UNIVERSITY OF SASSARI

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Dissertation for the Degree of Doctor of Philosophy in Environmental Biology presented at Sassari University in 2013

XXVI cycle

MOLECULAR CHARACTERIZATION OF HARMFUL ALGAL SPECIES

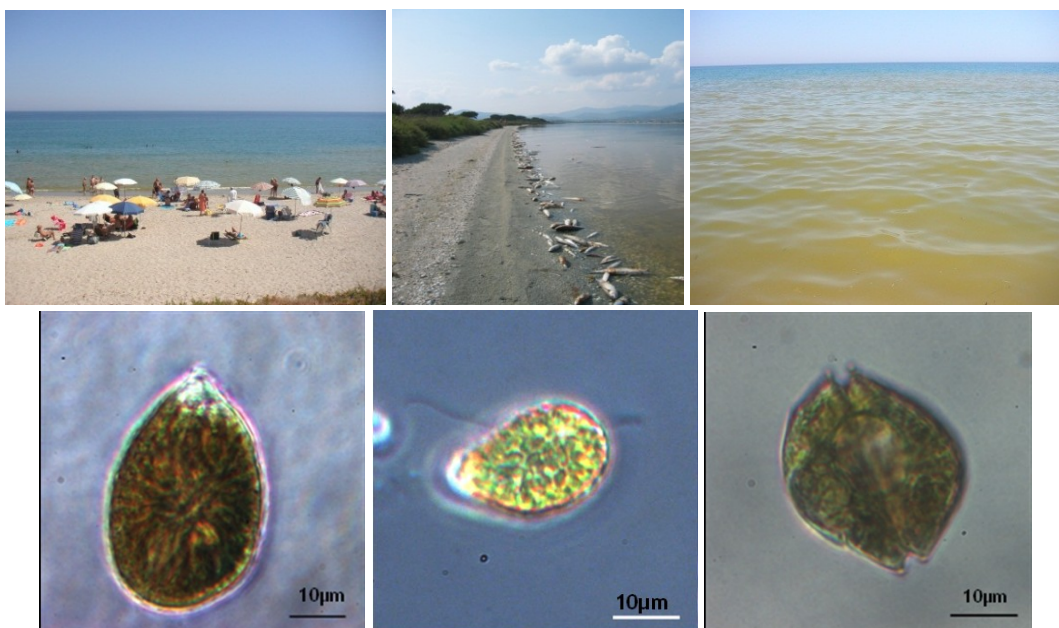PH.D. CANDIDATE: **Dr. Daniela Stacca**   DIRECTOR OF THE SCHOOL: **Prof. Marco Curini Galletti**

SUPERVISOR: **Prof. ssa Antonella Lugliè**

CO-SUPERVISOR: **Dr. Cecilia Teodora Satta**

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To my parents, thank you for everything and forever

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PREFACE

The present thesis, entitled *Molecular characterization of harmful algal species*, summarizes the activities and the results of the Dr. Daniela Stacca's Ph.D. project - Ph.D. degree in Enviromental Biology of the Ph.D. School in Natural Sciences of the University of Sassari (Italy), planned and supported by the Department of Sciences for Nature and Environmental Resources (DipNET) of the University of Sassari (Italy).

The study was performed under the Prof. Antonella Lugliè supervision and Dr. Cecilia Teodora Satta (DipNET) co-supervision. Other researchers significantly involved in the study were: Dr. Antonella Penna and Dr. Esther Garcés with their research groups.

In particular, during the first year, a training period was carried out under Dr. Garcés scientific supervision and with the support of Dr. René and Dr. Anglés, at the Institut de Ciéncies del Mar of Barcelona (Departament de Biologia Marina i Oceanografia). This period was fundamental to learn the basic techniques for the application of the most suitable molecular methods to achieve the expected objectives. At the beginning of the third year, another training period was done under Dr. Antonella Penna scientific supervision and with the support of Dr. Silvia Casabianca at the Department of Biomolecular Science, University of Urbino, to resolve methodological problems arose during the study and obtain the expected results. The collaboration with Dr. Anna Milandri and Dr. Elena Riccardi of the Fondazione Centro Ricerche Marine, Laboratorio di Riferimento Nazionale per le Biotossine Marine di Cesenatico, permitted to obtain information on the toxicity of *Alexandrium* strains.

The thesis consists of a general introduction, aims, four chapters (scientific papers) in accepted, submitted or manuscript form, and general conclusions.

The first part of the introduction summarizes a brief overview on the main aspects of the Harmful Algal Blooms (HABs). The second part is on the general knowledge of HABs in Sardinian aquatic ecosystems and presents the study cases considered in the thesis. The third part concerns the molecular approaches applied in this thesis to study Harmful Algal Species (HAS).

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Chapter 1 – Case study I: *Alexandrium* species

- a) The genus *Alexandrium* Halim in the Mediterranean Sea: new contributions from Sardinia (Italy)". Stacca D., Satta C.T., Riccardi E., Milandri A., Bazzoni A.M., Pulina S., Padedda B.M., Sechi N., Lugliè *A.* Manuscript form.

- b) "Dinoflagellate cyst assemblages in surface sediments from three shallow Mediterranean lagoons (Sardinia, North Western Mediterranean Sea)". Satta C.T., Anglès S., Garcés E., Sechi N., Pulina S., Padedda B.M., Stacca D., Lugliè A. *Estuaries and Coasts*, DOI 10.1007/s12237-013-9705-1.

Chapter 2 – Case study II: *Chattonella* species

- "Long-term *Chattonella* (Raphidophyceae) blooms: species identification on archived fixed samples from a Mediterranean lagoon". Stacca D., Satta C.T., Casabianca S., Penna A., Padedda B.M., Sechi N., Lugliè A.. Submitted to *Mediterranenan Marine Science*.

Chapter 3 – Case study III: Harmful algal species in beaches

"Interdisciplinary approaches to study harmful algal blooms (HABs) in beach environments: an experience from the Mediterranean Sea". Satta C.T., Padedda B.M., Stacca D., Simeone S., De Falco G., Penna A., Capellacci S., Pulina S., Perilli A., Sechi N., Lugliè A. To be submitted to *Advances in Oceanography and Limnology*.

Chapter 4 – Case study IV: Harmful dinoflagellate species in man-made lakes

"Potentially Harmful Algal Blooms in Mediterranean artificial lakes due to dinoflagellates: case studies from Sardinia". Satta C.T., Stacca D., Padedda B.M., Mariani M.A., Lai G., Sechi N., Lugliè *A.* Manuscript form.

In the final part a scientific paper published in Italian is attached:

- Aspetti dell'ecologia dei sistemi acquatici della Sardegna e loro principali problematiche. - Lugliè A., Padedda B.M., Bazzoni A.M., Caddeo T., Casiddu P., Farina P., Lai G., Manca B., Mariani M.A., Pulina S., Satta C.T., Stacca D., Sechi N., 2013. Atti dell'Assemblea generale e del *Symposium* I mari delle isole (Resèaux d'Excellence des Territoires Insulaires – RETI).

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ABSTRACT

The main purpose of the research activities carried out in this thesis was to give contributions to ecological studies on potentially harmful algal species (HAS) present in Sardinia's aquatic environments through the application of biomolecular techniques. In Sardinia, as well as globally in the world, the reports of blooms caused by HAS (Harmful Algal Blooms, HABs) have increased in recent decades, requiring specific studies and investigations.

The identification of the species involved is particularly important. Microalgae species identification based on cell morphology with the traditional microscopic methods is not a simple task. A valid support is represented by the recently developed biomolecular tecniques which can help in resolving species identification and detection problems.

In this thesis, several cases of study were investigated, regarding different types of environments, algal classes, genera, species within a same class and, therefore, different linked problems. Analyses were carried out on natural samples fresh or fixed and cell cultures obtained from isolated vegetative cells and/or cysts of resistance. The identification of HAS was carried out using molecular techniques, with nucleotide sequences of the LSU (or 28S rDNA) and 5.8S rDNA-ITS (internal transcribed spacer) regions such as genetic markers.

The confirm of taxonomic attribution was achieved for new isolates of the *Alexandrium* Halim genus, analysing cell cultures obtained by the germination of cysts isolated from sediments and the growth of vegetative cells isolated from the water column from different areas of Sardinia (Chapter Ia, Ib).

The presence of *Chattonella subsalsa* Biecheler was assessed in samples collected from the Santa Giusta Lagoon during events of fish kills in the summers of 1994, 1998, 1999 and 2010 by analyzing samples fixed with Lugol and conserved in the Department's collection (Chapter II).

It was possible obtain a preliminary framework of the HAS's presence in beach environments, including *Alexandrium taylorii* Balech, *Ostreopsis* cf. *ovata* Fukuyo,

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Barrufeta bravensis Sampedro et Fraga and *Gymnodinium instriatum* Freudenthal et Lee (Chapter III).

Finally, the first study on dinoflagellates species involved in blooms in reservoirs of Sardinia, was carried out (Chapter IV).

KEY WORDS: *Harmful algal blooms,* 5.8S-ITS rDNA, 28S rDNA*, Alexandrium*, *Ostreopsis*, *Chattonella*, Mediterranean Sea, man-made lakes.

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RIASSUNTO

L'attività di ricerca ha avuto lo scopo principale di offrire un contributo agli studi ecologici di specie algali potenzialmente nocive (Harmful Algal Species, HAS) presenti in ambienti acquatici della Sardegna, attraverso l'applicazione di tecniche di biologia molecolare. In Sardegna, così come nel resto del Mediterraneo e globalmente nel pianeta, la segnalazione di fioriture provocate dalle HAS (Harmful Algal Blooms, HABs) è aumentata negli ultimi decenni, richiedendo studi e approfondimenti specifici.

Di particolare importanza è il riconoscimento certo delle specie coinvolte, la cui identificazione è spesso problematica con le normali metodiche microscopiche, basate sulla morfologia cellulare. Recentemente le tecniche di biologia molecolare si sono rivelate davvero utile nel fornire rapidamente informazioni sulle HAS presenti in un'area.

In questa tesi si sono affrontati diversi casi di studio, prendendo in considerazione diverse classi algali, e all'interno delle classi, diversi generi e specie, provenienti da differenti tipologie di ambienti e quindi responsabili di problematiche diverse. Si è lavorato sia su campioni freschi che fissati, su campioni naturali e colture cellulari, su cellule vegetative e cisti di resistenza. L'identificazione delle HAS studiate è stata fatta con tecniche molecolari usando come marcatori genetici le sequenze nucleotidiche della regione LSU (o rDNA 28S) e dell'rDNA 5.8S con le regioni ITS (spaziatori trascritti interni). I risultati ottenuti hanno permesso di caratterizzare nuovi isolati del genere *Alexandrium* Halim, analizzando le colture cellulari ottenute con la germinazione di cisti isolate dal sedimento e la crescita di cellule vegetative isolate dalla colonna d'acqua di diverse aree della Sardegna (Capitolo I).

Si è potuta definire con certezza la presenza di *Chattonella subsalsa* Biecheler nei campioni prelevati dalla Laguna di Santa Giusta durante gli eventi di morie ittiche nelle estati del 1994, 1998, 1999 e 2010, analizzando campioni fissati con la soluzione di Lugol presenti nella collezione del Dipartimento (Capitolo II).

Si è ottenuto un quadro preliminare sulla presenza di HAS in ambienti di spiaggia, tra cui Balech, *Ostreopsis* cf. *ovata* Fukuyo, *Barrufeta bravensis* Sampedro e Fraga e *Gymnodinium instriatum* Freudenthal et Lee (Capitolo III).

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Infine si è iniziato uno studio sulle specie di dinoflagellati coinvolte in fioriture nei laghi artificiali della Sardegna.

PAROLE CHIAVE: Harmful algal blooms, 5.8S-ITS rDNA, 28S rDNA, *Alexandrium, Ostreopsis, Chattonella,* Mediterraneo, laghi artificiali.

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1. INTRODUCTION

1.1 Harmful Algal Blooms

Phytoplankton has a fundamental role in the food chain in aquatic environments and its natural ability to give rise to large proliferations (blooms) is considered a benefit for the secondary production, both in natural environments and aquatic farms. Nevertheless, in some instances, these events can have negative impacts on humans (both on health and economic activities), aquatic organisms and on the whole ecosystem. These types of blooms are known as Harmful Algal Blooms (HABs). This phenomenon is known from ancient times (e.g. Hallegraeff, 2003), but in recent decades it has been showing a strong increase, at least apparently. The expansion of HABs is correlated with different factors, such as eutrophication, changes in the stoichiometric ratios of nutrients (Anderson *et al.*, 2002; Heisler *et al.*, 2008), hydrodynamic modifications in coastal systems (e.g. creation of confinement areas; Vila *et al.*, 2001; Masó and Garcés, 2006), but also with the introduction of new species in a geographical area (Hallegraeff and Bolch, 1992), the over-exploitation of marine resources, especially with fisheries (Vasas *et al.*, 2001; Walsh *et al.*, 2011), and the possible effects of climate changes induced by human activities (Hallegraeff, 2010). Therefore, many causes appear to contribute and act contemporaneously on the increase of HABs, and each single effect is very difficult to be distinguished by the others. The increase in reports and recurrence of HAB events in the world can be explained with a variety of possible factors of influence (Anderson, 1989; Hallegraeff, 2003; Zingone, 2010), among which:

- the intensification of monitoring and a greater scientific interest that has developed in parallel in the world, with the advance of knowledge and skills study;
- the stimulation related to human activities on the coastal water (e.g. aquaculture);

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- the stimulation of blooms due to eutrophication and/or unusual climatic condition;

- the increase of HAS geographical distribution caused by the transport of resting cysts with the ballast waters and with the displacement of fishes stocks from one area to another (Garcés *et al.*, 2000; Zingone and Enevoldsen, 2000; Hallegraef 2003), but also with the transport on plastic debris by currents (Masó *et al.*, 2003).

The HAS are about 7% of the known marine species and belong to different algal groups: Dinophyceae, Bacillariophyceae, Raphidophyceae, Haptophyceae, Pelagophyceae and Cyanobacteria. Since each species has ecological characteristics and different physiological responses to environmental solicitations, there is a considerable heterogeneity in the typology of dynamics and impacts of the harmful blooms (Zingone and Enevoldsen, 2000).

These events occur with a broad range of associated phenomena (Zingone and Enevoldsen, 2000; Hallegraeff, 2010), which are summarized in Table 1. Their noxious effects can be divided into three groups on the basis of the impacts: a) on human health, b) on marine resources and/or ecosystems, c) on tourism and/or recreation use of water:

a) about 80 species are capable to produce toxins (Sournia, 1995). Certain algal toxins can cause different syndromes associated with a variety of neurological and/or intestine disorders in humans after consumption of contaminated organisms (such as bivalves, gastropods, crustaceans, fishes; Zingone and Enevoldsen, 2000), and they may be fatal in some cases. The most known syndromes for human health are: Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning, Amnesic Shellfish Poisoning (ASP) and Ciguatera Fish Poisoning (CFP). Other algal toxins can provoke respiratory problems and skin irritation due to aerosol or direct contact, e.g. during bathing (Sansoni *et al.*, 2003). The species mainly involved in this kind of impact are dinoflagellates and diatoms;

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b) in addition to the effects on human health, algal toxins can cause massive kills of fishes and others biota. They are implicated in death episodes of marine mammals, birds and other organisms related with the aquatic ecosystem in which toxic algal species develop (Van Dolah, 2000). The HAS can affect aquatic biota also with the production of mucilage, which can obstruct the gills. Further, the algal cells can be equipped with spines and other processes that can injury gills or other parts of the aquatic organisms. The responsible species can belong to dinoflagellates, diatoms, raphidophytes, prymnesiophytes and cyanobacteria. Another effect is related to high biomass blooms, which reduce the quantities of dissolved oxygen, due to organic matter degradation. The consequent hypoxic/anoxic conditions could cause devastating impacts on pelagic component (e.g., fishes) and benthonic ones (e.g., invertebrates and macrophytes) (Hallegraeff, 2003). Benthic macrophytes can suffer also in the case of intense and prolonged shading of the bottom due to high density blooms, such as those of pelagophytes;

c) the abnormal increase in abundance of one or more species can reduce transparency of water and alter its coloration, causing discoloration of waters (e.g., reddish, brownish). This phenomenon causes consequences on aesthetic characters of the landscape, impacting on tourism and recreational use of the waters.

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Table 1 Overview of the deleterious effects caused by HAS and HABs (Zingone and Enevoldsen, 2000).

EFFECTS	EXAMPLES OF CAUSATIVE ORGANISMS	
Human health		
Paralytic shellfish poisoning (PSP)	Dinoflagellates	*Alexandrium* spp., *Pyrodinium bahamense* var. *compressum*, G*ymnodinium catenatum*
	Cyanobacteria	*Anabaena circinalis*
Diarrhetic shellfish poisoning (DSP)	Dinoflagellates	*Dinophysis* spp., *Prorocentrum* spp.
Neurotoxic shellfish poisoning (NSP)	Dinoflagellates	*Karenia brevis*
Amnesic shellfish poisoning (ASP)	Diatoms	*Pseudo-nitzschia* spp.
Ciguatera shellfish poisoning (CFP)	Dinoflagellates	*Gambierdiscus toxicus*
Allergies, respiratory problems and skin irritation	Dinoflagellates Cyanobacteria	*Ostreopsis* spp. *Microcystis aeruginosa, Nodularia spumigena*
Natural and cultured marine resources		
Haemolytic, hepatotoxic, osmoregulatory effects and other unspecified toxicity	Dinoflagellates	*Gymnodinium* spp., *Cochlodinium polykrikoides*, *Pfiesteria piscicida, Gonyaulax* spp.
	Raphidophytes	*Heterosigma akashiwo, Fibrocapsa japonica*
	Prymnesiophytes	Chrysochromulina spp., Prymnesium spp.
	Pelagophytes Cyanobacteria	*Aureococcus anophagefferens* *Microcystis aeruginosa*
Mechanical damage	Diatoms	*Chaetoceros* spp.
Gill clogging and necrosis	Prymnesiophytes Diatoms	*Phaeocystis* spp. *Thalassiosira* spp.
Tourism and recreational activities		
Production of foams, mucilages, discoloration, repellent odours	Dinoflagellates Prymnesiophytes Diatoms Pelagophytes Cyanobacteria	*Noctiluca scintillans* *Phaeocystis* spp. *Cylindrotheca closterium* *Aureococcus anophagefferens* *Nodularia spumigena*
Marine ecosystem		
Hypoxia, anoxia	Dinoflagellates Diatoms Prymnesiophytes	*Noctiluca scintillans, Heterocapsa triquetra* *Skeletonema costatum* *Phaeocystis* spp.
Negative effects on feeding behaviour, reduction of water clarity	Pelagophytes	*Aureococcus anophagefferens,* *Aureoumbra lagunensis*
Toxicity to wild marine fauna	Dinoflagellates Diatoms	*Karenia brevis, Alexandrium* spp. *Pseudo-nitzschia australis*

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1.2 Harmful Algal Blooms in Sardinia and study cases

Since the 1970s, HABs have been recognized as one of the main ecological problems in coastal regions worldwide (Masó and Garcés, 2006). In recent decades the frequency and the areas affected by HABs have increased, interesting also the Mediterranean Sea (Garcés *et al.*, 1999, 2001; Vila *et al.*, 2005; Penna *et al.*, 2005; Giacobbe *et al.*, 2007; Genovesi *et al.*, 2011) and, within it, Sardinia (Lugliè *et al.*, 2011). The first HABs report in Sardinian waters regarded the Gulf of Olbia in 1987 (Sechi *et al.*, 1987) whereas the first study on HAS was carried out by Sannio *et al*. (1997), highlighting the presence of harmful species in different shellfish farming areas. Over the years and with the increase of controlled areas, the list of harmful algal species has increased (Lugliè *et al.*, 2011).

The main impacts caused by HABs in Sardinian coastal marine ecosystems were (Fig. 1):

- water discoloration, known more generally as "red tide" (Satta *et al.*, 2010), which could have important effects on water quality and, consequently on tourism;

- massive death of both fishes and others components of aquatic communities, caused by high biomass and toxic blooms, (Sechi *et al.*, 2001; Padedda *et al.*, 2012), with consequences on production and biodiversity;

- alarm for human health due to toxins through aerosols or by direct contact in areas of high tourist exploitation (ARPAS, 2009), also affecting the water recreational uses;

- block of harvesting and marketing of mussels and alarm for the human health, consequent to the accumulation of algal toxins in the tissues of shellfishes over the legal thresholds (Lugliè *et al.*, 2004), with economic consequences and possible arise of human syndromes.
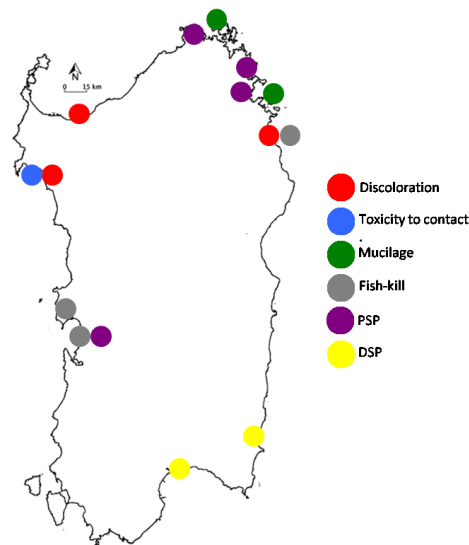
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Figure 1 Ascertained cases of HAB impacts along the Sardinian coast (Lugliè *et al.*, 2011).

In Sardinia, diarrhetic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP) positive cases have occurred in shellfish farms over the years (Tab. 3). The most frequent and problematic blooms were caused by *Alexandrium* genus Halim (Order: Gonyaulacales, Class: Dinophyceae). Species within this genus are associated with PSP in coastal waters worldwide, from boreal to tropical latitudes (Martins *et al.*, 2004). In the Mediterranean Sea, different species of this genus are reported, both toxic and not (Fraga *et al.*, 2004; Genovesi *et al.*, 2011). In Sardinia, eight toxic HABs had occurred in seven years (from 2002 to 2009), with a rapid expansion of both areas affected and seasonality of occurrence (Tab. 3) (Lugliè *et al.*, 2003; 2011). The first harmful bloom of *A. minutm* Halim and *A. catenella* (Whendon et Kofoid) Balech occurred in the Gulf of Olbia in 2002 and, then, in 2003, with saxitoxins (family of potent neurotoxins) values more than 800 μg kg^{-1} (Lugliè *et al.*, 2004; Milandri *et al.*, 2008). These high values resulted in health warning and block of mussels harvesting and marketing. In the following years, this kind of toxic HABs occurred in the Gulf of Oristano (2006 and 2008), Gulf of Cugnana (2005 and 2008) and in Porto Pozzo Lagoon (2008 and 2009). The most serious cases of water discoloration in beach environments were caused by dinoflagellates of the same genus, i.e. *Alexandrium taylorii* Balech (Satta *et al.,* 2010).

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In Sardinian beaches and/or other recreational marine sites, *Ostreopsis* cf. *ovata* Fukuyo bloomed with potential toxicity via direct contact (ARPAS, 2009).

Extended death of fishes and other components of the aquatic communities occurred more frequently in the Sardinian lagoons (Lugliè *et al*., 2013).

Table 3 Toxic HABs in shellfish farming areas of Sardinia from 1992 to 2010 (Lugliè *et al*., 2011).

Positivity	*Period*	*Locality*	*Species*
DSP	June 2001	Santa Gilla	-
	September-October 2003	Santa Gilla	*Dinophysis sacculus; D. caudata*
	January 2003	Feraxi	*Dinophysis fortii*
PSP	May-June 2002	Olbia	*Alexandrium catenella, A. minutum*
	April-May 2003		*Alexandrium catenella, A. minutum*
	January 2005	Cugnana	*Alexandrium minutum*
	December 2008		*Alexandrium catenella, A. minutum*
	November 2006	Ostistano	*Alexandrium catenella, A. minutum*
	October-November 2008		*Alexandrium catenella, A. minutum*
	December 2008	Porto Pozzo	*Alexandrium minutum*
	February 2009		*Alexandrium minutum*

The assessed HABs and their increasing trend in the last decade have highlighted the necessity to amplify the knowledge on the ecology of the involved species and their blooms, with the aim to develop strategies to reduce their negative impacts. On this basis, this thesis gives new information on HAS biodiversity in aquatic ecosystems of Sardinia, considering four study cases.

The first concerns the *Alexandrium* genus, which has caused, as reported previously, the more frequent and noxious toxic events in Sardinia. Different HAS of this genus were detected in the marine coastal zone of Sardinia in the last decade, e.g. *Alexandrium tamarense* (Lebour) Balech in Porto Torres harbour (Penna *et al*., 2008),

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A. taylorii in Platamona beach (summers 2007-2009; Satta *et al.*, 2010), *A. catenella* and *A. minutum* in shellfish farming in the Gulf of Olbia (Lugliè *et al.*, 2004) and in the Gulf of Oristano (Lugliè *et al.*, 2011), *A. tamutum* Montresor, Beran & John in the Gulf of Olbia (Fraga *et al.*, 2004). The species of *Alexandrium* are phenotypically very similar, so that discrimination of the morphological characters used for their identification is not a simple task. Over the last decades, their morphology-based taxonomy has been subjected to continuous revisions, and the application of molecular analysis to *Alexandrium* strains has proved useful (Penna *et al.*, 2008; Lilly *et al.*, 2007). In this thesis, the purpose was to increase the knowledge on isolates from different areas of Sardinia and characterize them in morphology, genetics and toxin productions.(Chapter Ia and Ib).

The second study case is on the genus *Chattonella* Biecheler (Raphidophyceae), which includes HAS causing fish kills in natural environments and aquaculture systems. In Sardinia, blooms of *Chattonella* species have been observed in lagoons, often concomitant with fish kills, such as in the cases of Santa Giusta Lagoon (Sechi *et al.*, 2001) and S'Ena Arrubia (Sannio *et al.*, 1997). During the blooms, difficulties in the taxonomic determination of *Chattonella* species due to the species pleomorphology (Khan *et al.*, 1995; Park *et al.*, 2012) and loss of morphological characteristics in fixed samples (Band-Schmidt *et al.*, 2004; Zingone *et al.*, 2006), prevented the definitive identification using traditional light microscopy. On a first analysis, on the base of alive cells observation, the species was attributed to *C. subsalsa* Biecheler. *C. subsalsa* shows cell morphology very similar to *C. marina* Hara et Chihara. Some of their taxonomic characters overlap, such as the length and the width of the cell (Hallegraef and Hara, 2003). Consequently, it was not excluded the hypothesis of mixed blooms of the two species. This also in consideration that *C. marina* is a well known HAS whereas harmfulness of *C. subsalsa* is more recent. Moreover, the harmful action's mechanisms are better known for *C. marina*, although the debate is still in progress (Endo *et al.*, 1992; Ishimatsu *et al.*, 1996; Khan *et al.*, 1996; Munday and Hallegraef, 1998; Bourdelais *et al.*, 2002; Shen *et al.*, 2010, 2011). Instead the potential harmful effects

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of *C. subsalsa* are less known and only few studies indicate the production of toxic substances (such for example: brevetoxins, radicals and peroxide) (Band-Schmidt *et al.*, 2012; Imai and Yamaguchi, 2012). Despite *C. subsalsa* has been described in a channel of the Salins de Villeroy (along the French coast) in the Mediterranean Sea, the first blooms of this species occurred in the Algeri harbour (Holland and Enjument, 1956) and in Spain, in Barcelona harbour (Margalef, 1968). During these events, only Holland and Enjument reported a contemporary presence of a large kill of fishes, molluscs and crustaceans. In their work the authors refer to *C. subsalsa* as a synonym for *Hornelia marine* Subrahmanyan (= *Chattonella marina* (Subrahmanyan) Hara and Chihara), thus generating confusion about the true nature of the species involved. Even if a cause-effect relationship between *Chattonella* blooms and fish kills in Santa Giusta Lagoon cannot be supported, the concomitance of *Chattonella* blooms and harmful events over the years can be highlighted (Fig. 2). In this thesis, the molecular analyses of archived samples (years 1994, 1998, 1999, 2010) was carried out to identify, with a retrospective approach, which *Chattonella* species occurred during harmful blooms in Santa Giusta Lagoon (Chapter II).
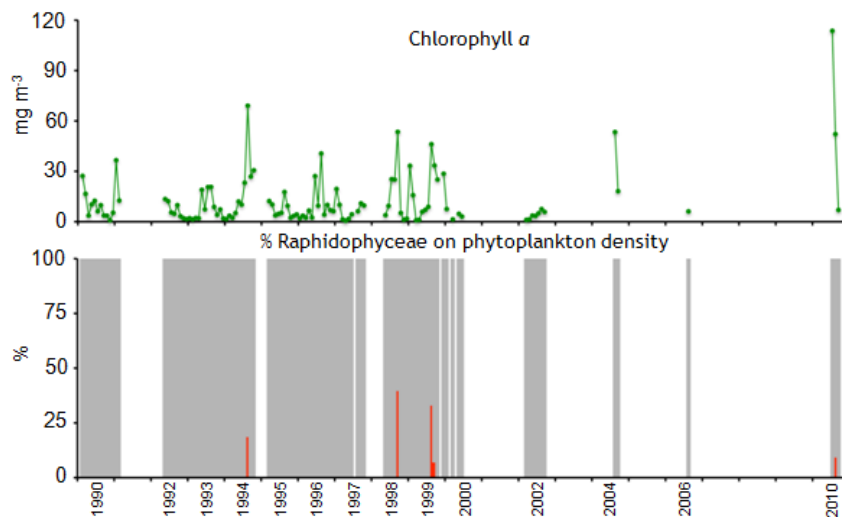
Figure 2 Percentages of Raphidophyceae species (in red) in the phytoplankton community during the years of massive fish kills (bottom graph) and values of chlorophyll *a* (upper graph).

The third study case is on blooms of noxious phytoplankton species in marine coastal waters, in particular in beach environments, where the ecosystem function of

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"recreation and (eco)tourism" has an important value (De Groot *et al.*, 2002). In fact, beaches are places where people can come for rest, relaxation, refreshment and recreation (De Groot *et al.*, 2002). The Mediterranean countries population is constantly increasing and this population pressure is exacerbated by tourism (EEA, 1999), which represents about a third of the world's international tourism. The related sewage generation is one of the most important pollution problems on the Mediterranean coast. It is able to influence environmental quality which, in turn, directly or indirectly affects human health, the stability of the marine ecosystem and the economy of the coastal zone (impact on tourism and fisheries) (EEA, 2006). With the people increasing numbers affluence and leisure-time, the demand for recreation in natural areas ('eco-tourism') will most likely continue to increase in the future (De Groot *et al.*, 2002). It is important to value the possible costs of long-term environmental degradation due to the coastal environments overexploitation c tourism. The activities of this case study have been part of a project funded by Autonomous Region of Sardinia ("Fioriture algali nocive in aree di particolare interesse economico della Sardegna: incremento delle conoscenze e nuovi approcci di studio finalizzati alla gestione e alla mitigazione" (RAS, CRP-26691), started in June 2012. The general aim of the project is to improve the ability to predict and mitigate impacts of HABs in areas of high economic interest (e.g. beaches for tourism), through the use of an interdisciplinary approach. During the summer months in Sardinia there is an increase of the possibility that ecologically sensitive areas on the island are over-used and even damaged (Hospers, 2003). In long time possible consequences of over-exploitation tourist on the island's ecosystem are waste and this may even result in a loss of biodiversity and wildlife habitats,.It runs the risk of threatening those very natural and cultural assets upon which the economy largely depends and tourism too (Hospers, 2003). In recent years, reports of HABs in beaches along Mediterranean coasts have increased significantly, likely due to a high vulnerability of marine environments to these events (Sampedro *et al.*, 2011). Species that have been more involved are *Alexandrium taylorii* and *Ostreopsis* cf. *ovata*, but the knowledge about

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HAS presence, distribution and bloom dynamics in Mediterranean beaches are very scarce. In this case study, several HAS from 74 Sardinian beaches were identified using molecular PCR methods, to support analyses carried out with microscopy traditional methods (Chapter III).

The fourth study case is a preliminary study on the issue of blooms of dinoflagellates potentially harmful in Mediterranean reservoirs. At the global level, in freshwater ecosystems, harmful blooms are mainly related to the affirmation of toxic cyanobacteria (CyanoHABs; Carmichael, 2008) and result in serious health consequences for humans and other biota (Chorus and Bartram, 1999; Codd *et al.*, 2005; Carmichael *et al.*, 2001). In Sardinia, water requirement satisfaction is based on the use of water accumulated in artificial lakes, which are eutrophic in the majority. Eutrophication favours the affirmation of cyanobacteria (Sechi and Lugliè, 1992, 1996) and the occurrence of harmful blooms (Messineo *et al.*, 2009; Mariani *et al.*, 2013). However, even some dinoflagellates can cause problems for drinking, e.g giving unpleasant tastes and odours, interfering with the filtration process for drinking water, and some species contain potentially toxic substances too (Hashimoto *et al.*, 1968; Oshima *et al.*, 1988; Kawataba and Hirano, 1995). Blooms of species attributed to the genus *Peridinium* Ehrenberg/*Peridiniopsis* Lemmermann and *Gymnodinium* Stein are frequently recorded in some lakes of Sardinia (e.g. Cedrino, Sos Canales and Torrei). Species of these genera are also reported as problematic because accelerates clogging of filter systems in drinking-water treatment, but may also break through these filters with the consequence of elevating the dissolved organic carbon (DOC) concentrations of the purified water and thus enhancing microbial growth (Hoehn, 2000; Hansen and Flaim, 2007; Zhang *et al.*, 2011). The availability of fixed samples taken in correspondence of blooms events (occurring in February 2012 in Cedrino Lake) or at high densities (in March 2011 in the Sos Canales Lake) and of resting cysts isolated from the sediments of Cedrino Lake and Sos Canales Lake have allowed to apply molecular techniques to confirm the identification and/or to ascertain the involved species (Chapter IV).

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1.3 Molecular approaches to study Harmful Algal Blooms

In the last decades a number of manuals on methods to study phytoplankton and HAS have been written (AFNOR, 2005; UNESCO, 2010). It has been due to the increased interest on HABs and to achieve a good standardization among analysts and to improve the data quality and their comparability. Basically, to study phytoplankton the methods are based on microscopy and molecular techniques. The microscopy methods can be used for a large part of the phytoplankton community and involve the identification of phytoplankton species based on their cell morphology. Whereas the majority of the molecular methods are aimed only at selected target species. The Utermöhl's method (Utermöhl, 1958) is probably the most widely used ones for determine both the quantity (density and biomass, determining individual cell size) and phytoplankton diversity. The method is based on the observation of sub-samples of variable volume (between 3 cm^3 up to 100 cm^3), which implies the detection of the species only when their cell density is higher than the limit of detection (e.g. 10 cells l^{-1} analysing 100 cm^3). Other disadvantages of the Utermöhl's method are the times very consuming and the request of considerable expertise and skills. In particular, in order to achieve reliable results, the analyst needs to have a good knowledge of the taxonomic literature. Furthermore, the recognition of species is anything but simple and in many cases also the use of epifluorescence microscopy, with the use of fluorochromes, or electron microscopy does not solve any doubts, nevertheless the considerable waste of resources (in terms of time, equipment and figures highly specialized). Moreover, HABs are complex events, with a wide array of taxa involved and, due to their negative impacts, the rapid and reliable detections of the species is often requested directly in natural samples from areas of interest (e.g., those with tourist and aquaculture). Many delicate algae do not survive collection and chemical preservation procedures, or they are altered such that recognition is impossible. Loss of pigmentation, cell shrinkage and detachment of distinctive flagella are common problems. Considering the difficulties and limitations of the microscopy methods, microalgae studies are increasingly exploring the use of molecular methods and their

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role is recognized as being of great importance in improving knowledge on HAS (Litaker and Tester, 2002). For this reason an increasing number of studies are employing molecular methods to define algal species as well as a more quickly and definitively identify members of an algal community. The pioneers were Medlin *et al.* (1991) using molecular information in characterization of a new diatom species. Scholin (1994) highlighted the importance of molecular probes as a tool for the identification of HAS. Then, from the late 90's onwards, there has been an increasing interest in this study area and now the use of molecular methods is a research field very active worldwide to identify and characterize HABs. Today, the description of new species, erection of new genera, or rearrangement of a species to a different genus is usually supported by molecular data in addition to morphological structures, ultrastructure, and information on biogeographic distribution (e.g. Fraga *et al.*, 2008). Thus, the understanding of evolutionary relationships among microalgal taxa has been really improved (Saldarriaga *et al.*, 2001). Spatially separated populations of microalgae species might display different properties, such as toxin production. By studying minor differences within the genome, populations can be confined to certain locations, and human assisted and/or natural migration of populations can be investigated (e.g., Persich *et al.,* 2006, Nagai *et al.*, 2007). Also, the increasing information on the structure of genes and new tools for investigating their expressions, have enhanced our understanding of algal physiological processes (Maheswari *et al.*, 2009). Penna *et al.* (2007) affirmed that the molecular tools could have many favourable characteristics with respect to the conventional methods:

a) the tests are rapid (few hours for processing phytoplankton material);

b) they are specific at the species and population level and some molecular probes can discriminate between toxic and non-toxic cells of the same species;

c) they require a lower level of expertise in the routine laboratory procedures compared with the expertise necessary to discriminate key morphological features indicative of HAS;

d) they can be applied in the screening of numerous field samples.

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Thanks to these techniques, the results obtained in the last decade have contributed to the genetic characterization of different HAS, to the correct comprehension of the relationship between genus, population and groups (strains), but also to a better definition of the geographical distributions and paths that have led to these (Scholin *et al.*, 1995, Hansen *et al.*, 2000; Edvardsen *et al.*, 2003; John *et al.*, 2004).

The number of probes available for algal identifications is rapidly increasing. The most used genetic markers for the identification of HAS are the rRNA gene family (Fig. 3), such as the D1/D2 regions of LSU, SSU, 5.8S and internal transcribed spacer (ITS) regions (Adachi *et al.*, 1996; Scholin *et al.*, 1994, 1997; Miller and Scholin, 1998; John *et al.*, 2005). Analysis of ribosomal RNA genes (rDNA) offered a potential explanation: morphologically defined species did not necessarily correlate with phylogenetic lineages (Scholin *et al.,* 1994, 1995; Spalter *et al.,* 1997; Walsh *et al.,* 1998). Recently, PCR based methods were also successfully employed to detect various HAS in environmental samples mostly preserved with fixatives (Connell, 2002; Godhe *et al.*, 2002).

So, these methods are becoming a support tool of great interest in ecological studies on HABs, just because they can give additional information and interpretations compared to the traditional methods.
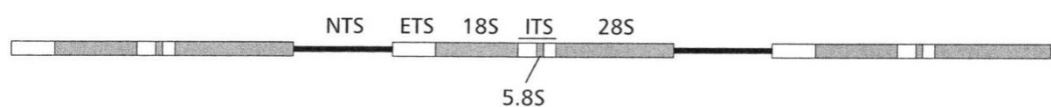

Figure 3 Ribosomial RNA genes and internal transcribed spacer regions whose sequences are widely used in molecular systematic. NTS = non-transcribed spacer, ETS = external transcribed spacer, 18S = small subunit (18S) rRNA gene, ITS = internal transcribed spacer, 5.8S rRNA gene, 28S = large subunit (28S) rRNA gene.

In the initial phase of the research carried out in this Ph.D. thesis, the methods developed on HAS and, among these the most suitable, were identified to achieve the planned objectives.

The methods described below were applied on different types of samples in the four cases study, using always as gene markers ITS-5.8S rRNA and/or 28S rRNA:

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- single cell method (Kai *et al.*, 2006) was used to analyze single cells obtained from field phytoplankton samples or culture (case study I and III). From the samples, observed under inverted microscope (Zeiss Axiovert 25), two or three cells were isolated with a micropipette and photographated. After a washing step with subsequent passages in filtered sea water or culture medium drops, each cell was positioned in a single drop on a slide. Then 1-2 cells were shifted directly into a PCR tube containing 5 µl of a Lysis Buffer previously prepared (0.005% SDS with 400 ng µl^{-1} proteinase K). The tube was then stored at -20 °C until it was decided to proceed with cell lysis. The lysis was carried out putting the PCR tubes with cells at -80 °C for 10', at 60 °C for 30' and finally at 95 °C for 10'. Subsequently PCR was done directly into the tube adding the PCR mix (Table 4). This method was applied also in the case study IV, but meeting notable difficulties in working with fixed cells. In fact, in the case with naked dinoflagellates, it was not possible remove all fixative, which interfered with the molecular analysis. Moreover, in the case of armoured dinoflagellates the normal number of cells used in the single cell analysis was not able to give enough DNA for the PCR.

