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XXVI CICLO

Identification of *Pseudo-nitzschia* (Bacillariophyceae) species using Whole-Cell Fluorescent In *Situ* Hybridization: from cultured sample to field test.

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Alla mia famiglia

Riassunto

L'identificazione precoce di diatomee fitoplanctoniche del genere *Pseudo-nitszchia* è di grande importanza per il fatto che questo genere annovera numerose specie in grado di produrre acido domoico, una neurotossina responsabile dell' ASP (Amnesic Shellfish Poisoning), sindrome che causa gravi disturbi neurologici nell'uomo e mortalità massiva della fauna marina.

Come per le altre diatomee, l'identificazione delle diverse specie di *Pseudo-nitzschia* avviene, ove possibile, mediante l'osservazione di caratteristiche morfologiche cellulari distinguibili al microscopio ottico, sebbene caratteri sistematici importanti si rilevano soprattutto dalle osservazioni ultrastrutturali del frustulo (parete cellulare silicea delle diatomee) attraverso il microscopio elettronico.

Negli ultimi anni, le analisi genetiche e molecolari, hanno rilevato la presenza di un' elevata diversità genetica nell'ambito del genere *Pseudo-nitzschia*, con la presenza di numerose specie geneticamente affini (cladi) definite specie *pseudo-criptiche* e *criptiche*, che presentano, rispettivamente, lievi o nessuna differenza ultrastrutturale, ma con apprezzabile diversità genetica. Queste differenze si riflettono anche nella capacità di queste specie di produrre o meno acido domoico e quindi di essere o meno neurotossiche.

Attualmente, l'identificazione di alcune alghe tossiche come anche l'individuazione di specie criptiche, è resa possibile attraverso l'impiego di markers molecolari usati su acidi nucleici estratti (analisi di regioni variabili del rDNA, sia nelle regioni codificanti che negli ITS, analisi degli

rRNA, tecniche di DNA microarray); di recente sono state sperimentate anche tecniche di identificazione molecolare su cellula intera (Whole Cell-WC) con uso di sonde fluorescenti (Fluorescent In Situ Hybridization-FISH). In particolare la WC-FISH, metodologia adottata in questo lavoro di tesi, prevede l'uso di brevi sequenze nucleotidiche (oligoprobes) marcate con fluorocromi, specifiche per regioni target dell'rRNA presenti nella subunità maggiore (LSU) o minore (SSU) del ribosoma. Queste regioni sono infatti molto variabili e riescono a identificare i diversi ceppi e/o cladi.

La metodologia WC-FISH è stata messa a punto partendo da protocolli già presenti in letteratura che sono stati da noi integrati e modificati. Nella prima fase di calibrazione e test delle diverse sonde, sono state impiegate monocolture di specie criptiche e/o pseudocripitiche del genere *Pseudo-nitzschia*, isolate e coltivate in laboratorio. Per ogni coppia oligoprobe/specie target è stata effettuata un'accurata fase di calibrazione allo scopo di ottenere un segnale di marcatura ottimale ed eliminare i fenomeni di cross-ibridazione delle diverse sonde con i cladi affini. Tali operazioni hanno consentito di validare, di tutte le sonde disponibili, 4 sonde in grado di individuare in maniera specifica, le specie criptiche *P. arenysensis*, *P. autumnalis* sp. nov., *P. delicatissima* e *P. dolorosa*, appartenenti originariamente al raggruppamento *P. delicatissima* (*P. delicatissima* complex).

La seconda parte del lavoro ha riguardato il trasferimento della tecnica su campioni naturali (Field-test) al fine di verificare la sua eventuale applicazione. Le sonde sono state impiegate anche su campioni ambientali in corrispondenza di fioriture di *Pseudo-nitzschia.*

Sui diversi campioni naturali, l'effettiva sensibilità e specificità della WC-FISH è stata valutata comparando i risultati ottenuti con quelli di

identificazione tradizionale in microscopia ottica (metodo Utermöhl). Complessivamente i field-test hanno evidenziato un buon accordo tra i due metodi dal punto di vista soprattutto qualitativo. Dal punto di vista quantitativo (stima delle abbondanze dopo WC-FISH vs metodo Utermöhl) i nostri dati mostrano una correlazione tra l'efficienza di detection della WC-FISH e numero di cladi presenti nel campione. In particolare, l'efficienza del metodo WC-FISH diminuisce con l'aumentare del numero di specie criptiche, come verificato da successivi esperimenti in laboratorio. Questa criticità del metodo rispetto alle stime quantitative, trova la sua spiegazione nel fatto che, nonostante l'accurato processo di calibrazione effettuato a monte, le diverse sonde, avendo sequenze molto simili, competono per gli stessi siti target delle specie affini del *P. delicatissima* complex.

Altri fattori possono inoltre influire sulla stima quantitativa con metodo WC-FISH: raccolta delle cellule per filtrazione che determina una perdita di densità cellulare; presenza di materiale organico nei campioni naturali che ostacola l'accesso delle sonde ai siti target; stato fisiologico delle cellule che influenza la quantità di rRNA disponibile e quindi l'intensità del segnale fluorescente.

Nonostante questi limiti, possiamo comunque concludere che la metodologia WC-FISH sia uno strumento economico e rapido per l'identificazione di specie criptiche e pseudo-criptiche, anche in campioni ambientali, e che le sonde da noi utilizzate siano un valido strumento per l'identificazione molecolare di specie potenzialmente tossiche del genere *Pseudo-nitzschia* e quindi utili per un monitoraggio precoce.

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1 Introduction and aim of work

The fast and secure identification of phytoplankton, especially of toxic species, is important from an ecological and economical point of view. Identification usually necessitates other time and cost-intensive techniques, such as electron microscopy, pigment analysis with high-performance liquid chromatography (HPLC), or sequencing of conserved genes sequences before a definitive identification can be made of particularly difficult taxa.

Diatoms are unicellular heterokont algae known most notably for their elaborately ornamented cell walls of opaline silica. The scanning electron microscope (SEM) revolutionized diatoms systematics, revealing taxonomically important ultrastructural features that were otherwise hidden from the light microscope. Most recently, the application of molecular biological techniques to systematic studies of diatoms has revealed still more variation, and these methods now play an important role in the discovery and delimitation of diatom species. This is evident in the increasingly common discovery of ‘‘cryptic species”, morphologically similar but genetically distinct species that, at some point, shared the same specific epithet. In some cases, DNA sequence data have indeed provided compelling evidence for the existence of cryptic species, in which the rates of morphological and molecular evolution have apparently been decoupled. In other cases, DNA sequence data corroborate the subtle morphological differences and allow to differentiate multiple species from what previously had been considered one. These species are sometimes referred to as ‘‘pseudo-cryptic’’.

Pseudo-nitzschia (Heterokonta, Bacillariophyceae) is a cosmopolitan genus of chain-forming, pennate, planktonic diatoms. The genus is highly

diverse, and is composed of several groups (clades) of genetically closely related species that are often indistinguishable in light microscopy (cryptic species). A number of species within this genus are associated with production of domoic acid, a neuroexcitatory amino acid responsible for a human illness known as Amnesic Shellfish Poisoning (ASP).

Pseudo-nitzschia are strongly elongated cells, which form distinctive stepped chains by overlapping cell ends-features that make the genus readily recognizable under the light microscope. However, individual species within the genus are much harder to discriminate, requiring detailed morphologic observations of valve fine structure. Characters such as cell length, valve outline and width in valve view may be observed in fresh or preserved material under the light microscope, but the finest features, which in many cases are diagnostic for making species determinations, are only revealed under scanning or transmission electron microscopy (SEM or TEM). Thus, identification and enumeration of particular *Pseudo-nitzschia*, especially in natural populations harboring potentially toxic and nontoxic species, is difficult and time consuming. In turn, correlating the abundance and distribution of particular *Pseudo-nitzschia* with manifestation of domoic acid in the plankton or food web is problematic.

In recent years, several new species have been circumscribed that are not easily distinguishable using light microscopy (LM) and often also using electron microscopy (SEM or TEM). All these species are referred to as pseudo-cryptic because of the subtle morphological dissimilarities between them, mainly consisting of slight differences in the ultrastructure of the poroids in the valve striae, a character that is only visible using TEM. Identification of pseudo-cryptic species is obviously critical when some of them are toxic, as in the case of *Pseudo-nitzschia*.

The ability to produce toxin that may be incurred in genetic diversity, but also in environmental changes such as nutrient status and growth. For this

reason, monitoring programs are needed able to identify and distinguish species pseudo-cryptic with different potential for toxicity.

While the *Pseudo-nitzschia* genus can be easily distinguished by the chains of cells joined by overlapping poles, even in live phytoplankton samples, species identification is much more difficult and often requires SEM and/or TEM in order to see the distinguishing morphological features. Electron microscopes are not readily available or appropriate for routine monitoring, and the identification of *Pseudo-nitzschia* species requires a high level of expertise. These impediments have led to the development of identification tools that require less specialized equipment and expertise. Therefore, all focus on development of new methods instead of traditional methods to facilitate the identification and quantification of harmful algae.

Presently, the most widely used detection methods include Fluorescence In Situ Hybridization (FISH) (Chen et al., 2008; Huang et al., 2008), real-time PCR (Shi et al., 2010; Park and Park, 2010), Sandwich Hybridization Assay (SHA) (Mikulski et al., 2008; Diercks et al., 2008), loop-mediated isothermal amplification (Zhang et al., 2009), Sandwich Hybridization integrated with Nuclease Protection Assay (Zhen et al., 2009) (NPA-SH) and Nucleic Acid Sequence-Based Amplification (NASBA) (Ulrich et al., 2010).

Phytoplankton species identification by Whole-Cell Fluorescent In Situ Hybridization (WC-FISH) with specific fluorochrome-labeled probes followed by fluorescence microscopy, offers a faster alternative for species identification. Based on conserved and variable region of the RNA of the ribosomal small and large subunit (SSU, LSU rRNA), signature sequences of varying specificity can be found, which has been used to develop probes for the identification of phytoplankton at various taxonomic levels from classes down to species or strains.

In this study we applied the Whole-Cell Fluorescent In Situ Hybridization (WC-FISH) on cryptic species and / or pseudo-cryptic of the genus *Pseudo-nitzschia*. We used fluorescent labeled probes directed against the ribosomal RNA (rRNA) of the 28S large subunit (LSU). The work was carried out with the aim to evaluate the possibility of applying the WC-FISH of natural samples and thus obtain the visual discrimination of all species cryptic/pseudo-cryptic of *Pseudo-nitzschia* in order to obtain a semi-quantitative estimation of the presence of potential toxic species.

2 Algae: general features

Something like two-thirds of the earth's surface are covered by ocean and sea and in them live the photosynthetic organisms calls “Algae”. Algae occur on shores and cost, attached to the bottom (benthic species), or live suspended in the water itself (planktonic species), down to a depth of around 150 m, depending on the transparency of the water. Altogether, the algae probably account for more than half the total primary production worldwide and virtually, all aquatic organism are dependent on this production.

Algae are extremely important not only ecologically, but also phylogenetically. It is thought that all the major groups (Phyla and Divisions) of animals and plant originated in the sea, and even today this is where one can find representatives of many ancient evolutionary lineages. Thus, if we are to be able to understand the diversity and the phylogeny of the plant world, it is of fundamental importance, indeed essential, to investigate the algae.

