UNIVERSITÀ DEGLI STUDI DI URBINO CARLO BO

Dipartimento di Scienze Pure e Applicate

Corso di Dottorato di Ricerca in Scienze di Base e Applicazioni

XXIX CICLO

High Resolution Melting: un metodo innovativo per l'identificazione molecolare di diatomee potenzialmente tossiche appartenenti al genere *Pseudo-nitzschia*

Settore Scientifico Disciplinare: BIO 07

Relatore:

Chiar.ma Prof.ssa Antonella Penna

Correlatore:

Dott.ssa Silvia Casabianca

Dottorando:

Dott.ssa Laura Pugliese

ANNO ACCADEMICO 2015 - 2016

Indice

Introduzione

La mia attività di dottorato di ricerca si è concentrata sull'individuazione di nuovi metodi di studio del fitoplancton; sull'applicazione di metodi molecolari per l'identificazione e la quantificazione di specie algali tossiche in campioni ambientali e sui relativi studi filogenetici.

Nel primo capitolo della presente tesi PhD, vengono descritte le diatomee da un punto di vista morfologico e tassonomico, ma anche da un punto di vista di ecologia quindi la loro distribuzione geografica, la presenza di fioriture algali nocive (HAB) nelle aree costiere e le problematiche per la salute umana legate alla produzione di tossine.

Nel secondo capitolo vengono descrittivi i metodi molecolari normalmente utilizzati per identificare, quantificare e studiare questi microrganismi nocivi nei campioni ambientali, fino a tecniche più innovative come il saggio di high resolution melting (HRM) messo a punto durante questo percorso di studio.

Il capitolo successivo riporta l'articolo, già sottomesso alla rivista scientifica Scientific report-Nature, dal titolo: "*A high resolution melting method for the molecular identification of the potentially toxic diatom Pseudo-nitzschia spp. in the Mediterranean Sea".* L'articolo descrive il saggio HRM messo a punto per l'identificazione di diatomee del genere *Pseudo-nitzschia* in campioni monoclonali isolati da acque costiere, prelevate durante le attività di monitoraggio eseguite dal laboratorio di biologia ambientale di Pesaro, con cadenza mensile nel corso del secondo anno di dottorato. Si è potuto così studiare la diversità genetica interspecie di *Pseudo-nitzschia* in colture monoclonali ottenuti da campioni ambientali raccolti lungo le coste pesaresi.

Nel quarto capitolo vengono introdotte a livello generale le dinoflagellate, la loro capacità di formare delle cisti e le problematiche per la salute umana dovute alla produzione di tossine da parte di questi microrganismi.

I due capitoli successivi presentano due progetti internazionali seguiti nel corso degli ultimi due anni di dottorato, per i quali è ancora in corso la stesura degli articoli scientifici.

Nel quinto capitolo, non potendo includere l'articolo scientifico in quanto ancora in fase di stesura, è stato allegato del materiale da cui è possibile evincere lo scopo generale del progetto M3-HABs (The Project Risk Monitoring, Modeling and Mitigation of Benthic Harmful Algal Blooms), finanziato dall'UE (nell'ambito del programma ENPI-CBCMED) per prevenire e ridurre i rischi legati alla dinoflagellata del genere *Ostreopsis*. Scopo generale del progetto è stato quello di sviluppare una strategia globale e comune per il monitoraggio di microalghe tossiche bentoniche, in particolare di *Ostreopsis* spp., dinoflagellate bentoniche responsabili di eventi di fioritura tossiche.

Nel sesto capitolo, non potendo includere l'articolo scientifico in quanto ancora in fase di stesura, viene riportata la relazione finale del lavoro svolto all'interno del progetto BALMAS (Ballast Water Management Plan and Strategy). Il principale obiettivo del progetto BALMAS è stato quello di stabilire un sistema comune di controllo e di gestione delle acque di zavorra delle imbarcazioni, e dei sedimenti, al fine di evitare i numerosi rischi dovuti allo sversamento nei porti delle acque di sentina di grandi navi con conseguente introduzione e/o trasferimento di organismi acquatici nocivi e di forme patogene (HAOP - Harmful Aquatic Organism and Patogenous) per l'ambiente e per la salute umana.

CAPITOLO 1

LE DIATOMEE

1. Le Diatomee

1.1. Generalità

Le Diatomee (Regno Protista, Divisione Bacillariophyta, Classe Bacillariophyceae) sono alghe brune, unicellulari, eucariotiche, generalmente delle dimensioni di pochi µm, possono vivere isolate o formare colonie e popolare ambienti diversi sia d'acqua dolce che salata. Sono organismi eucarioti ed autotrofi e rappresentano una delle principali componenti del fitoplancton acquatico, ritrovandosi nei diversi ambienti con generi e specie differenti a seconda delle caratteristiche chimico-fisiche, idrologiche e biogeografiche.

1.1.1. Strutture e forme principali

Caratteristica peculiare delle Diatomee è la parete cellulare, detta frustulo, composta principalmente da silice amorfa idrata (SiO_2nH_2O). Il frustulo è costituito da due valve, la parte superiore (epivalva) e quella inferiore (ipovalva) che vengono definite placche valvari e presentano sulla loro superficie una serie di ornamentazioni, le strie, costituite da coste, pori ed alveoli (Figura 1).

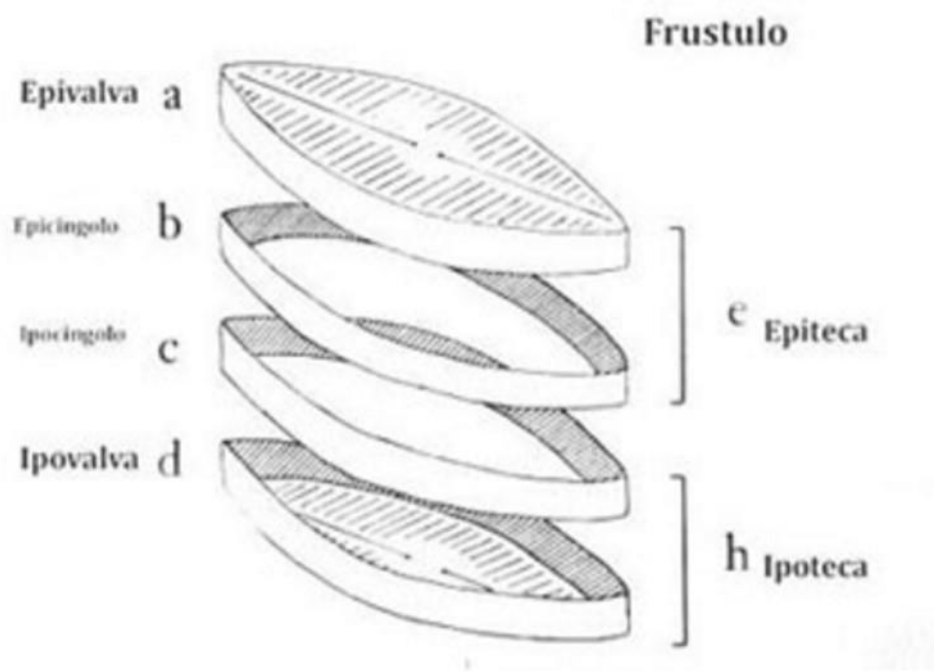


Figura 1. Rappresentazione della parete cellulare delle Diatomee: il frustulo. (http://www.isprambiente.gov.it/).

I bordi delle due valve si prolungano lateralmente a costituire le bande connettivali.

La dimensione, forma e disposizione di tutti gli elementi sopra citati risulta essere specie-specifica ed assume un notevole valore sistematico necessario per l'identificazione di ogni specie.
Al microscopio ottico è possibile quindi osservare due viste principali del frustulo: la vista valvare, che rappresenta la superficie superiore o inferiore del frustulo e la vista connettivale, in cui il frustulo viene visto di fianco, con le due valve che si sovrappongono (Figura 2).

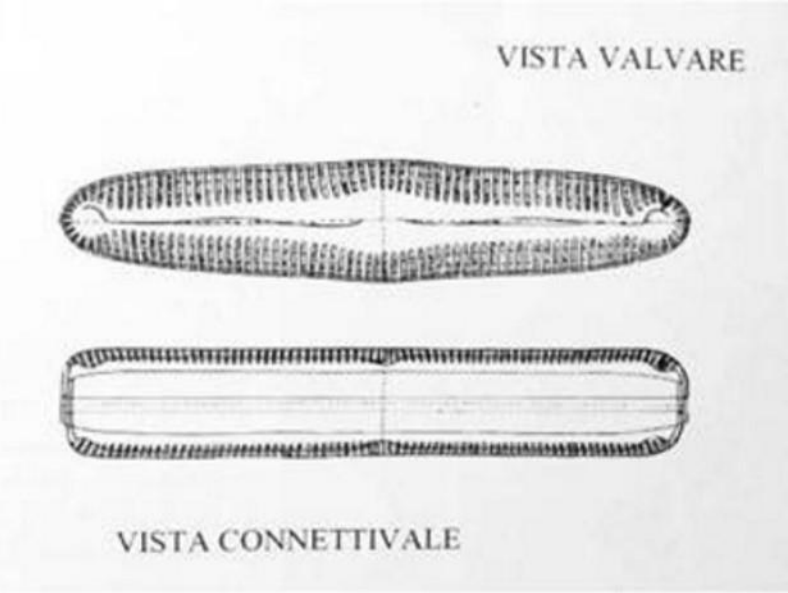


Figura 2. Frustulo al microscopio ottico (http://www.isprambiente.gov.it/).

In alcune specie di diatomee sul frustulo è presente anche il rafe, una fenditura longitudinale di struttura complessa che si interrompe a formare dei noduli centrali più o meno spessi e visibili a seconda della specie (http://www.isprambiente.gov.it/).

1.1.2. Aspetti di Ecologia

Le diatomee sono ubiquitarie e colonizzano tutti gli ambienti acquatici a qualsiasi latitudine. Rappresentano la componente principale del fitobenthos dei fiumi e sono presenti con molte specie nel fitoplacton lacustre e marino, sono i principali produttori primari (sia ambiente pelagico che bentonico) e si pensa che siano responsabili del 25% della produttività primaria globale (Jeffery & Hallegraeff 1990; Kelly et al., 1998; Deny, 2004; Kelly et al., 2006)
Le comunità di diatomee bentoniche sono usualmente distinte in base alla natura del substrato in:

- epifitiche, quando si sviluppano sulla superficie di altri vegetali, quali macroalghe, muschi e piante acquatiche;
- epipeliche, o epipsammiche quando vivono libere sul limo di fondo o sulla sabbia;

- epilitiche, se invece, aderiscono a substrati duri naturali o artificiali (per es. ciottoli, rocce, pilastri di ponti).

I fattori di crescita che influenzano la distribuzione e l'abbondanza delle diatomee planctoniche sono: parametri chimico fisici come la temperatura, il pH, la salinità e la velocità di corrente; parametri chimici quali le concentrazioni di ossigeno disciolto, silice, di sostanza organica e dei nutrienti (Round, 1981; Herbst & Blinn, 1998; Sabater, 2000). Molte specie risultano essere estremamente sensibili a questi fattori non tollerandone grandi variazioni, mentre poche sono quelle che mostrano un ampia adattabilità. La struttura delle comunità risente dunque delle condizioni ambientali proprie di ogni ecosistema e dei mutamenti dovuti ad eventi di disturbo, rendendole dei validi indicatori dello stato di qualità di un ecosistema.

Le diatomee presentano un ciclo vitale molto breve, a differenza degli altri indicatori biologici come macroinvertebrati, macrofite e pesci, rispondendo velocemente ai cambiamenti dei parametri ambientali (Rott,1991).

Sono stati sviluppati molti indici biotici basati sulla presenza, l'identificazione e la quantificazione di Diatomee per la valutazione dello stato di qualità degli ecosistemi fluviali (Descy, 1979; CEMAGREF, 1982; Leclerq & Maquet, 1987; Kelly & Whitton, 1995; Prygiel & Coste, 1999; Rott & Pipp, 1999; Kwandrans et al., 1999; Eloranta, 1999).

Elevate concentrazioni di nutrienti determinano la scomparsa di specie sensibili e la dominanza di specie più tolleranti nei corsi d'acqua e possono causare fenomeni di fioriture algali nei laghi e nelle acque marino costiere (Paerl 1988; Cloern 2001; Paerl et al., 2006).

1.2. Diatomee e rischio per la salute umana

Le fioriture algali sono processi naturali che si verificano nei laghi e negli ambienti marini (Tonolli, 1975) (Figura 3). Negli ultimi anni si è assistito ad un aumento delle fioriture algali nocive, tale fenomeno è in parte legato all'eutrofizzazione, data la stretta relazione tra la qualità dell'acqua e la salute, esse rappresentano una crescente preoccupazione per la salute umana (Anderson et al., 2002; Glibert et al., 2005; GeoHAB, 2006).

Figura 3. Tipica marea rossa in seguito a fioritura algale.

Le diatomee sono continuo oggetto di studio anche per la capacità di alcune specie planctoniche marine, la maggior parte appartenente al genere *Pseudo-nitzschia* (Figura 4), di produrre una neurotossina, l'acido domoico (Shimizu et al., 1989; Wright et al., 1989; Maranda et al., 1990; Forbes, & Denman 1991; Pan et al., 1996, Cangelosi et al., 1997; Bates et al., 1998; Vrieling et al., 1996; Amzil, 2001; Lapworth et al., 2001; Davidovich & Bates, 2002; Bargu et al., 2003; Bates et al., 2004; Lundholm et al., 2004; Lundholm et al., 2005).

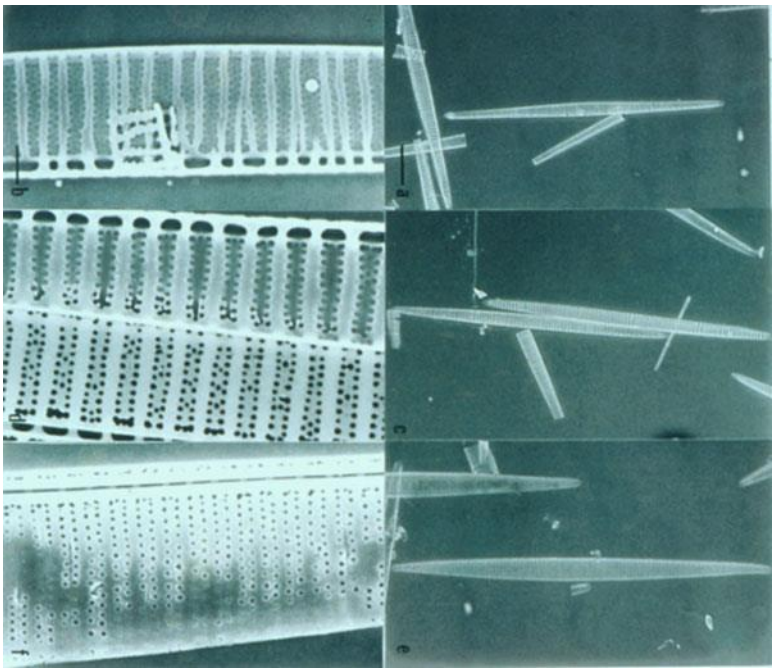


Figura 4. Immagine al SEM di Pseudo-nitzschia spp © Copyright WHOI 2007. All rights reserved.

In concomitanza con una fioritura algale di *Pseudo-nitzschia multiseries* (Hasle) nel 1987 in Canada è stata riportata un'intossicazione attribuita al consumo di mitili che coinvolse 153 persone (Wright et al., 1989).

Un'altra diatomea produttrice di acido domoico, *Pseudo-nitzschia australis*, è stata individuata lungo le coste delle California. In quest'area le tossine sono state rilevate nei molluschi e in alcuni pesci, in particolare nelle acciughe. Il consumo di questi pesci è stato ritenuto responsabile delle morie di uccelli marini osservate nella zona nello stesso periodo. Due specie bentoniche sono state segnalate per la produzione di questa tossina: *Nitzschia navisvaringica* (Lundholm and Moestrup) in acque marine e di transizione in Vietnam (Kotaki et al., 2004) e *Amphora coffeaeformis* (Agardh) (Shimizu et al., 1989; Maranda et al., 1990).

In Europa, benché non sia stato mai registrato alcun episodio di intossicazione, la presenza di acido domoico è stata riscontrata in mitili coltivati lungo la costa della Galizia e nella diatomea *Pseudo-nitzschia pungens* f. *multiseries*, raccolta nelle coste olandesi (Vrieling et al.,1996). La tossina, negli ultimi dieci anni, è stata rilevata in molluschi provenienti da molti paesi europei, in particolare nel Regno Unito, Irlanda e Francia (EFSA, 2009), determinando la chiusura degli impianti di raccolta nel Regno Unito, in Irlanda (Bogan et al., 2007a, b), in Francia (Klein et al, 2010), in Danimarca (Lundholm et al, 2005a), in Portogallo (Vale e Sampayo, 2001) e in Spagna (Fraga et al., 1998). Infatti diverse specie di *Pseudo-nitzschia* in ceppi europei si sono rivelate tossiche: *P. australis, P. calliantha, P. Galaxiae, P. multiseries, P. multistriata, P. pseudodelicatissima, P. pungens e P. seriata* (Lundholm et al., 1994; Sarno e Dahlmann, 2000; Fehling et al, 2004a, b.; Moschandreou et al., 2010).

In Italia, il problema del possibile rischio sanitario associato a questa tossina è stato sollevato quando, negli anni 1988-91, sono comparsi nell'alto e medio Adriatico aggregati mucillaginosi contenenti diatomee del genere *Nitzschia*. In realtà fu possibile escludere la presenza di acido domoico in questi aggregati. Tuttavia questa tossina rappresenta attualmente anche per l'Italia un problema sanitario. Le specie appartenenti al genere *Pseudo-nitzschia* produttrici di acido domoico, e quindi potenzialmente tossiche, hanno una distribuzione piuttosto ampia nella ecoregione mediterranea (Ade et al., 2003). In colture di *Pseudo-nitzschia multistriata* (Sarno & Dahlman, 2000) e *Pseudo-nitzschia galaxiae* (Cerino et al., 2005), originate da campioni provenienti dal Golfo di Napoli, è stata riscontrata la produzione di acido domoico, in basse concentrazioni.

Inoltre, colture di *Pseudo-nitzschia delicatissima* isolate in Mare Adriatico sono risultate positive per la presenza di acido domoico e in un caso questo raggiungeva quantità quantificabili di 0.063 fg $cell^{-1}$ (Penna et al., 2013).

1.2.1. ASP (Amnesic Shellfish Poisoning)

L'acido domoico (AD) è un aminoacido ciclico idrosolubile (Figura 5), prodotto principalmente dalle alghe marine rosse del genere *Chondria* e dalle diatomee del genere *Pseudo-nitschia*, è una molecola termostabile, analogo all'acido glutammico, in grado di attaccare dunque gli stessi recettori del Sistema Nervoso Centrale (Wright et al., 1989).

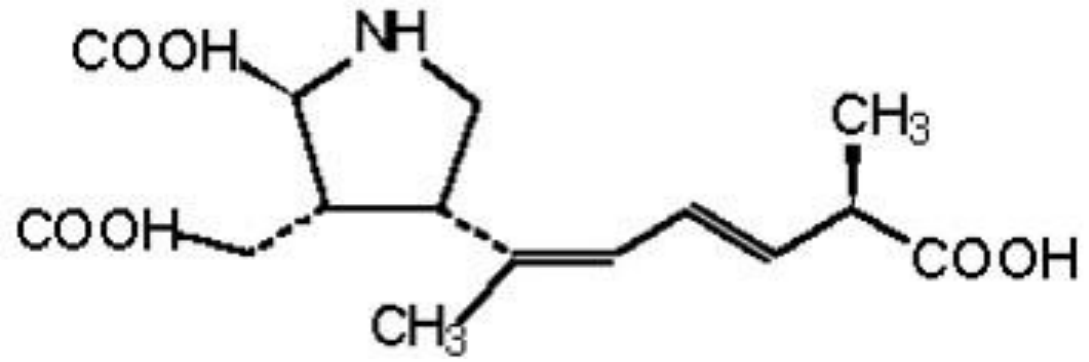


Figura 5. Struttura dell'acido domoico. http://medciclopedia.net/

L'assunzione di acido domoico tramite ingestione da mitili provoca in alcuni casi confusione e perdita di memoria, da cui il nome della sindrome che determina Amnesic Shellfish Poisoning (ASP), i cui sintomi sono di tipo gastrointestinale come vomito, diarrea, dolori addominali che si verificano nelle prime 24h e quelli di tipo neurologico quali confusione, perdita della memoria, disorientamento e nei casi più gravi coma e morte entro le 48h (Wright et al., 1989; EFSA, 2009).

L'acido domoico, l'aminoacido ritenuto responsabile di tale sindrome, è stato isolato per la prima volta dalla macroalga rossa *Chondria armata* (Daigo, K.; et al 1959) nell'ambito di studi sulle proprietà insetticida di estratti algali. Esso appartiene ad un gruppo di amminoacidi con attività eccitatoria sul SNC detti kainoidi, dal nome del capostipite, l'acido kainico, isolato dell'alga rossa *Digenea simplex* **(**Murakami, S. et al., 1953**)** ed usato in alcune regioni del sud-est asiatico per le sue proprietà antielmintiche. Attualmente, si ritiene che il principale produttore di acido domoico sia la diatomea pennata *Nitzschia pungens* f. *multiseries*, (Subba Rao, D. V. et al . 1988, Maranda, L. et al., 1989) ma anche altre specie, quali la *Amphora coffeiformis* (Shimizu, Y. et al., 1989) e la *Pseudo nitzschia australis* (Fritz, L.et al., 1992,) sono ritenute capaci di pro durre questa tossina.

In ogni caso, il contributo di certe diatomee al metabolismo secondario nelle macroalghe è soltanto speculativo, sebbene sia ben noto che la superficie di molte macroalghe è ricoperta proprio di diatomee. È stato, infatti, dimostrato che una cultura monoalgale dell'alga rossa *Palmaria palmata* (Laycock, M. V. e Bird, C. J1989) produce acido kainico a suggerire che la produzione di kainoidi è indipendente dalla specie di macroalghe.

CAPITOLO 2

METODOLOGIE MOLECOLARI INNOVATIVE PER LO STUDIO E IL MONITORAGGIO DI SPECIE HAB (HARMFUL ALGAL BLOOM)

2. Metodologie molecolari innovative per lo studio e il monitoraggio di specie HAB (Harmful Algal Bloom)

L'individuazione tempestiva delle specie algali nocive rappresenta una componente fondamentale della maggior parte dei piani di gestione delle fioriture algali potenzialmente tossiche (Harmful Algal Blooms, HABs). Se queste informazioni sono disponibili nelle prime fasi del processo di sviluppo HAB, è possibile raccomandare o intraprendere azioni idonee per ridurre al minimo gli effetti dell'evento di fioritura.

L'approccio classico per il rilevamento e la quantificazione delle specie HAB è l'osservazione diretta al microscopio ottico di campioni sul campo in vivo o di materiale conservato (Sournia 1978). Sebbene questa tecnica fornisca importante conferma visiva della presenza di una specie in un campione di acqua e generi stime ragionevolmente accurate di abbondanza cellulare, è un metodo che richiede un elevato livello di competenza nell'identificazione del fitoplancton e tempi lunghi di processamento. Per l'identificazione rapida, precisa e sensibile di specie fitoplanctoniche potenzialmente tossiche in campioni di acqua di mare nelle attività di monitoraggio di HAB sono stati quindi proposti e sviluppati numerosi approcci molecolari.

Le tecniche molecolari di rilevamento delle specie HAB prevedono l'identificazione di specifiche molecole target, come gruppi chimici situati sulla superficie cellulare e varie componenti del genoma di un organismo, queste classi di molecole vengono poi rilevate mediante anticorpi o sonde oligonucleotidiche. Ci sono due strategie attualmente utilizzate per la rilevazione di specie algali nocive con anticorpi, che coinvolgono la microscopia ad epifluorescenza e/o la citometria a flusso. In entrambi i casi, anticorpi specie-specifici che riconoscono antigeni di superficie vengono applicati a cellule intatte in combinazione con un sistema di segnalazione basate sull'uso di un fluoroforo, ottenendo un segnale fluorescente da cellule bersaglio marcate con un anticorpo che può essere rilevato con strumentazione appropriata.

L'altra classe di molecole bersaglio impiegata per la rilevazione altamente specifica di taxa HAB, mediante sonde oligonucleotidiche, sono gli acidi nucleici. In particolare, i componenti dei geni ribosomiali (rDNA) e dei loro prodotti di trascrizione, le corrispondenti molecole di RNA ribosomiale (rRNA). Le sequenze di geni ribosomiali contengono sia regioni altamente conservate che regioni altamente variabili, e questo consente l'individuazione di aree in grado di distinguere i taxa a vari livelli, compresi i ceppi, le specie, i generi, e sempre più ampi raggruppamenti filogenetici.

Ci sono due approcci principali per l'utilizzo di sonde oligonucleotidiche nella rilevazione di specie HAB. Il primo è denominato "whole cell hybridization" o "fluorescence in-situ hybridization" (FISH), in cui la sonda penetra, nelle cellule intatte e chimicamente fissate, si ibrida o si lega alla sua

sequenza bersaglio sulle molecole di rRNA, e viene visualizzata tramite un reporter fluorescente collegato direttamente alla sonda o applicato durante una fase secondaria.

L'approccio FISH è stato applicato ampiamente per la rilevazione di molte alghe nocive, comprese dinoflagellati (Adachi et al. 1996); *Dinophysis* spp. (Rehnstam-Holm et al. 2002); *Pfiesteria* spp. (Rublee et al. 1999), diatomee (Miller et al. 1998; Miller et al. 2000; Scholin et al. 1997), e rafidofite (Tyrrell et al. 2001).

2.1. Applicazione del saggio FISH

Eller et al. (2002) hanno sviluppato diverse sonde gerarchiche per il riconoscimento delle divisioni Haptophyta e Heterokonta in campioni di fitoplancton attraverso la tecnica di "fluorescence *in situ* hybridization". Le sonde sono state disegnate per l'identificazione di Haptophyta, Classi Prymnesiophyceae e Pavlovophyceae, per l'Ordine Coccolithales, per i cladi noti solo tramite dati di sequenza disponibili in librerie clonali e per il genere *Prymnesium*. La classificazione dei campioni di fitoplancton attraverso l'ibridazione con sonde gerarchiche, in grado di identificare livelli tassonomici successivi, sono utili durante l'ibridazione in parellelo. In particolare, vengono utilizzate due sonde, una per il riconoscimento del livello tassonomico più alto e l'altra per il riconoscimento del gruppo target. In questo modo si ottiene il conteggio simultaneo delle cellule di interesse a due livelli tassonomici differenti. In futuro, attraverso l'impiego di nuove sonde per la divisione Heterokonta (Stramenopiles) e di sonde per il riconoscimento a livello di classe, sarà possibile identificare, attraverso tecniche molecolari le specie più problematiche da classificare a causa della piccola taglia e della mancanza di caratteristiche morfologiche che contraddistinguono le specie. Sarà inoltre possibile identificare anche gruppi come le diatomee che non possono essere riconosciuti attraverso l'impiego di una singola sonda.

2.2. TSA (Tyramide signal amplification) - FISH

La "Tyramide Signal Amplification" (TSA) è una tecnica che permette di amplificare il segnale fluorescente delle sonde legate agli acidi nucleici mediante l'uso di tiramide in combinazione alla "fluorescence *in situ* hybridization". La sonda oligonucleotidica è marcata con l'enzima Horseradish perossidasi (HRP). L'enzima HRP catalizza la precipitazione di un composto fenolico, la tiramide fluoresceinata che si lega ai composti aromatici ricchi di elettroni come quelli delle molecule

proteiche. Il risultato della reazione TSA produce un'amplificazione del segnale fino a 30 volte superiore rispetto a quello ottenuto con l'impiego di sonde marcate con coloranti fluorescenti tradizionali (John et al. 2005). Tra le varie applicazioni, il saggio TSA-FISH è stato adattato per il rilevamento automatico attraverso citometria in fase solida (Töbe et al. 2006). Questo strumento permette il conteggio automatico di cellule marcate con coloranti fluorescenti su un un filtro a membrana. I risultati positivi ottentuti vengono quindi verificati tramite microscopia. In particolare, il microscopio a epifluorescenza guidato da un computer, identifica in maniera automatica i segnali positivi corrispondenti alle cellule (Töbe et al. 2010). Tra le applicazioni in ambito marino, la specie tossica di *Prymnesium parvum* è stata rilevata e contata attraverso lo sviluppo di sonde e l'applicazione di citometria in fase solida (Töbe et al. 2006).

Il nuovo metodo TSA-FISH accoppiato alla citometria in fase solida per la rilevazione e il conteggio di specie fitoplanctoniche, rappresenta un'analisi veloce, precisa, sensibile e semi-automatica ed ha un grande potenziale di applicazione nei programmi di monitoraggio di alghe tossiche.

Al momento, la tecnica è adatta solo per il riconoscimento di cellulee dalla forma rotonda e sferica, non per l'identificazione di specie di forma allungata e formanti colonie, come le diatomee appertenenti al genere *Pseudo-nitzschia*. Inoltre, viene raccomandata la conferma delle cellule positive attraverso le tecniche di microscopia. In aggiunta, i costi elevati delle attrezzature compreso il microscopio a epifluorescenza rappresentano un fattore limitante all'introduzione di questa metodologia nei laboratori.

2.3. Saggio di Sandwich Hybridization semi-automatico

Il saggio di "sandwich hybridization" (SHA) è un metodo molecolare rapido per l'identificazione e la quantificazione di cellule target di varie specie algali tossiche. Il saggio SHA è un potente strumento per valutare rapidamente la composizione delle comunità fitoplanctoniche HAB senza l'impiego di tecniche di microscopia che prevedono un'avanzata formazione tassonomica dell'operatore. In Nuova Zelanda il metodo SHA viene utilizzato nel monitoraggio di molluschi (Ayers et al. 2005) e per la rilevazione di invertebrati (es. Granchio verde europeo) e batteri marini (Preston et al. 2009).

Il saggio SHA utilizza due sonde di DNA che riconoscono sequenze di RNA ribosomiale (rRNA), quali le subunità maggiore e la subunità minore. Per il saggio SHA vengono utilizzate piastre da 96 pozzetti e un processore robotico (Marin and Scholin 2010). Campioni ambientali di 200-400 ml di acqua di mare vengono filtrati attraverso filtri PVDF (0.65-0.45 um). I filtri vengono trasferiti in

cryovials contenenti tampone di lisi. Quindi, i lisati ottenuti vengono usati per il saggio di ibridazione. Il principio del saggio SHA rappresenta un vero e proprio "sandwich" tra "sonda cattura" fissata su una matrice solida e complementare ad una sequenza target ribosomiale. Per rilevare queste molecole catturate è necessaria una seconda fase di ibridazione che prevede l'utilizzo di una sonda di DNA legata ad una sonda segnale. Questa seconda sonda di DNA riconosce una sequenza ribosomiale più conservata del frammento catturato. Il cosiddetto "sandwich" della sonda cattura/molecola target molecola/sonda segnale viene rilevato mediante una reazione colorimetrica in presenza di enzimi. La tecnica di SHA si basata sull'utilizzo di un processore semi-robotico, di un lettore di micropiastre, di un sistema di filtrazione e di micropipettatori come descritto da Marin e Scholin (2010). La densità ottica (O.D.) rilevata nei campioni, consente il calcolo della concentrazione cellulare target. Una curva standard costruita con un numero noto di cellulare è essenziale, oltre al tampone di lisi utilizzatoe al volume del campione processato, per determinare la stima del numero di cellule in ciascun campione ambientale, come descritto da Marin e Scholin (2010).

2.4. Applicazione del saggio di SHA con rilevamento automatico

Le applicazioni del test basato su SHA in campioni ambientali sono numerose (Greenfield et al., 2006, Scholin et al. 2009), e comportano l'impiego di un Environmental Sample Processor (ESP). L'ESP è un sistema robotico sviluppato per l'applicazione autonoma di test diagnostici molecolari per la rilevazione di specie HAB utilizzando sonde di DNA direttamente *in situ*. La rilevazione simultanea di specie fitoplanctoniche tossiche e delle loro tossine tramite l'impiego dell'ESP diventa fondamentale per la gestione degli effetti sulla salute umana e sull'ecosistema. Doucette et al. (2009) hanno sviluppato metodi per la determinazione e la quantificazione di acido domoico (DA) e delle cellule di *Pseudo-nitzschia* grazie all'ESP nella Baia di Monterey, California. L'ESP, in modo autonomo, è in grado di campionare e concentrare il particellato presente nell'acqua di mare, di rilevare molecole target specie-specifiche e di trasmettere i dati raccolti ad una nave o ad una base a terra. Un sistema molecolare basato sulla tecnica ELISA è stato sviluppato per il rilevamento di acido domoico utilizzando le IgG. Infatti, in altri studi le misurazioni della tossina erano limitate a materiale di archivio raccolto e conservato a bordo della piattaforma ESP per essere successivamente processato in laboratorio. Un approccio ELISA è stato utilizzato per la simultanea rilevazione dell'organismo produttore della tossina e della tossina stessa. Il limite di rilevazione del test era nel range dei ng/mL che corrispondeva a livelli di acido domoico espressi in ng/L relativi ai campioni di 0.5 L di acqua di mare prelevati dall'ESP.

2.5. Analisi di PCR

Infine, metodi di rilevamento di specie algali, rapidi, altamente specifici e sensibili, sono quelli basati sulla reazione a catena della polimerasi (PCR). Lo scopo della PCR è di generare numerose copie di un gene specifico, mediante reazioni cicliche, ripetute per 30-40 cicli all'interno di un termociclatore. Ogni ciclo prevede: una fase di denaturazione a 94-95°C, in cui il doppio filamento di DNA si denatura, generando un singolo filamento; una fase di annealing a 54-55°C, in cui si verifica l'appaiamento del primer con il filamento complementare, e questo permette alla polimerasi di attaccarsi e iniziare a copiare il DNA templato; una estensione finale a 72°C, in cui le basi complementari al templato sono accoppiate al primer nella direzione 3' (la polimerasi aggiunge i dNTP (insieme dei quattro tipi di deossinucleoside trifosfato) da 5' a 3', leggendo il templato in direzione 3'-5', le basi sono aggiunte in maniera co mplementare al templato). Poiché sono copiati entrambi i filamenti denaturati nella prima fase, si ottiene un incremento esponenziale del numero di copie del gene. Prima che l'amplificato di PCR sia utilizzato per successive applicazioni, è necessario verificarne la specificità, in quanto, per scarsa quantità o qualità del templato di origine, la reazione potrebbe generare frammenti di differente peso molecolare rispetto a quello atteso. Diversi studi hanno utilizzato primers taxa- o specie-specifici per amplificare regioni target di geni bersaglio di specie HAB, e gli amplificati sono stati successivamente visualizzati mediante elettroforesi su gel e colorazione con etidio bromuro o sono stati sequenziati (Penna et al. 1999, Godhe et al. 2001; Rublee et al. 2001) fornendo informazioni di tipo qualitativo.

Successivamente, sono state proposte tecniche sempre basate sul principio della PCR ma in grado di fornire una risposta quantitativa e non solo qualitativa, come la real-time PCR o PCR quantitativa (qPCR). Sono stati sviluppati diversi saggi di qPCR, per lo più basati sull'uso del colorante SYBR Green I e/o sonde come Taqman e Molecular beacons, per la quantificazione di un gran numero di specie microalgali tossiche (Galluzzi et al. 2004, Ghode et al. 2008, Erdner et al. 2010), attraverso la sola analisi della curva di melting per confermare la specificità del prodotto amplificato senza bisogno di ricorrere a tecniche come elettroforesi su gel o sequenziamento.

Il metodo utilizzato in questi studi si basa sull'uso di curve standard costruite usando plasmidi contenenti sequenze di DNA target o DNA genomico estratto da concentrazioni note di cellule in coltura.

La qPCR specie-specifica con successiva analisi della curva di melting ha permesso ad Andree et al. (2011) di differenziare i singoli prodotti di PCR, ampliconi, attraverso la rilevazione di piccoli

cambiamenti nella sequenza del gene ITS-1, 5.8S, e ITS-2 tra diversi ceppi della stessa specie di *Pseudo nitzschia* spp nel Mar Mediterraneo nord-ovest.

Penna et al. (2013) grazie al metodo qPCR hanno stimato il numero di copie rDNA e la variazione del numero di copie tra le specie di *Pseudo-nitzschia* nel Mar Adriatico.

2.6. Analisi di microarray

Un altro approccio usato per lo studio e l'identificazione delle *Pseudo-nitzschia* è stato quello delle tecniche d'ibridazione come il "microarray" e "dot-blot".

Il microarray di DNA è composto da un insieme di sonde di DNA attaccate ad una superficie solida come vetro, plastica o chip di silicio, che permette di esaminare simultaneamente la presenza di numerosi geni all'interno di un campione di DNA. Smith et al. (2012) hanno sviluppato un microarray per snellire l'identificazione dei microrganismi in campioni prelevati durante eventi di fioriture algali nocive, direttamente a bordo di navi di ricerca entro 7 ore dalla raccolta delle acque. In particolare, il protocollo prevede l'utilizzo di 307 sonde oligonucleotidiche specifiche per la regione interna trascritta (ITS1) tra il gene ribosomiale 18S e 28S, in grado di identificare specifici taxa e screenare 118 ribotipi di *Pseudo-nitzschia* da 15 specie disponibili al momento dello sviluppo del saggio.

Anche Barra et al. (2013) hanno valutato l'applicabilità della tecnologia microarray per il rilevamento di specie di *Pseudo-nitzschia* tossiche e non tossiche nel Golfo di Napoli. Sono state progettate 49 sonde di DNA genere- e specie-specifiche sui geni LSU (large subunit) e SSU (small subunit) rRNA di diverse specie di microalghe nocive appartenenti a dinoflagellati, rafidofite, primnesiofite e diatomee. Sono state rilevate 11 specie di *Pseudo-nitzschia*, di cui almeno 5 potenzialmente tossiche.

Nel 2014 lo stesso gruppo di ricerca, ha testato la funzionalità e la specificità di 23 delle sonde progettate contro dieci delle specie rilevate, utilizzando una procedura dot-blot. Nella procedura dot-blot applicata in questo studio, prodotti di PCR di circa 700 bp del gene nucleare LSU, generato da ceppi monoclonali di dieci specie di *Pseudo-nitzschia*, sono stati plottati su filtri di nitrocellulosa. Ciascun filtro è stato quindi incubato con una singola sonda specie-specifica. Undici delle sonde testate hanno mostrato risposte specifiche, individuando sette specie di *Pseudo-nitzschia*. Le altre sonde hanno mostrato risposte non specifiche o negative, questo perché le ibridazioni dot-blot sono più specifiche rispetto a quelle ottenute con l'approccio microarray e i falsi positivi sul microarray potrebbero dovuti alla presenza di identiche sequenze bersaglio in altre parti del genoma delle specie *Pseudo-nitzschia* considerate (Barra et al., 2014).

2.7. High Resolution Melting (HRM)

L’High Resolution Melting (HRM) è un metodo di analisi post-PCR utilizzato per identificare variazioni genetiche in sequenze di acidi nucleici. L’analisi HRM può discriminare sequenze di DNA in base alla loro composizione, lunghezza, contenuto GC, e filo complementarità. Questo metodo semplice e veloce, si basa sulla tecnica di PCR e sull’analisi della curva di dissociazione o curva di melting (dissociazione caratteristica del DNA a doppia elica durante il riscaldamento), utilizzando coloranti fluorescenti a concentrazioni più elevate, utilizzando strumenti che raccolgono i dati di fluorescenza a risoluzione di temperature più fine e software più sofisticati con nuovi algoritmi di scaling fluorescenti. Analisi HRM inizia con una PCR di amplificazione della regione di interesse in presenza di un colorante intercalante nel DNA a doppio filamento (dsDNA). Questo colorante ha un elevato grado di fluorescenza quando è legato al dsDNA e un basso grado di fluorescenza quando non è legato. L'amplificazione è seguita da una dissociazione ad alta risoluzione con una strumentazione in grado di catturare un gran numero di dati fluorescenti al cambiamento della temperatura, con altissima precisione. Quando il dsDNA si dissocia in singoli filamenti, il colorante viene rilasciato, provocando un cambiamento di fluorescenza. Il risultato che si ottiene è un profilo di melt caratteristico per ogni tipo di amplicone, che tiene conto anche di aspetti come il contenuto di GC, lunghezza, sequenza e eterozigosità.

L’analisi HRM successiva non richiede alcuna elaborazione manuale post-PCR, viene infatti eseguita in un sistema a tubo-chiuso, ed ha un costo di reazione relativamente basso rispetto ad altri metodi utilizzati per studiare la variazione genetica.

L’analisi HRM utilizza due modalità di analisi dei profili, una modalità distingue le curve sulla base delle differenze di temperatura di melting (Tm); l’altra distingue le curve sulla base della forma della curva caratteristica di ciascun amplicone.

2.7.1. Descrizione di un saggio HRM per Applied Biosystems® StepOne Real-Time PCR System

2.7.1.1. Progettazione e ottimizzazione della PCR

Questo passaggio comprende un'attenta progettazione del set di primers da utilizzare, la selezione dei reagenti della PCR e del colorante HRM, la definizione della libreria dei DNA di controllo da utilizzare.

a) Disegno dei primers

La lunghezza degli ampliconi potrebbe incidere sulla sensibilità e specificità dell'analisi HRM successiva. Durante la progettazione di primers per l'analisi di genotipizzazione sulla base di "single nucleotide polymorphism" (SNP), la lunghezza dell'amplicone dovrebbe essere contenuta per evitare il rilevamento di SNP al di fuori della regione di interesse. Tuttavia, ampliconi troppo corti potrebbero produrre segnali di fluorescenza inferiori, a causa della minore quantità di colorante che verrebbe incorporato. Al fine di garantire un adeguato segnale di fluorescenza, la lunghezza dell'amplicone dovrebbe essere approssimativamente 80-100 bp compresi i primers (es. lunghezza sequenza bersaglio di 30 bp in aggiunta a due sequenze di primer da 25 bp l'uno). Poiché i coloranti utilizzati per l'analisi HRM si legano genericamente a qualsiasi prodotto di DNA a doppio filamento, i primers devono essere specifici per la regione di interesse. La specificità viene valutata *in silico* attraverso un allineamento su BLAST (Base Locale Alignment Search Tool) delle sequenze dei primer con le sequenze presenti in banca dati. È inoltre buona pratica la progettazione di tre coppie di primers. Per ogni coppia deve essere testata la specificità del target, deve essere valutata la presenza di eventuali dimeri di primers e prodotti aspecifici mediante analisi della curva di melting; ed eventuali aspecifici devono essere valutati mediante elettroforesi su gel di agarosio.

b) Reagenti e coloranti

Per l'analisi HRM si utilizzano coloranti che si intercalano al DNA a doppia elica (dsDNA) che, anche a concentrazioni relativamente elevate, non inibiscono la reazione di PCR. Sono state testate concentrazioni di colorante leggermente superiori ed inferiori alla concentrazione raccomandata, al fine di determinare la concentrazione in grado di dare la massima fluorescenza senza inibire la PCR.
I coloranti comunemente utilizzati per l'analisi HRM includono:

• SYTO®9 Green Fluorescent Nucleic Acid Stain

• EvaGreen®Colorante

• SYBR®Green I

• SYBR®Greener ™

Numerosi studi affermano che le migliori prestazioni in HRM si ottengono con il colorante SYTO9, che è stato quindi utilizzato in questo studio.

c) Libreria controlli

In ogni esperimento di HRM è necessario includere un controllo negativo e almeno un controllo positivo per ciascuna variante attesa. Eseguire più repliche per ciascuna variante permette di

migliorare i risultati, e di definire in modo più efficace la variazione all'interno dei diversi campioni della stessa sequenza, o all'interno di repliche dello stesso genotipo.