- single cells isolated from field phytoplankton samples or viable cysts isolated from sediment were placed in individual wells, inside of plates containing the culture medium (L1, Guillard and Hergraves, 1993; salinity, 31). The plates were then maintained in culture chambers (temperature, 20 °C; 12:12 light-dark cycle; light intensity, 100 µmol photons m^{-2} s^{-1}) and checked every 3-4 days to verify the level of cell growth or germination of cysts and relative cell growth. The cultures obtained were used for the analysis (case study I). Genomic DNA was extracted from about 10-15 ml of culture in exponential growth phase. The cells were concentrated by a centrifugation at 3000 rpm for 15 min. The pellet was then transferred to a 2 ml Eppendorf tube and centrifuged again at 10,000 rpm for 5 min. The total genomic DNA was extracted by the resulting pellet using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA), following the

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manufacturer's instructions. The extracted DNA was immediately frozen at -80 ° C until use for PCR (Table 4). The major difference in these two types of study approaches, just described, is that with the single cell method was possible analyze only one gene marker for each analysis, but it is fast and simple, avoiding the DNA extraction from cultures. Instead, with the cultures, even if it was a more longer process, more gene markers could be studied. Moreover, others different aspects, as toxicology and morphology, could be investigated. With the preserved DNA is also possible deepening the study with further analyses, also after years. Another advantage is that with the genomic DNA if a first PCR fails it can be repeated. For example, for A*lexandrium minutum* species even if we had the cultures we had decided to use the Single Cell method on cells isolated from the cultures to speed up analysis. It is evident that the choice was done on the basis of the study that it was planned to perform. For the case study IV, the attempts to obtain a culture from the germination of cysts failed, even if germination experiments permitted the isolation of single cells and their consequent analyses;

- in the case study II, fixed archived samples with Lugol's iodine were successful analysed. The first step was to find the samples collected during the fish kills events in correspondence of *Chattonella* blooms in the last twenty years (1994, 1998, 1999 and 2010), since the beginning of ecological researches in Santa Giusta Lagoon, and conserved in Department's collection. Samples from at least two stations per sampling date were analysed, extracting the total DNA from 50 ml of fixed samples with DNeasy Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instruction. A first PCR was performed with 1 µl of extracted DNA. This PCR was performed to amplify the internal transcribed spacer (ITS) regions and 5.8S rDNA of all cells present in the sample. Subsequently, 1 µl of each PCR product was used into two distinct nested PCR amplifications, with specific primers for *C. marina* and for *C. subsalsa* (Connell, 2002) to obtain the DNA sequences of only ITS regions (Table

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4). The same treatment was done also on DNA extracted from culture strains of *Chattonella* (*C. subsalsa* CCMP 217 and *C. antiqua* NIES 1; Connell, 2002), used as positive controls for each PCR amplification. Also in this case the fixative gave some problems, which were surpassed with a number of washing passages with water or specific buffers.

A summary of methods used and related study cases with advantages/disadvantages of each one is reported in Table 4. All PCR amplifications were performed in a DNA Engine® Thermal Cycler. PCR products were always resolved on a 1.2% or 1.8% (80v) agarose gel depending by the length of the amplified fragments. PCR products were purified and sequenced by an external service (Macrogen Inc., Europe) using both primers, and a 3730XL DNA sequencer. All sequences were submitted to the BLAST database (Basic Local Alignment Search Tool) at NCBI (National Centre for Biotechnology Information) (BLAST Algorithm; http://www.ncbi.nlm.-nih.gov). Sequences obtained in the case study I and III were aligned with those obtained from GenBank using the MAFFT v.6 program (Katoh *et al*., 2002) under FFT-NS-i (slow iterative refinement method). The maximum-likelihood (ML) method and the GTRGAMMA evolution model of RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006) were used for phylogenetic relationships. Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap (BS) ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RAxML software or with the freely available University of Oslo Bioportal, http://www.bioportal.uio.no (Kumar *et al.*, 2009).

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Table 4 Summary of methods used in this PhD thesis. The number below the method is referred to the specific case study.

METHOD	PHASES				CONSIDERATION
Culture (I)	10-15 ml	DNA extraction	PCR (LSU and ITS-5.8S rDNA)	Electrophoresis and sequencing	The cultures of microalgae can derive from isolated vegetative cells and/or cysts from sediment samples. The method allows to obtain stocks of DNA which can be retained over time for further analyses.
Single Cell (I, III; IV)	Identification, photos and Isolation	One or two cells in 0.2 ml PCR tubes with 5µl of lysis buffer	80˚C for 10' 60˚C for 30' 95˚C for 10'	PCR (LSU and ITS-5.8S rDNA). Electrophoresis and sequencing	This method is very innovative. It was developed on Raphidophyceae and now it is applied also for other algal classes, like Dinophyceae. The great potential lies in being able to extract the DNA from a single cell found in natural samples or isolated from a culture. The application of the method is fast, avoiding the long passage of DNA extraction of the previous method.
Fixed samples (II)	DNA extraction from 50 ml of fixed achieved sample	PCR (ITS-5.8S rDNA)	Nested PCR with species specific primers	Electrophoresis and sequencing	This method allows to clarify doubts about the identification of species even after many years because it was applied to fixed samples (with Lugol's iodine or formalin) old up to twenty years.

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2. AIMS

The main purpose of this Ph.D. thesis was to increase the knowledge on the biodiversity of harmful algal species in aquatic environments of Sardinia, through the use of molecular techniques.

In particular, four case studies were investigated to obtain useful information on the ecology of harmful species:

- on species of the *Alexandrium* genus, considering new isolates from different marine ecosystems of Sardinia;

- on species of the *Chattonella* genus, analyzing fixed samples collected in Sardinian lagoons during past harmful events;

- on the presence of harmful species in beaches along the Sardinian coasts, which can cause negative consequences on tourism;

- on blooms of dinoflagellates species potentially harmful in Sardinian reservoirs.

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3. CHAPTER Ia

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The genus *Alexandrium* Halim in the Mediterranean Sea: new contributions from Sardinia (Italy).

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ABSTRACT Since the 1970s, harmful algal blooms (HABs) have been recognized as one of the main ecological problems in marine coastal zone worldwide. The dinoflagellate species of *Alexandrium* genus are among the major responsible for paralytic shellfish poisoning, a serious human syndrome caused by their toxins through chain food. The diversity of *Alexandrium* appears to be higher in the Mediterranean Sea than elsewhere. In this study, further contributions on the presence of potential toxic species in the Mediterranean Sea are reported from the Sardinian coast: *Alexandrium minutum* from Olbia, *A. catenella* from Santa Giusta Lagoon (Oristano) and *A. tamarense* from Alghero. *Alexandrium* strains were characterized for morphology and toxicity. Molecular analyses implied the sequencing of target rDNA regions (28S rDNA, 5.8S rDNA and ITS regions) of our isolates. The results of this study increase knowledge on these still debated species in the Mediterranean Sea with two new records in two new areas.

Key words: *Alexandrium minutum*, *Alexandrium catenella*, *Alexandrium tamarense*, harmful algal blooms, Mediterranean Sea

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INTRODUCTION

The dinoflagellate species of *Alexandrium* Halim are among the major responsible for harmful algal blooms (HABs) worldwide. Over the last three decades, both the frequency and geographic range of *Alexandrium* blooms have increased, as for HABs in general (Anderson, 1989; Hallegraeff, 1993; Penna *et al.*, 2008).

At least a half of the known *Alexandrium* species are toxic (Anderson *et al.*, 2012). The toxic compounds produced belong to saxitoxins, spirolides and goniodomins, but the most important in terms of impacts are the saxitoxins, responsible for the Paralytic Shellfish Poisoning (PSP) (Anderson *et al.*, 2012). PSP outbreaks cause dangerous effects on the human health and important economic implications for aquaculture and shellfish farms, impairment of tourism and recreational activities, alteration of marine trophic structure and death of marine mammals, fishes and seabirds (Anderson *et al.*, 2012). Some species in this genus are able to achieve high density and biomass, which may cause impacts on the water quality, causing discoloration (red tides) and an increase in turbidity, with consequent effects also on tourism and recreational use of the waters (Hallegraeff, 2003). Members of *Alexandrium* genus are detected worldwide. A great richness of species appears in the Mediterranean Sea (Tab. 1), probably also due to the notable level of taxonomic scrutiny (Penna *et al.*, 2008; Anderson *et al.*, 2012). Species as *Alexandrium minutum* Halim, *A. catenella* Balech and *A. taylorii* Balech have produced, respectively, toxic and high biomass blooms in many localities of the Mediterranean Sea (Garcés *et al.*, 1999; Vila *et al.*, 2001a, Giacobbe *et al.*, 2007; Penna *et al.*, 2005; Satta *et al.*, 2010; Garcés and Camp, 2012, and references therein).

Sardinia, which is the largest island in the western Mediterranean basin, has been affected by a high frequency of *Alexandrium*'s HABs in the last decade. The presence of toxic *A. catenella*, *A. minutum* and *A. tamarense* were reported in different years and areas (Penna *et al.*, 2007, 2008; Lugliè *et al.*, 2003a, 2004, 2011). *A. catenella* and *A. minutum* had been responsible for eight different events of PSP positivity in mussel farms from the 2002 to 2009 (Giacobbe *et al.*, 2003a; Lugliè *et al.*, 2003a, 2003b, 2004,

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2011). *A. taylorii* caused intense water discolorations assessed since 2007 (Satta *et al.*, 2010), causing alarm among tourists and local administrators (A. Lugliè, personal communication).

In this study, several strains of *Alexandrium* species, obtained by cells or resting cysts isolation from different sites along Sardinian coast were analysed by nucleotide sequencing of the 5.8S rDNA and internal transcribed spacer regions (Penna *et al.*, 2005, 2008), supporting the morphological results for their identification. In fact, the different species of *Alexandrium* are phenotypically very similar and the main criteria currently used to identify them are based upon the detailed study of thecal plate arrangement (Balech, 1995; Taylor *et al.*, 2003), which however does not always resolve the taxonomic attribution. Recently, various molecular techniques can be employed using different genetic markers for the identification of *Alexandrium* species (Adachi *et al.*, 1996; Scholin *et al.*, 1994, 1997; John *et al.*, 2005; Masseret *et al.*, 2009; Orr *et al.*, 2011).

MATERIAL AND METHODS

Origin of the strains and culture conditions

Alexandrium minutum strains (UNISS3 and UNISS4) were obtained from the isolation of vegetative cells from water samples collected in the Gulf of Olbia (north-east coast of Sardinia). *A. tamarense* (UNISS5) and *A. catenella* (UNISS6) strains were obtained by the germination of resting cysts isolated from sediment samples, respectively, from the Gulf of Alghero (north-western coast) and Santa Giusta Lagoon (central-western coast) (Fig. 1).

Cells and cysts were isolated with glass micropipettes, and then transferred into IWAKI tissue culture multiplates. Plates were filled with L1 medium (Guillard and Hargraves, 1993) prepared with filtered seawater adjusted to a salinity of 31.

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Table 1 Morphotaxonomic assignments among *Alexandrium* species, toxin type, ability to produce biomass blooms, presence in the Mediterranean Sea and records in Sardinia waters.

Species	Toxin type	Biomass bloom	Presence in Mediterranean Sea and in Sardinia	
			Site	References
A. acatenella (Whedon & Kofoid) Balech	Saxitoxins	Yes	ND	
A. affine (Inoue & Fukuyo) Balech	Saxitoxins	NR	Gibraltar, Alboran Sea, Spain	Penna *et al*., 2005
A. andersonii Balech	Saxitoxins	NR	Gulf of Naples, Tyrrhenian Sea	Montresor *et al*., 1998
			Saronikos, Aegean Sea, Greece	Penna *et al.*, 2008
A. balechii (Steidinger) Taylor	NK	Yes	Gulf of Salerno, Tyrrhenian Sea	Montresor *et al*., 1998
			Western Aegean Sea, Greece	Gotsis-Skretas *et al*., 2003
A. camurascutulum MacKenzie & Todd	NK	Yes *	Not detected	
			Balearic-Catalan Sea, Spain	Margalef and Estrada, 1987
			Valencia Harbour, Spain	Gomis *et al*., 1996
			Thau Lagoon, France	Abadie *et al*., 1999; Lilly *et al*., 2002
			Barcelona, Catalan Sea, Spain	Vila *et al*., 2001a
			Catalan coast, different harbours, Spain	Vila *et al*., 2001b
			Alexandria (eastern harbour), Egypt	Mikhail, 2001
			North Aegean Sea, Greece	Gotsis-Skretas *et al*., 2003
A. catenella (Whedon & Kofoid) Balech	Saxitoxins	Yes	Western Aegean Sea, Greece	Gotsis-Skretas *et al*., 2003
			Olbia, NW Tyrrhenian Sea, Italy	Lugliè *et al*., 2003a
			Andalusia coast, Spain	Fernández *et al*., 2004
			Tarragona, Catalan Sea, Spain	Vila *et al*., 2004
			Tunis Canal, Tunis	Penna *et al.*, 2005
			Algiers Harbour, Algeria	Penna *et al.*, 2005
			Gulf of Oristano, Sardinia, Italy	Milandri *et al*., 2008
			Sicily, Italy	Milandri *et al*., 2008
			Santa Giusta Lagoon, Sardinia, Italy	Satta *et al*., 2013
A. cohorticula[a] (Balech) Balech	Saxitoxins	NR	ND	
A. compressum (Fukuyo, Yoshida & Inoue) Balech	NK	NR	ND	
A. concavum (Gaarder) Balech	NK	NR	ND	
A. foedum Balech	NK	NR	Gulf of Salerno, Tyrrhenian Sea, Italy	Balech, 1990
A. fraterculus (Balech) Balech	NK	Yes **	ND	
A. fundyense Balech	Saxitoxins	Yes	ND	
A. gaarderae Nguyen-Ngoc & Larsen	NK	NR	ND	

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A. globulum Nguyen-Ngoc & Larsen	NK	NR	ND	
A. hiranoi Kita & Fukuyo	Goniodomins	NR	ND	
A. insuetum Balech	NK	Yes	Tunisia	Daly Yahia-Kefi *et al.*, 2001
			Corsica, France	Hansen *et al.*, 2003
			Gulf of Amvrakikos, Aegean Sea, Greece	Nikolaidis *et al.*, 2005
			Arenys Harbour, Catalan Sea, Spain	Penna *et al.*, 2008
A. kutnerae (Balech) Balech	NK	NR	Vilanova Harbour, Spain	Bravo *et al.*, 2006
A. leei Balech	NK	NR	ND	
A. margalefi Balech	NK	NR	Oliveri, Tyrrhenian Sea, Italy	Penna *et al.*, 2001
			Palamós Harbour, Catalan Sea, Spain	Bravo *et al.*, 2006
			Vilanova Harbour, Catalan Sea, Spain	Bravo *et al.*, 2006
			Alfacs Bay, Catalan Sea, Spain	Bravo *et al.*, 2006
A. minutum Halim	Saxitoxins	Yes	Alexandria Harbour, Egypt	Halim, 1960
			Izmir Bay, Aegean Sea	Korai and Buyukisik, 1988
			Ebre Delta, Catalan Sea, Spain	Delgado *et al.*, 1990
			Gulf of Naples, Tyrrhenian Sea, Italy	Montresor, 1990
			Trieste, North Adriatic Sea, Italy	Honsell, 1993
			Toulon Bay, France	Belin, 1993
			Sicily, Italy	Giacobbe and Maimone, 1994
			Kastela Bay, Adriatic Sea, Croatia	Marasovic *et al.*, 1995
			Ganzirri lagoon, Messina, Sicily	Giacobbe *et al.*, 1996
			Palma de Mallorca Harbour, Balearic Islands	Forteza *et al.*, 1998
			Valencia Harbour, Spain	Gomis *et al.*, 2000
			Vilanova Harbour, Catalan Sea, Spain	Vila *et al.*, 2001a
			Arenys Harbour, Catalan Sea, Spain	Vila *et al.*, 2001a
			Olimpic, Premià and Estartit harbours, Catalan Sea, Spain	Vila *et al.*, 2001a
			Tunisia	Daly Yahia-Kefi *et al.*, 2001
			Catalan coast, different harbours, Spain	Vila *et al.*, 2001a, 2005; Garces *et al.*, 2004; Bravo *et al.*, 2006
			Coasts of Greece	Gotsis-Skretas *et al.*, 2002
			Porto Torres Harbour, Tyrrhenian Sea, Sardinia, Italy	Giacobbe *et al.*, 2003b
			Olbia, Tyrrhenian Sea, Sardinia, Italy	Giacobbe *et al.*, 2003b
			Syracuse, Ionian Sea, Italy	Giacobbe *et al.*, 2003b
			Santa Giusta Lagoon, Oristano, Sardinia, Italy	Lugliè *et al.*, 2003b

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			Coast of Morocco	Tahri-Joutei *et al*., 2003
			Coast of the Turkish seas	Korai, 2004
			Coast of Andalusia, Spain	Fernandéz *et al*., 2004
			Corru S'Ittiri Lagoon, Sardinia, Italy	Satta *et al*., 2013
A. monilatum (Howell) Balech	Goniodomins	Yes	ND	
A. ostenfeldii (Paulsen) Balech & Tangen	Spirolides, saxitoxins	Yes	Alexandria Harbour, Egypt	Balech, 1995
			Adriatic Sea, Italy	Ciminiello *et al*., 2010
A. peruvianum (Balech & Mendiola) Balech & Tangen	Spirolides		Palamós Harbour, Catalan Sea, Spain	Bravo *et al*., 2006
A. pseudogonyaulax (Biecheler) Horiguchi ex Yuki & Fukuyo	Goniodomins	NR	Gulf of Trieste, North Adriatic Sea, Italy	Nichetto *et al*., 1995
			Ebre Delta, Catalan Sea, Spain	Bravo *et al*., 2006
			Platera Beach, Catalan Sea, Spain	Bravo *et al*., 2006
A. satoanum (Biecheler) Horiguchi ex Yuki & Fukuyo	NK	NR	ND	
			Catalan Coast, Spain	Margalef, 1969
			Gulf of Naples, Tyrrhenian Sea, Italy	Montresor, 1990
			Emilia Romagna coast, Adriatic Sea, Italy	Boni *et al*., 1983
			Coastal lagoons, Po River Delta, Italy	Sorokin *et al*., 1999
			Thau Lagoon, France	Masselin *et al*., 2001
			Olimpic and Premià harbours, Catalonia, Spain	Vila *et al*., 2001a
A. tamarense (Lebour) Balech	Saxitoxins (toxic and non-toxic strains)	Yes	North Aegean Sea, Greece	Gotsis-Skretas *et al*., 2002
			Western Aegean Sea, Greece	Giannakourou *et al*., 2005
			Porto Torres Harbour, Tyrrhenian Sea, Italy	Lugliè *et al*., 2003b
			Gulf of Naples, South Tyrrhenian Sea, Italy	John *et al*., 2003; Montresor *et al*., 2004
			Balearic Islands, Catalan Sea, Spain	Bravo *et al*., 2006
			Taranto, Ionian Sea, Italy	Penna *et al*., 2007
			Oliveri, North Tyrrhenian Sea, Italy	Penna *et al*., 2008
			Gulf of Oristano, Tyrrhenian Sea, Italy	Penna *et al*., 2008
A. tamiyavanichii Balech	Saxitoxins	NR	ND	
			Gulf of Naples, Tyrrhenian Sea, Italy	Montresor *et al*., 2004
A. tamutum Montresor, Beran & John	NK	NR	Gulf of Trieste, North Adriatic Sea, Italy	Montresor *et al*., 2004
			Sant Carles Harbour, Catalan Sea, Spain	Fraga *et al*., 2004
			Gulf of Olbia, Tyrrhenian Sea, Italy	Fraga *et al*., 2004
			La Fosca Beach, Catalan Sea, Spain	Delgado *et al*., 1997
			Palmira Beach, Mallorca, Spain	Delgado *et al*., 1997
A. taylorii Balech	Saxitoxins	Yes	Santa Panagia Bay, Ionian Sea, Sicily, Italy	Giacobbe and Yang, 1999
			Vulcano Island, South Tyrrhenian Sea, Italy	Penna *et al*., 2002
			Kavala, Aegean Sea, Greece	Penna *et al*., 2005

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				Gulf of Trieste, North Adriatic Sea, Italy	Penna *et al.*, 2008
				Paguera Beach, Catalan Sea, Spain	Penna *et al.*, 2008
				Platamona Beach, Sardinia, Italy	Satta *et al.*, 2010
A. tropicale Balech	NK	NR	ND		

NK = none known; NR = not reported; ND = not detected

[a]Japanese strains reportedly toxigenic, but possible misidentification of *A. tamiyavanichii* (Anderson *et* al., 2012); *a single case, but with *A. minutm*; **mixed blooms with *A. catenella* and *A. tamiyavanichii*

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Cultures were grown at 20±1 °C with a photoperiod of 12:12 light:dark cycle. Fluorescent tubes provided illumination with a photon irradiance of 100 µmol photons m^{-2} s^{-1}. Cultures were maintained in Iwaki flacks filled with L1 adjusted at a salinity of 31.

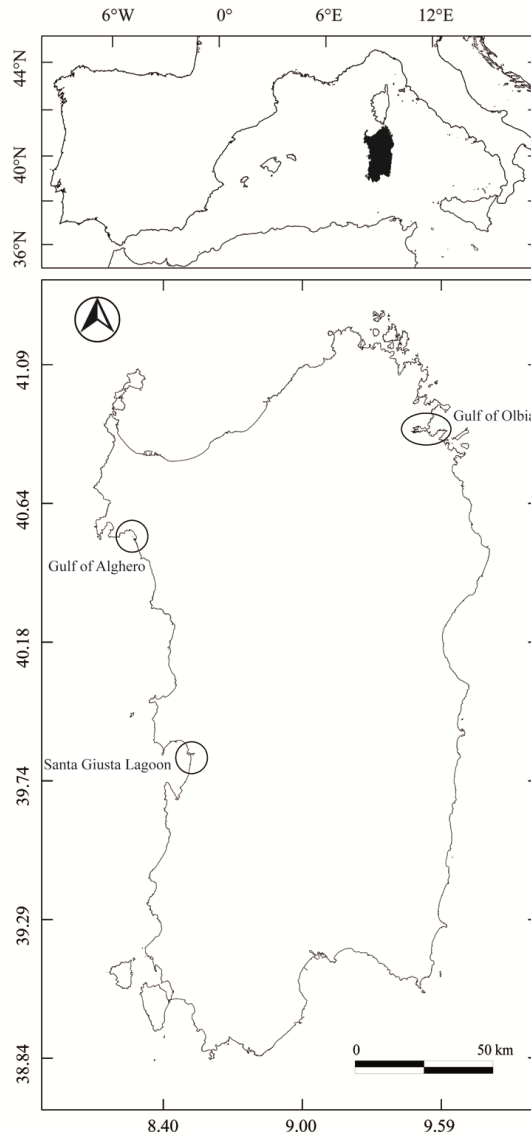**Figure 1** Geographical location of the sampling areas of the analyzed strains.

Morphological analysis

Cell size was determined under an inverted Zeiss Axiovert 25 microscope at 400X magnification. Plate patterns were studied following Balech (1995) after staining with

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Calcofluor white (Fluorescent Brightner 28, Sigma) and observation under UV epifluorescence (Fritz and Triemer, 1985).

DNA extraction

A. tamarense and *A. catenella* genomic DNA was extracted from approximately 10–20 ml of cultures in logarithmic growth phase. Cells were harvested by centrifugation at 3000 rpm for 15 min. The pellet was transferred to a 2 ml Eppendorf tube and centrifuged again at 10,000 rpm for 5 min. Total genomic DNA was extracted from the resulting pellet using a DNeasy Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The extracted DNA was immediately frozen at -80 °C. PCR primers D1R and D2C (Scholin *et al.*, 1994) were used to amplify the LSU rRNA gene. PCR was carried out in 25 µl reactions containing 1 µl of DNA extract, 0.625 µl of each primer (10 µM), 1.5 µl of dNTPs (200 µM of each), 1.5 µl of $MgCl_2$ (25 mM), 2.5 µl of 10X PCR buffer, and 0.125 µl of Taq DNA polymerase (Qiagen). The 5.8S rDNA and internal transcribed spacers (ITS1 and ITS2 regions) were amplified using ITSF01 and ITS4 primers (White *et al.*, 1990; Ki and Han, 2007). PCR was carried out in 50 µl reactions containing 1 µl of DNA extract, 4 µl of each primer (10 µM), 1 µl of dNTPs (200 µM of each), 1 µl of $MgCl_2$ (25 mM), 5 µl of 10X PCR buffer and 0.25 µl of Taq DNA polymerase (Qiagen).

A. minutum cultures were analyzed applying the single-cell PCR method (Kai *et al.*, 2006). Individual cells were isolated under an inverted microscope (Zeiss Axiovert 25) with a glass pipette and transferred in 200 µl PCR tubes containing 5 µl of lysis solution (0.005% SDS and 400 ng $µl^{-1}$ Proteinase K). Tubes were briefly centrifuged to ensure that the cells were at the bottom. Tubes were stored at -20 °C until the lysis. For the lysis the tubes were thawed and then frozen at -80 °C for at least 10 min, incubated at 60 °C for 30 min and finally at 95 °C for 10 min. The lysates were used immediately, or stored at -80 °C until use for the PCR. The PCR mixture is the same described above on 50 µl, both for LSU and 5.8S rDNA and internal transcribed spacer regions.

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Amplification

Thermocycling included one initial step at 95 °C for 5 min followed by 40 cycles at 95 °C for 20 s, than a cycle at 55 °C (for primers D1R and D2C) or 53 °C (for primers ITS-F01 and ITS4) for 30 s, and at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Aliquots of the PCR products were electrophoresed in 1.2% agarose gels. The remaining product was stored at 4 °C until sequenced.

Sequencing and phylogenetic analyses

Purification and sequencing were carried out by an external service (Macrogen Inc., Amsterdam) using both primers and a 3730XL DNA sequencer.

Sequences obtained were compared with sequences in the NCBI Nucleotide Collection (BLAST Algorithm; http://www.ncbi.nlm.-nih.gov) to determine the closest known sequences. Sequences alignment was carried out including other sequence data of *Alexandrium* species from GenBank (Appendix 1) with MAFFT v.6 program (Katoh *et al.*, 2002) under FFT-NS-i (slow; iterative refinement method). The sequence alignment analyses were manually checked with BioEdit v. 7.0.5 (Hall, 1999). Phylogenetic relationships, based on the LSU and ITS-5.8S rDNA, were inferred using maximum-likelihood (ML) method and the GTRGAMMA evolution model on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006). *Gonyaulax spinifera* (GenBank EU805591, EU532487) was used as outgroup. The tree with the best topology (the one with the greatest likelihood of 1000 alternative trees) was selected by repeated runs on distinct starting trees. Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with MrBayes (Huelsenbeck and Ronquist, 2001). For all analyses the freely available University of Oslo Bioportal, http://www.bioportal.uio.no (Kumar *et al.*, 2009) was employed.

Toxicity

PSP toxin analysis was conducted on exponentially growing (dependent on growth rate) strain cultures by high performance liquid chromatography (HPLC) post column oxidation method with fluorescence detection (OMA, 2005). Toxins were extracted

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from the cell pellet suspended in 0.1 mM acetic acid. The acidic pellet was sonicated (Ultrasonic® Liquid Processor Model XL2020, Heat Systems Inc.) for 30 min, in order to break the cells. The sonicated pellet was then centrifuged (at 4500 rpm for 10 min) and aliquots subjected to the analysis using both peroxide and periodate oxidation steps. The method allowed quantify individual PSP toxins, with the exception of the epimeric pairs (GTX1,4; GTX2,3) which form identical oxidation products and cannot be separated (Quilliam *et al.*, 1993). Toxins were quantified against linear calibrations of all currently available PSP toxin certified standard references. Toxicity equivalence factors (TEFs; EFSA, 2009) were used to calculate STX equivalence concentrations. No ion exchange fractionation was undertaken prior to quantification of the N-hydroxylated toxins as GTX1,4.

RESULTS

Morphology

A. catenella (UNISS6) culture was obtained by the germination of an ellipsoidal cyst (43 µm long and 30 µm wide). The cyst was greyish in colour with a granular content and a red accumulation body (Fig. 2a). *A. catenella* vegetative cells showed the absence of the ventral pore in the 1' plate , which was directly in contact with the Po (Fig. 2b). Cells were in chains of four-six cells. No size data was obtained due to the culture death.

A. tamarense (UNISS5) culture was obtained by the germination of an ellipsoidal cyst (50 µm long and 28 µm wide). The cyst was greyish in colour with a granular content and a yellow accumulation body. The archeoplyle extended for half-length of the cyst and was oval (Fig. 3a). *A. tamarense* cells showed the ventral pore in the 1' plate, the 6'' plate large (Fig. 3b) and 1' directly in contact with Po (Fig. 3c). Cell size ranged from 22.5 µm to 32.5 µm (mean = 27.7 µm; n= 20) in length and from 22.5 µm to 33.8 µm (mean = 28.7 µm; n = 20) in width. Cells were mainly single or in chains of two cells.

The two strains of *A. minutum* showed the 1' plate truncated anteriorly and posteriorly (Fig. 4a), a wide anterior sulcal plate (Fig. 4b) and the 2'''' plate of type B (trasversal axis more longer than the longitudinal axis) (Fig. 4c).
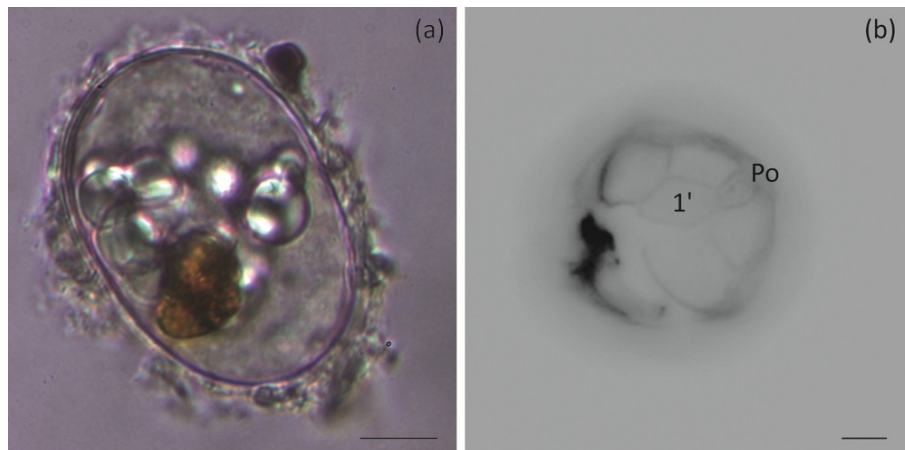
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Figure 2 *Alexandrium catenella* viable cyst (a) and vegetative cell (stained with calcofluor and observed using UV fluorescence) showing 1' plate without ventral pore and connected to Po plate (b). Scale bars: 10 μm.
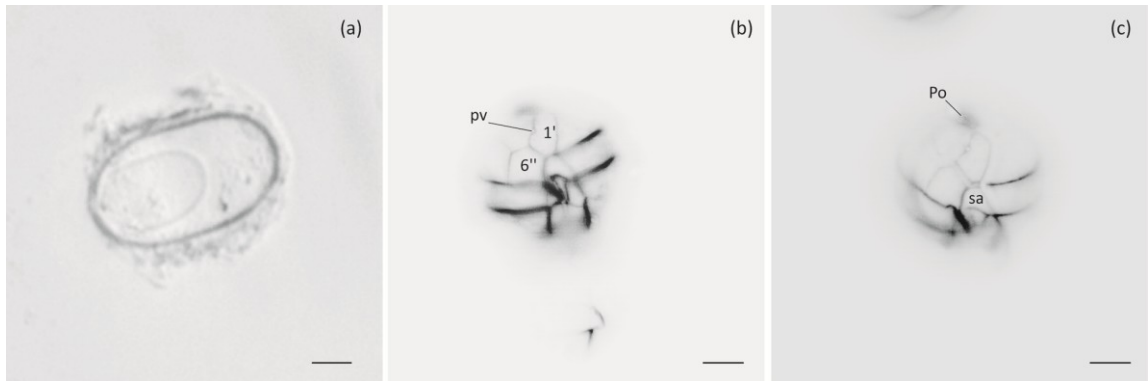

Figure 3 *Alexandrium tamarense* empty cyst showing the archeopyle (a), vegetative cell (stained with calcofluor and observed using UV fluorescence) showing 1' plate with ventral pore (pv) and 6'' plate (b), and vegetative cell showing sulcal anterior plate (sa) and Po plate. Scale bars: 10 μm.
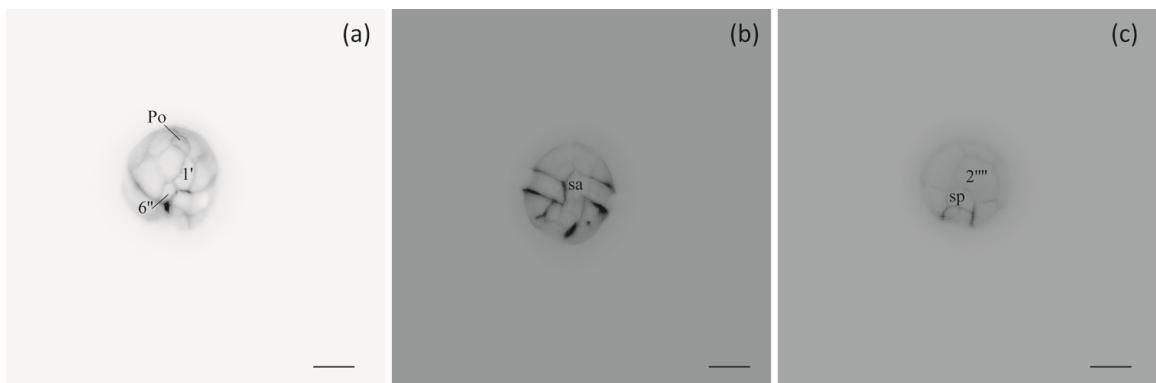
Figure 4 *Alexandrium minutum* vegetative cell (stained with calcofluor and observed using UV fluorescence) showing 1' plate directly connected to Po plate and 6'' plate (a), vegetative cell showing the sulcal anterior plate (sa) (b), and vegetative cell showing 2''' plate and sulcal plate (sp) (c). Scale bars: 10 μm.

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Cell size of UNISS3 strain ranged from 20 μm to 25 μm (mean = 22.2 μm; n = 20) in length and from 17.5 μm to 22.5 μm (mean = 20.1 μm; n = 20) in width. Cell size of UNISS4 strain ranged from 20 μm to 26.3 μm (mean = 22.4 μm; n = 20) in length and from 18.8 μm to 25 μm (mean = 21.1 μm; n = 20) in width. Cells were single.

Phylogenetic analyses

The sizes of PCR products for sequences obtained using D1R and D2C primers were 681 pb (GC = 39.94%) for *A. minutum* UNISS3, 683 pb (GC = 40.26%) for *A. minutum* UNISS4, 683 pb (GC = 42.31%) for *A. catenella* UNISS6, and 689 pb (GC = 40.78%) for *A. tamarense* UNISS5. Using ITSA and ITSB primers the lengths of PCR products were 711 pb (GC = 42.61%) for *A. minutum* UNISS3, 710 pb (GC = 42.11%) for *A. minutum* UNISS4, 732 pb (GC = 40.30%) for *A. catenella* UNISS6, and 733 pb (GC= 39.56%) for *A. tamarense* UNISS5. The analysed isolates showed identities of 99% or 100% with the different *Alexandrium* sequences present in GenBank in the respective clades of belonging.

The ITS-5.8S and LSU rDNA phylogeny showed the alignment of *A. tamarense* UNISS5 with the Group III or West European (WE Clade), *A. catenella* UNISS6 with the Group IV or Temperate Asian (clade TA), *A. minutum* UNISS3 and *A. minutum* UNISS4 with the Global Clade (Figs. 5-6). All sequences used in the trees with their respective clades are listed in Appendix 1 (Table 1 and Table 2).

Toxicity

Analysis of PSP toxin profile and overall toxicity were positive for GTX 2-3 and GTX 1-4 for the two strains of *A. minutum* from Olbia (Tab. 2). Toxins of *A. tamarense* were below the limit of detection.

DISCUSSION

The ecological and economic impacts of *Alexandrium* species have become relevant along the Mediterranean coasts during the last two decades, leading to numerous and detailed studies on this topic (Vila *et al.*, 2001a,b; Lugliè *et al.*, 2003a, 2003b; Bravo *et*

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al., 2006; Gomez, 2008). One of main issues remains the rapid species identification, absolutely necessary when potentially toxic species are involved in the blooms.

Alexandrium species identification is based on morphological criteria (cell size, shape, chain formation, theca ornamentation, cingular and sulcal excavation, presence of sulcal lists, shape of the apical pore complex (APC) and shape of 1' and 6''plates, and of sulcal plates), routinely assessed by microscopy methods during monitoring (Balech, 1985, 1995; Fukuyo, 1985). In some cases, the subtle morphological characteristics used for classification are not easily resolved, making difficult species attribution.

Table 2 Results of PSP toxin analysis. LOD = limit of detection, LOQ = limit of quantification

	STX	GTX 5	GTX 2,3	GTX 1,4	C 1,2	DcGTX 2,3
				fmol cell^{-1}		
A.tamarense UNISS5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
A. minutum UNISS3	<LOD	<LOD	**<LOQ**	**<LOQ**	<LOD	<LOD
A. minutum UNISS4	<LOD	<LOD	**<LOQ**	**<LOQ**	<LOD	<LOD
A.tamarense UNISS5*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
A. minutum UNISS3*	**<LOQ**	<LOD	**0.0042**	**0.013**	<LOD	**<LOQ**
A. minutum UNISS4*	**<LOQ**	**<LOQ**	**0.001**	**0.0182**	<LOD	**<LOQ**

* concentrated 1:10 times

A further complication is the possibility of toxic and non-toxic strains within the same species (Ichimi *et al.* 2001; Cordova *et al.*, 2003; Haya *et al.*, 2003). In this case, species identification is not sufficient to obtain information on the potentially harmfulness of the species and toxicological analysis are required (Masselin *et al.*, 2001; Milandri *et al.*, 2008). The potentially toxic species most frequently detected in the Mediterranean Sea have been *A. minutum*, *A. catenella* and *A. tamarense*. These species cluster into two different species-complexes. *A. minutum* into the "*A. minutum* complex" and *A. catenella* and *A. tamarense* into the "*A. tamarense* complex" (Adachi *et al.*, 1996; John *et al.*, 2003; Penna *et al.*, 2008; Lilly *et al.*, 2005). A species-complex is a genetically distinct cluster of strains sharing very similar morphological features (Anderson *et al.*, 2012).

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Figure 5 Maximum-likelihood phylogenetic tree of selected species based on the ITS-5.8S rDNA. Numbers on the nodes are the bootstrap values obtained after 1000 replicates. Only bootstrap values >50 are shown. *G. spinifera* was used as outgroup. Organisms sequenced in this study are written in red.