There had been an enormous increase of knowledge about the morphology and cytology of algae. These have led to a complete re-evaluation of the algal systematic and its phylogenetic interpretation. In particular, there has been an enormous increase in our knowledge of the cytoskeleton, especially in relation to the flagellar apparatus and the processes of mitosis and cytokinesis.

Also, ultrastructural and molecular genetic data, particularly the latter, have completely revisioned the systematic of Algae. For example, nucleotide sequence of ribosomal RNAs or their nuclear genes and the structure and sequences of the chloroplast genome are important new

sources of data, from which phylogenetic trees can be constructed and compared with trees derived from morphological data. It has been possible, for instance, to test the hypothesis of the endosymbiotic origin of chloroplast and mitochondria.

The names of the division and classes of Algae often contain a reference to the color of the organism included in them: Cyanophyta, blue-green algae; Rhodophyta, red algae; Chrysophyceae, golden algae; Phaeophyceae, brown algae; Chlorophyceae, green algae. The kinds and combinations of photosynthetic pigment present accordingly have an important role in algae classification.

The chemical nature of the storage products and cell walls also plays an important part in the definition of the various algal groups. The biochemical characters we have just mentioned are correlated with many other characters, drawn especially from the cytology and morphology of the organisms. Important criteria include, for instance, the presence or absence of flagellate cells, the structure of the flagella and flagellar roots, the pattern and course of mitosis (nuclear division) and cytokinesis (cell division), the presence or absence of an envelope of endoplasmic reticulum around the chloroplasts, and the possible existence of a connection between this envelope and the nuclear membrane. A summary of the mentioned characters and algal group is reported in the table 1.

Tab 1 Characteristics of selected Division of Algae					
	Brown Algae	Red Algae	Gree Algae	Dinoflagellates	Water Molds
Division	Phaeopphyta, Bacillariophyta	Rhodophyta	Chlorophyta	Dinoflagellata	Oomicota
Color	Brownish	Reddish	Green	Brownish	Colarless, white
Cell Wall	Cellulose, alginic acid, pectin, silica	Cellulose	Cellulose	Cellulose in menbrane	Cellulose
Cell Arrangement	Multicellular, unicellular	Most are multicellular	Unicellular multicellular	Unicellular	Multicellular
Photosynthetic Pigment	Chlorophyll a and c, carotene, xanthophylls	Chlorophyll a and c, phycobili-proteins	Chlorophyll a and b	Chlorophyll a and c, carotene xanthins	None
Sexual Reproduction	Yes	Yes	Yes	In a few (?)	Yes
Storage Material	Carbohydrate, oil	Glucose polymer	Glucose polymer	Strach	None

2.1 Division Heterokontophyta

The principal feature of the Heterokontophyta (Stramenopiles) is the flagellate cells heterokont, bearing a long pleuronematic flagellum, which is directed forwards during swimming, and a shorter smooth flagellum, which lacks the stiff hairs of the pleuronematic flagellum and points backwards along the cell. The pleuronematic flagellum is called mastigonemes. The mastigonemes are composed of glycoprotein (Bouck 1971) and are synthesized in cisternae of the endoplasmic reticulum (Dodge 1975; Moestrup 1982). The chloroplast is enclosed not only by its own double membrane, but also by a fold of endoplasmic reticulum. The chloroplast contain chlorophyll a, c_1 and c_2. The principal accessory pigment is fucoxanthin or vaucheriaxanthin. The main reserve polysaccharide is chrysolaminaran.

The Heterokontophyta constitute a natural group and includes diverse organisms with very different morphology: from the multicellular brown algae of Laminariales, to unicellular silica- walled diatoms. The division Heterokontophyta contains nine algal classes: Chrysophyceae, Parmaphyceae, Sarcinochrysidophyceae, Xanthophyceae, Eustigmatophyceae, Bacillariophyceae, Raphidophyceae,

Dictyochophyceae, Phaeophyceae. As *Pseudo-nitszchia* belong to the Bacillariphyceae, here we report some more informations about this Class.

2.2 Class Bacillariophyceae

Different characters distinguish the Bacillariophyceae (fig.1) from other classes within the Heterokontophyta. All species are unicellular or colonial coccoid algae. Each cell is encased by a unique type of cell wall, which is siliceous and takes the form of a box with an overlapping lid; this is termed the *frustule*. The only flagellate cells formed in the Bacillariophyceae are the male gametes of the centric diatoms. Here there is a single pleuronematic flagellum (Manton et al.1966). The chloroplasts are usually golden-brown, because the chlorophyll is masked by the accessory pigment fucoxanthin. Species that reproduce sexually, have a diplontic life cycle, with gametic meiosis.

Fig.1 Different cells of Diatoms.

The Bacillariophyceae are widespread in both marine and freshwater habitats. There are well over 250 genera of living diatoms, with around 100000 species (Round, Crawford 1990). Diatoms occur in the sea, in

freshwater, on damp rocks, or on soil. The phytoplankton of the oceans consist to a large extend of diatoms, and in temperate or cold parts of the oceans, particularly in nutrient- rich water, diatoms are largely responsible for the very high primary productivity that occurs. The constant 'rain' of dead diatom frustules to the bottom of these highly productive parts of the ocean results in the accumulation of diatoms oozes (Seibold and Berger 1982). Large fossil deposits from past geological periods are now mined as 'diatomite' or 'diatomaceus earth', which is used for filters, deodorants and decolouring agents, and as an abrasive, for instance, in toothpaste.

The very extend of the world's oceans makes it easy to see what an enormous role diatoms must play in the production of organic material through photosynthesis. This is underlined still further when one considers that almost all other marine life is directly or indirectly depended on this primary production for its food.

The class Bacillariophyceae contains two major groups, the centric diatoms (Centrales) (fig.3) and the pennate diatoms (Pennales) (fig.2), which distinguished each other on the basic differences in cell wall symmetry and structure. The silica shell (frustule) of a pennate diatom is elongate and usually bilaterally symmetrical in face (valve) view, with a lanceolate or elliptical outline. Te frustule consist of two halves: the hypotheca and epitheca (the overlapping lid). The epitheca consist in turn of two parts called epivalve and hoop-like side wall, epicingulum. Similarly, the hypotheca, consist of a hypovalve and hypocingulum. The epicinculum and epivalve are separated by a suture as are the hypocinculum and hypovalve. The valves are beautifully structured and ornamented, and these characters are species- specific and have a great systematic importance. Some pennate diatoms also exhibit a fissure along their longitudinal axis. This is known as a raphe, and is involved in gliding

movements made by diatom cells; motile diatoms always possess a raphe. In many other pennate diatoms the pattern appears at first sight to consist of lines (striae), which are regularly spaced and arranged roughly parallel to the transapical plane. The lines can sometimes be seen to be composed of many separate dots and the electron microscope reveals that the dots are pores (areolae) through the silica.

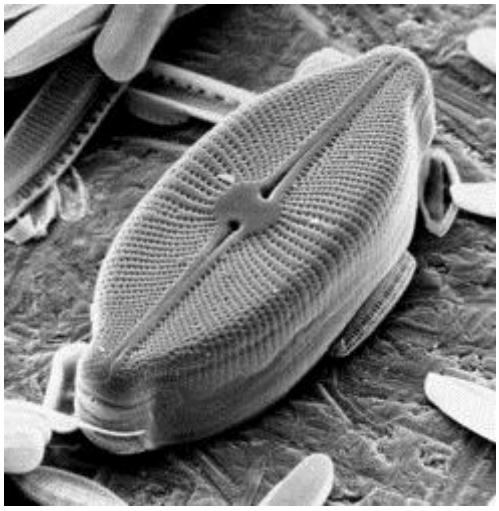


Fig.2 Pennales diatom. SEM photograph.

The cell wall of centric diatoms have the structure of the valve, and frequently radially symmetrical, the frustules often resembling a petri dish. As in pennate diatoms, the cells are ornamented with species-specific patterns and structures. In many centric diatoms the valves contain radial rows of small, more-or-less hexagonal chambers, called 'loculate areolae'.

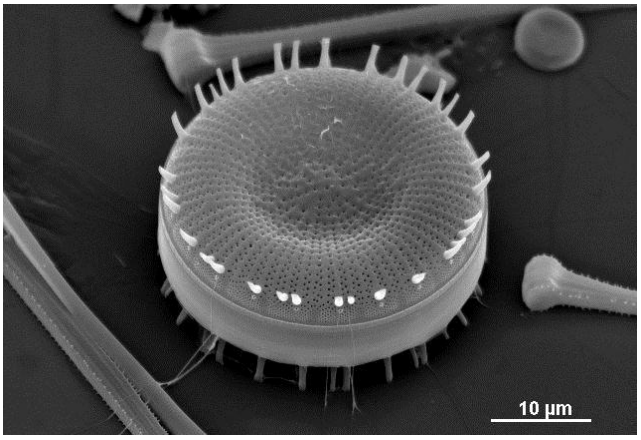


Fig.3 Centrales diatom. SEM photograph.

2.3 Genus *Pseudo-nitzschia*

The genus *Pseudo-nitzschia* (fig.4) was originally defined by Peragallo and Peragallo (1900) from the genus *Nitzschia* and has been subjected to many taxonomic changes over the last century based on frustule morphology.

Fig.4 *Pseudo-nitzschia*. Light Microscopy photograph.

Fifty years after being defined, *Pseudo-nitzschia* was reduced to a section of the genus *Nitzschia* on the basis of its raphe and motility. Eventually, *Pseudo-nitzschia* was again separated from *Nitzschia* as a distinct genus by Hasle (1994) based on morphological characters and later supported by analysis of the 18S ribosomal RNA (rRNA) (Douglas et al. 1994).

Pseudo-nitzschia, like many pennate diatoms, can reproduce sexually. Clonal cultures of *Pseudo-nitzschia* will gradually decrease in cell size over time and eventually die if they do not undergo sexual reproduction. This is due to vegetative cell division and splitting of the frustule between two daughter cells. The halves of the frustule fit together like a glass Petri dish, with one side slightly smaller than the other. The daughter cell that receives the smaller of the two frustules will grow a new second frustule inside the first. This cell will be smaller than the initial parent cell. In this way, the average dimensions of the cell gradually decrease until they become so

small the culture can no longer survive. However, if cells undergo sexual reproduction, cell size is restored.

Pseudo-nitzschia is a cosmopolitan genus, however, some tropical and polar species exist as well as coastal and oceanic species (Skov et al. 1999, Hasle 2002). The genus has also a global importance due to its production of the neurotoxin domoic acid that can cause Amnesic Shellfish Poisoning and Domoic Acid Poisoning. It has been recorded from nearly every major marine and estuarine environment and domoic acid has been found in the tissue or feces of organisms in multiple trophic levels in the oceans.

Studies show that blooms of *Pseudo-nitzschia* spp. are increasing in frequency and duration due to changes in coastal nutrients (Anderson et al. 2006, Parsons et al. 1999). It is often found in areas of upwelling or nutrient enrichment. Approximately 12 *Pseudo-nitzschia* species are documented domoic acid producers. The reason for domoic acid production is not fully understood. Laboratory analyses show cultures of *Pseudo-nitzschia* produce domoic acid under silicate or phosphate limitation, but not nitrogen or light limitation. Field studies in the Pacific Ocean and laboratory studies have found increased domoic acid production under conditions of iron limitation.