2.7.1.2. PCR e analisi della curva di melting

Si raccomanda l'uso di un sistema di PCR real-time per l'amplificazione, in quanto la reazione di amplificazione può essere monitorato in tempo reale, fornendo informazioni sulla qualità degli ampliconi. In alternativa, la PCR può essere eseguita su un termociclatore standard e successivamente trasferito in uno strumento di PCR real-time per effettuare il saggio di HRM. In un sistema di PCR real-time, i campioni vengono denaturati e i dati delle curve di melting direttamente raccolti. Quando invece viene utilizzato un termociclatore standard per la PCR, i campioni devono essere trasferiti in uno strumento PCR real-time per raccogliere i dati della curva di melting.

2.7.1.3. Analisi HRM

Il file dei dati dell'esperimento di HRM deve essere esportato dallo strumento di PCR real-time e importato nel software di analisi di HRM (High Resolution Melt software versione 3.0.1 Applied Biosystems) per eseguire l'analisi dei dati.

Per ogni campione di controllo positivo, è necessario inserire le relative informazioni nel software HRM e assegnare ogni controllo ai rispettivi pozzetti.

Successivamente, il software HRM utilizza le impostazioni di analisi predefinite per assegnare automaticamente una variante per ogni campione analizzato, e determina le diverse varianti basandosi sulle caratteristiche della curva di dissociazione (Tm), ossia sulle differenze nella forma delle curve e sulle differenze nei valori di Tm. Il profilo di fusione di un prodotto di PCR dipende dal suo contenuto di GC, lunghezza, sequenza ed eterozigosi.

Le curve di melting vengono visualizzate come "Aligned Melt Curves" in cui si nota la netta diminuzione della fluorescenza in funzione di un aumento di temperatura (Figura 6).

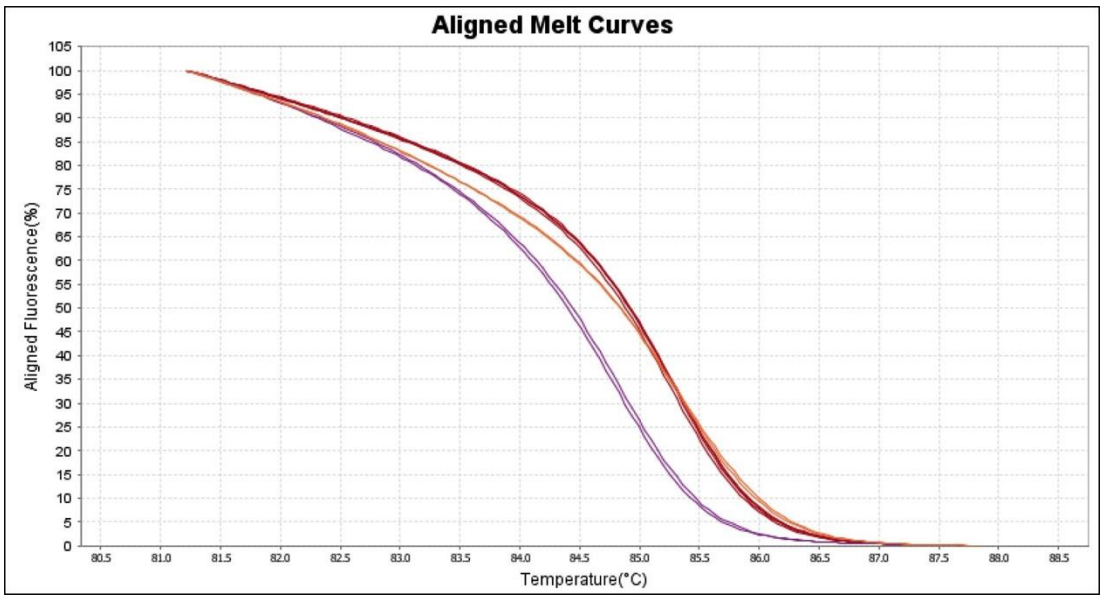


Figura 6. Rappresentazione grafica di un analisi HRM, curve di melting di tre varianti allineate allo stesso livello di fluorescenza.

Le curve di melting vengono allineate allo stesso livello di fluorescenza usando le regioni di pre- e post-melt. Le regioni pre e post-Melt sono definite da coppie di barre verticali poste prima e dopo la regione attiva di dissociazione (Figura 7), e l'area tra le coppie di barre è utilizzata dal software per designare il 100% di fluorescenza, ossia il punto in cui ogni amplicone è a doppio filamento. In particolare, il software HRM calcola automaticamente le regioni pre- e post- melting, ma è tuttavia possibile regolarle autonomamente, per ottimizzare l'analisi, posizionandole il più vicino possibile alla regione di analisi attiva.

Visualizzando la derivata delle curve di melting è possibile regolare le regioni di pre-e post-melt potendo così ottimizzare l'analisi delle varianti Sia per la regione pre-melt che post-melt la differenza di temperatura tra inizio e fine della regione dovrebbe essere compresa tra 0,2 e 0,5°C.

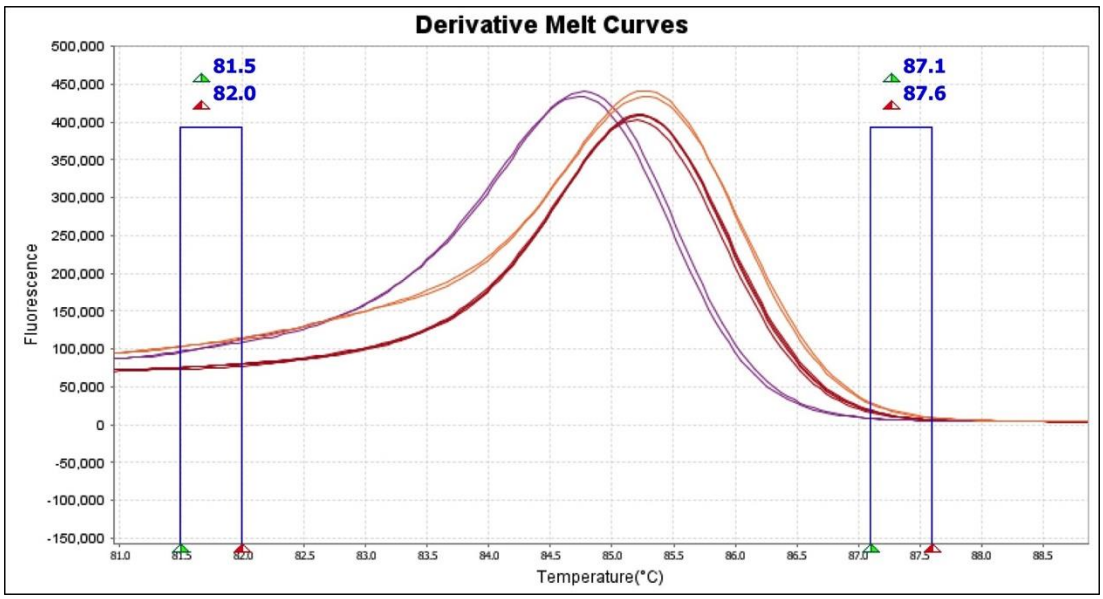


Figura 7. Derivata delle curve di melting di tre diverse varianti; le barre verticali indicano le regioni pre e post melt.

Se, al software HRM, sono state fornite le informazioni relative ad ogni campione di controllo incluso nell'esperimento e se sono stati assegnati i controlli ai rispettivi pozzetti, è possibile selezionare un controllo e i corrispondenti pozzetti come campioni di riferimento. In questo modo il software sottrae in automatico la curva di riferimento dalle altre curve, mostrando i dati come differenza della fluorescenza tra la curva di melting del campione di riferimento e le altre curve (Difference Plot). Questo tipo di visualizzazione consente di vedere più facilmente piccole differenze tra le curve e di identificare eventuali valori anomali (Figura 8).

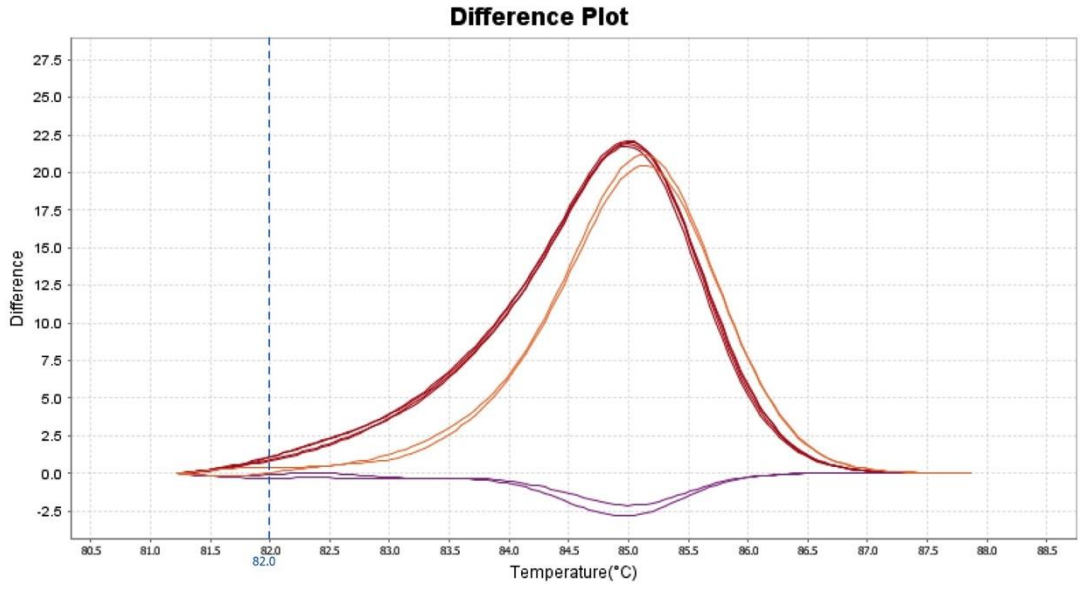


Figura 8. I dati mostrati come differenza della fluorescenza tra la curva di melting del campione di riferimento e le altre curve.

Il software HRM assegna automaticamente una variante per ogni campione presente nell'esperimento, secondo la % di somiglianza tra campioni e controlli, cioè in base alla forma delle curve di melting e alle Tm. Nel caso in cui nessuno dei campioni analizzati corrisponda ai controlli utilizzati, il software nomina le varianti in modo generico (esempio "*variant n*"). Una copertura totale delle possibili varianti con i relativi campioni di controllo, permette di sfruttare al massimo il potenziale del saggio HRM. Il metodo HRM oltre che in campo medico diagnostico è stato utilizzato anche per la genotipizzazione di microrganismi come *Symbiodinium* (Granados-Cifuentes et al. 2011) *Leishmania* (Ceccarelli et al. 2014), per la discriminazione intraspecie tra individui di diverse aree geografiche di *Karenia mikimotoi* (Al-Kandari et al. 2011).

In questo studio, per la prima volta, questo test molecolare basato sull' analisi della curva HRM è stato sviluppato e applicato per identificare le specie di *Pseudo-nitzschia*. Diversi ceppi in coltura di *Pseudo-nitzschia* spp., isolati da campioni di acqua di mare, sono stati analizzati,ed è stato possibile effettuare una precisa identificazione di tre specie diverse di *Pseudo nitzschia* basandosi sui diversi profili di curva di dissociazione generati dall'analisi HRM.

2.7.1.4. Limitazioni HRM

L'HRM è una tecnica relativamente semplice, rapida ed economica, ma dipende fortemente dall'uso di buoni strumenti per PCR e coloranti saturanti. La precisione è criticamente dipendente dalla risoluzione dello strumento utilizzato (Herrmann et al. 2006). Il tipo di colorante saturante utilizzato, pur non essendo rilevante per alcune applicazioni come l'analisi della metilazione, è fondamentale nel caso della genotipizzazione. Ad esempio, LCGreens Plus rileva eterozigoti meglio Sytos 9, che è meglio di EvaGreens, che è meglio di SYBR Green I (Wittwer, C.T. 2009).

La genotipizzazione è fortemente limitata dalla raccolta di genotipi di riferimento disponibili e dalla costruzione di una libreria di controlli idonea e ampiamente rappresentativa. Nei casi in cui i genotipi non possono essere identificati, questo potrebbe essere il risultato di una mancanza di un genotipo di riferimento appropriato al momento dell'analisi (Granados-Cifuentes et al. 2011).

Un ulteriore limitazione potrebbe essere rappresentata dalla necessità di lavorare con campioni monoclonali, ma nel nostro caso specifico questa procedura fa parte della routine essendo, il mantenimento di un'ampia collezione algale, uno dei servizi offerti dal Centro di biologia ambientale di Pesaro.

CAPITOLO 3

A HIGH RESOLUTION MELTING METHOD FOR THE MOLECULAR IDENTIFICATION OF THE TOXIC DIATOM SPECIES

3.1. HRM assay overview

The planktonic pennate diatom *Pseudo-nitzschia* (Bacillariophyceae) is a cosmopolitan genus commonly found in neritic and oceanic waters. The correct identification of *Pseudo-nitzschia* species has relevant implications for monitoring and management purposes, considering that toxigenic species may be morphologically similar or identical to non-toxigenic ones. Recent studies integrating molecular phylogenetic, morphological, ultrastructural and biological information have uncovered numerous cases of genetically distinct and at times reproductively isolated groups of strains that could not be distinguished easily or at all with microscopy. Molecular approaches constitute the most straightforward way to identify various taxa and elucidate their distribution over space and time. The high resolution melting (HRM) analysis can be an alternative technique to rapidly and accurately identity microalgal species and/or genotype cultured strains. Different melting profiles are obtained from the transition of double-strand (dsDNA) to single-strand DNA (ssDNA) as a result of a gradual temperature increase after PCR amplification. HRM has been used mainly for screening mutations linked to human diseases and for genotyping bacteria. To date, only one study applied HRM for microalgal species identification. The HRM analysis is faster, and less expensive than alternative approaches, such as microarray, or microsatellites. In the present study, we investigated the genetic diversity of several *Pseudo-nitzschia* species in monoclonal cultures obtained from environmental samples collected over one year in the Adriatic Sea using HRM based assay for species – specific identification. The HRM assay carried out on 21 strains of *Pseudo-nitzschia* spp. that were already identified, distinguished 3 different variants corresponding to the three different species of *Pseudo-nitzschia*, namely *P. pungens*, *P. delicatissima* and *P. calliantha*, as expected. The high resolution melting analysis is a powerful technique able to accurately identify *Pseudo-nitzschia* species that are difficult to be recognized by standard microscopy in environmental samples.

3.2. A high resolution melting method for the molecular identification of the potentially toxic diatom *Pseudo-nitzschia* spp. in the Mediterranean Sea

Laura Pugliese[1], Silvia Casabianca[1,2], Federico Perini[1], Francesca Andreoni[1], Antonella Penna[1,2,3*]

[1]Department of Biomolecular Sciences, University of Urbino, Viale Trieste 296, 61121 Pesaro, Italy
[2]Conisma, Consorzio Interuniversitario per le Scienze del Mare, Pz. Flaminio 9, 00196 Rome, Italy
[3]CNR–Institute of Marine Sciences (ISMAR), Largo Fiera della Pesca 60125 Ancona, Italy

in revision to Scientific Reports

A high resolution melting method for the molecular identification of the potentially toxic diatom *Pseudo-nitzschia* spp. in the Mediterranean Sea

Laura Pugliese[1], Silvia Casabianca[1,2], Federico Perini[1], Francesca Andreoni[1], Antonella Penna[1,2,3*]

[1]Department of Biomolecular Sciences, University of Urbino, Viale Trieste 296, 61121 Pesaro, Italy

[2]Conisma, Consorzio Interuniversitario per le Scienze del Mare, Pz. Flaminio 9, 00196 Rome, Italy

[3]CNR–Institute of Marine Sciences (ISMAR), Largo Fiera della Pesca 60125 Ancona, Italy

*corresponding author: antonella.penna@uniurb.it

Abstract

The aim of this study was to develop and validate a high resolution melting (HRM) method for the rapid, accurate and simultaneous identification of the harmful diatom *Pseudo-nitzschia* spp. in marine environment. The diatom *Pseudo-nitzschia* has worldwide distribution and some species are toxic, producing the potent domoic acid toxin, which poses a threat to human and animal health. Hence, species-specific identification of *Pseudo-nitzschia* spp. is relevant for toxic species detection. A pair of primers targeting the LSU rDNA of the genus *Pseudo-nitzschia* was designed for the development of the HRM assay. The analytical specificity of the assay was validated using 22 control DNAs of the *P. calliantha*, *P. delicatissima/P. arenysensis* complex and *P. pungens*. The HRM assay was applied to numerous unidentified *Pseudo-nitzschia* strains isolated from the northwestern Adriatic Sea (Mediterranean Sea). The post-PCR HRM analysis was able to detect and discriminate three distinct *Pseudo-nitzschia* taxa from unidentified samples. Further, the species-specific identification of *Pseudo-nitzschia* isolates by the HRM assay was consistent with phylogenetic analyses. The HRM assay was specific, robust and rapid when applied to high

numbers of cultured samples in order to taxonomically identity *Pseudo-nitzschia* isolates recovered from environmental samples.

Introduction

Diatoms (class Bacillariophyceae) are among the most productive eukaryotic microalgae. They are worldwide distributed in oceans and play a fundamental role in global biogeochemical cycles[1]. Within the Bacillariophyceae class, the genus *Pseudo-nitzschia* is found in polar, temperate, subtropical and tropical regions[2], although some *Pseudo-nitzschia* species are limited to distinct regional areas. At least 12 *Pseudo-nitzschia* species are toxic, as they produce domoic acid (DA), a neurotoxin causing amnesic shellfish poisoning (ASP), which was responsible for a dramatic intoxication event in 1987[3]. Blooms of *Pseudo-nitzschia* spp. have often been related to nutrient regimes modified by anthropogenic pressure[4]. The frequency of toxic blooms generated by different species of *Pseudo-nitzschia* is increasing in various coastal areas worldwide[2]. Multiple toxigenic *Pseudo-nitzschia* species frequently coexist in the same environment, even during bloom events that appear to be dominated by a single species[3]. Since the genus *Pseudo-nitzschia* includes a large number of species, their accurate taxonomical identification is important because they can be associated with domoic acid production[5]. To date, species identification or description has often been performed by integrating different methodological approaches based on scanning and transmission electron microscopy, and molecular analyses. However, the light microscopy does not always provide the resolution required for the identification of various *Pseudo-nitzschia* species[6,7]. Further, despite concerted efforts, the taxonomy of *Pseudo-nitzschia* is still bein updated, and new morphological species complexes and/or cryptic and pseudo-cryptic species (i.e. *P. delicatissima* or *P. pseudodelicatissima* complex) have recently been described within the genus[8,9]. Molecular taxonomy studies based on different genetic markers, including ribosomal RNA gene (LSU) and internal transcribed spacers (ITS regions), cytochrome oxidase 1 (cox 1) and chloroplast genes of ribulose 1,5 biphosphate carboxylase (rbcl), have uncovered numerous cases of genetically distinct,

and at times reproductively isolated, groups of strains or genetic lineages that could not be easily distinguished with light microscopy[10,11,12].

Recent molecular approaches, such as qPCR[13,14,15,16,17] ARISA[18], microarray[19,20,21] and dot blot hybridization systems[22] have been used for phytoplankton diagnostic species identification in seawaters[23]. All these methods have been developed for rapid, specific and sensitive screening of harmful algal bloom (HAB) species in monitoring activities. These methods are essentially based on the evaluation of sequence variation and design of oligonucleotide primers and/or probes in target nucleotide regions and they allow us to discriminate various species accurately. Among the molecular techniques used to analyze small genetic mutations, such as single nucleotide polymorphisms (SNPs), we have the post PCR high resolution melting (HRM) curve analysis. This technique is based on the melting properties of double-strand DNA (dsDNA). Different melting profiles are obtained from the transition of dsDNA to denaturated single-stranded DNA (ssDNA) as a result of a gradual temperature increase after PCR amplification. Both processes, PCR and gradual denaturation, take place in the same tube during a real-time run under two hours. The recent development of HRM was made possible by generation of new dyes designed for this technique and the technological improvements in real-time PCR instruments. The HRM method has been used mainly for genotyping microrganisms[24,25,26,27] and it is considered the simplest method for genotyping and detecting mutations because it can be performed immediately after qPCR.

In this study, the first molecular assay based on HRM curve analysis was developed and applied to detect various *Pseudo-nitzschia* species collected in the NW Adriatic Sea. Several cultured strains of *Pseudo-nitzschia* spp., isolated from seawater samples, were analyzed by the post PCR HRM assay. It was possible to make a precise accurate identification of three distinct species of *Pseudo-nitzschia* based on different melting curve profiles generated by the HRM analysis. The HRM-based identifications were also confirmed by phylogenetic analyses based on both LSU and ITS rDNA sequences.

This developed method proved to be specific, accurate and rapid in discriminating numerous cultured isolates of *Pseudo-nitzschia* species or complex, which are very difficult to identify using traditional light microscopy. Knowledge of diverse composition of potentially toxic *Pseudo-nitzschia* spp. can provide advanced strategies for the management of harmful algal blooms.

Methods

Sampling, isolation and cultures. The sampling sites were located 3000 m off the Italian coast (northwestern Adriatic Sea) at Tavollo (43°59'.30 N; 12°46'.42 E), Foglia (43°56'.55 N; 12°56'.18E) and Metauro (43°50'.54 N; 13°05'.9 E) river transects. Several strains of *P. calliantha, P. delicatissima, P.* cf. *arenysensis* and *P. pungens* isolated 500 m off the coast of Pesaro in 2009, 2010 and 2013, as described in Penna et al.[17], were used for the HRM assay development (Table S1). An additional 29 *Pseudo-nitzschia* strains were isolated from net samples using single cell isolation technique during the period from November 2014 to March 2015 (see Results section). All isolates were maintained in f/2 medium[28] at 16 ± 1°C on a 12:12 h light:dark cycle, at an irradiance of 100 µmoL photons m^{-2} s^{-1}.

Light microscopy (LM) observations of living cells were carried out using an Axiovert 40 CFL, Zeiss at 200x and 400x magnifications.

Genomic DNA extraction. Exponential phase cultures of *Pseudo-nitzschia* spp. were harvested by centrifugation at 4,000xg for 20 min at room temperature. Total genomic DNA was extracted from pellets using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA integrity was assessed by electrophoresis on agarose gel (0.8% w/v) and visualized by standard ethidium bromide staining under UV light. Quantification was performed using a Qubit fluorometer with a Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbard, CA, USA).

HRM primer design and specificity. A total of 21 LSU rDNA sequences of various *Pseudo-nitzschia* species mainly present in the Mediterranean Sea (Fig. 1)[29,30,31], available in GenBank, were aligned using the BioEdit Sequence Alignment Editor v. 7.0.5.3[32], to check for a SNP-carrying region flanked by highly conserved sequences suitable for primer design. The primers were designed using Primer-BLAST[33]. The primers for the amplification of 130 - 133 bp fragment, which was positioned from the 384 to 514 nucleotide position, were HRM_PSEUDO_F forward (5'-GCGAAGGAAACCAGTGTTTGT-3') and HRM_PSEUDO_R reverse (5'-GCTAGCAACAACAGACATCAACTCT-3'). The primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The *Pseudo-nitzschia* primer specificity was examined *in silico* using BLAST and tested in qPCR with both target and various purified genomic DNAs of *Pseudo-nitzschia* spp. among them: *P. calliantha, P. delicatissima, P.* cf. *arenysensis, P. pungens, P. fraudulenta, P. multistriata* and *P. pseudodelicatissima,* which are common in the northwestern Adriatic Sea. The specificity of the assay was also evaluated using the DNA of several other phytoplankton species. The PCR protocol is described below. The melting curve analysis was performed to check primer dimers and PCR products from misannealed primers. The PCR products were electrophoresed and analyzed on 1.8% (w/v) agarose gel. The amplified PCR fragments were sequenced by GATC Biotech AG, Köln, Germany to confirm the target specific DNA fragment. Each PCR reaction testing the specificity of the assay was performed in duplicate.

qPCR HRM assay. The qPCR HRM assay was performed in a final volume of 25 µl containing primers at final concentrations of 500 nM and 200 µM of each GeneAmp dNTP; 1.5 mM $MgCl_2$; 1X Reaction AmpliTaq Gold 360 Buffer; 1X Reaction MeltDoctor HRM Dye, (MeltDoctor™ HRM Dye, a stabilized form of the fluorescent SYTO® 9 double-stranded nucleic acid stain), 1.25 U of AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems, Foster City, CA, USA), and 1 ng of DNA template. PCR was carried out using the StepOne Real-Time PCR System (Applied

130 Biosystems, Foster City, CA, USA). The thermal cycling conditions consisted of 10 min at 95°C,
131 followed by 40 cycles at 95°C for 10 s and 60°C for 1min. HRM assay was performed from 60°C to
132 95°C with a ramp rate of 0.3%. All PCR experiments were performed in duplicate including target
133 positive controls of *P. calliantha* CBA72, *P. pungens* CBA100 and non-template controls (NTC).
134 As *P. delicatissima* and *P. arenysensis* shared the same LSU rRNA target amplicon, the *P.*
135 *delicatissima* CBA144 was used as a control for the *P. delicatissima/P. arenysensis* phylogenetic
136 complex.
137 The raw melting curve data were processed by the High Resolution Melt Software v. 3.0.1 (Applied
138 Biosystems, Foster City, CA, USA). The pre- and post-melt regions were set as close as possible to
139 the melting transition region. Positive controls, one for each species tested, were set in the HRM
140 software assigning each control to its corresponding well. Samples were analyzed and the software
141 automatically made a call for each sample according to the shape of the melt curves aligned to the
142 controls and the melting temperature (T_m).
143
144 **HRM assay validation and application for *Pseudo-nitzschia* species identification.** The method
145 was validated using several DNAs (n = 22) of *Pseudo-nitzschia*, already used as controls (as
146 above), such as the target species of the *P. calliantha*, *P. pungens* and *P. delicatissima*/*P.* cf.
147 *arenysensis* complex. In particular, 7 strains of each species, as *P. calliantha*, *P. pungens* and *P.*
148 *delicatissima*, and 1 strain of *P.* cf. *arenysensis*, were used (Table S1). The method was
149 subsequently applied to 29 strains of unidentified *Pseudo-nitzschia* spp. isolated from seawater off
150 the coast (northwestern Adriatic Sea) between November 2014 and March 2015, as previously
151 described. The HRM assay was applied for taxon-specific identification.
152
153 **Statistical analyses.** HRM data analysis was performed with Kruskall-Wallis and Mann-Whitney
154 tests to determine whether there were significant differences in the average Tm values among and

between *Pseudo-nitzschia* spp. isolates. All statistical calculations were performed using PAST ver. 2.17 with a $p < 0.05$ determining significance.

Molecular and phylogenetic analyses. Representative strains of *Pseudo-nitzschia* spp. identified by HRM assay were analyzed to confirm the species-specific taxonomical assignment by LSU and ITS-5.8S rDNA sequence alignment and phylogenetic analyses. The sequences of ribosomal genes obtained from new *Pseudo-nitzschia* cultured isolates were deposited in EMBL-EBI-ENA. Other ribosomal sequences of *Pseudo-nitzschia* spp. isolates were included in this study. All sequences were listed in Table S2. The LSU rDNA was amplified and sequenced using D1R or D2C primers targeting the D1-D2 region of the nuclear LSU rDNA[34]. The ITS region of the rDNA was amplified and sequenced using the universal primer ITSA and ITSB[35] or ITS1R and ITS1F[36]. The PCR reaction for the LSU rDNA was as follows: tubes contained 50 µl of mixture of 200 µM of dNTPs; 0.4 µM of each primer, 4 mM of $MgCl_2$, 1X reaction buffer (Master TaqBuffer, 5 PRIME, Germany), 1U Taq DNA Polymerase (5 PRIME, Germany) and 0.5-1 ng DNA template. PCR thermal cycling conditions were as follows: an initial denaturation at 95° C for 10 min, 35 cycles of 1 min at 95° C, 1 min at 50°C, and 2.5 min at 72°C and a final extension step of 7 min at 72°C. The PCR reaction for the ITS-5.8S rDNA using ITSA and ITSB primers was as follows: tubes contained 50 µl of mixture of 200 µM of dNTPs; 0.2 µM of each primer, 1 mM of $MgCl_2$, 0.75X TaqMasterPCR Enhancer (5 PRIME, Germany), 1X reaction buffer (Master TaqBuffer, 5 PRIME, Germany), 1U Taq DNA Polymerase (5 PRIME, Germany) and 0.5-1 ng DNA template. The PCR using ITS1R and ITS1F primers was carried out in a mixture as described above, with the following exceptions: 0.4 µM of each primer, 4 mM of $MgCl_2$ and 0.5X TaqMasterPCR Enhancer (5 PRIME, Germany). PCR thermal cycling conditions were as follows: an initial denaturation at 95°C for 10 min, 35 cycles of 30 s at 94° C, 30 s at 55°C min or 50°C, and 30 s at 72°C and a final extension step of 10 min or 2 min at 72°C.

All PCR amplified products were purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA), and the products were directly sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Kit v. 1.1 on the ABI 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Standard thermal cycling conditions were used for both templates setting the annealing temperature according to the template (60 °C and 50°C for ITS and LSU PCR specific primers, respectively). Difficult templates and repeated regions were solved increasing initial denaturation time and modifying thermal cycling condition as follows: denaturation at 96°C for 10 sec and annealing/extension at 50°C for 40 cycles.

The LSU and ITS-5.8S sequences were aligned using MAFFT software. Short aligned sequences and ambiguously aligned positions were excluded from the alignment manually or using Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks.html) with default settings. The neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses were performed in MEGA v. 6.06[37]. The robustness of NJ, MP and ML trees was tested by bootstrapping using 1000 pseudo-replicates. Distance and maximum likelihood trees were built based on the substitution model selected through the Akaike Information Criterion option implemented in MEGA v. 6.06. For LSU and ITS-5.8S gene rDNA alignment the most appropriate evolutionary models were found to be HKY + G and HKY + G +I, respectively. The MP analyses were performed using the Tree-Bisection-Redrafting (TBR) algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated. Bayesian analyses were performed using MrBayes 3.2.3[38] with the following settings: four Markov chains were run for 2,000,000 generations with a sampling frequency of 100 generations. Log-likelihood values for sampled trees were stabilized after almost 200,000 generations. The last 18,000 trees were used to estimate Bayesian posterior probabilities, whereas the first 2,001 were discarded as burn-ins. Results from two-independent runs were used to construct a majority-rule consensus tree containing the posterior probabilities.

The sequences of *Fragilariopsis rhombica* 5-17 AF7656 and *Fragilariopsis* sp. NL2010 GU170665 were used as an outgroup for the *Pseudo-nitzschia* LSU and ITS-5.8S gene phylogenetic analyses, respectively.

Results

HRM primer design and specificity. The *Pseudo-nitzschia* spp. primers, designed to amplify the target sequence of the LSU rDNA region, were examined *in silico* using BLAST and they were found to be specific to *P. calliantha, P. delicatissima, P.* cf. *arenysensis* and *P. pungens*. No non-specific products were detected, and amplification was not obtained in any template controls (NTCs). According to the *in silico* analysis, the PCR product size was 130 bp. The specificity of the qPCR assay was also tested by using DNA from other *Pseudo-nitzschia* and microalgal species. Negative amplifications were obtained. All these results show that the HRM assay was highly specific for targeting the *Pseudo-nitzschia* species.

Validation of the qPCR HRM assay. The HRM assay was found to successfully distinguish *Pseudo-nitzschia* species or complex such as *P. calliantha, P. pungens* and *P. delicatissima/P.* cf. *arenysensis*. All Ct values ranged from 20 to 24. The melting curve variation of the *Pseudo-nitzschia* spp. can be plotted in various ways, including aligned and difference plots, according to the melting behaviour of their amplicons determined using the High Resolution Melt software ver. 3.0.1 (Applied Biosystems) (Fig. S1). As shown in the plots, the melting curves of *Pseudo-nitzschia* spp. can be distinctly separated by both the silhouettes of the curves and the Tm for each species or complex. Three different average Tm values of 84.56 ± 0.18; 85.22 ± 0.06; 85.05 ± 0.09 were assigned to *P. calliantha*, *P. delicatissima/P.* cf. *arenysensis* and *P. pungens*, respectively. The Tm values were highly reproducible across 22 repeated melt curve runs (7 and 8 melt curve runs per *P. calliantha* and *P. pungens* and *P. delicatissima/P.* cf. *arenysensis,* respectively). The Kruskall Wallis test demonstrated that the three Tm values were significantly different (Hc = 22.32, p <

231 0.001). Further, the *a posteriori* pairwise Mann-Whitney test showed that the differences between
232 *Pseudo-nitzschia* species were significant with Bonferroni correction ($p<0.001$).
233
234 **Analysis of *Pseudo-nitzschia* spp. isolates.** The method was subsequently applied for the analysis
235 of unknown isolates of the *Pseudo-nitzschia* spp., collected between November 2014 and March
236 2015 in the northwestern Adriatic Sea. A total of 29 *Pseudo-nitzschia* spp. isolates were analysed
237 using the qPCR HRM assay. The melting profiles generated by PCR products of unidentified
238 isolates were evaluated, and it was observed that the *Pseudo-nitzschia* spp. in the unknown strains
239 showed consistency in their corresponding Tm values and curve silhouettes, which were similar to
240 those generated by DNA positive controls. In the end, the melting profile from the isolates can be
241 clustered into three groups using the auto-calling mode of the High Resolution Melt software
242 (Applied Biosystems), and identification of the *Pseudo-nitzschia* spp. can be made by comparing
243 their silhouettes and Tm values to those of controls. The confidence interval for auto-called results
244 ranged between 96–100% (Fig. 2). A total of 12 strains of *P. calliantha,* 10 strains of *P.*
245 *delicatissima/P.* cf. *arenysensis,* and 7 strains of *P. pungens* were identified (Table 1).
246 All melting curve peak Tm values, which included values of controls and isolates for each taxon,
247 such as *P. calliantha* (n = 19), *P. delicatissima/P.* cf. *arenysensis* (n = 18) and *P. pungens* (n = 14),
248 are illustrated in the box plot (Fig. 3). The Tm values among the three taxa were found to be
249 significantly different by the Kruskal-Wallis test ($Hc= 60.4$, $p<0.001$). Furthermore, a *posteriori*
250 pairwise Mann-Whitney comparisons showed that there was a significant difference between *P.*
251 *calliantha, P. delicatissima/P.* cf. *arenysensis,* and *P. pungens* ($p<0.001$ after Bonferroni
252 correction).
253 *Pseudo-nitzschia* spp. strains identified by the HRM assay were then verified by phylogenetic
254 analyses based on LSU and ITS-5.8S rDNA sequences. All the strains analyzed by the HRM assay
255 were sequenced and included in the phylogenetic analyses. Only, representative strains of the three
256 NW Adriatic *Pseudo-nitzschia* spp. are shown.

257

258 **Phylogenetic analyses of *Pseudo-nitzschia* spp. LSU and ITS-5.8S ribosomal genes.** The final
259 alignments of *Pseudo-nitzschia* spp. ribosomal gene sequences, as namely LSU and ITS-5.8S, with
260 *Fragilariopsis* as an outgroup, were as follows: LSU was 529 bp in length (A = 25.8%, T = 27.1%,
261 C = 17.2%, G = 30%) with 515 total informative sites, excluding gaps, and 99 polymorphic sites, of
262 which 65 were parsimony sites. ITS-5.8S was 890 bp in length (A = 27%, T = 34.8%, C = 18.2%, G
263 = 20%) with 468 total informative sites excluding gaps and 218 polymorphic sites, of which 245
264 were parsimony sites.
265 Based on single LSU and ITS-5.8S rDNA sequences, only minor differences between the NJ, MP,
266 ML and Bayesian inference analyses were found; therefore, only the ML phylogenetic trees are
267 presented. The LSU rDNA phylogeny that was obtained from 40 isolates of *Pseudo-nitzschia* spp.
268 showed that NW Adriatic representative strains, identified as *P. pungens* (CBA179 and CBA180)
269 and *P. calliantha* (CBA192 and CBA194) by the HRM assay, clustered in the clades of the
270 corresponding species. By contrast, Adriatic strains identified as *P. delicatissima/P.* cf. *arenysensis*
271 by the HRM assay and sharing identical LSU sequences, grouped together in a well-supported clade
272 of *P. arenysensis,* as sister to a clade including *P. delicatissima/P. micropora/P. dolorosa* within the
273 *P. delicatissima* complex (Fig. S2). Only four representative strains (CBA159, CBA165, CBA163,
274 CBA169) of Adriatic *P.* cf. *arenysensis* were shown. All these lineages were strongly supported by
275 high bootstrap and posterior probability values.
276 The ITS-5.8S rDNA phylogeny that was obtained from 31 isolates of *Pseudo-nitzschia* spp. showed
277 similar tree topology to the LSU rDNA phylogeny, confirming that the NW Adriatic representative
278 strains, identified as *P. pungens* (CBA179 and CBA180) and *P. calliantha* (CBA192 and CBA194)
279 by the HRM assay grouped with these corresponding taxa, and all strains identified as *P.*
280 *delicatissima/P.* cf. *arenysensis* by the HRM assay grouped into the clade of *P. arenysensis,* which
281 separated after *P. delicatissima*. Only three representative strains of Adriatic *P.* cf. *arenysensis* were

shown. Then, these two clades diverged after *P. micropora*. All these clades were supported by high bootstrap and posterior probability values (Fig. S3).

Discussion

Accurate identification of *Pseudo-nitzschia* species is of utmost importance since several species produce the potent neurotoxin ASP (amnesic shellfish poisoning), which is harmful to humans, some mammals and birds[2]. Traditionally, light microscopy is used in routine phytoplankton monitoring although inadequate, because it does not provide the necessary resolution required for the discrimination of *Pseudo-nitzschia* species, species complex and/or cryptic and pseudo-cryptic species already described within this genus[5,6,39]. In fact, the analyses of ultrastructural features characterizing a species are performed by electron microscopy. Therefore, molecular analyses are very often required to support or solve the species-specific identification from cultured isolates or field samples[40,41,23,22].

In the NW Mediterranean Sea, *Pseudo-nitzschia* spp. are present at high concentrations, commonly forming mixed blooms with other diatom species, as occurs in the NW Adriatic Sea[42,17] or with recurrent seasonal distribution, as occurs in the Tyrrhenian Sea and off the Catalan coast[43,44]. In the Mediterranean Sea, *Pseudo-nitzschia* spp. also show distinct distributions based on morphological and genetic characterization. Based on studies of seasonal succession, *P. calliantha, P. delicatissima* and *P. pungens* are the most commonly found species in the Adriatic Sea[45,17,46].