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Figure 6 Maximum-likelihood phylogenetic tree of selected species based on LSU rDNA. Numbers on the nodes are the bootstrap values obtained after 1000 replicates. Only bootstrap values >50 are shown. *G. spinifera* was used as outgroup. Organisms sequenced in this study are written in red.
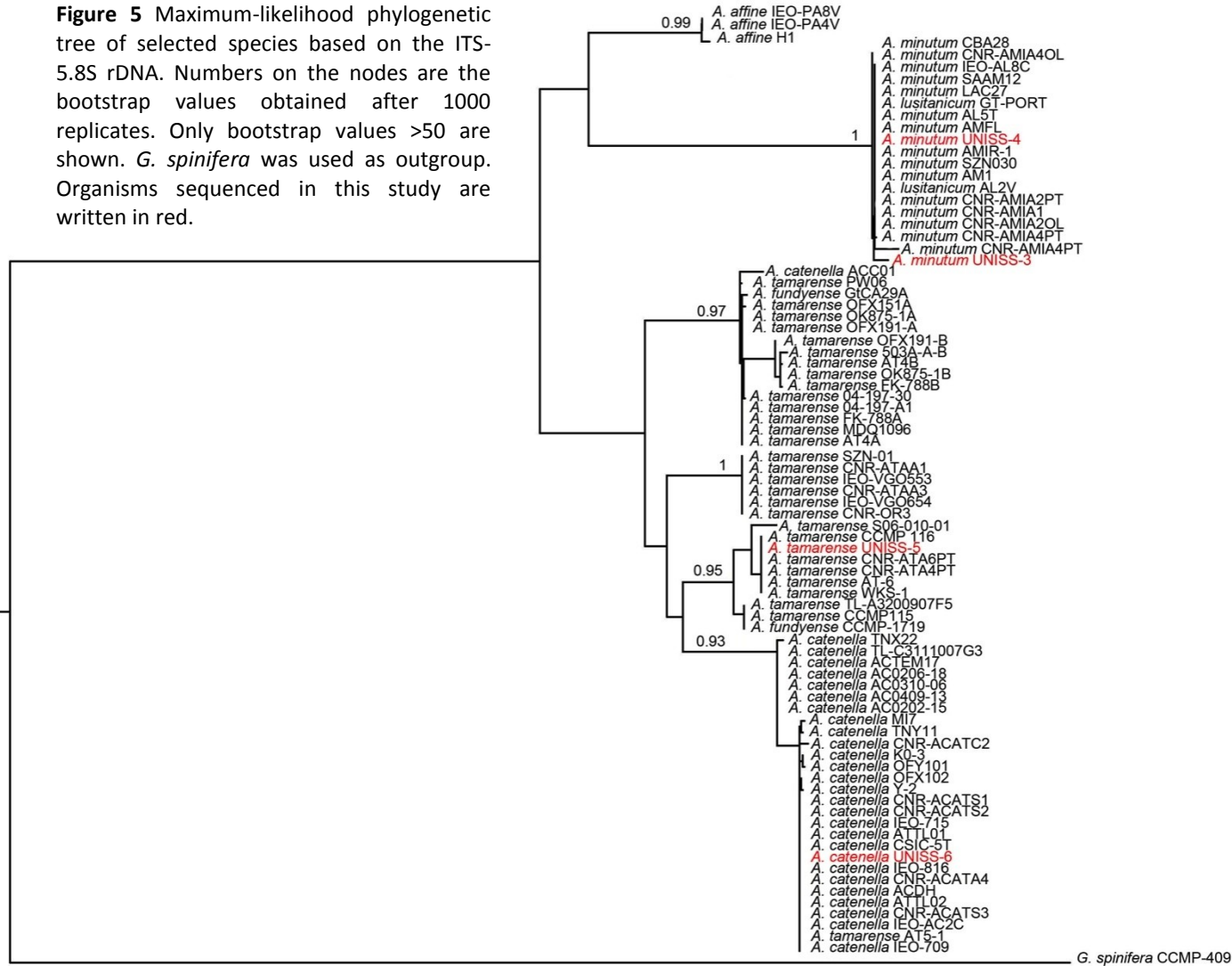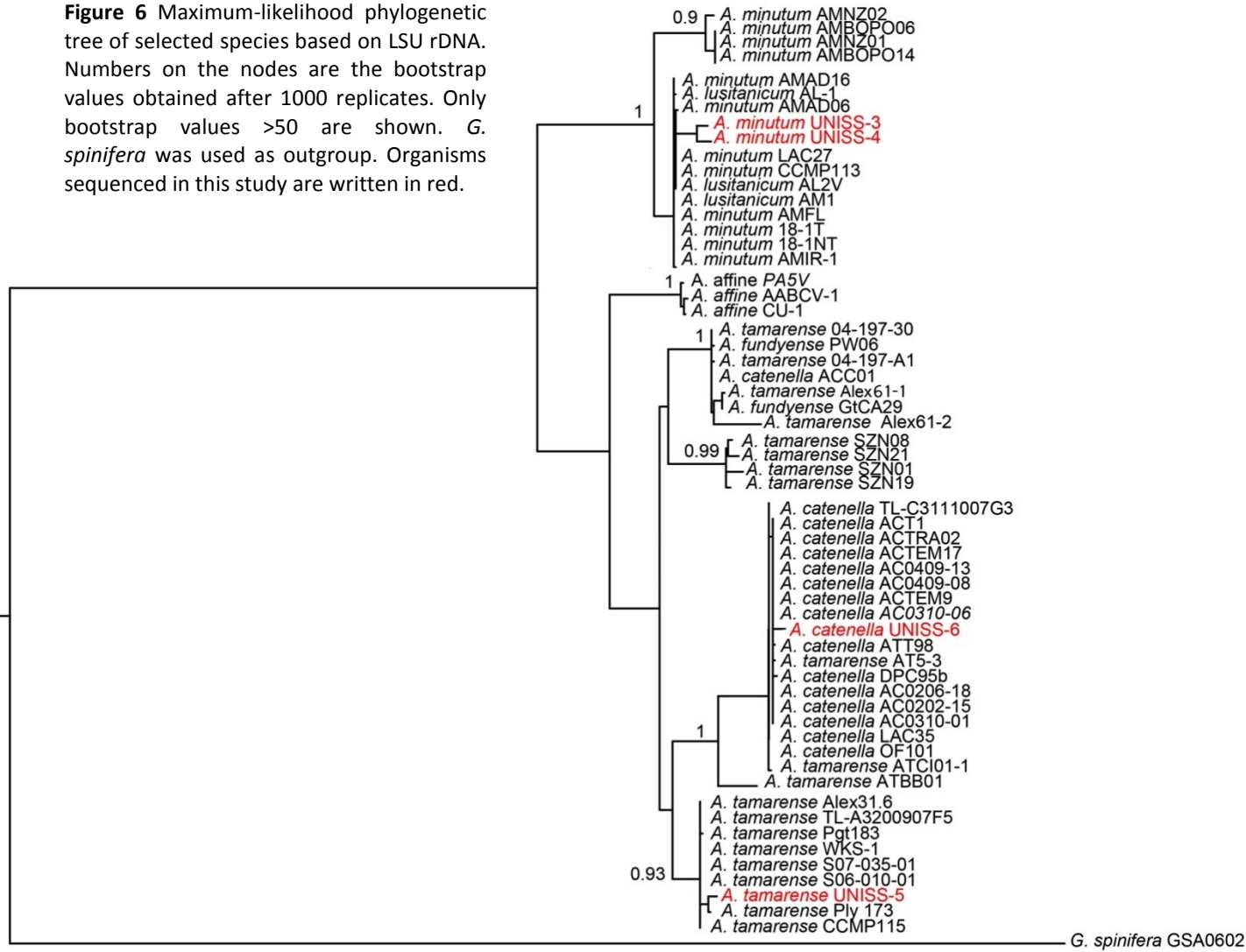

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Contributions to the knowledge of the A. tamarense species complex

A. tamarense, *A. catenella* and *A. fundyense* are distinguished based on the capability to form chains and the presence/absence of a ventral pore between plates 1' and 4' (Balech, 1995). Due to the high variability of morphological discrimination characters, toxicity, and molecular evidences that not confirm morphological differences used to identify a species, they were grouped in a species-complex (Medlin *et al.*, 1988; Sholin *et al.*, 1994; Adachi *et al.*, 1996; John *et al.*, 2003; Penna *et al.*, 2005). Nevertheless, recent studies on reproductive traits of members of the complex support the validity of the single species according to a biological concept (Brosnahan *et al.*, 2010). After a first distinction of ribotypes on the basis of geographical origins of the strains (North American, Western European, Temperate Asian, Tasmanian, and Tropical Asian; Scholin *et al.*, 1994), they are actually clustered into five phylogenetic well-supported clades, distinct with a numbering scheme (Lilly *et al.*, 2007; Collins *et al.*, 2009; Orr *et al.*, 2011). Each clade exclusively includes either toxic or non-toxic strains (Lilly *et al.*, 2007). In the Mediterranean Sea, *A. tamarense* of both II and III groups and *A. catenella* of group IV have been already reported (Penna *et al.*, 2008). Our study contributes to the knowledge on this species-complex with strains from two new sites along Sardinian coast, namely the Gulf of Alghero and Santa Giusta Lagoon.

A. tamarense from Alghero (UNISS5) grouped with other isolates of the group III, a clade still much debated. In fact, in general, Mediterranean *A. tamarense* strains were attributed to the group II (Mediterranean clade; John *et al.*, 2003; Lilly *et al.*, 2007; Penna *et al.*, 2008) and they are non-toxic. Penna *et al.* (2008) attributed for the first time two Mediterranean toxic *A. tamarense* strains from Porto Torres (North Western of Sardinia) to the group III (Western European clade). Recently, a new detection of *A. tamarense* of the group III from the Catalan coast was reported but no data on toxicity are available (Satta *et al.*, 2013). Our data highlight the necessity of further investigation on *A. tamarense* strains from different geographical areas in the Mediterranean Sea because of the general belief that Mediterranean *A. tamarense* belong only to the group II and, consequently, that they are non-toxic (Sholin *et al.*,

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1994; Lilly *et al.*, 2007). We reinforce the reports on the presence of *A. tamarense* of the group III and that they can be toxic or non-toxic. The alignment of *A. tamarense* UNISS5 with *A. tamarense* WKS 1 of the Pacific and with the European IEO-PE1V and CCMP 116 of the North Atlantic, respectively non-toxic and toxic, confirms our affirmation. In agreement with previous studies carried out on Sardinian strains from the Gulf of Olbia, *A. catenella* UNISS6 was added in group IV (Temperate Asian clade) which consists only of toxic strains isolated from around the world, e.g. China, Hong Kong, South Korea, Australia, Russia, France (Orr *et al.*, 2011; Collins *et al.*, 2009; Masseret *et al.*, 2009; Lilly *et al.*, 2007; Penna *et al.*, 2005 , 2008). In the summer of 1999, *A. catenella* was found for the first time in the Gulf of Olbia in Sardinia (Luglié *et al.*, 2003a, 2003b), and in May-June 2002 occurred the first case of toxicity in farmed mussels (STX> 800 kg^{-1} p.e.). In April-May 2003, a similar harmful event followed the first one in the Gulf of Olbia. Subsequently, other blooms occurred in other farming areas along Sardinian coast and with different seasonality: in autumn-winter months in 2005 and 2008 in the Gulf of Cugnana (North-Eastern Sardinia), and in autumn months 2006 and 2008 in the Gulf of Oristano (Western Sardinia)(Lugliè *et al.*, 2011). All Mediterranean isolates of *A. catenella*, including UNISS6, showed the same sequence of the Japanese ITS-5.8S rRNA genes, supporting the hypothesis of an alien character of the species in the Mediterranean Sea (Penna *et al.*, 2005). On the other hand, Masseret *et al.* (2009) supposed that the recent expansion of *A. catenella* in the Mediterranean could be explained not only with its recent introduction, probably mediated by human activities with the transport of vegetative or resting forms, but also with their presence from a long time in resistance forms or in abundances always very low (hidden flora). This hypothesis has to be yet proved and studied. If it results true, there would be the possibility that the detection of *A. catenella* in the Mediterranean Sea in the last 15 years may be linked to its more intense growth favoured by changing environmental conditions (e.g., related to anthropogenic pressure or global climate change). Our finding of *A. catenella*'s viable cysts in Santa Giusta Lagoon (Chapter Ib), a new site of detection, gives further indication on the potential affirmation of this species in a spatial contest larger than currently known.

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Further, it highlights the necessity of integrated studies between the planktonic/vegetative stages and benthic/resting stages to obtain more detailed information on the geographical distribution of HAS (Boero *et al.*, 1996; Rubino *et al.*, 2000, 2002).

Contributions to the knowledge of the A. minutum species complex

This species complex includes *A. minutum*, *A. lusitanicum* and *A. angustitabulatum* (Lilly *et al.*, 2005; McCauley *et al.*, 2009). These species show a great variability within and among species of the diagnostic characters used for their morphological distinction (length-width of the anterior sulcal plate, 1' shape and its connection with the apical pore complex, presence of a ventral pore), which is not supported by a corresponding genetic diversity (Lilly *et al.*, 2005). For this, *A. lusitanicum* and *A. angustitabulatum* should be considered as morphotypes within the morphological variability of *A. minutum* (Franco *et al.*, 1995; Hansen, 2003; Lilly *et al.*, 2005). *A. minutum* is the holotype of the genus *Alexandrium* and was described as responsible for a red tide in Alexandria harbour, Egypt (Halim, 1960). Actually, it has a worldwide distribution, from Europe to India, Malesya, Taiwan and New Zealand (Franco *et al.* 1994; Godhe *et al.*, 2000, 2001; Usup *et al.*, 2002; Hwang *et al.*, 1999; Chang *et al.* 1997, 1999), and it is largely reported also in the Mediterranean Sea (Tab. 1). The characterization of phylogenetic relationships between different strains of *A. minutum* to global level is fairly recent and is based on the LSU rDN*A*, showing the necessity of some clarifications (Lilly *et al.*, 2005; McCauley *et al.*, 2009). It is assumed that this species can be separated into two phylogeographic groups, one containing European and Australian isolates (Global clade), and the other comprising New Zealand and Taiwan isolates (Pacific clade; Lilly *et al.*, 2005). There is high level of similarity of the Mediterranean *A. minutum* strains based on LSU sequences (Hansen *et al.*, 2003; McKenzie *et al.*, 2004; Lilly *et al.*, 2005; Leaw *et al.*, 2005; Penna *et al.*, 2008). *A. minutum* UNISS3 and UNISS4 sequences aligned with those of the Global clade, showing a sequence homology equal to 100% with some isolates of *A. minutum* from Adriatic Sea (AMITA), Gulf of Trieste (AL8T), Porto Torres (CNR-AMIA4PT), Olbia (CNR-

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AMIA2OL) and Australia (AMD21) (Penna *et al.*, 2008; McCauley *et al.*, 2009). From the morphological point of view, *A. minutum* UNISS3 and UNISS4 responded fully to the diacritical characters of *A. lusitanicum*. The Global clade includes both toxic and non-toxic strains (Anderson *et al.*, 2012). *A. minutum* tends to produce primarily or exclusively gonyautoxins (GTX1–GTX4) (Cembella *et al.*, 1987). Toxin profiles found for *A. minutum* UNISS3 and UNISS4 indicated strain UNISS4 slightly more toxic (Table 2), but with lower values than those already assessed on a strain from Porto Torres (Lugliè *et al.*, 2003). *A. minutum* had been always present with *A. catenella* during the previously mentioned PSP events in Sardinian shellfish farms (Luglie *et al.*, 2011). One event of PSP was related to the detection of only *A. minutum* in Porto Pozzo Lagoon (North-Eastern Sardinia) in winter months at the ending of 2008 and the beginning of 2009. The ascertained toxicity of *A. minutum* UNISS3 and UNISS4 confirms the harmfulness of this species along Sardinian coast and the possibility of future harmful blooms in our waters.

CONCLUSION

Obtain detailed indication on the presence of potentially toxic species or strains in a geographic area represent an indispensable basis to develop management strategies of the risk (Hallegraef, 2003; Masó and Garcés, 2006). This study gives new information on the presence and harmfulness of potentially toxic species of the *Alexandrium* genus along the Sardinian coast. Our results encourage further investigations to increase knowledge on still debated species in the Mediterranean Sea, such as toxic and non-toxic strains of *A. tamarense* of the different groups.

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APPENDIX 1

Table 1 List of *Alexandrium* spp. isolates and sequences obtained from this study and GenBank for the ITS–5.8S rDNA regions.

Species	Strain	Sampling Location	Clade	Accession Number	Source	Toxicity
A. affine	IEO-PA4V	Spain: Ria de Vigo, Galicia		AJ632094	Penna *et al.*, 2005	no
A. affine	IEO-PA8V	Spain: La Linea, Gibraltar		AJ632095	Penna *et al.*, 2005	no
A. affine	H1	Japan: Harima Nada		AB006995	Adachi *et al.*, 1996	no
A. lusitanicum	GT-PORT	Portugal: Lagoa de Obidos	Global clade	EU707539	McCauley *et al.*, 2009	yes
A. lusitanicum	AL2V	Spain: Ria de Vigo, Galicia	Global clade	EU707499	McCauley *et al.*, 2009	yes
A. minutum	UNISS 3	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	ND	This study	/
A. minutum	UNISS 4	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	ND	This study	/
A. minutum	CBA28	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	FR668134	Casabianca *et al.*, 2012	yes
A. minutum	CNR-AMIA4OL	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	AJ514921	Penna *et al.*, 2008	yes
A. minutum	SAAM12	South Africa: Cape Town	Global clade	EU707547	McCauley *et al.*, 2009	yes
A. minutum	IEO-AL8C	Spain: Arenys, Catalan Sea, Mediterranean Sea	Global clade	AJ532914	Vila *et al.*, 2005	yes
A. minutum	SZN030	Italy: Gulf of Naples, Tyrrhenian Sea, Mediterranean Sea	Global clade	EU707549	McCauley *et al.*, 2009	yes
A. minutum	AMFL	England: Fleet Lagoon	Global clade	EU707521	McCauley *et al.*, 2009	yes
A. minutum	AM1	South Africa: Cape Town	Global clade	EU707511	McCauley *et al.*, 2009	yes
A. minutum	AL5T	Italy: Gulf of Trieste, Adriatic Sea, Mediterranean Sea	Global clade	EU707505	McCauley *et al.*, 2009	no
A. minutum	AMIR-1	Ireland: Cork Harbor	Global clade	EU707523	McCauley *et al.*, 2009	yes
A. minutum	CNR-AMIA1	Italy: Siracusa, Ionian Sea, Mediterranean Sea, Sicilia	Global clade	AJ621734	Vila *et al.*, 2005	yes
A. minutum	CNR-AMIA4PT	Italy: Porto Torres, Mediterranean Sea, Sardinia	Global clade	AJ514920	Penna *et al.*, 2008	yes
A. minutum	CNR-AMIA2OL	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	AJ532908	Penna *et al.*, 2008	yes
A. minutum	CNR-AMIA2PT	Italy: Porto Torres, Mediterranean Sea, Sardinia	Global clade	AJ532909	Penna *et al.*, 2008	yes
A. minutum	VGO663	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	AM238453	Penna *et al.*, 2008	yes
A. minutum	LAC27	Italy: Gulf of Trieste, Adriatic Sea, Mediterranean Sea	Global clade	AJ005050	Penna and Magnani, 1999	yes
A. catenella	ACC01	Chile	Group I (NA)	AJ272120	Marin *et al.*, 2001	yes
A. fundyense	GtCA29A	USA: Cape Ann, North Atlantic	Group I (NA)	NR	Adachi *et al.*, 1996	yes
A. tamarense	04-197-A1	United Kingdom: Scotland, Stonehaven	Group I (NA)	FJ042687	Collins *et al.*, 2009	yes
A. tamarense	04-197-30	United Kingdom: Scotland, Stonehaven	Group I (NA)	FJ042686	Collins *et al.*, 2009	yes
A. tamarense	FK-788A/B	Japan: Funka Bay	Group I (NA)	AB006994; AB006993	Adachi *et al.*, 1996	yes
A. tamarense	MDQ1096	Argentina: Mar de la Plata, West Pacific	Group I (NA)	AM292306	Penna *et al.*, 2008	yes
A. tamarense	PW06	USA: Port Benny, North Pacific	Group I (NA)	NR	Adachi *et al.*, 1996	yes
A. tamarense	OK875-1A/B	Japan: Okirai Bay, East Pacific	Group I (NA)	NR	Adachi *et al.*, 1996	yes

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A. tamarense	AT4A/B	Japan: Harima Nada, East Pacific	Group I (NA)	NR	Adachi *et al.*, 1996	yes
A. tamarense	OFX191-1A/B	Japan: Ofunato Bay, East Pacific	Group I (NA)	NR	Adachi *et al.*, 1996	yes
A. tamarense	At503A-A/B	Japan: Mikawa Bay, East Pacific	Group I (NA)	NR	Adachi *et al.*, 1996	yes
A .tamarense	OFX151A	Japan: Ofunato Bay, East Pacific	Group I (NA)	NR	Adachi *et al.*, 1996	yes
A. tamarense	CNR-ATAA1	Italy: Taranto, Ionian Sea, Mediterranean Sea, Puglia	Group II,(ME)	AJ491152	Penna *et al.*, 2007	no
A. tamarense	CNR-ATAA3	Italy: Taranto, Ionian Sea, Mediterranean Sea, Puglia	Group II,(ME)	AM292308	Penna *et al.*, 2008	no
A. tamarense	IEO-VGO654	Spain: Maiorca, Balearic Sea coast	Group II,(ME)	AM238650	Penna *et al.*, 2008	no
A. tamarense	IEO-VGO553	Greece: Kavala, Aegean Sea coast	Group II,(ME)	AM238651	Penna *et al.*, 2008	no
A. tamarense	SZN-01	Italy: Gulf of Naples, Tyrrhenian Sea, Mediterranean Sea	Group II,(ME)	AM238652	Penna *et al.*, 2008	no
A. tamarense	CNR-OR3	Italy: Oristano, Mediterranean Sea, Sardinia	Group II,(ME)	AM292307	Penna *et al.*, 2008	no
A. fundyense	CCMP1719	USA	Group III, (WE)	DQ444290	Ki and Han, 2007	yes
A. tamarense	CCMP115	United Kingdom: England, Plymouth	Group III, (WE)	JF521640	Orr *et al.*, 2011	no
A. tamarense	S06-010-01	United Kingdom: Scotland, Weisdale, Shetland	Group III, (WE)	FJ042685	Collins *et al.*, 2009	no
A. tamarense	TL-A3200907F5	France: Thau Lagoon, Mediterranean Sea	Group III, (WE)	FR686537	Genovesi *et al.*, 2011	no
A. tamarense	CNR-ATA4PT	Italy: Porto Torres, Mediterranean Sea, Sardinia	Group III, (WE)	AJ514906	Penna *et al.*, 2008	yes
A. tamarense	CNR-ATA6PT	Italy: Porto Torres, Mediterranean Sea, Sardinia	Group III, (WE)	AJ514907	Penna *et al.*, 2007	yes
A. tamarense	CCMP 116	Spain: Ria de Vigo, Galicia	Group III, (WE)	AJ005047	Penna and Magnani, 1999	yes
A. tamarense	WKS-1	Japan: Kushimoto, East Pacific	Group III, (WE)	AB006991	Adachi *et al.*, 1996	no
A. tamarense	UNISS 5	Italy: Gulf of Alghero, Mediterranean Sea, Sardinia	Group III, (WE)	ND	This study	/
A. tamarense	AT-6	China	Group III, (WE)	DQ176666	Tang *et al.*, 2005*	no
A. catenella	OFX102	Japan: Ofunato Bay, East Pacific	Group IV, (TA)	NR	Adachi *et al.*, 1996	yes
A. catenella	OFY101	Japan: Ofunato Bay, East Pacific	Group IV, (TA)	NR	Adachi *et al.*, 1996	yes
A. catenella	TNY11	Japan: Tanabe Bay, East Pacific	Group IV, (TA)	NR	Adachi *et al.*, 1996	yes
A. catenella	TNX22	Japan: Tanabe Bay, East Pacific	Group IV, (TA)	NR	Adachi *et al.*, 1996	yes
A. catenella	Y-2	Japan: Yamakawa, East Pacific	Group IV, (TA)	NR	Adachi *et al.*, 1996	yes
A. catenella	K0-3	Japan: Uranouchi Inlet,East Pacific	Group IV, (TA)	NR	Adachi *et al.*, 1996	yes
A. catenella	IEO-709	Spain: Tarragona, Catalan Sea, Mediterranean Sea	Group IV, (TA)	AJ968683	Penna *et al.*, 2005	yes
A. catenella	IEO-715	Spain: Tarragona, Catalan Sea, Mediterranean Sea	Group IV, (TA)	AJ968681	Penna *et al.*, 2005	yes
A. catenella	IEO-816	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	AJ968680	Penna *et al.*, 2005	yes
A. catenella	IEO-AC2C	Spain: Barcelona, Catalan Sea, Mediterranean Sea	Group IV, (TA)	AJ532912.	Penna *et al.*, 2005	yes
A. catenella	CNR-ACATA4	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Group IV, (TA)	AJ532910	Penna *et al.*, 2005	yes
A. catenella	ATTL02	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	AJ608264	Penna *et al.*, 2005	yes
A. catenella	ATTL01	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	AJ608263	Penna *et al.*, 2005	yes
A. catenella	CSIC-5T	Spain: Tarragona, Catalan Sea, Mediterranean Sea	Group IV, (TA)	AJ580325	Penna *et al.*, 2005	yes
A. catenella	CNR-ACATS3	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Group IV, (TA)	AJ580320	Penna *et al.*, 2005	yes
A. catenella	CNR-ACATS1	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Group IV, (TA)	AJ580319	Penna *et al.*, 2005	yes
A. catenella	CNR-ACATS2	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Group IV, (TA)	AJ580318	Penna *et al.*, 2005	yes

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A. catenella	CNR-ACATC2	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Group IV, (TA)	AJ580317	Penna *et al.*, 2005	yes
A. catenella	ACTEM17	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	FM211470	Masseret *et al.*, 2009	yes
A. catenella	AC0409-13	Japan: Akasaki Seaport	Group IV, (TA)	FM211466	Masseret *et al.*, 2009	yes
A. catenella	AC0310-06	Japan: Kita Nada, Kagawa	Group IV, (TA)	FM211462	Masseret *et al.*, 2009	yes
A. catenella	AC0206-18	Japan: Ago Bay, Mie	Group IV, (TA)	FM211460	Masseret *et al.*, 2009	yes
A. catenella	AC0202-15	Japan: Uchino-Umi, Tokushima	Group IV, (TA)	FM211457	Masseret *et al.*, 2009	yes
A. catenella	ACDH	China	Group IV, (TA)	DQ176658	Tang *et al.*, 2005	yes
A. catenella	MI7	Japan: Harima Nada	Group IV, (TA)	AB006990	Adachi *et al.*, 1996	yes
A. catenella	TL-C3111007G3	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	FR686536	Genovesi *et al.*, 2011	yes
A. tamarense	AT5-1	China	Group IV, (TA)	DQ176662	Tang *et al.*, 2005	yes
A. catenella	UNISS 6	Italy: Oristano, Mediterranean Sea, Sardinia	Group IV, (TA)	ND	This study	/
G. spinifera	CCMP 409	USA: West Boothbay Harbor, MA		EU532487	Howard *et al.*, 2009	yes

Note: The following acronyms, abbreviations and symbols indicate respectively:

NA = North American clade, ME = Mediterranean clade, WE = Western European clade, TA = Temperate Asian clade

/ = Toxicity of strain not studied

NR = GenBank sequence not reported

ND = Sequence not yet deposited in GenBank

*= Unpublished

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Table 2 List of *Alexandrium* spp. isolates and sequences obtained from this study and GenBank for the LSU rDNA region.

Species	Strain	Sampling Location	Clade	Accession Number	Source	Toxicity
A. affine	AABCV-1	Mexico: Gulf of California, Notrh Pacific Ocean		AY152706	Band-Schmidt *et al.* 2003	no
A. affine	PA5V	Spain: Ria de Vigo, Galicia		NR	Scholin *et al.*, 1994	no
A. affine	CU-1	Gulf of Thailand, Pacific Ocean		U44935	Scholin *et al.*, 1994	no
A. lusitanicum	18-1T	Portugal: Lagoa de Obidos	Global clade	EU707456	McCauley *et al.*, 2009	yes
A. lusitanicum	18-1NT	Portugal: Lagoa de Obidos	Global clade	EU707455	McCauley *et al.*, 2009	no
A. lusitanicum	AL2V	Spain: Ria de Vigo, Galicia	Global clade	EU707460, AY962837	McCauley *et al.*, 2009, Lilly *et al.*, 2005	yes
A. lusitanicum	AL-1	Portugal	Global clade	JF906999	Tang *et al.*, 2012	no
A. minutum	UNISS 3	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	ND	This study	/
A. minutum	UNISS 4	Italy: Olbia, Tyrrhenian Sea, Mediterranean Sea, Sardinia	Global clade	ND	This study	/
A. minutum	AMAD16	Australia: Adelaide	Global clade	JF521633	Orr *et al.*, 2011	yes
A. minutum	AMIR-1	Ireland: Cork Harbor	Global clade	EU707473, AY962848	McCauley *et al.*, 2009, Lilly *et al.*, 2005	yes
A.minutum	AMFL	England: Fleet Lagoon	Global clade	EU707471	McCauley *et al.*, 2009	yes
A. minutum	AM1	France: Morlaix Bay	Global clade	EU707466 ; AY962843	McCauley *et al.*, 2009, Lilly *et al.*, 2005	yes
A. minutum	AMAD06	Australia: Port River	Global clade	U44936	Scholin *et al.*, 1994	yes
A. minutum	CCMP113	Spain: Ria de Vigo, Galicia	Global clade	JF521634	Orr *et al.*, 2011	yes
A. minutum	LAC27	Italy: Gulf of Trieste, Adriatic Sea, Mediterranean Sea	Global clade	AY962842	Lilly *et al.*, 2005	yes
A.minutum	AMNZ02	New Zeland: Anakoha Bay	Pacific Clade	EU707477	McCauley *et al.*, 2009	yes
A.minutum	AMNZ01	New Zeland: Croisilles Harbor	Pacific Clade	EU707476	McCauley *et al.*, 2009	yes
A.minutum	AMBOPO06	New Zeland: Tauranga Harbor	Pacific Clade	EU707468,AY962846	McCauley *et al.*, 2009, Lilly *et al.*, 2005	yes
A.minutum	AMBOPO14	New Zeland	Pacific Clade	EU707469,AY962847	McCauley *et al.*, 2009, Lilly *et al.*, 2005	yes
A. catenella	ACC01	Chile	Group I (NA)	AY268597	Lilly *et al.*, 2007	yes
A. fundyense	GtCA29	USA: Cape Ann, North Atlantic	Group I (NA)	NR	Scholin *et al.*, 1994	yes
A. tamarense	PW06	USA: Port Benny, North Pacific	Group I (NA)	U44927	Scholin *et al.*, 1994	yes
A. tamarense	04-197-A1	Scotland	Group I (NA)	FJ042682	Collins *et al.*, 2009	yes
A. tamarense	04-197-30	Scotland	Group I (NA)	FJ042681	Collins *et al.*, 2009	yes
A. tamarense	Alex61-1	Scotland: Firth of Forth	Group I (NA)	AJ303445	Hingman *et al.*, 2001	yes
A. tamarense	Alex61-2	Scotland: Firth of Forth	Group I (NA)	AJ303446	Hingman *et al.*, 2001	yes
A. tamarense	SZN01	Italy: Gulf of Naples, Tyrrhenian Sea, Mediterranean Sea	Group II,(ME)	AJ535368	John *et al.*,2003	/
A. tamarense	SZN08	Italy: Gulf of Naples, Tyrrhenian Sea, Mediterranean Sea	Group II,(ME)	AJ535369	John *et al.*,2003	/

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A. tamarense	SZN19	Italy: Gulf of Naples, Tyrrhenian Sea, Mediterranean Sea	Group II,(ME)	AJ535370	John *et al.*,2003	/
A. tamarense	SZN21	Italy: Gulf of Naples, Tyrrhenian Sea, Mediterranean Sea	Group II,(ME)	AJ535374	John *et al.*,2003	/
A. tamarense	CCMP115	England: Plymouth	Group III, (WE)	JF521640	Orr *et al.*, 2011	no
A. tamarense	Pgt183	England: Plymouth	Group III, (WE)	U44930	Sholin *et al.*, 1994	no
A. tamarense	S06-010-01	Scotland: Shetland, Weisdale	Group III, (WE)	FJ042673	Collins *et al.*, 2009	no
A. tamarense	Alex31.6	Ireland: Cork Harbour	Group III, (WE)	AJ303433	Hingman *et al.*, 2001	no
A. tamarense	WKS-1	Japan: Kushimoto, East Pacific	Group III, (WE)	NR	Scholin *et al.*, 1994	no
A. tamarense	S07-035-01	Scotland: Laxfirth, Shetland	Group III, (WE)	FJ042680	Collins *et al.*, 2009	no
A. tamarense	Ply 173	England: Plymouth	Group III, (WE)	AJ308587	Hingman *et al.*, 2001	no
A. tamarense	TL-A3200907F5	France: Thau Lagoon	Group III, (WE)	FR686537	Genovesi *et al.*, 2011	no
A. tamarense	UNISS 5	Italy: Gulf of Alghero, Mediterranean Sea, Sardinia	Group III, (WE)	ND	This study	/
A. catenella	DPC95b	South Korea: Dadaepo	Group IV, (TA)	AY082051	Kim and Kim, 2004	/
A. catenella	OF101	North Japan: Ofunato Bay	Group IV, (TA)	U44931	Sholin *et al.*, 1994	yes
A. catenella	ACT1	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	FM211468	Masseret *et al.*, 2009	yes
A.catenella	ATT98	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	AF318220	Guillou *et al.*, 2002	yes
A.catenella	ACTRA02	Australia: Triabunna, Tasmania	Group IV, (TA)	AY338754	Mackenzie *et al.*, 2004	yes
A. catenella	AC0206-18	Japan: Ago Bay, Mie	Group IV, (TA)	FM211460	Masseret *et al.*, 2009	yes
A. catenella	AC0409-13	Japan: Akasaki Seaport	Group IV, (TA)	FM211466	Masseret *et al.*, 2009	yes
A. catenella	AC0409-08	Japan: Akasaki Seaport	Group IV, (TA)	FM211465	Masseret *et al.*, 2009	yes
A. catenella	AC0310-06	Japan: Kita Nada, Kagawa	Group IV, (TA)	FM211462	Masseret *et al.*, 2009	yes
A.catenella	AC0310-01	Japan: Kita Nada, Kagawa	Group IV, (TA)	FM211461	Masseret *et al.*, 2009	yes
A. catenella	ACTEM17	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	FM211470	Masseret *et al.*, 2009	yes
A. catenella	ACTEM9	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	FM211469	Masseret *et al.*, 2009	yes
A. catenella	AC0202-15	Japan: Uchino-Umi, Tokushima	Group IV, (TA)	FM211457	Masseret *et al.*, 2009	yes
A. catenella	TL-C3111007G3	France: Thau Lagoon, Mediterranean Sea	Group IV, (TA)	FR686536	Genovesi *et al.*, 2011	yes
A. catenella	UNISS 6	Italy: Oristano, Mediterranean Sea, Sardinia	Group IV, (TA)	ND	This study	/
A. catenella	LAC35	Australia: Port Phillip Bay	Group IV, (TA)	AY268610	Lilly *et al.*, 2007	/
A. tamarense	ATCI01-1	China: Dai Ya Bay	Group IV, (TA)	AY268612	Lilly *et al.*, 2007	yes
A. tamarense	AT5-3	South China	Group IV, (TA)	JF906993	Tang *et al.*, 2012	yes
A. tamarense	ATBB01	Australia: Bell Bay, Tasmania	Group V , (TASM)	U44933	Scholin *et al.*, 1994	no
G. spinifera	GSA0602	Italy: Cesenatico, Adriatic Sea, Mediterranean Sea		EU805591	Riccardi *et al.*, 2009	yes

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Note: The following acronyms, abbreviations and symbols indicate respectively:

NA = North American clade, ME = Mediterranean clade, WE = Western European clade, TA = Temperate Asian clade, TASM = Tasmanian clade

/ = Toxicity of strain not studied

NR = GenBank sequence not reported

ND = Sequence not yet deposited in GenBank

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4. CHAPTER Ib

Published on Estuaries and Coast

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Dinoflagellate Cyst Assemblages in Surface Sediments from Three Shallow Mediterranean Lagoons (Sardinia, North Western Mediterranean Sea)

**Cecilia T. Satta · Sílvia Anglès · Esther Garcés ·
Nicola Sechi · Silvia Pulina · Bachisio Mario Padedda ·
Daniela Stacca · Antonella Lugliè**

Abstract The present study identified and quantified dinoflagellate cysts in surface sediments from three Mediterranean lagoons. Sediment samples were recovered from 11 stations in May 2009 at Cabras Lagoon, eight stations in May 2010 at Corru S'Ittiri Lagoon, and five stations in May 2011 at Santa Giusta Lagoon. Fifty-three dinoflagellate cyst morphotypes were identified. Sixteen species are first reports for the lagoons, and two for the Mediterranean Sea. Moreover, a new *Scrippsiella* species was discovered in Cabras. Seven harmful algal species were identified, primarily belonging to the potentially toxic genus *Alexandrium*. Total cyst abundance, number of morphotypes, and assemblages varied among lagoons, and each lagoon showed a distinct morphotype composition. A degree of heterogeneity was also detected within lagoon. Cabras and Santa Giusta cyst assemblages were characterised by morphotypes belonging to the autotrophic genus *Scrippsiella*, whereas Corru S'Ittiri assemblages showed dominance of heterotrophic morphotypes, including *Protoperidinium* cf *tricingulatum*. Differentiation among lagoons was also evident according to environmental conditions. Salinity proved to be a fundamental variable in determining total cyst abundance, morphotype number, and composition.

This study was among the first to examine dinoflagellate cyst composition in coastal lagoons, especially from the Mediterranean region, and contributed data that increased our knowledge of cyst-producing dinoflagellates in these environments.

Introduction

Dinoflagellates constitute an important component of phytoplankton, and are commonly found in freshwater, brackish, and marine ecosystems. During their life cycle, many dinoflagellate species are capable of producing resting cysts prevalently through sexual reproduction (Figueroa et al. 2008 and references therein; Walker 1984). Resting cysts have considerable ecological importance for cyst-producing species, as they ensure survival, favour dispersion, provide a source of genetic diversity, and promote bloom initiation and its recurrence (Anderson and Wall 1978; Dale 1983). Once formed, cysts settle in sediments and remain viable for months, years, or even up to one century (Lundholm et al. 2011). Consequently, cysts serve as a proxy of plankton populations. Therefore, cysts in the sediments are a valuable tool to increase our knowledge on dinoflagellate diversity and distribution, overcoming the problems associated with the fugacity of plankton populations (Dale 1983; Godhe and McQuoid 2003). Moreover, cyst assemblage composition generally reflects temperature, salinity, productivity, and nutrient conditions (Zonneveld et al. 2013 and references therein), and several cyst assemblage signals can indicate eutrophication and pollution conditions (Dale 2009 and references therein; Shin et al. 2011). In recent years, a growing interest in cyst studies resulted in many reports associated with coastal,

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estuarine, and oceanic areas worldwide (Zonneveld et al. 2013). These studies are numerous in transitional waters, such as estuarine environments; however, less information is available for coastal lagoons (Pospelova et al. 2004).

Coastal lagoons are fundamental ecosystems since they provide key ecosystem goods and services, including water quality improvement, reducing transportation pollutant loads from rivers to coastal marine areas, fisheries resources, human recreational areas, and habitat and food for migratory and resident animals (Levin 2001). However, the rich goods and services provisions available in lagoons have resulted in human over-exploitation of these ecosystems, and consequently, major structural changes have occurred to optimise anthropogenic activities. In addition, lagoons suffer indirect human pressures resulting from intense activities on lagoon watersheds (e.g. intensive agriculture and farms, industry), as well as global climate changes (Lloret et al. 2008). Cloern and Jassby (2010) indicated the coastal lagoon phytoplankton exhibited intricate responses to the complexity of influences and processes that act on the environment. Therefore, phytoplankton assemblage composition can vary widely based on geological, hydrological, and ecological factors, and is also influenced by geographic location. In addition, Phlips et al. (2010) observed significant intra-site heterogeneity as well as at small scale. Dinoflagellates also exhibit a wide response to environmental conditions, and for cyst-producing species, additional information can be obtained from resting stages stored in sediments. Therefore, obtaining data from poorly known cyst assemblages of coastal lagoons can serve to identify cyst responses from these ecosystems, and establish an important foundation for ecological characterisation and comparison.

The first objective of this study was to identify and quantify dinoflagellate cysts in recent sediments from three Mediterranean lagoons located in the same geographical area (Gulf of Oristano, Sardinia, North Western Mediterranean Sea). Second, long-term environmental data availability for these lagoons enabled us to compare the cyst assemblages with specific environmental conditions among the lagoons.