Many species of *Pseudo-nitzschia* are found over a wide range of salinity and temperature (*P. pungens*) while other species are restricted to a narrow environmental regime (*P. prolongatoides* and *P. turgiduloides*). Blooms of *Pseudo-nitzschia* happen relatively frequently, in some regions seasonally, and in a wide variety of locations (fig.5).

Pseudo-nitzschia blooms can be stimulated by nutrients from two sources: upwelling or mixing events and riverine inputs. Both sources stimulate *Pseudo-nitzschia* blooms at concentrations of 8-22 μM $NO3^{-}$,

2.4-35 µM Si, 0.2-2 µM $PO4^{-}$ (Dortch et al. 1997, Scholin et al. 2000, Trainer et al. 2000, Loureiro et al. 2005), but in different temperature and salinity regimes.

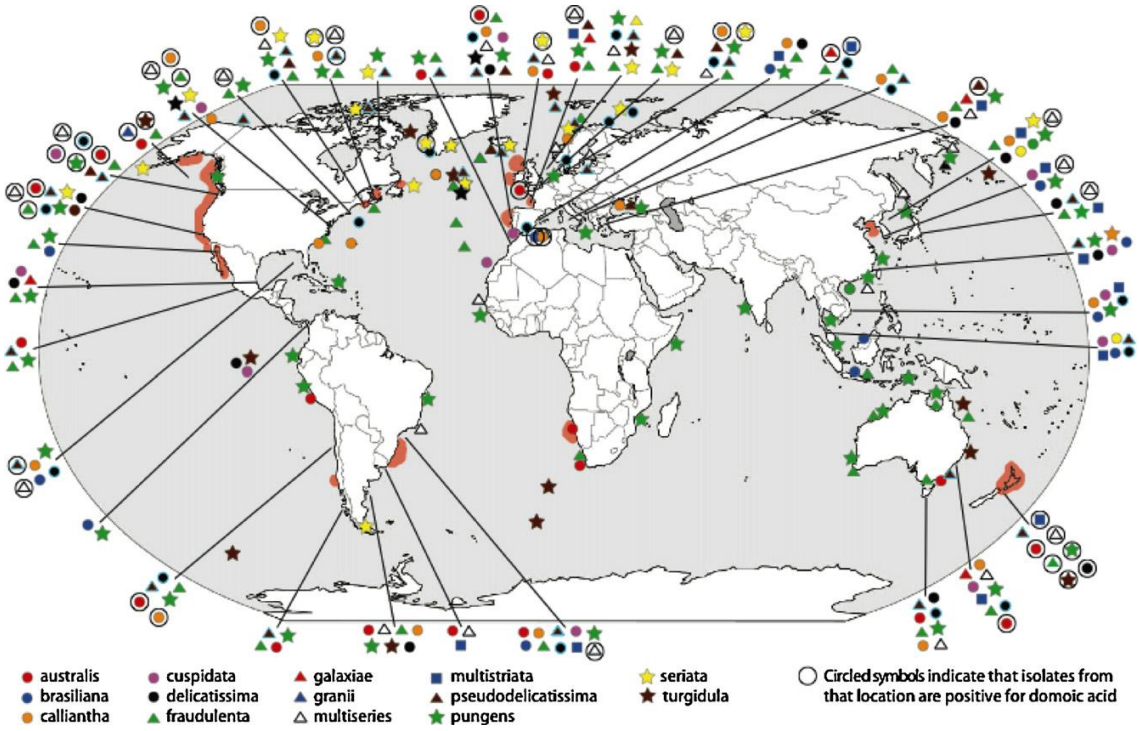


Fig.5 Toxigenic species of *Pseudo-nitzschia*. Symbols of those species that have been demonstrated to produce domoic acid in culture are circled, and are shown at the locations from which they were isolated. Areas along coastlines marked with red are locations where closures of shellfish harvesting due to elevated levels of domoic acid (>20 mg DA g_1 wet weight of shellfish tissue), or animal mortalities, have occurred. Modified and updated from Thessen (2007) and Trainer et al. (2008). Symbols outlined in blue indicate a species description that was made before major taxonomic revisions were implemented for *P. delicatissima* and *P. pseudodelicatissima* (From Trainer, 2012).

Different species in natural populations can demonstrate distinct correlations with environmental characteristics, which suggests seasonal succession of species or regional specificity (Fryxell et al. 1997). Many species may coexist, but different growth and loss rates can lead to complex bloom dynamics and seasonal succession. Several studies looking at molecular data have found potential cryptic species or ecotypes within morphological species (Amato et al. 2007, Amato et al. 2008).

Pseudo-nitzschia is an important primary producer at the base of the food web. It is consumed directly by a wide variety of organisms from heterotrophic dinoflagellates to planktivorous fish. It can form dense blooms and be an important source of food for these primary consumers, thereby introducing domoic acid into higher trophic levels. As a hydrophilic molecule, domoic acid (DA) does not bioaccumulate. Instead, DA is concentrated in the digestive system with little transfer to surrounding tissues and can be quickly eliminated from the body. The toxin is moved through the food chain during blooms when primary consumers with guts full of *Pseudo-nitzschia* are eaten by secondary consumers.

During the last decade, an increasing number of studies combining morphological and molecular characters for studying taxonomy and evolution of *Pseudo-nitzschia* have been published and species have been newly described or emended (Lundholm et al., 2003, 2006; Amato et al., 2007; Amato and Montresor, 2008; Churro et al., 2009). This has revealed that cryptic and pseudo-cryptic species are a more commonly encountered phenomenon than previously considered (Orsini et al., 2004; Lundholm et al., 2003, 2006; Amato et al., 2007; Quijano- Scheggia et al., 2009b). Cryptic species are morphologically identical, but genetically different, whereas pseudo-cryptic species, apart from the genetic diversity, show minor morphological differences that are only detected by detailed examination (Mann and Evans, 2007). However, correct species assignment provides a possibility for understanding complex patterns of physiological parameters, e.g. toxin production, biogeographical patterns and species succession in field studies.

Today, species identification is easier with the developments of PCR tools, e.g. microarray or simpler methods like ARISA (Automated

Ribosomal Intergenic Spacer Analysis) analyses (McDonald et al., 2007; Hubbard et al., 2008; Medlin and Kooistra, 2010; Kudela et al., 2010).

Different regions of the genome have been targeted to assess the phylogeny of *Pseudo-nitzschia*: LSU, ITS1, 5.8S and ITS2 of the ribosomal DNA and rbcL, the large subunit of RuBisCo (Lundholm et al., 2002a,b, 2006; Orsini et al., 2004; Amato et al., 2007). The nuclear-encoded internal transcribed spacers (ITS), comprising ITS1, 5.8S and ITS2, have been the most widely used, as they give highly supported separation of species, and ITS2 is useful as a barcode sequence (Evans et al., 2007; Moniz and Kaczmarska, 2009). So, in the last decade, a number of species complexes have been identified in *Pseudo-nitzschia* (Trainer et al., 2012). For example, *Pseudo-nitzschia delicatissima* has been split into well separated pseudo-cryptic species: *P. delicatissima, P. decipiens* and *P. dolorosa*. However, it was recently recognized that *P. delicatissima*, as previously defined, actually consists of two separate clades, representing two cryptic species: *P. delicatissima and P. arenysensis* (Orsini et al., 2004; Lundholm et al., 2006; Amato et al., 2007; Quijano-Scheggia et al., 2009b). The two cryptic species are morphologically indistinct, but comparisons of the secondary structure of ITS2 support that the species are separate entities. Furthermore, the species differ with respect to physiological parameters, e.g. temperature required to sexualize the genotypes and differences in growth rates (Kaczmarska et al., 2008; Quijano- Scheggia et al., 2009b).

2.4 Toxic *Pseudo-nitzschia* and domoic acid

The ecological position of *Pseudo-nitzschia* is in part related to its ability to produce domoic acid (DA). While most studies on DA production are focused on the harmful effects and gross levels of DA produced, the possible role of DA in the intracellular regulation of *Pseudo-nitzschia* physiology has also been considered.

Pseudo-nitzschia was not recognized as a toxic diatom until the first documented incident of Amnesic Shellfish Poisoning (ASP) occurred in Prince Edward Island, Canada in 1987 when residents ate domoic acid contaminated mussels (*Mytilus edulis*) from Cardigan Bay estuaries (Bates et al. 1989). Out of 250 reported illnesses, 107 met the case definition for ASP (Perl et al. 1990). Common symptoms were vomiting, abdominal cramps, diarrhea, incapacitating headache and loss of short-term memory (Perl et al. 1990).

Due to its variable toxicity and cosmopolitan distribution, *Pseudo-nitzschia* poses a unique management challenge worldwide. An effective monitoring program must include *Pseudo-nitzschia* identification and enumeration, domoic acid quantification and testing of potential seafood vectors. Since the presence of *Pseudo-nitzschia* does not guarantee the presence of domoic acid, abundance data alone are rarely a sufficient basis for management decisions. Instructions on how to monitor for *Pseudo-nitzschia* spp. and domoic acid can be found in the Manual on Harmful Marine Microalgae, edited by G. M. Hallegraeff, D. M. Anderson and A. D. Cembella. Traditional plankton sampling techniques are typically useful for *Pseudo-nitzschia* monitoring; however, there are situations when this sampling method may be inadequate. Molecular probes have come into use to increase speed of identification and resolution between genetically

distinct strains. There are several types of rRNA targeted probes: whole-cell hybridization (labeling of intact cells), fluorescent in-situ hybridization (FISH), sandwich hybridization (measuring DNA in cell homogenate) and PCR methods (PCR replication of targeted genome).

Domoic acid (MW 311) (fig.6) is a water-soluble, heat stable analogue of the amino acid glutamate (Hatfield et al. 1995, Leira et al. 1998). It was first isolated from *Chondria armata* and named after the Japanese word for seaweed – domoi (Mos 2001). DA is toxic to vertebrates because it binds to neurons twenty times more powerfully than ordinary neurotransmitters, resulting in a massive depolarization of the neuron.

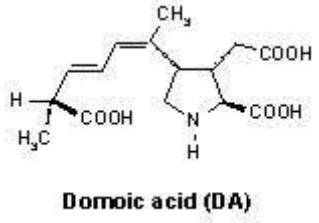


Fig. 6 Molecular structure of Domoic acid.

DA is quantified by HPLC and UV detection. If shellfish contain more than 20 mg kg^{-1} DA, harvesting is stopped until three consecutive samples spaced out over at least 2 weeks have < 20 mg DA kg^{-1} (Todd 2003). Countries with limits for DA in shellfish are Canada, USA, New Zealand, Chile, Peru and EU member states. The limit is 20 mg kg-1 edible meat. A limit of 30 mg DA kg-1 in cooked viscera of Dungeness crab has been set by the FDA in the United States.

In order to minimize economic impact of DA contamination, the EU allows harvest of scallops with whole body burdens of DA between 20 and 250 mg kg^{-1} if they are sold after total removal of the hepatopancreas.