In this study, a reliable, rapid and robust molecular qPCR HRM assay was developed in order to rapidly and accurately detect harmful *Pseudo-nitzschia* species in cultured samples obtained from coastal water survey. This HRM method is based on a post PCR analysis, which differs from previous qPCR melt curve analyses[13,15,47], because the amplicons produced by the qPCR are subjected to a thermal gradient with temperature increments of 0.1°C/sec using sensitive instrumentation that ensures absolute precision of the temperatures increments. By continuously monitoring the fluorescence emitted by the Meltdoctor HRM Dye, it is possible to assess the exact

308 melting temperature of the amplicon with a precision of 0.1°C. Base differences and/or insertions or
309 deletions of one or more bases are revealed, and this makes it possible to discriminate between
310 amplicons and, consequently, between species.
311 The genus specific primers were designed on a partial domain (D1/D2 domains) LSU rDNA
312 sequence alignment including most representative *Pseudo-nitzschia* spp. species from the
313 Mediterranean region in order to include a high level of genetic diversity. The variable D1–D3
314 region of LSU has been widely used for species-specific identification using various molecular
315 approaches such as qPCR[15,17], microarray[19,21,48], and FISH or sandwich hybridization assay[49,50,51].
316 Within the LSU gene, we identified a variable inter-specific target, flanked by highly conserved
317 regions, which was suitable for primer design and the relative production of specific amplicons of
318 each HRM variant. The ITS regions were also explored for primer design, but, they showed too
319 much variability to encompass target *Pseudo-nizschia* species. In fact, ITS regions of diatom
320 species are also known to be highly variable at intra-species level[39,52]. The amplicon length was 130
321 bp satisfying the HRM analysis conditions. The specificity of the amplicons was shown by i*) in*
322 *silico* using BLAST; ii) qPCR performed on target DNAs of *P. calliantha*, *P. pungens, P.*
323 *delicatissima* and *P.* cf. *arenysensis* isolates; iii) qPCR carried out on several DNAs of other
324 *Pseudo-nitzschia* and microalgal species. The primers were found to identify exclusively *Pseudo-*
325 *nitzschia* spp., since non-specific products and/or dimers were never obtained. Thus, the
326 identification of *Pseudo-nitzschia* species by our HRM assay was species-specific and accurate. The
327 HRM method was developed and validated on 22 previously identified control isolates previously
328 identified[17]. Hence, the sequence variations within the analyzed region of the LSU gene allowed us
329 to use the HRM assay for the identification of these isolates. In particular, *P. delicatissima* and *P.*
330 cf. *arenysensis* constituting the *P. delicatissima* complex, showed no differences in their nucleotide
331 sequences and their high resolution melting curves were identical. Other molecular approaches,
332 such as dot-blot hybridization[22], have also shown that these species share identical target LSU
333 regions because of incomplete lineage sorting[7]. When the *P. delicatissima* complex sequence was

334 selected as a reference in the pairwise alignment analysis, *P. calliantha* and *P. pungens* showed 5
335 and 7 nucleotides of difference, respectively. These sequence features allowed us to distinguish
336 these species and/or complex by the HRM assay. The melting curves of all the isolates could be
337 clustered into three groups using the auto-calling mode of the High Resolution Melt software. The
338 identification of the *Pseudo-nitzschia* spp. was made by comparing their values to those of controls,
339 with a confidence interval of between 96–100% for auto-called results. The resulting melt profile
340 reflected the difference in the amplicons and/or GC content. In fact, the different Tm_c of 84.56 ±
341 0.18, 85.22 ± 0.06 and 85.05 ± 0.09, obtained for *P. calliantha*, *P. delicatissima*/*P.* cf. *arenysensis*
342 and *P. pungens* respectively, and the different silhouettes of the curves were due to the presence of
343 the nucleotide differences in the 130 bp amplicons as highlighted above.
344 The HRM assay, which used target *Pseudo-nitzschia* spp. controls, was applied to 29 *Pseudo-*
345 *nitzschia* spp. unidentified isolates collected in the NW Adriatic Sea during a period of survey.
346 Distinct species were identified among isolates, specifically *P. calliantha* (12 isolates), *P. pungens*
347 (7 isolates) and *P. delicatissima*/*P.* cf. *arenysensis* (10 isolates). No cross-reactivity or melt curve
348 overlapping among various species-specific DNAs were obtained. The Tm values were
349 significantly different among the three identified variants of the *Pseudo-nitzschia*.
350 Furthermore, phylogenetic analyses of the LSU and ITS-5.8S rDNA sequences of *Pseudo-nitzschia*
351 spp. isolates identified by the HRM assay confirmed their species-specific taxonomical designation.
352 The phylogenetic inference obtained from rDNA sequences was robust demonstrating that the
353 distinct clades of *P. calliantha, P. pungens, P.* cf. *arenysensis* that were included in the *P.*
354 *delicatissima* complex were supported by high bootstrap values and Bayesian inferences. *P.*
355 *delicatissima* is a cryptic species complex, comprising different genetic lineages[7,11,53], which also
356 includes *P. arenysensis*. Therefore, the HRM assay was able to identify the *P. delicatissima*
357 complex without discriminating the species because of a lack of SNPs in the amplicon between
358 toxic *P. delicatissima* and non toxic *P. arenysensis*. However, it has already been proved that in the
359 NW Adriatic Sea, *P. delicatissima,* as well as *P. arenysensis* strains are non- toxic or have low

360 toxicity[17,44]. In any case, further investigation is needed to better characterize the Adriatic *P.* cf.
361 *arenysensis*. In fact, this phylogenetic clade was distinct from the *P. arenysensis* of other
362 Mediterranean areas, showing that the *P. delicatissima* complex still includes crytptic or
363 semicryptic species.
364 The *Pseudo-nitzschia* taxa detected in the NW Adriatic Sea may be potentially toxic producing
365 species since they are included in the IOC Taxonomy List of Harmful Algae. Further, they are
366 always retrieved in blooming events and/or occurrences of mixed diatom species [54,55] in this area of
367 the Mediterranean where aquaculture farming is widespread.
368 Therefore, a specific detection method able to discriminate harmful *Pseudo-nitzschia* species is a
369 crucial component in the monitoring of HAB species and the potential risk of real toxic events in
370 target coastal areas.
371 In conclusion, the post PCR HRM assay developed in this study appears to be a promising tool for
372 simultaneous detection and discrimination of the *Pseudo-nitzschia* spp. The assay offers several
373 advantages: it is specific, reproducible and rapid when applied to several simultaneously processed
374 cultured samples.
375 The HRM assay depends on available reference genotypes, and it analyzes monoclonal cultures of
376 the species under investigation. In the future, the sampling of potentially numerous harmful species
377 in the Mediterranean Sea could make use of the application range of the HRM assay.
378

379 **Acknowledgments**

380 Financial supports were provided by Regione Marche - Project of coastal monitoring n. 49 of
381 23/12/2013 and ENPI (European Neighborhood and Partnership Instrument) CBCMED (Cross
382 Border Cooperation in the Mediterranean) M3-HABs II-B/2.1/0096. The authors are really grateful
383 to Dr. Luca Galluzzi for his role in the stimulating scientific discussion and Dr. Samuela Capellacci
384 for providing some strains.
385

386 **Author contributions statement**

387 L.P. and A.P. contributed to the conception and design of the study; L.P. carried out the study. L.P.,
388 S.C. and F.P. performed the statistical analyses. F.A. carried out the sequence analysis. A.P.
389 performed the phylogenetic analyses. All authors were involved in the manuscript preparation and
390 revision approval of the final version of the manuscript.

391

392 **Competing financial interests**: the authors declare no competing financial interests.

393

394 **References**
395

396 1. Jin, X., Gruber, N., Dunne, J.P., Sarmiento, J.L. & Armstrong, R.A. Diagnosis the
397 contribution of phytoplankton functional groups to the production and export of particulate
398 carbon, CaCO3, and opal from global nutrient and alkalinity distributions. *Global*
399 *Biogeochem. Cycles*, 20, GB2015 (2006).
400 2. Trainer, V.L. *et al. Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring
401 and impacts on ecosystem health. *Harmful Algae* **14,** 271-300 (2012).
402 3. Bates, S. S. *et al.* Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a
403 toxin in shellfish from eastern Prince Edward Island, Canada. *Can. J. Fish. Aquat. Sci.* **46**,
404 1203-1215 (1989).
405 4. Glibert, P.M. & Burkholder J.M. Harmful algal blooms and eutrophication: "strategies" for
406 nutrient uptake and growth outside the Redfield comfort zone. *Chinese J. Oceanol. Limnol.*
407 **29**, 724-738 (2011).
408 5. Lundholm, N. *et al.* Cryptic and pseudo-cryptic diversity in diatoms-with descriptions of
409 *Pseudo-nitzschia hasleana* sp. nov. and *P. fryxelliana* sp. nov. *J. Phycol.* **48**, 436-454
410 (2012).
411 6. Hasle, G.R. & Lundholm, N. *Pseudo-nitzschia seriata f. obtuse* (Bacillariophyceae) raised in
412 rank based on morphological, phylogenetic and distributional data. *Phycologia* **44**, 608-619
413 (2005).
414 7. Lundholm, N. *et al.* Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima*
415 complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and
416 phylogenetic analyses. *J. Phycol.* **42**, 464–481 (2006).
417 8. Ajani, P., Murray, S., Hallegraeff, G., Brett, S. & Armand, L. First reports of *Pseudo-*
418 *nitzschia micropora* and *P. hasleana* (Bacillariaceae) from the Southern Hemisphere:

419 Morphological, molecular and toxicological characterization. *Phycol. Res.* **61**, 237–248
420 (2013).
421 9. Ruggiero, M.V. *et al.* Diversity and temporal pattern of *Pseudo-nitzschia* species
422 (Bacillariophyceae) through the molecular lens. *Harmful Algae* **42**, 15-24 (2015).
423 10. Sarno, D., Kooistra, W.H.C.F., Medlin, L.K., Percopo, I. & Zingone, A. Diversity in the
424 genus *Skeletonema* (Bacillariophyceae). II. An assessment of the taxonomy of *S. costatum*-
425 like species with the description of four new species. *J. Phycol.* **41**, 151–176 (2005).
426 11. Amato, A. *et al.* Reproductive isolation among sympatric cryptic species in marine diatoms.
427 *Protist* **158**, 193–207 (2007).
428 12. Nanjappa, D., Audic, S., Romac, S., Kooistra, W.H.C.F. & Zingone, A. Assessment of
429 species diversity and distribution of an ancient diatom lineage using a DNA metabarcoding
430 approach. *PLoS One* **9** (8): e103810 (2014).
431 13. Park, T.G. *et al.* Identification of the dinoflagellate community during *Cochlodinium*
432 *polykrikoides* (Dinophyceae) blooms using amplified rDNA melting curve analysis and real-
433 time PCR probes . *Harmful Algae* **8**, 430–440 (2009).
434 14. Fitzpatrick, E., Caron, D.A. & Schnetzer, A. Development and environmental application of
435 a genus-specific quantitative PCR approach for *Pseudo-nitzschia* species. *Mar. Biol.* **157**,
436 1161-1169 (2010).
437 15. Andree, K. B. *et al.* Quantitative PCR coupled with melt curve analysis for detection of
438 selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the northwestern Mediterranean
439 Sea. *Appl. Environ. Microbiol.* 77, 1651-1659 (2011).
440 16. Perini, F. *et al.* New approach using the real-time PCR method for estimation of the toxic
441 marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. *PLoS One* **6** (3): e17699
442 (2011).

443 17. Penna, A. *et al*. Toxic *Pseudo-nitzschia* spp. in the northwestern Adriatic Sea:
444 characterization of species composition by genetic and molecular quantitative analyses. *J.*
445 *Plankton Res.* **35**: 352-366 (2013).
446 18. Hubbard, K.A., Rocap, G. & Armbrust, E.V. Inter- and intraspecific community structure
447 within the diatom genus *Pseudo-nitzschia* (Bacillariophyceae). *J. Phycol.* **44**, 637–649,
448 (2008).
449 19. Galluzzi, L. *et al*. Development of an oligonucleotide microarray for the detection and
450 monitoring of marine dinoflagellates. *J. Microbiol. Meth.* **84,** 234–242 (2011).
451 20. Smith, M.W. *et al*. High resolution microarray assay for rapid taxonomic assessment of
452 *Pseudo-nitzschia* spp. (Bacillariophyceae) in the field. *Harmful Algae* **19,** 169–180 (2012).
453 21. Barra, L., Ruggiero, M.V., Sarno, D., Montresor, M. & Kooistra, W.H.C.F. Strengths and
454 weaknesses of microarray approaches to detect *Pseudo-nitzschia* species in the field.
455 *Environ. Sci. Poll. Res.* **20**, 6705-6718 (2013).
456 22. Barra, L., Ruggiero, M.V., Chen, J. & Kooistra, W.H.C.F. Specificity of LSU rRNA-
457 targeted oligonucleotide probes for *Pseudo-nitzschia* species tested through dot-blot
458 hybridization *Environ. Sci. Poll. Res.* **21**, 548–557 (2014).
459 23. Penna, A. & Galluzzi, L. The quantitative real-time PCR applications in the monitoring of
460 marine harmful algal bloom (HAB) species. *Environ. Sci. Poll. Res.* **20**, 6851–6862 (2013).
461 24. Koyuncu, F., Tas, S., Müftüoğlu, A.E. & Uzonur, I. Genetic heterogeneity characterization
462 of a *P. minimum* bloom sample using DNA melt-curve analysis, high resolution melt-curve
463 analysis and real-time RAPD-PCR *Fresenius Environ. Bull.* **19**, 2404-2410 (2010).
464 25. Al-Kandari, M.A., Highfield, A.C., Hall, M.J., Hayes, P. & Schroeder, D.C. Molecular tools
465 separate harmful algal bloom species, *Karenia mikimotoi*, from different geographical
466 regions into distinct sub-groups. *Harmful Algae* **10**, 636-643 (2011).

467 26. Granados-Cifuentes, C. & Rodriguez-Lanetty, M. The use of high-resolution melting
468 analysis for genotyping *Symbiodinium* strains: a sensitive and fast approach. *Mol. Ecol. Res.*
469 **11**, 394–399 (2011).
470 27. Ceccarelli, M., Galluzzi, L., Migliazzo, A. & Magnani M. Detection and Characterization of
471 *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by SYBR green- based real-time PCR
472 and high resolution melt analysis targeting kinetoplast minicircle DNA. *PLoS One* 9 (2):
473 e88845 (2014).
474 28. Guillard R.R.L. Culture of phytoplankton for feeding marine invertebrates in Culture of
475 Marine Invertebrate Animals. (eds W.L. Smith & M.H Chanley) 26–60 (Plenum Press
476 1975).
477 29. Moschandreou, K.K. *et al.* Inter- and intra-specific diversity of *Pseudo-nitzschia*
478 (Bacillariophyceae) in the northeastern Mediterranean, *Eur. J. Phycol.*, **47**, 321-339 (2012).
479 30. Ruggiero, M.V. *et al.* Diversity and temporal pattern of *Pseudo-nitzschia* species
480 (Bacillariophyceae) through the molecular lens. *Harmful Algae*, **42**, 15–24 (2015).
481 31. Relini, G. Checklist della Flora e della Fauna dei Mari Italiani (Parte II). *Biol. Mar.*
482 *Mediterr.* **17** (Suppl. 1) 387-828 (2010).
483 32. Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis
484 program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**, 95-98 (1999).
485 33. Ye, J. *et al.* Primer-BLAST: a tool to design target-specific primers for polymerase chain
486 reaction. *BMC Bioinformatics* **13**, 134 (2012).
487 34. Scholin, C.A., Herzog, M., Sogin, M. & Anderson, D.M. Identification of group and strain-
488 specific genetic markers from globally distributed *Alexandrium* (Dinophyceae). II. Sequence
489 analysis of fragments of the LSU rRNA gene. *J. Phycol.* **30**, 999-1011 (1994).
490 35. Adachi, M., Sako, Y. & Ishida, Y. Restriction fragment length polymorphism of ribosomal
491 DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species
492 (Dinophyceae). *J. Phycol.* **30**, 857–863, (1994).

493 36. Pin, L.C., Teen, L.P., Ahmad, A. & Usup, G. Genetic Diversity of *Ostreopsis ovata*
494 (Dinophyceae) from Malaysia. *Mar. Biotechnol.* **3**, 246–255 (2001).
495 37. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum
496 likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**,
497 2731-2739 (2011).
498 38. Ronquist, F. & Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed
499 models. *Bioinformatics* **19**, 1572-1574 (2003).
500 39. D'Alelio, D. *et al.* Internal transcribed spacer polymorphism in *Pseudo-nitzschia*
501 *multistriata* (Bacillariophyceae) in the Gulf of Naples: recent divergence or intraspecific
502 hybridization? *Protist* **160**, 9-20 (2009).
503 40. Metfies, K. & Medlin, L.K. Feasibility of transferring fluorescent in situ hybridization
504 probes to an 18S rRNA gene phylochip and mapping of signal intensities. *Appl. Environ.*
505 *Microbiol.* **74**, 2814-2821 (2008).
506 41. Kudela, R.M., Howard, M.D.A., Jenkins, B.D., Miller, P.E., & Smith, G.J. Using the
507 molecular toolbox to compare harmful algal blooms in upwelling systems. *Progr.*
508 *Oceanogr.*, **85**, 108–121 (2010).
509 42. Totti, C. *et al.* Seasonal variability of phytoplankton populations in the middle Adriatic sub-
510 basin. *J. Plankton Res.* **22**, 1735-1756 (2000).
511 43. Cerino, F. *et al.* The alternation of different morphotypes in the seasonal cycle of the toxic
512 diatom *Pseudo-nitzschia galaxiae*. *Harmful Algae* **4**, 33-48 (2005).
513 44. Quijano-Scheggia, S. *et al. Pseudo-nitzschia* species on the Catalan coast: characterization
514 and contribution to the current knowledge of the distribution of this genus in the
515 Mediterranean Sea. *Sci. Mar.* **74**, 395–410 (2010).
516 45. Aubry, B. F., Berton, A., Bastianini, M., Socal, G. & Acri, F. Phytoplankton succession in a
517 coastal area of the NW Adriatic, over a 10-year sampling period (1990–1999). *Cont. Shelf*
518 *Res.* **24**, 97–115 (2004).

519 46. Caroppo, C. *et al.* Phytoplankton dynamics with a special emphasis on harmful algal blooms
520 in the Mar Piccolo of Taranto (Ionian Sea, Italy). *Environ. Sci. Pollut. Res.* **23**, 12691–
521 12706 (2015).
522 47. Zhang, F. & Li, Z. Detection and quantification of cultured marine *Alexandrium* species by
523 real-time PCR. *World J. Microbiol. Biotechnol.* **28**, 3255–3260 (2012).
524 48. Dittami, S. M., Pazos, Y., Laspra, M. & Medlin, L. K. Microarray testing for the presence of
525 toxic algae monitoring programme in Galicia (NW Spain). *Environ. Sci. Pollut. Res.* **20**,
526 6778–6793 (2013).
527 49. Scholin, C.A. *et al.* DNA probes and a receptor-binding assay for detection of *Pseudo-*
528 *nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural
529 samples. *J. Phycol.* **35**, 1356-1367 (1999).
530 50. Toebe, K. Whole cell hybridisation for monitoring harmful marine microalgae. *Environ. Sci.*
531 *Pollut. Res.* ***20***, 6816–6823 (2013).
532 51. Scholin C. *et al.* Remote detection of marine microbes, small invertebrates, harmful algae,
533 and biotoxins using the environmental sample processor (ESP). *Oceanography* **22**, 158-167
534 (2009).
535 52. McDonald, S.M., Sarno, D. & Zingone, A. Identifying *Pseudo-nitzschia* species in natural
536 samples using genus-specific PCR primers and clone libraries. *Harmful Algae* **6**, 849–860
537 (2007).
538 53. Quijano-Scheggia, S.I. *et al.* Morphology, physiology, molecular phylogeny and sexual
539 compatibility of the cryptic *Pseudo-nitzschia delicatissima* complex (Bacillariophyta),
540 including the description of *P. arenysensis* sp. Nov. *Phycologia* **48**, 492-509 (2009).
541 54. Caroppo, C., Congestri, R., Bracchini, L. & Albertano, P. On the presence of *Pseudo-*
542 *nitzschia calliantha* Lundholm, Moestrup et Hasle and *Pseudo-nitzschia delicatissima*
543 (Cleve) Heiden in the Southern Adriatic Sea (Mediterranean Sea, Italy). *J. Plankton Res.* **27**,
544 763–774 (2005).

545 55. Maric, D. *et al*. Blooms of the potentially toxic diatom *Pseudo-nitzschia calliantha*
546 Lundholm, Moestrup & Hasle in coastal waters of the northern Adriatic Sea (Croatia).
547 *Estuar. Coast. Shelf Sci.* **92**, 323–331 (2011).
548
549

550 **Table 1.** List of *Pseudo-nitzschia* spp. strains isolated from the NW Adriatic
551 Sea 3000 m off the coast. The strains were analyzed and subsequently identified
552 by the HRM assay.

Strain	Species identified by HRM	Collection site	Sampling date
CBA 172	*P. calliantha*	Tavollo	20 November 2014
CBA 173	*P. calliantha*	Tavollo	20 November 2014
CBA 181	*P. calliantha*	Tavollo	19 March 2015
CBA 183	*P. calliantha*	Tavollo	19 March 2015
CBA 189	*P. calliantha*	Tavollo	19 March 2015
CBA 191	*P. calliantha*	Metauro	20 March 2015
CBA 192	*P. calliantha*	Metauro	20 March 2015
CBA 193	*P. calliantha*	Tavollo	20 March 2015
CBA 194	*P. calliantha*	Metauro	20 March 2015
CBA 198	*P. calliantha*	Tavollo	13 January 2015
CBA 203	*P. calliantha*	Tavollo	13 January 2015
CBA 205	*P. calliantha*	Tavollo	13 January 2015
CBA 159	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	20 November 2014
CBA 161	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	20 November 2014
CBA 163	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	20 November 2014
CBA 165	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	20 November 2014
CBA 166	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	20 November 2014
CBA 167	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	13 January 2015
CBA 168	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	13 January 2015
CBA 169	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	13 January 2015
CBA 170	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	13 January 2015
CBA 171	*P. delicatissima/P.* cf. *arenysensis*	Tavollo	13 January 2015
CBA 179	*P. pungens*	Metauro	20 March 2015

CBA 180	*P. pungens*	Tavollo	20 March 2015
CBA 182	*P. pungens*	Tavollo	19 March 2015
CBA 184	*P. pungens*	Tavollo	19 March 2015
CBA 186	*P. pungens*	Tavollo	19 March 2015
CBA 2S	*P. pungens*	Foglia	10 February 2015
CBA 3S	*P. pungens*	Foglia	10 February 2015

553

554

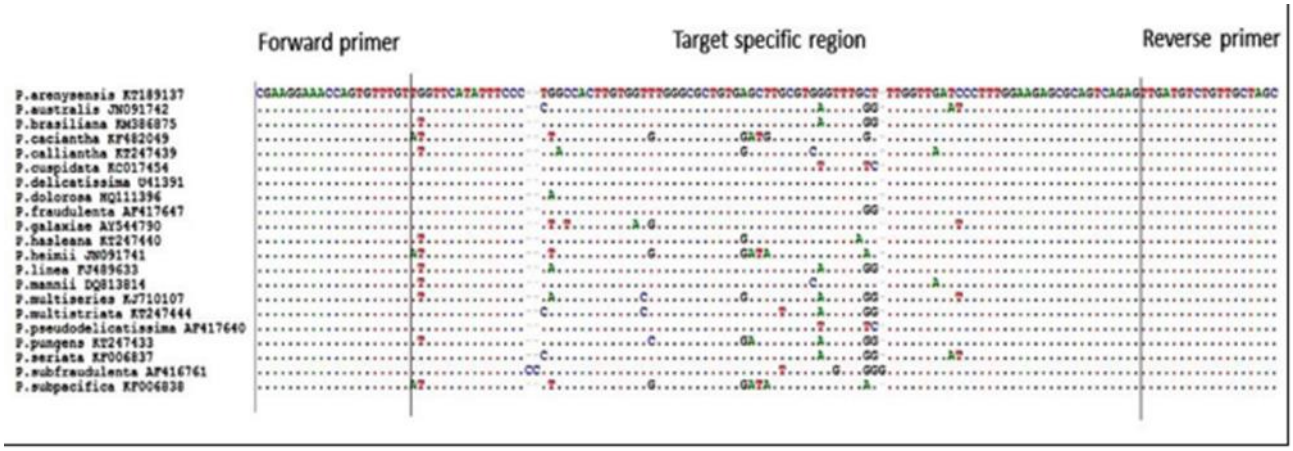


Figure 1. The alignment of consensus LSU rDNA sequences of 21 *Pseudo-nitzschia* species. The sequences were aligned using the Bioedit Sequence Alignment v. 7.0.5.3. The common forward and reverse primers flanked species-specific regions of the 21 *Pseudo-nitzschia* species considered under investigation. The target LSU rDNA amplified region was 130-133 bp in length and located in the 384 to 514 nucleotide position.

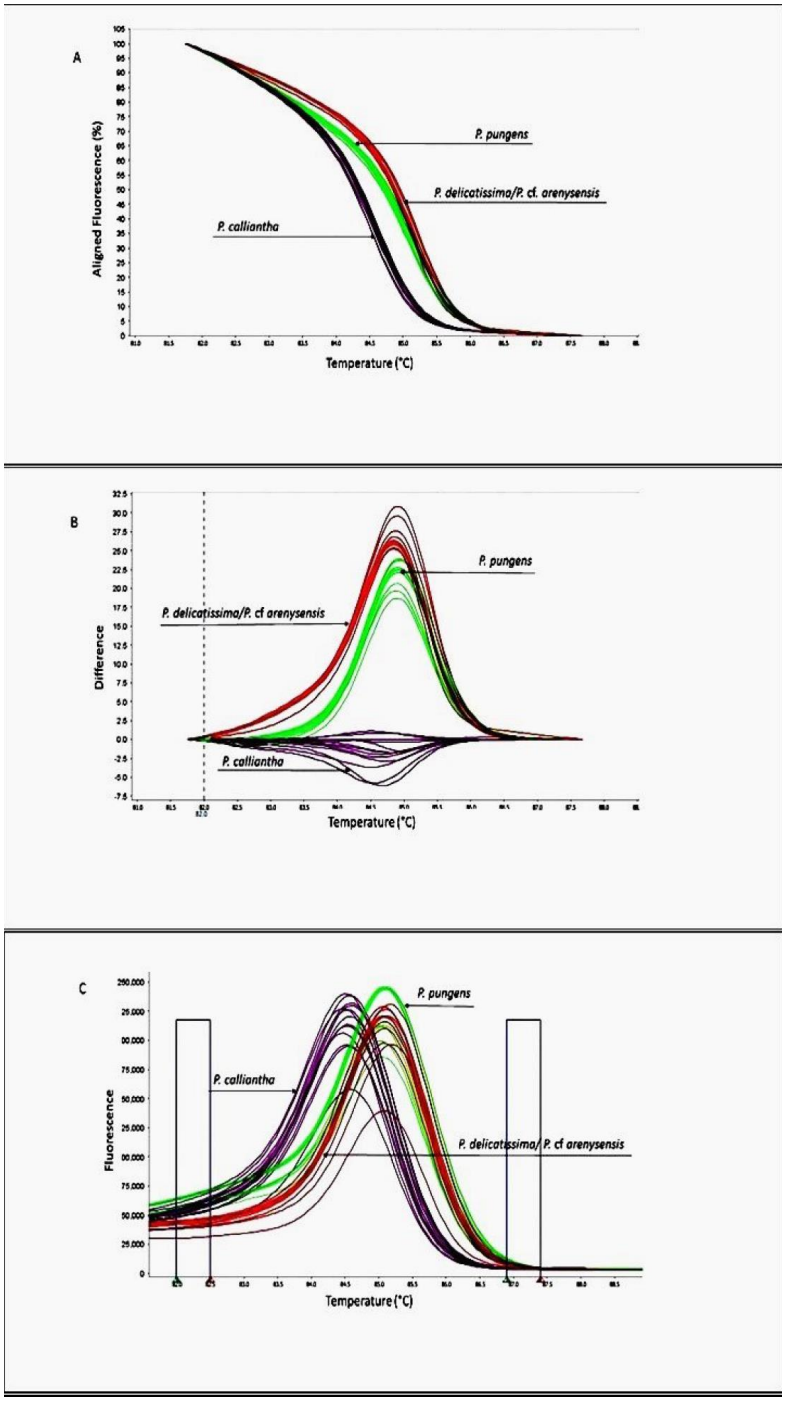


Figure 2. Melting curve variance of the *P. calliantha* (n = 12 strains), *P. delicatissima/P.* cf. *arenysensis* (n = 10 strains) and *P. pungens* (n = 7 strains) in **(A)** aligned, **(B)** difference and **(C)** derivative plot analyses; from left to right, vertical bars represent the pre and post – melt regions. A melting curve generated by a DNA positive control for each taxon was also included. Only one replicate of the HRM assay experiment for each strain is shown.

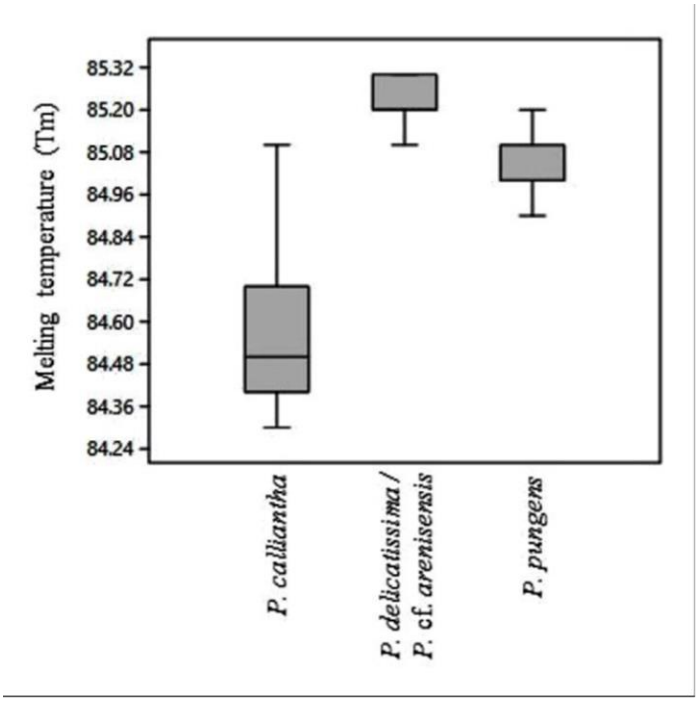


Figure 3. Box plot showing melting curve temperatures (Tm) of *Pseudo-nitzschia* spp. isolates collected in the NW Adriatic Sea and used in this study. The Kruskall Wallis test shows significant differences in average Tm values among species ($p<0.001$).

Supplementary material

Scientific Reports

A high resolution melting method for the molecular identification of the potentially toxic diatom *Pseudo-nitzschia* spp. in the Mediterranean Sea

Laura Pugliese[1], Silvia Casabianca[1,2], Federico Perini[1], Francesca Andreoni[1], Antonella Penna[1,2,3]*
[1]Department of Biomolecular Sciences, University of Urbino, Viale Trieste 296, 61121 Pesaro, Italy
[2]Conisma, Consorzio Interuniversitario per le Scienze del Mare, Pz. Flaminio 9, 00196 Rome, Italy
[3]CNR–Institute of Marine Sciences (ISMAR), Largo Fiera della Pesca 60125 Ancona, Italy

*corresponding author: email: antonella.penna@uniurb.it

Table S1. List of *Pseudo-nitzschia* spp. strains isolated from the NW Adriatic Sea, at Pesaro, 500 m off the coast, used in the HRM development assay.

Species	Strain	Sampling date
P. cf. *arenysensis*	CBA 160	10 February 2013
P. calliantha	CBA 59	18 September 2009
P. calliantha	CBA 62	18 September 2009
P. calliantha	CBA 70	24 October 2009
P. calliantha	CBA 71	24 October 2009
P. calliantha	CBA 72	24 October 2009
P. calliantha	CBA 73	24 October 2009
P. calliantha	CBA 74	24 October 2009
P. delicatissima	CBA 131	19 March 2010
P. delicatissima	CBA 133	19 March 2010
P. delicatissima	CBA 144	15 April 2010
P. delicatissima	CBA 145	15 April 2010
P. delicatissima	CBA 150	12 May 2010
P. delicatissima	CBA 152	12 May 2010
P. delicatissima	CBA 153	12 May 2010
P. pungens	CBA 94	30 December 2009
P. pungens	CBA 100	20 January 2010
P. pungens	CBA 101	20 January 2010
P. pungens	CBA 102	20 January 2010
P. pungens	CBA 103	20 January 2010
P. pungens	CBA 105	20 January 2010
P. pungens	CBA 111	20 January 2010

Table S2. List of *Pseudo-nitzschia* spp. isolates, sampling location, LSU and ITS-5.8S gene sequence accession numbers. Isolates and sequence codes in bold are from this study.

Species	Strain ID	Geographical origin	Accession no. LSU	Accession no. ITS
P. arenysensis	14V	Spain	AY764136	AY764136
P. arenysensis	2b	California, USA	KT189137	-
P. arenysensis	AL-24	Naples, Italy, Tyrrhenian Sea	DQ813811	-
P. arenysensis	AL-11	Naples, Italy, Tyrrhenian Sea	-	DQ813840
P. cf. *arenysensis*	**CBA159**	Pesaro, Italy, Adriatic Sea	**LT596179**	**LT596202**
P. cf. *arenysensis*	**CBA160**	Pesaro, Italy, Adriatic Sea	**LT596193**	-
P. cf. *arenysensis*	**CBA161**	Pesaro, Italy, Adriatic Sea	**LT596190**	-
P. cf. *arenysensis*	**CBA163**	Pesaro, Italy, Adriatic Sea	**LT596180**	**LT596194**
P. cf. *arenysensis*	**CBA165**	Pesaro, Italy, Adriatic Sea	**LT596181**	-
P. cf. *arenysensis*	**CBA166**	Pesaro, Italy, Adriatic Sea	**LT596200**	-
P. cf. *arenysensis*	**CBA167**	Pesaro, Italy, Adriatic Sea	**LT596192**	**LT596195**
P. cf. *arenysensis*	**CBA168**	Pesaro, Italy, Adriatic Sea	**LT596201**	-
P. cf. *arenysensis*	**CBA169**	Pesaro, Italy, Adriatic Sea	**LT596182**	-
P. cf. *arenysensis*	**CBA170**	Pesaro, Italy, Adriatic Sea	**LT596189**	-
P. cf. *arenysensis*	**CBA171**	Pesaro, Italy, Adriatic Sea	**LT596191**	-
P. arenysensis	ICMB130	Barcelona, Spain	-	EU367952
P. caciantha	AL-56	Naples, Italy, Tyrrhenian Sea	DQ813812	DQ813834
P. calliantha	AL-112	Naples, Italy, Tyrrhenian Sea	DQ813815	DQ813841
P. calliantha	CBA62	Pesaro, Italy, Adriatic Sea	**LT596176**	HE663423
P. calliantha	CBA72	Pesaro, Italy, Adriatic Sea	**LT596175**	HE663433
P. calliantha	CBA192	Pesaro, Italy, Adriatic Sea	**LT596184**	-
P. calliantha	CBA193	Pesaro, Italy, Adriatic Sea	-	**LT596205**
P. calliantha	CBA194	Pesaro, Italy, Adriatic Sea	**LT596183**	**LT596206**
P. cuspidata	AL-17	Naples, Italy, Tyrrhenian Sea	DQ 813809	DQ813827
P. cuspidata	PA7	Sydney, Australia	KC017453	-
P. delicatissima	1001 2 B	Copenhagen, Denmark	AF417645	-
P. delicatissima	ICMB134	Tarragona, Spain	-	EU327383
P. delicatissima	AL 22	Naples, Italy, Tyrrhenian Sea	DQ813810	DQ813832
P. delicatissima	AY4	Gauteng, South Africa	EF522107	-
P. delicatissima	CBA144	Pesaro, Italy, Adriatic Sea	**LT596187**	HE650934
P. delicatissima	CBA145	Pesaro, Italy, Adriatic Sea	**LT596188**	HE650935
P. delicatissima	CLA1.A1	Gauteng, South Africa	EF522114	-
P. dolorosa	AL-59	Naples, Italy, Tyrrhenian Sea	DQ813813	DQ813835
P. fraudulenta	LIMENS1	Copenhagen, Denmark	AF 417647	AY257840
P. fukuyoi	PnTb72	Sarawak, Malaysia	KC147537	-
P. hasleana	HAWK3/1	Sydney, Australia	KC 017446	-
P. hasleana	HAWK4	Sydney, Australia	-	KC017468
P. hasleana	NWFSC186	Copenhagen, Denmark	JN050298	-
P. inflatula	No7	Copenhagen, Denmark	-	DQ329204
P. mannii	AL-101	Naples, Italy, Tyrrhenian Sea	DQ813814	DQ813839
P. micropora	VPB-B3	Copenhagen, Denmark	AF 417649	AY257847

P. multiseries	OFPm984	Copenhagen, Denmark	AF417655	DQ062664
P. multistriata	**CBA174**	Pesaro, Italy, Adriatic Sea	**LT596185**	**LT596196**
P. multistriata	PSM11	Primorsky krai, Russian Federation	-	KT247444
P. multistriata	SZN-B31	Naples, Italy, Tyrrhenian Sea	AF416756	-
P. multistriata	SZN-B32	Naples, Italy, Tyrrhenian Sea	AF416757	-
P. pseudodelicatissima	8A 14	Thermaikos Gulf, Greece	FJ859054	-
P. pseudodelicatissima	9A 1	Thermaikos Gulf, Greece	FJ859055	-
P. pseudodelicatissima	AL-15	Naples, Italy, Tyrrhenian Sea	DQ 813808	DQ813826
P. pseudodelicatissima	**CBA175**	Pesaro, Italy, Adriatic Sea	**LT596186**	-
P. pseudodelicatissima	P11	Helsingor, Denmark	AF 417640	AY257854
P. pseudodelicatissima	SZN-B545	Naples, Italy, Tyrrhenian Sea	KF241716	-
P. pungens	CBA100	Pesaro, Italy, Adriatic Sea	**LT596177**	HE650958
P. pungens	CBA111	Pesaro, Italy, Adriatic Sea	**LT596178**	HE650968
P. pungens	CBA179	Pesaro, Italy, Adriatic Sea	**LT596197**	**LT596203**
P. pungens	CBA180	Pesaro, Italy, Adriatic Sea	**LT596198**	**LT596204**
P. pungens	CBA182	Pesaro, Italy, Adriatic Sea	**LT596199**	-

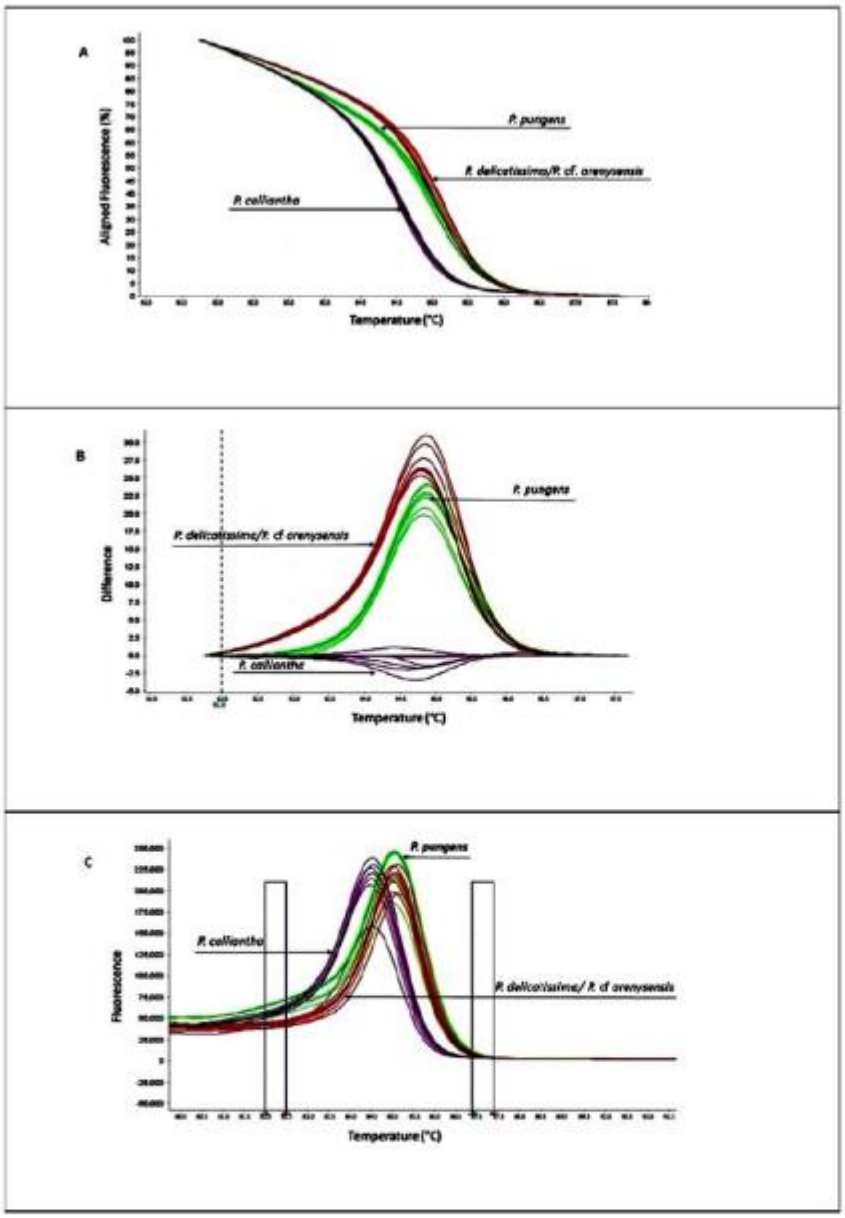


Figure S1. Melting curve variance of the three *Pseudo-nitzschia* species controls (*P. calliantha,* n = 7 strains, *P. delicatissima/P. cf. arenysensis,* n = 8 strains, *P. pungens* n = 7 strains) in (A) aligned, (B) difference and (C) derivative plot analyses; from left to right vertical bars represent the pre and post – melt regions. Only one replicate of the HRM assay experiment for each strain is shown.

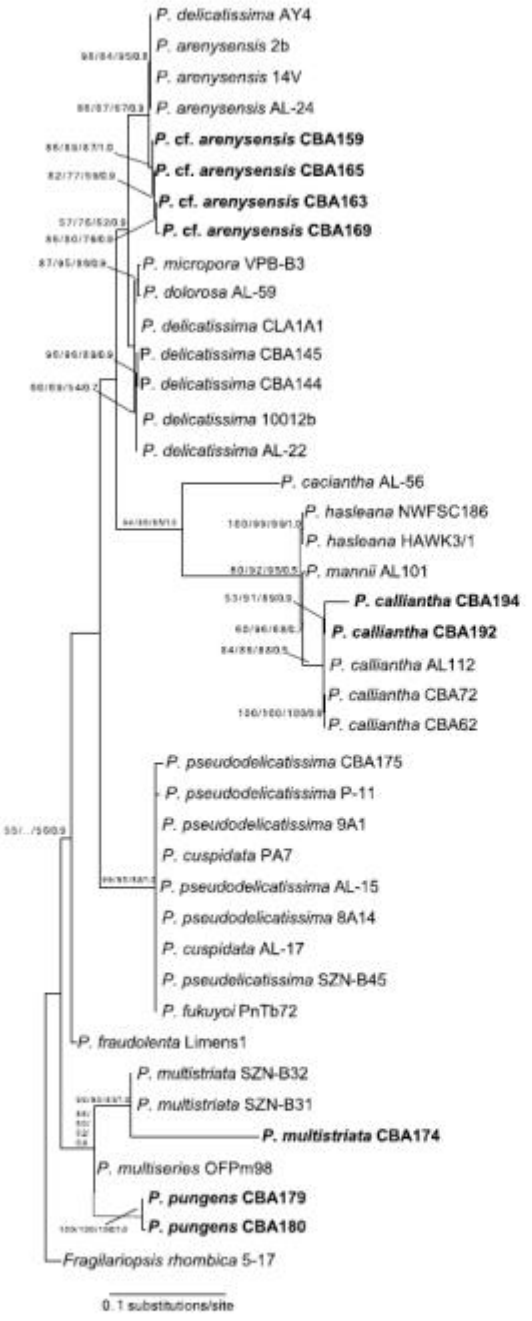


Fig. S2. Maximum likelihood phylogenetic tree of the genus *Pseudo-nitzschia* inferred from LSU rDNA. The tree was rooted with *Fragilariopsis rhombica* 5-17 as outgroup. Numbers of the major nodes represented from left to right or from upper to lower NJ (1000 pseudo-replicates), MP (1000 pseudo-replicates), ML (1000 pseudo-replicates) bootstrap and Bayesian posterior probability values. Only bootstrap values > 50% were shown. All sequences of bold isolates were obtained in this study.

