyaulax	H1	Harima Nada, Japan	Adachi et al. 1996	AB006997	No-PSP
A. tamarense	CU-15	Gulf of Thailand, Thailand	Adachi et al. 1996	AB006992	Yes-PSP
A. tamarense	FK-788	Funka Bay, Japan	Adachi et al. 1996	AB006993	Yes-PSP
A. tamarense	AT-A	Chinhae Bay, Korea	Cho 2001	AF374224	PSP?
A. tamarense	AT-B	Chinhae Bay, Korea	Cho 2001	AF374225	PSP?
A. tamarense	AT-2	Chinhae Bay, Korea	Cho 2001	AF374227	PSP?
A. tamarense	AT-6	Chinhae Bay, Korea	Cho 2001	AF374228	PSP?
A. tamarense	AT-10	Chinhae Bay, Korea	Cho 2001	AF374226	PSP?
A. tamarense	[e]CCMP116	Vigo, Spain	Penna et al. 1999	AJ005047	Yes-PSP
A. tamarense	WKS-1	Kushimoto, Japan	Adachi et al. 1996	AB006991	Yes-PSP
A. tamiyavanichii	MS-01	Sebatu, Malaysia	Usup et al. 2002	AF145224	No-PSP

[a] who sequenced the strain

[b] CNR = Consiglio Nazionale delle Ricerche, Messina, Italy

[c] IEO = Instituto Español de Oceanografia, Vigo, Spain

[d] CSIC-ICM = Institut de Cienciès del Mar, Barcelona, Spain

[e] CCMP = Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, Maine, US

Fig. 1 Spring-summer values of (a) *Alexandrium taylorii* densities, salinities, and (b) nutrients in Vulcano-St.1, Sicily. Years 2000–2003
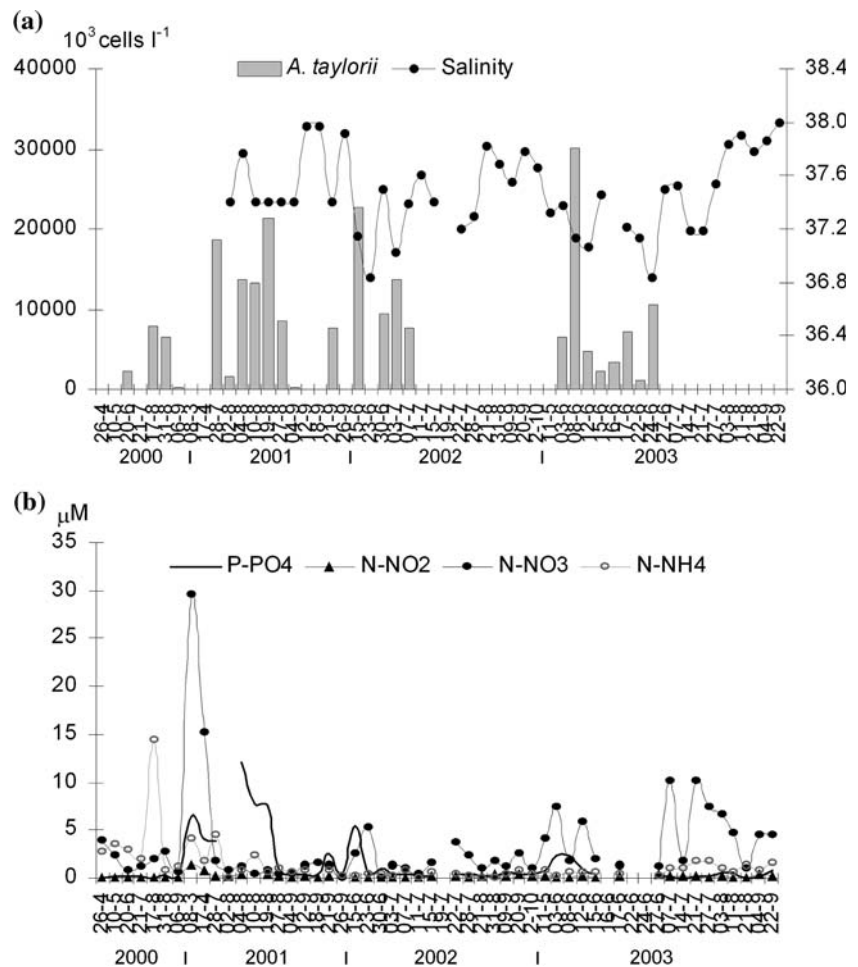
in 2001 in water samples, showed an incidence of resting vegetative stages of 7–11% over the total *A. taylorii* population, with the highest percentages near the sediment. These values increased to 22% in late summer when the bloom was declining.