Study Areas

Cabras, Santa Giusta, and Corru S'Ittiri lagoons are located along the western coast of Sardinia (North Western Mediterranean Sea) and adjacent to the Gulf of Oristano (Fig. 1). The lagoons belong to a complex system of wetlands and transitional waters of high economic and natural value, which are protected by international agreements (Ramsar Convention; Site of Community Importance and Special Protection Areas). The lagoons are primarily exploited for fishing (Cabras, Santa Giusta, and Corru S'Ittiri) and shellfish harvesting (Corru S'Ittiri). Furthermore, the catchments suffer the impacts of

urban, agricultural, and industrial activities, and several engineering projects were completed in the lagoons with different objectives. Consequently, the lagoons are highly eutrophic (Pulina et al. 2012; Sechi et al. 2001). Phytoplankton and nutrient monitoring have been performed since the early 1990s, particularly in Cabras and Santa Giusta, due to recurrent dystrophic crises impacts on lagoon fish productivity. Due to the availability of long-term data, the two lagoons were accepted as research sites in part of site "14 Sardinian marine ecosystems" of the Italian network Long Term Ecological Research (www.lteritalia.it).

Cabras Lagoon

Cabras is the largest lagoon in Sardinia, occupying 23.8 km^2, with a mean depth of 1.6 m. The most important freshwater input is derived from the Rio Mare e' Foghe, located in the northern lagoon area (Fig. 1a). During the twentieth century, lagoon–sea exchange was highly modified by human intervention. A connection with the adjacent Mistras Lagoon was eliminated, and the Scolmatore (= spillway) canal was constructed in late 1970s. Then, in the early 1980s, the canal was barred with a dam to prevent further increases in lagoon salinity, and artificial barriers were constructed to control fishing. Consequently, the only exchanges with the sea occur through a small system of narrow channels in the southern area of the lagoon. Phytoplankton is the most important primary producer, and the lagoon biotic structure has faced considerable variation in recent years (Pulina et al. 2012). The Cabras sediments exhibit very high total organic carbon (TOC) and organic matter (OM) values, and also high percentage of silt size fraction (De Falco et al. 2004).

Santa Giusta Lagoon

Santa Giusta occupies an area of 8 km^2, with a mean depth of 1 m. Rio Pauli Maiori and Rio Pauli Figu are the two primary freshwater inputs, both located on the lagoon's east side (Fig. 1b). Santa Giusta also experienced substantial human modification during the twentieth century, resulting in profound ecosystem alterations. The Pesaria channel, which originally connected the lagoon to the sea through the Tirso River outlet, was deepened, widened, and separated from the river. An industrial harbour was subsequently built, which was connected to the lagoon through an industrial canal controlled by bulkheads. A fish catch system was constructed in the final portion of the Pesaria channel. In 1995, a diversion canal for Oristano urban wastes (the main urban town in the catchment) was built. Despite the canal, the high inorganic nutrient concentrations and algal biomass remained unchanged (Sechi et al. 2001). Macrobenthic algae and phytoplankton are the most important primary producers in Santa Giusta. Sechi et al. (2001) reported several fish kill events associated with

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Fig. 1 Location of Cabras (**a**), Santa Giusta (**b**), and Corru S'Ittiri (**c**) lagoons and localization of sampling stations; *black filled cycles*: sediment sampling stations, *white filled cycles*: sediment and environmental sampling stations, *stars*: environmental sampling stations, *black arrows*: freshwater inputs, *white arrows*: inlets
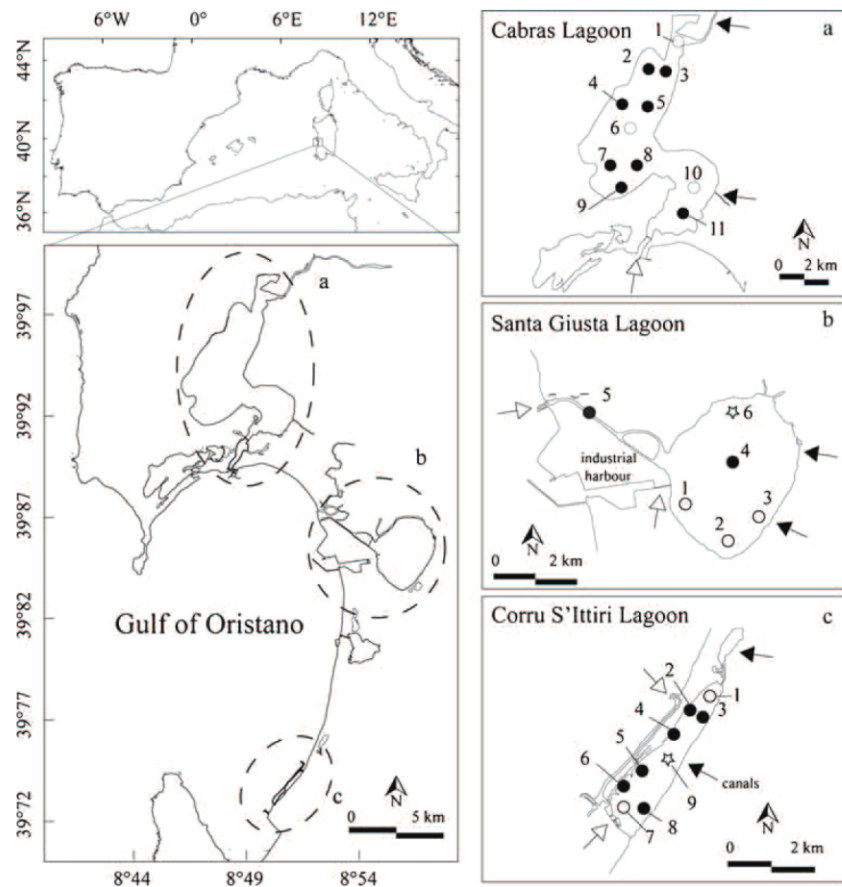
harmful algal species blooms in this lagoon. Santa Giusta sediments show high levels of TOC and OM, especially in surface layers (1–2 cm) in the north and south lagoon areas (Magni et al. 2008). Lugliè et al. (2002) showed Santa Giusta sediment grain sizes exhibited a degree of heterogeneity, but the finer fractions were primarily located in the south-central lagoon area, consistent with central and peripheral canals dredged in the 1970s to facilitate seawater flow into the lagoon.

Corru S'Ittiri Lagoon

Corru S'Ittiri is the smallest of the three lagoons, with a total area of 1.5 km^2 and a mean depth of 0.8 m. This lagoon, unlike the others, originated from a coastal area confined by shore-parallel sand spit, which was artificially connected to the land along its southern end. Inputs from the catchment emerge from the small Pauli Pirastru Pond located to the north. In addition, freshwater from a nearby irrigation area drainage system flows into the lagoon by six canals along the east

coastline (Fig. 1c). The sea connection occurs through two inlets, one to the north and the other larger one, at the lagoon's southern end (Fig. 1c). The lagoon sediment is primarily sandy, and characterised by the strong presence of calcareous reef-like aggregates built up by the serpulid polychaete *Ficopomatus enigmaticus* Fauvel (B.M. Padedda, personal communication). Finer sediments are distributed in the underwater canal along the west coast.

Materials and Methods

Sediment Sampling and Dinoflagellate Cyst Analysis

Sampling surveys were conducted in May 2009 (Cabras Lagoon), May 2010 (Corru S'Ittiri Lagoon), and May 2011 (Santa Giusta Lagoon). Sediment cores were collected from 11 stations in Cabras, eight stations in Corru S'Ittiri, and five stations in Santa Giusta (Fig. 1). Sampling stations were chosen based on the distribution of finer grain size fraction of the sediments.

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Moreover the localization of the stations at Santa Giusta and Corru S'Ittiri was forcibly limited by the presence of calcareous aggregates produced by the polychaete *F. enigmaticus* that covered the bottom of both lagoons. Samples were collected using a hand-core connected to cylindrical plastic tubes (40 cm long, 5 cm in diameter). Samples were stored in the dark at 4 °C and processed within 6 months of collection.

The top 1 cm of each core sample from each station was sectioned. Subsamples (2–3 cm^3) of each section were suspended into filtered seawater (FSW) and sonicated for 2 min using a Bandelin Sonoplus ultrasonic homogeniser. Following sonication, samples were filtered through a 100-μm mesh and collected on a 10-μm mesh. The fraction obtained was washed with FSW and collected in a 50-ml tube. Subsamples (5–7 ml) of the slurry were processed for cyst concentration and separation from inorganic particles using the sodium polytungstate (SPT) method (Bolch 1997), adding the SPT solution at the second centrifugation step following Bravo et al. (2006). The resulting sample was rinsed in a 10-μm mesh and collected in 5–15 ml of FSW. Final cyst sample aliquots were counted in 3-ml sedimentation chambers with a Zeiss Axiovert 10 inverted microscope at ×400 magnification.

Cyst identification, nomenclature, and terminology applied to describe morphotypes were based on the literature reported in Table 1. The *Scrippsiella trochoidea* complex was established for all morphotypes referable to this species (as reported in Satta et al. 2013). The designation "round brown" was established to include spherical/oval protoperidinioid cysts that could not be identified due to an absence of information on the archeopyle. Round brown smooth types included the smooth typology, indicative of *Protoperidinium*, *Diplopsalis*, and related genera. Round brown spiny types included all morphotypes that presented spinous processes. Some of these morphotypes might belong to the cyst-based genera *Echinidinium* and *Islandinium*. Cysts with unknown affinity were classified as Unidentified. Cyst abundances were expressed as relative abundances (per cent) of the entire cyst assemblage and as the number of cysts per gram dry weight sediment. Dry weight was obtained by drying wet sediment subsamples (2–3 cm^3) at 105 °C for 24 h.

Excystment Experiments

To confirm the identification, a number of the cyst morphotypes were photographed, isolated with a glass micropipette, and then transferred into IWAKI tissue culture multiplates. Plates were filled with L1 medium (Guillard and Hargraves 1993) prepared with FSW adjusted to a salinity of 31. The plates were incubated at 20±1 °C for a 12:12 light:dark cycle under an irradiance of 100 μmol m^{-2} s^{-1}. Plates were checked every 2–4 days to confirm cyst germination. Thecae of armoured germinated cells were stained with Calcofluor white, and examined under fluorescence microscopy.

Genetic Analyses

Genetic analyses were conducted to confirm species identification of several cyst morphotypes (Table 1). DNA was extracted from ~10 ml of each culture in the logarithmic growth phase. Cells were centrifuged at 3,000 rpm for 15 min, the pellet transferred to a 2-ml Eppendorf tube, and centrifuged at 10,000 rpm for 5 min. The final pellet was used to extract total DNA using a DNeasy Plant MiniKit (Qiagen) following the manufacturer's instructions. Extracted DNA was immediately frozen at −80 °C. PCR primers D1R and D2C (Scholin et al. 1994) were used to amplify the D1–D2 regions of the large subunit (LSU) rRNA gene, and ITS1 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer (ITS) 1, the 5.8S rRNA gene, and ITS 2. PCR reactions (total 50 μl) were performed using 1 μl of DNA, 0.625 μl of each primer (10 μM), 1.5 μl of dNTPs (200 μM of each), 1.5 μl of MgCl$_2$ (25 mM, only in LSU reactions), 2.5 μl of 10x PCR buffer, and 0.125 μl of Taq DNA polymerase (Qiagen). PCR thermocycle parameters included an initial denaturation cycle of 95 °C for 5 min; followed by 40 cycles of 95 °C for 20 s, 55 °C (for LSU PCR) or 53 °C (for ITS and 5.8S PCR) for 30 s, and 72 °C for 1 min; followed by a final extension of 72 °C for 10 min. Aliquots of PCR products were electrophoresed in 1.2 % agarose gels and visualised under UV illumination. The remaining PCR products were frozen at −20 °C until sequenced. An external service (GenoScreen, France) was contracted to conduct purification and sequencing using the specified primers under a 3730XL Sanger sequencer. The generated sequences were queried against NCBI nucleotide sequences using BLAST analysis (http:// www. ncbi.nlm.nih.gov) to ascertain species sequences with the highest identity values.

Statistical Approaches and Environmental Data

Variation in cyst assemblage composition among lagoons was analysed applying a cluster analysis on a similarity matrix based on the Bray–Curtis similarity index with the PRIMER software. This matrix was constructed on log$_{10}$ $(x+1)$ transformed cyst abundance data. Based on morphotype mean per cent composition in each lagoon, morphotypes contributing <1 % were excluded from the analysis to reduce the influence of rare (i.e. very low abundance) species. Differences in cyst assemblage composition among lagoons were tested using the one-way analysis of similarities (ANOSIM).

Temperature (Tem), salinity (Sal), dissolved inorganic nitrogen (DIN), total phosphorous (Ptot), and chlorophyll *a* (Chl*a*) data for Cabras and Santa Giusta were provided from long-term datasets (7 and 10 years for Cabras and Santa

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Table 1 Biological names, mean percentage abundances (P <1.5 %, *1.5–10 %, **10–15 %, ***>15 %), and references of the dinoflagellate cyst morphotypes identified in Cabras (CA), Corru S'Ittiri (CI), and Santa Giusta (SG) lagoons

	CA	CI	SG	References
Organic Peridiniales				
Bysmatrum sp.[a]	–	*	–	Satta et al. 2013
Kryptoperidinium foliaceum (Stein) Lindemann	–	–	P	Figueroa et al. 2009
Pentapharsodinium cf *daleii* Indelicato et Loeblich III	–	–	P	Lewis 1991
Protoperidinium compressum (Abé) Balech	–	P	–	Wall and Dale 1968
Protoperidinium cf *divergens* (Ehrenberg) Balech	–	P	–	Dale 1983
Protoperidinium leonis (Pavillard) Balech	–	–	P	Wall and Dale 1968
Protoperidinium cf *tricingulatum* Kawami, van Wezel, Koeman et Matsuoka	–	***	–	Kawami et al. 2009
Protoperidinium sp.	–	P	–	
Round brown spiny type 1	*	*	–	
Round brown spiny type 2	*	–	–	
Round brown spiny type 3	–	*	–	
Round brown spiny type 4	–	*	–	
Other round brown spiny cysts	–	*	P	Radi et al. 2013 and references therein
Round brown smooth type 1	–	*	–	
Round brown smooth type 2	–	*	–	
Round brown smooth type 3	–	–	*	
Other round brown smooth cysts	–	*	*	
Scrippsiella donghaienis[a] Gu	–	P	–	Gu et al. 2008
Scrippsiella sp. 1[a]	***	–	–	
Scrippsiella sp. 2	–	–	**	
Calcareous Peridiniales				
Pentapharsodinium tyrrhenicum[a] (Balech) Montresor, Zingone at Marino	–	*	*	Montresor et al. 1993
Scrippsiella kirschiae Zinssmeister	–	*	*	Satta et al. 2010; Zinssmeister et al. 2012
Scrippsiella lachrymosa Lewis	–	–	P	Lewis 1991
Scrippsiella precaria Montresor et Zingone	**	*	P	Montresor and Zingone 1988
Scrippsiella rotunda Lewis	–	–	P	Lewis 1991
Scrippsiella trochoidea complex[a] (Stein) Balech ex Loeblich III	*	**	***	Montresor et al. 2003
Scrippsiella sp. 3	–	*	P	Montresor et al. 1994
Scrippsiella sp. 4	*	–	–	
Scrippsiella sp. 5	–	–	*	
Other *Scrippsiella* spp.	–	P	P	
Calcareous type 1	–	*	–	
Calcareous type 2	–	P	P	
Gonyaulacales				
Alexandrium catenella[a] (Whedon et Kofoid) Balech	–	–	*	Fukuyo 1985
Alexandrium minutum Halim	–	*	**	Bolch et al. 1991; Bravo et al. 2006
Alexandrium tamarense (Lebour) Balech	–	–	*	Fukuyo 1985
Alexandrium cf taylorii Balech	–	P	–	Bravo et al. 2006
Fragilidium sp.	*	–	–	
Gonyaulax cf *verior* Sournia	–	–	*	Matsuoka et al. 1988
Gonyaulax sp. 1	–	–	*	
Other *Gonyaulax* spp.	–	P	*	
Pyrophacus sp.	–	–	*	
Gymnodiniales				
Gymodinium impudicum[a] (Fraga et Bravo) Hansen et Moestrup	–	–	*	Kobayashi et al. 2001
Gymnodinium litoralis[a] Reñé	–	**	–	Reñé et al. 2011
Gymnodinium/Gyrodinium sp.	–	–	P	

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Table 1 (continued)

	CA	CI	SG	References
Polykrikos sp.	P	–	–	
Gymnodiniales type 1	P	–	*	
Gymnodiniales type 2	–	P	–	
Gymnodiniales type 3	–	*	–	
Suessiales				
Biecheleria cincta[a] (Siano, Montresor et Zingone) Siano comb. nov.	–	P	*	Siano et al. 2009; Satta et al. 2013
Unidentified cyst type 1	***	–	–	
Unidentified cyst type 2	**	–	–	
Unidentified cyst type 3	–	*	–	
Unidentified cyst type 4	–	–	*	
Other unidentified cysts	P	*	*	

Harmful algal species are reported in *bold*

[a] Species confirmed by genetic analysis

Giusta, respectively). Data for Corru S'Ittiri were provided from sampling surveys conducted from February 2010 through December 2011 within the Zoumgest project. All parameters were obtained routinely, every 14 or 30 days, from three sampling stations in Cabras and Corru S'Ittiri and from four sampling stations in Santa Giusta (Fig. 1). Laboratory analyses were performed according to the methods described in Pulina et al. (2012). Mean multi-annual values for the above environmental parameters are reported for winter (December–January–February) and summer (June–July–August) for each station and lagoon (Table 2). Principal component analysis (PCA) was used to identify patterns of variation in environmental parameters and express data similarities and differences among lagoons. Data were $\log_{10}(x+1)$ transformed before analysis with PRIMER software. In addition, differences in summer and winter environmental data among lagoons were assessed through a one-way analysis of variance (ANOVA) using R program. When significant differences were observed ($P<0.05$), a Tukey's pairwise comparison test was also performed.

Table 2 Multiannual mean values and standard errors of surface temperature (Tem), salinity (Sal), dissolved inorganic nitrogen (DIN), total phosphorous (Ptot), and chlorophyll a (Chl$a$) in summer and winter in Cabras (CA), Corru S'Ittiri (CI), and Santa Giusta (SG) environmental sampling stations

Season	Stations	Tem (°C)	Sal	DIN (μM)	Ptot (μM)	Chl$a$ (μg l^{-1})
Summer	1 CA	25.6±0.6	21.3±1.9	5.2±0.7	11.1±1.0	38.5±7.4
	6 CA	25.9±0.5	24.4±1.6	9.7±4.0	8.4±0.7	35.4±6.0
	10 CA	25.9±0.5	25.6±1.6	8.5±3.5	7.2±0.6	24.6±3.3
	1 CI	27.0±0.5	38.2±0.5	5.8±1.5	4.9±0.5	22.0±5.5
	7 CI	26.5±0.5	39.5±0.5	1.8±0.4	3.4±0.4	21.0±5.9
	9 CI	26.7±0.5	39.1±0.5	2.1±0.4	4.4±0.5	26.4±8.1
	1 SG	25.9±0.4	33.3±0.9	4.5±0.9	6.0±0.4	17.1±2.9
	2 SG	26.3±0.4	36.4±0.6	6.3±2.3	4.5±0.4	10.1±2.1
	3 SG	26.6±0.4	33.1±0.9	3.7±0.9	6.2±0.5	26.7±6.6
	6 SG	26.5±0.4	32.6±0.9	4.5±1.0	6.4±0.5	22.4±3.4
Winter	1 CA	10.6±0.5	13.9±1.9	24.9±7.0	10.0±1.3	50.5±8.0
	6 CA	10.3±0.4	17.8±1.7	10.3±2.7	8.2±0.9	61.1±10.3
	10 CA	10.3±0.5	18.1±1.8	12.1±3.6	8.2±0.8	67.5±12.0
	1 CI	11.4±0.4	30.4±1.0	34.9±7.5	17.2±3.9	16.6±5.1
	7 CI	10.9±0.5	30.8±0.8	29.4±7.6	12.1±3.2	17.3±6.0
	9 CI	11.0±0.5	30.3±1.0	28.8±9.0	12.4±3.3	21.5±7.3
	1 SG	11.0±0.3	25.1±1.2	17.6±3.8	4.9±0.3	10.7±2.4
	2 SG	11.0±0.4	25.7±1.5	9.9±3.2	4.1±0.3	11.9±2.5
	3 SG	11.7±0.3	25.0±1.3	27.0±8.8	6.3±0.9	9.9±1.9
	6 SG	11.2±0.3	24.7±1.2	16.9±2.5	5.0±0.3	12.1±2.3

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Results

Dinoflagellate Cyst Morphotypes

Fifty-three cyst morphotypes were found in surface sediments among the three lagoons, the majority representing 13 genera and four orders (Peridiniales, Gonyaulacales, Gymnodiniales, and Suessiales; Table 1). Eleven morphotypes were observed in Cabras, and 29 were identified in Corru S'Ittiri and Santa Giusta, some of which are shown in Figs. 2 and 3. Seven morphotypes were unique to Cabras, 15 to Corru S'Ittiri, and 16 to Santa Giusta. Moreover, 12 morphotypes were shared between at least two lagoons, while only two morphotypes were common to all three lagoons. Seven morphotypes referable to harmful algal species were observed. *Alexandrium minutum* was detected in Corru S'Ittiri and Santa Giusta. *Alexandrium catenella*, *Alexandrium tamarense*, *Gymnodinium impudicum*, and *Kryptoperidinium foliaceum* were only observed in Santa Giusta. Finally, *Alexandrium* cf *taylorii* and *Gymnodinium litoralis* were only detected in Corru S'Ittiri (Table 1; Figs. 2 and 3).

Below we provide descriptions of four morphotypes that are poorly represented in the literature:

Protoperidinium cf tricingulatum

P. cf *tricingulatum* (Fig. 2f) represented >15 % of the total cyst abundance in Corru S'Ittiri (Table 1). The cyst was spherical and 21–27 μm in diameter (n=7). The double-layered wall was covered by numerous capitate spines 5–7 μm long, and cyst content was granular and pale brown in colour. The archeopyle was theropylic. The morphotype largely resembled *P. tricingulatum* cysts described from Wadden Sea samples (Kawami et al. 2009), although our cysts were slightly smaller. Germinated vegetative cells were clearly heterotrophic *Protoperidinium*-like cells; however, no further morphologic or genetic characterisation was possible.

Scrippsiella donghaienis

S. donghaienis (Fig. 2k) was observed at only one Corru S'Ittiri station with low percentage abundance (<1.5 %, Table 1). The cyst was non-calcareous and spherical (22 μm in diameter), and the cyst content was granular and greyish/brownish in colour. A yellow accumulation body was present at one side of the cyst. Germination experiments were successful, and a culture was established. Genetic analyses generated nucleotide sequences with high identity values to *S. donghaienis* (94–100 % identity), a species described from the East China Sea (Gu et al. 2008).

Scrippsiella sp. 1

Scrippsiella sp. 1 (Fig. 2l) represented >15 % of the total cyst abundance in Cabras (Table 1). The cyst was non-calcareous and spherical (20–26 μm in diameter; n=10), and cyst content was granular and pale grey in colour. A ruby red accumulation body was present at one side of the cyst. Germination experiments produced a culture, and genetic analyses of *Scrippsiella* sp. 1 generated nucleotide sequences that differed significantly from the sequences of other *Scrippsiella* species deposited in the NCBI database.

Scrippsiella sp. 2

Scrippsiella sp. 2 (Fig. 2m) represented 10–15 % of the total cyst abundance in Santa Giusta (Table 1). The cyst was non-calcareous and spherical (20–23 μm in diameter; n=3), and the content was granular and greenish-grey in colour. A yellow accumulation body was present. This morphotype largely resembled a type described by Satta et al. (2010) as *Scrippsiella* sp. 2.

Total Cyst Abundance and Dinoflagellate Assemblage Composition

Cyst abundances were lower in Cabras compared to Corru S'Ittiri and Santa Giusta lagoons (Table 3). In Cabras, the highest total cyst abundance was 287 cysts g^{-1} (station 6) and the lowest 46 cysts g^{-1} (station 10). Cyst abundance in Corru S'Ittiri ranged from 61 cysts g^{-1} (station 3) to 1,072 cysts g^{-1} (station 8), whereas Santa Giusta, with abundances ranging from 144 cysts g^{-1} (station 5) to 2,317 cysts g^{-1} (station 2), showed the highest cyst abundance.

The Peridiniales exhibited the highest taxon representation in all dinoflagellate assemblages among the three lagoons, with a 66 %, 78 %, and 58 % mean relative percentages, in Cabras, Corru S'Ittiri, and Santa Giusta. Peridiniales were comprised primarily of organic morphotypes in Cabras and Corru S'Ittiri, whereas calcareous morphotypes prevailed in Santa Giusta. The other groups were less represented (<10 %), with the exception of unidentified morphotypes in Cabras, Gonyaulacales in Santa Giusta, and Gymnodiniales in Corru S'Ittiri (Table 3).

The Cabras cyst assemblage was characterised by dominance of the organic *Scrippsiella* sp. 1 and the Unidentified cyst types 1 and 2 (Table 3). In particular, *Scrippsiella* sp. 1 was recorded at all stations, with station 6 exhibiting the highest abundance (Fig. 4).

Heterotrophic organic morphotypes were dominant at Corru S'Ittiri. *P.* cf *tricingulatum* was the most represented, with station 2 exhibiting the highest abundance (Fig. 4). Round brown spiny and smooth morphotypes were also well represented. Among the calcareous Peridiniales, the *S.*
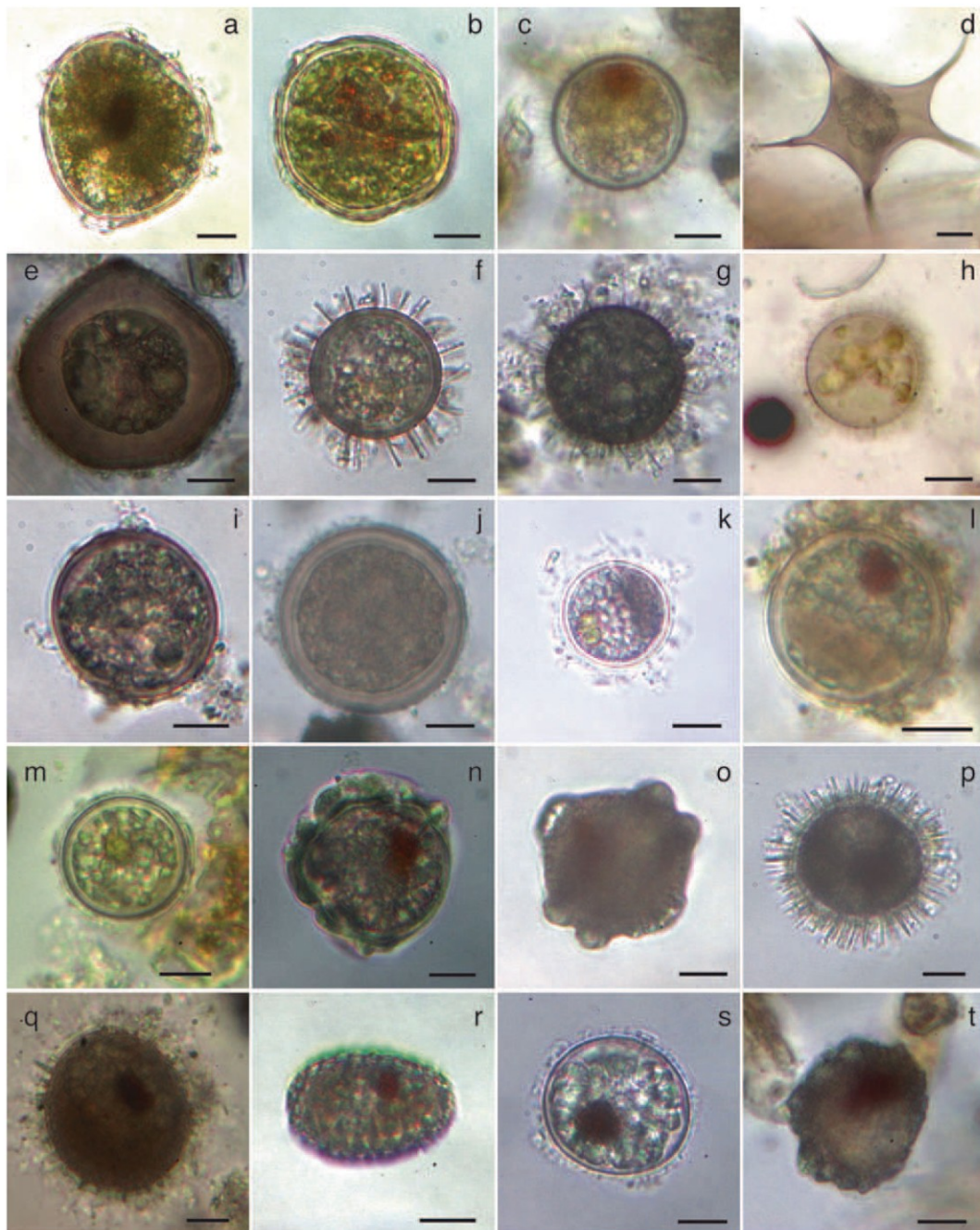
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Fig. 2 Photomicrographs of some dinoflagellate cysts in surface sediments from Cabras, Corru S'Ittiri, and Santa Giusta lagoons: (**a**) *Bysmatrum* sp., (**b**) *Kryptoperidinium foliaceum*, (**c**) *Pentapharsodinium* cf *daleii*, (**d**) *Protoperidinium compressum*, (**e**) *Protoperidinium* cf *divergens*, (**f**) *Protoperidinium* cf *tricingulatum*, (**g**) Round brown spiny type 1, (**h**) Round brown spiny type 2, (**i**) Round brown smooth type 1, (**j**) Round brown smooth type 2, (**k**) *Scrippsiella donghaiensis*, (**l**) *Scrippsiella* sp. 1, (**m**) *Scrippsiella* sp. 2, (**n**) *Pentapharsodinium tyrrhenicum*, (**o**) *Scrippsiella kirskiae*, (**p**) *Scrippsiella precaria*, (**q–s**) *Scrippsiella trochoidea* complex, and (**t**) Calcareous type 2. *Scale bars*, 10 μm

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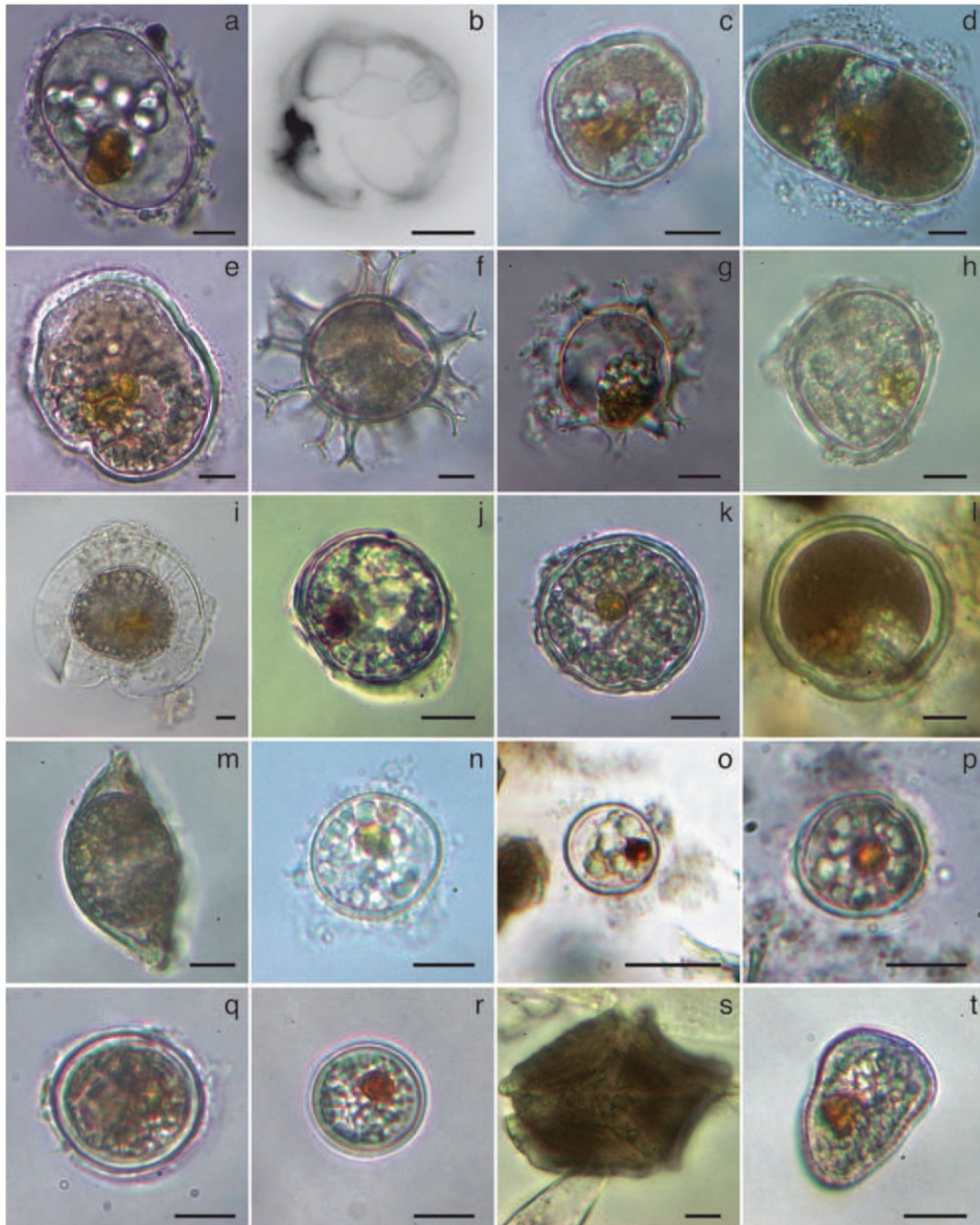
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Fig. 3 Photomicrographs of some dinoflagellate cysts in surface sediments from Cabras, Corru S'Ittiri, and Santa Giusta lagoons: (**a**, **b**) *Alexandrium catenella*: (**a**) resting cyst, (**b**) vegetative cell stained with Calcofluor, (**c**) *Alexandrium minutum*, (**d**) *Alexandrium tamarense*, (**e**) *Alexandrium* cf *taylorii*, (**f**–**h**) *Gonyaulax* spp., (**i**) *Pyrophacus* sp., (**j**) *Gymnodinium impudicum*, (**k**) *Gymnodinium litoralis*, (**l**) *Gymnodinium/Gyrodinium* sp., (**m**) Gymnodiniales type 1, (**n**) *Biecheleria cincta*, (**o**) Unidentified type 1, (**p**) Unidentified type 2, (**q**) Unidentified type 3, (**r**) Unidentified type 4, and (**s**, **t**) other Unidentified cysts. *Scale bars*, 10 μm

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Table 3 Absolute abundances (cysts per gram dry weight) and relative abundance percentages (per cent) of dinoflagellate morphotypes and cyst groups at each station of the three lagoons

	Cabras												Corru S'ittiri									Santa Giusta					
	1	2	3	4	5	6	7	8	9	10	11	Mean	1	2	3	4	5	6	7	8	Mean	1	2	3	4	5	Mean
Bysmatrum sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	67	0	35	13	0	0	0	0	0	0
Kryptoperidinium foliaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	1
Pentapharsodinium cf daleii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Protoperidinium compressum	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	0	0	0	3	0	0	0	0	0	0
Protoperidinium cf divergens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	51	0	6	0	0	0	0	0	0
Protoperidinium leonis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	3
Protoperidinium cf tricingulatum	0	0	0	0	0	0	0	0	0	0	0	0	76	309	25	269	12	67	51	0	101	0	0	0	0	0	0
Protoperidinium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	1	0	0	0	0	0	0
Round brown spiny type 1	29	0	15	6	0	0	14	0	0	0	0	6	0	0	0	0	0	0	153	35	23	0	0	0	0	0	0
Round brown spiny type 2	21	0	0	39	0	0	20	0	36	5	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Round brown spiny type 3	0	0	0	0	0	0	0	0	0	0	0	0	38	0	4	90	0	201	51	69	57	0	0	0	0	0	0
Round brown spiny type 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	3	0	0	0	0	0	0
Other round brown spiny cysts	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	54	0	0	0	0	7	5	0	0	0	0	1
Round brown smooth type 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	12	0	0	0	2	0	0	0	0	0	0
Round brown smooth type 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	134	0	35	21	0	0	0	0	0	0
Round brown smooth type 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	59	154	137	0	0	70
Other round brown smooth cysts	0	0	0	0	0	0	0	0	0	0	0	0	19	77	0	18	0	0	0	69	23	2	84	41	18	9	31
Scrippsiella donghaienis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	51	0	6	0	0	0	0	0	0
Scrippsiella sp. 1	36	37	61	19	27	118	41	83	36	14	66	49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scrippsiella sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39	335	130	117	0	124
Total Org Peridiniales																											
Cysts per gram dry weight	86	37	76	65	27	118	75	83	72	18	66	66	133	413	34	431	59	468	357	242	267	107	586	316	135	9	231
Per cent	71	42	29	48	45	41	33	57	44	40	0	45	50	39	56	56	38	70	47	23	47	44	25	37	18	6	26
Pentapharsodinium tyrrhenicum	0	0	0	0	0	0	0	0	0	0	0	0	0	129	2	36	48	0	51	104	46	7	126	14	72	0	44
Scrippsiella kirschiae	0	0	0	0	0	0	0	0	0	0	0	0	0	103	2	18	0	0	51	35	26	7	56	69	9	0	28
Scrippsiella lachrymosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	3
Scrippsiella precaria	0	23	0	0	22	42	0	0	0	82	0	15	0	26	0	36	0	0	0	69	16	0	14	0	0	0	3
Scrippsiella rotunda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	2
Scrippsiella trochoidea complex	7	9	31	0	0	0	41	0	18	0	0	10	57	52	8	126	0	67	204	173	86	48	251	62	81	54	99
Scrippsiella sp. 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	51	35	11	7	0	7	0	0	3
Scrippsiella sp. 4	0	9	0	0	5	17	7	0	9	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scrippsiella sp. 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	237	82	90	0	82
Other Scrippsiella spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	0	0	0	3	2	0	7	0	0	2
Calcareous type 1	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	12	0	0	35	9	0	0	0	0	0	0

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Table 3 (continued)

	Cabras												Corru S'ittiri									Santa Giusta					
	1	2	3	4	5	6	7	8	9	10	11	Mean	1	2	3	4	5	6	7	8	Mean	1	2	3	4	5	Mean
Calcareous type 2	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	0	0	0	3	0	14	0	0	0	3
Total Cal Peridiniales																											
Cysts per gram dry weight	7	41	31	0	27	59	48	0	18	9	82	29	57	387	11	215	59	67	357	450	200	73	712	240	262	54	268
Per cent	6	47	12	0	45	21	21	0	11	20	50	21	21	37	19	28	38	10	47	42	30	30	31	28	34	38	32
Alexandrium catenella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	36	0	8
Alexandrium minutum	0	0	0	0	0	0	0	0	0	0	0	0	19	0	0	18	0	0	0	35	9	5	84	0	90	54	47
Alexandrium tamarense	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	14	7	27	0	11
Alexandrium cf taylorii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35	4	0	0	0	0	0	0
Fragilidium sp.	0	0	0	0	5	17	0	0	0	5	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gonyaulax cf verior	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	209	0	0	0	42
Gonyaulax sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	56	21	18	0	22
Other Gonyaulax spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	18	0	0	0	0	2	5	98	21	9	0	26
Pyrophacus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	251	21	9	0	57
Total Gonyaulacales																											
Cysts per gram dry weight	0	0	0	0	5	17	0	0	0	5	0	2	19	0	2	36	0	0	0	69	16	39	712	69	189	54	213
Per cent	0	0	0	0	9	6	0	0	0	10	0	2	7	0	3	5	0	0	0	6	3	16	31	8	25	38	23
Gymnodinium impudicum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	2
Gymnodinium litoralis	0	0	0	0	0	0	0	0	0	0	0	0	57	103	8	72	36	0	51	138	58	0	0	0	0	0	0
Gymnodinium/Gyrodinium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	1
Polykrikos sp.	0	0	15	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gymnodiniales type 1	0	0	0	6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	209	75	90	0	75
Gymnodiniales type 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Gymnodiniales type 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	67	0	0	8	0	0	0	0	0	0
Total Gymnodiniales																											
Cysts per gram dry weight	0	0	15	6	0	0	0	0	0	0	0	2	57	103	9	72	36	67	51	138	67	2	209	82	90	9	79
Per cent	0	0	6	5	0	0	0	0	0	0	0	1	21	10	16	9	23	10	7	13	14	1	9	10	12	6	8
Biecheleria cincta	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	0	0	0	3	2	0	21	9	9	8
Total Suessiales																											
Cysts per gram dry weight	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	0	0	0	3	2	0	21	9	9	8
Per cent	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	2	1	6	2
Unidentified cyst type 1	0	9	61	26	0	0	61	62	72	9	0	27	0	0	0	0	0	0	0	0	0	2	0	21	9	9	8
Unidentified cyst type 2	29	0	69	39	0	93	34	0	0	5	16	26	0	0	0	0	0	0	0	0	0	1	0	2	1	6	2
Unidentified cyst type 3	0	0	0	0	0	0	0	0	0	0	0	0	0	52	0	0	0	67	0	69	23	0	0	0	0	0	0
Unidentified cyst type 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	89	72	0	35

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Table 3 (continued)

| | Cabras | | | | | | | | | | | | Corru S'ittiri | | | | | | | | | Santa Giusta | | | | | |
	1	2	3	4	5	6	7	8	9	10	11	Mean	1	2	3	4	5	6	7	8	Mean	1	2	3	4	5	Mean
Other unidentified cysts	0	0	8	0	0	0	7	0	0	0	0	1	0	77	4	18	0	0	0	104	25	5	98	27	9	9	30
Total unidentified																											
Cysts per gram dry weight	29	9	137	65	0	93	102	62	72	14	16	55	0	129	4	18	0	67	0	173	49	21	98	117	81	9	65
Per cent	24	11	53	48	0	32	45	43	44	30	10	31	0	12	6	2	0	10	0	16	6	8	4	14	11	6	9
Total cyst abundances (cysts per gram dry weight)	121	88	259	136	60	287	225	145	163	46	165	154	267	1,057	61	772	154	669	766	1,072	602	244	2,317	844	767	144	863

trochoidea complex was the most abundant (Fig. 4 and Table 3).