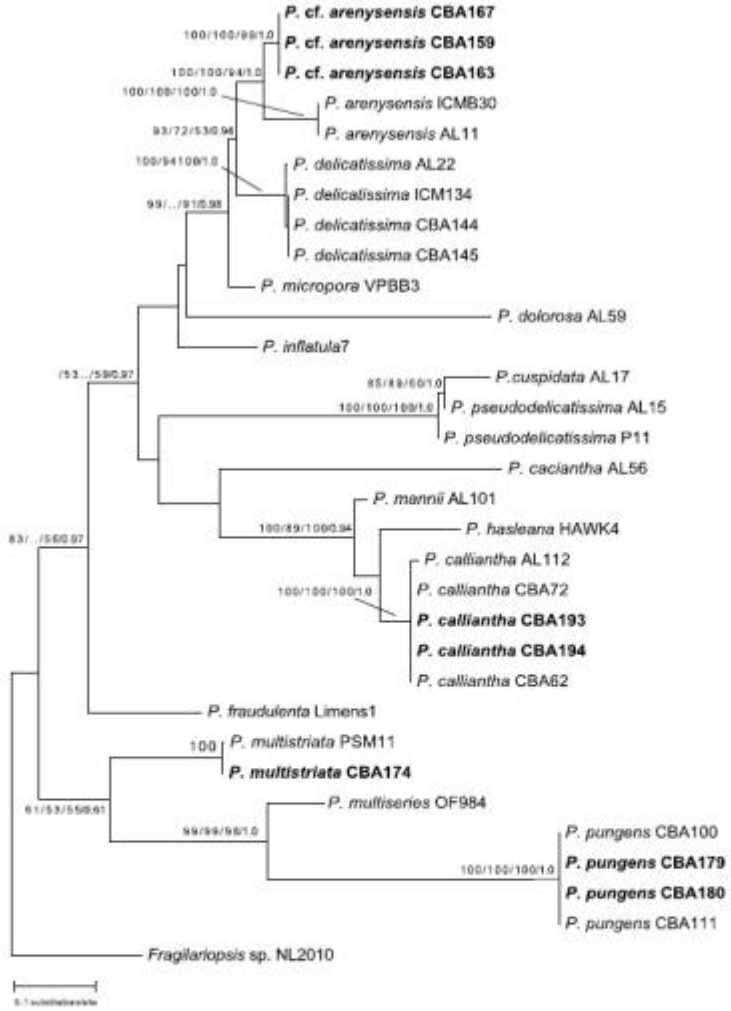


Fig. S3. Maximum likelihood phylogenetic tree of the genus *Pseudo-nitzschia* inferred from ITS-5.8S rDNA. The tree was rooted with *Fragilariopsis* sp. NL2010 as outgroup. Numbers of the major nodes represented from left to right NJ (1000 pseudo-replicates), MP (1000 pseudo-replicates), ML (1000 pseudo-replicates) bootstrap and Bayesian posterior probability values. Only bootstrap values > 50% were shown. All sequences of bold isolates were obtained in this study.

CAPITOLO 4

LE DINOFLAGELLATE

4. Le Dinoflagellate

4.1. Generalità

Le dinoflagellate sono organismi eucarioti e unicellulari, componenti principali del fitoplancton sia marino che di acqua dolce. Sono presenti a tutte le latitudini, prevalentemente in acque costiere temperate e in condizioni di stabilità della colonna d'acqua. Le loro dimensioni variano da 10 μm a 200 μm. Le dinoflagellate sono anche note per la loro importanza nella datazione e nella correlazione stratigrafica, in quanto il loro ciclo vitale prevede lo sviluppo di una forma di resistenza (cisti) che può depositarsi nel sedimento e lì diventare fossile.

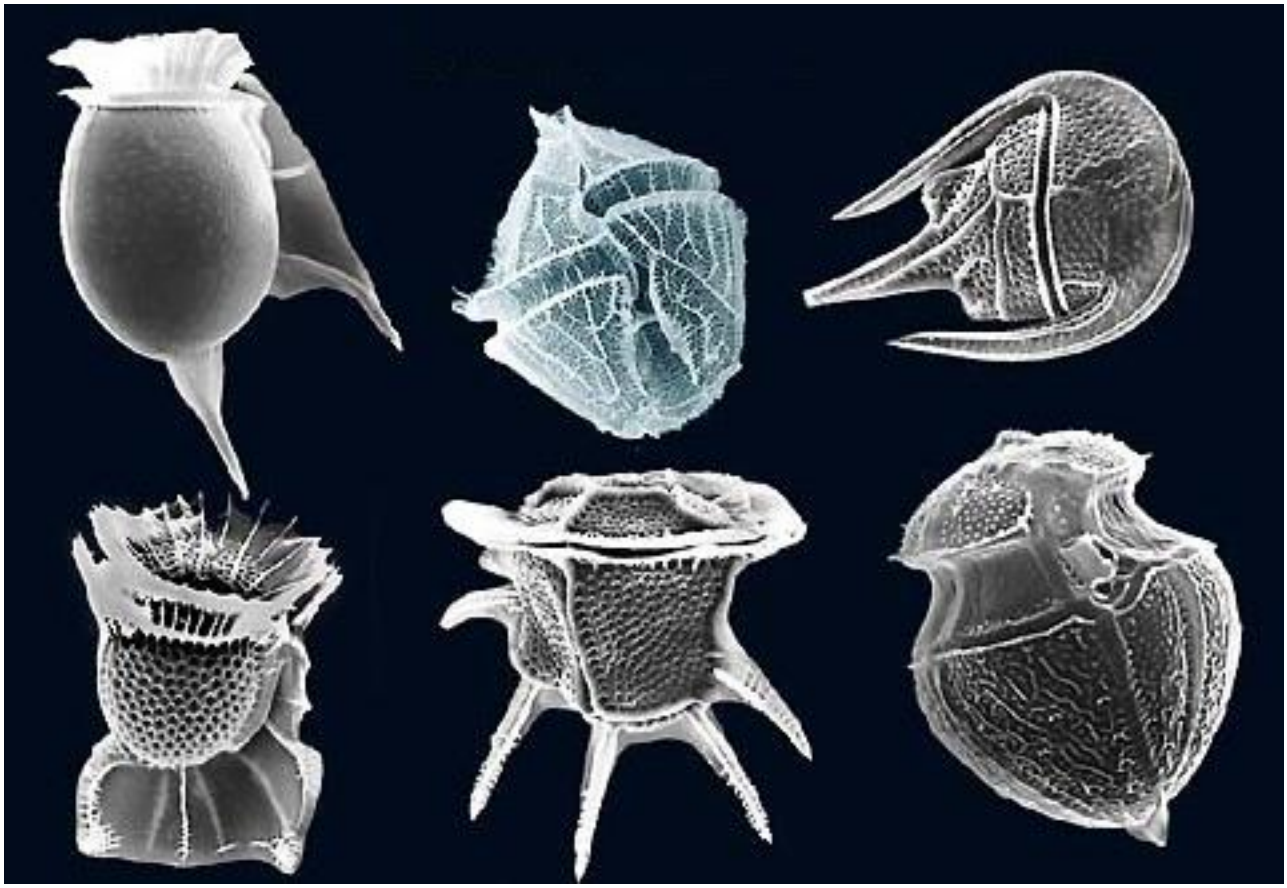


Figura 9. Immagine di varie dinoflagellate. www.flickr.com

4.1.1. Descrizione morfologica

Le dinoflagellate possono presentare diverse morfologie. La struttura morfologica tipica è caratterizzata dalla presenza di due solchi che attraversano il corpo cellulare: il solco trasversale (cingolo), che divide la cellula in una parte anteriore (episoma/epiteca) e in una parte posteriore

(iposoma/ipoteca); il solco longitudinale che incide ventralmente la parte posteriore della cellula dividendola in due metà.

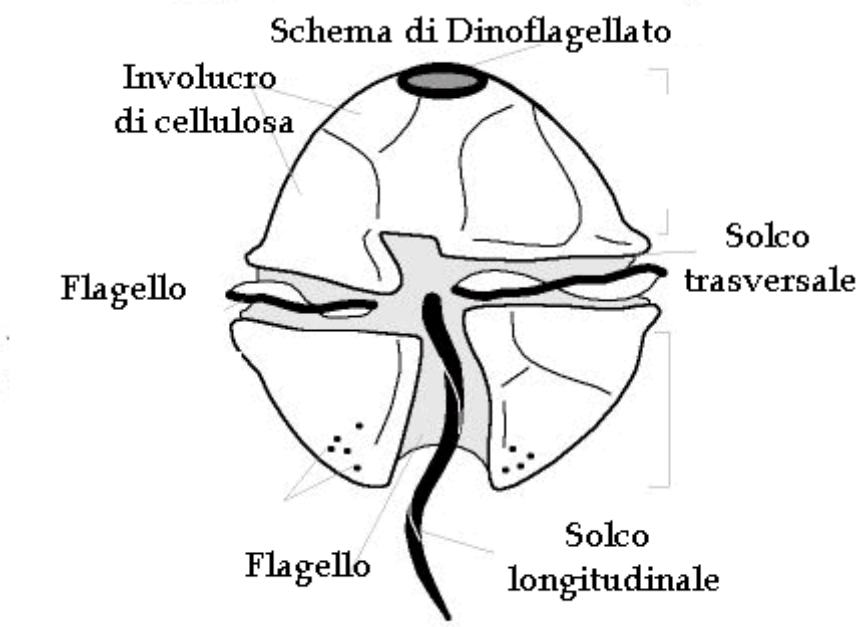


Figura 10: Struttura tipica delle dinoflagellate. http://omodeo.anisn.it/

Le dinoflagellate si muovono grazie alla presenza di due flagelli: il flagello trasversale disposto circolarmente attorno alla cellula e contenuto nel cingolo, e il flagello longitudinale diretto posteriormente e inserito nella sua parte prossimale nel solco. Il flagello longitudinale determina la direzione della cellula mentre il flagello trasversale funge da propulsore. L'azione combinata dei due flagelli rende così possibile un avanzamento a spirale tipico di queste cellule.

Il citoplasma delle dinoflagellate contiene tutti gli organuli delle cellule eucariote. Il numero e la forma dei cloroplasti, nelle forme fotosintetizzanti, sono molto variabili e contengono clorofilla a e clorofilla c2, β-carotene e xantofille (peridinina, neoperidinina, dinoxantina e neodinoxantina). Una caratteristica peculiare delle cellule delle dinoflagellate è la presenza della pusula, che è costituita da un sistema di vacuoli specializzati. La funzione della pusula è quella di aprirsi verso l'ambiente esterno attraverso il canale flagellare, ed è probabilmente correlata a fenomeni di osmoregolazione, escrezione e assorbimento (Dodge e Crawford, 1970). La pusula è presente in tutte le forme marine ed è più sviluppata nelle forme eterotrofe, ma è assente nelle forme simbionti. Numerose tricocisti sono spesso presenti nelle dinoflagellate; si tratta di corpi di natura proteica probabilmente sviluppati da vescicole derivate dall'apparato del Golgi, di forma bastoncellare, avvolte da una membrana e generalmente situate nella parte periferica del citoplasma (Leadbeater e Dodge, 1966; Leadbeater e Dodge, 1967; Dodge, 1987, Honsell et al., 2013).

Queste strutture vengono espulse verso l'esterno, con un meccanismo legato alla modificazione della loro conformazione sterica, che determina un allungamento pari a otto volte la loro lunghezza. Con l'espulsione delle tricocisti, la cellula subisce una spinta in direzione opposta; sembra che questa espulsione venga adottata dalla cellula come meccanismo di difesa.

4.1.2. Formazione delle cisti

Al momento della formazione della cisti, la cellula della dinoflagellata è interessata da cambiamentimetabolici considerevoli. Inizialmente, Bibby & Dodge (1972) hanno descritto un incremento diamido nei tilacoidi e un accumulo di lipidi nei plastidi (riserve energetiche), la scomparsa dei corpi di Golgi, un incremento del numero di vacuoli e un accumulo di corpi poliedrici. Tutti questi fenomeni sono associati a un abbassamento dei processi metabolici della cellula, particolarmente nei plastidi. L'accumulo di corpi colorati (eye-spots) è dovuto alla concentrazione di pigmenti (carotenoidi e xantofille) che sono i maggiori prodotti della degenerazione dei plastidi. Gli eye-spots possono essere completamente liberi nel citoplasma (Dodge, 1973) oppure di forma globulare e costituiti da diversi strati lipidici, circondati da una tripla membrana, come in *Peridinium balticum* (LEVANDER) LEMMERMAN (Dodge, 1969; Tomas e Cox, 1973). Una volta sintetizzati, questi carotenoidi, sono composti che apparentemente non possono essere demoliti, probabilmente perché nelle cellule mancano gli enzimi deputati a tale processo. Le clorofille, ricche di azoto, sono invece verosimilmente catabolizzate e possono eventualmente diventare componenti dei cristalli che si accumulano in grande numero nei vacuoli. I corpi poliedrici accumulati nei vacuoli hanno funzione di riserva di azoto per la cellula dormiente. Il fatto che le cisti di *Gonyaulax tamarensis var. excavatum* BRAARUD sono dieci volte più tossiche della loro cellula motile (Dale et al., 1978) rappresenta il fondamento dell'ipotesi sul ruolo dell'azoto nella tossina PSP.

Tali cambiamenti metabolici si completano con la perdita dei flagelli: la cellula diventa non motile (ipnozigote) e si formano una o due membrane che si consolidano formando la parete della cisti. Da questo momento la cisti sedimenta attraverso la colonna d'acqua e diventa componente stessa del sedimento. La cisti rimane nel sedimento in questa fase dormiente fino alla comparsa dei fattori che ne indurranno la germinazione. La produzione di cisti sembra essere conseguenza di condizioni ambientali sfavorevoli date da cambiamenti chimico-fisici (diminuzione di temperatura, differenti condizioni di salinità) o dalla pressione ecologica, mentre è un fenomeno generalmente raro in situazioni ambientali stabili. Dopo un periodo di dormienza, se si verificano condizione ambientali favorevoli la cisti germina e il ciclo si chiude con una divisione meiotica, producendo nuove cellule, ognuna delle quali svilupperà flagelli e teca

4.2. Dinoflagellate e tossine

4.2.1. Tossine DSP

DSP (Diarrhetic Shellfish Poisoning): sindrome di avvelenamento associata all'ingestione di tossine costituite da un gruppo di molecole liposolubili (polieteri ciclici) divisi in tre classi strutturali, associate a differenti effetti tossicologici, che sono l'acido okadaico e i suoi derivati, le pectenotosssine e le yessotossine. Le biotossine accumulate attraverso il filter-fedding nell'epatopancreas dei molluschi eduli lamellibranchi (mitili, vongole, ostriche) (Cabrini et al., 1994), possono arrivare attraverso la catena alimentare ad avvelenare anche l'uomo (nessun trattamento con alte o basse temperature ha la capacità di inattivare la tossina). L'avvelenamento è caratterizzato da disturbi a carico dell'apparato gastrointestinale che comprendono nausea, vomito, diarrea, dolori addominali, mal di testa e febbre. Non si sono verificati mai casi mortali e il recupero è completo. L'insorgenza della sindrome DSP (il limite massimo di contenuto di acido okadaico nei mitili è di 160 µg/kg, mentre il limite massimo di contenuto del gruppo delle yessotossine è di 3,75 mg/kg) può verificarsi in un lasso di tempo che va dai 30 minuti alle 2-3 ore, con sintomi che si protraggono generalmente per 2-3 giorni (Adema, 1978; Yasumoto et al., 1985). Le tossine DSP sono prodotte da dinoflagellate appartenenti ai generi *Dinophysis* (*Dinophysis fortii* PAVILLARD; *Dinophysis sacculus* STEIN; *Dinophysis acuta* EHRENBERG; *Dinophysis caudata* SAVILLE-KENT; *Dinophysis rotundata* CLAPARÈDE et LACHMANN) e *Prorocentrum lima*. Nel Mare Adriatico è stato dimostrato che altre due specie di dinoflagellate producono tossine DSP e precisamente *Lingulodinium polyedrum* (roduce homo-yessotossina (un analogo della yessotossina) (Satake et al.,1996; Tubaro et al.,1997) e *Gonyaulax grindleyi* REINECKE produce yessotossina.

4.2.2. Tossine PSP

PSP (Paralytic Shellfish Poisoning): sindrome di avvelenamento associata all'ingestione di saxitossina e dei suoi 21 analoghi che sono neurotossine idrosolubili termostabili aventi effetto paralizzante. Le saxitossine si concentrano in diverse specie di molluschi bivalvi e alcuni crostacei (aragoste) e sono trasferite all'uomo attraverso la catena alimentare. La dose di saxitossina letale per l'uomo è di 0.5 mg per kg di peso corporeo. L'estrema tossicità delle saxitossine è dovuta a un'azione di blocco a livello dell'assone sui canali del sodio. Le biotossine PSP possono uccidere direttamente i pesci; alcune specie di interesse commerciale (aringhe, acciughe e merluzzi) possono fungere da

indicatori della presenza della biotossina PSP dato che muoiono prima che nei loro tessuti muscolari la tossina raggiunga concentrazioni pericolose per la salute umana. La legislazione italiana ha posto il limite di tolleranza per le tossine PSP nei molluschi bivalvi di 800 μg/kg (EFSA European Food Safety Authority, 2009). I primi sintomi di avvelenamento possono insorgere entro 10-30 minuti, in seguito al consumo di prodotti ittici contaminati, con vomito, diarrea dolori addominali, parestesia e bruciore alle labbra, alle gengive, alla lingua, al viso, al collo e alle estremità. In seguito possono manifestarsi brevità di respiro, secchezza delle fauci, disturbi della parola e perdita di coordinazione; infine, può sopraggiungere la morte tra le 2-12 ore. Per impedire la morte si può provare l'unico intervento possibile che è rappresentato da somministrazioni per via orale di sospensioni di carbone attivo in acqua, al fine di tentare una rimozione meccanica per adsorbimento del tossico prima che questo possa essere assorbito dall'organismo (Jellet et al., 1992; Lipkind e Fozzard, 1994) e una terapia sintomatica per combattere la paralisi respiratoria (Auerbach, 1988). Le tossine di tipo PSP sono prodotte da specie algali appartenenti al genere *Alexandrium* (*Alexandrium tamarense*; *Alexandrium minutum*; *Alexandrium pacificum Alexandrium fundyense*. *Gymnodinium catenatum* è un'altra specie che produce tossine PSP che ha un'ampia distribuzione geografica, dalla costa atlantica della Spagna alla costa pacifica dell'America, al Giappone e all'Australia. Nel Mediterraneo sono presenti specie del genere *Alexandrium*.

4.2.3. Tossine NSP

NSP (Neurotoxic Shellfish Poisoning): sindrome di avvelenamento associata all'ingestione di un gruppo di poliesteri liposolubili, le cosiddette brevetossine, che possiedono una spiccata azione neurotossica. Le strutture molecolari comprendono più di 10 anelli e oltre 10 stereoisomeri per ciascun tipo di brevetossina. La tossicità delle brevetossine si esplica mediante azione sui canali del sodio presenti sulle membrane cellulari del nervo o del muscolo, con conseguente influsso di questi ioni nella cellula. I sintomi che caratterizzano l'avvelenamento a carico del sistema nervoso e dell'apparato gastrointestinale includono formicolio e insensibilità delle labbra, della lingua e della gola, dolori muscolari, vertigini, diarrea e vomito. L'insorgenza della sindrome da brevetossina compare nell'arco di tempo dai 30 minuti alle 2-3 ore (il limite di tossicità riferito alla parte edibile è 0.8 ppm), con una durata dei sintomi per due o tre giorni; generalmente non costituisce un pericolo per la vita dell'uomo, dal momento che il recupero è completo (Mattei et al., 1999). L'avvelenamento neurotossico è stato osservato fino a ora lungo le coste del Messico ed è stato associato al consumo di molluschi bivalvi. Durante la proliferazione di *Karenia brevis*, si possono verificare disturbi di tipo

respiratorio dovuti all'inalazione di aerosol di acqua marina contenente cellule e loro frammenti o brevetossine disciolte. Le tossine di tipo NSP sono prodotte dalla dinoflagellata *Karenia brevis* e da altre specie appartenenti al genere *Karenia.*

4.2.4. Tossine CFP

CFP (Ciguatera Fish Poisoning): sindrome di avvelenamento associata all'ingestione di tossine diverse (ciguatossina, scaritossina, maitotossina, gambiertossine) tipica delle regioni costiere tropicali e subtropicali.
I primi sintomi insorgono entro 6 ore dal consumo del pesce contaminato, causando disordini gastrointestinali, neurologici e cardiovascolari, quali insensibilità e formicolio del cavo orale e delle estremità del corpo, nausea, vomito e diarrea, parestesia, vertigini e debolezza muscolare e infine aritmia, bradicardia e abbassamento della pressione sanguigna (Jellet et al., 1992; Paulliac et al., 2000). Le tossine di tipo CFP sono prodotte da diverse specie di dinoflagellate appartenenti al genere *Gambierdiscus* (Adachi and Fukuyo, 1979).

4.2.5. Palitossina

La palitossina (Moore e Sceuer, 1971) è una tossina non proteica, avente un peso molecolare variabile da 2659 a 2680. La molecola base della palitossina è costituita da una lunga catena alifatica parzialmente insatura contenente eteri ciclici, 64 centri chirali, 40-42 gruppi idrossilici e 2 gruppi ammidici (Moore et al., 1981). La palitossina è una delle più potenti e letali tossine marine non proteiche conosciute. Il meccanismo molecolare attraverso cui agisce sulle cellule di mammifero è un legame diretto con l'enzima di membrana Na-K-ATPasi, che determina un aumento della permeabilità ionica; a concentrazioni maggiori agisce anche sulle pompe ioniche della membrana cellulare, con influsso di sodio e calcio ed efflusso di potassio e conseguente depolarizzazione (Habermann, 1989). Gli effetti tossici prodotti sono una potente vasocostrizione, depressione della funzione cardiaca, ischemia e danno al miocardio, fibrillazione ventricolare e blocco cardiaco.Sono stati segnalati casi di disturbi alle prime vie respiratorie e talvolta stati febbrili in persone vicine al mare (Sansoni et al., 2003; Gallitelli et al., 2005, Ciminiello et al., 2006). Nell'estate del 2005 a Genova, 240 persone che avevano frequentato aree costiere, senza immergersi in acqua, sono ricorse alle cure ospedaliere perché accusavano sintomi quali: febbre, faringodinia, tosse, dispnea, cefalea,

nausea, rinorrea, congiuntivite, vomito e dermatite. La palitossina è prodotta dalle dinoflagellate bentoniche appartenenti al genere *Ostreopsis* (Penna et al., 2005).

CAPITOLO 5

INTERCALIBRATION OF COUNTING METHODS FOR THE TOXIC BENTHIC DINOFLAGELLATE *OSTREOPSIS OVATA* BLOOMS IN THE MEDITERRANEAN SEA

5.1. ENPI CBCMED M3-HABs Project overview

www.m3-habs.net

The Project of Risk Monitoring, Modeling and Mitigation of Benthic Harmful Algal Blooms (M3-HABs) was funded under the European Union ENPI-CBC Med Program. The aim of the Project was to develop a global and a common pan-Mediterranean strategy for monitoring benthic toxic microalgae, particularly *Ostreopsis* spp. along Mediterranean coasts. *Ostreopsis* spp. are benthic dinoflagellate responsible of toxic bloom events as these species are likely to produce palytoxin and palytoxin-like compounds. In recent years, HABs events, related to the presence of *Ostreopsis* species, were found to be more frequent and extend to wider latitudes. They were held responsible for some health issues, in addition to the damages caused to the ecosystem, thus, adequate monitoring programs to manage these events have become necessary. Upon request from the scientific community studying harmful algal blooms, the M3-HABs project initiated to foster research on these events and to develop a global and a common pan-Mediterranean strategy for monitoring *Ostreopsis* spp. The project included infrastructure development, monitoring tools and strategies guide lines, as well as the establishment of common sampling techniques and counting methods to the entire Mediterranean basin. To decide on effective monitoring procedures, an understanding of the environmental drivers and of the implications of *Ostreopsis* blooms on human health, had to be widespread among the countries. Universities, research institutes, environmental agencies and technological enterprises based along Mediterranean coasts joined, under this EU funded project (ENPI-CBCMED Program) to prevent and reduce the hazards related to *Ostreopsis*.

5.2. M3-HABs Project - FINAL DELIVERABLE of the Workpackage 6: Common and Intercalibrated Sampling Strategies and Protocols.

M3-HABs

Deliverable 6.2

Improved and common laboratory protocols regarding preserving and microscopy and molecular counting techniques

Lead Beneficiary	**PP3 LOV**
Deliverable authors	**Cécile Jauzein, Silvia Casabianca, Laura Pugliese, Antonella Penna and Rodolphe Lemée**
Deliverable version	**1.0**

Delivery date in DoW	**Month 20**
Actual delivery date	**21/12/2015**

"This publication has been produced with the financial assistance of the European Union under the ENPI CBC Mediterranean Sea Basin Programme. The contents of this document are the sole responsibility of implementing partner and can under no circumstances be regarded as reflecting the position of the European Union or of the Programme's management structures."

Project funded by the EUROPEAN UNION

ENPI CBCMED
CROSS-BORDER COOPERATION IN THE MEDITERRANEAN

CONTENTS

ENPI CBCMED
CROSS-BORDER COOPERATION IN THE MEDITERRANEAN

1. INTRODUCTION

The main goal of the WP6 was to standardize strategies and processes for sampling, preserving and counting *Ostreopsis* cells in order to produce an international monitoring protocol. This working package involved all partners of the M3HABs project, located in France (LOV, UNS), Italy (CoNISMA, ARPAL), Lebanon (CNRS) and Tunisia (INSTM). All these associated countries have experienced *Ostreopsis* blooms since many years, consequently gaining a large experience in managing both sampling and laboratory activities for the monitoring of benthic harmful algal blooms. Deliverables of the WK6 largely benefited from sharing such experience between partners during inter-calibration sessions that were held in Rome (Italy) and Nice (France) in 2014 and during a Summer School organized in Batroun (Lebanon) in 2014 that was focused on monitoring protocols. Specific experiments and analyses were also performed during summer seasons of 2014 and 2015 in order to define potential improvements of common protocols. All research efforts and results from sharing experiences are summarized below. For sampling methods, classical methods based on benthic and planktonic sampling are described in details, as well as sampling using artificial substrates and a prototype named as "BEDI", two new methodologies that were defined and tested during the M3HABs project. Main improvements and recommendations are also listed for the treatment of samples, including steps such as fixative addition or separation of *Ostreopsis* cells from their substrates. For the counting techniques, analyses allowed for an estimation of counting efficiency, using the classical microscopical method, as well as other alternative methods that were based on molecular techniques using qPCR (quantitative PCR) or a new methodology of automatic optical counting using 3D representation of cells.

The present document describes in details the common protocol defined between partners of the M3-HABs project. It also identifies new promising methodologies that could improve approaches that are currently used for surveys of benthic harmful algal blooms. Specific recommendations for sampling, preserving and counting procedures are listed, considering procedures suitable for ecological studies, monitoring and data collection for modelling of *Ostreopsis* blooms.

2. TREATMENT AND STORAGE

2.1 How to preserve samples?

For classical methods, each partner has been using a specific preservative in order to keep benthic and planktonic samples before processing: it could be either formaldehyde or Lugol, at various concentrations. For the intercalibration between molecular and microscopy methods for counting *Ostreopsis* abundances, samples were fixed with 1% (vol/vol) of acidic Lugol solution (final concentration). For benthic samples from biotic substrates, this fixative concentration is adapted to fast treatment of samples, when the separation step (between macroalgal substrate and epiphytic microalgal cells) is done few minutes after Lugol addition, however. If the separation step has to be delayed, macroalgae quickly absorb part of the Lugol and an extra-addition of Lugol should be performed. More or less, the benthic samples of isolated *Ostreopsis* cells should have at least a "tea color".

2.2 How to separate benthic Ostreopsis from substrates?

As a common protocol defined between partners of the M3HABs project, the isolation of epiphytic *Ostreopsis* cells comes from different agitation steps. Benthic samples should be vigorously shaken for 10 secondes, then rinsed with 100 mL of filtered seawater (0.2 µm) and shaken again. Similar protocols can be used for both biotic and artificial substrates. The use of a sieve of 500 µm is useful for removing macroalgal fragments or artificial substrates. However, in presence of macroflocs of *Ostreopsis*, the use of sieving may lead to loss of cells, making the sieving step discretionary. The total volume (sample + rinsing water) should be measured to allow for estimation of *Ostreopsis* densities. Abundances have to be normalized per unit of macroalgal weight or surface of artificial substrates. In order to characterize biotic substrate quantity, only fresh weight of macroalgal substrate has been usually measured. However, both fresh and dry weights of macroalgal substrate quantity should be performed in order to get an appropriate estimation for standardization of benthic *Ostreopsis* concentrations. A suitable drying of high weighted samples of macroalgae might require more than 48h at 70°C.

Specific tests were run during summers 2014 and 2015 by the partner LOV (France) in order to analyse the efficiency of the separation between marcroalgal substrate and epiphytic microalgal cells according to *(i)* the addition (or not) of fixative and *(ii)* the number of washing steps performed. Results showed that the addition of acidic lugol before the separation step significantly helps ($p < 0.001$) for the isolation of *Ostreospsis* cells from macroalgal substrates (*Figure 1*). According to these tests, when acidic lugol was added before the separation step, the completion of 10 seconds of agitation and one washing step allows for the collection of more than 96% of the epiphytic *Ostreopsis* cells. Interestingly, a separation of

Ostreopsis cells in seawater (without fixative addition) is also efficient but definitely requires two successive washing steps in order to do not underestimate cell abundances.

Four sets of experiments were done and similar results were obtained under either low (~ 20,000 cell/gFW) or high (~ 200,000 cell/gFW) bloom conditions. Thus, the following recommendations can be used for the whole bloom survey: for the common protocol, it is recommended to add acidic lugol fixative before the separation step because it helps making the mucilage looser, and at least one washing step is recommended in order to collect most of the cells from benthic samples. If the used fixative for the survey is formaldehyde, it should be added after the separation step and the completion of two successive washing steps is recommended for *Ostreopsis* cell isolation in seawater.

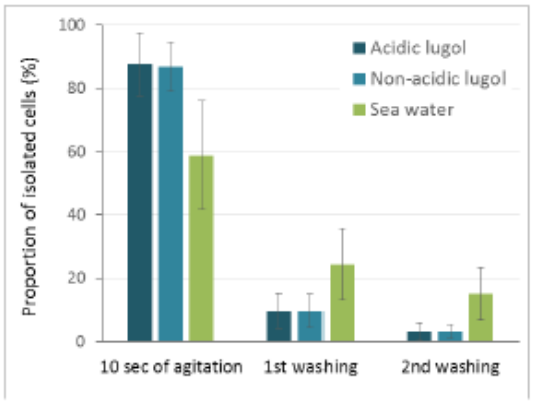


Figure 1: Proportion of isolated Ostreopsis cells from a macroalgal sample after 10 sec of agitation and one or two washing steps under three different conditions: when acidic lugol or non-acidic lugol was added before the separation step or when no fixative was added.

2.3 How long between sample collection and processing?

The duration of the storage period before sample processing is dependent on the type of studies conducted: it is restricted to 1 or 2 days for monitoring, but can be extended to several months for ecological studies, if stored in dark conditions at +4°C. For molecular studies the maximum storage period is one month to avoid material deterioration.

3. COUNTING PROCEDURES

3.1 How to count using classical microscopy methods?

In order to optimize time for sampling and processing, partners agreed to limit the number of counting per sample to one (except for the samples dedicated to intercalibration): the maximum effort should be focused on analyzing variability at small scale, monitoring as much stations as possible and analyzing benthic and planktonic populations separately as often as possible.

The easiest way to count benthic populations requires the use of a Sedgewick Rafter Counting cell (1 mL) and a classical microscope. Some partners use the Utermöhl method, where a subsample (1-25 mL) is poured in a cylinder/chamber complex and left to settle before observation at the inverted microscope. For planktonic samples, all partners use the Utermöhl method settling volumes from 10 to 100 mL. For monitoring, a sedimentation volume of 50 mL is recommended, with a 24h settling time. For all samples, at least 200 cells have to be counted per sample during the bloom duration.

In order to optimize the use of the Utermöhl method for the monitoring of planktonic *Ostreopsis* cells, a test was run during the summer seasons 2014 and 2015 in order to compare the counting efficiency when different volumes of sedimentation are used. Results revealed a good efficiency of the counting using a 10 mL column, even when only 30 cells were counted per slide (*Figure 5*). Most importantly, they showed that the use of a 100 mL-column induces an underestimation of the cell counts of ~10% (compared to counting using a 50 mL column, *Figure 2*). This is probably due to the fact that, when the column is too high, a significant amount of *Ostreopsis* cells get stuck on the edges of the column and do not sediment.

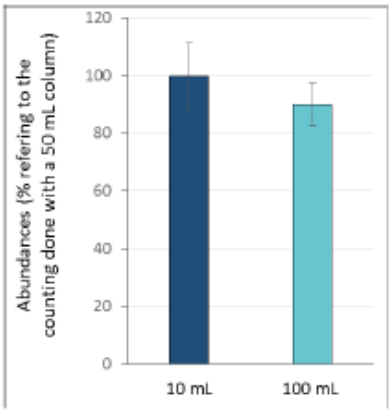


Figure 2: Comparison of counting efficiency using sedimentation columns of 10 mL, 50 mL and 100 mL. Data are expressed as percentages of the abundance of Ostreopsis cells estimated with a 50 mL-column

3.2 Alternative methods for counting

3.2.1 Automatic optical counts

The development of an automatic opto-electronic system for counting *Ostreospsis* cell abundances is part of Work Package 4. A specific algorithm, capable of recognising *Ostreopsis* cells, was defined for this approach, using 3D representation of phytoplankton cells. This methodology involved three main steps: the segmentation (identifying objects out of the background and main debris), the identification (including species identification) and the counting. This algorithm was optimized in order to identify objects even in aggregates and with an identification at the species level.

3.2.2 Counts using molecular techniques

Molecular technological advances in real time quantitative PCR (qPCR) make it possible to identify toxic algae more rapidly and accurately. These molecular methods, mostly based on SYBR Green, directly quantify various HAB (harmful algal bloom) microalgal species in different environmental matrices, such as seawater, hard and soft substrata. A limitation of HAB species quantification stems from the use of target molecular marker. The challenges with using a marker is its copy number stability within a target species, and ensuring specificity. *Ostreopsis* spp. showed high copy number variability within ribosomal genes including LSU gene. The strategy of using a standard curve generated from a DNA scalar dilution of pooled cell samples collected during periodically summer bloom of toxic *O.* cf. *ovata* in Mediterranean coastal sites was developed. This allowed for normalization of *O.* cf. *ovata* copy number variability, thereby obtaining an accurate and rapid quantification of cells in field samples. Environmental standard curves for specific geographical sites were generated to quantify cell abundance and determine the species-specific identification of *Ostreopsis* in water and macroalgal samples. The standard curves were created based on the amplification of target ribosomal LSU sequences using species-specific primers Ovata rt forward (5′TTTGATCACTTTGGCAATCT-3′) and Ovata rt reverse (5′-TGAACTTTACCATGCCATTAG-3′) from Perini et al. (2011). The species-specific primers for *O.* cf. *ovata* were applied for the amplification of 204 bp of LSU rDNA. Therefore, field samples collected during summer blooms in 2014 and 2015 were used to run qPCR for the identification and quantification of *Ostreopsis* species. The molecular qPCR analyses were applied to *Ostreopsis* cf. *ovata* and also *Ostreopsis* cf. *siamensis*, using species-specific primers, Siamensis rt forward (5′-CACCACTGAGTGTGCGTACTG-3′) and Siamensis rt reverse (5′-GTTGGTGCGTACATTACTTCA-3′) from Casabianca et al. (2013), as part of the Work Package 6. Thus, quantification of *Ostreopsis* cells was obtained using environmental site-specific standard curves generated in order to obtain *Ostreopsis* LSU copy number/cell at each Mediterranean coastal site. Testing the efficiency of this approach, qPCR showed a high sensitivity, high specificity and high rapidity. As a main advantage, qPCR method allows species-specific identification and a counting efficiency often more sensitive than light microscopy both in water and substrata samples (Figs. 3 and 4).

The qPCR assays run from benthic, planktonic and aerosol samples collected along the Mediterranean coast showed that mainly *O.* cf. *ovata* was found in the study sites. Interestingly, samples collected from Lebanese coastal areas revealed the existence of a potential new species of *Ostreopsis* that is now currently characterized.

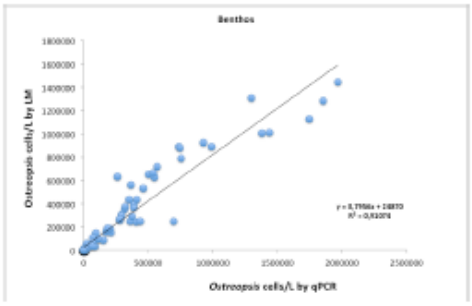


Figure 3. Linear relationship between Ostreopsis abundance (cells/L) counted by molecular qPCR and light microscopy (LM) from benthic substrata of Mediterranean coastal sites.

The qPCR assays run from benthic, planktonic and aerosol samples collected along the Mediterranean coast showed that mainly *O*. cf. ***ovata*** was found in the study sites. Interestingly, samples collected from Lebanese coastal areas revealed the existence of a potential new species of ***Ostreopsis*** that is now currently characterized. Monoclonal strains of ***Ostreopsis*** spp. samples isolated from Lebanese water were analyzed for the molecular taxonomical assignment. The sequence analysis revealed the presence of a new potential species of ***Ostreopsis*** that has to be further characterized. However, new species-specific primers were designed to be used in the qPCR analyses for molecular quantification of ***Ostreopsis*** species.

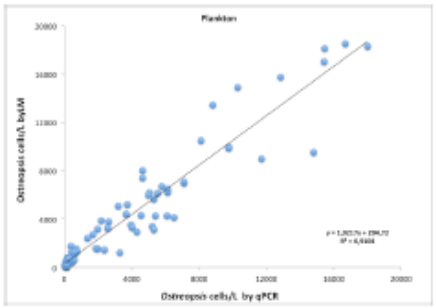


Figure 4. Linear relationship between Ostreopsis abundance (cells/L) counted by molecular qPCR and light microscopy (LM) from water samples of Mediterranean coastal sites.

3.2.3 Intercalibration among counting methods

In 2015, all partners agreed to take sub-samples, at different phases of the bloom (development, peak and decline of the bloom), in order to help for optimization of counting methods and intercalibration between them. These sub-samples were stored according to the standard protocol: they were fixed with 1% (vol/vol) of acidic Lugol solution (final concentration), stored at +4°C and sent for analyses in less than one month in order to avoid any material deterioration. The same field samples (benthic or planktonic) were analysed by the three different methods: classical microscopy method, automatic optical counting and molecular qPCR method. All measurements (optical, automatic and molecular) were performed in triplicates and counted by the same operator. Results revealed good correlations between the different counting methods ($R^2 > 0.86$). Considering the use of the opto-electronic system *versus* the classical counting methodology, automatic procedures tend to underestimate *Ostreopsis* abundances compared to classical microscopic counts. Comparing microscopic counts and counting using qPCR, it appears that molecular procedures tend to overestimate plankton samples, but underestimate benthic ones.

3.3 What to count?

For both benthic and planktonic samples, all partners are currently analyzing the abundance of *Ostreopsis* species and of other benthic microalgal taxa, with particular attention to potentially toxic dinoflagellates (e.g., *Coolia*, *Prorocentrum*, *Amphidinium*). Moreover, microphytobenthos dominant taxa (e.g., diatoms, cyanobacteria etc.) are also counted in some laboratories, to collect additional and crucial information about the environmental conditions promoting or limiting *Ostreopsis* bloom development. If such sampling efforts are currently made, they should be maintained (if possible) for ecological studies and monitoring performed in 2015.