The NJ phylogenetic tree of *A. taylorii* and other *Alexandrium* species based on the 5.8S rDNA-ITS regions is shown in Fig. 4. In respect to the other species of *Alexandrium* examined to date, *A. taylorii* appeared to diverge. Within the genus *Alexandrium*, the Mediterranean *A. taylorii* isolates formed a homogeneous clade, distinct from the others. These isolates shared identical nucleotide sequences.

Species-specific PCR assays of fixed field samples are shown in Fig. 5. By using species-specific primers designed for *A. taylorii*, PCR amplifications of the 5.8S rDNA-ITS regions yielded

products of 297 base pair size for the field samples containing *A. taylorii* cells. Species-specific PCR amplifications for *A. taylorii* revealed positive results for the presence of this species in the Vulcano phytoplankton population samples in agreement with the microscopic examination.

Discussion

Without understimating the setbacks to the tourism industry, the ecological impact of *A. taylorii* blooms may be important considering the high levels of biomass produced, as in the case of the West Bay, Vulcano (up to 3×10^7 cells l^{-1}) and coincident conditions of oxygen supersaturation of the waters, that may threaten marine life. Trophic trends in the Tyrrhenian target site

Fig. 2 Horizontal distribution of *A. taylorii* cells in the West Bay, Vulcano. Surfer plot of 22 July 2001. Triangles (1–3) indicate routine sampling stations. Asterisks indicate additional cruise stations
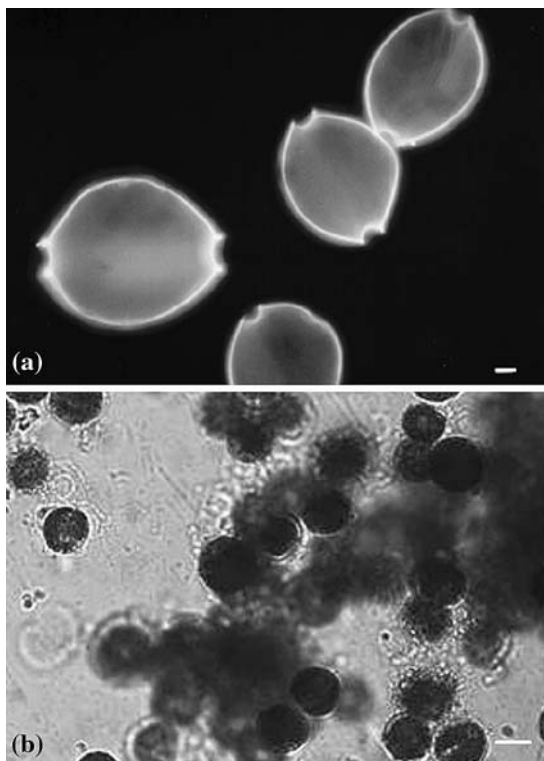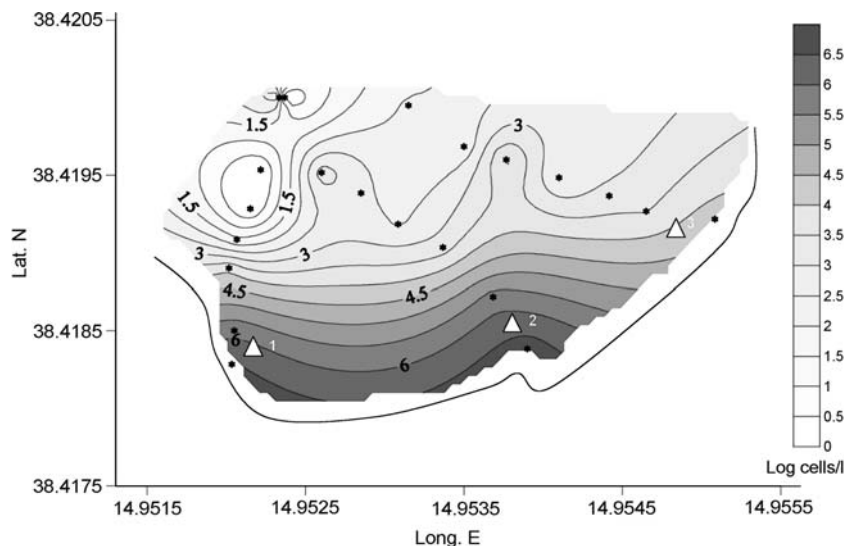