There are many methods of detection of DA. However, the limit of detection of the mouse bioassay for ASP toxin is 40 mg DA kg^{-1} shellfish, above the regulatory limit of 20 mg DA kg^{-1} shellfish (Fernandez et al. 2009).

A heavily studied aspect of *Pseudo-nitzschia* physiology is domoic acid (DA) production. It is commonly known that growth phase, nutrients, temperature, irradiance and bacteria play a role in DA production. Many studies show *Pseudo-nitzschia* cultures produce little DA until cell division has stopped. In batch cultures, DA production often starts at the onset of stationary phase and DA content of the cells peaks about one week later. Some cultures produce DA during late exponential phase, possibly because this is a period of transition when some cells have stopped growing and are producing DA while other cells are still dividing. In continuous culture, toxin content increases when growth is slowed by decreasing the dilution rate (Pan et al. 1996). This growth effect means that many factors that slow growth would also indirectly increase toxin production. Studies showing that an increased pH will increase toxin production also show that growth rate decreases under these circumstances, making it difficult to know the effect of pH alone (Lundholm et al. 2004). Cultures grown on urea have higher DA production, but also have a slower growth rate. The increase in toxin production when growth slows must be taken into account when investigating factors that affect DA production (Trainer 2012).

Nutrient limitation is widely used to induce DA production in culture, with Si and P limitation commonly used. Metabolism typically decreases with lower temperatures, but many enzymes involved in the production of DA have differing temperature optima. For example, RUBISCO, the enzyme that fixes atmospheric carbon, operates optimally at a higher temperature than nitrate reductase, which transforms $NO3^-$ into $NO2^-$

(Trainer 2012). Both fixed carbon and reduced nitrogen are required for DA synthesis.

Temperature has an obvious effect on DA production by regulating the speed of multiple enzyme reactions within the cell. No studies have examined the effects of rapid changes in temperature on DA production, although rapid temperature changes can uncouple the light and dark reactions of photosynthesis.

Irradiance is, of course, a very important control on DA production since it provides the energy necessary for biosynthesis. Irradiances below 100 μmol photons m-2sec-1 can lead to decreased DA production, a trait that has consequences for mass culture of toxic *Pseudo-nitzschia* (Whyte et al. 1995).

In the over two decades since the first deaths associated with the DA outbreak in Prince Edward Island, Canada, considerable progress has been achieved in all areas of the science of *Pseudo-nitzschia*. The rapid initial characterization of the toxin, and the clear establishment of the causative genus, have opened the door to extensive, hypothesis-driven research on factors that lead to toxic bloom development and ways to avoid future incidence of ASP.

3 Methodologies for the study of phytoplankton

3.1 Traditional methods of analysis of the phytoplankton

The research on phytoplankton ecology are generally based on counting and identifications of phytoplankton cells on natural samples. In this way, a lot of natural samples are collected, fixed and conserved for successively microscopic analysis. The type of sampling is chosen in relation to the program objective: for qualitative or semi-quantitative analysis, the net sampling are used (fig.8); for quantitative estimation in discrete depth along the profile vertical, samples are collected with Niskin or Vandhorn type bottles (fig.7).

Fig.7 Niskin bottles.

Fig.8 Plankton net sampling.

The sampling by net allows to filter a large volumes of water, but it is also selective and, depending from the size of the mesh, a determinate fraction of phytoplankton can be sampled. Samples collected with nets have the advantage of containing a large amount of material on which to conduct the preliminary taxonomic investigations. A sampling carried out in this way makes it possible to filter a large amount of water by concentrating the phytoplankton organisms in a reduced volume. However,

quantitative studies conducted on samples collected with this procedure have shown poor reliability because many species of small size they escape through the meshes, even in the case in which the meshes are particularly fine.

The quantitative sampling by Niskin and/or Vandhorn bottles, allows to estimate parameters such as cell density and biomass. The fixed samples remain virtually unchanged, if kept in appropriate conditions, making it a major database system that can be employed at a later date. For longer period of preservation, some problems occur for the siliceous frustules of diatoms and for the coccoliths (Zingone et al., 1990). One of the most widely used fixatives is formalin at final pH neutral or slightly basic. Lugol's solution, less toxic than formalin, is suitable for very long periods of conservation. Lugol's solution is appropriate for the preservation of small flagellates, and for slightly silicified diatoms, while it is less suitable for Coccolithophores.

The successive cells counting is performed on a sub-sample and the more traditional method involves the observation and counting of phytoplankton cells by an inverted microscope (Fig.9).

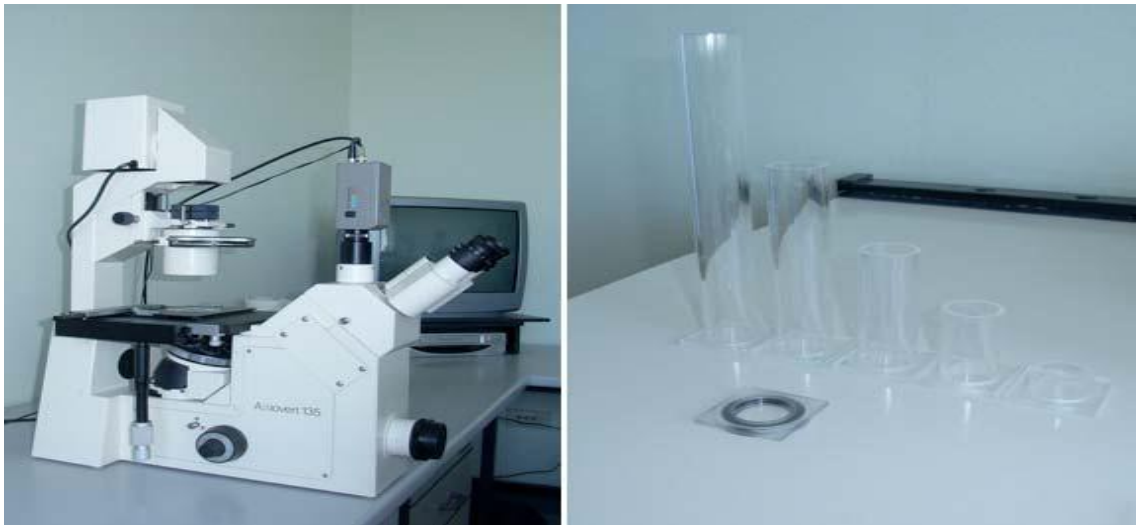


Fig.9 Inverted microscope and settling chamber.

This method, as first described by Utermohl (1958), is utilized to identify and enumerate the phytoplankton community from many different types of aquatic habitats. In this method the algae are allowed to settle onto the base of the settling chamber.

A recent evaluation of the quality control of the Utermohl method was performed by Rott et al. (2007). Similar estimates on the precision of the method (determination of the number of cells to be classified, often around 200 cells per sample) as well as the criteria for the validation of the analysis have been discussed in the guides European standards (EN 15204, 2006).

During the microscopic analysis, the taxonomic identification of algal cells is realized through dichotomous keys that allow the identification of algal organisms in the sample.

The Utermohl method is difficult to apply for some taxa. In fact, the ultrastructural morphological characteristics on which is based the taxonomy of some group (e.s. diatoms, dinophyta, ecc.) needs the scanning electron microscope (SEM) and / or transmission (TEM) (Fig.10).

Fig.10 SEM and TEM

These morphological characteristics concern mainly the structure of the wall or cell envelope which presents a considerable variety in different taxonomic groups (Hasle et al., 1997).

The choice of the use of a SEM and/or a TEM is linked to the type of structure that it will be analyze. The SEM shows a lower power of resolution than the TEM, but allows to obtain three-dimensional images and is therefore particularly useful for the observation of the external surface of whole cells such as the theca of dinoflagellates, the frustule of diatoms or coccoliths of Coccolithophores. Alternatively, the TEM allows the observation of samples of reduced thickness (ultrathin sections) and is therefore suitable for observing cellular content and organules features. In this case the high power of resolution displays morphological details important for algal taxonomical studies. The disadvantages of the use of electron microscopy are that these techniques require time-consuming preparation sample and are often laborious and expensive.

3.2 Molecular tools

Small photosynthetic organisms are responsible for the bulk of primary production both in oceanic as in neritic waters. These organisms play also a great roles in many biogeochemical processes that regulate our global climate.

Approximately less than 10% of the known biodiversity in the marine protistan community is known and new groups being discovered regularly (Kim et al. 2011). Many cosmopolitan species are very difficult to identified, with little or no morphological markers to separate them (Medlin, 2013). Their small size and paucity of morphological markers, the inability to bring many into culture, and the difficulty of obtaining samples for long term seasonal studies in open ocean environments has hampered our knowledge of phytoplankton diversity and population structure (Medlin

and Kooistra, 2010). The advent of molecular biological techniques has greatly enhanced our ability to analyze phytoplankton populations.

Molecular tools, in general, offer the possibility to estimate biodiversity at all levels, e.g., kingdom/class/family/species level, in a comparatively small environmental sample. In some cases, even a few milliliters of seawater may be enough. Moreover, some of the techniques are very sensitive, e.g., they offer the possibility to detect single cells in a sample.

Depending on the question(s) being asked, the molecular tools to answer them differ greatly (tab.2).

In general, molecular techniques have some significant advantages over traditional methods:

1. High sensitivity, enabling the researcher to detect even single specific cells among thousands of others.
2. Dead or non-culturable cells can be analyzed.
3. Species-specific data (such as sequences) can be obtained without the need to culture or even isolate a species (Medlin and Kooistra, 2010)

As with all methods, molecular ones also contain certain disadvantage. The harvesting of cells through filtration or centrifugation may be harmful for fragile organisms, which thus may escape the analysis. In PCR-based approaches, biases are evident concerning the choice of (universal) primers, PCR conditions (e.g., the amount of DNA or primers used, annealing temperature, cycle number, *etc.*), machines or enzymes used., Hybridization experiments are susceptible to hybridization conditions (temperature, salt concentration, time) or base composition and subsequent detection of fluorescence may be hampered by autofluorescence (Medlin and Kooistra, 2010).

Tab.2 Molecular tools for study phytoplankton. From: ISPRA "Manuale del fitoplancton"2009.