References cited in the text:

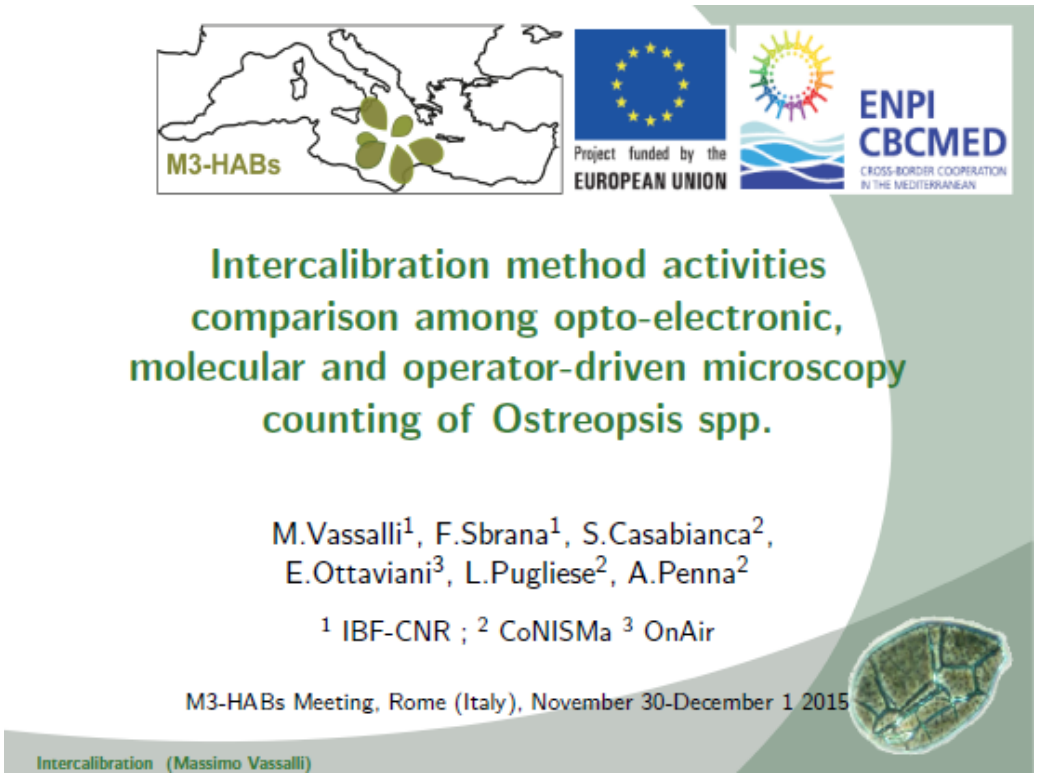
ENPI CBCMED
CROSS-BORDER COOPERATION IN THE MEDITERRANEAN

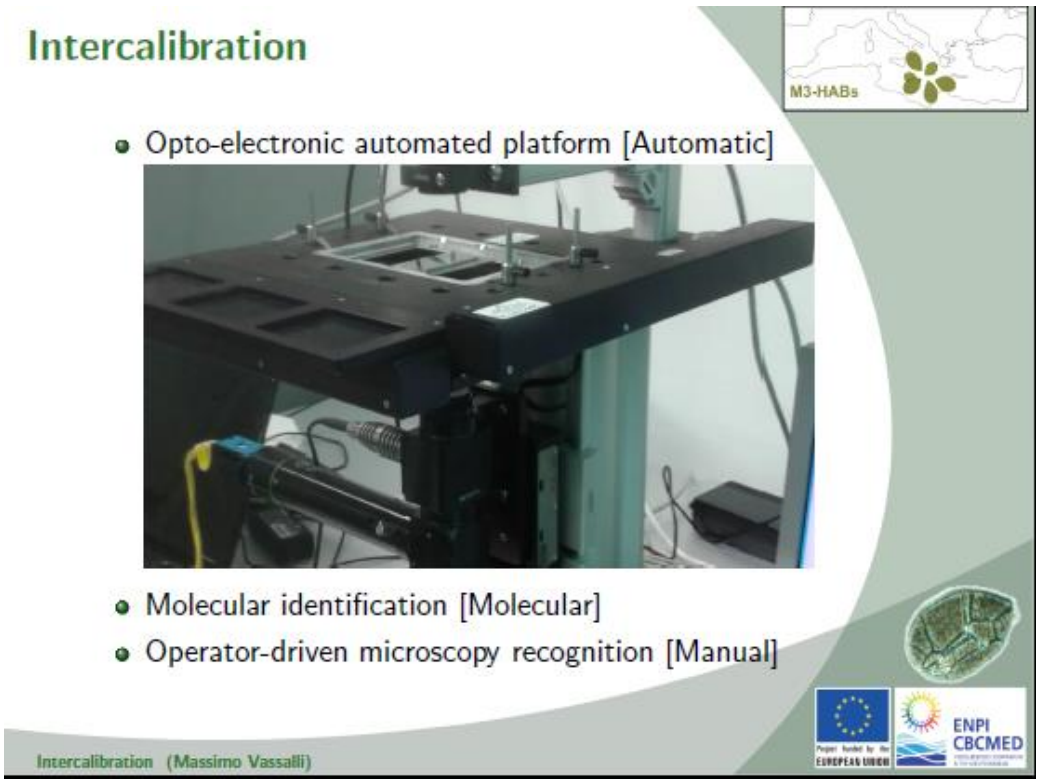
Casabianca S., Casabianca A., Riobò P., Franco J.M., Vila M., Penna A. 2013. Quantification of the Toxic Dinoflagellate *Ostreopsis* spp. by qPCR Assay in Marine Aerosol. ***Environ. Sci. Technol.*** 47, 3788−3795.

Perini F., Casabianca A., Battocchi C., Accoroni S., Totti C., Penna A. 2011. New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. ***Plos One***, 6: e17699: 1-9.

5.3. Final meeting ENPI CBCMED M3-HABs, CNR, Roma, 30th November – 1st December 2015

Presentation within the WP4 – Opto-electronic system and algorithm for cell identification and counting.





Intercalibration

- Opto-electronic automated platform [Automatic]
- Molecular identification [Molecular]

- Operator-driven microscopy recognition [Manual]

Intercalibration

- Opto-electronic automated platform [Automatic]
- Molecular identification [Molecular]
- Operator-driven microscopy recognition [Manual]

Common sampling and treatment procedure

Intercalibration (Massimo Vassalli)

Distributed processing

- Automatic [Genova (Italy) @CNR]
- Molecular [Urbino (Italy) @CoNISMa]
- Manual [@partner]

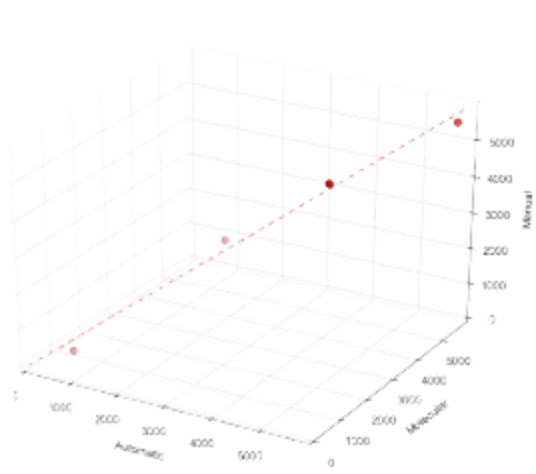
Intercalibration (Massimo Vassalli)

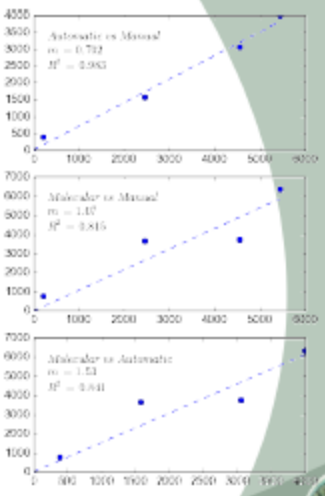
Processed sites

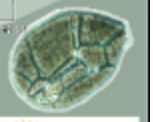
- Salammbô
- Haliotis
- Genova
- Batroun
- Rochambeau
- Ancona

Data were processed for each site, dividing **Plankton** from **Benthos** only for **2015** blooms, collected on the basis of the stndardized protocol. Other sites and datasets are still being analyzed. **This is a first look at the data that needs a further discussion**

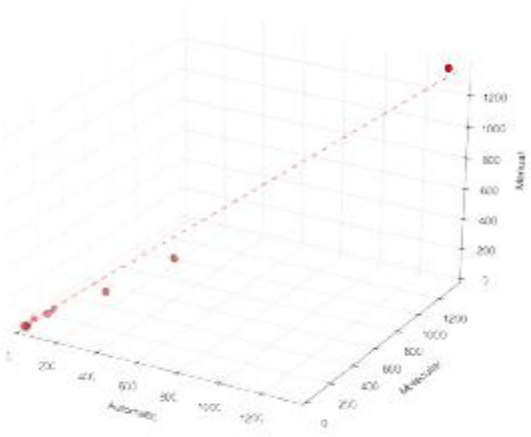
Rochambeau - Plankton

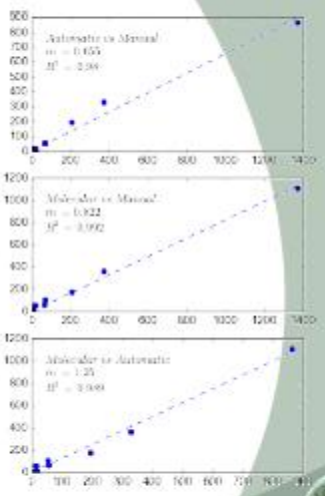






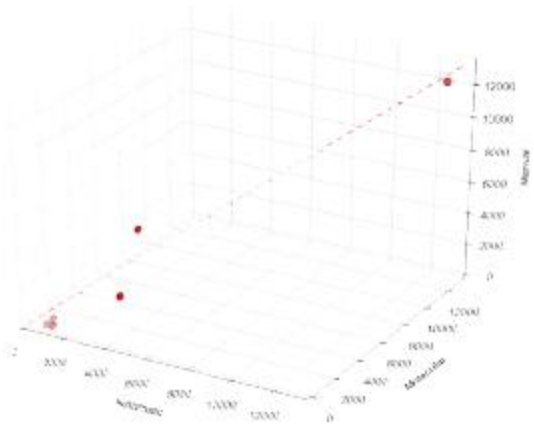
Rochambeau - Benthos

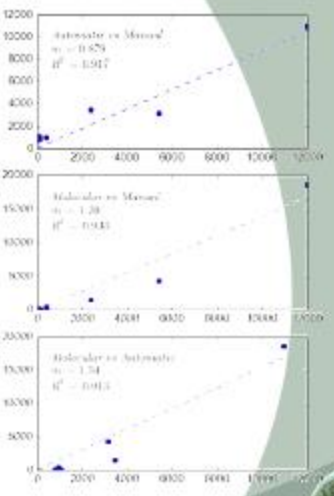




Intercalibration (Massimo Vassalli)

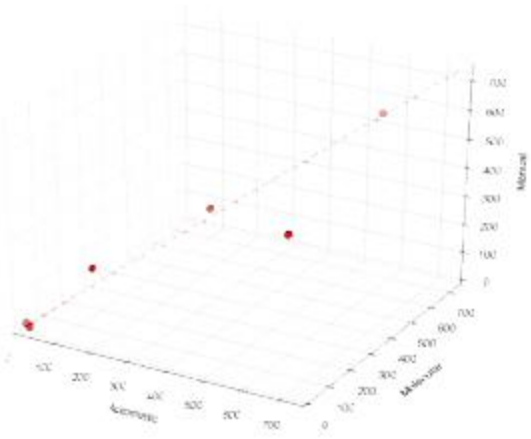
Genova - Plankton

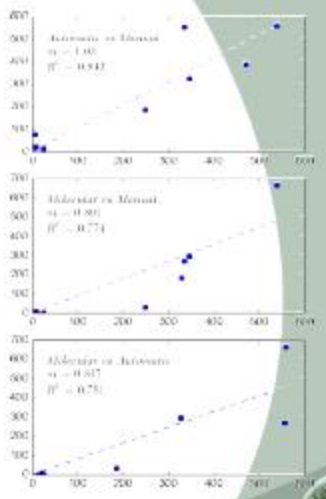


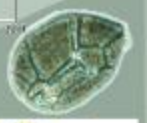


Intercalibration (Massimo Vassalli)

Genova - Benthos

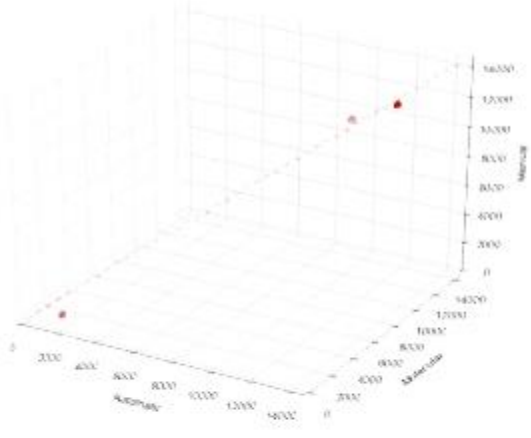


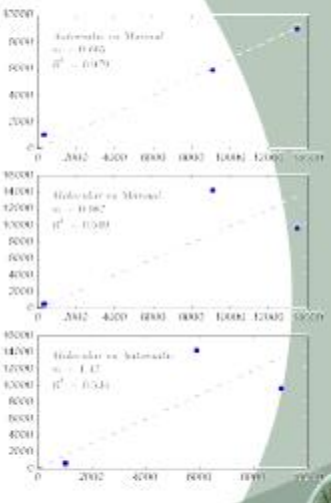




Intercalibration (Massimo Vassalli)

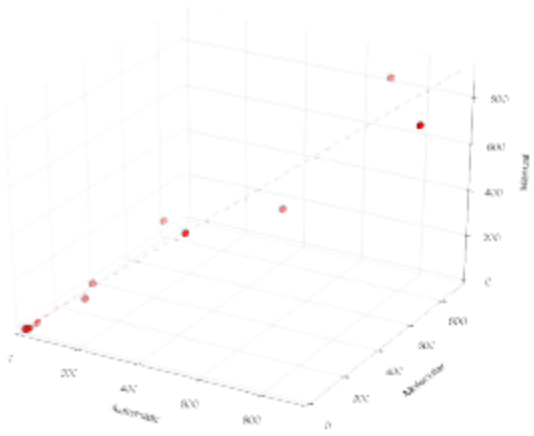
Haliotis - Plankton

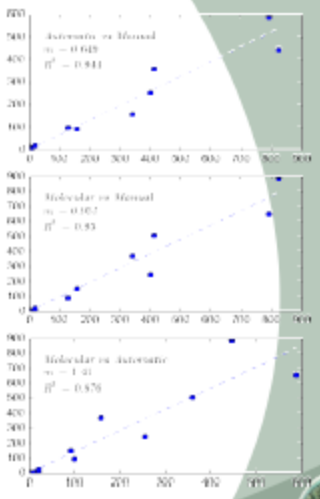


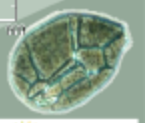


Intercalibration (Massimo Vassalli)

Haliotis - Benthos

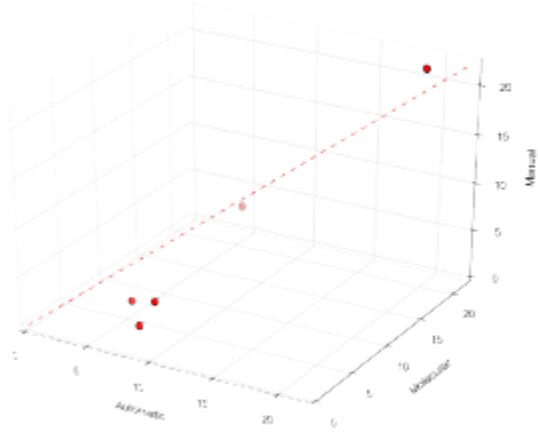


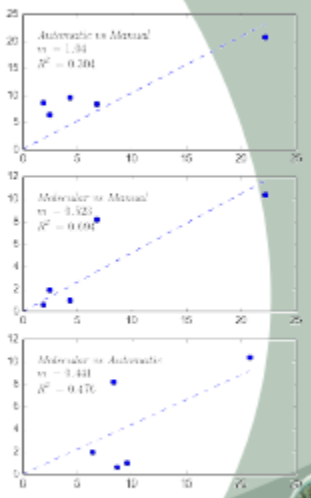


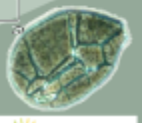


Intercalibration (Massimo Vassalli)

Batroun - Benthos

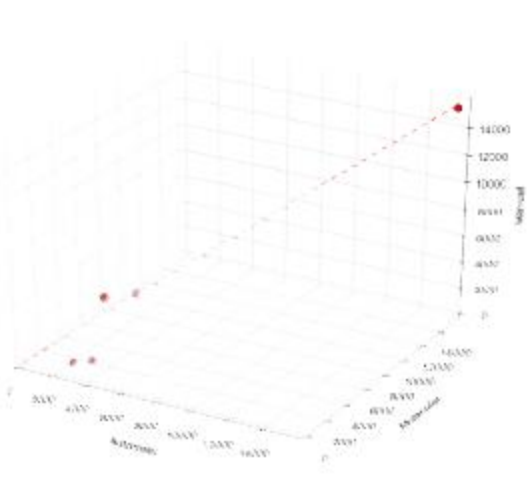


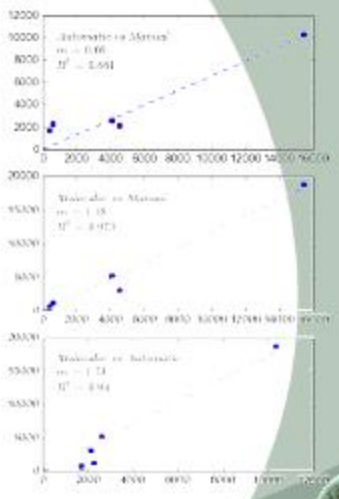




Intercalibration (Massimo Vassalli)

Ancona - Plankton

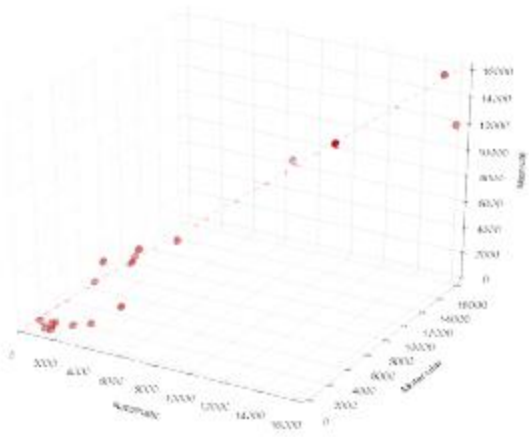


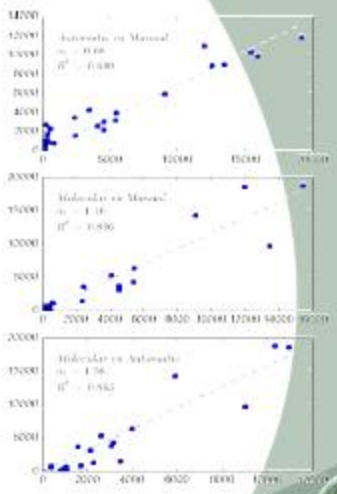


Global correlation

Is the result of the intercalibration consistent among all territories, methods and partners ?

ALL sites - Plankton



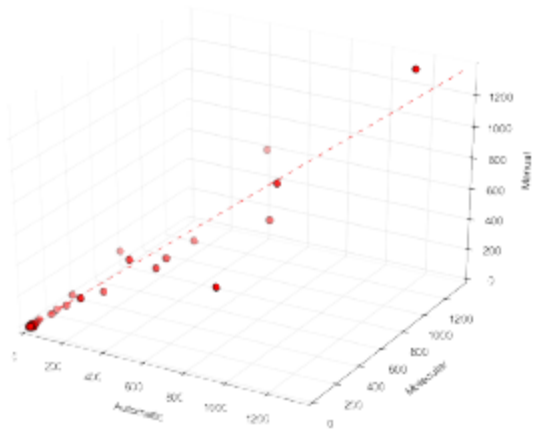


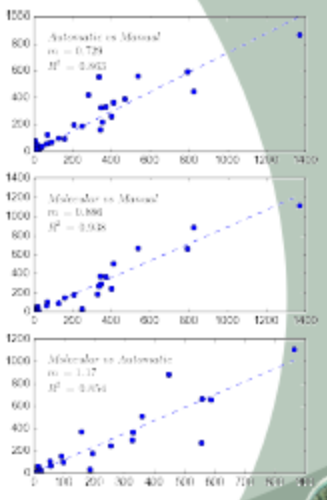
M3-HABs

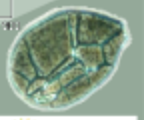
Intercalibration (Massimo Vassalli)

ENPI CBCMED

ALL sites - Benthos







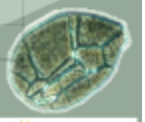
M3-HABs

Intercalibration (Massimo Vassalli)

ENPI CBCMED

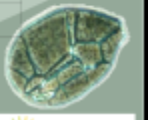
Data interpretation

Location	m_{auto}	R^2_{auto}	m_{mol}	R^2_{mol}
Plankton	0.68	0.899	1.16	0.866
Salamboo	0.645	0.833		
Haliotis	0.605	0.979	0.987	0.509
Genova	0.879	0.917	1.39	0.833
Batroun	1.82	0.696		
Rochambeau	0.702	0.983	1.07	0.813
Ancona	0.66	0.881	1.18	0.973
Benthos	0.729	0.863	0.886	0.938
Salamboo	1.35	0.881		
Haliotis	0.619	0.944	0.937	0.95
Genova	1.03	0.891	0.812	0.771
Batroun	1.04	0.304	0.523	0.691
Rochambeau	0.635	0.98	0.822	0.992
Ancona	0.943	0.661		



Data interpretation

Location	m_{auto}	R^2_{auto}	m_{mol}	R^2_{mol}
Plankton	0.68 < 1	0.899	1.16	0.866
Salamboo	0.645	0.833		
Haliotis	0.605	0.979	0.987	0.509
Genova	0.879	0.917	1.39	0.833
Batroun	1.82	0.696		
Rochambeau	0.702	0.983	1.07	0.813
Ancona	0.66	0.881	1.18	0.973
Benthos	0.729 < 1	0.863	0.886	0.938
Salamboo	1.35	0.881		
Haliotis	0.619	0.944	0.937	0.95
Genova	1.03	0.891	0.812	0.771
Batroun	1.04	0.304	0.523	0.691
Rochambeau	0.635	0.98	0.822	0.992
Ancona	0.943	0.661		

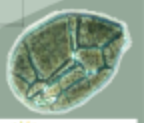


The automatic procedure underestimates the concentration

Data interpretation

Location	m_{auto}	R^2_{auto}	m_{mol}	R^2_{mol}
Plankton	0.68	0.899	1.16 > 1	0.866
Salamboo	0.645	0.833		
Haliotis	0.605	0.979	0.987	0.509
Genova	0.879	0.917	1.39	0.833
Batroun	1.82	0.696		
Rochambeau	0.702	0.983	1.07	0.813
Ancona	0.66	0.881	1.18	0.973
Benthos	0.729	0.863	0.886 < 1	0.938
Salamboo	1.35	0.881		
Haliotis	0.619	0.944	0.937	0.95
Genova	1.03	0.891	0.812	0.771
Batroun	1.04	0.304	0.523	0.691
Rochambeau	0.635	0.98	0.822	0.992
Ancona	0.943	0.661		

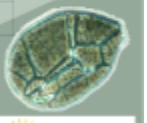
The molecular procedure overestimates plankton samples and underestimates benthos samples



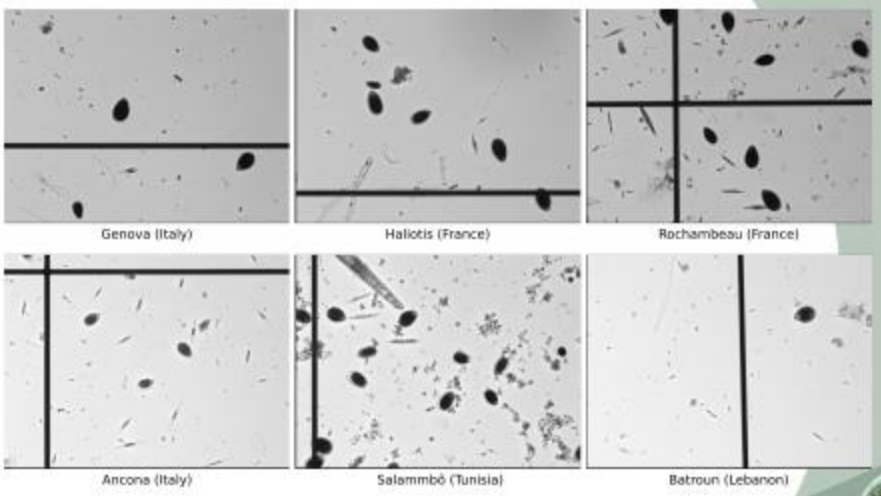
Data interpretation

Location	m_{auto}	R^2_{auto}	m_{mol}	R^2_{mol}
Plankton	0.68	0.899	1.16	0.866
Salamboo	0.645	0.833		
Haliotis	0.605	0.979	0.987	0.509
Genova	0.879	0.917	1.39	0.833
Batroun	1.82	0.696		
Rochambeau	0.702	0.983	1.07	0.813
Ancona	0.66	0.881	1.18	0.973
Benthos	0.729	0.863	0.886	0.938
Salamboo	1.35	0.881		
Haliotis	0.619	0.944	0.937	0.95
Genova	1.03	0.891	0.812	0.771
Batroun	1.04	0.304	0.523	0.691
Rochambeau	0.635	0.98	0.822	0.992
Ancona	0.943	0.661		

The only large disagreement is for Batroun samples



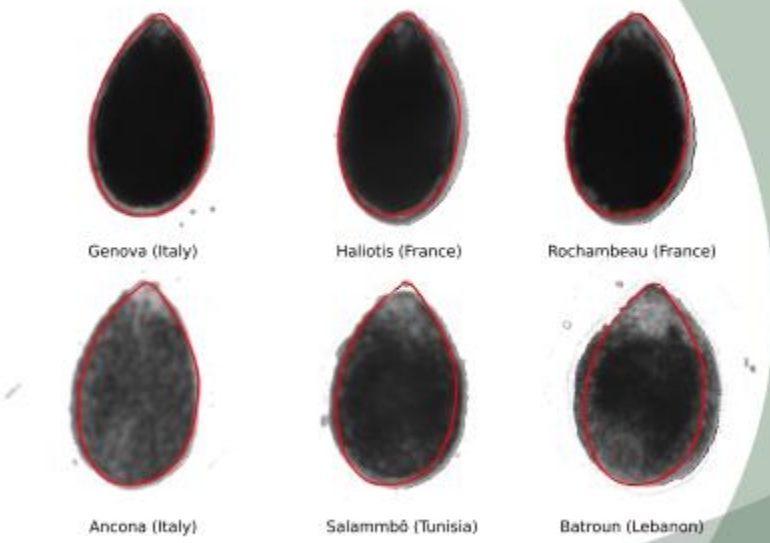
Data interpretation



ENPI CBCMED

Intercalibration (Massimo Vassalli)

Data interpretation



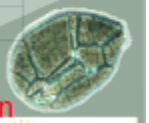
ENPI CBCMED

Intercalibration (Massimo Vassalli)

Data interpretation: take-home message

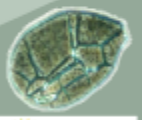
Location	m_{auto}	R^2_{auto}	m_{mol}	R^2_{mol}
Plankton	0.68	0.899	1.16	0.866
Salamboo	0.645	0.833		
Haliotis	0.605	0.979	0.987	0.509
Genova	0.879	0.917	1.39	0.833
Batroun	1.82	0.696		
Rochambeau	0.702	0.983	1.07	0.813
Ancona	0.66	0.881	1.18	0.973
Benthos	0.729	0.863	0.886	0.938
Salamboo	1.35	0.881		
Haliotis	0.619	0.944	0.937	0.95
Genova	1.03	0.891	0.812	0.771
Batroun	1.04	0.304	0.523	0.691
Rochambeau	0.635	0.98	0.822	0.992
Ancona	0.943	0.661		

All methods linearly correlate: I can perform the intercalibration

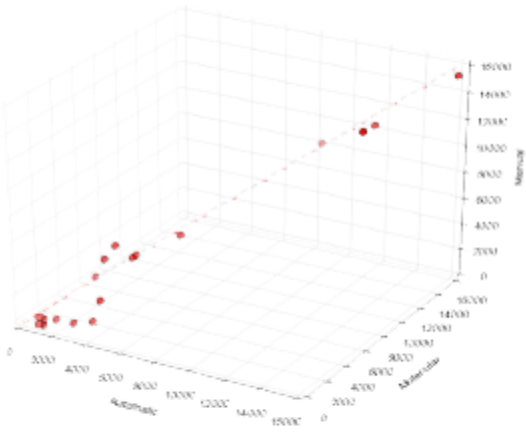


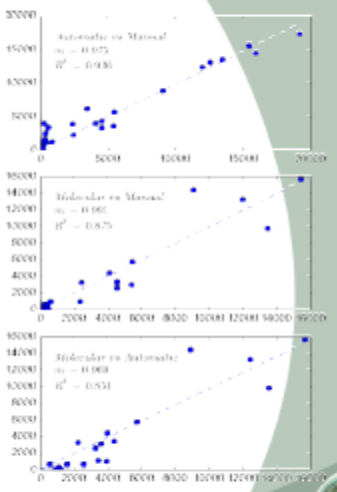
Intercalibration

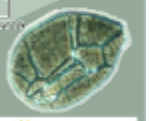
- There is a linear correlation among all methods
- The correlation coefficient is different for all the sites, both for automatic (more) and molecular (less) approaches, but still linear
- The identification is sensitive to the species (Batroun case)
- Automatic count was performed using the same template for all sites (trained on Genova samples), thus having a slightly larger variation.
- Site-specific molecular LSU standard curves
- It is possible to apply a site-specific intercalibration



ALL sites intercalibrated - Plankton

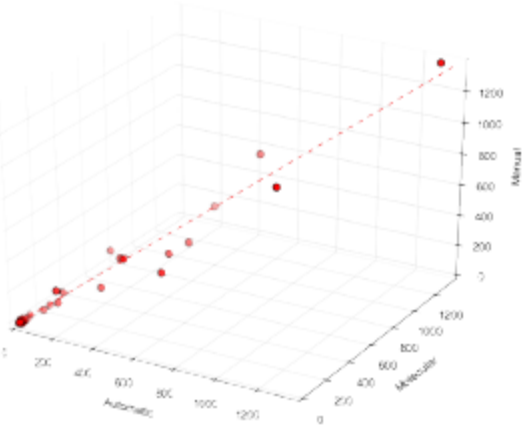


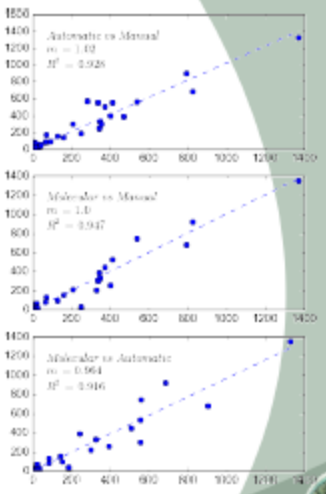


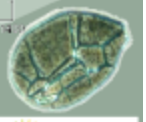


Intercalibration (Massimo Vassalli)

ALL sites intercalibrated - Benthos







Intercalibration (Massimo Vassalli)

CAPITOLO 6

HAB DINOFLAGELLATE RESTING CYSTS MONITORING

6.1. EU CBC IPA ADRIATIC BALMAS Project overview

www.balmas.eu

The United Nations have recognized the transfer of harmful organisms and pathogens across natural barriers as one of the four greatest pressures to the world's oceans and seas, causing global environmental changes, and posing threat to human health, property and resources. Ballast water (BW) transfer by vessels was recognized as a prominent vector of such species, and was regulated by the International Convention for the Control and Management of Ship´s Ballast Water and Sediments, 2004 (BWM Convention). The BWM Convention sets the global standards on ballast water management (BWM) requirements, while recognizing that regional and local specifics have to be considered for its effective implementation. The Adriatic Sea is a unique and highly sensitive ecosystem. The economic development and social existence of the coastal States strongly depend on the clean and preserved Adriatic Sea. However, the Adriatic Sea is also a seaway mainly used by international shipping transporting goods to or from Europe as hinterland, with also intense local shipping. Increasing, serious concern is the introduction of harmful aquatic organisms and pathogens (HAOP) by ships'BW. By developing a joint Adriatic Ballast Water Management Decision Support System, Ballast Water Management Plan and Strategy, BALMAS will ensure uniform BWM requirements to ease shipping and at the same time to maximize environmental and economic protection of all sea users. The general BALMAS objective is to establish a common cross-border system, which will link all researchers, experts and responsible national authorities from Adriatic countries in order to avoid unwanted risks to the environment from the transfer of HAOP. This can be achieved through control and management of ships' BW and sediments. Further, long-term effective BWM in the Adriatic will be set at the cross-border level utilizing this project's related knowledge and technology.

6.2. Final Report CBC IPA ADRIATIC BALMAS Project

Workpackage 5: HAB dinoflagellate resting cysts monitoring and implication of their dispersion through ballast waters. Molecular Identification of Potentially Toxic Cysts in Sediments.

Perini Federico, Pugliese Laura, Penna Antonella

DISB, University of Urbino, Viale Trieste 296, Pesaro

Bastianini Mauro

CNR ISMAR, Arsenale Tesa 104, Castello 2737/F, Venezia

Cabrini Marina

OGS, via Auguste Piccard, 54, Trieste

Trieste

Pompei Marinella

CRM, Centro Ricerche Marine, Via A. Vespucci 2, Cesenatico

Marini Mauro

CNR ISMAR, Largo Fiera della Pesca 2, Ancona

The project is co-funded by the European Union
Instrument for Pre-Accession Assistance

INTRODUCTION

Many HAB (harmful algal bloom) species of dinoflagellates produce resting stage cells or cysts depositing in the bottom sediments. It is known that these cysts play an important role in bloom initiation as the seed population for long time. In addition, high cyst abundances in the bottom sediments have been reported from the areas, where dense toxic target algal species blooms have been observed frequently, and these areas are mainly located in the temperate seas. Resting cyst production is related to the life cycles of dinoflagellates, which consist of asexual reproduction by cell division and sexual reproduction by conjugation. During this process, two vegetative cells conjugate to form a planozygote, which then become cysts, which exist in the sediment until their germination. Dormant resting stages of phytoplankton are considered a common strategy that confers various ecological advantages, such as genetic recombination, dispersal, and seeding or regulation of the seasonal succession of dinoflagellates. Germination of toxic cysts, that often can contribute to generate bloom events, occurs under suitable environmental conditions, such as salinity, light intensity, nutrients, and endogenous factors. Therefore, monitoring the distribution of cyst densities in the coastal areas prior to an outbreak is important so that damage by toxic bloom can be minimized. The potential of ballast water as a major vector for marine introductions has been long recognised. It has been already demonstrated that ballast waters can be transport vector for global dispersal of HAB species. The dispersion or re-introduction of HAB species mainly at coastal sites also through artificial vectors, i.e. ballast water and aquaculture traffic, is relevant to understand the reasons and mechanisms underlying their spread to many areas worldwide. The evidence for ballast water as a vector for transport of *Alexandrium* cysts from Japanese waters into Australia is very strong. Cysts of toxic *Alexandrium* could be isolated, and germinated, from the ballast tanks of several vessels. The PSP-producing *Gymnodinium catenatum* has been considered an invasive species in the NE Atlantic suggesting that it could have been introduced by transport of viable cysts in ballast tanks, even if this species should be included in the list of cryptogenic species. In the Mediterranean Sea (Catalan and Tyrrhenian Seas), the controversial origin of the toxic PSP-producing *Alexandrium pacificum* is being long debated. Some authors retained that it is an introduced species, based on molecular and historical series data, while other evidences, but not exhaustive, demonstrated a potential endemic origin. Anyway, it is clear the range of expansion of this species from the western to central areas of the Mediterranean Sea (Ionian Sea); these records have been recently documented in harbor or bays affected by maritime traffic or aquaculture activities. In particular, the NW Adriatic Sea is subjected to frequent algal blooms and in the last 25 years, mussels farmed along the North-central Adriatic coasts were contaminated by harmful toxins causing significant economic losses due to closures of these farms. In most cases, marine lipophilic toxins (MLTs) contaminated mussels. MLTs include

okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins(PTXs), yessotoxins (YTXs) and azaspiracids (AZAs).

2. MATERIALS AND METHODS

2.1 Sampling of resting cysts in harbor sediments

The sediment samples were taken in 4 harbors, such as Ancona, Bari, Trieste and Venice, in the Adriatic Sea at different sampling sites (Fig. 1 and Table 1).

Table1. Harbours, sampling stations and geographical coordinates.

Sampling harbour	Sampling Station	Geographical Coordinates
BARI	BA1	Lat. 41° 8.536’N; Long. 16° 52.064'E
	BA2	Lat. 41° 8.061'N Long. 16° 52.134'E
	BA3	Lat. 41° 8.473'N Long. 16° 51.143'E
ANCONA	DS	Lat. 43°37'12.97"N Long. 13°29'48.20"E
	LR	Lat. 43°37'30.91"N Long. 13°29'40.00"E
	API	Lat. 43°40'29.72"N Long. 13°24'34.37"E
TRIESTE	St C1	Lat. 45°42’03”N Long. 13°42’36”E
	St 1	Lat. 45°38’1.08”N Long. 13°45’9.24”E
	St 2	Lat. 45°37’47.22”N Long. 13°46’10.98”E
	St 3	Lat. 45°37’4.08”N Long. 13°46’30.96”E
	St 4	Lat. 45°36’41.04”N Long. 13°47’3.78”
VENICE	BAL PORT 1	Lat. 45°26'13.17"N Long. 12°18'39.98"E
	BAL WAT 2	Lat. 45°26'54.19"N Long. 12°15'29.87"E
	PTF	Lat. 45°18'49.79"N Long. 12°30'31.79"E

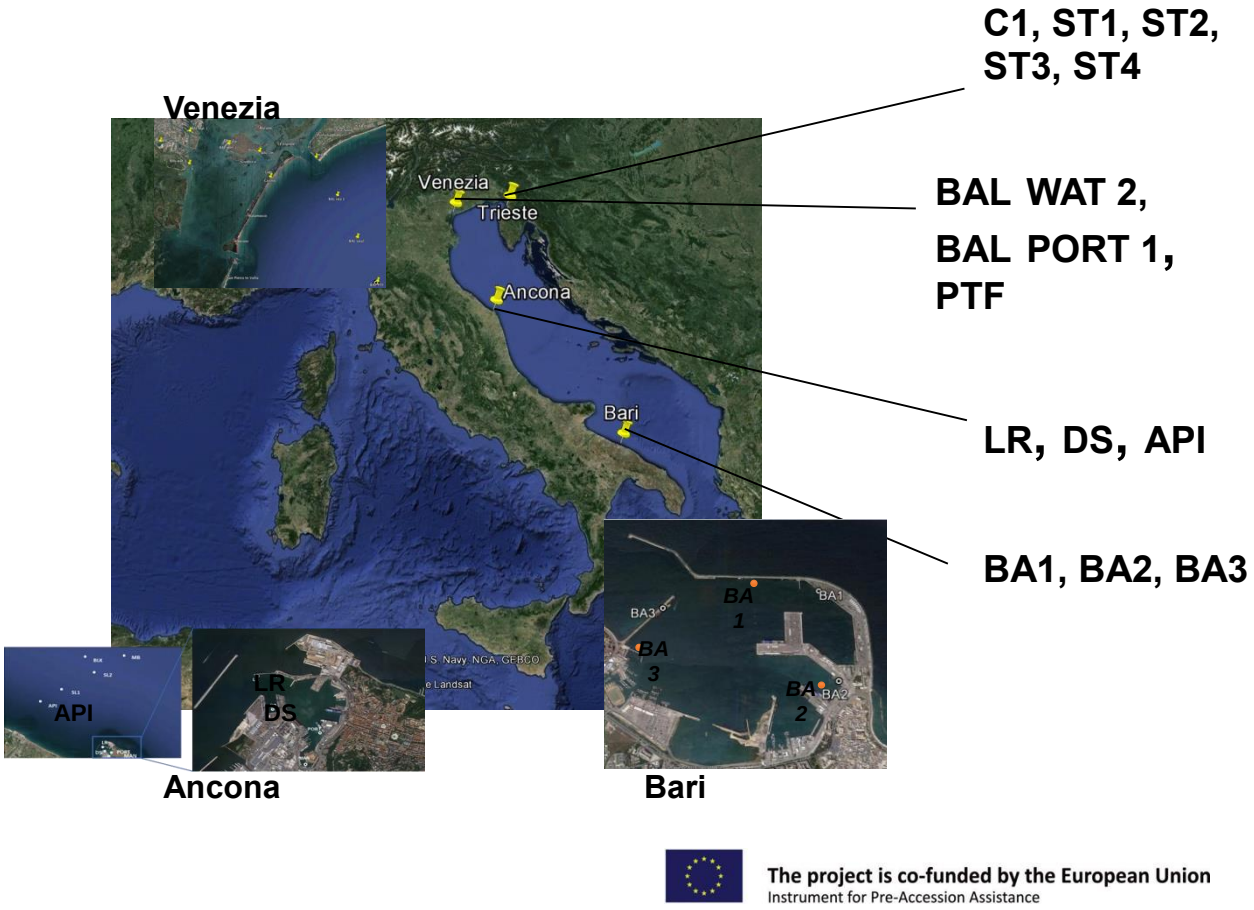


Fig. 1. Map of the four harbours and sampling sites in the Adriatic Sea (Trieste, Venice, Ancona, Bari).

Sediment samples were taken with gravity core. At the each sampling site, 3 gravity cores were taken. From each core the upper 2 cm of sediment, 2 subsample cores (ϕ = 2.5 cm), were used for optical microscopy and molecular PCR analysis.

2.2 Dinoflagellate cysts isolation

Subsamples of surface sediment (5-6 g fresh weight) were used to purify cysts using method described by Bolch (1997) with some modification. Subsamples were sonicated in water bath at room temperature for 2 min to disaggregate cysts from sediment particles and then, washed with filtered

seawater (0.45 μm pore size) through 100 μm and 20 μm sieves. The fraction in between was collected in a smaller beaker and brought at 7 ml final volume with sterilized seawater. This washed sample was placed in a 15 ml falcon tube containing 3 ml of sodium metatungstate monohydrate (2.05 g/cm3 density) on the bottom and was centrifuged for 10 min at 3600 rpm on room temperature. Dinoflagellate cysts were isolated from the sediment with gradient centrifugation in polytungstate. After centrifugation the interphase between polytungstate and seawater is clearly visible. The water was carefully removed using a pipette, and then the interphase was collected. Interphase was centrifuged with seawater at 3000 r.p.m. for 10 min to achieve a pellet on the bottom. This washing procedure was repeated twice and finally, the pellet was stored at -80°C until the DNA extraction.
DNA purification was carried out by using Ultra Clean Soil DNA Kit (MoBio Lab. In., Solana beach, CA,USA) following the manufacturers' instructions. Genomic DNA was quantified using Qubit fluorometer with a Quant-iT dsDNA HS Assay Kit, as recommended by the manufacturer (Invitrogen, Carlsbard, CA, USA).

2.3 Molecular PCR assay

Genus (*Alexandrium*) and species-specific (*Alexandrium minutum, A. mediterraneum, A. pacificum, Lingulodinium polyedrum, Protoceratium reticulatum, Fibrocapsa japonica, Gonyaulax spinifera, Ostreopsis cf. ovata*) primers were designed on the 5.8S rDNA-ITS regions and/or were derived from Penna et al. (2007). Amplification reactions from sediment samples for the detection of different target taxa cysts were performed in an Applied Biosystems DNA Thermo Cycler 2720 (Foster City, CA, USA). Direct PCR using genus-or species-specific primers was as follows: reaction tubes contained a 50 μL mixture of 200–400 μM of each dNTP; 0.2–0.4

mM of each primer; 3.0-6.0 mM $MgCl_2$; 1X Reaction Buffer (Eppendorf, Germany); 0.5-1X Enhancer Buffer (Eppendorf, Germany); 0.2-1.5 mg/µl BSA (Table 2); 0.5 U of Taq Polymerase (Eppendorf, Germany) and 0.5-1.0 ng of sediment template DNA. PCR conditions were as follows: an initial denaturation step of 10 min at 95°C, 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, and a final extension step of 7 min at 72 °C. The PCR products (20 µL) were resolved on a 1.8% (w/v) agarose, 1X TAE (Tris/Acetic acid/EDTA) buffer gel and were visualized by standard ethidium bromide staining under UV light.