Fig. 3 General morphology of motile cells (fluorescence) and temporary cysts (bright field). Scale bars = 4 μm (**a**) and 25 μm (**b**)

apparent shift of the marked eutrophic conditions towards a slighter eutrophy. This could be due to a recent improvement of the sewage treatment. Previous studies in this area suggested that nutrients play a relevant role in the development and maintenance of *A. taylorii* blooms, the high concentrations in summer of inorganic nitrogen and phosphorus being probably due to the hotel sewage, when the anthropogenic pressure increases in relation to the tourism (Penna et al., 2002). Then, climatic conditions such as high water temperature, calm sea and weak tide, seem to favour the bloom development. Garcés et al. (2002) suggested that under short-term environmental perturbations, for instance storms or swells, *A. taylorii* encysts to withstand the unfavourable conditions and restores the population after a few days of calm weather, thus functioning as a population stock. In addition, encystment would also reduce the population losses from the area, also through cell aggregation in clusters of cysts. This, together with the physical characteristics (relatively low water renewal) and favourable wind conditions, may favour population growth and persistence (Garcés et al., 1999, Basterretxea et al., 2005).

Many factors, natural or anthropogenic, are known to contribute to the spreading worldwide of HAB species. Such phenomena are amplified by human activities, such as the wrong

indicate high amounts of nutrients linked to the increased anthropogenic activity in summer (see also Penna et al., 2001), although we observed an