Finally, in Santa Giusta, the organic Peridiniales *Scrippsiella* sp. 2 and round smooth cysts were highly important in terms of abundance, reaching their maximum at station 2 (Fig. 4 and Table 3). Of the calcareous Peridiniales, the *S. trochoidea* complex and other species belonging to this genus were the most represented. Furthermore, *Gonyaulax* species were also well represented, with station 2 exhibiting the highest abundance (Fig. 4 and Table 3).

Among the harmful species, *A. minutum* was the most widely distributed morphotype (Table 3), whereas *A. catenella* and *A. tamarense* were only detected in Santa Giusta, and *G. litoralis* was only observed in Corru S'Ittiri, showing a wide distribution among stations (Table 3). The other harmful species were detected in the sediments in minimal to low abundance. *G. impudicum* and *K. foliaceum* were observed in Santa Giusta, and *A.* cf *taylorii* was detected in Corru S'Ittiri (Table 3).

Comparing cyst abundances and composition of the assemblages among the three lagoons, the ANOSIM analysis confirmed significant differences (Global R, 0.941; $P = 0.1$ %). The cluster analysis showed that cyst assemblages from each lagoon divided at a level of 30 Bray–Curtis similarity coefficient. In addition, some variation is evident even among stations within each lagoon (Fig. 5).

Environmental Conditions

The first two PCA components, PCA 1 and PCA 2, explained 85 % of the total variation (57.4 % and 27.6 % for the first and the second components, respectively). Temperature data were not included in the final PCA, since the variable did not influence the results in preliminary analyses. The environmental parameter eigenvectors showed that PCA 1 was positively correlated with winter and summer Sal, and negatively correlated with winter Chl*a*, summer DIN, and Ptot. PCA 2 showed a negative correlation with winter DIN, Ptot, and summer Chl*a* (Table 4). The two component sample scores showed notable differences among the three lagoons, but certain heterogeneity was also observed within stations for Cabras and Santa Giusta (Fig. 6). The Cabras stations had a strong negative correlation with PCA 1, and were characterised by low winter and summer Sal values and higher summer nutrients and winter Chl*a* concentrations compared to Corru S'Ittiri and Santa Giusta stations. In addition, Cabras station 1, Santa Giusta station 3, and all Corru S'Ittiri stations were negatively correlated with PCA 2, characterised by increased winter nutrients and summer Chl*a* (Fig. 6).

According to ANOVA results, the studied lagoons differed significantly in Sal and Ptot, both in summer and winter (Table 5). In particular, Tukey's test highlighted that Cabras was significantly different from Santa Giusta and Corru S'Ittiri in summer Sal, and in summer Ptot, while all the lagoons

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Fig. 4 Absolute cyst abundances of the most important morphotypes among stations in surface sediments of the three lagoons (*Ptri*, *Protoperidinium* cf *tricingulatum*; *RBSp*, round brown spiny morphotypes; *RBSm*, round brown smooth morphotypes; *Scri1*, *Scrippsiella* sp. 1; *Scri2*, *Scrippsiella* sp. 2; *Spre*, *Scrippsiella precaria*; *Stro*, *Scrippsiella trochoidea* complex; *Scrspp*, other calcareous *Scrippsiella* species; *Gonspp*, *Gonyaulax* species)
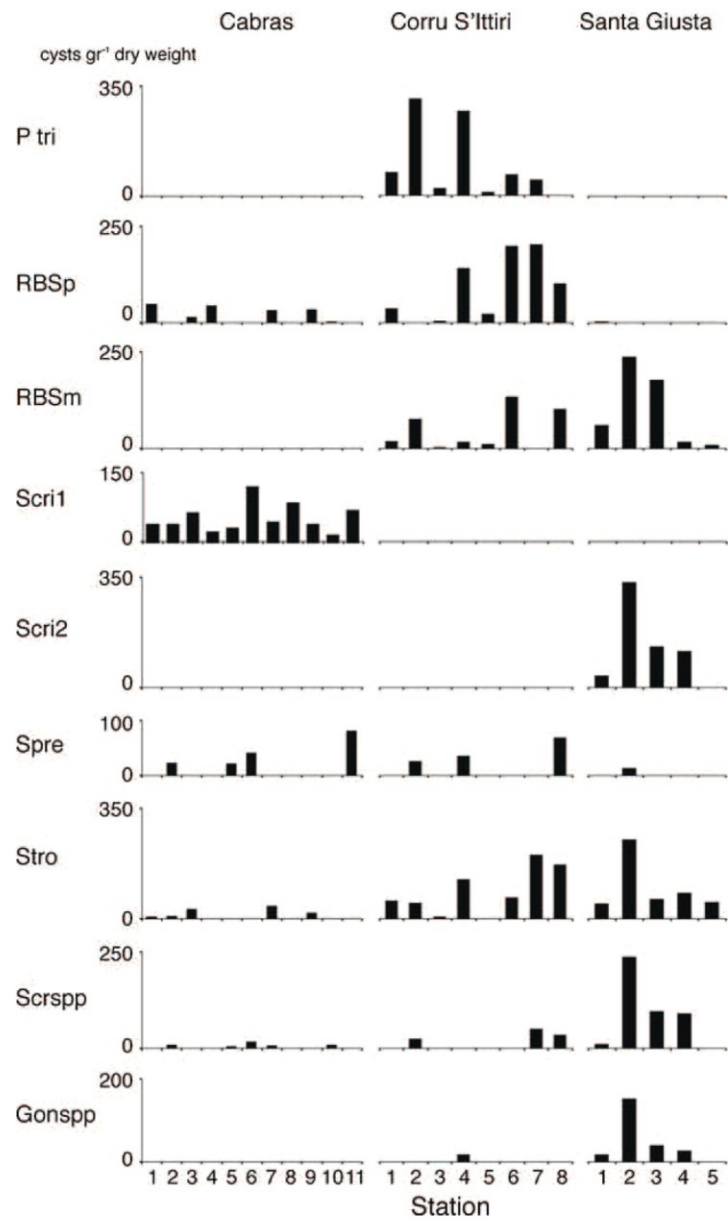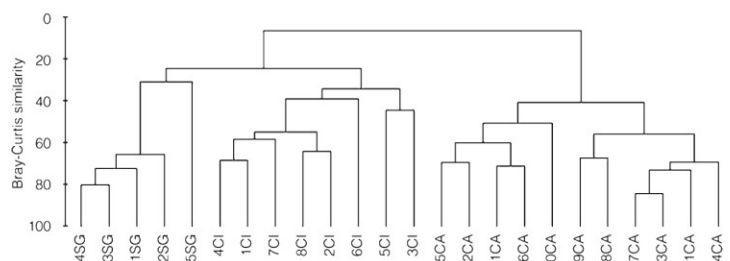

Fig. 5 Cluster diagram of sampling stations based on cyst assemblage composition. The scale indicates the Bray–Curtis similarity coefficient. *CA* Cabras Lagoon, *CI* Corru S'Ittiri Lagoon, *SG* Santa Giusta Lagoon

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Table 4 Results of the PCA (eingenvalues, eingenvectors, and scores) applied to environmental parameters

Parameters	Summer		Winter		Stations	Scores	
	PCA1	PCA2	PCA1	PCA2		PCA1	PCA2
Sal	0.459	0.034	0.452	−0.004	1 CB	−2.412	0.389
DIN (μM)	−0.346	0.276	0.231	−0.530	2 CB	−3.134	0.155
Ptot (μM)	−0.434	−0.039	0.049	−0.598	3 CB	−3.272	−1.548
Chl$a$ (μg l^{-1})	−0.277	−0.487	−0.38	−0.214	1 CI	1.5	−1.385
					7 CI	2.605	−1.19
					9 CI	1.893	−1.521
					1 SG	0.705	1.311
Eingenvalues	4.59	2.21			2 SG	1.107	3.013
Variation (%)	57.4	27.6			3 SG	0.724	−0.149
					6 SG	0.284	0.924

differed significantly among each other in winter Sal. Furthermore, Santa Giusta and Corru S'Ittiri differed significantly in winter Ptot. Cabras was also significantly different from Santa Giusta and Corru S'Ittiri in winter Chl$a$ (Table 6).

Discussion

The results of our study provide much needed data on dinoflagellate resting cysts in shallow coastal lagoons worldwide. In addition, our data are the first to compile dinoflagellate species assemblages derived from cysts in Mediterranean coastal lagoons. Cyst studies conducted in other lagoons primarily examined toxic *Alexandrium* species (Genovesi

Fig. 6 Results of principal component analysis (PCA) based on environmental parameters of the three lagoons. *CA* Cabras Lagoon, *CI* Corru S'Ittiri Lagoon, *SG* Santa Giusta Lagoon

et al. 2011), while other research on dinoflagellate assemblages in Mediterranean regions evaluated coastal, estuarine, and open waters (e.g. Giannakourou et al. 2005; Montresor et al. 1998; Rubino et al. 2010; Satta et al. 2010, 2013; Zonneveld et al. 2012), enclosed seas (Mudie et al. 2001), and semi-enclosed basins (Rubino et al. 1996). Our study also revealed a pattern in the composition and distribution in cyst species assemblages among the three lagoons.

Morphotype Composition

Morphotype composition in the lagoons showed that, in terms of number of morphotypes, Peridiniales was the most represented group, and within it, the calcareous types prevailed, as already noted in previous studies in Mediterranean coastal areas (Montresor et al. 1998; Satta et al. 2010, 2013). Nevertheless, the composition of each cyst assemblage was fairly specific to each lagoon. This inter-lagoon heterogeneity, also observed at the intra-lagoon level, was comparable to that reported for macroinvertebrates in a considerable number of Italian lagoons (Basset et al. 2006). Basset et al. (2006) also emphasised how among lagoons taxonomic similarities are typically low, even in lagoons that are close geographically,

Table 5 Results of one-way ANOVA on environmental variables among the three studied lagoons

Significant values (P<0.05) are marked in *bold*

	Variable	F	P
Summer	Tem	0.19	0.828
	Sal	**12.37**	**0.007**
	Ptot	**13.99**	**0.005**
	DIN	3.15	0.116
	Chl$a$	2.72	0.144
Winter	Tem	1	0.422
	Sal	**52.79**	**<0.001**
	Ptot	**8.42**	**0.018**
	DIN	4.26	0.071
	Chl$a$	**8.52**	**0.018**

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Table 6 Tukey's test P values

	Variable	CA–SG	CA–CI	SG–CI
Summer	Sal	**0.024**	**0.007**	0.562
	Ptot	**0.007**	**0.002**	0.434
Winter	Sal	**0.001**	**<0.001**	**0.039**
	Ptot	0.181	0.181	**0.015**
	Chl*a*	**<0.001**	**0.003**	0.427

Significant values ($P<0.05$) are marked in *bold*

and taxa shared among lagoons is decidedly less than within individual lagoons.

The cyst assemblages described in our study included the discovery of a new *Scrippsiella* species (*Scrippsiella* sp. 1; Satta et al., in preparation) found in Cabras. We also identified previously unreported species from the Mediterranean basin (*P.* cf *tricingulatum* and *S. donghaiensis*), thereby expanding the geographic distribution of these species. In addition, we confirmed several species of *Pentapharsodinium* (*Pentapharsodinium tyrrhenicum* and *Pentapharsodinium* cf *daleii*), *Scrippsiella* (*S. donghaiensis*, *Scrippsiella kirschiae*, *Scrippsiella lachrymosa*, *Scrippsiella precaria*, and *Scrippsiella rotunda*), and *Gymnodinium* (*G. litoralis* and *G. impudicum*). Previous plankton studies in these lagoons reported *Scrippsiella* and *Gymnodinium* species, but the taxa were allied at the genus level (Pulina et al. 2012). In our study, the identification of *G. litoralis* and *G. impudicum* was obtained by the application of genetic methods. Germination experiments and genetic analyses have been also the key in the discovery of the species *Bysmatrum* sp. and *Biecheleria cincta*, as already reported in Satta et al. (2013) and *S. donghaiensis*. Therefore, the use of different tools in cyst studies is confirmed as decisive to generate as much dinoflagellate diversity data as possible.

Of the cysts of harmful species observed in the lagoons, most belong to the *Alexandrium* genus, as already observed in other coastal areas of the NW Mediterranean Sea (Satta et al. 2013). The negative impact of these species is widely known worldwide, particularly in the Mediterranean basin (Garcés and Camp 2012 and references therein) and also in lagoon environments (Genovesi et al. 2011; López-Flores et al. 2006). Penna et al. (2007) applied molecular approaches and identified *A. tamarense* and other *Alexandrium* in the Santa Giusta plankton. However, *A. catenella* and *A. minutum* were not detected in the lagoon until this study.

In Sardinian waters, *A. minutum* and *A. catenella* were the primary species responsible for several toxic outbreaks, which caused considerable economic aquaculture losses and human health alerts (Lugliè et al. 2011). Species detection in lagoons converted to clam breeding farms (Corru S'Ittiri), and particularly in the harvest areas outside the controlled breeding sites (Santa Giusta), emphasises the potential risk of toxic events.

Regarding the high biomass-producing species, the finding of *G. litoralis* in Corru S'Ittiri contributed to the description of this species (Reñé et al. 2011). Finally, *K. foliaceum* and *G. impudicum* in Santa Giusta represent the first report of these species in the lagoon.

Total Cyst Abundance and Assemblage Composition versus Environmental Context

Total cyst abundances broadly reflect dinoflagellate cyst production in certain geographic areas (Dale 2001), although other factors can influence cyst deposition (e.g. sedimentation rates) and cyst permanence in sediments (e.g. germination, grazing, bioturbation, and degradation). Salinity strongly influences dinoflagellate planktonic cell distribution, and consequently cyst production, in transitional waters and coastal marine areas. Concurrently, oligohaline environments are reported to present low species number. Therefore, lower cyst abundance and morphotype number are found at lower salinities (e.g. Mudie et al. 2001; Pospelova et al. 2004). This is consistent with the lower total cyst abundance and morphotype numbers recorded in Cabras, which was characterised by low salinity (annual mean ~20).

Other studies also show a connection between the decrease of cyst abundances and morphotype number and conditions of heavy pollution and eutrophication (Pospelova et al. 2005). In general, lagoons are strongly affected by eutrophication and water quality deterioration. The lagoons from our study are no exception. Pulina et al. (2012) reported Cabras exhibited conditions indicative of a highly degraded environment, and Magni et al. (2004) showed the macrofaunal benthic component was poorly diversified and characterised by the dominance of only a few species. Then, the lower total cyst abundances and number of morphotypes found in Cabras sediments in the present study is consistent also with high eutrophic conditions. However, also Santa Giusta experienced strong nutrient enrichment (Sechi et al. 2001; Lugliè et al. 2002), and data reported in this study from Corru S'Ittiri outline a clear eutrophic condition (N. Sechi, personal communication).

Statistical analyses indicated that salinity, during summer and winter seasons, was one of the main discriminating factors among the lagoons. Moreover, among nutrients, total phosphorous resulted the most important. Interestingly, long-term analyses of phytoplankton and environmental variables in Cabras showed phytoplankton composition and environmental conditions have changed. In particular, decreased salinity was consistent with the replacement of the dominant taxonomic classes Bacillariophyceae and Dinophyceae with Cyanophyceae (Pulina et al. 2012). Therefore, reduced cyst abundance and morphotype number in Cabras can primarily be associated with low Dinophyceae presence in the lagoon phytoplankton rather than severe eutrophication, a condition that is common among the three lagoons.

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Cyst assemblage abundance and composition among Cabras, Corru S'Ittiri, and Santa Giusta lagoons showed considerable diversification and significant differences among them. Cabras cyst assemblage was much distinctive, with the dominance of the new species belonging to the autotrophic genus *Scrippsiella*. Corru S'Ittiri and Santa Giusta cyst assemblages appeared to be more similar to each other, although characterised by different morphotype proportions. Autotrophic calcareous morphotypes such as *Scrippsiella* species were more abundant in Santa Giusta, whereas heterotrophic morphotypes were most highly represented in Corru S'Ittiri.

Interestingly, Cabras and Santa Giusta cyst assemblages showed almost the same cyst signal of predominance of small autotrophic *Scrippsiella* species. This observation is congruent with those from other Mediterranean coastal areas, including sites characterised by intense anthropogenic impacts and eutrophication (Giannakourou et al. 2005; Montresor et al. 1998; Satta et al. 2010, 2013). Shallow, highly stratified, and nutrient-enriched areas are particularly favourable habitats for colonist dinoflagellate species (Smayda and Reynolds 2003), such as small autotrophic peridinioids like *Scrippsiella* species (Montresor et al. 1998; Ribeiro and Amorim 2008).

In addition to the autotrophic signal, Santa Giusta shows considerable importance of heterotrophic morphotypes, mainly referable to *Protoperidinium*, *Diplopsalis*, and related genera (round brown smooth cysts). The heterotrophic signal is still more evident in the Corru S'Ittiri assemblages. Numerous studies demonstrated that the proportion of cysts produced by heterotrophic dinoflagellates tends to increase with increasing nutrient enrichment, providing a suitable indication of eutrophication in transitional waters (Dale 2009 and references therein; Pospelova and Kim 2010). The high nutrient levels observed in Santa Giusta, and particularly in Corru S'Ittiri, are consistent with these previous reports.

Godhe and McQuoid (2003) emphasised that coastal areas characterised by higher salinity can favour heterotrophic species (e.g. *Protoperidinium* sp.). Therefore, the higher proportion of heterotrophs in Corru S'Ittiri might be associated with increased salinity at the Corru S'Ittiri Lagoon compared to Cabras and Santa Giusta, and a greater influence of the adjacent marine waters.

In summary, our study indicated that salinity was a fundamental driver in determining dinoflagellate cyst assemblage abundance and composition, confirming that observed in other studies (Pospelova et al. 2004). The role of specific nutrients in determining morphotype distribution among our eutrophic lagoons needs further insights in order to obtain a more comprehensive picture.

Furthermore, we confirmed that cyst responses to eutrophication varied at small geographic scale in three Mediterranean lagoons, depending on local environmental conditions, as already demonstrated by studies in other eutrophic areas around the world (Zonneveld et al. 2012 and references therein).

Conclusions

This study characterised cyst assemblages in highly variable environments, such as coastal lagoons, which provided additional evidence for complex lagoon environments, and contributed to a growing body of knowledge on cyst-producing dinoflagellates. Our accurate characterisation of lagoon taxa using germination experiments and genetic analyses generated valuable data on dinoflagellate biodiversity, and more specifically on regional harmful species. Our simultaneous evaluation of environmental data facilitated additional insights on assemblage composition, emphasising dinoflagellate variability also at small geographical scale, and the relationship between species/taxon distribution, and environmental factors, among which salinity was of particular importance.

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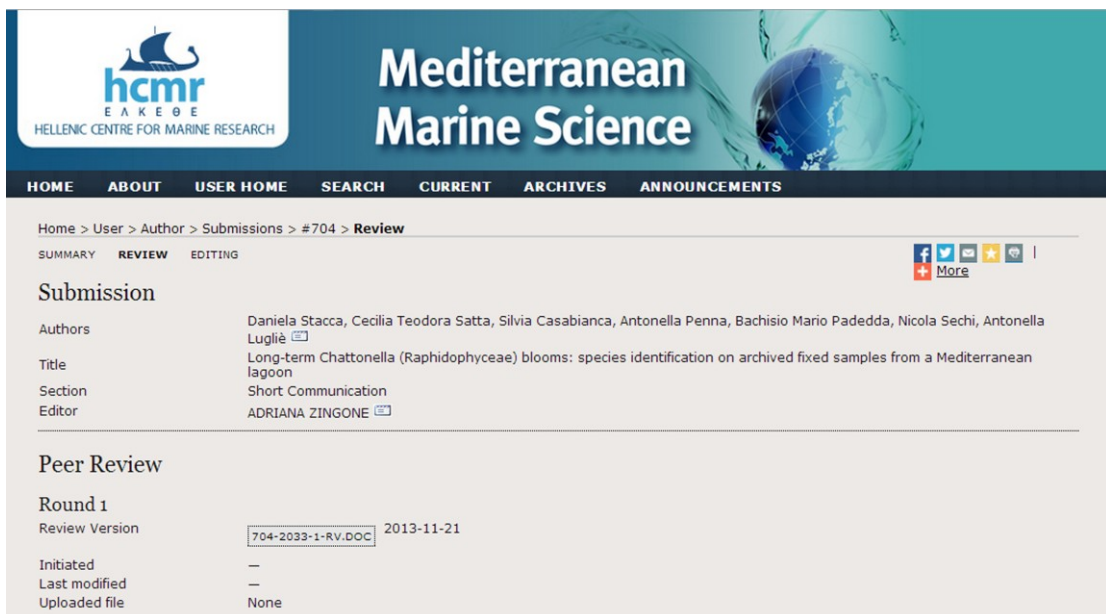
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5. CHAPTER II

Long-term *Chattonella* (Raphidophyceae) blooms: species identification on archived fixed samples from a Mediterranean lagoon.

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ABSTRACT The present study applied molecular approach on archived fixed phytoplankton samples (old up to twenty years) to determine *Chattonella* species (i.e. *C. subsalsa* and/or *C. marina*) present during past blooms, associated with fish kills. Samples were collected during the blooms occurred in the summer months of 1994, 1998, 1999, and 2010 in a Mediterranean lagoon (Santa Giusta Lagoon, Sardinia Island, Italy). Based on PCR amplification of nuclear internal transcribed spacer (ITS) regions, we established the occurrence of *C. subsalsa* in all analysed samples. These results provide valuable data that increase knowledge on the long-term occurrence of *Chattonella*-blooms in a Mediterranean transitional ecosystem.

Key words: *Chattonella subsalsa*, *Chattonella marina*, harmful algal blooms, archived fixed samples, ITS regions, Sardinia, LTER-Italy.

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INTRODUCTION

Chattonella Biecheler (Raphidophyceae) exhibits a worldwide distribution, and includes deleterious species causing fish kills in natural environments and aquaculture systems (Imai and Yamaguchi, 2012 and references therein). Imai and Yamaguchi (2012), in their recent review, recognized five species, i.e. *Chattonella antiqua* (Hada) Ono, *C. marina* (Subrahmanyan) Hara et Chihara, *C. minima* Hara et Chihara, *C. ovata* Hara et Chihara, and *C. subsalsa* Biecheler, although, previously, Demura *et al.* (2009) proposed three species, and determined *C. antiqua* and *C. ovata* were varieties of *C. marina*.

As the other raphidophytes, *Chattonella* species lack rigid cell walls. Consequently, their cellular shape and morphology are lost with fixation (Band-Schmidt *et al.*, 2004; Zingone *et al.*, 2006), making their identification particularly difficult. Molecular techniques permitted the identification of raphidophytes in archived fixed samples, allowing their use in retrospective studies (Bowers *et al.*, 2006). The objective of this study was to identify *Chattonella* species in archived fixed natural phytoplankton samples, up to twenty-years old, using a molecular PCR based assay. The samples were collected during past blooms concomitant with fish kills in a Mediterranean lagoon.

MATERIALS AND METHODS

Phytoplankton samples analyzed in this study were collected from Santa Giusta Lagoon (Sardinia Island, Italy, Western Mediterranean Sea). Santa Giusta Lagoon is located along the west central coast of Sardinia (Fig. 1) and it is a research station in part of site "14 Sardinian marine ecosystems" of LTER-Italy network (www.lteritalia.it).

Analysed samples were collected from the water surface layer (-30 cm), from 3 to 5 stations (Fig. 1) during *Chattonella* blooms in summers 1994, 1998, 1999 and 2010. All samples were immediately fixed with Lugol's iodine solution. *Chattonella* cell densities were obtained following Utermöhl method (1958) using an inverted Axiovert Zeiss 25 microscope within ten days from sampling.

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PCR analyses were performed on samples from at least two of the sampled stations for each sampling date (Table 1) for a total of 24 analyzed samples. DNA was extracted with the DNeasy Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions from 50 ml of fixed natural samples (Fig. 2).
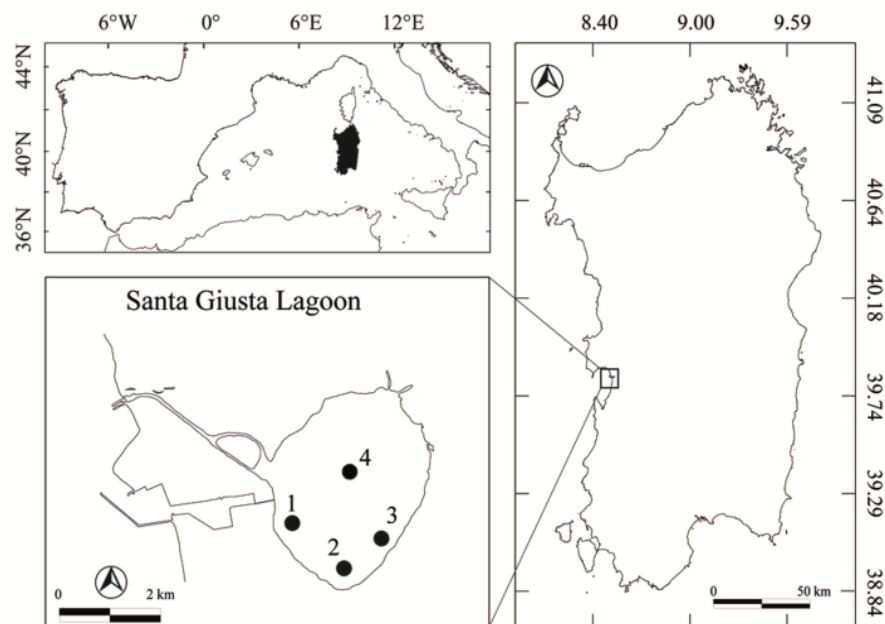
Figure 1 Santa Giusta Lagoon and sampling stations.

A first PCR was performed with 1 μl of extracted DNA in a total 40.5 μl reaction mixture containing the following: 0.25 mM of each dNTP; 0.1μM of each primer; 2.5 mM $MgCl_2$; 1x HotMaster Taq Buffer (PRIME, Hamburg, Germany); 2.5 U Taq DNA polymerase (PRIME). PCR was performed using ITSA and ITSB primers (Adachi *et al.*, 1994) to amplify the internal transcribed spacer (ITS) regions and 5.8S rDNA. PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min; followed by 35 cycles at 94 °C for 20 s, 57 °C for 10 s, and 70 °C for 30 s; and a final elongation step at 70 °C for 5 min. Six PCR replicates for each sample were performed, three with undiluted DNA, and three with 1:10 diluted DNA (Fig. 2). Subsequently, 1 μl of each PCR product was used in two distinct nested PCR amplifications with the same mixture condition of above written. These two distinct nested PCR were done with specific primers: oBTG-005-F and oBTG-027-R for *C. marina*, and oBTG-005-F and oBTG-028-R

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for *C. subsalsa* (Connell, 2002). Nested PCR conditions were as follows: an initial denaturation step at 95 °C for 5 min; then 35 cycles at 95 °C for 30 s, 55 °C for 10 s (using species-specific primers for *C. marina*) or 45 °C for 10 s (using species-specific primers for *C. subsalsa*), and 72 °C for 30 s; a final elongation step at 72 °C for 10 min. The same treatment was done also on DNA extracted from culture strains of *Chattonella* (*C. subsalsa* CCMP217 and *C. antiqua* NIES 1; Connell, 2002), used as positive controls for each PCR amplification. All PCR amplifications were performed in a DNA Engine® Thermal Cycler.
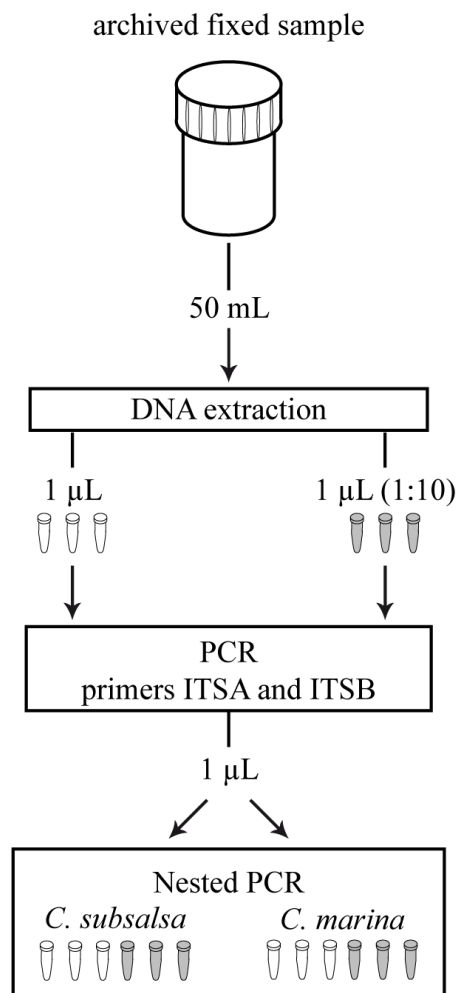
Figure 2 Scheme of the protocol used.

PCR products were resolved on a 1.8% (80v) agarose gel. Nested PCR products were purified and sequenced by an external service (Macrogen Inc., Europe) using both

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primers, and a 3730XL DNA sequencer. All sequences were submitted to the BLAST database (Basic Local Alignment Search Tool) at NCBI (National Centre for Biotechnology Information).

Table 1 List of analysed samples, *Chattonella* densities, and PCR amplification assay results. +, positive amplification; -, negative amplification.

Sampling date	Station	*Chattonella* density (cells 10^3 L^{-1})	*Chattonella subsalsa* Not diluted DNA	*Chattonella subsalsa* Diluted DNA	*Chattonella marina* Not diluted DNA	*Chattonella marina* Diluted DNA
03/08/1994	1	12,927	- - -	+ + +	- - -	- - -
	3	30,243	- - -	+ + -	- - -	- - -
25/08/1994	5	187	+ + -	+ + -	- - -	- - -
06/09/1994	3	831	+ + -	+ + -	- - -	- - -
	5	256	+ + -	+ - -	- - -	- - -
03/09/1998	1	179	+ + +	+ - -	- - -	- - -
	5	3,249	+ + +	+ + -	- - -	- - -
26/08/1999	2	3,692	+ + -	+ + -	- - -	- - -
	3	11,931	+ + -	+ + -	- - -	- - -
07/09/1999	1	1,278	- - -	+ + -	- - -	- - -
	2	1,315	+ - -	+ - -	- - -	- - -
19/07/2010	3	399	+ + -	+ + -	- - -	- - -
	5	390	+ + -	+ - -	- - -	- - -
22/07/2010	2	474	+ + +	+ + -	- - -	- - -
	3	2,191	+ + -	+ + +	- - -	- - -
	4	584	+ + -	+ - -	- - -	- - -
24/07/2010	1	1,600	+ + +	+ + +	- - -	- - -
	3	1,827	+ + -	+ + +	- - -	- - -
04/08/2010	2	898	+ - -	+ + -	- - -	- - -
	3	2,511	+ + -	- - -	- - -	- - -

RESULTS AND DISCUSSION

Chattonella is one of the raphidophyte genera, which includes species associated with fish kills (Hallegraeff and Hara, 2003). The tracking of these taxa in recent investigations and in time series data has been very difficult due to the loss of necessary morphological characteristics in fixed samples (Klöpper *et al.,* 2013), and presumably due to low cell abundance in coastal areas (Imai *et al.,* 2006). Consequently, *Chattonella* species become often evident only when harmful events occur. The use of molecular methods to detect the presence of *Chattonella* species is a viable alternative approach to expedite and facilitate identification in fixed natural

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samples (Connell, 2002; Bowers *et al.*, 2006; Marin and Scholin, 2010), similarly as experienced for other harmful species (Penna *et al.*, 2007).

Between *C. marina* and *C. subsalsa*, the former is the most notorious fish-killing species that has caused severe damage to fish farming and wild fish populations, with very important economic losses (Imai and Yamaguchi, 2012). *C. subsalsa* exhibits relatively more recent history as a deleterious species, and data on this species is scarce (Imai and Yamaguchi, 2012). Cell morphology show overlapping characters between *C. subsalsa* and *C. marina*, as emphasized by Hallegraeff and Hara (2003). Consequently, species identification with microscopic methods is uncertain, particularly on fixed samples.

The sequencing and BLAST analysis of PCR products, revealed that *C. subsalsa* was detected in all field phytoplankton samples analysed (Table 1). The high specificity of the employed primers (Connell, 2002) allowed to obtain PCR products unequivocally belonging to this species, with control DNA of 399 bp length for *C. subsalsa* (Fig. 3a) and 181 bp for *C. marina* (Fig. 3b). *C. subsalsa*'s type locality is a Mediterranean lagoon (Thau Lagoon, Salins de Villeroy, Sète; Biecheler, 1936). Bowers *et al.* (2006) already reported the species analysing one isolate from a non-bloom sample from Santa Giusta Lagoon (Lugliè A., personal communication).

ITS sequences comparison between *C. subsalsa* sequences obtained in this study and those deposited in GenBank (Table 2) yielded interesting results. BLAST analysis showed a 99-100% similarity among all *C. subsalsa* sequences, with the exception of Adriatic *C. subsalsa* (96-97% sequence identity).

Klöpper *et al.* (2013) recently reported clear differences among strains from the Adriatic Sea, and other *C. subsalsa* isolates from worldwide localities, which appeared to form a globally homogenous group (Bowers *et al.*, 2006). Our data support the need for further investigations using a plurality of genetic markers for *C. subsalsa*, and strains from additional Mediterranean sites, including transitional ecosystems.

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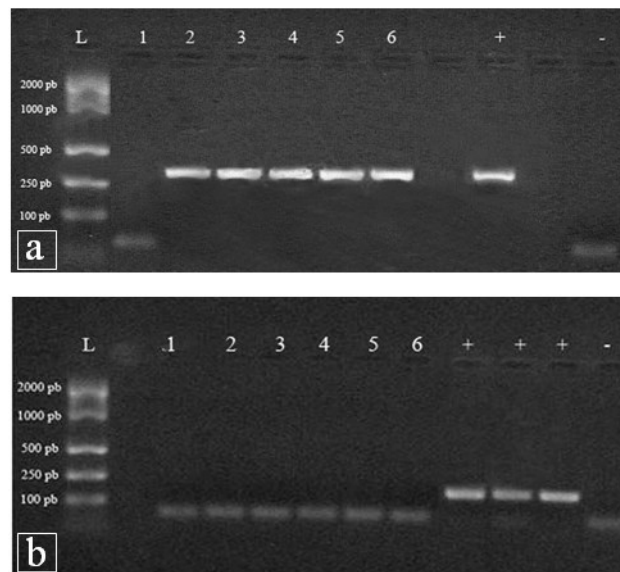
Figure 3 Nested PCR amplification products using an EasyLadder I - Bioline (L) for *C. subsalsa* and *C. marina*: *C. subsalsa*, six replicates of 22-7-2010 (station 2) sample with five positive PCR amplifications (2-6), the positive control using CCMP217 culture (+), and the negative control (-) (a); *C. marina,* six replicates of 22-7-2010 (station 3) sample with six negative PCR amplifications (1-6), three replicates of NIES I culture as positive controls (+), and the negative control (-) (b).

Table 2 *Chattonella* cf. *subsalsa* and *C. subsalsa* sequences obtained from GenBank.

Accession number	Geographical origin	Strain code or source	References
AB334367.1	Gulf of Mexico, America	CCMP 217	Demura *et al.* 2009
AB334368.1	Indian River Bay, USA, America	CCMP 2191	Demura *et al.* 2009
AF153196.1	Gulf of Mexico, America	CCMP 217	Connell, 2000 (Unpublished)
AF409126.1	/	/	Ben Ali *et al.* 2002
AY858864.1	Japan	C. Tomas Japan	Bowers *et al.* 2006
AY858866.1	California, America	C. Tomas California	Bowers *et al.* 2006
AY858868.1	North Carolina, America	C. Tomas North Carolina	Bowers *et al.* 2006
AY858869.1	Santa Giusta Lagoon, Sardinia, Italy	C. Tomas Sardinia	Bowers *et al.* 2006
DQ191680.1	Delaware Inland Bays, USA, America	CCMP 2191	Zhang *et al.* 2006
JF896101.1	Iran	CHPI36	Attaran-Fariman and Bolch, 2011 (Unpublished)
JF907041.1	Bahia de Navachiste, Messico, America	CSNAV-1	Band-Schmidt *et al.* 2012 (Unpublished)
JX067584.1	Adriatic Sea, Rimini, Italy	CRIM_F	Klöpper *et al.* 2013
JX067585.1	Adriatic Sea, Rimini, Italy	CRIM_E	Klöpper *et al.* 2013
JX067586.1	Adriatic Sea, Rimini, Italy	CRIM_C	Klöpper *et al.* 2013
JX067587.1	Adriatic Sea, Rimini, Italy	CRIM_A	Klöpper *et al.* 2013
JX067588.1	Adriatic Sea, Rimini, Italy	CRIM_B	Klöpper *et al.* 2013
JX067589.1	Adriatic Sea, Rimini, Italy	CRIM_D	Klöpper *et al.* 2013

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Our results document the presence of *C. subsalsa* during four past harmful events in a Mediterranean lagoon, using molecular investigative techniques on stored natural phytoplankton samples collected over time (also of twenty years old), contributing to increment knowledge on this species. In fact, whereas harmful *Chattonella* blooms have been well documented in East Asian coasts (Japan, Korea, China, India), South Australia, south-east USA coast (California) in the last decades (Imai and Yamaguchi, 2012 and references therein), they have been less frequently reported from Mediterranean coastal areas, including lagoons and other transitional ecosystems (Mikhail, 2007).