Tecnique	Markers	Applications	Taxonomic level	Reference
AFLP (Amplified Fragment Length Polymorphism)	detecting polymorphisms in DNA using restriction enzymes to digest genomic DNA, followed amplification with PCR	Population genetics, space-time evolution of diversity	Isolated, population	John et al., 2004
DGGE (Denaturing Gradient Gel Electrophoresis)	gene sequences	Studios of amplification of polymorphisms in DNA target by PCR	Between isolated, population	Larsen at al.,2001; McDonald et al.,2007a
RAPD (Random Amplification of Polymorphic DNA)	non-specific variable regions by amplification with PCR	Population genetics, time evolution of diversity	Isolated, population	Bolch et al.,1999
SSR or Microsatellites (Simple Sequence Repeats)	repeat sequences and specific by amplification with PCR	Population genetics, time evolution of diversity	Isolated, population	Evans et al.,2004,2005; Rynearson and Arbrust, 2004
RFLP (Restriction Fragment Length Polymorphism)	specific regions obtained using restriction enzymes	Population genetics, time evolution of diversity	Isolated, population, species	Scholin at al.,1994
PCR qualitative and sequences analysis	Target regions by amplification in PCR	Identification, phylogenesis, taxonomy, Bar-cording	Genus, species	Penna et al.,2007; Kooistra et al.,2008
Real Time PCR	Target regions aplification in quantitative PCR	Identification, quantification	Genus, species	Bowers et al.,2000; Galuzzi et al.,2004, 2008
DNA ARRAY with fluorescence	genes sequences and ribosomal genes	Identification	Specific taxon	Metfies and Medlin, 2004
Electrochemical detection	Ribosomal genes	Identification	Specific taxon	Metfis et al.,2005
SHA (Sandwich Hybridization Assay)	Sequences ribosomal genes	Identification	Specific taxon	Scholin et al.,1999
TSA-FISH (Tyramide Signal Amplification- Fluorescent In Situ Hybridization)	Ribosomal genes	Identification	Specific taxon	Not et al.,2002; Anderaaon et al.,2005; john et al.,2005; Tobe et al., 2006

General assessment of comparative biodiversity in a larger number of samples can be achieved with DNA fingerprinting methods based on denaturing or temperature gradient gel electrophoresis (DGGE, TGGE) (Muyzer and De Wall 1993; Wright and Bentzen 1994) or single strand conformation polymorphisms (SSCP) (Schwieger and Tebbe 2008). These methods can discern strains, that differ by only a few DNA base pairs, by performing first a PCR, followed by a polyacrylamide gel electrophoresis

with a denaturant or temperature gradient (Coyne et al. 2001; Etscheid and Riesner 1998; Godhe 2002; Muyzer et al. 1993). The method is based on the melting behavior of DNA sequences in the gel with increasing concentrations of denaturing substances or increasing temperature (Etscheid and Riesner 1998; Van Hannen et al. 1998). The benefits are obviously the rapidness, the high sensitivity, specificity, and good resolution to study a population structure, whereas the drawbacks are the non-quantitative detection due to the PCR and the utilization of neurotoxin acrylamide (Biegala et al. 2003; Godhe 2002; Van Hannen et al. 1998).

Real-Time PCR or Quantitative PCR (QPCR) is a modification of conventional PCR protocols and allows the product formation in the PCR reaction to be monitored *in-situ* by fluorescence (Marie et al. 2006). This PCR approach can be used to determine the abundance of specific groups. The simple approach is the utilization of nucleic acid dyes (e.g., SYBR Green or Ethidiumbromide), which bind to the newly synthesized double-stranded DNA as soon as it is formed during PCR. The incorporation is measured in a special thermocycler device and compared to a standard. The major drawback is that the dye will unspecifically bind to all double-stranded DNA including any unexpected PCR products, which may lead to errors in the calculation. The main advantage are the detection accuracies over a large dynamic range, the fast analysis and the large sample throughput (Countway and Caron 2006; Johnson et al. 2006; Zhu et al. 2005). However, it destroys cells, and the equipment and components are expensive (Godhe 2002). It requires sophisticated controls and calibrations (Johnson et al. 2006).

Presence or absence of a known species can be monitored with species-specific probes using chemiluminescent detection and DNA dot blot techniques or, more sophisticated, with fluorescent *in-situ* hybridization (FISH). DNA microarrays (fig.11) or so-called DNA chips are one of the

most powerful innovations in microbiology. Microarrays were introduced in the mid 1990s primarily for the detection and monitoring of gene expression (Schena et al. 1995; Schena et al. 1996). The application of sequences onto the surface of a glass slide with special surface properties in an ordered array is based on a minimized, but high throughput form of a dot-blot (Gentry et al. 2006; Ye et al. 2001). The DNA microarray experiment is performed by chip production, sample isolation and preparation, hybridization and data analysis. Prior to the hybridization, the target nucleic acid is labeled with a fluorescent dye, which can be incorporated directly to the nucleic acid or via indirect labeling of other substances (Cheung et al. 1999; Metfies et al. 2006; Southern et al. 1999).

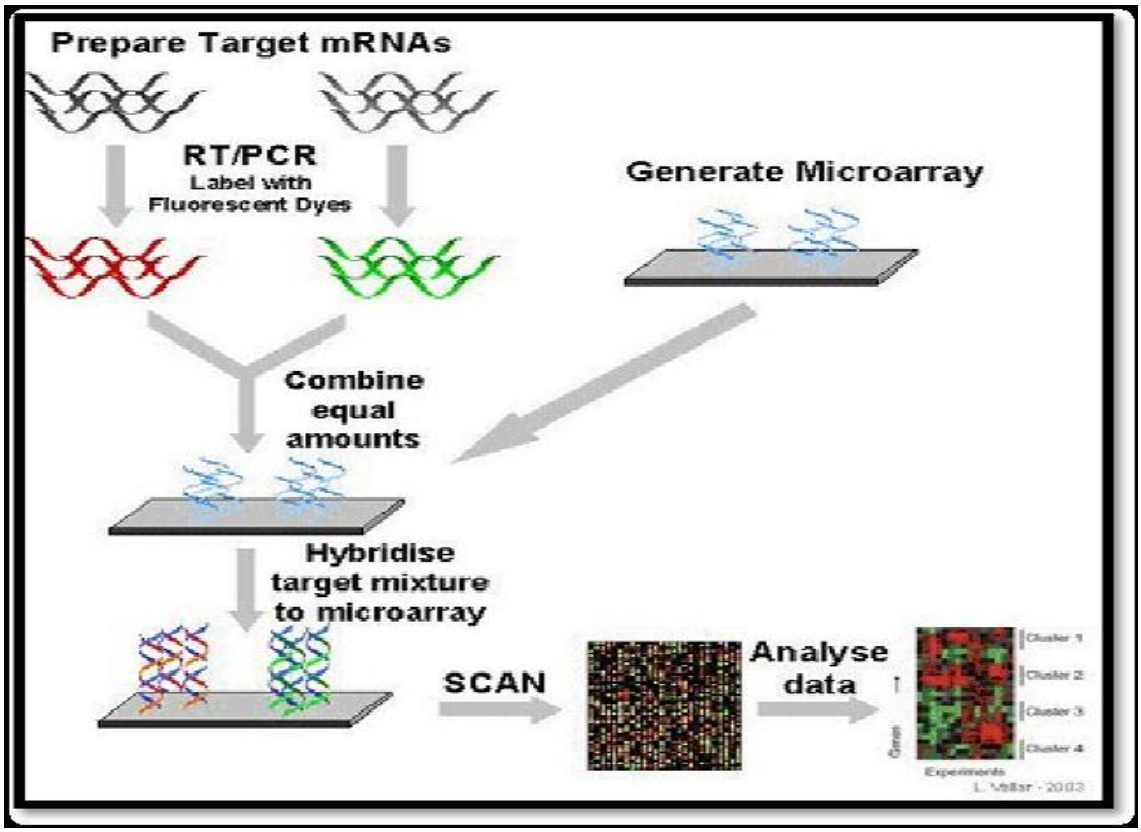


Fig.11 Schematic representation of Microarray experiment.

The hybridization pattern is captured via fluorescent excitation in a special device, the microarray scanner (Ye et al. 2001). One of the major drawbacks of this method is a possible cross-reaction from unspecific binding. The probes are designed to be specific to known sequences but there are a high number of unknown environmental sequences. As a consequence, species without a probe on the chip can also be overlooked (Gentry et al. 2006). Furthermore, the development of a functional chip is time-consuming, expensive and all probes on one chip need to work specifically under the same hybridization conditions (Boireau et al. 2005; Feriotto et al. 2002; Metfies et al. 2006).

3.3 Molecular marker

A molecular marker is a genetic trait of DNA or in the case of proteins (isozymes) used to distinguish between individuals or groups by the marker's different alleles (Medlin and Koistra 2010).

Photosynthetic organisms usually contain three different genomes: the nuclear, the plastid and the mitochondrial genomes (fig.12). Each has its own unique set of genes, each of which evolves at different rates.

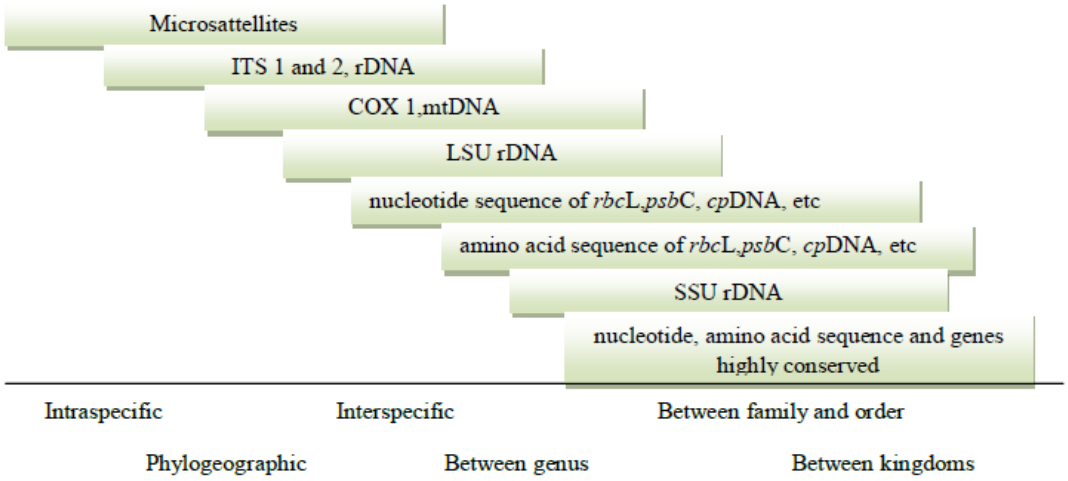


Fig. 12 Regions of DNA used as molecular markers and levels analysis.

Microsatellites (MS) or simple sequence repeats (SSR), are the most powerful molecular markers available (Burke et al., 1991; Wright et al., 1994). Microsatellites are short sequences of one to six nucleotides, e.g., (CT)n or (CAG)n, that are repeated five to dozens and sometimes hundreds of times and are found in great abundance dispersed all over the genomes of all organisms investigated so far. This abundance together with the large number of alleles, resulting from high mutation rates because of their special, regular structure, makes them highly useful molecular markers at the population level (Alpermann 2009).

Distinction of individuals at the species level can be obtained using highly variable molecular markers, such as ITS sequences (internal-transcribed spacer) or microsatellites.

At higher taxonomic levels, slower evolving coding regions, such as the ribosomal RNA genes (small subunit or SSU, large subunit or LSU) and the large subunit of RUBISCO (ribulose 1,5-bisphosphate carboxylase, *rbcL*), are commonly used, but other genes, such as the plastid *psaA*, *psaB*, *psbC* and *tufA* genes, are increasing (Medlin and Koistra 2010).

The SSU rRNA gene is often the gene of choice for cloning and is the gene most commonly used as a phylogenetic yardstick. The method allows the exhaustive description of biodiversity in a sample down to the species level. Also the resulting sequence information may serve as a basis for developing specific oligonucleotide probes necessary for subsequent methods like FISH (Woese 1987).