3. RESULTS

Genus and specie specific PCR amplifications using primers designed in the ribosomal genes of one dinoflagellate genus and eight species gave amplified fragments of different base pair lengths as described in Table 2.

Table 2. List of primers used in qualitative PCR based assay.

Target taxa	Forward primer name	Reverse primer Name	Forward primer sequence (5'- 3') → Reverse primer sequence (5'- 3') ←	Amplification size (bp)	G + C %	Primer Locations
Alexandrium spp.	5.8S-3'	5.8S-5'	F'-GCAADGAATGTCTTAGCTCAA R'-GCAMACCTTCAAGMATATCCC	135	38.0 42.8	5.8S(5'→3') 5.8S (3'←5')
Alexandrium pacificum	ITS1c	5.8S-3'	F'-AGCATGATTTGTTTTTCAAGC R'-GCAMACCTTCAAGMATATCCC	226	33.3 42.8	ITS1 (5'→3') 5.8S (3'←5')
Alexandrium minutum	ITS1m	5.8S-3'	F'-CATGCTGCTGTGTTGATGACC R'-GCAMACCTTCAAGMATATCCC	212	52.3 42.8	ITS1 (5'→3') 5.8S (3'←5')
Alexandrium mediterraneum	5.8S-5'	ITS2t	F'- TGTTACTTGTACCTTTGGGA R'- ACAACACCCAGGTTCAAT	134	40.0 44.4	5.8S (5'→3') ITS2 (3'←5')
Fibrocapsa japonica	Fibrocapsa F	Fibrocapsa R	F'-GCAGAGTCCAGCGAGTCATCA R'-TAATATCCCAGACCACGCCAGA	180	57.1 50.0	5.8S (5'→3') ITS2 (3'←5')
Ostreopsis ovata	Ovata F	Ostreopsis R	F'-CAATGCTCATGTCAATGATG R'-CCAGGAGTATGCCTACATTCAA	210	40.0 45.5	ITS1 (5'→3') 5.8S (3'←5')
Lingulodinium polyedrum	L.Poly GF	L.Poly GR	F'-ATGTGTTCTCATCGGATGTTG R'-CACAGTACCGCTGCCACTTAAA	383	45.5 50.0	ITS1 (5'→3') ITS2 (3'←5')
Protoceratium reticulatum	P.ret F	P.ret. R	F'-TGCTGATTGCCATCTATCTT R'-CAGAAGCGCGTTAAACAG	382	40.0 50.0	ITS1 (5'→3') ITS2 (3'←5')
Gonyaulax spinifera	GonyspinF_for	GonyspinR_rev	F'- GAAACTCCTTCTGTGGATGC R'-TCACAGTTCCCTCATGGTACT	154	50.0 47.6	LSU (D1-D2)

The specificity of the primers and the efficiency of the PCR assay were assessed both in the study of Penna et al 2007. The new primers pair for the *Gonyaulax spinifera* was tested for specificity by multiple alignments on *silico* BLAST platform and by PCR amplification of the genomic DNA in the presence of mixed non-target taxa clonal strain DNA together with target microalgal species. These species-specific primers showed high specificity in all PCR experiments and no other detectable bands were observed. The sensitivity and the absence of inhibitors of the PCR-based assay were assessed on the plasmid containing target-cloned sequence. The sensitivity of the PCR assay was also assessed using genomic DNA as a template. The sensitivity of the PCR-based assay carried out on genomic DNA corresponded to the specific PCR amplification of 1pg. The presence of 1 ng of background genomic DNA from the sediment sample, containing mixed dinoflagellate resting cysts, did not have an effect on the sensitivity of any of the species-specific PCR-based assays. Assays using genomic DNA were inhibited by an amount $\geq$ 5 ng of the background DNA from the sediment sample.

3.1 Detection of microalgal resting cysts in Italian harbors using the molecular PCR based assay

A total of 28 sediment samples were collected during the survey of BALMAS project. The PCR-based assay was effective for the qualitative detection of the target cysts in sediment. These samples contained mixed cyst communities including the target morphotypes. PCR detection of the target species was compared with the microscopy analyses of the same sediment samples. The PCR assay detected the presence of the resting stages belonging to different dinoflagellate taxa, even if target cysts were not observed in the sediment samples by microscopy examinations (47 cases). The PCR assays were positive for the presence of cysts of the genus *Alexandrium* and the species *A. minutum, A. mediterraneum* (ex Group II), *Protoceratium reticulatum, Gonyaulax spinifera, Lingulodinium polyedrum* and *Fibrocapsa japonica.* The resting cysts of species *A. pacificum,* which is a potential NIS (non indigenous species) species, was detected in 6 trials in the sediment samples examined by PCR assay in the first and second campaigns of all 4 harbours, but, in particular, it was present in Bari harbour (stations BA1 in first sampling, and BA2 and BA3 in the second campaign (Tables 5 and 6). The positive detection of one genus and 8 species obtained by PCR assay and microscopy were compared. Samples positive using PCR methods were 27/28 for *Alexandrium* sp, 27/28 for *A. minutum*, 9/28 for *A. mediterraneum*, 6/28 for *A. pacificum*, 5/28 for *F. japonica*, 15/28 for *P. reticulatum*, 21/28 for *L. polyedrum* and 23/28 for *G. spinifera*. In contrast, the species-specific identification of *O.* cf. *ovata* was undetected by the molecular method in accordance with microscopy. In general, the number of positive detections obtained by PCR assay was higher than microscopy determinations in samples with 59% of positive determinations by molecular methods than positive detection events by microscopy (41%) (Fig. 1).

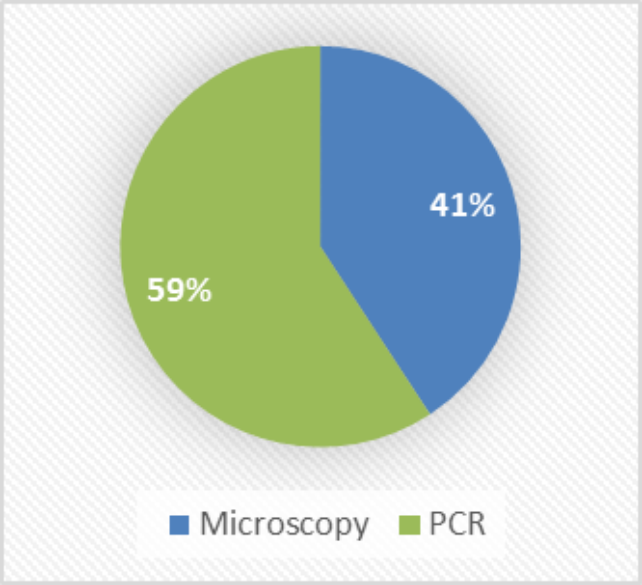


Fig. 2. Percentage of positive determinations obtained by both microscopy and PCR methods in the activity of BALMAS project.

False negatives were checked by inhibitory PCR control experiments adding plasmid containing DNA target or gDNA with sample template in a new reaction (Penna et al. 2010). The lack of PCR-amplified fragments was observed in some sediment samples containing a low fraction of target taxa cysts (a total of 16 cases). For more details, concerning results of single harbours, see Tables and Figures as below.

Table 3. PCR based assay on the ITS-5.8S and LSU rDNA and microscopy analyses of target taxa resting stages from sediments at Trieste harbour, 1st campaign (14 May 2014).

*potential NIS HAB (harmful algal species) species.

Taxon	Station C1		Station ST1		Station ST2		Station ST3		Station ST4	
	Microscopy	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
A. minutum	positive	negative	positive	positive	positive	positive	positive	positive	positive	positive
A. cf. *catenella/tamarense*	positive	*not reported*	negative	*not reported*	positive	*not reported*	negative	*not reported*	positive	*not reported*
A. mediterraneum (ex Group II)	*not reported*	negative	*not reported*	positive	*not reported*	negative	*not reported*	negative	*not reported*	positive
*A. pacificum** (ex Group IV)	*not reported*	negative	*not reported*	negative	*not reported*	negative	*not reported*	negative	*not reported*	positive
Fibrocapsa japonica	negative	negative	negative	positive	negative	negative	negative	negative	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	negative	positive	negative	positive	negative	negative	positive	negative	negative
Lingulodinium polyedrum	positive	positive	positive	negative	positive	positive	positive	positive	positive	positive
Gonyaulax spinifera^	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive

Table 4. PCR based assay on the ITS-5.8s and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Trieste harbour, 2nd campaign (31 March 2015).
*potential NIS HAB (harmful algal species) species.

Taxon	Station C1		Station ST1		Station ST2		Station ST3		Station ST4	
	Microscopy	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
A. minutum	positive	positive	positive	positive	negative	positive	positive	positive	positive	positive
A. cf. *catenella/tamarense*	negative	*not reported*	positive	*not reported*	positive	*not reported*	negative	*not reported*	negative	*not reported*
A. mediterraneum (ex Group II)	*not reported*	negative	*not reported*	negative	*not reported*	negative	*not reported*	negative	*not reported*	negative
*A. pacificum** (ex Group IV)	*not reported*	negative	*not reported*	negative	*not reported*	negative	*not reported*	negative	*not reported*	negative
Fibrocapsa japonica	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	positive	positive	positive	positive	negative	positive	negative	positive	negative	negative
Lingulodinium polyedrum	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Gonyaulax spinifera	positive	positive	positive	positive	positive	negative	positive	negative	positive	positive

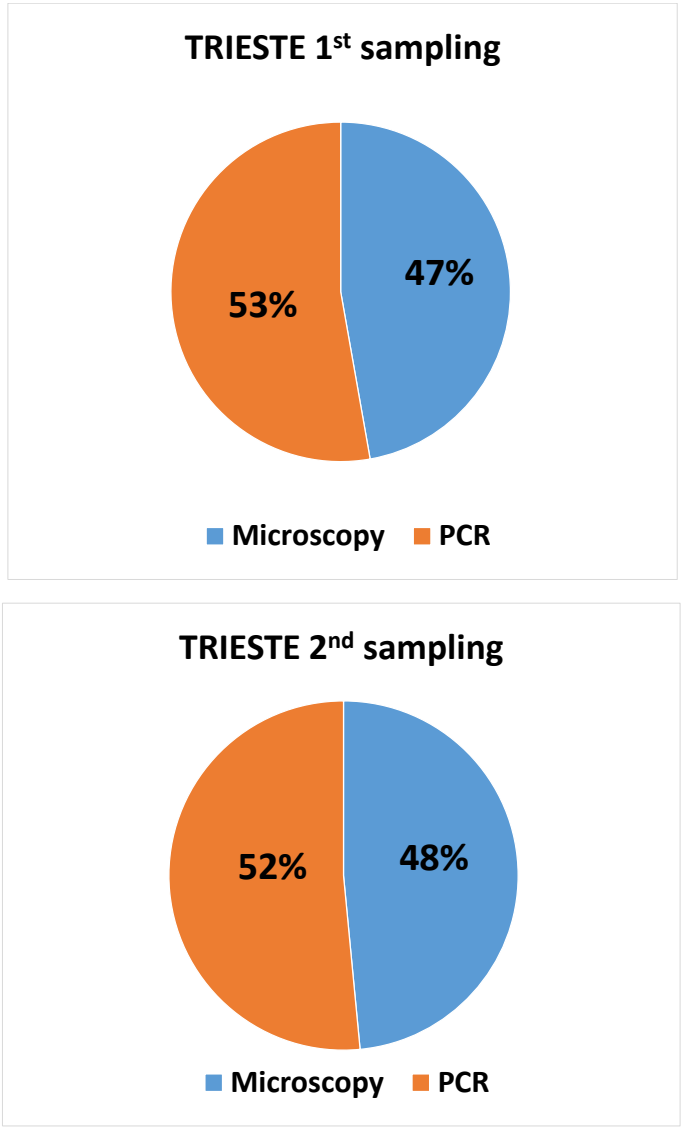


Fig. 3. Percentage of positive determinations obtained by microscopy (blue) and PCR assay (orange).

Table 5. PCR based assay on the ITS-5.8s and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Bari harbour, 1st campaign (28 May 2014).

*****potential NIS HAB (harmful algal species) species.

Taxon	Station BA1		Station BA2		Station BA3	
	Microscopy	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	positive
A. minutum	positive	positive	positive	positive	positive	positive
A. mediterraneum (ex Group II)	positive	positive	positive	positive	positive	negative
*A. pacificum** (ex Group IV)	negative	negative	negative	positive	negative	negative
Fibrocapsa japonica	negative	negative	negative	positive	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	negative	negative	negative	negative	negative
Lingulodinium polyedrum	negative	positive	positive	negative	positive	negative
Gonyaulax spinifera	positive	positive	positive	negative	positive	positive

Table 6. PCR based assay on the ITS-5.8s and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Bari harbour, 2nd campaign (19 November 2014).

Taxon	Station BA1		Station BA2		Station BA3	
	Microscopy	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	positive
A. minutum	positive	positive	positive	positive	positive	positive
A. mediterraneum (ex Group II)	positive	negative	positive	positive	positive	negative
A. pacificum (ex Group IV)*	negative	negative	negative	positive	negative	positive
Fibrocapsa japonica	negative	negative	negative	negative	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	positive	negative	positive	negative	positive
Lingulodinium polyedrum	negative	negative	positive	positive	positive	positive
Gonyaulax spinifera	positive	positive	positive	positive	negative	positive

*potential NIS HAB (harmful algal species) species.

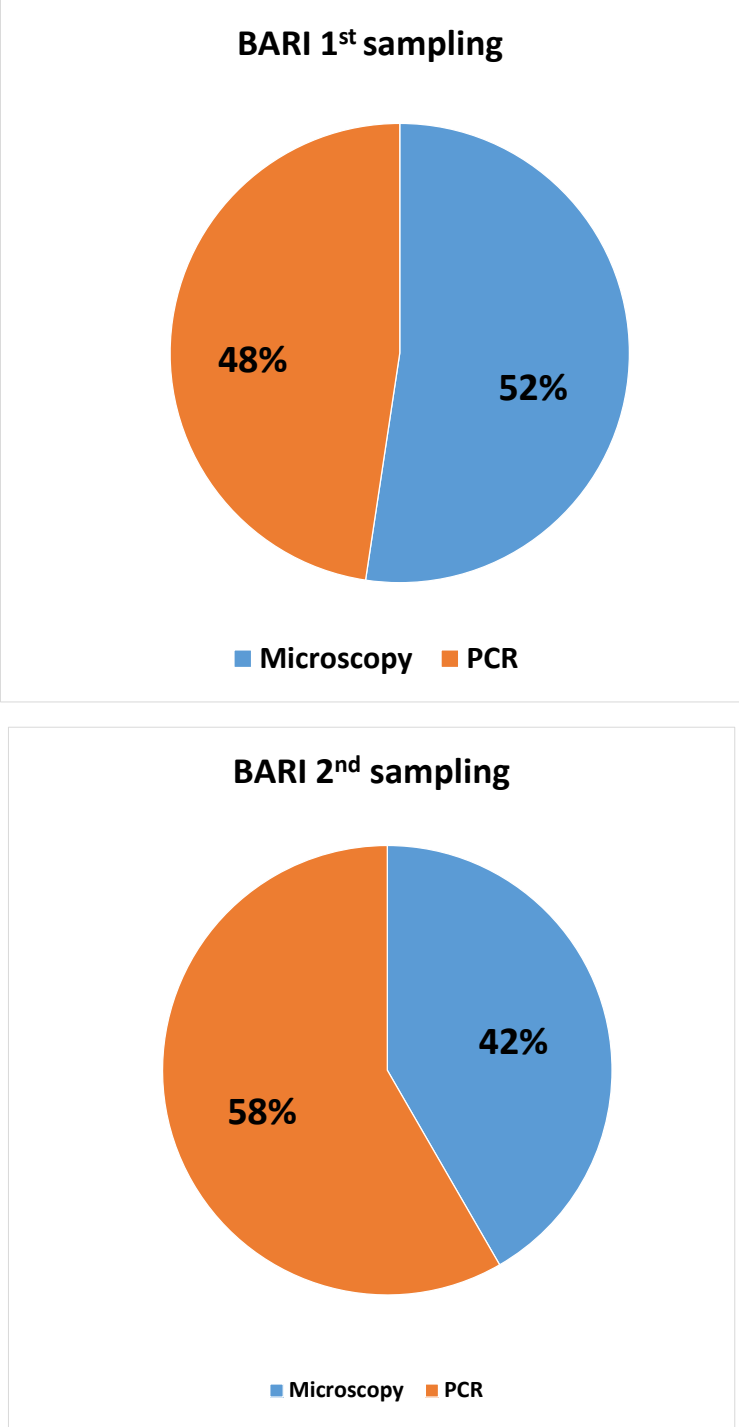


Fig. 4. Percentage of positive determinations obtained by microscopy (blue) and PCR (orange).

Table 7. PCR based assay on the ITS-5.8S and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Venice harbour, 1st campaign (21 May 2014).

	Station BAL PORT 1		Station BAL WAT 2		Station PTF	
Taxon	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	negative
A. minutum	positive	positive	positive	positive	positive	negative
A. mediterraneum (ex Group II)	negative	negative	negative	negative	negative	negative
*A. pacificum** (ex Group IV)	negative	negative	negative	negative	negative	negative
Fibrocapsa japonica	negative	negative	negative	negative	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	positive	negative	negative	negative	negative
Lingulodinium polyedrum	negative	positive	negative	negative	positive	negative
Gonyaulax spinifera	negative	positive	negative	negative	negative	negative

*potential NIS HAB (harmful algal species) species.

Table 8. PCR based assay on the ITS-5.8s and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Venice harbour, 2nd campaign (21 October 2014).

	Station BAL PORT 1		**Station BAL WAT 2**		**Station PTF**	
Taxon	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	negative	positive	positive	positive
A. minutum	positive	positive	negative	positive	positive	positive
A. mediterraneum (ex Group II)	negative	negative	negative	negative	negative	negative
*A. pacificum** (ex Group IV)	negative	negative	negative	positive	negative	negative
Fibrocapsa japonica	negative	negative	negative	negative	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	positive	negative	negative	negative	negative
Lingulodinium polyedrum	positive	positive	positive	positive	positive	negative
Gonyaulax spinifera	negative	positive	negative	positive	negative	positive

*****potential NIS HAB (harmful algal species) species.

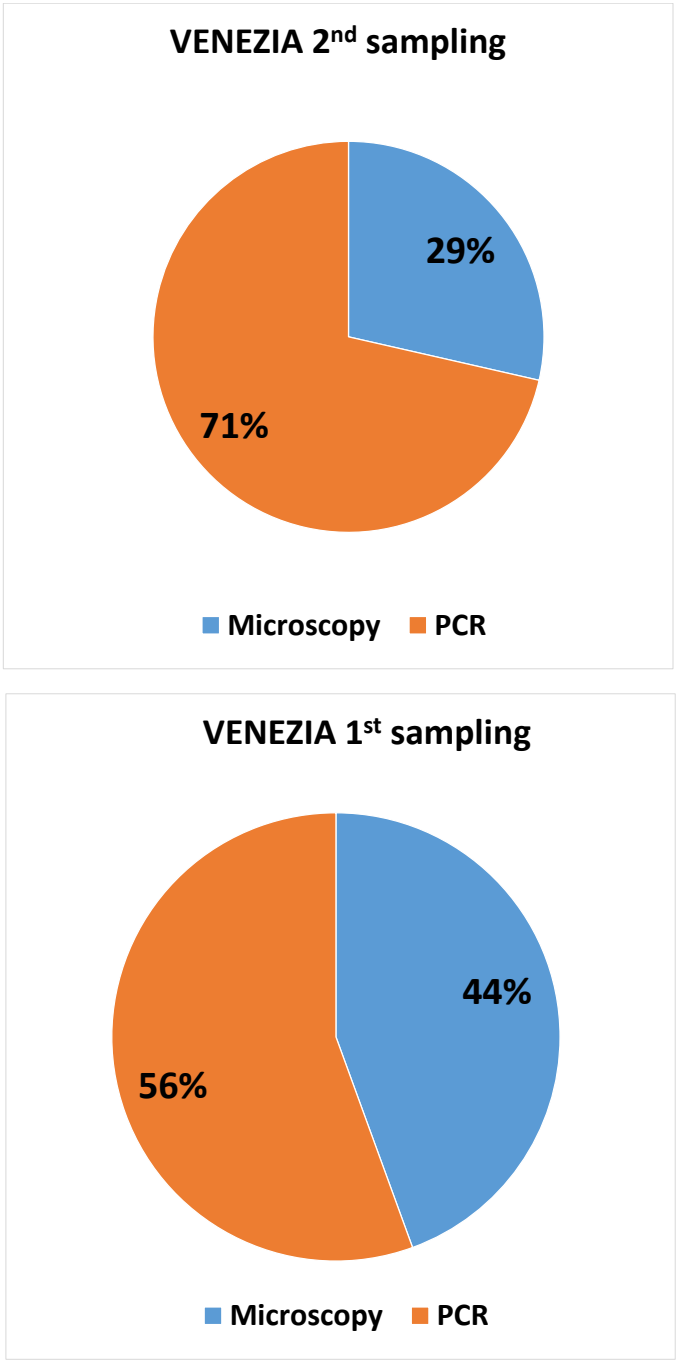


Fig. 5. Percentage of positive determinations obtained by microscopy (blue) and by PCR (orange).

Table 9. PCR based assay on the ITS-5.8s and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Ancona harbour, 1st campaign (7 May 2014).

	Station LR		Station DS		Station API	
Taxon	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	positive
A. minutum	positive	positive	positive	positive	positive	positive
*A. mediterraneum** (ex Group II)	negative	positive	negative	positive	negative	negative
A. pacificum (ex Group IV)	negative	negative	negative	positive	negative	negative
Fibrocapsa japonica	negative	positive	negative	positive	negative	positive
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	positive	negative	positive	negative	positive
Lingulodinium polyedrum	negative	positive	positive	positive	positive	positive
Gonyaulax spinifera	negative	positive	negative	positive	negative	positive

*****potential NIS HAB (harmful algal species) species.

Table 10. PCR based assay on the ITS-5.8s and LSU rDNA and microscopy analysis of target taxa resting stages from sediments at Ancona harbour, 2 nd campaign.

*****potential NIS HAB (harmful algal species) species.

	Station LR		**Station DS**		**Station API**	
Taxon	**Microscopy**	**PCR**	**Microscopy**	**PCR**	**Microscopy**	**PCR**
Alexandrium spp.	positive	positive	positive	positive	positive	positive
A. minutum	positive	positive	positive	positive	positive	positive
*A. mediterraneum** (ex Group II)	negative	positive	negative	positive	negative	negative
A. pacificum (ex Group IV)	negative	negative	negative	negative	negative	negative
Fibrocapsa japonica	negative	negative	negative	negative	negative	negative
Ostreopsis cf. *ovata*	negative	negative	negative	negative	negative	negative
Protoceratium reticulatum	negative	negative	negative	positive	negative	positive
Lingulodinium polyedrum	positive	positive	positive	positive	positive	positive
Gonyaulax spinifera	negative	positive	negative	positive	negative	positive

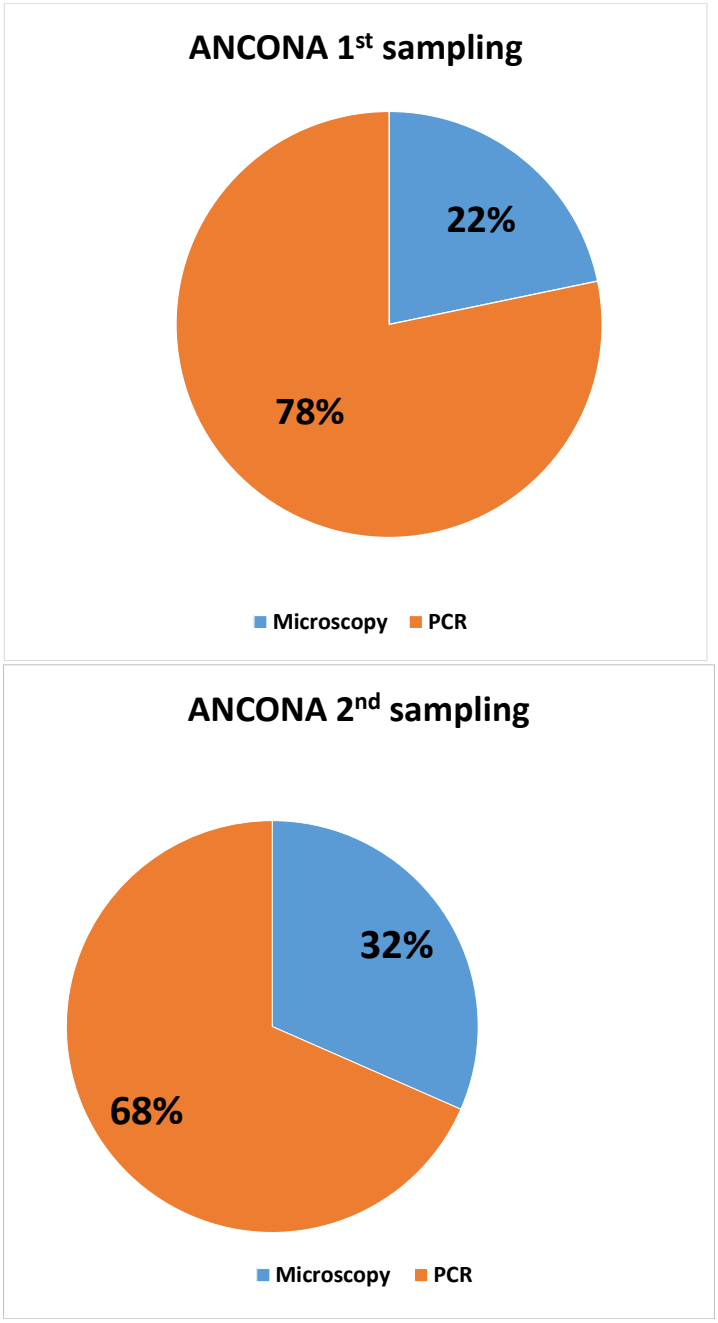


Fig. 6. Percentage of positive determinations obtained by microscopy (blue) and by PCR (orange).

DISCUSSION

Molecular methodologies are highly specific, sensitive, and rapid techniques for the diagnostic identification of microbial eukaryotes in marine environments, including sediments. Moreover, gene amplification techniques (PCR methods) with taxon-specific oligonucleotide primers have been extensively developed and have shown great potential with regard to the identification and enumeration of many harmful dinoflagellate species, including potential NIS HAB species. The PCR technique has mostly been utilized for the identification of vegetative cells in the water column rather than other life cycle stages, such as the resting stages in sediments, but with new

contributions by several authors (Godhe et al. 2002, Erdner et al. 2010, Penna et al. 2010).

This activity proposes the application of the PCR method to sediments for the rapid and specific detection of dinoflagellate cysts in numerous sediment samples during monitoring activity. The molecular PCR techniques based on the amplification of targeted ribosomal genes allow also the accurate and rapid identification of species-specific resting stages, which are difficult to recognize by microscopy methods for the morphological features of the resting cysts. In our case, the *A. tamarense* species complex, *A. mediterraneum* and *A. pacificum* or the raphydophyte *F. japonica* can be detected unambiguously.

Based on the result obtained in this study, the technique produced higher detection efficiency than the microscopic method, as shown by the higher positive percentage of the target harmful cysts in sediment.

The highly variable ITS and LSU regions with more conserved rDNA genes permits discriminations at the inter-species level in sediment samples containing various cyst populations, as demonstrated for other genera and species in seawater samples in our previous study (Penna et al 2007; 2010).

It is likely that the presence of same inhibitory substances, which are not completely eliminated from sediment during extraction and purification using the commercial kit, can negatively affect the PCR reaction. Inhibitor substances, such as humic acid, polyphenols, polysaccharides and metal, and nuclease activity are the major concern when extracting genomic DNA from marine sediments (Stults et al., 2001). In fact, the co-precipitation of compounds that inhibits PCR confuse the molecular analysis of field samples by producing false negative results (Tebbe and Vahjen, 1993). In this monitoring program, we applied a total DNA extraction and purifying procedures using a commercial kit to eliminate the potential inhibitors of the PCR reaction.

CONCLUSION

Based on the results obtained of the monitoring activity, the molecular technique provided a higher positive detection rate of target cysts than microscopy or the same rate of detection. It can be argued that our results were based on the single species -

specific detection, not rather on the complex or group. Therefore, the cysts detection percentage of both methods was accomplished based on this assumption nearby the molecular taxonomic recognition.

In this activity of monitoring resting cysts by molecular method, cysts of the potential NIS and HAB phytoplankton species, such as *A. pacificum*, was detected in six samples.

References

Bolch, C. J. S.; The use of sodium polytungstate for the separation and concentration of living dinoflagellate cysts from marine sediments. *Phycologia*, (1997) 36(6), 472-478

Erdner DL, Percy L, Keafer B, Lewis J, Anderson DM. A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. *Deep Sea Res Part 2 Top Stud Oceanogr*. (2010) ; 57(3-4): 279–287

Godhe A.,. AndersonD.M., Rehnstam-Holm A.S.. PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. (2002) *Harmful Algae* 375–382

Penna, A., Bertozzini, E., Battocchi, C., Galluzzi, L., Giacobbe, M.G., Vila, M., Esther Garces, E., Lugliè, A., Magnani, M.; Monitoring of HAB species in the Mediterranean Sea through molecular methods. *J. Plankton Res*. (2007) 29 (1), 19-38

Penna A., Fraga S., Battocchi C., Casabianca S., Giacobbe M.G., Riobó P., Vernesi C. - A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt. *J. Biogeogr.* (2010, 37: 830-841.

Stults JR, Snoeyenbos-West O, Methe B, Lovley DR, Chandler DP. Application of the 5′ fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Appl Environ Microbiol*.(2001) 67:2781–2789

Tebbe .C C. and Vahjen W.. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol*. (1993) vol. 59 no. 8 2657-2665.

6.3 CATALOGUE OF HARMFUL AQUATIC ORGANISMS AND PATHOGEN (HAOP) SPECIES

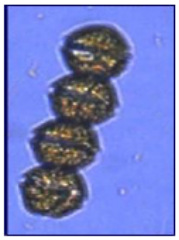
Appendix

BALMAS
Ballast water management system for Adriatic Sea protection

1.11.2013 – 30.9.2016
Project number: 1°str./0005
IPA Adriatic Cross-border Cooperation Programme 2007 – 2013

Catalogue of HAOP species producing resting cysts in selected Adriatic ports.

Alexandrium pacificum
R.W. Litaker, 2014



Indigenous or non indigenous or cryptogenic: Non indigenous.

Recipient region by BALMAS project: The resting cysts of toxic species *A. pacificum* were found in all port sediment samples examined by molecular PCR assay (Bari, Ancona and Venice).

Presence-Abundance: Regular, mostly in spring and summer.

Habitat and ecology: Planktonic, distributed in temperate coastal waters; it has been frequently reported in the western, and now, in Central Mediterranean Sea. Ecology related to high salinities and high concentrations of NO_3 and NH_4.

Documented blooms: Many blooms are associated with eutrophic, warm water surface temperatures, in semi-confined areas and lagoons, with local accumulation of cysts in the western Mediterranean Sea.

Geographic distribution in the Mediterranean Sea: Western and Central Mediterranean Sea.

Harmful effects and documented impacts: Impact on community, habitat, ecosystem (harmful algal bloom), health (PSP Toxin contamination of shellfish).

Introduction pathways: Anthropogenic pathways (ship ballast waters or aquaculture activities).

Management measures: Monitoring of coastal waters programs; reducing nutrient content of riverine inputs (urban waste water, agricultural and industrial discharge); treatment of sediments, plastic debris; control of ballast waters.

Alexandrium pacificum

Classification

Phylum Miozoa
Class Dinophyceae
Order Gonyaulacales
Family Gonyaulacaceae
Genus Alexandrium

Identification

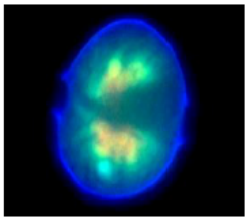
This species was misidentified as *Gonyaulax catenella* or *Alexandrium catenella* in temperate waters of western Pacific Ocean and Mediterranean Sea. Genetically, it was named as Temperate Asian (TA) clade and Group IV. Now, the taxonomical name is *Alexandrium pacificum*.

References: Bravo, I. et al. (2008) *Alexandrium catenella* and *Alexandrium minutum* blooms in the Mediterranean Sea: Toward the identification of ecological niches. *Harmful Algae* 7: 515-522. Collos, Y. et al. (2004) Nitrogenous nutrition of *Alexandrium catenella* (Dinophyceae) in cultures and in Thau lagoon, Southern France. *J. Phycol.* 40: 96-103. Jauzein et al. (2008) Short-term temporal variability of ammonium and urea uptake by *Alexandrium catenella* (Dinophyta) in cultures. *J. Phycol.* 44:1136-1145. Masseret, E. et al. (2009) Unexpected genetic diversity among and within populations of the toxic dinoflagellate *Alexandrium catenella* as revealed by nuclear microsatellite markers. *Appl. Environ. Microbiol.* 75: 2037-2045. Penna, A. et al. (2005) *Alexandrium catenella* (Dinophyceae), a toxic ribotype expanding in the NW Mediterranean Sea. *Mar. Biol.* 148: 13-23. Penna A., et al. 2015. The *sxt* gene and paralytic shellfish poisoning toxins as markers for the monitoring of toxic *Alexandrium* species blooms. *Environ. Sci. Technol.* 49: 14230–14238. Vila, M. et al. (2001) Is the distribution of the toxic dinoflagellate*Alexandrium catenella* expanding along the NW Mediterranean coast? *Mar. Ecol. Progr. Ser.* 222: 73-83.

Project co-found by UE
Instrument for Pre-Accession Assistance

www.balmas.eu

Alexandrium minutum
Halim, 1960



Indigenous or non indigenous or cryptogenic: Indigenous.

Recipient region by BALMAS project: The resting cysts of toxic species *A. minutum* were found in the Port sediment samples examined: Bari, Ancona, Koper, Venice, Sibenik, Split.

Presence-Abundance: Regular, mostly in spring and autumn.

Habitat and ecology: Planktonic in warm, temperate, coastal and estuarine waters. In particular, found in coastal enriched sites, harbours, estuaries or lagoons. Ecology related to low salinities and nutrient-rich freshwater inputs; euryhaline and eurytherm.

Documented blooms: Many blooms are associated with local accumulation of cysts in confined water areas.

Geographic distribution in the Mediterranean Sea: Broadly distributed.

Harmful and documented impacts: Producer of paralytic shellfish poisoning toxins (GTXs, STX, neoSTX), possible toxins accumulation in marine food webs, high-biomass blooms, water discolouration events. Impact on human health, zooplankton, fisheries, aquaculture, tourism.

Introduction pathways: Association with vessel vector (ballast water).

Management measures: Monitoring of coastal waters programs; reducing nutrient content of riverine inputs (urban waste water, industrial and agricultural discharges); treatment of sediments, plastic debris; control of ballast waters.

Alexandrium minutum

Classification

Phylum Miozoa

Class Dinophyceae

*Order*Gonyaulacales

*Family*Dinophyta

*Genus*Alexandrium

Identification

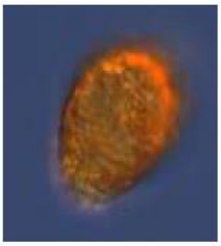
Cysts are roughly hemispherical in shape, almost circular when observed from above and kidney-shaped from the side. The cyst wall is pale in color and is covered by a thin layer of mucilage. The cytoplasm is granular with numerous lipid globules and an orange-red accumulation body.

References: Anglés, S., et al. (2010) *Alexandrium minutum* resting cyst distribution dynamics in a confined site. *Deep Sea Res. II*: 57: 210-221; Figueroa, R.I., Garcés, E., and Camp, J. (2010) Reproductive plasticity and local adaptation in the host parasite system formed by the toxic *Alexandrium minutum*and the dinoflagellate parasite *Parvilucifera sinerae*. *Harmful Algae* 10: 56-63; Estrada, et al. (2010) The role of resting cysts in *Alexandrium minutum*population dynamics. *Deep Sea Res. II:* 57: 308-321; Lilly, E.L. et al. (2005) Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. *Harmful Algae* 4: 1004–1020; Penna, A. et al. (2008) Phylogenetic relationships among the Mediterranean *Alexandrium*(Dinophyceae) species based on sequences of 5.8S gene and internal transcribed spacers of the rRNA operon. *Eur. J. Phycol.* 43: 163-178; Vila, M. et al. (2005) A comparative study on recurrent blooms of *Alexandrium minutum*in in two Mediterranean coastal areas. *Harmful Algae* 4: 673-695. Penna A., et al. 2015. The *sxt* gene and paralytic shellfish poisoning toxins as markers for the monitoring of toxic *Alexandrium* species blooms. *Environ. Sci. Technol.* 49: 14230–14238.

Project co-found by UE
Instrument for Pre-Accession Assistance

www.balmas.eu

Fibrocapsa japonica
Toriumi & Takano 1973



Indigenous or non indigenous or cryptogenic: Indigenous.

Recipient region by BALMAS project: Cysts of *F. japonica* were detected in the ports of Bari, Trieste, Ancona.

Presence-Abundance: NA.

Habitat and ecology: Planktonic in warm, temperate, coastal and estuarine waters. In particular, found in coastal enriched sites, estuaries. Ecology, low nutrient uptake efficiency, and its growth is favoured in high-nutrient conditions, which are frequently encountered in the stratified shallow coastal and brackish waters.

Documented blooms: Occasionally blooms are associated with eutrophic, warm water surface temperatures, coastal areas and lagoons.

Geographic distribution in the Mediterranean Sea: Central Mediterranean Sea.

Harmful effects and documented impacts: Fishery effect. Impact on fisheries and tourism.

Introduction pathways: NA.

Management measures: Monitoring of coastal waters programs; reducing nutrient content of riverine inputs (urban waste water, industrial and agricultural discharges); treatment of sediments, control of ballast waters.

Fibrocapsa japonica

Classification

Phylum Ochrophyta
Class Raphidophyceae
Order Chattonellales
Family Chattonellaceae
Genus Fibrocapsa

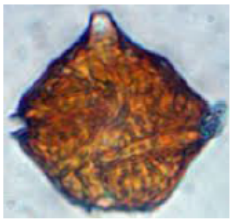
Identification

At the microscope, cells appear ovate, mainly 'raspberry' shaped, eventually aggregated into mucous nets.

References: Cucchiari E. et al. 2010. Resting cysts of *Fibrocapsa japonica*(Raphidophyceae) from coastal sediments of the northern Adriatic Sea (Mediterranean Sea). *Harmful Algae,* 10: 81-87; de Boer et al., 2012. The toxic effect of the marine raphidophyte *Fibrocapsa japonica* on larvae of the com-mon flatfish sole (Solea solea). *Harmful Algae,* 17 (1), 92- 101; de Boer et al. 2004. Effects of salinity and nutrient conditions on growth and haemolytic activity of *Fibrocapsa japonica* (Raphidophyceae). *Aquat. Microb. Ecol.,* 37 (2), 171-181. Fani F. et al. 2014. *Fibrocapsa japonica* (Raphidophyceae) occurrence and ecological features within the phytoplankton assemblage of a cyclonic eddy, offshore the Eastern Alboran Sea. *Med. Mar. Sci.* 15: 250-262; Pezzolesi, L. et al., 2010. Toxicity evaluation of *Fibrocapsa japonica* from the Northern Adriatic Sea through a chemical and toxicological approach. *Harmful Algae,* 9 (5), 504-514.

Project co-found by UE
Instrument for Pre-Accession Assistance
www.balmas.eu

Gonyaulax spinifera (Claparède & Lachmann) Diesing 1866



Indigenous or non indigenous or cryptogenic: Indigenous.

Recipient region by BALMAS project: The resting cysts of toxic species *G. spinifera* were found in the Port sediment samples examined: Bari, Ancona Venice, Trieste, Sibenik, Split, Koper, Pula, Rijeka.

Presence-Abundance: Regular, mostly in summer and autumn.

Habitat and ecology: Planktonic in temperate, coastal waters. Both heterotrophic (eat other organisms) and autotrophic (photosynthetic). Predators of marine microorganisms and animal larvae; reproduction both sexual and asexual.

Documented blooms: It is a recurrent species in the northern Adriatic Sea forming also blooms.

Geographic distribution in the Mediterranean Sea: Broadly distributed.

Harmful effects and documented impacts: Producer of yessotoxin shellfish poisoning toxins (YTXs), possible toxins accumulation in marine food webs, high-biomass blooms, water discolouration events (particularly when in large numbers, called "red tides" because the cells are so abundant they make water change colour). Impact on human health and aquaculture.

Introduction pathways: NA

Management measures: Monitoring of coastal waters and intensive analyses of mussel toxicity at shellfish farms.

Gonyaulax spinifera

Classification

Phylum Miozoa
Class Dinophyceae
Order Gonyaulacales
Family Gonyaulacaceae
Genus Gonyaulax

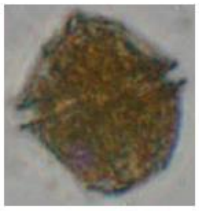
Identification

G. spinifera cells are slightly longer than broad. The epictheca has convex sides and a small epical horn. The hypotheca has 2-4 antapical spines. The sulcus extends almost the whole length of the cell. The cingulum is deeply excavated and displaced by 2 or more widths. G. spinifera is sometimes confused with *Gonyaulax digitale*. It is likely that *Gonyaulax spinifera* is actually a species complex rather than a single species.

References: Gárate-Lizárraga et al. (2014). Bloom of *Gonyaulax spinifera* (Dinophyceae: Gonyaulacales) in Ensenada de La Paz Lagoon, Gulf of California. *CICIMAR Oceanides* 29(1): 11-18; Ciminiello, P. et al. (2007). Desulfoyesso toxins from Adriatic mussels: A new problem for seafood safety control. *Chem. Res. Toxicol.,* 20(1), 95–98; Penna, A. et al. (2006). Potentially harmful microalgal distribution in an area of the NW Adriatic coastline: Sampling procedure and correlations with environmental factors. *Estuar.Coastal Shelf Sci.,* 70(1-2), 307–316; Riccardi, M. et al. (2009). *Gonyaulax spinifera* from the Adriatic Sea: Toxin production and phylogenetic analysis. Harmful Algae, 8(2), 279–290.

Project co-found by UE
Instrument for Pre-Accession Assistance
www.balmas.eu

Lingulodinium polyedrum (Stein)Dodge, 1989



Indigenous or non indigenous or cryptogenic: Indigenous.