Although we cannot support a cause-effect relationship between *C. subsalsa* blooms and fish kills, which have been observed concurrently over the years, our results can confirm that when harmful events occurred, *C. subsalsa* was present. On-going studies, integrating our long-term ecological data (Sechi *et al.,* 2001; Lugliè *et al.,* 2002), will offer further detailed scenarios on the environmental conditions accompanying these events. Further, they will offer the opportunity to model *Chattonella* blooms for improving the capabilities to predict and/or to mitigate them, with a better management of the affected environments.

Acknowledgements

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6. CHAPTER III

To be submitted to Advances in Oceanography and Limnology

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1 **Interdisciplinary approaches to study harmful algal blooms (HABs) in beach**

2 **environments: an experience from the Mediterranean Sea**

3

4

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25   **Abstract**

26   The presence and distribution of harmful algal species in beach environments were investigated

27   along the coasts of Sardinia, the second largest island in the Mediterranean Sea, in summer 2012.

28   Fourteen potentially noxious taxa were observed in the 74 sampled beaches. The majority of

29   recovered taxa is potentially toxin and/or high biomass bloom producer. *Alexandrium taylorii*,

30   *Gymnodinium instriatum* and *Ostreopsis* cf. *ovata* were the most frequent and abundant taxa,

31   although the maximum of abundance was of *Barrufeta bravensis* (4.4×10^{6} cells L^{-1}). At the time

32   of samplings, *B. bravensis*, *A. taylorii* and *G. instriatum* caused an intense water discoloration in

33   two Sardinian beaches: Bosa Marina and Villasimius. Molecular PCR analyses supported species-

34   specific identification and were decisive for *B. bravensis* identification. Furthermore, PCR assay

35   increased informations on species distribution and contributed to identify *O.* cf. *ovata*. The

36   considered beaches were heterogeneous for environmental and morphodynamic conditions. Gravel

37   and medium-fine sands seem to be the most important factor in driving harmful species

38   distribution.

39   The data obtained represent a base of knowledge on the distribution of harmful species in beach

40   environments, poorly known worldwide. The relationship identified between noxious species and

41   grain size hypothesizes a crucial role of vegetative cell recruitment from cyst beds also in beaches.

42

43

44   **Keywords:** Harmful algal blooms; Mediterranean Sea; beaches; tourism; interdisciplinary

45   approach.

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99

1. Introduction

52

53 Marine coastal ecosystems around the world have been suffering for a wide set of

54 environmental problems, among which Harmful Algal Blooms (HABs) is one of the most

55 impactful [1]. Species involved in HABs give rise to different types of noxious impacts,

56 depending on species-specific characteristics [1,2]. In terms of harmful effects, two typologies

57 of causative organisms can be recognized: the toxin producers and the high-biomass

58 producers [1]. Some harmful algal species (HAS) are related to both characteristics. Toxin

59 producer species can cause toxic symptoms in marine and non-marine fauna or poisoning in

60 humans by consuming organisms contaminated by algal toxins, as well as cause irritation and

61 respiratory problems. Toxin events can result also from very low abundances of the causative

62 organisms [3]. Instead, high-biomass producers give rise to remarkable proliferations that

63 could have severe impacts on the water quality (e.g., higher turbidity, water discolorations,

64 unpleasant odours etc.) and also on the biota of the region (e.g., due to anoxia or shaded

65 conditions, community and food-web changes).

66 The global increase of HABs is worldwide recognized and it has been confirmed also in the

67 Mediterranean Sea [4], where this phenomenon is considered as one of the major emerging

68 issue [5]. A general consensus on the link between human activities and this increase has been

69 achieved (e.g. eutrophication [6]). However, many of the causes are yet to be understood. In

70 fact, the factors that influence HABs are numerous and cover many aspects of biology,

71 physics and chemistry [4,7]. The complexity of HABs highlights the importance of the

72 multidisciplinary approach in the study and the mitigation of these events through the

73 analyses of the continuum between land and aquatic ecosystems, pelagic and benthonic

74 communities, environmental and socio-economic drivers.

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75   On this basis, we designed a project to improve the ability to predict and mitigate impacts of

76   HABs in areas of high economic interest through the use of an interdisciplinary approach.

77   Given the economic importance of tourism and marine production in the Mediterranean

78   context, the project has been oriented on HABs that impact beach environments and shellfish

79   farming areas (Figure 1).

80   The objective of this paper is to report the early results of the project, obtained from the first

81   year of sampling surveys in beach environments (Figure 1). Presence and distribution of

82   potentially HAS were determined in 74 beaches. The traditional microscopy methods were

83   combined with PCR methods to improve the ability of harmful species-specific identification

84   and to obtain more detailed informations on the distribution of target species. Moreover,

85   during samplings, data on different environmental variables were acquired (nutrients and

86   chemical-physical parameters of the water, and sediment grain size). Beaches were also

87   characterized on the basis of their morphology (both emerged and submerged) and wave

88   dynamic.

89

90   **2. Methods**

91   *2.1 Study areas and samplings*

92   Seventy-four beaches were selected on the basis of basic characteristics (e.g., geographical

93   localization, morphology, wind exposure), human pressures (presence of villages and/or

94   settlements; artificial structures, e.g., harbours), inputs of freshwater, and information on past

95   bloom events (Figure 2, Table 1).

96   Samplings were conducted in the first year of the project (from July to August 2012),

97   providing a sampling at each beach. Surface water samples (approximately 0.2 m depth) were

98   taken at about 3 m from the beachfront, collecting one sample for beach. In each sampling,

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99   500-mL aliquots of seawater were fixed with Lugol's solution for harmful algal species and

100 phytoplankton identification and counting. Net (10-μm mesh) phytoplankton samples were

101 also collected and Lugol fixed to perform molecular analysis.

102 Samples for nutrients (2000-mL) and chlorophyll *a* (5000-mL) analyses were collected and

103 maintained under dark and in refrigerated conditions (4 °C), and analysed within a few hours

104 after collection. Chorophyll *a* samples (calibration samples) were collected in at least 3

105 beaches for sampling to calibrate the fluorimetric instrument.

106

107 ***2.2 Harmful algal species and phytoplankton counting and identification***

108 Aliquots (10-50-100 mL) of the Lugol fixed samples were analysed to estimate the

109 abundances of harmful species and other phytoplankton species, after sedimentation in

110 settling chambers following the Utermöhl's technique [8]. Larger and more easily identifiable

111 cells were counted at 100× magnification from the entire bottom of the sedimentation

112 chamber, whereas smaller species were counted at 200× and 400× magnification in an

113 adequate number of fields. All counts were performed using an inverted microscope Zeiss

114 Axiovert 25.

115 The harmful species were identified according to Balech [9] and Faust and Gulledge [10],

116 while the other species in according to Tomas [11]. Armoured dinoflagellates were stained

117 with Fluorescent Brightner 28 (Sigma) and examined under a Zeiss Axiovert 100 microscope

118 with UV epifluorescence [12], to determine the thecal plate arrangement.

119

120 ***2.3 Molecular analysis***

121 *2.3.1 Single cell PCR*

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122 One or two cells of selected harmful species from several beaches were isolated with a

123 micropipette under an inverted microscope (Zeiss Axiovert 25), washed in several drops of

124 filtered seawater and placed in 200-µL PCR tubes. Each tube was previously filled with 5 µL

125 of the lysis buffer (0.005% SDS with 400 ng μL^{-1} Proteinase K; [13]). The reaction mixtures

126 were frozen for at least 10 min at -80 °C, incubated at 60 °C for 30 min, and at 95 °C for 10

127 min. The lysates were used immediately, or stored at -80 °C until use.

128 PCR was conducted directly using 5 µL of the lysate in a PCR mixture containing 5 µL of 10x

129 buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8

130 mM of the primers ITSF01 and ITS4 [14, 15] or D1R and D2C [16]. The PCR conditions

131 were as follow: an initial denaturation for 5 min at 95 °C, 40 cycles for 20 s at 95 °C, 30 s at

132 53 °C, and 60 s at 72 °C, followed by a final elongation step for 10 min at 72 °C. PCR was

133 carried out in a DNA Engine® Thermal Cycler.

134 Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% (80v)

135 agarose gel and visualized under UV illumination. Purification and sequencing were carried

136 out by an external service (Macrogen Inc., Europe) using the appropriated primers, and a

137 3730XL DNA sequencer. All sequences were submitted to the Basic Local Alignment Search

138 Tool (BLAST database) at the National Centre for Biotechnology Information (NCBI).

139 Sequences were aligned with those obtained from GenBank using the MAFFT v.6 program

140 [17] under FFT-NS-i (slow; iterative refinement method). The maximum-likelihood (ML)

141 method and the GTRGAMMA evolution model of RAxML (Randomized Axelerated

142 Maximum Likelihood) v. 7.0.4 [18] were used for the phylogenetic relationships. Repeated

143 runs on distinct starting trees were carried out to select the tree with the best topology (the one

144 with the greatest likelihood of 1000 alternative trees). Bootstrap (BS) ML analysis was done

145 with 1000 pseudo-replicates and the consensus tree was computed with the RAxML software.

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146 *2.3.2 PCR based assay*

147 Net fixed samples were analyzed. Total DNA extraction was performed on 30-50 mL of each

148 sample, as described in Penna *et al*. [19] and Battocchi *et al*. [20]. PCR amplification assays

149 for genus and species-specific detection of *Alexandrium* sp., *A. catenella*, *A. minutum*, *A.*

150 *tamarense*, and *A. taylorii* were carried out as described by Penna *et al.* [19], with exception

151 of 4 pmol of each primer and 1 U of Taq polymerase (Diatheva, Italy). PCR amplification

152 reactions for the species-specific *Ostreopsis* (*O.* cf. *ovata* and *O.* cf. *siamensis*) identification

153 were done following the protocol of Battocchi *et al.* [20]. Genus and species-specific

154 oligonucleotide primers used in the PCR based assay derived from Penna *et al.* [19].

155

156 *2.4 Environmental variables and chlorophyll a*

157 Temperature (Tem), salinity (Sal), pH and dissolved oxygen were measured in situ using a

158 handheld multiparameter instrument (YSI 556MPS). Concentrations of nitrate (N-NO$_3$),

159 ammonium (N-NH$_4$) and nitrite (N-NO$_2$), reactive (P-PO$_4$) and total phosphorous (P-Ptot),

160 total nitrogen (N-Ntot) and reactive silica (Si-SiO$_4$) were determined following Strickland and

161 Parsons [21] and using a 200 DMS (Varian) spectrophotometer. The sum of nitrate,

162 ammonium and nitrite is reported as dissolved inorganic nitrogen (DIN).

163 Chlorophyll *a* was determined with fluorimetric analysis (Trilogy). The instrument was

164 calibrated using the data obtained with the spectrophometric method [22] on the collected

165 calibration samples.

166

167 *2.5 Grain size analysis and morphodynamic classification*

168 Sediment samples for grain size characterization were collected with hand cores (three

169 replicates) on the foreshore at each beach. Sediment was dried (24 h at 80 °C) and sieved for

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104

170   grain size determination. Data were grouped in the following classes: gravel (> 2 mm; GR),

171   coarse sand (0.5 ÷ 2 mm; CS) and medium fine sand (< 0.5 mm; MFS). It was not possible

172   collect samples from Cala Fuili (55) because this beach consists of boulders, and Porto Botte

173   (36) for the compactness of the beach surface.

174   Morphological features of beaches were determined analyzing the aerial images derived from

175   various sources (www.sardegnageoportale.it, Google heart and National Cartographic Portal).

176   The indentation index (IND index) was determined following Bowman *et al.* [23], as the ratio

177   of the length of the coast between the headlands that limit the beach and the chord linear

178   distance between the same headlands. This value can be considered representative of the

179   degree embayment of each beach. The number of bars of the submerged domain of each

180   beach was determined and reported as: multibarred (MB, more than one bar in the shoreface),

181   single barred (SB, one bar in the shoreface), no bar (NB, no bar in the shoreface). Moreover,

182   beaches presenting shoreface with extended rocky outcrops or *P. oceanica* meadows were

183   indicated as rocky (R). The mean annual wave energy (AWE) for each beach derived from

184   Atzeni *et al.* [24].

185

186   ***2.6 Statistical analysis***

187   Principal component analysis (PCA) was performed on environmental (Tem, Sal, $P-PO_4$, P-

188   Ptot, DIN, N-Ntot and $Si-SiO_4$) and morphodynamic (GR, CS, MFS, AWE and IND index)

189   parameters to visualise patterns of variation and express data similarities and differences

190   among beaches. For PCA on morphodynamic parameters, beaches were grouped on the basis

191   of the submerged morphology. Grain size data resulted from the average of the three

192   replicates. All data were $\log_{10} (x+1)$ transformed before analysis with PRIMER software.

193   A Detrended Correspondence Analysis (DCA) was first conducted on phytoplankton data. It

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194   indicated a linear distribution (gradient length < 2), thus validating the use of a direct linear

195   methodology such as Redundancy analysis (RDA) [25]. RDA was used to assess relationships

196   between phytoplankton composition and the abovementioned environmental and

197   morphodynamic parameters. Phytoplankton species were grouped according to the Class of

198   belonging (Prasinophyceae, *Pra*; Cryptophyceae, *Cry*; Chlorophyceae, *Chl*; Euglenophyceae,

199   *Eug*) and for some classes (Bacillariophyceae and Dinophyceae) in accordance with specific

200   characteristics (*solitary diatoms* and *colonial diatoms*) or size (*big dinoflagellates* and *small*

201   *dinoflagellates*). Harmful algal taxa were grouped in *bloom-forming species* (including all

202   bloom-forming species less *Alexandrium minutum*) and *other harmful species* (including all

203   toxin-producing species less *A. taylorii*) (see Table 2 below). *Ostreopsis* cf. *ovata* (*O. cf.*

204   *ovata*) was not included in any group because of its mainly benthic life strategy. Taxa not

205   attributable to any group were reported as *others*. Data were $\log_{10}$ (x+1) transformed before

206   analysis with CANOCO software.

207

208   **4. Results**

209   ***4.1 Presence and distribution of harmful algal species and the related environmental***

210   ***contest***

211   Fourteen potentially harmful taxa were detected in the 74 beaches in summer 2012. Eleven

212   were identified at species level, while 3 at genus level. The majority of taxa belonged to the

213   class of Dinophyceae (Table 2).

214   The highest number of the observed taxa (10) can produce harmful impacts on human health,

215   seven taxa can produce harmful impacts on water quality in different types of environments

216   and one can produce impacts on marine biota. Several taxa can produce two or more harmful

217   impacts (Table 2).

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218   The presence of one or more harmful species was found in fifty-five beaches (74% of the

219   total). The most frequent species were *Alexandrium taylorii*, *Gymnodinium instriatum* and

220   *Ostreopsis* spp. (Table 2). The distribution maps for these species showed a general trend of

221   greater observation throughout the north (Asinara Gulf, Strait of Bonifacio, Tyrrhenian Sea

222   and Sardinia Sea sides) area of the island and a more limited detection in the south,

223   particularly in the southwest (Sardinia Sea side) and southeast (Tyrrhenian Sea side) (Figure

224   3). Considering microscopy results, *A. taylorii* and *G. instriatum* were observed in 24 and 21

225   beaches, respectively. These species reached abundance values of 8.7×10^5 cells L^{-1} at

226   Villasimius (45) and 7.3×10^5 cells L^{-1} at Bosa Marina (21), respectively (Table 2). However,

227   *Barrufeta bravensis* was the species that reached the highest abundances (4.4×10^6 cells L^{-1}),

228   despite its only one observation (at Bosa Marina, 21). The high cell densities of these species

229   resulted in intense discolorations of the water. One of these events was a mixed bloom of the

230   three species and was registered at Bosa Marina (21). The other event was caused by the only

231   presence of *A. taylorii* and was recorded at Villasimius (45). *Ostreopsis* species were

232   observed in 18 beaches and showed rather low abundance values with a maximum of 1060

233   cells L^{-1} (registered at Balai, 8) (Table 2).

234   Lesser frequently observed species were *Alexandrium minutum*, *Alexandrium* spp.,

235   *Gymnodinium impudicum*, *Lingulodinium polyedrum* and *Protoceratium reticulatum*. *A.*

236   *minutum* and *Alexandrium* spp. were detected in 5 and 6 beaches, respectively (Figure 4,

237   Table 2), mainly located in the northeast (Tyrrhenian Sea side) and northwest areas (Sardinia

238   Sea side). Both species showed similar maximum abundance values ($\approx 1 \times 10^3$ cells L^{-1}),

239   registered at Tortolì harbour (54) and Baia Sardinia (71), respectively. *G. impudicum* was

240   observed in 5 beaches (Figure 5; Table 2) and the largest number of observations were in the

241   Oristano Gulf (Figure 5). This species reached a maximum of 7.4×10^3 cells L^{-1} at

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242   Torregrande II (29). *L. polyedrum* and *P. reticulatum* were observed in 5 and 7 beaches,

243   respectively (Figure 5, Table 2), without a specific area of detection. These species reached

244   maximum of 355 cells L^{-1} (Lido II, 17) and 623 cells L^{-1} (Marina Tertenia, 50).

245   The remaining harmful taxa showed a narrower distribution and low abundance values (Table

246   2).

247   All harmful species observed were present at a fairly broad range of environmental variables

248   (Table 2). During the two bloom events, temperature and salinity were, respectively, of 25.1

249   °C and 37.2 at Bosa Marina (21) and 27.3 °C and 37.3 at Villasimius (45 $P-PO_4$ was below

250   the detection level at both sites ($P-PO_4 < 0.03$ μM), while P-Ptot was 0.9 μM (Bosa Marina,

251   21) and 0.7 μM (Villasimius, 45). DIN and N-Ntot were, respectively, 1.1 μM and 31 μM at

252   Bosa Marina (21), and 0.5 μM and 25.9 μM at Villasimius (45). Chlorophyll *a* was 8.2 mg m^{-3}

253   and 2.6 mg m^{-3}, respectively at Bosa Marina (21) and Villasimius (45).

254

255   **4.2 Molecular analysis of PCR assay**

256   *4.2.1 Single cell PCR*

257   Eleven isolates of *A. taylorii* cells were analysed with the single-cell method (Table 3). All

258   obtained sequences had the 99-100% of similarity with those obtained from different areas of

259   the Mediterranean (Catalan coast, Adriatic, Tyrrhenian and Aegean seas, Balearic Islands)

260   deposited in GenBank (data not shown).

261   Four isolates of *Ostreopsis* were analyzed and identified as *O.* cf. *ovata* Fukuyo (Table 3).

262   The obtained sequences showed 99% of similarity with other isolates from the Atlantic and

263   Mediterranean areas deposited in GenBank (data not shown).

264   The unique isolate of *Barrufeta bravensis* showed a sequence homology of 99% with the five

265   sequences deposited in GenBank for this species (data not shown).

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266 The sequence obtained for *Gymnodinium instriatum* showed sequence homology of 99% with

267 both the sequences of *G. instriatum* and *G. uncatenum* deposited in GenBank, deriving from

268 different areas of the world (China, Iran, Spain and the USA) (data not shown).

269

270 *4.2.2 PCR based assay*

271 PCR based assay was applied on 59 field net samples (extracted DNA enough for the

272 analysis) and it was positive for the presence of genus *Alexandrium* and species *A. taylorii*, *A.*

273 *minutum, A. tamarense* and *O.* cf. *ovata,* while PCR amplification resulted negative for the

274 presence of *A. catenella* and *O.* cf. *siamensis. A. taylorii* was the species that showed the

275 highest number of PCR positives (42), while *A. minutum* was found in 12 samples (Figure 6b)

276 and *A. tamarense* in three samples (data not shown). Finally, *Ostreopsis* cf. *ovata* was found

277 in 8 samples (Figure 6c).

278

279 **4.3 Environmental and morphodynamic characterization of beaches**

280 Temperature and salinity among the sampled beaches varied from 17.4 °C (Argentiera, 12) to

281 28.9 °C (Marina Tertenia, 50), and from 27.8 (Torregrande II, 29) to 39.4 (Saline, 66),

282 respectively. Regarding nutrients, P-PO$_4$ and P-Ptot minima were below the detection limit of

283 the method and maxima reached 1.1 µM (Torregrande II, 29) and 15.6 µM (Lido II, 17),

284 respectively. DIN and N-Ntot ranged from 0.4 µM (La Caletta, 62) to 45.2 µM (Porto Ferro,

285 13), and from 7.3 µM (Foxi e' Sali, 40) to 46.3 µM (Porto Ferro, 13), respectively. Finally,

286 Si-SiO$_4$ varied from 0.03 µM (Cala Brandinchi, 65) to 41.5 µM (Torregrande II, 29).

287 The preliminary PCA analysis on the environmental parameters led to the exclusion from the

288 analysis of two beaches (Porto Ferro, 13; Lido II, 17) because of the excessive out of values

289 (respectively, DIN due to N-NO$_3$ and P-Ptot). The first two axes (PCA Axis 1 and PCA Axis

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290   2) explained together 83.1% of the total variation (Table 4, Figure 7). The resulting PCA plot

291   showed a distribution of the beaches mainly according to Si-SiO$_4$ and DIN (Table 4, Figure

292   7).

293   Beach sediments were mainly sandy (from coarse to fine). Only in five cases the gravel

294   content was higher than 50%. The silt and pelitic fractions were not relieved in the analysed

295   samples. Forty-three out of 72 beaches were unindented (IND index < 1.69), the majority

296   resulting MB or SB beaches. On the contrary, the indented beaches (IND index > 2.34) were

297   less frequent (10 up to 72 beaches) accounting 4 SB, 3 NB and 3 R.

298   The first two axes of the PCA performed on the morphodynamic parameters, explained

299   together 75.8% of the total variation (Table 5, Figure 8). The environmental parameter

300   eigenvectors showed that PCA axis 1 was positively correlated with GR and negatively

301   correlated with MFS. PCA axis 2 showed a positive correlation with AWE (Table 5, Figure

302   8). Regarding the submerged beach morphology the MB beaches resulted more energetic in

303   respect to the NB and R, whilst the SB results more heterogeneous. La Pelosa beach (11) was

304   excluded from PCA because submerged morphology was not easy to be determined.

305

306   ***4.4 Relationships among phytoplankton assemblages, harmful algal species and***

307   ***environmental variables***

308   RDA analysis provided insight into the relationship between the phytoplankton groups and

309   environmental parameters. Together, all environmental variables accounted for 59.3% of the

310   first two axes variation (Figure 9). AWE and GR were the significant environmental factors

311   explaining variability in the phytoplankton composition (F = 0.14, p = 0.008 and F = 0.10, p =

312   0.018 for AWE and GR, respectively). Both the *bloom-forming species* and the *other harmful*

313   *species* showed a negative correlation with GR and a positive correlation with MFS, although

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314   this variable is not significant. *O.* cf. *ovata* instead did not seem to have relationships with the

315   significant variables identified (Figure 9a). Beaches positively related to GR were mainly

316   located in the south coast (Cagliari Gulf, Tyrrhenian Sea and Sardinia Sea sides), while those

317   positively related to AWE were of more heterogeneous geographical localization (Figure 9b).

318

319   **5. Discussion**

320   The results of the first year of our project provide useful data on the presence of HAS in

321   beach environments along the coasts of Sardinia, covering a length of about 2,000 km and

322   concerning different water bodies within the Mediterranean Sea (Tyrrhenian Sea, Sardinia

323   Sea, Strait of Bonifacio, and the main gulfs of Cagliari and Asinara). Beaches are, in general,

324   poorly studied environments regarding the presence and distribution of harmful species. Most

325   of the informations along the Mediterranean coasts derive from studies on specific species,

326   e.g. *Alexandrium taylorii* [26-30], naked dinoflagellates [31-32], *Ostreopsis* species [20, 33-

327   35 and references therein] or groups of species [36-37]. Only a few data are available on the

328   entire set of harmful species [38]. Our results emphasize the presence of potentially noxious

329   species either typical of beaches (e.g. *Alexandrium taylorii*), or most frequently reported from

330   deeper coastal waters (e.g. *Dinophysis* and *Pseudo-nitzschia* species). The list of species

331   observed is similar to those of other beaches in the Mediterranean Sea [38], with some new

332   reports. Overall, the found taxa are able to impact on water quality and/or human health,

333   respectively due to their high-biomass production and/or toxicity.

334

335   *5.1 High-biomass producers*

336   *Alexandrium taylorii* was the most widely distributed species in the studied beaches. The

337   combination of molecular method (PCR) with microscopy provided extended distribution of

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338 the species, confirming the usefulness of a multi-method approach in analyses of wild

339 samples. In Sardinia, this species was prior reported at Platamona [39] but its presence was

340 already assessed also at Valledoria and La Speranza beaches (Satta, personal communication).

341 *A. taylorii* is the species that has produced more issues in beaches along the Mediterranean

342 coast in recent years [26-30, 39]. Our results confirm its ability to cause harmful events either

343 alone or in mixed blooms.

344 *Barrufeta bravensis* and *Gymnodinium instriatum* accompanied *A. taylorii* in the observed

345 mixed bloom (Bosa Marina, 21). Recurrent mixed blooms with *B. bravensis* had been

346 reported in the Catalan beach of La Fosca [32], leading to the description of this species.

347 Consequently, our discovery of *B. bravensis* is the first record in a different geographic area

348 of its certain description. The use of molecular methods was decisive for its sure

349 determination. In fact, especially for naked dinoflagellates, the morphological taxonomic

350 criteria require specific insights, not always easily investigable. *G. instriatum* is a well known

351 red tide species worldwide [40] but only recently reported in Mediterranean beaches (A. Reñé

352 personal communication). Anyway, cysts of *G. instriatum* were also reported from sediments

353 of other coastal areas [41-42]. Our results indicate its wide distribution in Mediterranean

354 beaches and its capability to contribute to discolouration events. The high homology of

355 genetic sequence obtained with both the sequences of *G. instriatum* and *G. uncatenum* in

356 GenBank highlights that taxonomic definition of these species needs clarifications.

357 *Gymnodinium impudicum* has been widely reported in the Mediterranean Sea, both in beaches

358 and others environments [38], both in the vegetative and resting stages [41]. Vila et al. [38]

359 indicated a significative negative relationship between *G. impudicum* and salinity, with the

360 highest abundances values reached in harbours. Our findings were especially in areas close to

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361   transitional environments, as already reported by Sannio *et al*. [43], confirming its preference

362   for low salinity and enclosed sites.

363   Also *Kryptoperidinium foliaceum* blooms in transitional environments (e.g., lagoons and

364   estuaries). *K. foliaceum* has been rarely reported in the Mediterranen basin, both in the

365   vegetative and resting stages, although high abundance blooms were observed in Sardinian

366   and Catalan lagoons [44]. In fact, our detection regarded only a beach near to a lagoon (Lido

367   I, 16), where this species produces recurrent blooms (Calich Lagoon, Pulina personal

368   communication).

369   *Alexandrium minutum* and *Ostreopsis* cf. *ovata* reported as high biomass bloom producers,

370   express their harmfulness also via toxins and for this they are considered in the following

371   paragraph.

372

373   **5.2 Toxin producers**

374   *Ostreopsis* is a genus that includes benthic species capable to produce palytoxin-like toxins,

375   which can affect humans directly, through aerosol or contact, especially during recreational

376   activities [35 and references therein]. Further, high biomass and toxic blooms can seriously

377   affect aquatic biota [45] and productions [46]. In the last fifteen years, *Ostreopsis* blooms

378   have become more frequent, intense and widely distributed worldwide, including the

379   Mediterranean Sea [35]. The presence of these species, although not in bloom events, had

380   already been also reported in several Sardinian coastal areas [47]. In our samples, the

381   application of molecular methods (PCR) provided the identification of *O.* cf. *ovata*. This

382   species was responsible for a harmful event along Sardinian coast in Alghero in summer 2009

383   (http://www.sardegnaambiente.it/documenti/21_162_20090916152500.pdf).

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384   The other potentially toxic species were detected in a lesser number of beaches and in low

385   abundances (<1000 cells l^{-1}), as already reported in Catalan beaches [38]. In the case of

386   *Alexandrium minutum*, the PCR allowed to obtain more detailed pictures of species-specific

387   distribution. Moreover, this technique permitted the detection of *A. tamarense*, not identified

388   with microscopy methods. *Alexandrium*, *Dinophysis* and *Pseudo-nitzschia* toxins can

389   accumulate in shellfish and potentially harm humans throughout the food chain.

390   Consequently, their monitoring regards mainly farming areas while other near shore coastal

391   sites remain out of the normal monitoring programs [38]. However, toxins can accumulate

392   also in wild shellfish representing a potential harmful if gathered for food. This type of impact

393   is widely known in other countries and includes specific controls on HAS [48]. Our data

394   contribute to define a first scenario of their presence along Sardinian coast out of the normal

395   areas of controls, highlighting their potential impacts.

396

5.3 Harmful algal species vs environmental and morphodynamic variables

398   Environmental and morphodynamic data from the sampled beaches ranged widely, indicating

399   that the considered beaches were representative of wide case studies. No evident relationships

400   were assessed between HAS groups and AWE, IND index and nutrients, despite they

401   appeared important drivers in favouring HAS affirmations in a number of other cases [49].

402   Sediment grain size resulted the most important variable in driving HAS groups. In particular,

403   HAS groups had a positive relationship with the finer fractions and were at the opposite side

404   of coarser sediments. This finding might be related to the influence of resting dormant stages.

405   All the HAS found, in fact, are able to produce resting dormant stages in their life cycles.

406   These non-motile stages once formed, settle in sediments and join with fine sized particles

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407 [50]. Then, the high abundance and number of resting stage producing species are observed in

408 association with finer sediments rather than coarse sediments [50].

409 Finally, *O.* cf. *ovata* showed no relation with the variables considered, in accordance with the

410 benthic life and, therefore, probably influenced by other factors.

411

412 **6. Conclusions and future perspective**

413 The informations obtained in this study provide useful knowledge basis on the presence and

414 distribution of HAS in the poorly studied beach ecosystems of the Mediterranean Sea. After

415 Sicily, Sardinia is the second largest island in the Mediterranean Sea but it is the first as

416 development of the coastline. Moreover, its central location in the western Mediterranean

417 makes Sardinia a natural laboratory to evaluate the potentialities of HABs development. Our

418 findings emphasize the wide distribution of the species that make explicit their harmful

419 potential through the development of high biomass directly in beaches. In addition, the

420 mapping of the toxin-producing species with indirect action (linked to food chains) in these

421 environments provides useful information still poorly available along the coasts of the

422 Mediterranean basin. The use of an interdisciplinary approach has proved successful.

423 Molecular techniques contribute to increase knowledge on identification and distribution of

424 species, and the grain size data seem explain species distribution. Precisely the relationship

425 between the grain size and the HAS opens an interesting scenario of major interest for the

426 understanding of the dynamics of the species in the beaches.

427 Detailed data on spatial and temporal dynamics of these species are need as well as data on

428 the presence and distribution of their resting cysts in the sediments. These data will be

429 fundamental to identify the localization of the source of bloom inoculum and consequently

430 understand more in detail the phenomena.

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571 Table 1 List of the selected beaches and main characteristics.
572

Geographical localization	Beach name	Code	Sampling date	Beach typology	Brackish and/or freshwater inputs	Villages and/or settlements	Harbours and/or artificial structures
North	Rena Bianca	1	July 25	Pocket	-	+	-
North	Cala Sarraina	2	July 25	Pocket	-	-	-
North	Marinedda	3	July 25	Pocket	-	+	-
North	Badesi	4	July 25	Linear	+	+	-
North	Valledoria	5	July 25	Linear	+	+	-
North	Castelsardo	6	July 17	Man-made	+	+	+
North	Platamona	7	July 17	Linear	+	+	-
North	Balai	8	July 17	Pocket	-	+	-
North	Fiume Santo	9	July 17	Linear	+	-	-
North	Saline	10	July 17	Linear	+	+	-
North	Pelosa	11	July 17	Pillow	-	+	-
Northwest	Argentiera	12	July 17	Pocket	-	+	-
Northwest	Porto Ferro	13	July 10	Pocket	-	-	-
Northwest	Mugoni	14	July 10	Pocket	+	-	-
Northwest	Lazzaretto	15	July 10	Pocket	-	+	-
Northwest	Lido I	16	July 10	Linear	+	-	+
Northwest	Lido II	17	July 10	Linear	+	+	-
Northwest	Lido III	18	July 10	Linear	-	+	+
Northwest	Lido IV	19	July 10	Linear	+	+	+
Northwest	Speranza	20	July 10	Pocket	+	-	-
Northwest	Bosa Marina	21	August 6	Pocket/ Man-made	-	+	+
Northwest	Porto Alabe	22	August 6	Linear	-	+	-
Southwest	Is Arenas	23	August 6	Linear	+	-	-
Southwest	Su Pallosu	24	August 6	Pocket	+	+	-
Southwest	Putzu Idu	25	August 6	Pocket	+	+	-
Southwest	San Giovanni I	26	July 5	Semi-Pocket	-	-	-
Southwest	San Giovanni II	27	July 5	Linear	-	-	-

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Southwest	Torregrande I	28	July 5	Linear	+	+	-
Southwest	Torregrande II	29	July 5	Linear	+	-	+
Southwest	Arborea	30	August 8	Linear	+	-	-
Southwest	Funtanazza	31	July 30	Linear	-	-	-
Southwest	Bugerru	32	July 30	Pocket/ Man-made	-	+	+
Southwest	Cala Domestica	33	July 30	Pocket	-	-	-
Southwest	Portoscuso	34	July 30	Linear	-	+	-
South	Porto Botte	35	July 30	Linear	+	+	-
South	Porto Pino	36	July 31	Linear	+	+	+
South	Tuaredda	37	July 31	Pillow	-	+	-
South	Cala Cipolla	38	July 31	Pocket	-	-	-
South	Chia	39	July 31	Linear	+	+	-
South	Foxi e' Sali	40	July 31	Linear	+	+	-
South	Nora	41	July 31	Bay	+	-	-
South	Perd'e Sali	42	August 21	Linear	-	+	+
South	La Maddalena	43	August 21	Linear	+	+	-
South	Poetto	44	August 21	Linear	+	+	-
South	Villasimius	45	August 21	Linear	+	+	+
Southeast	Cala Pira	46	August 21	Pocket	-	+	-
Southeast	Cala Sinzias	47	August 21	Linear	-	+	-
Southeast	Colostrai	48	August 20	Linear	+	-	-
Southeast	Foce Flumendosa	49	August 20	Linear	+	+	+
Southeast	Marina Tertenia	50	August 20	Linear	-	+	-
Southeast	Barisardo	51	August 20	Linear	+	+	-
Southeast	Cea	52	August 20	Pocket	-	+	-
Southeast	Porto Frailis	53	August 20	Pocket	-	+	-
Southeast	Tortolì harbour	54	August 20	Linear/ Man-made	+	+	+
Southeast	Lotzorai	55	August 20	Linear	+	+	-
Northeast	Cala Fuili	56	August 28	Pocket	-	-	-
Northeast	Cala Gonone	57	August 28	Man-made	-	+	+
Northeast	Osalla	58	August 28	Pocket	+	-	+
Northeast	Marina di Orosei	59	August 28	Linear	+	+	+
Northeast	Porto Corallo	60	August 28	Pocket	+	+	-

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Northeast	Berchida	61	August 28	Linear	+	-	-
Northeast	La Caletta	62	August 29	Linear	+	+	-
Northeast	Ottiolu	63	August 29	Pocket	-	+	+
Northeast	La Cinta	64	August 29	Linear	+	+	-
Northeast	Cala Brandinchi	65	August 29	Pocket	+	-	-
Northeast	Saline	66	August 29	Pocket	+	+	-
Northeast	Pittulongu	67	August 8	Pocket	-	+	-
Northeast	Golfo Aranci	68	August 8	Pocket	-	+	-
Northeast	Marinella	69	August 8	Pocket	-	+	-
Northeast	Liscia Ruia	70	August 8	Pocket	-	-	-
Northeast	Baia Sardinia	71	August 8	Pocket	-	+	-
Northeast	Cannigione	72	August 8	?	-	+	-
Northeast	Palau	73	August 8	Pocket	-	+	+
Northeast	Porto Pozzo	74	July 25	Pocket	+	+	-

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Table 2 Potentially harmful algal species observed along the Sardinian beaches, harmful impacts (D: impact on water quality, T: impact on human health, P: impact on marine invertebrates), number of observations (obs.), cell abundance and environmental variable ranges in correspondence of observations. bdl: below the detection limit of method (detection limits, P-PO$_4$: 0.03 µM; P-Ptot: 0.08 µM).

Genus / species	Class	harmful impact	obs.	abundance (cells l^{-1})	Tem °C	Sal	P-PO$_4$ µMP	P-Ptot µMP	DIN µMN	N-Ntot µM N
Alexandrium taylorii Balech	Dinophyceae	D, T*	24	163 - 866434	21.1 - 27.3	37.0 - 39.0	bdl - 0.2	bdl - 0.9	0.5 - 45.2	11.3 - 46.3
Alexandrium minutum Halim	Dinophyceae	D, T	5	163 - 958	21.1 - 26.3	37 - 38.9	bdl - 0.04	0.1 - 0.2	0.5 - 45.2	14.3 - 46.3
Alexandrium spp.	Dinophyceae	T	6	82 - 973	21.6 - 25.4	35.9 - 38.6	bdl - 0.04	0.1 - 0.4	1.0 - 4.1	16.1 - 25.8
Barrufeta bravensis Sampedro et Fraga	Dinophyceae	D	1	4436588	25.1	37.2	bdl	0.9	1.1	31.0
Dinophysis caudata Claparède et Lachmann	Dinophyceae	T	4	20 - 400	24.0 - 25.6	37.7 - 38.8	bdl	bdl - 0.5	0.5 - 1.7	11.1 - 27.4
Dinophysis rotundata Claparède et Lachmann	Dinophyceae	T	2	20 - 687	23.7 - 25.0	37.6 - 38.8	bdl	bdl - 0.2	0.5 - 0.9	11.1 - 18.7
Dinophysis sacculus Stein	Dinophyceae	T	2	168 - 186	23.7 - 25.2	38.5 - 38.6	bdl	0.3 - 0.4	1.1 - 2.4	17.1 - 22.5
Gymnodinium impudicum (Fraga et Bravo) Hansen et Moestrup	Dinophyceae	D	5	80 - 7430	23.3 - 25.6	27.8 - 38.8	bdl - 1.1	bdl - 2.8	0.5 - 3.4	11.1 - 29.3
Gymnodinium instriatum (Freudenthal et Lee) Coats	Dinophyceae	D	21	10 - 727938	22.3 - 26.4	36.5 - 39.4	bdl - 0.2	bdl - 0.9	0.4 - 5.8	7.3 - 31.0
Kryptoperidinium foliaceum (Stein) Lindemann	Dinophyceae	D	1	140763	22.9	37.1	0.2	1.2	1.7	19.0
Lingulodinium polvedrum (Stein) Dodge	Dinophyceae	T	5	20 - 355	21.3 - 24.4	36.5 - 38.8	bdl - 0.1	0.2 – 15.5	0.7 - 5.8	12.5 - 27.5
Ostreopsis spp.	Dinophyceae	D, T, P	18	10 - 1060	21.3 - 27.6	37.1 - 39.1	bdl - 0.3	bdl - 0.8	0.9 - 7.1	7.3 - 28.6
Protoceratium reticulatum (Claparède et Lachmann) Butschli	Dinophyceae	T	7	159 - 623	21.3 - 28.9	37.2 - 38.9	bdl - 0.04	bdl - 15.6	0.5 - 1.4	14.3 - 20.2

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| *Pseudo-nitzschia* spp. | Bacillariophyceae | T | 3 | 163 - 860 | 24.7 - 25.7 | 37.2 - 38.3 | bdl | bdl - 0.4 | 1.2 - 4.1 | 16.9 - 20.1 |

* toxin production detected only in non-Mediterranean strains.

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Table 3 List of isolates subjected to single-cell analysis, beaches of origin, beach codes and marker genes.