This molecule, besides the availability of a huge dataset (the current ARB database contains over 300,000 prokaryotic and eukaryotic sequences aligned by secondary structure), offers some advantages:

1. Universally present with the same function in all organisms;
2. Variable regions of conservation, which enables design of primers or probes to be designed;

3. Many copies, which makes PCR easy or genome application for later PCR also easy;
4. No evidence for lateral gene transfer, so vertical descent is analyzed by phylogenetic methods (Woese 1987).

Also the resulting sequence information may serve as a basis for developing specific oligonucleotide probes necessary for subsequent methods like FISH. Probes are short oligonucleotides of normally 16-24 bp length that are hundred percent homologous only to a complementary sequence in a gene of the species of interest and differ by at least one position to all other organisms. In hybridization experiments, these probes can therefore be used to identify species of interest by binding to the target's sequence and later detection by a probe-attached label, e.g., Digoxigenin (DIG) or a fluorochrome like Fluorescein (Toebe 2013).
The next section reports extensively about this technique.

3.4 Fluorescent In Situ Hybridization (FISH) and Whole Cell-FISH (WC-FISH)

In situ hybridization is a powerful technique that enables the visualization of nucleic acid probes on target tissue, cells, nuclei and chromosomes, so that the location of the nucleic acid (DNA, RNA) can be determined as *in vivo*. The ability to detect nucleic acid *in situ* enables: construction of physical maps of chromosomes; analysis of chromosome structure and aberrations; determination of the spatial and temporal expression of genes (Toebe 2013)

Understanding *in situ* hybridization requires knowledge of molecular biology, genetics, immunochemistry ad histochemistry.

The main steps are outlined in figure 13 and initially involve the preparation of biological material and the labeling of a nucleic acid sequence to from the probe. Labeling a nucleic acid involves the incorporation of either a radioactive or non-radioactive marker which can be detected. Both probe and material are then denatured to make all nucleic acid single- stranded. Finally the sites of hybridization are detected and visualized; the detection methods depend on the type of label attached to the probe.

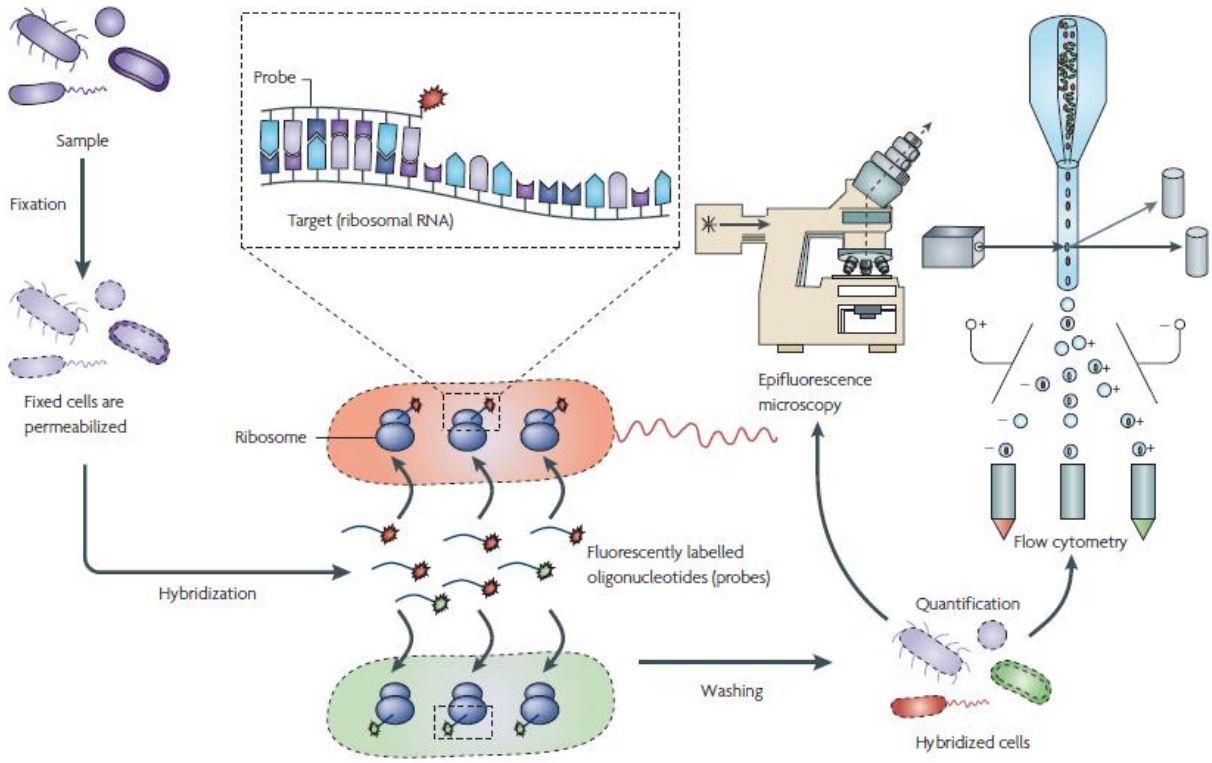


Fig.13 Basic steps of fluorescence *in situ* hybridization. The sample is first fixed to stabilize the cells and permeabilize the cell membranes. The labelled oligonucleotide probe is then added and allowed to hybridize to its intracellular targets before the excess probe is washed away. The sample is then ready for single-cell identification and quantification by either epifluorescence microscopy or flow cytometry (Aman and Fuchs 2008).

Two types of non- radioactive *in situ* hybridization procedures can be distinguished: the direct and the indirect methods. In the direct method the

label that has been incorporated into the nucleic acid can be visualized directly once *in situ* hybridization has been completed. The recently introduced fluorochrome- labelled nucleotides (fluorescein isothiocyanate FITC, rhodamine TRITC, Cyanine Cy3), which can be enzymatically incorporated into the nucleid acid, are becoming extremely important because of the simplicity of detection.

Successful *in situ* hybridization experiment require that the material is adequately preserved and that the target sequences and tissue morphology are maintained. In addition, the tissue has to be permeable to probe and detection reagents. Once harvested the material should be fixed quickly to minimize endogenous nuclease activity and other degradation processes. Rapid fixation is particularly important when RNA is to be detected because RNA is very sensitive to the degrading activity of RNase. The fixation step serves to preserve tissue morphology and minimize loss of nucleic acids. Two categories of fixative are predominantly used: cross-linking fixative (glutaraldehyde, formaldehyde) or protein- precipitating fixatives (ethanol or methanol mixed with acetic acid). The fixative chosen depends on the material and probe being used, the method of imaging probe hybridization sites and the level of sensitivity required.

The *in situ* hybridization reaction exploit the kinetics of nucleic acid duplex formation. Nucleic acid duplexes form by hydrogen bonding between two complementary nucleic acid strands. The stability of the duplex under defined conditions can be determined by calculating its melting temperature (T_m). The T_m is the temperature at which one-half of the duplex molecules become dissociated or "melted" into single strands.

The stability of the hybrid nucleic acid is dependent on a number of factors:

- The proportion of guanine and cytosine (%GC). GC pairs are more stable than AT pairs because they are bonded together by three as opposed to two hydrogen bonds. For this reason, DNA with a high GC content is more stable than DNA which is rich in AT, and more energy is required to separate the two strands.

- Length of hybrid nucleic acid. In general, a long duplex is more stable than a shorter one because it is held together by more hydrogen bonds. Oligonucleotide probes have fewer hydrogen bonds binding the duplex, and this reduces the duplex stability.

Typically, labeled nucleic acid probes are made up in a hybridization mix containing formamide , salts, dextran sulphate, with optional incorporation of blocking DNA or tRNA, sodium dodecyl sulphate and bovine serum albumin. Formamide is used in denaturation and hybridization solution to enable a reaction temperature that is not damaging to tissue morphology. Formamide also regulates stringency. Salts in solution are used to regulate the ionic strength of hybridization and denaturation solution and to help stabilize the nucleic acid duplex. Dextran sulphate can increase the hybridization reaction rate by factor of 3. It functions by forming a matrix in the hybridization mixture which concentrates the probe without affecting the stringency. Unlabelled blocking DNA or RNA is included to block probe hybridization to non- specific sites. Sodium dodecyl sulphate (SDS) helps in probe penetration by acting as a wetting agent. Bovine serum albumin (BSA) can reduce some non-specific probe hybridization.

The precise amount of target nucleic acid available for *in situ* hybridization is difficult to assess because of unknown effects of the nucleic acid conformation and its interaction with associated molecules, particularly protein. The rate hybridization depends on the probe length,

complexity of sequence and concentration. In general, long probes may result in a slower rate because of limited diffusion into the material.

Post-hybridization washes are usually carried out in a slightly more stringent solution that the hybridization mixture to denature and remove weakly bound probe, leaving only perfectly or nearly perfectly matched nucleotides in the duplex.

FISH with rRNA-targeted oligonucleotide probes was first used for identification, localization, and quantification of defined microbial populations in complex samples (Amann et al.,1995). The thousands of ribosomes provide enough targets for probe binding and therefore, strong enough signals to be detected. In picoplankton and also in bacterial cells, which often show weaker signals because of their small size and therefore lower ribosome content, techniques like catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) can be used to boost the signal strength up to a detectable level (Schönhuber et al., 1997; 1999).

Higher group level probes, species, or even strain specific oligonucleotide probes for FISH are available for many taxa and enable the differentiation of morphological similar co-occurring species, especially for harmful algal bloom (HAB) species (Mikulski et al. 2008; Anderson et al. 2005; Miller and Scholin 1998; Chen 2011).

In these paper the FISH is applied to the whole algal cells and so we can refer as Whole Cell FISH. For the successful detection it is necessary to fix the cells on a filter or in a culture for preservation and to permeabilize the membrane before the application of probes. The advantages are definitely the rapid quantitative detection and visualization of algal species in a mixed field sample and the discrimination of closely related species or strains with even similar appearance (Godhe 2002;

Groben et al. 2004; John et al. 2003a; John et al. 2005; Metfies et al. 2006). Furthermore the morphology of the cells is conserved and thus also different external life stages can be recognized (Godhe 2002).

One drawback of this method is that cellular rRNA content may vary under different environmental conditions and could have an impact on the fluorescence signal. Further challenges are the autofluorecence of photosynthetic cells, non-specific binding of probes, difficulties with penetration of thick cell walls of resting cysts and non-stability of rRNA molecules (DeLong 1998; Garcés et al. 1998; Godhe 2002; Medlin and Simon 1998; Rice et al. 1997).

In the last years , several works have performed WC-FISH for detection of HAB specie. Among these species, notably members belonging to the dinoflagellate genera *Alexandrium* (Toebe et al. 2013a; Tang et al. 2012; Touzet et al. 2011; Hosoi-Tanabe and Sako 2006; Anderson et al. 2005), *Prorocentrum* (Hou et al. 2009), *Cochlodinium* (Mikulski et al. 2008) and *Karenia* (Mikulski et al. 2005), or species of the diatom genus *Pseudo-nitzschia* (Parsons et al. 1999; Rhodes et al. 1998; Miller and Scholin 1998), as well as members of the haptophyte genus *Prymnesium* (Toebe et al. 2006) were monitored by FISH analysis in their marine environment.