Fig. 4 Neighbor-Joining (NJ) tree inferred from the 5.8S rDNA and ITS sequences of *Alexandrium* species. Sequences of *A. taylorii* from this study are in bold. *G. sanguineum* was used as an outgroup. Numbers on the major nodes represent NJ (10000 pseudo-replicates) bootstrap values. Only bootstrap values ≥ than 50% are shown
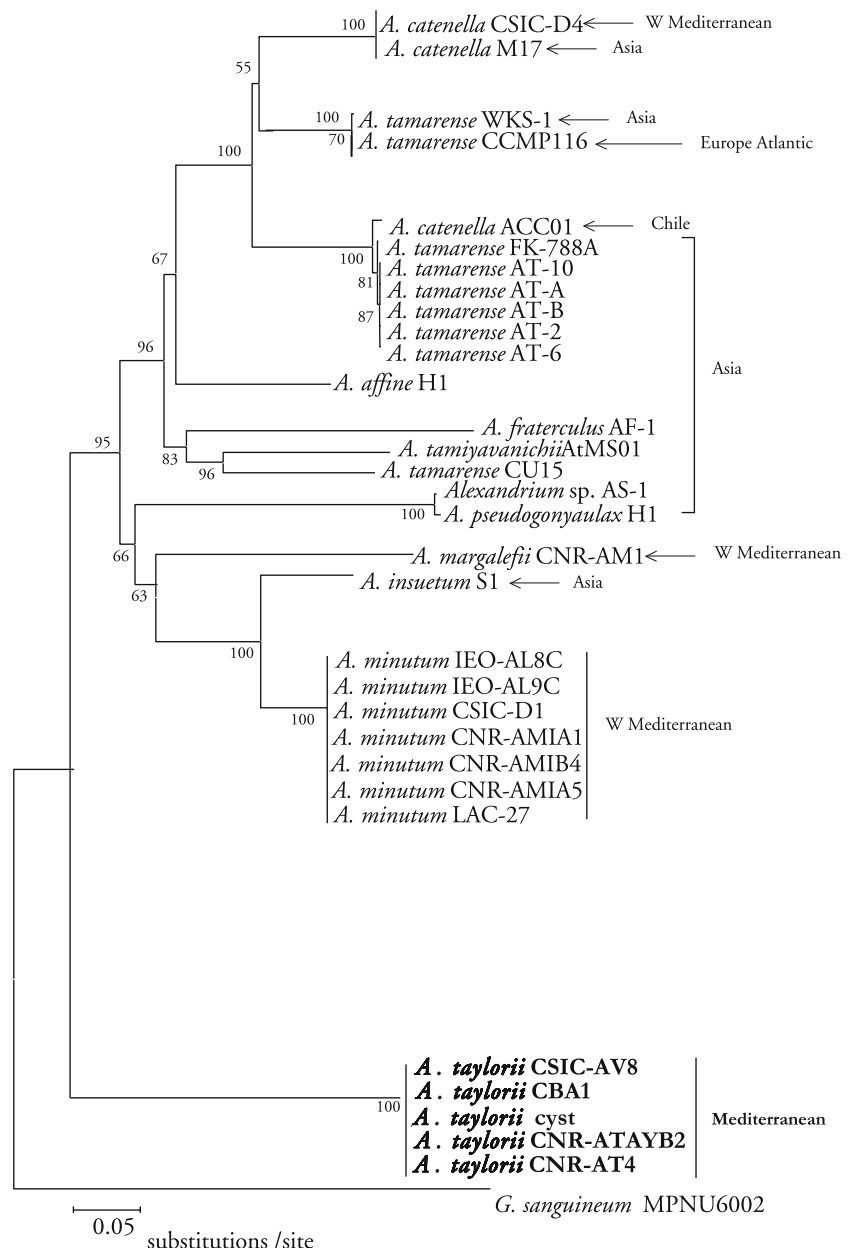
management of the coastal area leading to increased amounts of nutrients in the waters and eutrophy, lack of seawage treatments, use of fertilizers in agriculture, the sea discharge of ship's ballast water favouring the introduction of non-indigenous organisms, the so-called "alien species". Cell dispersion can also be favoured by anthropogenic means, as plastics (Masò et al., 2003) or movement of recreational boats in summer. Therefore, areas previously free by harmful species can later become subject to HABs, if such species find in the new environment conditions favouring cell growth.