Recipient region by BALMAS project: The resting cysts of toxic species *L. polyedrum* were found in the Port sediment samples examined: Venice, Trieste, Sibenk, Split, Koper, Ancona, Bari, Pula.

Presence-Abundance: Regular, mostly in spring and autumn.

Habitat and ecology: Planktonic, found in estuarine waters. It is a bioluminescent planktonic species commonly found in neritic waters. It is responsible for magnificent displays of phosphorescence at night in warm coastal waters. This warm-water species is a red tide former that has been associated with fish and shellfish mortality events.

Documented blooms: It is a recurrent species in the northern Adriatic Sea forming also blooms.

Geographic distribution in the Mediterranean Sea: Widely distributed.

Harmful effects and documented impacts: YTXs producer, shellfish farm contamination. Shellfish farm contamination and related economic losses.

Introduction pathways: NA.

Management measures: Monitoring of coastal waters and intensive analyses of mussel toxicity at shellfish farms.

Lingulodinium polyedrum

Classification

Phylum Miozoa
Class Dinophyceae
Order Gonyaulacales
Family Gonyaulacaceae
Genus Lingulodinium

Identification

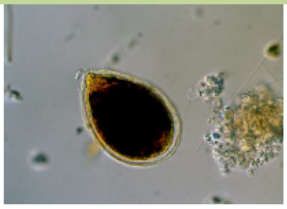
Cells are angular, roughly pentagonal and polyhedral-shaped. Cells range in size from 40-54 µm in length and 37-53 µm in transdiameter width. It is a photosynthetic species with dark orange-brown chloroplasts.

References: Rubino. et al. Dinoflagellate cysts from surface sediments of Syracuse Bay (Western Ionian Sea, Mediterranean). *Deep Sea Res.* II, 57: 243-247; Paz, B., Riobó, P., Fernández, M. L., Fraga, S., Franco, J. M. (2004). Production and release of yessotoxins by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* in culture. *Toxicon*, 44(3), 251–8.

Project co-found by UE
Instrument for Pre-Accession Assistance

www.balmas.eu

Ostreopsis spp.
Johs. Schmidt, 1901



Indigenous or non indigenous or cryptogenic: *O. ovata* and *O. siamensis* are considered NIS in the Mediterranean Sea (http://www.marinespecies.org/).

Recipient region by BALMAS project: Bari, Pula.

Presence-Abundance: Rare.

Habitat and Ecology: Marine coastal regions of tropical and subtropical oceans; two species (*O. ovata*, *O. siamensis*, *O. fattorussoi*) in the Mediterranean Sea (Taylor, 1979; Tognetto et al., 1995; Accoroni et al. 2016). Living epiphytic or benthic on macroalgae, corals or detritus.

Geographic distribution in the Mediterranean Sea: *Ostreopsis* cf. *ovata* has been reported in Italy (along all coastal areas with few exceptions), Spain, France, Croatia, Albania, Greece, and along the Lebanese and north African coasts (Penna et al. 2014).

Harmful effect and documented impacts: *O. cf. ovata* and *O. cf. siamensis* are producer of palytoxin analogues (Ciminiello et al. 2011). In the last few years, serious cases of human intoxication associated with *Ostreopsis* spp. blooms have been recorded along the Italian, French and Spanish coastlines (Durando et al. 2007; Barroso Garcia et al. 2008; Tichadou et al. 2010).

Documented blooms: The presence of *Ostreopsis* spp. in tropical and sub-tropical areas has been described for over a century; during the last two decades, increasing occurrences of *O.* cf. *ovata* and *O.* cf. *siamensis* in temperate areas, such as New Zealand and the coast of Japan, have been reported (Shears and Ross, 2009). Marked increase of toxic blooms and widespread of *O.* cf. *ovata* has occurred in the Mediterranean Sea since 2005, where it has been reported as the most widespread and abundant between the two species (Battocchi et al., 2010; Totti et al. 2010; Mangialajo et al., 2011).

Introduction pathways: Association with vessel vector (ballast water).

Management measures: Italian guidelines to assess and manage the risk associated to bathing waters and recreational activities (Funari et al., 2015).

Ostreopsis

Classification

Phylum Miozoa

Class Dinophyceae

Order Gonyaulacales

Family Goniodomataceae

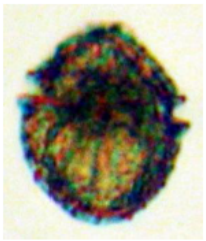
Genus Ostreopsis

Identification

Benthic, phototrophic, unicellular biflagellate heterodynamic cells of medium size (30-110 µm) with cell wall of cellulosic plates. Cells are apical-antapically compressed, ovoid in apical and antapical view but lens-shaped in ventral or dorsal view. The apical pore is a long slit, displaced to the left dorsal side. The cingulum is deeply impressed, the small recessed sulcus is restricted to the hyposome, with one sulcal list. Nucleus of the dinokarotic type, spherical, located posteriorly. Chloroplasts present.
Nine morphospecies have been described in the genus, based only on size, morphology, and thecal plate pattern. The identification at the species level is problematic, because there have been imprecisions in the original descriptions which led to misunderstandings and confusions in subsequent interpretations (Hoppenrath et al., 2014).

References: Accoroni et al. *Ostreopsis fatturussoi* sp. nov. (Dinophyceae), a new benthic toxic *Ostreopsis* species from the eastern Mediterranean Sea. *J. Phycol.* (in press). Barroso Garcia et al. (2008) An epidemic outbreak with respiratory symptoms in the province of Almeria [Spain] due to toxic microalgae exposure. *Gac. Sanit.* 22, 578–584. Battocchi et al., 2010. Monitoring toxic microalgae *Ostreopsis* (dinoflagellate) species in coastal waters of the Mediterranean Sea using molecular PCR-based assay combined with light microscopy. *Mar. Pollut. Bull.* 60, 1074–1084; Ciminiello et al. (2010) Complex palytoxin-like profile of *Ostreopsis ovata*. Identification of four new ovatoxins by high-resolution liquid chromatography/mass spectrometry. *Rapid Commun. Mass. Spectrom.* 24, 2735–2744. Durando et al. (2007) *Ostreopsis ovata* and human health: epidemiological and clinical features of respiratory syndrome outbreaks from a two-year syndromic surveillance, 2005–06, in northwest Italy. *Euro Surveill.* 12 (23), 3212. Funari et al. 2015. *Ostreospis cf. ovata* blooms in coastal water: Italian guidelines to assess and manage the risk associated to bathing waters and recreational activities. *Harmful Algae* 50, 45–56; Hoppenrath et al. 2014. Marine benthic dinoflagellates - unravelling their worldwide biodiversity. KleineSenckenberg-Reihe Vol. 54. pp. [1]-276. Frankfurt am Main & Stuttgar: V. Mosbrugger& E. Schweizerbartsche Verlagsbuchhandlung; Mangialajo et al. 2011. Trends in Ostreopsis proliferation along the Northern Mediterranean coasts. *Toxicon* 57, 408–420; Penna et al. 2014. Mitochondrial, but not rDNA, genes fail to discriminate dinoflagellate species in the genus *Ostreopsis*. *Harmful Algae* 40: 40-50. Totti et al. (2010). *Ostreopsis ovata* bloom along the Conero Riviera (northern Adriatic Sea): Relationships with environmental conditions and substrata. *Harmful Algae*, 9, 233–239. Tichadou et al. (2010) Health impact of unicellular algae of the Ostreopsis genus blooms in the Mediterranean Sea: experience of the French Mediterranean coast surveillance network from 2006 to 2009. Clin. Toxicol. 48 (8), 839–844.

Protoceratium reticulatum (Claparède & Lachmann) Bütschli, 1885



Indigenous or non indigenous or cryptogenic: Indigenous.

Recipient region by BALMAS project: The resting cysts of toxic species *Protoceratium reticulatum* were found in the Port sediment samples examined: Bari, Ancona, Venice, Trieste, Koper, Split.

Presence-Abundance: Regular, mostly in spring and autumn.

Habitat and ecology: Planktonic, found in estuarine waters. Is a heterotrophic dinoflagellate. It reproduces asexually.

Documented blooms: It is a recurrent species in the northern Adriatic Sea forming also blooms.

Geographic distribution in the Mediterranean Sea: Widely distributed.

Harmful effects and documented impacts: YTXs producer, shellfish farm contamination. Impact shellfish farm contamination and related economic losses.

Introduction pathways: NA.

Management measures: Monitoring of coastal waters and intensive analyses of mussel toxicity at shellfish farms.

Protoceratium reticulatum

Classification

Phylum Miozoa
Class Dinophyceae
Order Gonyaulacales
Family Gonyaulacaceae
Genus Protoceratium

Identification

This dinoflagellate exists as unicells with an oval shape, occasionally becoming somewhat polygonal. The cysts are about 30-40 µm in diameter.

References: Ciminiello et al. (2003). Complex yessotoxins profile in *Protoceratium reticulatum* from north-western Adriatic Sea revealed by LC–MS analysis. *Toxicon* 42: 7-14; Guerrini et al. 2007. Influence of temperature, salinity and nutrient limitation on yessotoxin production andrelease by the dinoflagellate *Protoceratium reticulatumin* batch-cultures. *Harmful Algae* 6:707-717; Paz et al. 2004. Production and release of yessotoxins by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrumin* culture. *Toxicon* 44: 251-58; Paz et al. 2006. Study of the effect of temperature, irradiance and salinity on growth and yessotoxin production by the dinoflagellate *Protoceratium reticulatum* in culture by using a kinetic and factorial approach. *Mar. Env. Res.* 62: 286-300.

Bibliografia

Adachi, R., Fukuyo, Y. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. Bull. Japan. Soc. Sci. Fish., 45 (1), 67–71 (1979).

Adachi, M., Sako, Y., Ishida, Y. Identification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (Dinophyceae) using DNA probes and whole-cell hybridization. J. Phycol., 32, 1049–1052 (1996).

Ade, P., Funari, E., Poletti, R. Il rischio sanitario associato alle tossine di alghe marine. Annali Istituto Superiore di Sanità, 39 (1), 53-68 (2003).

Adema, D.M.M. *Daphnia magna* as a Test Animal in Acute and Chronic Toxicity Tests. Hydrobiologia, 59, 125-134 (1978).

Al-Kandari, M.A., Highfield, A.C., Hall, M.J., Hayes, P., Schroeder, D.C. Molecular tools separate harmful algal bloom species, *Karenia mikimotoi*, from different geographical regions into distinct sub-groups. Harmful Algae, 10, 636-643 (2011).

Amzil, Z., Fresnel, J., Le Gal, D., Billard, C. Domoic acid accumulation in French shellfish in relation to toxic species of *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima*. Toxicon, 39, 1245-1251 (2001).

Anderson, D.M., Glibert, P.M., Burkholder, J.M. Harmful algal blooms and eutrophication: nutrient sources, composition and consequences. Estuaries, 25, 562– 584 (2002).

Anderson, D.M., Keafer, B.A., Kulis, D.M., Connell, L., Scholin, C.A.Application of molecular probes in studies of *Alexandrium* in the Gulf of Maine: success and problem areas. Abstract, 10th International Conference on Harmful Algae, St. Pete Beach, Fla., 14, (2002).

Andree, K. B., Fernández-Tejedor, M., Elandaloussi, L. M., Quijano-Scheggia, S., Sampedro, N., Garcés, E., ... & Diogène, J.. Quantitative PCR coupled with melt curve analysis for detection of selected Pseudo-nitzschia spp.(Bacillariophyceae) from the northwestern Mediterranean Sea. Applied and environmental microbiology, 77(5), 1651-1659 (2011).

Atlantic Research Laboratory Technical Report 56, NRCC 29083; 57, NRCC 29086.

Ayers, K., Rhodes, L.L., Tyrrell, J., Gladstone, M., Scholin, C. International accreditation of sandwich hybridisation assay format DNA probes for micro-algae. New Zealand Journal Of Marine And Freshwater Research, 39, 6 (2005).

Barra, L., Ruggiero, M.V., Sarno, D., Montresor, M., Kooistra, W.H.C.F. Strengths and weaknesses of microarray approaches to detect *Pseudo-nitzschia* species in the field. Environ. Sci. Poll. Res., 20, 6705-6718 (2013).

Barra, L., Ruggiero, M.V., Chen, J., Kooistra, W.H.C.F. Specificity of LSU rRNA-targeted oligonucleotide probes for *Pseudo-nitzschia* species tested through dot-blot hybridization. Environ. Sci. Poll. Res., 21, 548–557 (2014).

Bates, S.S., Garrison, D.L., Horner, R.A. Bloom dynamics and physiology of domoic-acid-producing *Pseudo-nitzschia* species. In Anderson D.M., Cembella A.D., Hallegraef G.M. Physiological Ecology of Harmful Algal Blooms. G 41, 267-292. Springer-Verlag, Berlin. (1998).

Bates, S.S., Gaudet, J., Kaczmarska, I., Ehrman, J.M. Interaction between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle; can bacteria produce domoic acid autonomously? Harmful Algae, 3, 11-20 (2004).

Bibby, B.T., Dodge, J.D. The encystment of a freshwater dinoflagellate: a light and electronmicroscopical study. Br. Phycol. J., 7, 85-100 (1972).

Bogana, Y.M., Harkina, A.L., Gillespiea, J., Kennedya, D.J., Hessb, P., Slatera, J.W. The influence of size on domoic acid concentration in king scallop, *Pecten maximus* (L.). Harmful Algae, 6 (1), 15-28 (2007).

Bolch, C. J. S. The use of sodium polytungstate for the separation and concentration of living dinoflagellate cysts from marine sediments. Phycologia, 36(6), 472-478 (1997).

Cabrini, M., Del Negro, P., Predonzani, S. Distribution of DSP toxins in different tissues of *Mytylus galloprovincialis*. Measure for success. Bordeaux Acquaculture, 19. EAS, Spec. Pubbl. 21, 234-235 (1994).

Cangelosi, G.A., Hamlin, A.M., Marin, R., Scholin C.A. Detection of stable prerRNA in toxigenic *Pseudo-nitzschia* species. Appl. Environ. Microbiol., 63, 4859-4865 (1997).

Casabianca, S., Casabianca, A., Riobò, P., Franco, J.M., Vila, M., Penna, A. Quantification of the Toxic Dinoflagellate *Ostreopsis* spp. by qPCR Assay in Marine Aerosol. Environ. Sci. Technol., 47, 3788−3795 (2013).

Ceccarelli, M., Galluzzi, L., Migliazzo, A., Magnani, M. Detection and Characterization of *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by SYBR green- based real-time PCR and high resolution melt analysis targeting kinetoplast minicircle DNA. PLoS One, 9 (2), e88845 (2014).

CEMAGREF 1982. Étude des méthodes biologiques d'appreciation quantitative de la qualité des eaux. Rapport Q. E. Lyon- A. F. Bassin Rhône- Mediterranée Corse. Lyon: CEMAGREF. (1982).

Cerino, F., Orsini, L., Sarno, D., Dell'Aversano, C., Tartaglione, L., & Zingone, A. The alternation of different morphotypes in the seasonal cycle of the toxic diatom *Pseudo-nitzschia galaxiae*. Harmful Algae, 4, 33-48 (2005).

Ciminiello, P., Dell'aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C., Melchiorre, N. The Genoa 2005 Outbreak. Determination of Putative Palytoxin in Mediterranean *Ostreopsis ovata* by a New Liquid Chromatography Tandem Mass Spectrometry Method. Anal. Chem., 78 (17), 6153–6159 (2006).

Cloern, J.E. Our evolving conceptual model of the costal eutrophication problem. Mar. Ecol.-Prog. Ser., 210, 223-253 (2001).

Daigo, K. Studies on the constituents of *Chondria armata*. II. Isolation of an anthelmintical constituent. Yakugaku Zasshi (J. Pharm Soc Jpn), 79, 353-356 (1959).

Dale, B., Yentsch, C. M. and Hurst, J. Toxicity in resting cysts of the red-tide dinoflagellate *Gonyaulax excavata* from deeper water coastal sediments. Science, 201, 1223-1225 (1978).

Davidovich, N. A., Bates, S.S. *Pseudo-nitzschia* life cycle and the sexual diversity of clones in diatom populations. In Garces, E., Zingone, A., Dale, B., Montresor, M., Reguera, B. (eds.) Proc of the Life HAB workshop: Life history of microalgal species causing of harmful algal blooms. Office Publ Eur Comm, Luxemborug, 27-30 (2002).

Deny, L. Relation of abundance-weighted averages of diatom indicator values to measured environmental conditions in standing freshwaters. Ecol. Indic., 4, 225-275 (2004).

Descy, J.P. A new approach to water quality estimation using diatoms. Nova Edwigia, 64, 305-323 (1979).

Dodge, J.D. A review of the fine structure of algal eyespots. Br. Phycol. J., 4, 199-210 (1969).

Dodge, J.D., Crawford, R.M. The morphology and fine structure of *Ceratium hirundinella* (Dinophyceae). J. Phycol., 6, 137-149 (1970).

Dodge, J.D. The Fine Structure of Algal Cells. Accademic Press, London (1973).

Dodge, J.D. General ultrastructure. In: The Biology of the Dinoflagellates, ed. F.J.R. Taylor, Blackwell Scientific Publications, Oxford, 93-119 (1987).

EFSA, 2009. Marine biotoxins in shellfish-domoic acid. Scientific Opinion of the Panel on Contaminants in the Food Chain (Question No EFSA-Q-2006-065H). The EFSA Journal, 1181, 1-61 (2009).

Eller, G., Medlin, L. Molecular probes for the rapid detection of toxin marine microalgae. Abstract, 10th International Conference on Harmful Algae, St. Pete Beach, Fla., 83 (2002).

Eloranta, P. Applications of diatom indices in Finnish rivers. In: Prygiel J., Whitton B.A. & Bukowska J. (eds) Use of Algae for Monitoring Rivers III, 138- 144 (1999).

Erdner, D.L., Percy, L., Keafer, B., Lewis, J., Anderson, D.M. A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep Sea Res. Part II: Topical Studies in Oceanography, 57 (3-4), 279–287 (2010).

Fehling, J., Green, D.H., Davidson, K., Bolch, C.J., Bates, S.S. Domoic acid production by *Pseudo-nitzschia seriata* (bacillariophyceae) in Scottish waters. J. Phycol., 40 (4), 622–630 (2004).

Forbes, J. R., Denman, K.L. Distribution of *Nitzschia pungens* in coastal waters of British Columbia. Can. J. Fish. Aquat. Sci., 48, 960-967 (1991).

Fraga, S., Alvarez, M.J., Miguez, A., Fernandez, M.L., Costas, E., Lopez-Rodas, V. *Pseudo-nitzschia* species isolated from Galician waters: toxicity, DNA content

and lectin bindingassay. In Harmful Algae (Reguera, B., Blanco, J., Fernandez, M.-L. & Wyatt, T., editors), 270-273. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Fritz, L., Quilliam, M.A., Wright, J.L.C., Beale, A.M., Work, T.M. An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudo-nitzschia australis*. J. Phycol., 28(4), 439-442 (1992).

Gallitelli, M., Ungaro, N., Addante, L.M., Silver, N.G., Sabbà, C. Respiratory illness as reaction to tropical algal blooms occurring in a temperate climate. JAMA-J. Am. Med. Assoc. 293, 2599-2600 (2005).

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garcés, E., & Magnani, M. Development of a real-time PCR assay for rapid detection and quantification of Alexandrium minutum (a dinoflagellate). Applied and Environmental Microbiology, 70(2), 1199-1206 (2004). GEOHAB (Global Ecology and Oceanography of Harmful Algal Blooms Programme). HABs in Eutrophic Systems. P. Glibert (ed). IOC and SCOR, Paris and Baltimore. (2006).

Glibert, P.M., Anderson, D.A., Gentien, P., Graneli, E., Sellner, K.G. The global, complex phenomena of harmful algal blooms. Oceanography, 18 (2), 136–147 (2005).

Godhe, A., Anderson, D.M., Rehnstam-Holm, A.S. PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. Harmful Algae, 375–382, (2002).

Godhe, A., Asplund, M. E., Härnström, K., Saravanan, V., Tyagi, A., & Karunasagar, I. Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. Applied and environmental microbiology, 74(23), 7174-7182(2008).Granados-Cifuentes, C. Rodriguez-Lanetty, M. The use of high-resolution melting analysis for genotyping *Symbiodinium* strains: a sensitive and fast approach. Mol. Ecol. Res., 11, 394–399 (2011).

Godhe, A., Otta, S.K., Rehnstam-Holm, A-S., Karunasagar, I., Karunasagar, I. Polymerase chain reaction in detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in field samples from southwest India. Mar. Biotechnol., 3, 152–162 (2001).

Granados-Cifuentes, C., Rodriguez-Lanetty, M. The use of high-resolution melting analysis for genotyping *Symbiodinium* strains: a sensitive and fast approach. Molecular ecology resources, 11(2), 394-399 (2011).

Greenfield, D. I., Marin III, R., Doucette, G. J., Mikulski, C., Jones, K., Jensen, S., ... & Scholin, C. Field applications of the second-generation Environmental Sample Processor (ESP) for remote detection of harmful algae: 2006-2007. Limnology and Oceanography: methods, 6, 667-679 (2008). Haberman, E. Palytoxin acts through the Na+/K+ ATPase. Toxicon. 27, 1171-1187 (1989).

Greenfield, D. I., Marin, R., Jensen, S., Massion, E., Roman, B., Feldman, J., & Scholin, C. A. Application of environmental sample processor (ESP) methodology for quantifying *Pseudo-nitzschia australis* using ribosomal RNA-targeted probes in sandwich and fluorescent in situ hybridization formats. Limnology and Oceanography: Methods, 4(11), 426-435 (2006).

Herbst, D.B., Blinn D.W. Experimental mesocosm studies of salinity effects on the benthic algal community of a saline lake. J. Phycol., 34, 772-778 (1998).

Hernroth, B. E., Conden-Hansson, A. C., Rehnstam-Holm, A. S., Girones, R., & Allard, A. K. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Applied and Environmental Microbiology, 68(9), 4523-4533 (2002).

Herrmann, M.G., Durtschi, J.D., Bromley, L.K., Wittwer, C.T., Voelkerding K.V. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clin Chem 52, 494–503 (2006).

Honsell, G., Bonifacio, A., De Bortoli, M., Penna, A., Battocchi, C., Ciminiello, P., ... & Tubaro, A. New insights on cytological and metabolic features of *Ostreopsis* cf. *ovata Fukuyo* (Dinophyceae): a multidisciplinary approach. PLoS One, 8(2), e57291 (2013).

http://www.isprambiente.gov.it/

Jeffrey, S.W., Hallegraeff G.M. Phytoplankton ecology of Australasian waters. In: Clayton M.N., King R.J. (eds), Biology of Marine Plants. Longman, Australia. 310-348 (1990).

Jellet, J.F., Marks, L.J., Steward, J.E., Dorey, M.L., Watson-Wright, W., Lawrence, J.F. Paralytic Shellfish Poison (saxitoxin family) Bioassay: Automated Endpoint Determination and Standardization of the in vitro Tissue Culture Bioassay, and Comparison with the Standard Mouse Bioassay. Toxicon, 36, 1143-1156 (1992).

John, U., Medlin, L.K., Groben, R. Development of specific rRNA probes to distinguish between geographic clades of the *Alexandrium tamarense* species complex. J. Plankton Res., 27(2), 199-204 (2005).

Kelly, M.G., Whitton B.A. The Trophic Diatom Index: a new index for monitoring eutrophication in rivers. J. Appl. Phycol., 7, 433-444 (1995).

Kelly, M. G., Cazaubon, A., Coring, E., Dell'Uomo, A., Ector, L., Goldsmith, B., ... & Kwandrans, J. Recommendations for the routine sampling of diatoms for water quality assessments in Europe. J. Appl. Phycol., 10, 215-224 (1998).

Kelly, M.G., Bennett, C., Coste, M., Delmas, F., Denys, L., Ector, L., Fauville, C., Ferreol, M., Golub, M., Kahlert, M., Lucey, J., B.Ni Chathain, Pardo, I., Pfister, P., Picinska-Faltynowicz, J., Schranz, C., Schaumburg, J., Tison, van Dam J.H., Vilbaste, S. Central/Baltic GIG Phytobenthos Intercalibration Exercise - final report. Bowburn Consultancy Durham (2006).

Klein, C., Claquin, P., Bouchart, V., Le Roy, B., & Véron, B. Dynamics of *Pseudo-nitzschia* spp. and domoic acid production in a macrotidal ecosystem of the Eastern English Channel (Normandy, France). Harmful Algae, 9 (2), 218-226 (2010).

Kotaki, Y., Lundhol, N., Onodera, H., Kobayashi, K., Bajarias, F.F.A., Furio, E.F., Iwataki, M., Fukuyo, Y., Kodama, M. Wide distribution of *Nitzschia navisvaringica*, a new domoic-acid-producing benthic diatom found in Vietnam. Fish. Sci., 70, 28-32 (2004).

Kwandrans, J., Eloranta, P., Kavecka, B., Wojtan, K. Use of benthic diatom communities to evaluate water quality in rivers of southern Poland. In: Prygiel J., Whitton B.A. & Bukowska J. (eds). Use of Algae for Monitoring Rivers III: 154-165 (1999).

Lapworth, C., Hallegraeff, G.M.J., Ajani, P.A. Identification of domoic-acidproducing *Pseudo-nitzschia* species in Australian waters. In Hallegraef G.M., Blackburn

S.I., Bolch C.J., Lewis D., (eds). Harmful Algal Blooms 2000: Proceedings of the Ninth International Conference on Harmful Algal Blooms. 38-41 (2001).

Laycock, M.V., Bird, C.J. Abstract of papers, Vancouver, Canada, p. 87 (1989).

Leadbeater, B.S.C., Dodge, J.D. An electron microscope study of dinoflagellate flagella. J. Gen. Microbiol., 46, 305-314 (1966).

Leadbeater, B.S.C., Dodge, J.D. The fine structure of *Woloszynskia micra* sp. nov., a new marine dinoflagellate. Br.Phycol. Bull., 3, 1-17 (1967).

Leclerq L., Maquet B., Deux nouveaux indices chimiqua et diatomique de qualità d'eau courante. Application au Samson et à ses affluents. Institut royal des sciences naturelles de Belgique, document de travail. 38, 1-13 (1987).

Lipkind, G.M., Fozzard, H.A. A Structural Model of the Tetrodotoxin and Saxitoxin Binding Site of the Na^+ Channel. Biophys. J., 66, 1-13 (1994).

Lundholm, N., Skov, J., Pocklington, R., & Moestrup, Ø. Domoic acid, the toxic amino acid responsible for amnesic shellfish poisoning, now in *Pseudo-nitzschia seriata* (Bacillariophyceae) in Europe. Phycologia, 33, 6475-478 (1994).

Lundholm, N., Hansen P. J., Kotaki Y. Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo-nitzschia* and *Nitzschia*. Mar. Ecol.-Prog. Ser., 273, 1-15 (2004).

Lundholm, N., Hansen P.J., Kotaki Y. Lack of allelopathic effects of the domoic acid-producing marine diatom *Pseudo-nitzschia multiseries*. Mar. Ecol.-Prog. Ser., 288, 21-33 (2005).

Maranda, L., Wang, R., Masuda, K., Shimizu, Y. Investigation of the source of domoic acid in mussels. In Granéli, E., Sundström, B., Edler, L., Anderson, D.M., (eds.). Toxic Marine Phytoplankton. Elsevier, New York, 300–304 (1990).

Marin III, R., Scholin C.A. 12 Toxic algal detection using rRNA-targeted probes in a semi-automated sandwich hybridization format. Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis, p. 87.

Mattei, C., Mologo, J., Marquais, M., Vernoux, J., Benoit, E. Hyperosmolar D-mannitol reverses the increased membrane excitability and the nodal swelling caused by Caribbean ciguatoxin- 1 in single frog myelinated axons. Brain Res., 847 (1), 50-58 (1999).

Miller, T. A., Rapp, J., Wastlhuber, U., Hoffmann, R. W., & Enzmann, P. J. Rapid and sensitive reverse transcriptase-polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cells. Dis. Aquat. Org., 34 (1), 13-20 (1998).

Moore, R.E., and Scheuer P.J. Palytoxin: a new marine toxin from a coelenterate. Science, 172, 495-498 (1971).

Moore, R.E., Bartolini, G. Structure of palytoxin. J. Am. Chem. Soc., 103, 2491-2494 (1981).

Moschandreou, K. K., Papaefthimiou, D., Katikou, P., Kalopesa, E., Panou, A., & Nikolaidis, G. Morphology, phylogeny and toxin analysis of *Pseudo-nitzschia pseudodelicatissima* (Bacillariophyceae) isolated from the Thermaikos Gulf, Greece. Phycologia, 49 (3), 260-273 (2010).

Murakami, S., Takemoto, T., & Shimizu, Z. Studies on the effective principles of *Digenea-simplex* aq. 1. separation of the effective fraction by liquid chromatography. Yakugaku Zasshi-Journal of the pharmaceutical society of Japan, 73(9), 1026-1028 (1953).

Paerl, H.W. Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. Limnol. Oceanogr., 33, 823–847 (1988).

Paerl, H.W., Valdes L.M., Peierls B.L., Adolf J.E., Harding L.W. Anthropogenic and climatic influences on the eutrophication of large estuarine systems. Limnol. Oceanogr., 51, 448–462 (2006).

Pan Y.L., Rao D.V.S., Mann K. H. Acclimation to low light intensity in photosynthesis and growth of *Pseudo-nitzschia multiseries* Hasle, a neurotoxigenic diatom. J. Plankton Res., 18, 1427-1438 (1996).

Parsons, M.L., Scholin, C.A., Miller, P.E., Doucette, G.J., Powell, C.L., Fryxell, G.A., Dortch, Q., Soniat, T. *Pseudo-nitzschia* species (Bacillariophyceae) in Louisiana coastal waters: molecular probe field trials, genetic variability, and domoic acid analyses. J. Phycol., 35, 1368–1378 (1999).

Paulliac, S., Sasaki, M., Inoue, M., Naar, J., Branaa, P., Chinain, M., Tachibana, K., Legrand, A.M. Chararcterization of mice antisera elicited with a ciguatoxin tetracyclic synthetic ring fragment (JKLM) conjugated to carrier proteins. Toxicon, 38 (5), 669-685 (2000).

Penna, A., Magnani, M. Identification of *Alexandrium* (Dinophyceae) species using PCR and rDNA-targeted probes. J. Phycol., 35, 615–621 (1999).

Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobó, P., Vernesi, C. Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the Western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. J. Phycol., 41, 212-225 (2005).

Penna, A., Bertozzini, E., Battocchi, C., Galluzzi, L., Giacobbe, M.G., Vila, M., Garces, E., Lugliè, A., Magnani, M.. Monitoring of HAB species in the Mediterranean Sea through molecular methods. J. Plankton Res. 29 (1), 19-38 (2007).

Penna, A., Fraga, S., Battocchi, C., Casabianca, S., Giacobbe, M. G., Riobó, P., & Vernesi, C. A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt. Journal of biogeography, 37(5), 830-841 (2010)

Penna, A., Casabianca, S., Perini, F., Bastianini, M., Riccardi, E., Pigozzi, S., & Scardi, M. Toxic *Pseudo-nitzschia* spp. in the northwestern Adriatic Sea: characterization of species composition by genetic and molecular quantitative analyses. Journal of plankton research, 35(2), 352-366 (2013).

Perini, F., Casabianca, A., Battocchi, C., Accoroni, S., Totti, C., Penna, A. New approach using the real-time PCR Method for estimation of the toxic marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. Plos One, 6, e17699 (2011).

Prygiel, J., Coste, M., Bukowska, J. Review of the major diatom-based techniques for the quality assessment of rivers-State of the art in Europe. In J. Prygiel, B. A. Whitton, & J. Bukowska (Eds.), Use of algae for monitoring Rivers III (pp. 224–238). Douai: Agence de l'Eau Artois-Picardie (1999).

Rott, E., Methodological aspects and perspectives in the use of periphyton for monitoring and protecting rivers. In: Whitton, B.A., Rott E., Friedrich G. (eds). Use of algae for monitoring rivers. 9-16 (1991).

Rott, E., Pipp E. Progress in the use of benthic algae for monitoring rivers in Austria. In: Prygiel J., Whitton B.A., Bukowska J. (eds) Use of Algae for Monitoring Rivers III, pp. 110-112 (1999).

Round, F.E., Crawford R.M., Mann D.G. The diatoms: biology and morphology of the genera. Cambridge University Press. (1990).

Rublee, P.A., Kempton, J.W., Schaefer, E.F., Burkholder, J.M., Glasgow, H.B., Oldach, D.W. PCR and FISH detection extends the range of *Pjiesteria piscicida* in estuarine waters. Virginia J. Science, 50 (4), 325-336 (1999).

Rublee, P.A., Kempton, J.W., Schaefer, E.F., Allen, C., Harris, J., Oldach, D.W., Bowers, H., Tengs, T., Burkholder, J.M., Glasgow, H.B. Use of molecular probes to assess geographic distribution of *Pfiesteria* species. Environ. Health Perspect., 109, 765–767 (2001).

Sabater, S. Diatom communities as indicators of environmental stress in the Guadiamar River, S-W Spain, following a major mine tailings spill. J. Appl. Phycol., 12, 113-24 (2000).

Sansoni, G., Borghini B., Camici G., Casotti M., Righini P., Rustighi C. Fioriture algali di *Ostreopsis ovata* (Gonyaulacales: Dinophyceae): un problema emergente. Biologia ambientale, 17(1), 17-23(2003).

Sarno, D., Dahlman, J. Production of domoic acid in another species of *Pseudo-nitzschia*: *P. multistriata* in the Gulf of Naples (Mediterranean Sea). Harmful Algae News, 21(5) (2000).

Satake, M., Terasawa, K., Kadowaki, Y., Yasumoto, T. Relative configuration of yessotoxin and isolation of two new analogs from toxic scallop. Tetrahedron Lett. 37, 5955-5958 (1996).

Scholin, C.A., Buck, K.R., Britschgi, T., Cangelosi, G., Chavez, F.P. Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. Phycologia, 35, 190–197 (1996).

Scholin, C.A., Miller, P., Buck, K., Chavez, F., Harris, P., Haydock, P., Howard, J., Cangelosi, G. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. Limnol. Oceanogr., 42, 1265–1272 (1997).

Scholin, C. A., Marin, R., Miller, P. E., Doucette, G. J., Powell, C. L., Haydock, P., ... & Ray, J..DNA probes and a receptor-binding assay for detection of *Pseudo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. J. Phycol., 35, 1356-1367 (1999).

Scholin, C., Doucette, G., Jensen, S., Roman, B., Pargett, D., Marin III, R., ... & Harris, A. Remote detection of marine microbes, small invertebrates, harmful algae, and biotoxins using the environmental sample processor (ESP). Oceanography, 22, 158-167 (2009).

Shimizu, Y., Gupta, S., Masuda, K., Walker, C.K., Wang, R. Dinoflagellate and other microalgal toxins: chemistry and biochemistry. Pure Appl. Chem., 61, 513-516 (1989).

Smith, M. W., Maier, M. A., Suciu, D., Peterson, T. D., Bradstreet, T., Nakayama, J., & Simon, H. M. High resolution microarray assay for rapid taxonomic assessment of *Pseudo-nitzschia* spp. (Bacillariophyceae) in the field. Harmful Algae, 19, 169–180 (2012).

Sournia, A. Catalogue des espèces et taxons infraspécifiques de dinoflagellés marins actuels publiés depuis la révision de J. Schiller. III (Complément). Rev. Algol., 13, 3–40 (1978).

Stults, J.R., Snoeyenbos-West, O., Methe, B., Lovley, D.R., Chandler, D.P. Application of the 5′ fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. Appl. Environ. Microbiol., 67, 2781–2789 (2001).

Subba, R.D.V., Quilliam, M.A., Pocklington, R. Domoic acid-a neurotoxic amino acid produced by the marine diatom *Nitzschia pungens* in culture. Can. J. Fish. Aquat. Sci., 45 (12), 2076-2079 (1988).

Tebbe, C.C., Vahjen, W. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol., 59 (8), 2657-2665 (1993).

Töbe, K., Eller, G., Medlin, L.K. Automated detection and enumeration for toxic algae by solid-phase cytometry and the introduction of a new probe for *Prymnesium parvum* (Haptophyta: Prymnesiophyceae). J. Plank Res., 28, 643–657 (2006).

Töbe, K., Kullis, D., Gladstone, M., Anderson, D., Medlin, L.K. Detecting intact algal cells with whole cell hybridisation assays, microscopic and molecular methods for quantitative phytoplankton analysis. In: Karlson B., Cusack C, Bresnan E (eds) Microscopic and molecular methods for quantitative phytoplankton

analysis. Intergovernmental Oceanographic Commission of UNESCO IOC Manuals and Guides. 55 Paris, 55–66 (2010).

Tomas, R.N., Cox, E.R. Observations on the symbiosis of *Peridinium balticum* and its intracellular alga. I. Ultrastructure. J. Phycol., 9, 304-323 (1973).

Tonolli, V. Introduzione allo studio della limnologia: ecologia e biologia delle acque dolci. Edizioni dell'Istituto Italiano di Idrobiologia, Verbania Pallanza. (1975).

Tubaro, A., Sidari, L., Della Loggia, R., Yasumoto, T. Occurence of yessotoxin-like toxins in phytoplankton and mussels from northern Adriatic Sea. In: Reguera, B., Blanco, J., Fernandez, M.L. & Wyatt, T. (Ed.). Proceedings of the VIII International Conference on Harmful algae. Vigo, Spain 25-29 June. Paris: Xunta de Galicia and IOC of UNESCO, 470-472 (1997).

Tyrrell, J.V., Bergquist, P.R., Bergquist, P.L., Scholin, C.A. Detection and enumeration of *Heterosigma akashiwo* and *Fibrocapsa japonica* (Raphidophyceae) using rRNA-targeted oligonucleotide probes. Phycologia, 40, 457–467 (2001).

Vale, P. Sampayo, M.A. Domoic acid in Portuguese shellfish and fish. Toxicon, 39, 893-904 (2001)

Vrieling, E.G., Koeman, R.P.T., Peperzak, L., Veenhuis, M., Scheerman, P., Gieskes, W.W.C. *Pseudo-nitzschia pungens* cf. *multiseries* and other *Pseudo-nitzschia* species in the Dutch Wadden Sea. In: Yasumoto T, Oshima Y, Fukuyo Y (Ed.). Harmful and toxic algal blooms. Proceedings of the VII International Conference on Toxic Phytoplankton. Sendai, Japan, 12-16 July 1995. Paris: Intergovernmental Oceanographic Commission of UNESCO: 139-142 (1996).

Wittwer, C. T. High-resolution DNA melting analysis: advancements and limitations. Human mutation 30(6), 857-859 (2009).

Wright, J. L. C., Boyd, R. K., Freitas, A. D., Falk, M., Foxall, R. A., Jamieson, W. D., ... & Pathak, V. P. Identification of domoic acid, a neuroexcitatory amino acid, toxic mussels from eastern Prince Edward Island. Can. J. Chem., 67, 481-90 (1989).

Yasumoto, T., Murata, M., Oshima, M., Sano, M., Tatsumoto, G.K., Clardy, J. Diarrhetic Shellfish Toxins. Tetrahedron, 41, 1019-1025 (1985).

Ringraziamenti

Ringrazio la Professoressa Antonella Penna in qualità di Tutor e la Dott.ssa Silvia Casabianca in qualità di Co-Tutor del mio dottorato di ricerca.

Un sentito grazie va a tutto il gruppo della Sezione di Biologia Ambientale di Pesaro, che è stato un punto di riferimento certo.
Grazie alla Dott.ssa Samuela Capellacci per gli isolamenti di campioni microalgali, indispensabili per il mio lavoro di ricerca, ma anche per avermi insegnato tutto con infinita disponibilità e soprattutto per il continuo incoraggiamento ricevuto in questi 3 anni; alla Dott.ssa Valentina Sparvoli per il supporto e il sostegno ricevuti nella fase iniziale di questo percorso, indispensabili per proseguire; al Dott. Federico Perini per aver contribuito con il suo spessore alla mia formazione scientifica e per aver costantemente stimolato in me il ragionamento scientifico; al Dott. Fabio Ricci per le uscite in mare di campionamento periodico, che hanno reso possibile la raccolta del materiale di partenza del mio lavoro di ricerca.

Ringrazio il Dott. Luca Galluzzi e il Dott. Marcello Ceccarelli della Sezione di Biotecnologie di Fano, per la loro disponibilità e per la formazione ricevuta, indispensabile per l'avvio del mio progetto di ricerca.

Appendice

Partecipazione a progetti internazionali

1. 2014- 2015 Progetto EU ENPI CBCMED M3-HABs II-B/2.1/0096 "Risk monitoring, modelling and mitigation of benthic harmful blooms along Mediterranean coast".
2. 2014-2015 Progetto EU CBC IPA Adriatic BALMAS 1° STR/0005 "Ballast water management system for Adriatic Sea protection".

Partecipazioni a conferenze nazionali e internazionali

1. Perini F., **Pugliese L.,** Bastianini M., Finotto S., Pompei M., Di Poi E., Fornasaro D., Cabrini M., Marini M., Penna A. (2015) Molecular methods and potentially toxic resting cyst dynamic in the harbour sediments. 4th Meeting of IPA INTERREG ADRIATIC BALMAS Project, 24-26 March 2015, Durazzo, Albania.
2. Casabianca S., Perini F., **Pugliese L.,** Casabianca A., Giussani A., Abboud Abi Saab M., Penna A. Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay. 46° Congresso della Società Italiana di Biologia Marina Roma, 10-12 giugno 2015.
3. **Pugliese L.,** Casabianca S., Perini F., Penna A. High resolution melting PCR assay for rapidly discriminating the diatom *Pseudo-nitzschia*. XI Incontro dei Dottorandi in Ecologia e Scienze Ambientali. Roma ,17-19 Settembre 2015.
4. **Pugliese L.,** Casabianca S., Perini F., Andreoni F., Penna A. High Resolution Melting: a new approach for molecular characterization of *Pseudo-nitzschia* spp. (Bacillariophyceae) 47° Congresso della Società Italiana di Biologia Marina, Torino, 13-17 giugno 2016.
5. Casabianca S., Perini F., **Pugliese L.,** Casabianca A., Giussani A., Abboud Abi Saab M., Penna A. Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay. 46° Congresso della Società Italiana di Biologia Marina Roma, 10-12 Giugno 2015.
6. **Pugliese L.,** Casabianca S., Perini F., Andreoni F., Penna A. High Resolution Melting: a new approach for molecular characterization of *Pseudo-nitzschia* spp. (Bacillariophyceae). 47° Congresso della Società Italiana di Biologia Marina, Torino, 13-17 Giugno 2016.
7. Casabianca S., Asnaghi V., Penna A., Chiantore M., Giussani V., **Pugliese L.,** Ottaviani E., Sbrana F., Vassalli M. Intercalibrazione di metodi innovativi per l'identificazione ed il conteggio di *Ostreopsis* spp.. 47° Congresso della Società Italiana di Biologia Marina, Torino, 13-17 Giugno 2016.
8. Casabianca S., Perini F., Casabianca A., **Pugliese L.,** Giussani A., Chiantore M., Penna A. Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay. 41th CIESM Congress, Kiel, Germany, 12-16 Settembre

2016.