Species	Beach	Code	Marker gene
Alexandrium taylorii	Marinedda	3	ITS – 5.8S rDNA
Alexandrium taylorii	Badesi	4	ITS – 5.8S rDNA
Alexandrium taylorii	Platamona	7	ITS – 5.8S rDNA
Alexandrium taylorii	Speranza	20	ITS – 5.8S rDNA
Alexandrium taylorii	Bosa Marina	21	ITS – 5.8S rDNA
Alexandrium taylorii	Cala Cipolla	38	ITS – 5.8S rDNA
Alexandrium taylorii	Chia	39	ITS – 5.8S rDNA
Alexandrium taylorii	Nora	41	ITS – 5.8S rDNA
Alexandrium taylorii	Porto Corallo	60	ITS – 5.8S rDNA
Alexandrium taylorii	Liscia Ruia	70	ITS – 5.8S rDNA
Alexandrium taylorii	Baia Sardinia	71	ITS – 5.8S rDNA
Ostreopsis **cf.** *ovata*	Cala Sarraina	2	ITS – 5.8S rDNA
Ostreopsis **cf.** *ovata*	Balai	8	ITS – 5.8S rDNA
Ostreopsis **cf.** *ovata*	Lido Rafael	18	ITS – 5.8S rDNA
Ostreopsis **cf.** *ovata*	Cala Cipolla	38	ITS – 5.8S rDNA
Barrufeta bravensis	Bosa Marina	21	ITS – 5.8S rDNA

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Table 4 Results of the PCA (eingenvalues and eingenvectors) applied to environmental parameters.

Parameters	PCA Axis 1	PCA Axis 2
Tem	-0.005	-0.044
Sal	-0.029	0.007
P-PO$_4$	0.072	-0.042
P-Ptot	0.164	-0.140
DIN	0.279	-0.867
N-Ntot	0.028	-0.370
Si-SiO$_4$	0.942	0.296
Eingenvalues	0.09	0.02
Variation (%)	69.1	14.0

Table 5 Results of the PCA (eingenvalues and eingenvectors) applied to grain size and morphodynamic parameters.

Parameters	PCA Axis 1	PCA Axis 2
GR	0.606	-0.190
CS	0.322	0.136
MFS	-0.720	-0.233
AWE	-0.101	0.938
IND index	0.009	-0.14
Eingenvalues	0.86	0.30
Variation (%)	56	19.8

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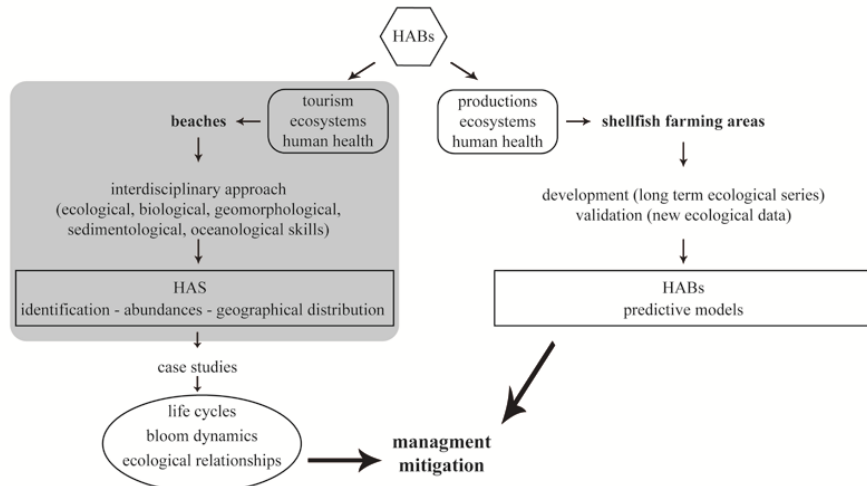
Figure 1 Structure of the project. The gray square encloses the activities object of the present paper.
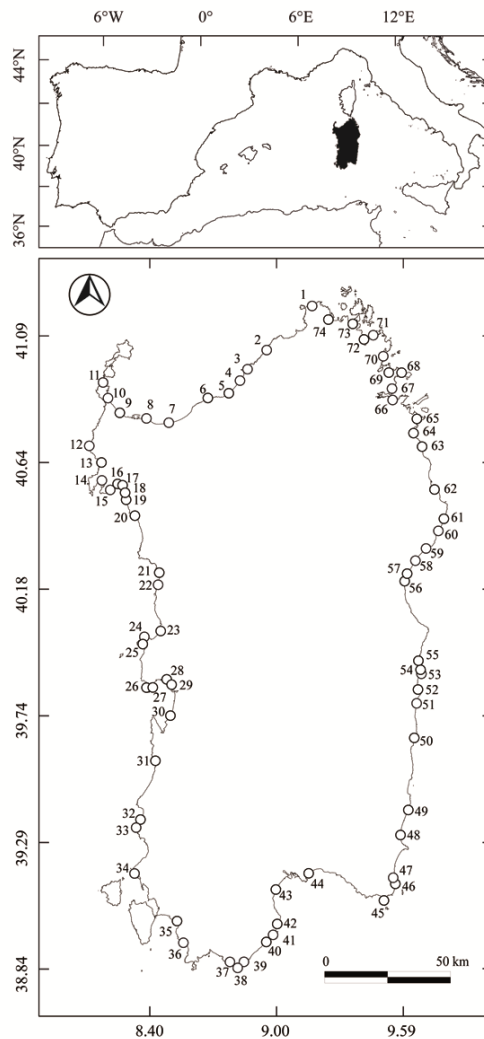
Figure 2 Geographical localization of sampled beaches.

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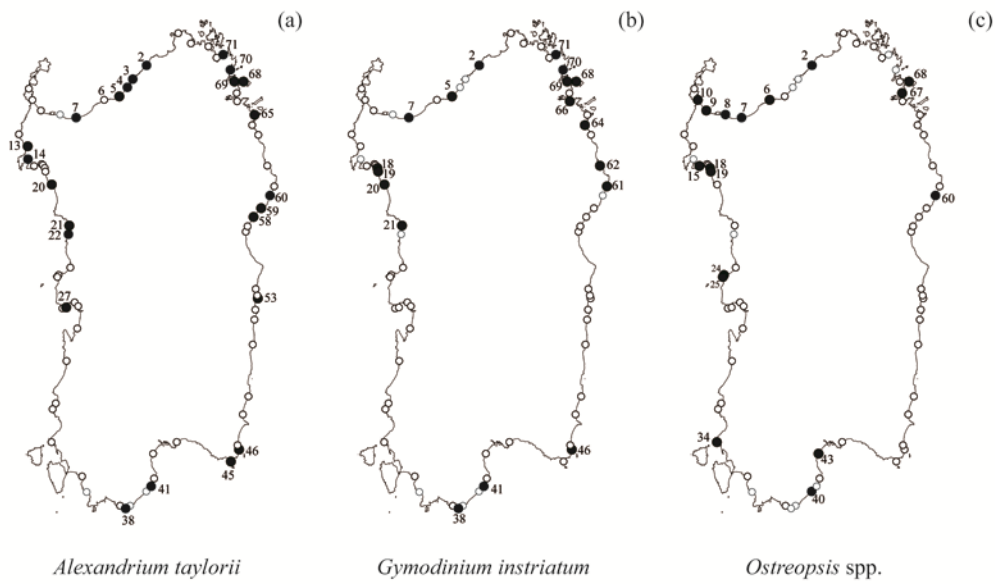
Figure 3 Maps of distribution for *Alexandrium taylorii* (a), *Gymnodinium instriatum* (b), and *Ostreopsis* spp. (c). Black cycle: finding of the species, white cycle: not finding.
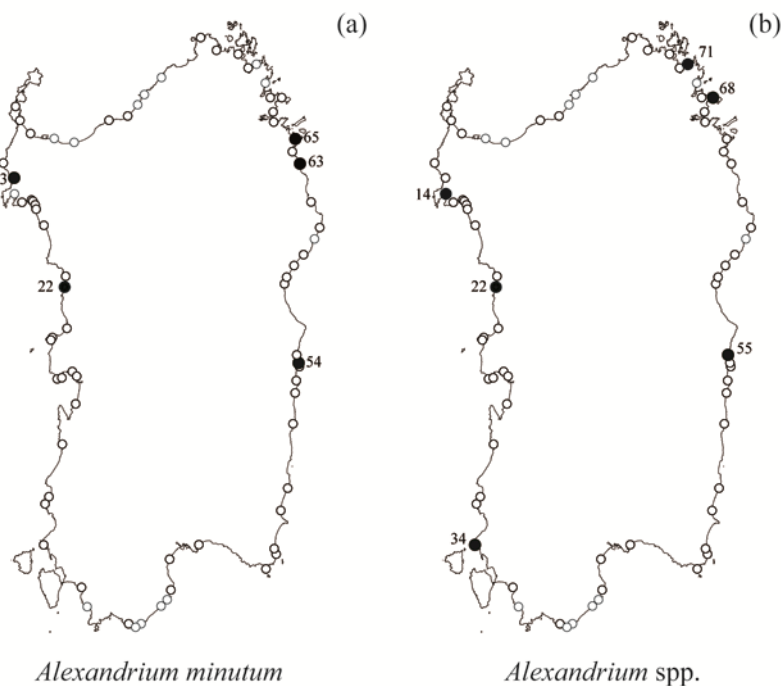
Figure 4 Maps of distribution for *Alexandrium minutum* (a), and *Alexandrium* spp. (b). Black cycle: finding of the species, white cycle: not finding.

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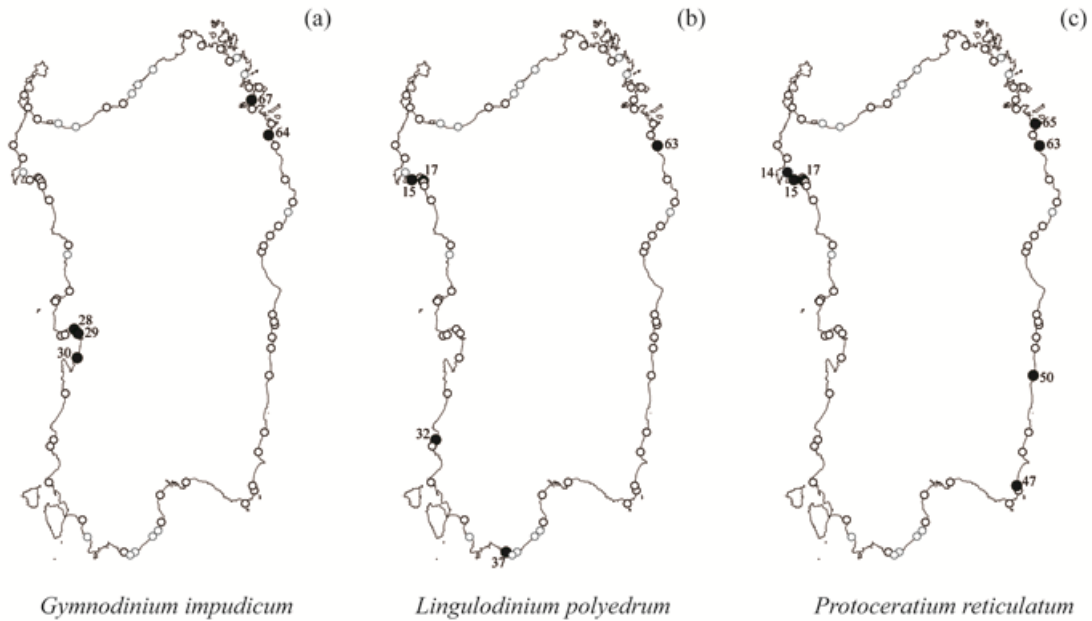
Figure 5 Maps of distribution for *Gymnodinium impudicum* (a), *Lingulodinium polyedrum* (b), and *Protoceratium reticulatum* (c). Black cycle: finding of the species, white cycle: not finding.
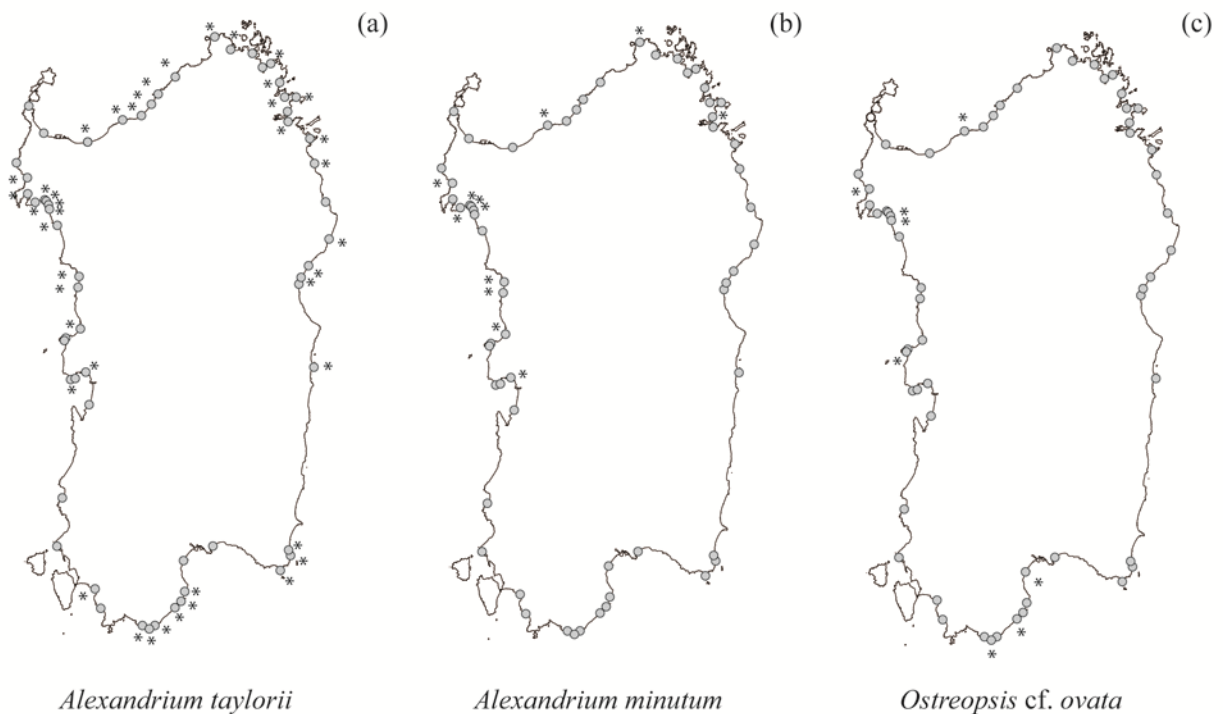
Figure 6 Results of PCR based assay for *Alexandrium taylorii* (a), *A. minutum* (b), and *Ostreopsis* cf. *ovata* (c). Grey cycle: sample tested with PCR, *: species detection.

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6. CHAPTER III - Interdisciplinary approaches to study harmful algal blooms (HABs) in beach environments: an experience from the

Mediterranean Sea
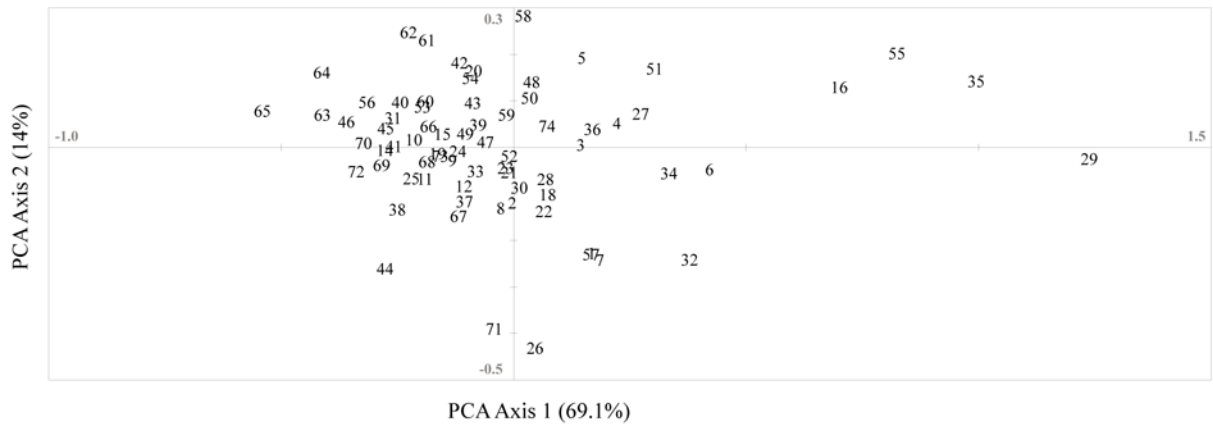
Figure 7 Results of principal component analysis (PCA) based on environmental parameters of the sampled beaches.
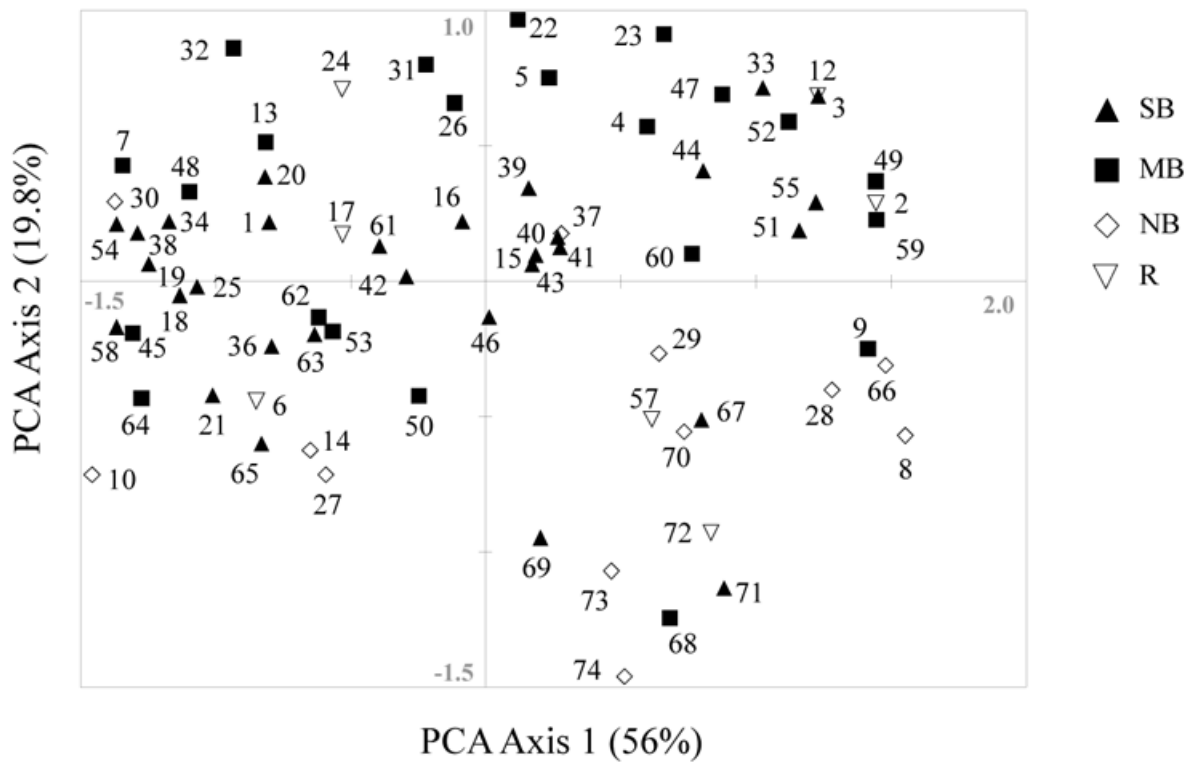
Figure 8 Results of principal component analysis (PCA) based on morphodynamic parameters of the sampled beaches.

SB: one bar, MB: one or more bars, NB: no bar, R: rocky.

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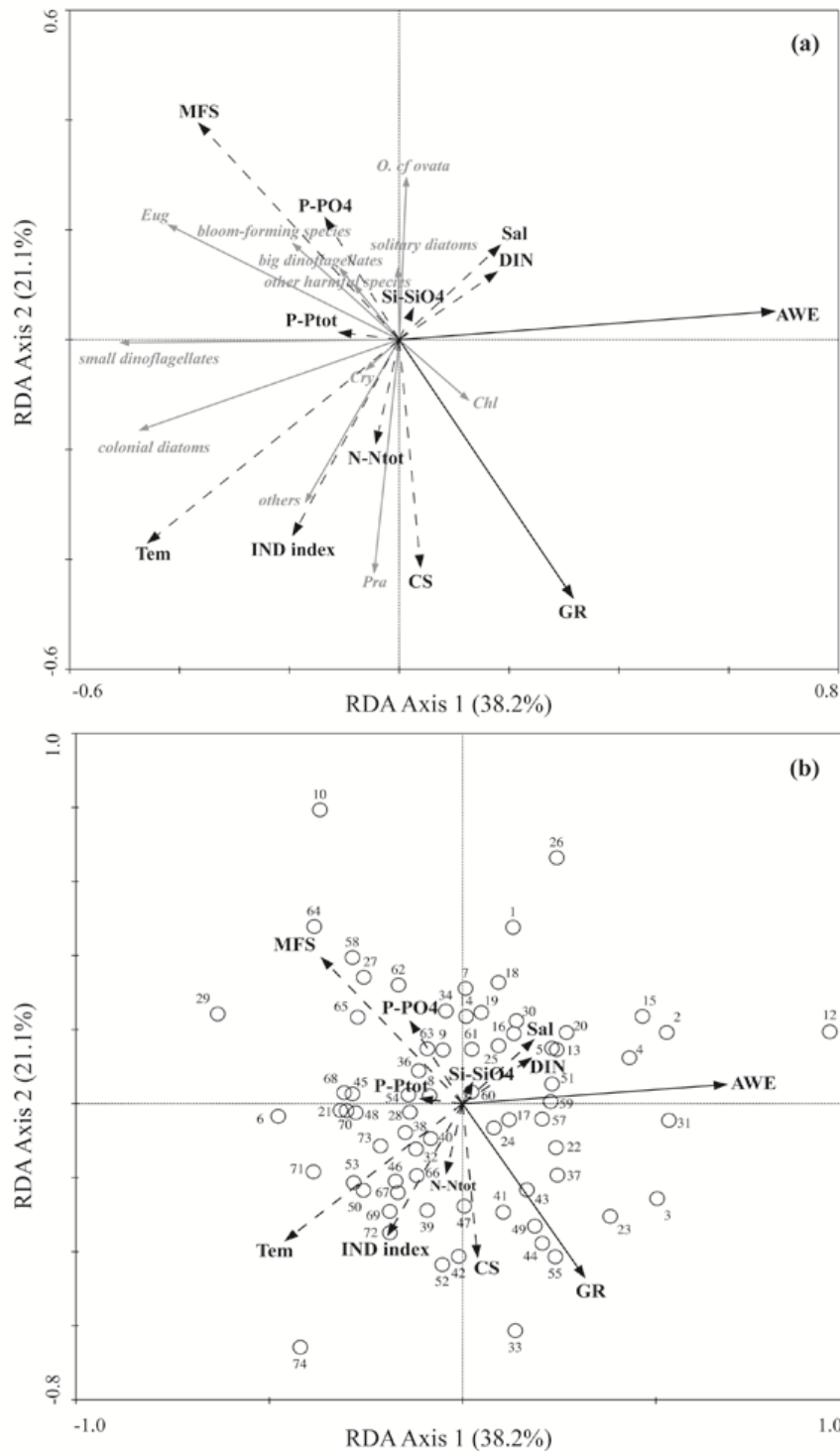
Figure 9 Redundancy analysis (RDA) results showing ordination of (a) phytoplankton groups and environmental variables. Statistically significant (p<0.05) environmental variables are shown with solid arrows, and (b) samples scores.

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7. CHAPTER IV

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Potentially Harmful Algal Blooms in Mediterranean artificial lakes due to dinoflagellates: case studies from Sardinia.

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ABSTRACT In the Mediterranean region, artificial lakes represent the main sources of water supply for various human demands. It is especially in the southern areas and islands, where the scarce environmental quality favours toxic blooms, predominantly due to cyanobacteria. Freshwater red tides caused by dinoflagellates are less common, but they have been more recently observed in many reservoirs and lakes, becoming a new emergent issues for water treatments. Sardinia is the richest Italian region in number of dams and water volume stored. More than 90% of the drinking water derives from the artificial lakes, requiring specific investigation on the water quality for drinking use. The aim of this study was to give a contribution in the ascertainment of the dinoflagellates species involved in bloom events in two Sardinian artificial lakes, Sos Canales and Cedrino. We analysed fixed samples taken in correspondence of bloom events and viable resting cysts isolated from the sediments. Molecular analysis, scanning electron microscopy (SEM) and light microscopy were used. Preliminary results deriving from SEM analysis identified as a *Peridiniopsis* species the armoured red tide dinoflagellate of the Cedrino Lake, while biomolecular analysis determined as a *Gymonodinium* species (most probably *G. uberrimum*, as supposed on the morphological features) the naked dinoflagellate from Sos Canales Lake. Our study emphasizes the importance of the investigation on the different phases of the dinoflagellate life cycle and the necessity of an approach with multiple analysis techniques.

Key words: *Gymnodinium, Peridiniopsis*, harmful algal blooms, artificial lakes, drinking-water.

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INTRODUCTION

In the Mediterranean basin, the recently developed models to predict impacts of the climate change indicate an increase in temperatures in the summer months and a decrease in precipitations (Giorgi and Lionello, 2008; Spyropoulou *et al.*, 2013). These impacts will be exacerbated in the southern region due to the increase of desertification processes (Gao and Giorgi, 2008). The expected reduction of water resources will be also accompanied by a water quality deterioration, particularly with the increase in toxic cyanobacteria blooms (Naselli-Flores *et al.*, 2007) with associated risks for human health (Chorus and Bartram, 1999; Codd *et al.*, 2005; Carmichael *et al.*, 2001) and costs for the water treatments for the different uses (e.g. drinking water, agriculture, livestock, industry, electricity production).

On the national scenario, the artificial lakes are the main sources of water supply in the southern regions and islands. In fact, in the last century numerous dams have been built, to answer to the increasing demands of the growing human population and the related socio-economic development, as well as globally in the world (Jørgensen *et al.* 1991).

Sardinia is the richest Italian region in number of dams (44 with a volume > 0.5 x 10^6 m^3) and volume of potentially stored water (about 2 x 10^9 m^3; Lugliè *et al.*, 2013). Moreover, in Sardinia, more than 90% of the drinking water derives from the artificial lakes whereas in the other southern Italian regions and Sicily the same percent value derives from ground water sources. Consequently, Sardinia appears a natural laboratory where verify hypotheses on the future scenarios of water quality for drinking use.

The majority of the Sardinian artificial lakes are eutrophic, favouring the affirmation of Cyanobacteria (Sechi and Lugliè, 1992, 1996) and the occurrence of toxic blooms (Messineo *et al.*, 2009; Mariani *et al.*, 2013). Blooms of Cyanobacteria (CyanoHABs; Charmicael, 2008) have been directly involved in adverse events in Sardinia since the 1980, both in fish kills and in possible effects on human health due to the production of potent toxins, cyanotoxins (Messineo *et al.*, 2009, and references therein). On the

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basis of the more recent data (Mariani *et al.*, 2013), abundance of cyanobacteria, cyanotoxins (microcystins) and trophic status appear closely linked in Sardinia.

However, several other algal groups, such as diatoms, dinoflagellates and chrysophytes, can affect the quality of drinking water, causing problems in the treatment plants for their removal and increasing related costs (Niesel *et al.*, 2007), or giving anomalous tastes and odours (Hargesheimer and Watson, 1996). Even if freshwater red tides due to dinoflagellates are less common than their marine analogues, they were observed in many reservoirs and lakes (Kawabata *et al.*, 1995; Cantonati *et al.*, 2003; Zhang *et al.*, 2011). They are usually a serious problem causing foul odours, taste and the reduction of the aesthetic value in these environments, so often countermeasures must be taken since they can be toxic (Fukuju *et al.*, 1998). In artificial lakes of Sardinia, dinoflagellates can be an important component of the phytoplankton assemblages (e.g., Sos Canales Lake, Lugliè *et al.*, 1996; Alto Flumendosa Lake, Meregalli *et al.*, 2002; Torrei Lake, M.A. Mariani personal communication), given in some occasion harmful blooms (Sechi, 1983; 1986). Species identification was sometimes uncertain at the time of the blooms, because of the difficulty in the observation of all the required diacritical characteristics. In fact, classification is mainly based on the use of traditional microscopy methods for the observation and/or measurement of morphologic characteristics not always easily detected, such as cell size, shape or position of chloroplasts, or, in the case of armoured dinoflagellates, their plate arrangement. In addition, many morphological features vary in response to the environmental conditions as well as during different stages of the life cycles (Ki and Han, 2005, 2007). Consequently, analyses require considerable taxonomic experience and skills (Godhe *et al.*, 2001; Ki and Han, 2005).

The aim of this study was to give a contribution in the ascertainment of the dinoflagellate species involved in bloom events in Mediterranean artificial lakes, considering two Sardinian artificial lakes (Cedrino and Sos Canales lakes) where dinoflagellates can achieve notable abundance in the phytoplankton. In particular, specific objectives were to confirm *Gymnodinium uberrimum* (Allman) Kofoid & Swezy

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as the dominant naked dinoflagellate in Sos Canales Lake and the attribution, at least at genus level, of the red tide armoured dinoflagellate in Cedrino Lake.

To achieve the objectives, we analysed fixed samples taken in correspondence of bloom events and viable resting cysts isolated from sediments. We used a multi-method approach, performing molecular analyses and observations with scanning electron microscopy (SEM) and light microscopy on the different kinds of samples.

MATERIAL AND METHODS

Study area and sampling

Cedrino Lake (CD) and Sos Canales Lake (SC) are two artificial lakes of Sardinia (Western Mediterranean Sea), located in the northern part of the island. CD and SC belong to the Italian Network of Long Term Ecological Research (LTER-Italy; www.lteritalia.it). They are managed by the Ente Acque della Sardegna (ENAS), the regional manager of dams and lakes. In both lakes, there is a Remote System Monitoring in Real Time, which allows obtain data on a number of basic variables (temperature, pH, conductivity, dissolved oxygen and fluorimetric chlorophyll *a*) along a vertical profile throughout the water column. A mobile device for selecting water of higher quality was installed to reduce the costs of drinking water treatment. In both the lakes, phytoplankton is the main primary producer.

CD was built in 1984 by damming the upper stream of River Cedrino (Fig. 1). Since its early formation, the lake has shown bad environmental conditions, due to its eutrophication (Padedda *et al.*, 2012). Cyanophyceae have become the dominant phytoplankton group, with the affirmation of potentially toxic species (Messineo *et al.*, 2009). Another environmental issue has been recently observed, linked to the massive affirmation, with water discolouration (red tide), of armoured dinoflagellate species, preliminary attributed to the genus *Peridinium*.

SC was built in 1956 by damming the upper stream of River Tirso (Fig. 1). It is located in a small, relatively pristine watershed, surrounded by cork-oaks woods. The water catchment is small and granitic. Though difficult to quantify, there are a small number

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of anthropogenic activities in the watershed, mainly associated with cork production and wild breeding. Despite the low impact of human activities, and a relatively low (theoretical) phosphorus load (Sechi, 1989), SC is classified as meso-eutrophic according to OECD criterions (Sechi, 1989; Sechi and Lugliè, 1992; Lugliè *et al*., 1996; Marchetto *et al*., 2009). Long-term studies started in 1978, have shown that the phytoplankton is co-dominated by Cyanobacteria, Dinophyceae, Chlorophyceae and Bacillariophyceae (Sechi and Lugliè, 1996). The naked dinoflagellate *Gymnodinium uberrimum* is reported as the dominant species for biomass.

Samplings for dinoflagellate studies were undertaken at one station in each lake, located at about 500 m from the dam. Water samples (100 ml) were collected from six depths (0, 1, 2.5, 5, 7.5, 10 m) and immediately fixed in the field with Lugol's solution. Samples submitted to the analyses were chosen amongst those collected over the years (since 1994 for CD and 1978 for SC) and archived in the collections of the Department of Sciences for Nature and Environmental Resources of the University of Sassari.

Sediment sampling, cyst analysis and germination experiments

Surface sediment samples were collected from Cedrino Lake in May 2013 and Sos Canales Lake in June 2013, using a grab. The samples were stores in the dark at 4 °C until processed.

Aliquots (3-4 cm^3) of the sediment samples were suspended in filtered deionized water and sonicated for 2 min using a Bandelin Sonoplus ultrasonic homogenizer. The sonicated material was filtered through a 100-µm mesh and collected on a 10-µm mesh. The fraction obtained was washed with filtered deionized water and collected in a 50-ml tube. Subsamples (7-10 ml) of the slurry were processed for cyst concentration and separation from inorganic particles using the sodium polytungstate (SPT) method (Bolch, 1997), modified by Bravo *et al.* (2006). The obtained supernatant was washed and collected in 5-10 ml of filtered deionized water. Aliquots of the resulting sample were counted in 3-ml sedimentation chambers with a Zeiss Axiovert 10 inverted microscope at 400x magnification.

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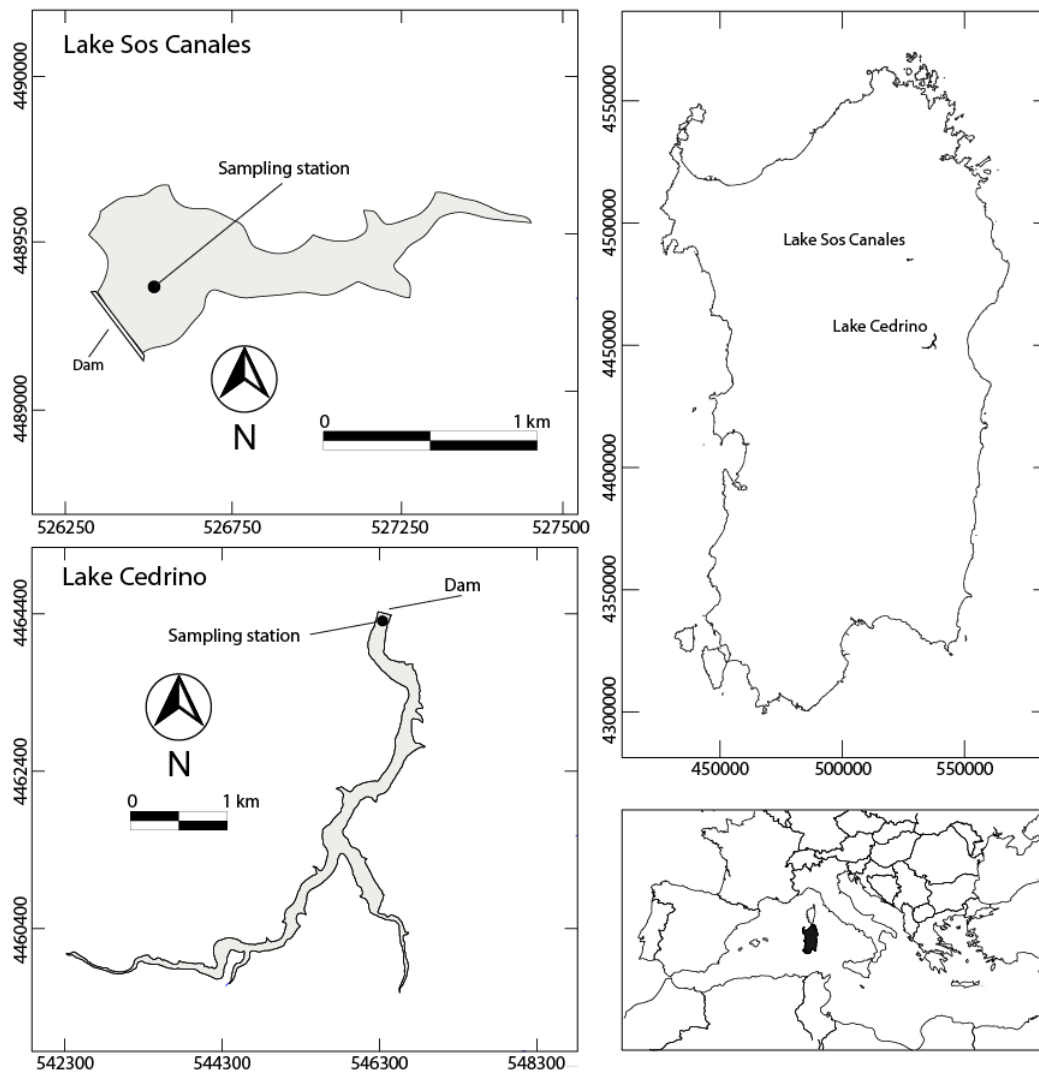
Figure 1 Geographical localization of Cedrino and Sos Canales lakes.

Cyst abundances were expresses as the number of cysts per gram dry weight sediment. Dry weight was obtained by drying wet sediment subsamples (2-3 cm^3) at 105 °C for 24 h.

Germination experiments, both on single cysts and on aliquots of the pre SPT sample, were conducted to identify species. Single cysts and filtered sediments (1 ml) were placed in tissue culture multiplates filled with L1 medium (Guillard and Hargraves, 1993) prepared with filtered deionized water. The plates were incubated at 20±1 °C, with 12:12 light:dark cycle (Sos Canales Lake), and 10±1 °C, with 10:14 light:dark cycle (Cedrino Lake), under an irradiance of 100 µmol m^{-2} s^{-1} (both lakes). Plates were verified every 2-4 days to check cyst germination.

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Morphological analyses

Fixed cells and cysts from both lakes were photographed and measured under a Zeiss Axiovert 10 inverted microscope equipped with a Zeiss AxioCam photo camera. The measurements of cell sizes were made using a calibrated eyepiece micrometer in light microscopy (LM) at 400X magnification.

Fixed cells from Cedrino Lake were also observed with scanning electron microscopy (SEM). The fixed cells were filtered onto a nuclepore filter (10 μm pore size; Millipore) using a syringe and a Swinnex filter holder. For the subsequent steps, the special device proposed by Chomerat and Couté (2008) was used to prevent cell losses. The device was transferred through an increasing concentration ethanol series (25, 50, 75, 95, and 100%; 10 min in each concentration). The filter was rinsed in 100% ethanol and critical point dried in liquid CO_2, using a Polaron Jumbo critical point drying apparatus. The filter was glued to a SEM stub using colloidal silver, sputter-coated with gold palladium and examined using a Zeiss EVO 10 SEM.

Biomolecular Approaches

<u>Fixed cells</u>

Fixed cells derived from bloom samples for CD (27 February 2012), and from a sample collected in spring for SC (30 March 2011), characterised by high cell abundance of the investigated gymnodinioid species. The cells were isolated using an inverted Axiovert Zeiss 25 microscope and the Single Cell method was applied (Kai *et al.*, 2006).

To bypass the presence of the fixative, the following preventive treatments were tested on cells:

- before the isolation of the cells, 1 ml of sterile water was added to 1 ml of sample in 2 ml eppendorf tubes, in order to remove Lugol's iodine fixative. After mixing, a centrifugation at 2000 rpm for 1' was done. These passages were repeated for five times;

- isolated cells (from a minimum of six to a maximum of 15 cells) were washed using drops of a sodium thiosulfate solution to decrease the inhibiting effect of

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the fixative on the PCR (Auinger *et al.* 2008), before transfer to a 0.2 ml PCR tube containing 3 µl of double distilled water (ddH$_2$O). PCR tubes were stored at -20°C before the direct PCR amplification;

- 10 ml of sample were transferred in a 50 ml falcon, and left to settled for 24 hours. Then, 8 ml of supernatant were removed and replaced with 8 ml of WC medium (Guillard and Lorenzen, 1972). This step was repeated from one to three times. The washed cells were then isolated and filled in lysis buffer solution or not. Cells with lysis buffer were submitted to temperature shock (Kai *et al.*, 2006). Tubes with the lysate and cells not submitted to lysis were filled with 10 µl of thiosulfate working solution (Auinger *et al.*, 2008), and then warmed at 95 °C for 5' and frozen at -80 °C until the PCR.

Cyst and germinated cells

Single cysts isolated from sediments were analysed following Kai *et al.* (2006) or Bolch *et al.* (2001). Cells germinated from filtered sediments were processed directly with the single cell method (Kai *et al.*, 2006).

PCR conditions and sequencing

The PCR protocol included 40.5 µL reaction mixture containing 0.25 mM of each dNTP; 0.1 µM of each primer (ITSA and ITSB, Adachi *et al.*, 1996); 2.5 mM MgCl$_2$; 1x HotMaster Taq Buffer (PRIME, Hamburg, Germany); 2.5 U Taq DNA polymerase (PRIME). PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min; followed by 35 cycles at 94 °C for 20 s, 57 °C for 10 s, and 70 °C for 30 s; and a final elongation step at 70 °C for 5 min. With PCR products a second PCR was carried out with the same conditions of the former, using 1 µl as or 1 µl diluted 1:15.