3.5 Molecular probes ribosomal RNA

The continually growing number of available algal 18S and 28S rDNA sequences, e.g. from GenBank (Benson et al. 2011), makes it possible to design rRNA oligonucleotide probes which specifically target the SSU (or 18S) or LSU (or 28S) ribosomal DNA (rDNA) from higher group down to species level (Groben et al. 2004; Lange et al. 1996).

The rRNA provides some characteristics, which predestines this molecule as a phylogenetic marker. First of all, the rRNA is an integrated part of the ribosome, the protein factory of each cell, and has the same function in every organism. The rRNA molecules must have developed in early stages of life and thus are evolutionarily conserved even in its two- and three-dimensional structures. Although the primary structure is highly conserved throughout all organisms, some regions in the rRNA are more conserved than others (Thiele et al., 2011).

The conserved sequence stretches on the rRNA provide a variety of technical advantages, it is highly abundant in cells, making it suitable as a target for in situ hybridization studies. Also the resulting sequence information may serve as a basis for developing specific oligonucleotide probes necessary for subsequent methods like FISH. Probes are short oligonucleotides of normally 16-24 bp length that are hundred percent homologous only to a complementary sequence in a gene of the species of interest and differ by at least one position to all other organisms. In hybridisation experiments, these probes can therefore be used to identify species of interest by binding to the target's sequence and later detection by a probe-attached label, e.g., Digoxigenin (DIG) or a fluorochrome like Fluorescein.

The full-cycle rRNA approach was first developed as a phylogeny-based toolbox for cultivation-independent studies of microbial diversity and ecology. After DNA extraction, the 16S rRNA genes are amplified by polymerase chain reaction (PCR) using conserved primers. The amplified rRNA genes are singularized by cloning in a plasmid vector and transformation in competent *Escherichia coli* cells. They are then sequenced and submitted to sequence databases. Comparative sequence analysis is the basis for the design of oligonucleotide probes. Finally, these

probes can be applied to environmental samples using FISH techniques (Amann et al., 1995).

For FISH, a probe length of 15–25 nucleotides, most often 18 nucleotides is common. The GC content of a newly designed probe should be between 50% and 70%, since a higher GC content could result in unspecific binding. The GC content of probe sequences influences their melting behavior (Thiele et al., 2011). A problem that should be considered during the design of FISH probes is target site accessibility (Fuchs et al., 2001). Although the secondary structure of the ribosome is highly conserved and a consensus map could be developed from the 16S rRNA accessibility studies, each probe should be checked on their respective target group of organisms to ensure high probe signals (Behrens et al., 2003). In the last step of synthesis, a fluorochrome is added to the 5^{I} end of the oligonucleotide. A range of fluorochromes is available for the labeling of nucleic acids. Standard labels for in situ hybridization are the green fluorescein and the red tetramethylrhodamine derivatives (Tab.3).

The optimal hybridization conditions need to be established to guarantee high specificity and good sensitivity of the probe. For this, a series of hybridizations is performed at increasing stringency either by increasing the temperature of hybridization or by increasing the concentrations of a denaturing agent such as formamide in the hybridization buffer.

Table 3 Dye labels frequently used for oligonucleotide probes and their characteristics.

	Excitation (±10 nm)	Emission (±10 nm)	Molecular weight (Da)	ε (mol^{-1} cm^{-1})
Carboxy-fluorescein (FAM)	492	518	376	79 000
Fluorescein	490	520	389	77 000
Alexa 488	494	517	643	71 000
Atto 488	501	523		90 000
CY3	512/552	565/615	766	150 000
Alexa 546	554	570	1079	112 000
Carboxytetramethyl-rhodamine (TAMRA)	540	565	466	91 000
Alexa 594	590	617	820	92 000
Atto 590 (rhodamine-derivative)	594	624		12 000
CY5	625–650	670	792	250 000
Alexa 647	651	672	1250	270 000

4 Materials and methods

4.1 Algal cultures and culture conditions

Pure cultures of *P. dolorosa, P. arenysensis, P. autumnalis, P. delicatissima, P.galaxia, P. fraudolenta, P. manni, P. pseudodelicatissima,* and *P. multisriata* employed in this study were kindly provided from Stazione Zoologica "Anton Dohrn" (Naples). All cultures were grown in 50 ml flasks containing 35 ml Guillard's f/2 medium (Guillard, 1975) (Table 4). The cultures were kept at 22°C on a 16:8 h light:dark cycle with light provided by cool white fluorescent tubes at a photon flux density of 100µmol m^{-2} s^{-1}.

Tab.4 Composition of Guillard's f/2 medium.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82×10^{-4} M
NaH_2PO_4 H_2O	5 g/L dH_2O	1 mL	3.62×10^{-5} M
Na_2SiO_3 $9H_2O$	30 g/L dH_2O	1 mL	1.06×10^{-4} M
trace metal solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe below)	0.5 mL	---

The growth of monocultures was monitoring at intervals of 24 h by left to sediment a 1ml of culture in a Sedgwick-Rafter slide (a rectangular chambers 50x20 mm, depth 1 mm area of 10 cm^2) and counting. The figure 14 shows an example of a monoculture's growth. When the population reaches the stage neighbor to the plateau phase (about 600-800 cells/ml), it needs to proceed for sub-cultures or, alternatively, the culture was processed for in situ hybridization experiments.

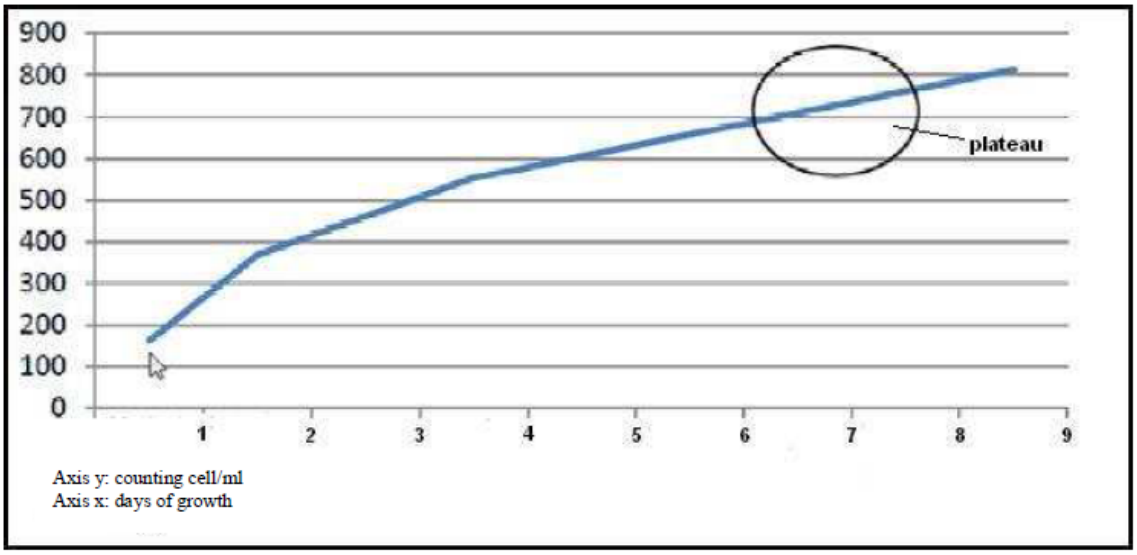


Fig. 14 Growth of monoculture on standard medium

4.2 Whole Cell Fluorescent In Situ Hybridization (WC-FISH): application of probes to algal cultures

Mid-exponential growth cultured cells (25 mL) were gently collected by vacuum (ca. 100 mmHg) onto 47 mm 0,8 μm isopore polycarbonate membrane filters Millipore™ by a manifold filter (fig. 15).

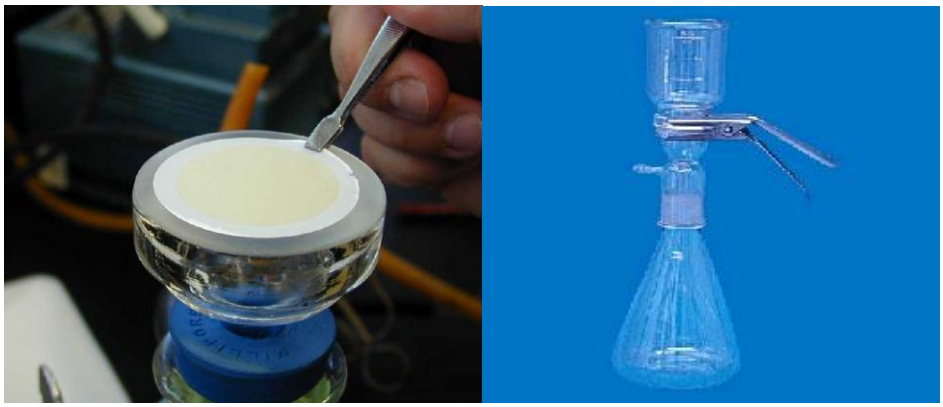
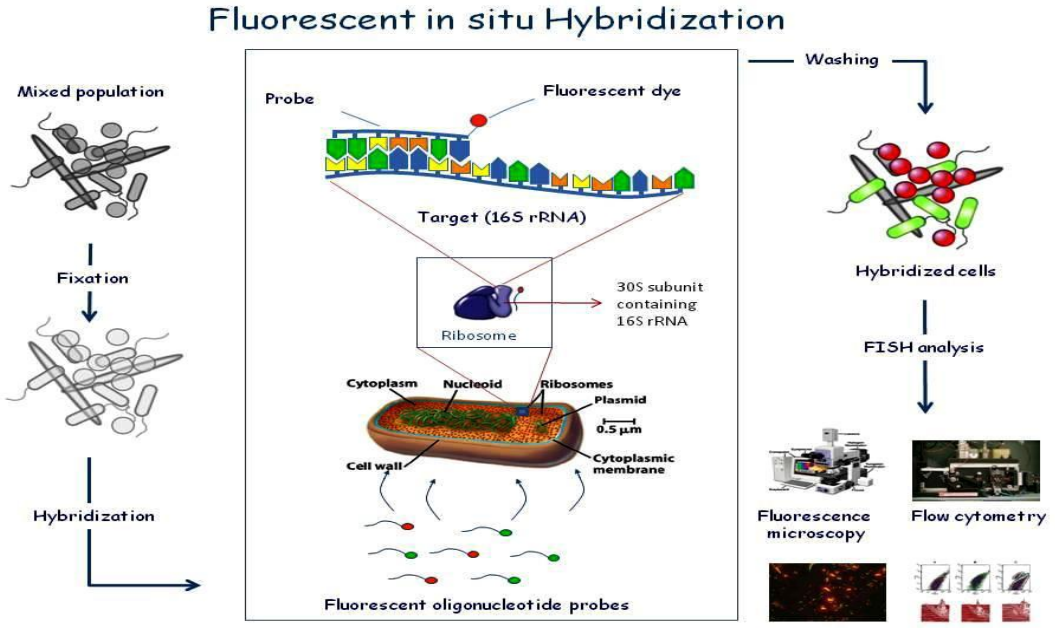


Fig. 15 Isopore polycarbonate membrane filters Millipore™ 47 mm 0,8 μm and vacuum filtration apparatus.