In the specific case of *A. taylorii*, we obtained further evidence for its spreading in the Mediterranean region, from the western side (the Catalan coast of Spain) to the eastern area (Greece).
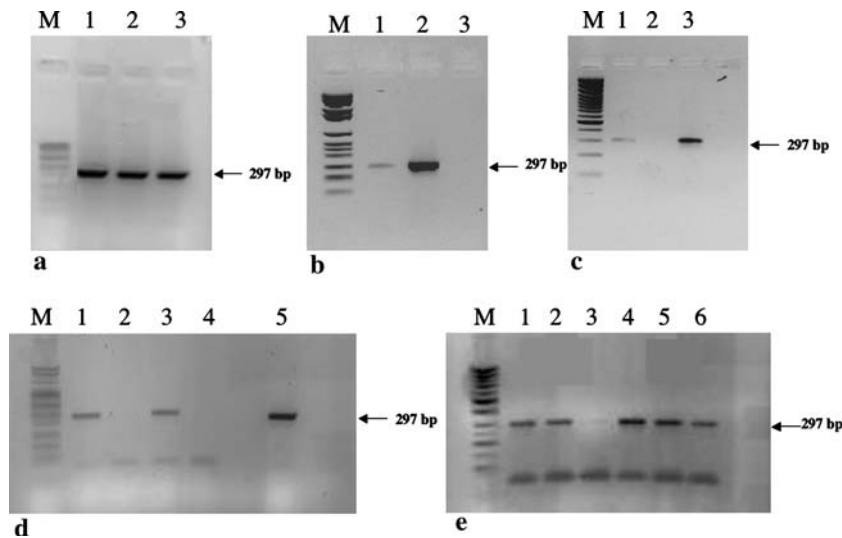
Fig. 5 *A. taylorii* specific PCR assays of mixed phyto-plankton population samples from different Mediterranean localities. (**a**) template plasmid containing *A. taylorii* 5.8S rDNA-ITS regions at different concentrations: lane 1, 10 ng; lane 2, 1 ng; lane 3, 0.1 ng. (**b**) Saronikos Gulf (Greece) field sample: lane 1, phytoplankton template DNA (1 ng); lane 2, template plasmid containing *A. taylorii* 5.8S rDNA-ITS regions (1 ng); lane 3, negative control (no template DNA). (**c**) St. Carles field sample, Catalan Sea (Spain): lane 1, phytoplankton template DNA (1 ng); lane 2, negative control (no template DNA); lane 3, template plasmid containing *A. taylorii* 5.8S rDNA-ITS regions (1 ng). (**d**) Vulcano West Bay field samples, Tyrrhenian Sea (Italy), 15th June 2003: lane 1, Station1 phytoplankton template DNA (1 ng); lane 2, Station

3 phytoplankton template DNA (1 ng); lane 3, Station2 phytoplankton template DNA (1 ng); lane 4, Station Capo Testa Grossa phytoplankton template DNA (1 ng); lane 5, template plasmid containing *A. taylorii* 5.8S rDNA-ITS regions (1 ng). (**e**) Vulcano field sample, West Bay Station1: lane 1, phytoplankton template DNA (10 ng), 16th June 2003; lane 2, phytoplankton template DNA (0.1 ng), 16th June 2003; lane 3, phytoplankton template DNA (0.01 ng), 16th June 2003; lane 4, phytoplankton template DNA (10 ng), 17th June 2003; lane 5, phytoplankton template DNA (0.1 ng), 17th June 2003; lane 6, phytoplankton template DNA (0.01 ng), 17th June 2003. M, size standards; arrow indicates reactions that produced a single species-specific PCR product

Aiming at the improvement of the HAB detection tools, species-specific primers for *A. taylorii* were applied in this study in parallel to conventional techniques. This allowed the detection of the target organism also when it occurred as resting stage and the exact species identity was more difficult to be determined. The application of the PCR analysis to field seawater samples, evidenced the presence of *A. taylorii* in all mixed phytoplankton population samples collected in different sampling points in the Vulcano West Bay, with exception of the Station 3, where the cell abundance was often extremely low. Further, the species-specific primer application to the PCR analyses resulted also suitable for the Greek and Spanish field samples containing the target species. These results on the species-specific detection of *A. taylorii* by PCR assay were always in agreement with the microscopic examination.

The phylogenetic analyses of geographically distinct isolates of *A. taylorii* (Italy, Spain, Greece) indicated the existence of a unique, homogeneous Mediterranean population, constituted by isolates with identical ITS-5.8S rDNA sequences.

In this study, the confirmation of the specific identity through the coupling of microscopic and molecular analyses provided new insights into the biogeography of *A. taylorii*, showing the extension of its geographic range from the western Mediterranean to the eastern area. Future researches will give priority to the advancement in both control and prevention strategies as safeguard of the coastal marine environment and public health, and development of advanced technologies for the rapid detection and mitigation of HAB events through laboratory experiments and in situ application.

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