Only for the cells germinated by cysts, another PCR protocol was used: 50 µL mixture containing 0.8 µM of each primer (D1R and D2C, Scholin *et al.*, 1994), 200 µM of dNTPs (Qiagen mix), PCR Buffer 1X, and 1.25 U of Taq DNA polymerase (Qiagen). PCR conditions were as follows: initial step at 95 °C for 5 min followed by 40 cycles of 95 °C for 20 s, than a cycle at 55 °C for 30 s, and at 72 °C for 1 min, followed by a final extension of 72 °C for 10 min.

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All aliquots of the PCR products were electrophoresed in 1.8% agarose gels and the remaining product conserved at 4 °C until sequenced. PCR products were purified and sequenced by an external service (Macrogen Inc., Europe) using both primers, and a 3730XL DNA sequencer. All sequences were submitted to the BLAST database (Basic Local Alignment Search Tool) at NCBI (National Centre for Biotechnology Information).

RESULTS

Cyst morphology, counts and germination experiments

Cedrino Lake

One cyst morphotype was identified from Cedrino Lake sediments. Most of these cysts maintained the external theca (Fig. 2a), while some of them lost the theca (Fig. 2b). Cysts were 29.8 µm long and 28.7 µm wide (n=9), the content was grainy with yellowish to reddish accumulation bodies. Cyst abundances were 2807 cysts gr^{-1} and 792 cysts gr^{-1} for the thecate and non-thecate type respectively. Germination experiments both on single cysts and filtered sediments failed.
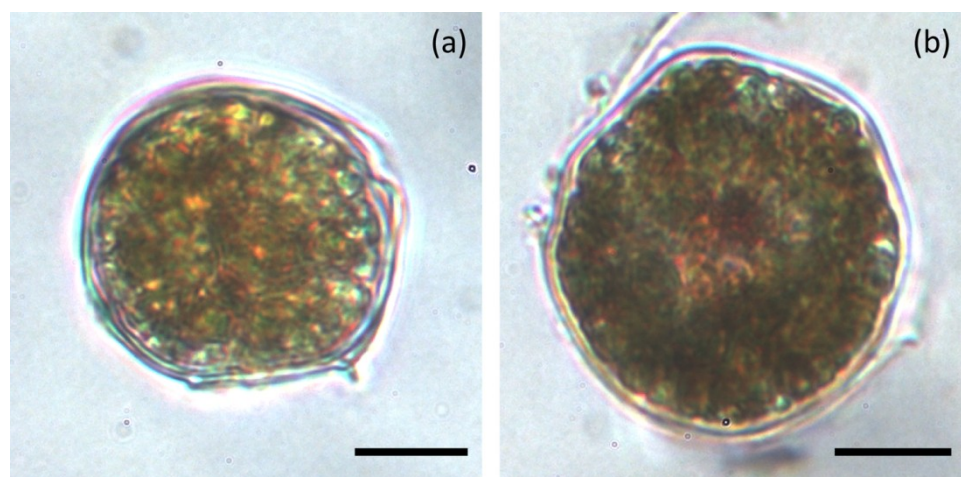
Figure 2 Cysts from Cedrino Lake, with the theca (a), and without the theca (b). Scale bars: 10 µm.

Sos Canales Lake

Three morphotypes were identified from Sos Canales Lake sediments. The first morphotype (Fig. 3a) was rounded in shape (mean diameter: 51.5 µm; n=6), with a double wall. In numerous cysts the double wall was broken and/or lost (Fig. 3b). The

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cyst content was granular and greyish in colour, with a red accumulation body. The second morphotype (Fig. 3c) resembled the first in shape and content but was smaller (mean diameter: 35 µm; n=4), and always without double wall. The third morphotype showed a peridinioid shape (Fig. 3d) measuring 30 µm in length and 29 µm in width (n=1). The content was granular and greenish in colour, with an opaque reddish accumulation body. Cyst abundances were 513 cysts gr^{-1}, 96 cysts gr^{-1} and 27 cysts gr^{-1} for the first, second and third morphotype respectively. Germination experiments were successful for the filtered sediments.
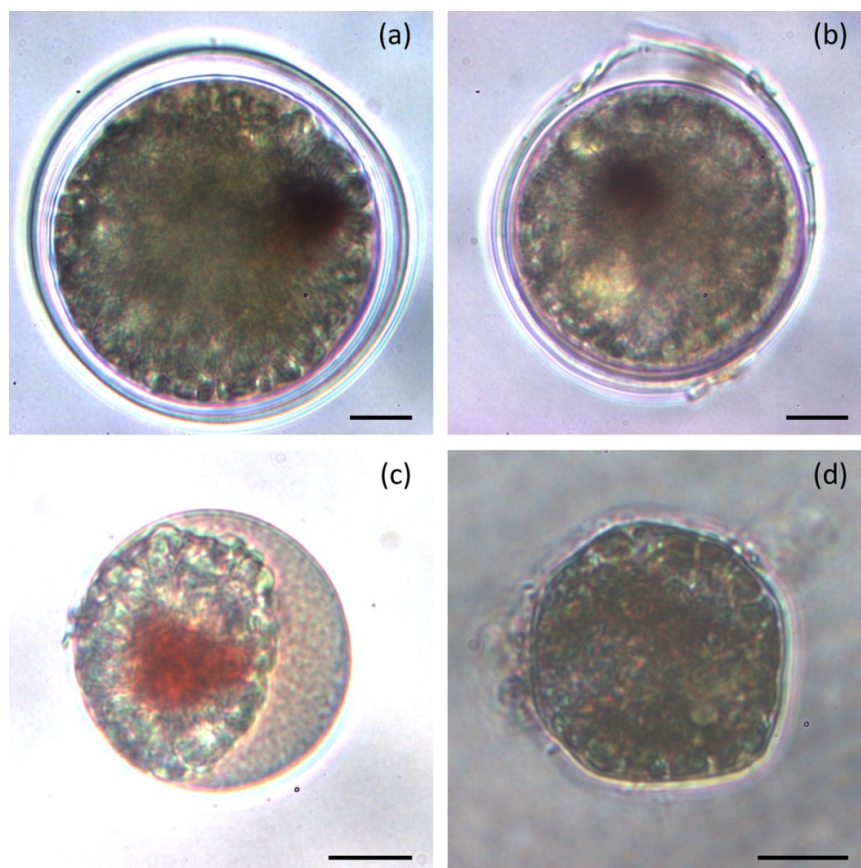
Figure 3 Cysts from Sos Canales Lake, the first morphotype (a), first morphotype loosing the double wall (b), the second morphotype (c), and the third morphotype (d). Scale bars: 10 µm.

Cell morphology

<u>Cedrino Lake</u>

The cells were biconical and subcircular in apical view, 25-42.5 µm long and 23.8-35 µm wide (n=20). Epitheca was conical and hypotheca was from conical to rounded,

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adorned by numerous spines (Fig. 4a-e). Theca ornamentation consisted in numerous scattered pores, though arranged in regular rows at the upper and lower cingular borders.
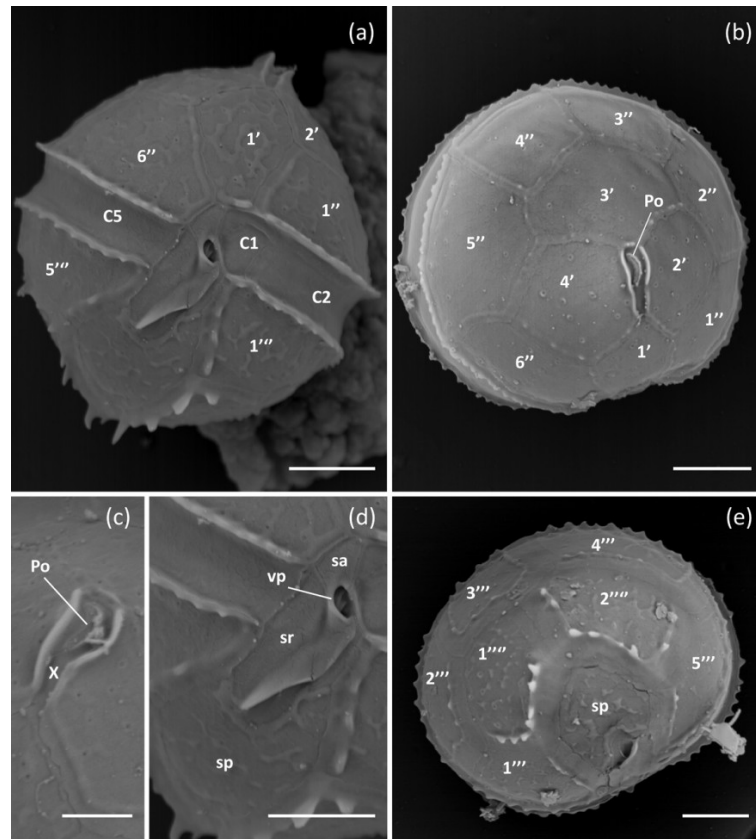
Figure 4 Scanning electron micrographs of the armoured dinoflagellate from Cedrino Lake, ventral view (a), apical view (b), particular of the APC plate (c), sulcal plates (d), and antapical view (e). Scale bars: 5 μm.

The plate formula was Po, X, 4', 6'', 5C, 4S, 5''' and 2''''. The apical pore complex (APC) was fairly in the centre of the epitheca, showing two protruded rims on its sides (Fig. 4b-c). The pore plate (Po) and the canal plate (X) formed the APC. Po was comma-shaped, 1.8 μm long (n=5), with the upper edge oriented towards the right side of the cell. The X canal was rectangular and 1.98 μm long (n=5). The first apical plate (1') was pentagonal and in contact with APC, the fifth cingular plate (C5) and the sulcal anterior plate (sa). The 3' plate was six sided (Fig. 4b). Intercalary plates were absent (Fig. 4b). The precingular plates (from 1'' to 6'') were four or five sided. The cingulum was slightly displaced, 1 time its width and provided of thorny rims more or less protruded.

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There were 5 cingular plates, of which C5 was the narrowest. Sulcus seemed to be formed by four plates. The sulcal anterior plate (sa) was the smallest, whereas the sulcal posterior plate (sp) the largest. The right sulcal plate (sr) was elongated and provided of a sulcal list. The sulcal list slightly covered the ventral pore (vp) and significantly covered the left sulcal plate (Fig. 4d). The two antapical plates (1'''' and 2'''') were rather centred with respect to the 5 five postcingular plates (1'''-5''') and the posterior sulcal plates (Fig. 4e). An ordered series of spines was present in the antapical plates, however, not coinciding with the edge of the plates but moved toward the center. This type of ornamentation, although less bulging, was also present in the post cingular plates (Fig. 4e). The plate arrangement placed this dinoflagellate in the genus *Peridiniopsis*.

<u>Sos Canales Lake</u>

The cells showed the typical gymnodinioid shape, with epitheca and hypotheca almost equal in size (Fig. 5a). Cells were often surrounded by abundant mucus and showed a wide variability in size (Fig. 5b), varying from 26.3 µm to 45 µm in length and from 25 µm to 40 µm in width (n=20).
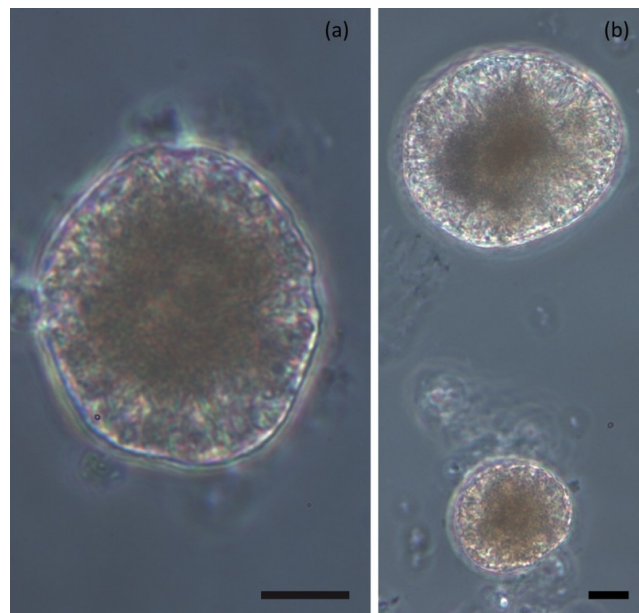
Figure 5 Light photomicrographs micrographs of *Gymnodinium uberrimum.* Ventral view of a Lugol fixed cell (a), and cells showing the wide variation in size (b). Scale bars: 10 µm.

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Genetic analysis

<u>Cedrino Lake</u>

None of the analyses performed gave positive results, on both fixed vegetative cells and alive cysts. Moreover, cyst germination experiments failed, with any possibility to isolate alive vegetative cells

<u>Sos Canales Lake</u>

None of the analyses performed gave positive results, on both fixed vegetative cells and alive cysts. Two LSU rDNA sequences (D1/D2 regions, 730 base pairs) were obtained from two single cells isolated by cyst germination from filtered sediments. BLAST analysis showed a low identity values (84%) with *Gymnodinium aureolum* (E.M.Hulburt) Gert Hansen sequences reported in GenBank.

DISCUSSION

Freshwater dinoflagellates are less studied than marine dinoflagellates; there are more than 1700 described species of free-living marine dinoflagellates and only about 220 described freshwater species. Moreover, many of the known freshwater dinoflagellates have been described before electron microscopy and molecular genetic methods became available, thus identifying freshwater dinoflagellates from different groups and geographic locations is challenging (Annekova, 2013). Moreover, for unarmoured species there is an absence of a satisfactory taxonomy (Hallegraef, 1993). On the other hand, blooms of the genus *Peridiniopsis* Lemmermann and *Gymnodinium* Stein species have been documented in numerous lakes (e.g. Meyer et al., 1997; Rodriguez *et al.*, 1999; Takano *et al.*, 2008; Annekova, 2013). In Sardinian artificial lakes (Cedrino and Sos Canales lakes), species of these genera were involved in bloom events or represented the dominant species at least as biomass in the phytoplankton. Consequently, their affirmation can represent a further issue for the treatment of drinking water (Niesel *et al*., 2007).

The dinoflagellates are present in large lakes (surface area more than 500 km^2), but blooms are generally formed in small natural and man-made water bodies (Pollingher,

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1990). In most cases, the blooms are formed by *Ceratium* spp. and less frequently by *Peridinium* spp. and *Peridiniopsis* spp..

In Sardinian artificial lakes dinoflagellate blooms have been detected since the seventies and were mainly due to intense growths of *Ceratium hirundinella* (Müller) Dujaedin (Sechi, 1983; Sechi *et al.*, 1986). Only recently, blooms due to others dinoflagellates have been observed, such as in Cedrino Lake, where the water appeared reddish during the bloom occurred in February 2012 (cell density up to 25 x 10^6 cells l^{-1}).

Turbolence is necessary for the initiation of bloom (resuspension of cysts) (Pollingher and Hickel, 1991). In deep Mediterranean artificial lakes, such as Cedrino and Sos Canales, water mixing due to homeothermy generally occurs at the ending of autumn and beginning of winter, whereas external inputs from watersheds are relevant during the months from late autumn to late spring (filling phase). Consequently, these are the periods during which resuspension phenomena can mainly occur, probably promoting also the intense growth observed in the Cedrino Lake.

Based on tabulation features the dinoflagellate from Cedrino Lake can be assigned to the *Peridiniopsis* genus. This genus is quite variable and heterogeneous with regard to the number and position of ephitecal plates and several groups (or sections) were distinguished within the genus (Popovsky and Pfiester, 1990). *Peridiniopsis* from Cedrino Lake can be classified into the 'penardii' section due to the presence of four apical and six subcingular plates, and no intercalary plates. The symmetry of epithecal plates and the 3' plate hexangular (Ten-Hage *et al.*, 2007) suggested the possible belonging to *P. penardii* or *P. durandii*. The discriminating character between these two species is reported to be the shape of the cells (oval: *P. penardii*, rhombic: *P. durandii*; Ten-Hage *et al.*, 2007). However, the cells of *P. penardii* are not reported to be oval in other reports on this species (Hansen and Flaim, 2007). The majority of the cysts from Cedrino Lake appeared from oval to irregular in shape and with the theca surrounding. These observations do not coincide with those reported for *P. penardii* in culture by Sako *et al.* (1987). However, the possibility of morphological variations of cyst morphotypes within the same species cannot be excluded (e.g. *Bysmatrum*

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species, Satta *et al.*, 2013a, 2013b), and needs further investigations. Therefore further studies are necessary for the correct identification of the species from Cedrino Lake. Moreover, the failure of biomolecular approaches on boh fixed vegetative cells and cysts suggests their application on alive vegetative cells, obtained from both cyst germination or directly collected in the field.

On the contrary, molecular analysis on vegetative cells obtained from cysts germination from Sos Canales Lake showed their belonging to the *Gymnodinium* genus. *Gymnodinium* species from freshwaters are poorly studied through the modern biological methods (Annekova, 2013), and few sequences have been deposited in GenBank. Our sequences as well as the only other freshwater sequences reported for *G. baicalense* (not yet deposited in Genbank), are more similar to those of *G. aureolum* than others *Gymnodinium* species. Resting cysts produced by *G. uberrimum* (reported as *G. limneticum*) were described in 1935 (Wołoszyńska, 1935 in Mertens *et al.*, 2012). The biggest cyst morphotype from Sos Canales Lake reflects this description, indicating however the likely not-gelatinous origin of the second coating of cysts. The second cyst morphotype is a very interesting discovery, resulting morphologically similar to the former, but considerably smaller. This finding may emphasize the possibility of the presence of two species, or indicate the presence of the same species that is capable of producing large and small cysts, such as large and small vegetative cells. So, only on the base of morphological data, we can affirm that one of the possible species present in Sos Canales Lake is *G. uberrimum*. Our future purposes will be to obtain more isolates of these species and analyze different genetic markers in order to its characterization.

In any case, our study emphasizes the importance to investigate on the different phases of the dinoflagellate life cycle and the necessity of an approach with multiple analysis techniques. In fact, many dinoflagellate species show a complex life cycle, which involves the production of resting stages with a prolonged dormancy period (Dale, 1983). Resting cysts may function for survival during unfavourable environmental conditions, reproduction, genetic recombination, and dispersal (Dale, 1983). Studies on cysts have increased considerably in recent years, especially in

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marine environments, whereas freshwater dinoflagellate cysts have received much less attention (Mertens *et al.*, 2012 and references therein). For this reason, our study on the lake sediments is an important source of information for the Mediterranean basin. Our results underline a significant presence of cysts in the sediments of the two investigated lakes and the presence of different morphotypes, although the attribution of morphotypes to species was not currently possible.

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8. GENERAL CONCLUSIONS

This Ph.D. thesis gives novel contributions to the ecological knowledge of potential harmful algal species (HAS) in Sardinian aquatic ecosystems. New molecular data on different taxa and from different kinds of environments were provided, supporting and/or clarifying the data obtained with classical research approaches. The used techniques were developed relatively recently. We applied the PCR (with 28S and ITS-5.8S rDNA as genetic markers) on fixed samples, on cultures obtained from phytoplankton cells or germination of resting cysts, on single cells from natural samples and germinated from cysts. These techniques allowed to obtain insights on specific issues, identified as study cases.

In particular, the considered studied cases were:

- on the biodiversity of the potential toxic dinoflagellate genus *Alexandrium*, in Sardinian marine and transitional environments. Species belonging to this genus, in the last decade, have caused eight harmful algal blooms in Sardinian mussel farms (Giacobbe *et al.*, 2003a; Lugliè *et al.*, 2003a, 2003b, 2004, 2011), causing alarm for human health and economic losses. In this thesis, new data on the presence and toxicity of several *Alexandrium* species (namely, *A. minutum*, *A. tamarense* and *A. catenella*) along the Sardinian coasts were obtained analysing new isolates from Olbia, Alghero and Santa Giusta Lagoon (chapter Ia). New isolates of *A. catenella*, *A. tamarense* and *A. minutum*, from cysts germination or from vegetative cells growth from different marine coastal ecosystems, have been analysed (Chapter Ia). *A. minutum* had been involved in many toxic blooms in Sardinian shellfish farming areas during the last decade. The new strains considered in this thesis were obtained from vegetative cells isolation from samples collected in the Gulf of Olbia, the main shellfish farming site in Sardinia. The obtained results give useful support to the new emerging theories about the *A. minutum*-group morphology and phylogeny on a global scale. *A. tamarense* strain derived from a cyst germination isolated from the sediments collected in the Gulf of Alghero. Until 2008, all Mediterranean strains were grouped in a single lineage (on the base of nucleotide sequences of 5.8S gene and Internal

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Transcribed Spacer regions of the rRNA operon), considered non-toxic. Therefore, this species was considered harmless in the Mediterranean basin for a long time. Penna *et al.* (2008) for the first time reported the detection of toxic strains from Porto Torres harbour. Consequently, the new *A. tamarense* strain analyzed in this study allowed deepening knowledge about the presence of toxic and non-toxic strains in the Sardinian coast. Also *A. catenella* strain was obtained from the germination of a cyst taken from Santa Giusta Lagoon's sediment. This is the first report of this species in a Sardinian lagoon. Its detection in an area out of the normal monitoring for toxic algae in shellfish farming areas emphasizes the potential risk of toxic events linked to the harvesting of wild shellfishes. This result was a part of a wider study on dinoflagellate cysts composition in Mediterranean coastal lagoons and contributed to increase the global knowledge on cyst producing dinoflagellates in transitional ecosystems (chapter Ib);

- on the identification of potential harmful raphidophytes species present during past bloom events coinciding with fish kills in Santa Giusta Lagoon. The retrospective analysis on fixed archived samples with Lugol's iodine old up to twenty years, allowed to assess the presence of *C. subsalsa* in coincidence of these harmful events (chapter II). On-going studies, integrating long-term ecological data available for this lagoon (which is part of the LTER-Italy network), will offer further detailed scenarios on the environmental conditions accompanying these events. Further, they will offer the opportunity to model *Chattonella* blooms to improve the capabilities to predict and/or to mitigate them, with a better management of the affected environments;

- on the presence and distribution of potential HAS in the poorly studied beach environments, which represent areas of high economic interest due to tourism. Also in this case, molecular methods supported species identification, and were decisive for new reports of species whose geographical distribution is not yet well known. They also have permitted to increase information on species distribution. In particular, the more frequent and/or abundant species were *Alexandrium taylorii*, *Barrufeta bravensis*, *Gymnodinium instriatum* and *Ostreopsis* cf. *ovata* (chapter III). These species

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can affect water quality and human health, with negative impacts on the recreational function of the beaches, requiring particular attention in their touristic exploitation;

- finally, this thesis reports the preliminary results of a study on dinoflagellates blooms in Mediterranean reservoirs, considering the Sardinian artificial lakes, Sos Canales and Cedrino (chapter IV). In fact, even if Cyanobacteria are considered the most harmful group in freshwater environments, other phytoplankton component can affect water quality, conferring for example unpleasant odours and tastes, and increasing water treatment costs. Numerous difficulties have been met in the application of molecular methods to Lugol's iodine fixed samples, collected during blooms and in the germination of cysts isolated from the sediments. SEM analysis allowed identify as a *Peridiniopsis* species (likely, *P. penardii*) the armoured red tide dinoflagellate of the Cedrino Lake, while biomolecular analyses have determined as a *Gymonodium* species (most probably *G. uberrimum*, as supposed on the morphological features) the naked dinoflagellate from Sos Canales Lake. The absence of registered sequences for freshwater *Gymnodinium* in Genbank not allowed confirm species attribution.

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Annex 1

Atti dell'Assemblea generale e del Symposium I mari delle isole

(Resèaux d'Excellence des Territoires Insulaires – RETI)

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Aspetti dell'ecologia dei sistemi acquatici della Sardegna e loro principali problematiche.

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ABSTRACT Eutrophication and Harmful Algal Blooms (HABs) are discussed in this paper as two of the main environmental issues of aquatic ecosystems of Sardinia. Eutrophication is caused by increased nutrient concentrations (largely nitrogen and phosphorous) in water bodies, which stimulate algal growth and lead to degraded water quality and impairments of aquatic ecosystems. It is the major issue of Sardinian reservoirs and lagoons. The HABs, which are often linked to eutrophication, are events that produce damage to the environment and/or risk for the economic human interests. Different typologies of HABs are signaled along Sardinian coast.

RIASSUNTO In questo contributo si analizzano due delle principali problematiche ambientali degli ecosistemi acquatici della Sardegna: l'eutrofizzazione e le fioriture algali nocive. L'eutrofizzazione è il processo causato dall'incremento dei nutrienti (per lo più fosforo ed azoto) nei corpi idrici, che stimola la crescita vegetale e modifica la comunità, portando ad una degradazione della qualità ambientale e ad una perdita del valore degli ecosistemi. Questa è la principale problematica dei laghi artificiali e delle lagune sarde. Le fioriture algali nocive, spesso collegate all'eutrofizzazione, sono invece l'insieme di eventi che producono danni all'ambiente e/o agli interessi dell'uomo. Differenti tipologie di fioriture nocive sono segnalate anche lungo le coste sarde.

Keywords: Eutrophication, harmful algal blooms, ecosystem functions, reservoirs, lagoons.

Parole chiave: Eutrofizzazione, fioriture algali nocive, funzioni ecosistemiche, laghi artificiali, lagune.

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INTRODUZIONE

La Sardegna è la regione italiana con la maggiore estensione della linea di costa (circa 1900 km), rappresentando poco più di un quarto delle coste italiane, ed è una delle mete turistiche marine più ambite nei mesi estivi (Osservatorio Nazionale per il Turismo, 2013), grazie alla cultura e alla ricchezza delle bellezze naturali (Hospers, 2003). Della fascia marina costiera fanno parte anche le lagune, di cui la Sardegna è particolarmente ricca (Abbiati *et al.*, 2010), e che rappresentano aree di rilevante interesse naturalistico e produttivo.

La Sardegna è anche la regione che, nel panorama nazionale, ospita il maggior numero di dighe e la maggior quantità di acqua invasata, per un volume complessivo pari a circa 2×10^9 m^3 d'acqua. I laghi artificiali costituiscono la principale fonte di approvvigionamento idrico per tutte le utilizzazioni, da quella potabile, all'agricoltura, all'allevamento, all'industria, alla produzione di energia elettrica. L'unico lago naturale, il Lago di Baratz, nella Nurra nordoccidentale, è caratterizzato da una specificità dei caratteri ecologici e naturalistici, che lo rendono pressoché unico anche nel contesto nazionale.

Turismo, pesca, approvvigionamento idrico sono solo una piccola parte delle opportunità d'uso dei servizi e dei beni derivanti dagli ecosistemi acquatici della nostra regione. Per definizione, i servizi ed i beni ecosistemici sono i benefici, diretti ed indiretti, che l'uomo ottiene dagli ecosistemi per soddisfare le proprie necessità. Servizi e beni sono riconducibili a quattro categorie di funzioni ecosistemiche (habitat, production, regulating, information; De Groot *et al.*, 2002), la cui valutazione e mantenimento su definiti livelli di equilibrio rappresenta una condizione fondamentale per continuare a beneficiarne (Peterson *et al.*, 2009).

Il loro sfruttamento intensivo può limitare la disponibilità di servizi e beni a discapito non solo dell'ambiente, ma delle popolazioni stesse, interferendo negativamente sulle condizioni sociali ed economiche. Esiste quindi l'esigenza di comprendere meglio sia il funzionamento degli ecosistemi attraverso l'analisi delle strutture e lo studio dei

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processi, sia come le alterazioni delle funzioni ecosistemiche si riflettano sui servizi e beni forniti (Peterson *et al.*, 2009).

Con questo contributo si riporta il quadro generale di due delle principali problematiche dei sistemi acquatici della Sardegna: l'eutrofizzazione e le fioriture algali nocive.

L'EUTROFIZZAZIONE

L'apporto di nutrienti in un ambiente acquatico è un processo naturale, indispensabile per il sostentamento degli organismi viventi, per il flusso dell'energia e la ciclizzazione della materia, cioè per il funzionamento dell'ecosistema. Lo stato trofico di un ecosistema acquatico è determinato dalla concentrazione di nutrienti nelle acque, derivanti dalle deposizioni atmosferiche, dalla ciclizzazione interna e dagli scambi con il sedimento, e, soprattutto, dal bacino imbrifero. Quando le concentrazioni dei nutrienti, in particolare dell'azoto e del fosforo, aumentano, spesso per l'incremento degli inputs dal bacino imbrifero, si verificano nel tempo dei profondi cambiamenti, corrispondenti al processo di eutrofizzazione. La diretta conseguenza dell'aumentata disponibilità di nutrienti è l'incremento della produttività, l'accumulo di biomassa algale, il cambiamento delle comunità, del flusso dell'energia e della ciclizzazione dei materiali (Wassmann e Olli, 2005). Le attività antropiche accelerano il processo di eutrofizzazione: se allo sviluppo socioeconomico, con l'intensificazione di attività quali il disboscamento, l'uso di concimi, l'allevamento zootecnico, le attività industriali nei bacini imbriferi, non si abbina un'attenta gestione dei nutrienti in ingresso nei sistemi acquatici (spesso in forma di reflui), si instaura il così detto processo di eutrofizzazione culturale (Salmaso *et al.*, 2006). Un concetto importante è quello dell'interconnessione dei sistemi acquatici e della loro disposizione a cascata, del *continuum* tra acque interne, quelle di transizione e sistemi marini costieri, per cui le problematiche a monte, come l'eutrofizzazione dei laghi, hanno effetti che si propagano a valle, per esempio nelle lagune e negli ambienti costieri. In particolare, nel contesto sardo, l'eutrofizzazione, è il problema ambientale più grave e diffuso nei laghi artificiali (Fig. 1a). I dati raccolti negli anni, hanno evidenziato che l'eutrofia è accompagnata da un

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ruolo dominante dei cianobatteri all'interno del fitoplancton (Marchetto *et al.*, 2009). La loro abbondanza diventa maggiore nel semestre caldo anche se, talvolta, sono presenti in maniera rilevante anche nel resto dell'anno, compresi i mesi invernali più freddi. Considerando che in Sardegna l'acqua potabile deriva per il 90% dai laghi artificiali e che questi sono in maggioranza eutrofici e dominati dai cianobatteri, il rischio di esposizione a sostanze ossiche prodotte dai cianobatteri (le cianotossine), è elevato. A cascata, l'eutrofizzazione interessa anche la maggior parte delle lagune sarde (Fig. 1b), i cui equilibri ecosistemici dipendono oltre che dalle relazioni con il bacino imbrifero, dagli scambi con l'area marina antistante e dalle modificazioni subite nel secolo scorso. L'interesse economico per questi ecosistemi (prevalentemente per la pesca) si è manifestato con interventi di tipo ingegneristico, per il riassetto delle relazioni con il mare. È emblematico il caso del distretto dei sistemi lagunari dell'oristanese, dove gli interventi hanno radicalmente modificato la fisionomia e l'ecologia delle lagune e del paesaggio complessivo (es., lagune di S'Ena Arrubia e di Santa Giusta).

LE FIORITURE ALGALI NOCIVE: HARMFUL ALGAL BLOOMS.

Il fitoplancton è composto da microalghe e cianobatteri ed è responsabile del 45% della produzione primaria del pianeta. Il fitoplancton costituisce la base per lo sviluppo dei successivi livelli trofici ed è quindi fondamentale per le produzioni animali, cioè per quelle che l'uomo maggiormente sfrutta, sia in ambiente naturale che negli allevamenti. Negli ultimi decenni sono diventate più frequenti e diffuse le segnalazioni di eventi in cui le microalghe hanno effetti nocivi sugli interessi dell'uomo e sugli ecosistemi (Masò e Garcés,2006 e riferimenti all'interno). Questi eventi sono conosciuti come Harmful Algal Blooms (HABs). Le cause dell'aumento possono essere diverse e tra queste si ipotizza che l'eutrofizzazione abbia una notevole importanza, così come gli impatti antropici sulla linea di costa, con modificazioni dell'idrodinamismo costiero, per esempio con la costruzione di porti o di sistemi di protezione nelle spiagge (Masò e Garcés, 2006 e riferimenti all'interno). Nell'incremento degli HABs appare rilevante anche l'introduzione di nuove specie in

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un'area geografica, per esempio con le acque di zavorra o con l'acquacoltura, con un ruolo chiave giocato dalle forme di resistenza delle specie nocive (Garcés *et al.*, 2002).

La tendenza all'incremento delle segnalazioni di specie microalgali nocive (Harmful Algal Species), capaci di determinare HABs, a livello regionale è stata pressoché parallela alla dinamica riscontrata a livello globale (Lugliè *et al.*, 2011).

Negli ambienti marini costieri sardi, i principali impatti delle proliferazioni algali nocive, sono stati (Fig. 2):

• le discolorazioni dell'acqua, conosciute più in generale come "red tide", che possono avere importanti effetti sia sull'estetica del paesaggio, quindi sul turismo e l'uso ricreativo, che sul funzionamento degli ecosistemi; • le morie, sia di pesci che di altre componenti delle comunità biotiche, causate dalle elevate biomasse prodotte dalle microalghe o dall'azione di tossine o con altre modalità, con conseguenze sulle produzioni, sulla biodiversità e sul funzionamento degli ecosistemi;

• l'allarme per la salute dell'uomo, per il possibile impatto di tossine microalgali attraverso l'aerosol o per contatto diretto in aree ad elevata fruizione turistica;

• il blocco della raccolta e la commercializzazione di molluschi bivalvi e l'allarme per la salute dell'uomo, per l'accumulo di tossine microalgali nei tessuti dei molluschi oltre le soglie consentite. Le tossine trasmesse all'uomo con l'alimentazione possono infatti provocare sindromi anche mortali.

Per quanto riguarda quest'ultimo impatto, le specie che negli anni hanno generato le maggiori problematiche in Sardegna appartengono ai dinoflagellati, con i generi *Alexandrium* (*A.catenella* (Whendon e Kofoid) Balech e *A. minutum* Halim) e *Dinophysis* (*D. caudata* Saville-Kent, *D. sacculus* Stein, *D. fortii* Pavillard) (Lugliè *et al.*, 2011). Sempre ai dinoflagellati appartengono anche le specie che hanno generato i più gravi eventi di discolorazione in ambiente di spiaggia (in particolare, *Alexandrium taylorii* Balech) e di potenziale tossicità per contatto diretto (*Ostreopsis ovata* Fukuyo). Le morie di pesci e di altre componenti delle comunità biologiche si sono invece verificate più frequentemente negli ambienti lagunari. L'accertamento della relazione

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di causa-effetto tra la presenza di specie microalgali potenzialmente nocive e il verificarsi delle morie è un percorso complesso, che richiede il coinvolgimento di diverse competenze. Spesso non è stato possibile chiarire definitivamente il ruolo effettivamente svolto dalle microalghe a causa del coinvolgimento tardivo della ricerca scientifica, avvenuto praticamente quando gli eventi, con tutte le conseguenze economiche, sociali ed ecologiche associate, si erano già conclusi (es., Pulina *et al.*, 2012). Per questo tipo d'impatto, accanto al possibile coinvolgimento di specie di dinoflagellati dei generi *Prorocentrum* e *Amphidinium*, un ruolo di particolare rilevanza è stato evidenziato, sin dagli anni '90, per specie del genere *Chattonella* (Raphidophyceae). La determinazione di queste specie è particolarmente problematica, soprattutto nei campioni fissati. Le cellule, prive di un rivestimento rigido, per l'azione dei fissativi perdono la loro forma e morfologia cellulare, fondamentali per il riconoscimento al microscopio. Solo grazie all'applicazione di tecniche molecolari è stato possibile determinare le specie di *Chattonella* presenti durante le morie (Stacca *et al.*, sottomesso). Infine, nei laghi artificiali sardi, le fioriture di cianobatteri, sin dagli anni '80, appaiono direttamente coinvolte in eventi nocivi, sia per le morie di pesci che per i possibili effetti sulla salute dell'uomo per la produzione di cianotossine (Messineo *et al.*, 2009 e riferimenti all'interno). I dati più recenti permettono di affermare che i blooms di ciano batteri (CyanoHABs) interessano i 2/3 dei laghi sardi e che esiste uno stretto collegamento tra l'abbondanza di cianobatteri, le cianotossine (microcistine) e lo stato trofico (Mariani *et al.*, accettato).

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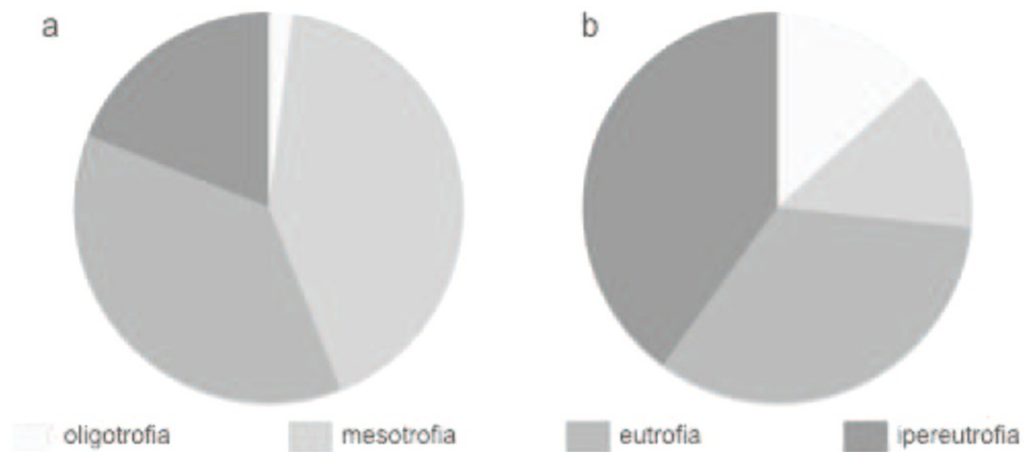
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Figura 1 – Classificazione trofica di 36 laghi artificiali (a) e di 15 lagune (b) della Sardegna.
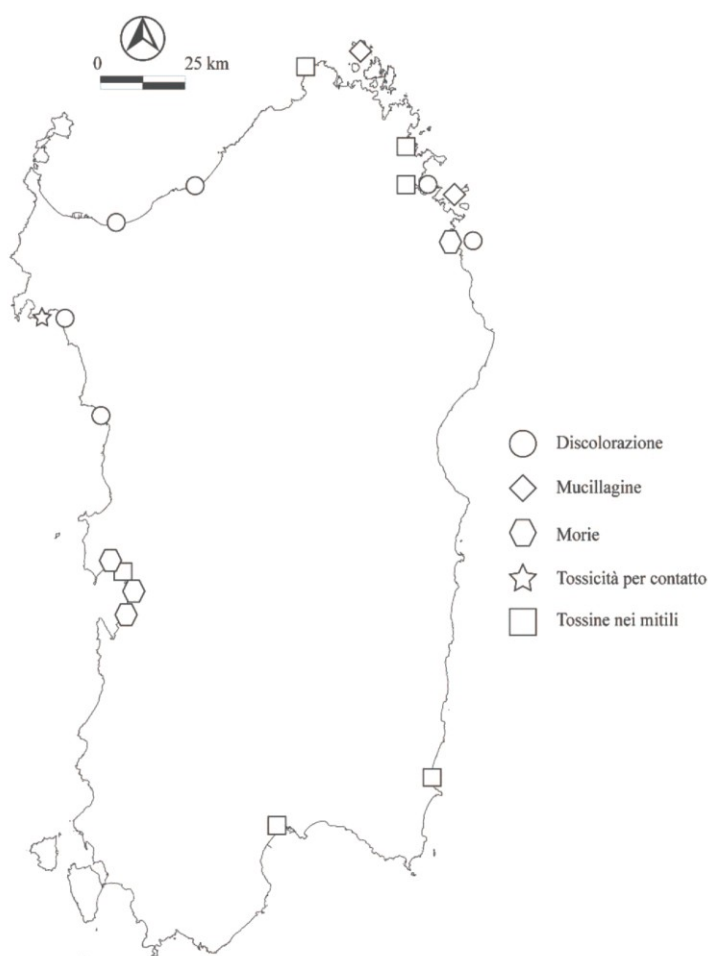

Figura 2 – Impatti documentati di HABs negli ambienti costieri della Sardegna.