9. Casabianca S., **Pugliese L.,** Perini F., Andreoni F., Penna A. High Resolution Melting: an innovative approach for molecular characterization of *Pseudo-nitzschia* spp. (Bacillariophyceae). Società Botanica Italiana. Gruppo di Algologia. Riunione scientifica annuale, Ravenna, 18-19 Novembre 2016.

Pubblicazioni sottomesse per la pubblicazione su riviste ISI

1. **Pugliese L.,** Casabianca S., Perini F., Andreoni F., Penna A. High Resolution Melting method for the molecular identification of potential toxic diatom *Pseudo-nitzschia* spp. in the Mediterranean Sea. *Scientific reports* (July 2016)

Pubblicazioni in preparazione per la pubblicazione su riviste ISI

2. Perini F., Bastianini M., **Pugliese L.,** Pompei M., Cabrini M., Marini M., Penna A. HAB dinoflagellate resting cysts monitoring and implication of their dispersion through ballast waters. *(Marine Pollution Bulletin)*
3. Vassalli M., Penna A., Sbrana F., Casabianca S., Gjeci N., Asnaghi V., Ottaviani E., Giussani V., **Pugliese L.,** Capellacci S., Chiantore M.C., Intercalibration of counting methods for toxic benthic dinoflagellate *Ostreopsis ovata* blooms in the Mediterranean Sea. (*Ecological Indicators*)

Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay

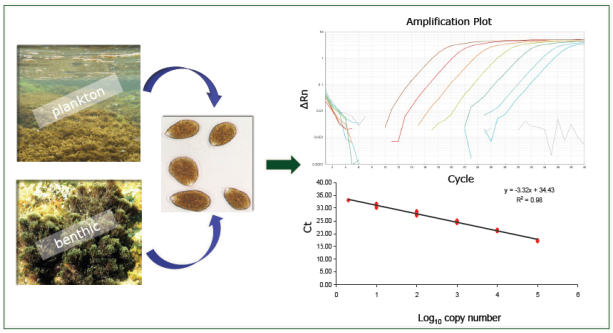
Casabianca S.[1], Perini F.[1], Casabianca A.[1], Pugliese L.[1], Giussani V.[2], Chiantore M.[2], Penna A.[1]

[1]Dep. of Biomolecular Sciences (DISB), University of Urbino, Pesaro, Italy
silvia.casabianca@uniurb.it - antonella.penna@uniurb.it
[2]DISTAV, University of Genoa, Italy

Introduction:

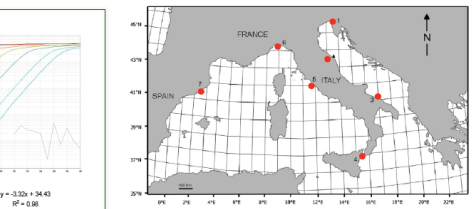
Ostreopsis cf. *ovata* is a toxic benthic dinoflagellate that causes high biomass blooms involving risks for human health, with negative impacts on marine biota, aquaculture activities and coastal seawater quality.

The aim of this study was to apply a rapid and sensitive qPCR method to quantify *Ostreopsis* cf. *ovata* abundance in environmental samples collected from Mediterranean coastal sites and to develop site-specific environmental standard curves.

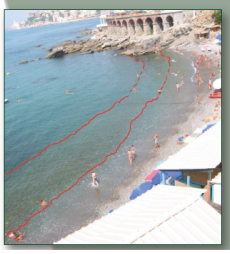


Environmental site-specific standard curves characterization

Sampling locality	Mean LSU-STD curve	Mean Ct cell^{-1}	LSU copy No.[a] (cell^{-1} ± SD)
Llavaneres (Catalan Sea, Spain)	y = –3.3113x + 22.921	23.42 ± 0.5	2137 ± 190[b]
Civitavecchia (Tyrrhenian Sea, Italy)	y = –3.3253x + 22.337	22.33 ± 0.14	4429 ± 424
Trieste Canovella (Adriatic Sea, Italy)	y = –3.3409x + 24.979	24.99 ± 0.09	699 ± 41
Passetto-Portonovo (Adriatic Sea, Italy)	y = –3.3759x + 18.861	18.87 ± 0.03	48,617 ± 908
Genova (Tyrrhenian Sea, Italy)	y = –3.3626x + 23.026	23.02 ± 0.11	2736 ± 204
Bari (Adriatic Sea, Italy)	y = –3.3438x + 22.906	22.92 ± 0.13	2929 ± 168
Taormina (Ionian Sea, Italy)	y = –3.359x + 23.072	23.06 ± 0.06	2649 ± 108



Location of sampling stations in Mediterranean Sea



Toxic algal blooms of *Ostreopsis* cf. *ovata* with toxic aerosol production

Materials and Methods:

Due to the potential variation in the rRNA gene copy number, environmental standard curves (one for each Mediterranean sampling site) from pooled cell samples collected during a bloom of toxic *O.* cf. *ovata*, were generated. This allowed the normalization of *O.* cf. *ovata* copy number variability, thereby obtaining an accurate and rapid quantification of cells in field samples.

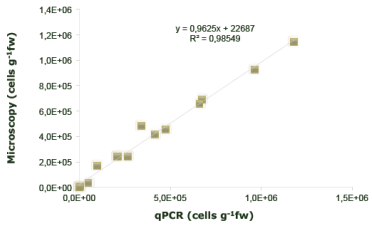
O. cf. *ovata* by SEM (top) and light microscopy

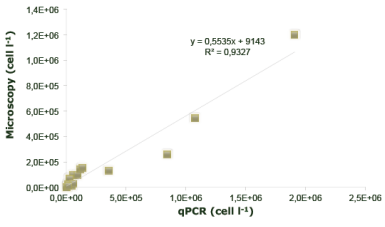
Results:

Environmental samples of macroalgae and surface seawater collected at the seven Mediterranean coastal sites were analysed by both qPCR assay, using LSU environmental standard curves, and light microscopy.

In macroalgal samples, the higher abundance of *O.* cf. *ovata* was 1.18 x 10^6 ± 6.33 x 10^5 cells g^{-1}fw, while the minimum abundance was 772 ± 41 cells g^{-1}fw by qPCR.

The abundance of *O.* cf. *ovata* in seawater samples was generally lower than cell concentrations on macroalgae samples, with the exception of one sample (1.90 x 10^6 ± 8.45 x 10^5 cells l^{-1}). Notably, the qPCR reaction of one sample, in which no *Ostreopsis* cells were found by microscopy resulted in a positive amplification (1106 ± 426 cells l^{-1}).





- Significant positive correlation between *O.* cf. *ovata* cell densities on macroalgal samples and in water column (n = 16, Spearman r = 0.8386, p < 0.0001).
- Significant correlation between *Ostreopsis* cf. *ovata* abundance determined by light microscopy and qPCR assays (n = 16, Spearman r = 0.9808, p < 0.0001 and n = 15, Spearman r = 0.9263, p < 0.0001 for macroalgal and seawater samples, respectively).

Conclusions:

✧ Generation of environmental standard curves for each sampling site to normalize the LSU rDNA variability of *Ostreopsis* cf. *ovata*.
✧ The molecular method showed accuracy and reliability, confirmed by the significant correlation between *Ostreopsis* cf. *ovata* abundance determinations by qPCR and light microscopy.

Finally:

➢ The qPCR approach was effective in assessing beach water quality during the survey activity of the study period.
➢ The qPCR approach proved to be a powerful tool for rapid and efficient quantification of toxic *Ostreopsis* cf. *ovata* cells.

Reference:

Casabianca S., Perini F., Casabianca A., Battocchi C., Giussani V., Chiantore M., Penna A. (2014). Monitoring of toxic *Ostreopsis* cf. *ovata* in recreational waters using qPCR based assay. Marine Pollution Bulletin 88 (1-2): 102-109.

For more information about M3-HABs:
http://www.m3-habs.net
https://twitter.com/M3HABs
https://www.facebook.com/M3HABs

Presentato presso 47° Congresso della Società Italiana di Biologia Marina, Torino, 13-17 Giugno 2016

Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay

Casabianca S.[1], Perini F.[1], Pugliese L.[1], Casabianca A.[1], Giussani V.[2], Abboud-Abi Saab M.[3], Penna A.[1]

[1]Dep. of Biomolecular Sciences (DISB), University of Urbino, Viale Trieste, 296 - 61121 Pesaro, Italy
silvia.casabianca@uniurb.it - antonella.penna@uniurb.it
[2]DISTAV, University of Genoa, Italy
[3]National Council for Scientific Research / National Center for Marine Sciences Batroun, Lebanon

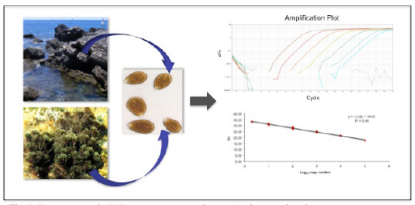


Fig. 1. Environmental qPCR to estimate toxic *Ostreopsis* cf. *ovata* abundance

Abstract - *Ostreopsis* sp. is a toxic marine benthic dinoflagellate that causes high biomass blooms dangerous for human health, marine biota, aquaculture activities and negatively impacting coastal seawater quality.

Aim - The aim of this study was to apply a rapid and sensitive qPCR method to quantify *Ostreopsis* cf. *ovata* abundance in environmental samples collected from different Mediterranean coastal sites based on site-specific environmental standard curves.

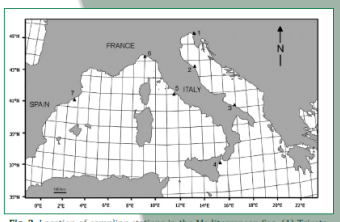


Fig. 2. Location of sampling stations in the Mediterranean Sea. (1) Trieste, NW Adriatic Sea, Italy. (2) Ancona (Passetto–Portonovo), NW Adriatic Sea, Italy. (3) Bari, SW Adriatic Sea, Italy. (4) Taormina, Ionian Sea, Italy. (5) Civitavecchia, Tyrrhenian Sea, Italy. (6) Genova, Tyrrhenian Sea, Italy. (7) Llavaneres, Catalan Sea, Spain.

Introduction - The benthic dinoflagellate *Ostreopsis* sp. (Fig. 1) is an epi-benthic microalga and it includes *Ostreopsis* cf. *ovata* that can produce potent non-protein toxins. Recently, massive blooms of *Ostreopsis* spp. have become frequent also in the Mediterranean Sea (Penna *et al.*, 2010).

In this study, seawater affected by *Ostreopsis* spp. blooms at various Mediterranean beaches (Fig. 2) was monitored using a qPCR assay based on site-specific environmental standard curves (Casabianca *et al.*, 2014).

Moreover, as new strains were isolated from the eastern area of the Mediterranean Sea and their sequences were different from *O.* cf. *ovata*, new primer sets were designed for the identification of the genus *Ostreopsis* spp. and *Ostreopsis* sp. from eastern Mediterranean Sea.

Table 1
LSU-STD environmental standard curve, mean Ct cell^{-1} and LSU gene copy number cell^{-1} determined by qPCR assay from the macroalgal samples collected in 2011–2013 at different Mediterranean coastal sites.

Sampling locality	Mean LSU-STD curve	Mean Ct cell^{-1}	LSU copy No.[a] (cell^{-1} ± SD)
Llavaneres (Catalan Sea, Spain)	$y = -3.3113x + 22.921$	23.42 ± 0.5	2137 ± 190[b]
Civitavecchia (Tyrrhenian Sea, Italy)	$y = -3.3253x + 22.337$	22.33 ± 0.14	4429 ± 424
Trieste Canovella (Adriatic Sea, Italy)	$y = -3.3409x + 24.979$	24.99 ± 0.09	699 ± 41
Passetto–Portonovo (Adriatic Sea, Italy)	$y = -3.3759x + 18.861$	18.87 ± 0.03	48,617 ± 908
Genova (Tyrrhenian Sea, Italy)	$y = -3.3626x + 23.026$	23.02 ± 0.11	2736 ± 204
Bari (Adriatic Sea, Italy)	$y = -3.3438x + 22.906$	22.92 ± 0.13	2929 ± 168
Taormina (Ionian Sea, Italy)	$y = -3.359x + 23.072$	23.06 ± 0.06	2649 ± 108

[a] Mean LSU gene copy number calculated in triplicates ± standard deviation (SD). These data were obtained by plotting the Ct value per cell against pLSUO plasmid standard as the two curves showed the same PCR efficiencies ($\Delta S < 0.1$).
[b] From Casabianca et al. (2013).

Table 2
QPCR assay and microscopy analysis of *Ostreopsis* cf. *ovata* abundance from macroalgae samples collected in 2011 and 2013 at various Mediterranean coastal sites.

Sample No.	Locality	Sampling date	Abundance[a] (cells g^{-1}fw ± SD)	
			qPCR	Microscopy
* 1	Llavaneres (Spain)	22.06.2011	n.d.[b]	n.d.
* 2	Llavaneres (Spain)	22.06.2011	n.d.	n.d.
3	Llavaneres (Spain)	21.07.2011	98,677 ± 1091	169,120 ± 2039
4	Llavaneres (Spain)	22.07.2011	342,459 ± 43,691	484,086 ± 3913
5	Civitavecchia15888 (Italy)	03.10.2013	49,484 ± 1688	36,548 ± 2673
6	Civitavecchia15892 (Italy)	03.10.2013	5408 ± 115	6733 ± 1708
7	Trieste Canovella 1 (Italy)	16.09.2013	212,797 ± 66,631	241,067 ± 28,706
8	Trieste Canovella 2 (Italy)	16.09.2013	208,733 ± 23,336	240,816 ± 6136
9	Trieste Canovella 3 (Italy)	16.09.2013	265,722 ± 16,939	239,811 ± 34,194
10	Ancona, Portonovo (Italy)	10.09.2013	850 ± 232	1173 ± 774
11	Ancona, Passetto 1 (Italy)	10.09.2013	415,062 ± 29,170	417,572 ± 15,025
12	Ancona, Passetto 2 (Italy)	10.09.2013	673,835 ± 42,287	689,281 ± 40,586
13	Genova a (Italy)	22.07.2013	473,598 ± 30,455	455,607 ± 71,592
* 14	Genova c (Italy)	26.07.2013	1,180,043 ± 63,326	1,143,652 ± 125,136
15	Genova e (Italy)	31.07.2013	661,039 ± 10,950	656,814 ± 9676
16	Genova g (Italy)	02.08.2013	965,409 ± 79,188	925,962 ± 97,907
17	Taormina St.1 (Italy)	13.09.2012	2286 ± 111	1734 ± 270
* 18	Taormina St. 2 (Italy)	13.09.2012	772 ± 41	713 ± 59

[a] Mean abundance determined in triplicates ± standard deviation (SD).
[b] Not detected.

Table 3
QPCR assay and microscopy analysis of *Ostreopsis* cf. *ovata* abundance in seawater samples collected in 2011 and 2013 at various Mediterranean coastal areas.

Sample No.	Locality	Sampling date	Abundance[a] (cells l^{-1} ± SD)	
			qPCR	Microscopy
19	Civitavecchia 15,887 (Italy)	03.10.2013	1832 ± 90	1260 ± 85
* 20	Llavaneres (Spain)	22.06.2011	n.d.[b]	n.d.
21	Llavaneres (Spain)	20.07.2011	56,320 ± 10,478	97,667 ± 12,503
22	Llavaneres (Spain)	22.07.2011	28,405 ± 5226	66,000 ± 5657
* 23	Ancona, Portonovo (Italy)	10.09.2013	1106 ± 426	n.d.
24	Ancona, Passetto (Italy)	10.09.2013	92,600 ± 8414	98,400 ± 19,819
25	Genova b (Italy)	22.07.2013	44,353 ± 4590	14,500 ± 2121
26	Genova d (Italy)	26.07.2013	17,000 ± 1044	23,500 ± 707
27	Genova f (Italy)	31.07.2013	58,000 ± 7071	29,778 ± 5441
28	Genova h (Italy)	02.08.2013	17,500 ± 707	11,776 ± 1275
29[c]	Bari, Trullo 1 (Italy)	18.09.2013	115,750 ± 19,363	142,000 ± 19,305
30[c]	Bari, Trullo 2 (Italy)	18.09.2013	135,250 ± 27,476	154,000 ± 26,969
31[c]	Bari, S. Spirito 1 (Italy)	19.09.2013	1,077,265 ± 34,479	542,250 ± 148,572
* 32[c]	Bari, S. Spirito 2 (Italy)	19.09.2013	1,907,088 ± 84,540	1,198,750 ± 226,729
33[c]	Bari, Giovinazzo 1 (Italy)	19.09.2013	357,255 ± 24,375	131,000 ± 26,255
34[c]	Bari, Giovinazzo 2 (Italy)	19.09.2013	841,710 ± 39,675	256,250 ± 25,617

[a] Mean abundance determined in triplicates ± standard deviation (SD).
[b] Not detected.
[c] Samples collected with the syringe method, cell abundance is expressed as cells l^{-1}.

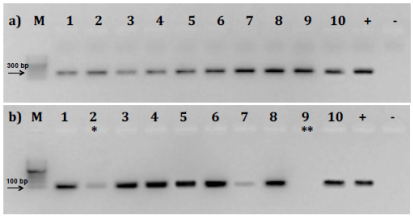


Fig. 4. Genus specific PCR amplification of *Ostreopsis* in macroalgae samples from Lebanon using primers designed on COI mt sequences (a); clade specific PCR amplification of *Ostreopsis* sp. 2 in macroalgae samples from Lebanon using primers designed on ITS-5.8S rDNA sequences (b)

1: TRI 20 A1 18.08.14
2: TRI 20 A2 13.10.14 *
3: BAT 14 A1 23.08.14
4: BAT 14 A1 29.8.14
5: BEY II A1 20.08.14
6: BYB 14 A1 13.10.14
7: NAQ B A1 19.08.14
8: NAQ B A1 14.10.14
9: BAT 14 A1 18.08.14 **
10: NAQ B A3 14.10.14
+: CNT+
-: CNT-

*: also positive *O.* cf. *ovata*
**: sample degradation

Results - The *O.* cf. *ovata* LSU rDNA copy number per cell in environmental samples were different at each of the seven sampled Mediterranean coastal sites. Thus, for the quantification of *O.* cf. *ovata* in environmental samples seven different site-specific LSU-STD curves were used (Tab. 1).

***O.* cf. *ovata* abundance by qPCR in macroalgae (Tab. 2).**
Max: sample n. 14 from Genova ($1.18\times10^6\pm6.33\times10^5$ cells g^{-1}fw); Min: sample n. 18 from Taormina (772±41 cells g^{-1}fw); Negative samples n° 1 and n° 2 from Llavaneres.

***O.* cf. *ovata* abundance by qPCR in seawater (Tab. 3).**
Generally lower than cell concentrations on macroalgae samples. The only exception was sample n. 32 from Bari ($1.90\times10^6\pm8.45\times10^5$ cells l^{-1}). Sample n. 23 from Ancona, negative by microscopy, showed 1106±426 cells l^{-1} of *O.* cf. *ovata* by qPCR. Negative sample n° 20 from Llavaneres.

There was a significant positive correlation between cell densities on macroalgal samples and in water column (n=16, Spearman r=0.8386, p <0.0001).
A significant correlation was found between *O.* cf. *ovata* abundance determined by light microscopy and qPCR assays (n=16, Spearman r=0.9808, p <0.0001 and n=15, Spearman r=0.9263, p <0.0001 for macroalgae and surface seawater samples, respectively).

Due to the presence of new potential *Ostreopsis* species belonging to Atlantic/Mediterranean clade (David *et al.* 2013), genus-specific primers, designed on COI mt sequences, were used for the amplification of environmental samples collected in the eastern Mediterranean coastal areas (Lebanon) (Fig. 3). Results showed positive amplification for the presence of genus *Ostreopsis* (Fig. 4 a).

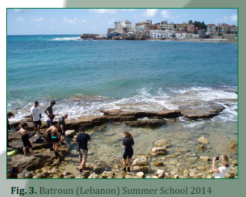


Fig. 3. Batroun (Lebanon) Summer School 2014

Furthermore, positive amplifications were obtained in all samples for *Ostreopsis* sp. 2 Atlantic/Mediterranean clade, using new species-specific primers designed on ITS-5.8S rDNA sequences with the only exception of one degraded sample (Fig. 4 b).

Conclusions - New primer sets were applied for the identification of the genus *Ostreopsis* and *Ostreopsis* sp. 2 Atlantic/Mediterranean clade from eastern Mediterranean Sea (Lebanon). Moreover, LSU rDNA gene was used to accurately quantify *Ostreopsis* spp. abundance in natural samples by qPCR method. This approach, based on the generation of environmental site-specific standard curves, was validated in several Mediterranean coastal areas and its application allowed a rapid and correct quantification of *O.* cf. *ovata* in field assessing beach water quality during the survey activity of the study period (Casabianca *et al.*, 2014).

References - Penna A., Fraga S., Battocchi C., Casabianca S., Riobò P., Giacobbe M.G., Vernesi C. (2010) - A phylogeography study of the toxic benthic genus *Ostreopsis* Schmidt, *J. Biogeogr.*, **37**: 830-841.
Casabianca S., Perini F., Casabianca A., Battocchi C., Giussani V., Chiantore M., Penna A. (2014) - Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay. *Mar. Pollut. Bull.*, **88**: 102-109.
David H., Laza-Martinez, A. Miguel I., Orive E. (2013). *Ostreopsis* cf. *siamensis* and *Ostreopsis* cf. *ovata* from the Atlantic Iberian Peninsula: morphological and phylogenetic characterization. *Harmful Algae*, **30**: 44-55.

For more information about M3-HABs:
http://www.m3-habs.net
https://twitter.com/M3HABs
https://www.facebook.com/M3HABs

INTERCALIBRAZIONE DI METODI INNOVATIVI PER L'IDENTIFICAZIONE ED IL CONTEGGIO DI *OSTREOPSIS* SPP.

S. Casabianca [1,2*], V. Asnaghi[1,3*], A. Penna[1,2], M. Chiantore[1,3], V. Giussani[3,4], L. Pugliese[2], E. Ottaviani[5], F. Sbrana[6], M. Vassalli[6]

Il principale obiettivo del progetto biennale **M3-HABs**, finanziato nell'ambito del programma ENPI-CBCMED, è stato quello di fornire una comune ed efficace strategia pan-mediterranea per il monitoraggio delle microalghe bentoniche tossiche (HABs), con particolare attenzione alla dinoflagellata *Ostreopsis* sp.

Introduzione:

Uno degli **obiettivi specifici** del progetto è stato quello di sviluppare metodologie e strumenti per rendere il processo di identificazione e conteggio delle microalghe più rapido ed efficiente.

Nell'ambito del progetto M3-HABs **due metodologie innovative** per l'identificazione ed il conteggio di *Ostreopsis* spp. sono state proposte e confrontate con il metodo classico di microscopia ottica, che richiede lunghi tempi di processamento e riconoscimento ad opera di operatori specializzati, con un certo grado di esperienza nella tassonomia delle microalghe.

Fig. 1 Localizzazione dei siti di campionamento

Materiali e metodi:

40 campioni di macroalghe e acqua di mare sono stati raccolti durante l'estate 2015 in vari siti del Mediterraneo: Salammbô (Tunisia), Batroun (Libano), Haliotis e Rochambeau (Francia), Genova e Ancona (Italia). I campioni sono stati fissati in lugol 1% (v/v) e analizzati con il metodo di microscopia ottica (Utermöhl, 1958). In parallelo i campioni sono stati contatati con i due metodi innovativi: il sistema opto-elettronico e l'analisi molecolare di qPCR.

qPCR ASSAY:

Termociclatore Step-one Real-time PCR System (Applied Biosystems) per le reazioni di amplificazione specie-specifica di geni target per la quantificazione di cellule di *O. cf. ovata* e di *Ostreopsis* sp.

SISTEMA OPTO-ELETTRONICO:

Composto da un microscopio dotato di piattaforma motorizzata per acquisire immagini su tutta la superficie e collegato ad un software dedicato di elaborazione di immagini e riconoscimento automatico (addestrato solo su campioni provenienti da Genova attraverso apprendimento automatico e poi testato sul dataset complessivo).

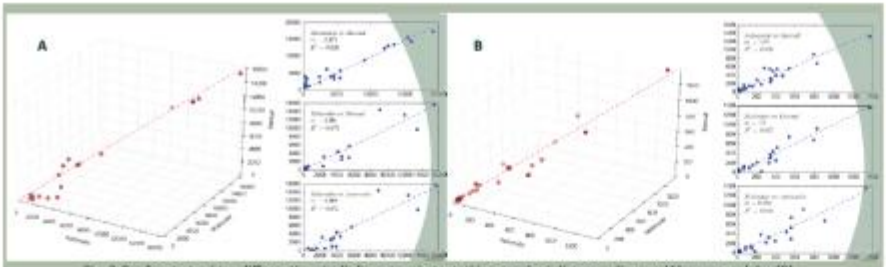


Fig. 2 Confronto tra i tre differenti metodi di conteggio testati in campioni di acqua di mare (A) e macroalghe (B).

Risultati:

Complessivamente, il confronto tra le concentrazioni cellulari ottenute attraverso i tre diversi metodi mostra una correlazione lineare significativa (Fig. 2, in tutti i casi R^2>0.85, p-value <10^{-12}).

Il metodo di qPCR, precedentemente validato (Casabianca et al., 2014) e basato sulla costruzione di curve standard ambientali sito-specifiche e di curve standard plasmidiche (Perini et al. 2011), ha permesso una quantificazione rapida e corretta di tutte le specie di *Ostreopsis* che si sviluppano in Mar Mediterraneo. Anche il sistema opto-elettronico, oltre ad essere in grado di fornire conteggi rapidi e ragionevolmente accurati della concentrazione di *Ostreopsis* cf. *ovata*, su cui è stato addestrato, è stato in grado di riconoscere, al pari della qPCR, la presenza di una specie nuova (Fig. 3), attualmente in fase di descrizione (Accoroni et al., *submitted*), nei campioni raccolti in Libano.

Il metodo di qPCR tende a sovrastimare i conteggi a causa della rilevazione anche di alcune cellule rotte e quindi di molecole di DNA, mentre il sistema opto-elettronico normalmente sottovaluta la stima, in quanto l'algoritmo di riconoscimento automatico è stato addestrato per ridurre il numero di falsi positivi.

Fig. 3 *Ostreopsis* spp. riconosciute dal sistema opto-elettronico in base alla forma e confermate dalla qPCR.

Conclusioni:

I tre metodi di conteggio hanno mostrato un'ottima correlazione, incoraggiando la loro ulteriore implementazione nell'ottica dell'identificazione e conteggio di altre specie microalgali nocive (e non), fornendo un utile strumento in un'ampia prospettiva di monitoraggio, come previsto dalla direttiva quadro sulla Strategia per l'ambiente marino (Marine Strategy, 2008/56/CE).

Bibliografia:

Casabianca S., Perini F., Casabianca A., Battocchi C., Giussani V., Chiantore M., Penna A. (2014) - Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay. Mar. Pollut. Bull., 88: 102-109.

Perini, F., Casabianca, A., Battocchi, C., Accoroni, S., Totti, C., Penna, A., 2011. New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. PLoS ONE 6, e17699.

Web site: **http://www.m3-habs.net/**

Follow us also on:

Dedicated section on the web TV TRIWù:
http://www.triwu.it/sezione-ostreopsis-nel-mediterraneo

***Corresponding authors:** valentina.asnaghi@unige.it; silvia.casabianca@uniurb.it

This publication has been produced with the financial assistance of the European Union under the ENPI CBC Mediterranean Sea Basin Programme.

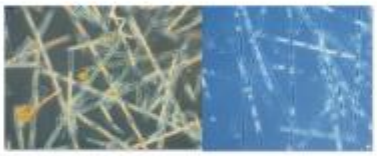
ıtification of *Pseudo-nitzschia* spp. (Bacillariophyceae) in coastal waters

Laura Pugliese, Silvia Casabianca, Federico Perini, Francesca Andreoni, Antonella Penna

Department of Biomolecular Sciences, University of Urbino, Viale Trieste 296, Pesaro, Italy

Aim

The planktonic pennate diatom *Pseudo-nitzschia* (Bacillariophyceae) is a cosmopolitan genus commonly found in neritic and oceanic waters. The correct identification of *Pseudo-nitzschia* species has relevant implications for monitoring and management purposes, considering that toxigenic species may be morphologically similar or identical to non-toxigenic ones. Genetic approaches can play a key role in solving taxonomic problems, as well as geographic distributions, and level of genetic differentiation of populations [1]. The aim of this study was to develop a high resolution-melting (HRM) assay for the rapid, accurate and simultaneous identification of various *Pseudo-nitzschia* spp. from monoclonal cultures obtained from several water samples during a period of survey (November 2014-June 2015).



Pseudo-nitzschia spp. assemblages.

Fig. 1. Map of sampling site of this study in the northwestern Adriatic

Table 1. List of the known strains of *Pseudo-nitzschia* spp. used to validate the HRM assay

Species	Strains	HRM variant
P. calliantha	CBA 78	*P. calliantha*
P. calliantha	CBA 79	*P. calliantha*
P. calliantha	CBA 62	*P. calliantha*
P. calliantha	CBA 70	*P. calliantha*
P. calliantha	CBA 71	*P. calliantha*
P. calliantha	CBA 72	*P. calliantha*
P. calliantha	CBA 73	*P. calliantha*
P. calliantha	CBA 74	*P. calliantha*
P. delicatissima	CBA 131	*P. delicatissima*/*P. cf. arenysensis*
P. delicatissima	CBA 133	*P. delicatissima*/*P. cf. arenysensis*
P. delicatissima	CBA 144	*P. delicatissima*/*P. cf. arenysensis*
P. delicatissima	CBA 145	*P. delicatissima*/*P. cf. arenysensis*
P. delicatissima	CBA 150	*P. delicatissima*/*P. cf. arenysensis*
P. delicatissima	CBA 152	*P. delicatissima*/*P. cf. arenysensis*
P. delicatissima	CBA 153	*P. delicatissima*/*P. cf. arenysensis*
P. pungens	CBA 94	*P. pungens*
P. pungens	CBA 100	*P. pungens*
P. pungens	CBA 101	*P. pungens*
P. pungens	CBA 102	*P. pungens*
P. pungens	CBA 103	*P. pungens*
P. pungens	CBA 108	*P. pungens*
P. pungens	CBA 111	*P. pungens*

Table 2. List of *Pseudo-nitzschia* spp. strains isolated from the northwestern Adriatic Sea used in the HRM assay.

Species	Strains	HRM variant
Pseudo-nitzschia spp.	CBA 58	*P. pungens*
Pseudo-nitzschia spp.	CBA 59	*P. pungens*
Pseudo-nitzschia spp.	CBA 109	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 110	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 141	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 143	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 146	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 147	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 148	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 149	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 154	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 155	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 156	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 157	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 158	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 159	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 160	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 170	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 171	*P. delicatissima*/*P. cf. arenysensis*
Pseudo-nitzschia spp.	CBA 172	*P. calliantha*
Pseudo-nitzschia spp.	CBA 173	*P. calliantha*
Pseudo-nitzschia spp.	CBA 174	*P. multistriata*
Pseudo-nitzschia spp.	CBA 176	*P. pungens*
Pseudo-nitzschia spp.	CBA 180	*P. pungens*
Pseudo-nitzschia spp.	CBA 181	*P. calliantha*
Pseudo-nitzschia spp.	CBA 182	*P. pungens*
Pseudo-nitzschia spp.	CBA 183	*P. calliantha*
Pseudo-nitzschia spp.	CBA 184	*P. pungens*
Pseudo-nitzschia spp.	CBA 186	*P. pungens*
Pseudo-nitzschia spp.	CBA 189	*P. calliantha*
Pseudo-nitzschia spp.	CBA 191	*P. calliantha*
Pseudo-nitzschia spp.	CBA 192	*P. calliantha*
Pseudo-nitzschia spp.	CBA 193	*P. calliantha*
Pseudo-nitzschia spp.	CBA 194	*P. calliantha*
Pseudo-nitzschia spp.	CBA 195	*P. calliantha*

Introduction

- The high resolution melting (HRM) analysis can be an alternative technique to rapidly and accurately identify microalgal species and/or genotype [2].
- Different melting profiles are obtained from the transition of double-strand (dsDNA) to single-strand DNA (ssDNA) as a result of a gradual temperature increase after PCR amplification.
- The HRM analysis is faster, and less expensive than alternative approaches, such as microarray [3], or microsatellites [4].
- In the this study, a molecular assay based on HRM curve analysis was developed and applied to detect various species *Pseudo-nitzschia* species collected in the NW Adriatic Sea (Fig. 1).

Results

- Design of genus *Pseudo-nitzschia* specific primers on LSU rDNA for qPCR HRM analysis.
- Strains of *Pseudo-nitzschia* spp. (n = 22) were analyzed by HRM assay to validate the HRM method (Table 1). **The HRM assay distinguished three different variants corresponding to two different species namely *P. calliantha*, and *P. pungens* and one complex namely *P. delicatissima*/*P. cf. arenysensis* (Fig. 2).**
- The HRM assay has been applied for assigning the species of unknown strains (n = 31) of *Pseudo-nitzschia* spp. (Table 2): **12 strains were identified as *P. calliantha*, 11 strains were identified as *P. delicatissima*/*P. cf. arenysensis*; 7 strains were identified as *P. pungens* and one strain as "variant 1" (Fig. 3).**
- The confidence interval for auto-called results of 96–100% indicated that the species discrimination was robustly convincing. The average difference in Tm values was sufficient enough to enable discrimination between the species during the melting curve analysis. The Tm values among species were significantly different by Kruskal-Wallis test (Hc= 70.96, p<0.001). Mann-Whitney pairwise comparisons showed that *Pseudo-nitzschia* spp. Tm values were significantly different (p<0.001) (Table 3). Subsequently, the LSU rDNA genes of these strains were sequenced to verify the species. This in silico analysis also allowed to assign the recognized strain in HRM as "variant 1" belonging to the species *P. multistriata* (see also Fig. 3).

Table 3. The average Tm ± (SD) for each *Pseudo-nitzschia* species analyzed by HRM assay. n.d. = not detected

Species	Control Tm range (C°)	Samples Tm range (C°)	Average Tm ± SD (C°)	P-value Kruskal-Wallis
P. calliantha	84.3-84.7	84.3-85.1	84.56±0.33	Hc= 69.72 Hc=70.96 P<0.001
P. delicatissima/*P. cf. arenysensis*	85.2-85.3	85.0-85.3	85.05±0.09	
P. pungens	84.9-85.1	85.0-85.2	85.11±0.06	
Pseudo-nitzschia variant 1	n.d.	86.9-86.4	86.66 ± 0.05	

Conclusions

The qPCR-HRM assay developed in this study appears to be a promising tool for rapid and simultaneous detection and discrimination of the species *Pseudo-nitzschia* spp. by using only a single pair of primers, compared to the qPCR classic assay, which used one pair the primers for each species. The high resolution melting analysis is a powerful technique able to accurately identify *Pseudo-nitzschia* species that are difficult to be recognized by standard microscopy in environmental samples. This innovative methodology can be useful in the survey activity of HAB monitoring programs.

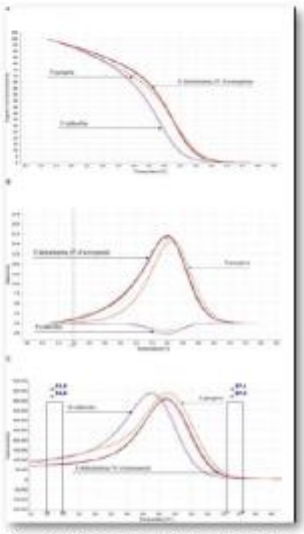


Fig. 2. Melting curve variance of the three controls *Pseudo-nitzschia* species in (A) aligned, (B) difference and (C) derivative plot analyses.

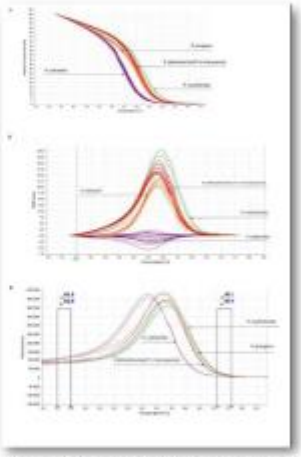


Fig. 3. Melting curve variance of the four *Pseudo-nitzschia* species in (A) aligned, (B) difference and (C) derivative plot analyses. The melting curve and Tm for each species can be very well discerned.

References

1. Penna A., Casabianca S., Perini F., Pigozzi S., Riccardi E., Bastianini M., Scardi M. (2012) - Toxic *Pseudo-nitzschia* spp. in the northwestern Adriatic Sea: characterization of species composition by genetic and molecular quantitative analyses. *J. Plank. Res.* 35: 362-366.
2. Granados-Cifuentes C. and Rodriguez-Lanetty M. (2011) - The use of high-resolution melting analysis for genotyping Symbiodinium strains: a sensitive and fast approach. Mol. Ecol. Res. 11: 394–399.
3. Smith M.W., Maier M.A., Suciu D., Peterson T., Bradstreet T., Nakayama J.,Simon H. (2012) - High resolution microarray assay for rapid taxonomic assessment of Pseudo-nitzschia spp. (Bacillariophyceae) in the field. Harmful Algae 19: 169–180.
4. Casteleyn G., Leliaert F., Backeljau T., Debeer A., Kotaki Y., Rhodes L., Lundholm N., Sabbe K., Vyverman W. (2010) - Limits to gene flow in a cosmopolitan marine planktonic diatom. *PNAS* 29: 12952–12957.

Intercalibration of counting methods for *Ostreopsis* spp. blooms in the Mediterranean Sea

M. Vassalli[a],_ A. Penna[b], F. Sbrana[a], S. Casabianca[b], N. Gjeci[c], V. Asnaghi[d], E. Ottaviani[c], V. Giussani[d], L. Pugliese[b], S. Capellacci[b], M.C. Chiantore[d]

[a]Institute of Biophysics, CNR IBF, Via De Marini 6, Genova, Italy

[b]DISB, University of Urbino, Viale Trieste 296, Pesaro (PU), Italy

[c]OnAir srl, Via Barabino 26/4B, Genova, Italy

[d]DISTAV, University of Genova, Corso Europa 16, Genova, Italy

(manuscript in contribution to Ecological Indicators)

M3-HABs Project Flyer

COORDINATION TEAM

Project Coordinator
Mariachiara Chiantore

Project co-coordinator
Valentina Asnaghi

Project Financial Manager
Maddalena Laggini

ENPI Project Officer
Renato Botti

CONTACT

Prof. Mariachiara Chiantore
Tel: + 39 010 3538384
e-mail: chiantor@dipteris.unige.it

This publication has been produced with the financial assistance of the European Union under the ENPI CBC Mediterranean Sea Basin Programme. The contents of this document are the sole responsibility of CoNISMa and its implementing partners, and can under no circumstances be regarded as reflecting the position of the European Union or of the Programme's management structures.

PARTNERS

ITALY

CoNISMa (Applicant)
National Interuniversity Consortium for Marine Sciences

CNR-IBF - Institute of Biophysics of the National Research Council

ARPAL

On Air

DHI Italy

FRANCE

LOV - National Center of the Scientific Research and Université Pierre et Marie Curie - Laboratoire d'Océanographie de Villefranche

UNS - University of Nice-Sophia Antipolis ECOMERS laboratory

TUNISIA

INSTM - National Institute of Marines Sciences and Technologies

LEBANON

CNRS - National Council for Scientific Research

+ 15 ASSOCIATES PARTNERS

Research Institutions, Environmental Agencies, Municipalities, Health Institutions, Aquaculture enterprises and media from 7 Mediterranean Countries

M3-HABs

Risk Monitoring, Modelling and Mitigation of benthic Harmful Algal Blooms along Mediterranean coasts

www.m3-habs.net

Follow us on:

Project funded by the EUROPEAN UNION

ENPI CBCMED

Harmful Algae Blooms: a threat for beach users, a challenge for a EU project

Universities, research institutes, environmental agencies and technological enterprises based along Mediterranean coasts joined, under the umbrella of a EU funded project (ENPI-CBCMED program), to reduce the hazards related to benthic harmful algae, particularly *Ostreopsis*, a toxic dinoflagellate of tropical origin.

The project M3-HABs has started in January 2014 and will help to increase awareness and implement measures to manage benthic harmful algal blooms in the Mediterranean basin through the development of common monitoring procedures, automated counting methods and modeling predictive tools.

Funding
The project is funded by ENPI CBCMED Programme.
Total budget: €1.998.060
Programme contribution: €1.798.254 (90%)
Project co-financing: € 199.806 (10%)

Activities in 7 work packages

WP1: Coordination and Management (CoNISMa, Italy)

WP2: Communication (CNRS, Lebanon)

WP3: Capitalization (INSTM, Tunisia)

WP4: Opto-electronic system and algorithm for cell identification and counting (CNR-IBF, Italy)

WP5: Predictive tool for *Ostreopsis* blooms (CoNISMa , Italy)

WP6: Common and inter-calibrated sampling strategies and protocols (LOV, France)

WP7: Common risk detection and management strategies (ARPAL, Italy)

The initiatives adopted aim at:

- ✓ Developing an opto-electronic system coupled with innovative molecular techniques for automatic cell identification and counting
- ✓ Developing a predictive tool for *Ostreopsis* blooms, on the basis of identified environmental drivers
- ✓ Developing an improved and common protocol for monitoring *Ostreopsis* abundances and related environmental drivers
- ✓ Setting up of common risk detection and management strategies

To capitalize project results, two Summer Schools for PhD students and young researchers will be organized and an *Ostreopsis* network will be consolidated, in order to promote collaborations and exchanges of knowledge among scientists working on harmful algal blooms.

INVOLVING STAKEHOLDERS

M3-HABs envisages a large stakeholders' involvement of environmental local agencies, municipalities, research institutions, but the final beneficiaries of the project are all the people using the coastal environment for both recreational and economic purpose.