30 ml of freshly prepared saline- EtOH fixative was added so that the whole filter was covered and was incubated at temperature 4°C for 2 h. Saline- EtOH fixative was prepared by adding 25mL 95% ethanol, 2mL deionized H_2O and 3mL 25X SET buffer (3.75 M NaCl, 25mM EDTA, 0.5 M Tris HCl; pH 7.8) (Miller e Sholin 2000). The solution will normally showed strong precipitation, but this does not influence the fixation. Afterwards, the fixative was removed by vacuum filtration and the filters were processed immediately or stored at -20 ° C for a maximum 4 months.

The WC-FISH protocol consists in a series of steps summarized below:

- Pre-hybridization;
- Hybridization;
- Post-hybridization;
- Detection of the signal.



http://www.biovisible.com/indexRD.php?page=fish

The step of pre-hybridization involves washing the filter with 5X SET buffer (25XSET buffer: 10,95 NaCl, 2,5 ml EDTa, 25 ml TRIS HCl) for 5 min at RT. Subsequently, to eliminate the autofluorescence of chlorophyll into cells, the filter was treated with 50% dimetil-formamide (DMF) and incubate at room temperature for 1h and then washed whit 5X SET buffer for 5 min at RT.

Before the hybridization step the filter were cut into small pieces (e.g was cut a filter of 47 mm diameter easily was cut into 12 pieces). Each piece of filter was put onto microscope slide, and was apply 54 µl of hybridization buffer (5X SET, 0.1% v/v, IGEPAL-CA630 o Nonidet-P40,

30 µg/ml $^{-1}$ polyA, X% v/v Formamide) with 6 µl of probe specific (50 ng/µl^{-1} stock). The percentage of formamide (FA) in the buffer depends on the probe requirements. Formamide concentration for probes needs to be determined empirically. In the hybridization buffer we tested different concentration FA and without FA for give clear positive signal of the probe each target species. The probes using in this work (tab.5) was provided by researchers of Stazione Zoologica "Anton Dohrn" (Naples).

Tab. 5 Probes list and target species.

	Probe name	Sequenza	Specie/clade
P.delicatissima complex	Pdel1D01_25	TTGACAACGACTCACTCCACCAGG	*P.dolorosa*
	Pdel3B_25	TAATGTTAAAGTCTATAGACCACAA	*P.arenysensis*
	Pdel3A_25	GACAAAAACTCACTCTACCAGGCGG	*P.arenysensis*
	Pdel4D03_25	TGACAACGACTCACTCTACCAGGC	*P.autumnalis*
	Pdel4D02_25	GATTGTGCAAATATCCAACCACTGT	*P.autumnalis*
	Pdel2D01_25	TCCAACCACTGTTACTTTCATTACG	*P.delicatissima*
P.pseudodelicatissima complex	PpdeD02_25	CCCGGCAGATAACGTCAAGGTCTAT	*P.pseudodelicatissima*
	PgalaD02_25	CCAAAGGAATCAACCAAAGCAAACC	*P.galaxiae*
	PfrauD04_25	ACGGGAGTTTCACCCTCTCAGCTGTC	*P.fraudulenta*
	Pman2D03_25	CTTCAGACCACAATTCGGCGCTTAAA	*P.manni*
Controls	PmulaD03_25	AACCCAAACTCACGAAAGCTCACAG	*P.multistriata*
	UniC	GWATTACCGCGGKGCTG	Eukaryotic cell
	UniR	CAGCMGCCGCGGUAAUWC	Prokaryotes cell

The DNA oligonucleotides extended from 18- 26 pb were labeled at the 5' fluorochrome with fluorescein isothiocyanate (FITC) and, in some cases, Cyanine (Cy3) (fig. 16).

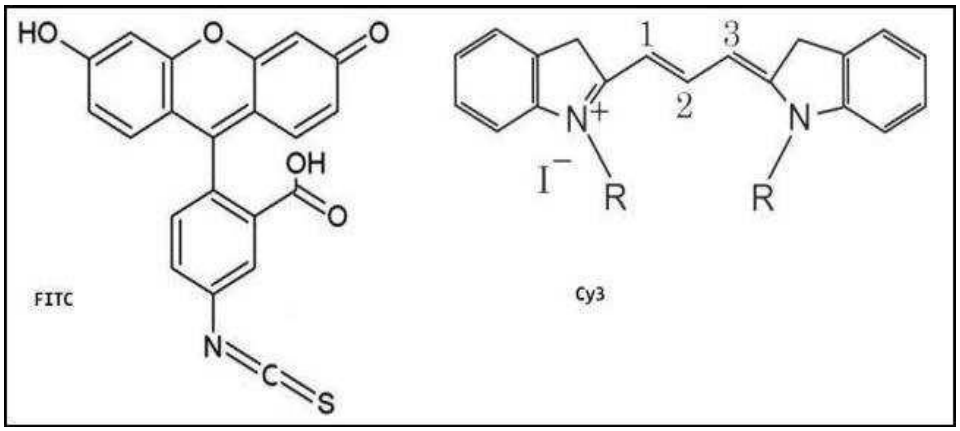


Fig.16 Fluorochrome fluorescein isothiocyanate (FITC) and Cyanine (Cy3).

These oligoprobes recognize target sites of 28S large subunit (LSU). We also used a positive (UniC) and a negative (UniR) controls. Oligonucleotide UniC is targeted toward a universally conserved eukaryotic sequences of 18S subunit rRNA; oligonucleotide UniR was directed towards 16S subunit rRNA and is specific for prokaryotes (Miller and Scholin, 2000).

The fluorescent-labeled probe is light sensitive, so were kept the filters in the dark for the rest of the procedure and minimize exposure to light when handling them.

The filter pieces assembly was placed in an incubator at 45 °C for 2h. The post-hybridization step was performed by several washing for removing the excess of unbound probes. Temperatures, times and concentration buffer of post-hybridization wash were optimized to obtain an intense and specific fluorescence signal, as reported in the section results. The detection of the fluorescent signal was performed by laying the filter onto the microscope slides and applying a mixture of 150 µl of Citifluor-4',6-diamidin-2-fenilindolo (DAPI) directly onto filter. Slides were analysed by Leica DMRB epifluorescence microscopy and digital images of probed cells were captured using a Leica camera system.

4.3 Cross-reactivity test

In order to evaluate the sensitivity and the specificity of our probes, the ten microalgae monocultures were used for cross-hybridization test with all the probes according to the established FISH procedure. For remove nonspecific signal, the temperatures, times and concentration buffer washes were all optimized and the stronger probes were screened.

4.4 WC-FISH on simulated field sea-water sample

In order to further verify the accuracy and specificity of the screened probes and our FISH method, some tests were performed on simulated field samples. To prepare the test material, field seawater was sampled from the Calabrian coast (Northern Tyrrhenian Sea) in which no *Pseudonitzschia* cells was detected. The obtained seawater was directly used to mix evenly with the exponential cultures of the target species. After counting the target cells in the mixture under the light microscope (LM), the artificial sample was fixed and filtered as described in the FISH protocol. The FISH test was applied as described above and the labeled cells were examined and counted under an epifluorescence microscope.

4.5 WC-FISH applied to field sample

Seawater samples were collected monthly at a Long-Term Ecological Research station Mare Chiara in the Gulf of Naples (40°48.5′N, 14°15′E), from May 2011 to April 2012. The station is located two nautical miles from the coast, and the bottom is at ca 70 m. Five liter of seawater was collected using a 12-L Niskin bottle mounted on an automatic Carousel sampler. The seawater was transported in a coolbox to the lab. Samples for cell enumeration in light microscopy were fixed with neutralized formaldehyde at a final concentration of 0.6 % and stored in the dark at 4 °C. A volume ranging from 1 to 50 ml was left to settle in an Utermöhl chamber for counting and enumeration (Utermöhl 1931). Cells from 50 or 75 ml of sample were fixed for FISH as previously described.

4.6 WC-FISH on Calabrian field sample

Seawater samples along Northern Ionian Sea were collected by net during a research cruises in June and October 2012. Samples for cell enumeration in light microscopy were fixed with neutralized formaldehyde at a final concentration of 0.6 % and stored in the dark at 4 °C. Seawater was processed on board ship to provide sufficient material to allow application of probes, cell counts by LM. The seawater was transported in a coolbox to the lab. A volume was left to settle in an Utermöhl chamber for counting and enumeration (Utermöhl 1931), and other 50ml of seawater was processed for the successively WC-FISH.

5 Results

5.1 Establishment and optimization of WC-FISH protocol

The Whole-Cell Fluorescent In Situ Hybridization technique used in this work followed the protocol of Miller and Scholin (1998, 2000) which we modified with some adjustments and we also integrated with the protocol of Groben and Medlin (2005). These adjustments concerned essentially the following points:

- Collection and fixation of the algal cells;
- Amount of probe;
- Addition and percentage of formamide;
- Ratio between volume of the hybridization mix/ volume of the probe;
- Calibration of the post-hybridization washing (temperature, stringency, time washings) .

As of the collecting and fixing the algal cells, the protocols of Miller and Scholin (1996) and Simon (2000), work on the whole cell processed in centrifuge tubes; otherwise other protocols (Medlin e Simon, 1998; Groben and Medlin, 2005; Scholin et al., 1999; Metfies et al., 2006) work on filter-based method. In our preliminary trials on *Pseudo-nitzschia* cells, we compared these two methodologies in order to establish what was working better. The results are shown in figure 20. It is possible to see how the filter-based method that immobilize the algal cells on filters, retains better the cell morphology (fig.20); moreover this method reduces the cells depletion in the sample respect to the centrifugation - based method that causes a consistent depletion because of the multiple cycles of centrifugation and resuspension. So, we choosed as collection method the filter-based method using polycarbonate filters through vacuum pump.

Before application of the probes it was necessary to fix the cells on filter. For the hybridization protocols, the fixation is the first and crucial step for the subsequent hybridization. Various protocols are in use, and they are mostly derived from those developed for bacteria that often employ paraformaldehyde (PFA) as a fixative. Comparing different protocols, we have verified the one used by Scholin et al. (1996, 1998) gave the best results for the most species tested. We took into consideration a saline ethanol fixative (Scholin et al., 1996), which preserves the integrity of the fragile cells and affords a good signal from the fluorescent probes (Miller and Scholin, 1998, 2000). This method eliminates the paraformaldehyde and replaces it with ethanol saline. In addition, the presence of ethanol in this fixative, allows the extraction of chlorophyll from the cells. This is advantageous for experiments in situ hybridizations with phytoplankton autofluorescence, that can be a problem, and instead it allows a good visualization of the probe signal. We added a pre-wash with hybridization buffer without FA and probe, after fixation steps.

Regarding the hybridization and post-hybridization steps, the filters were divided into small pieces and processed on microscope slides and / or multiwell plates (for cell culture). The hybridization was carried out by replacing the water bath with hot air oven in the appropriate humid chamber. The hybridization buffer was prepared according to Groben and Medlin (2005), modified by us as regards the ratio between volume of the hybridization mix/ volume of the probe and the quantity of the probe itself. We at last used 600 ng of probe for each slide.

The Sodium Dodecylsulfate (SDS) is a component that can be used in the buffer hybridization (Simon et al., 2000); but it seems to destroy the more fragile cells, as the dinoflagellates. We used IGEPAL-CA630 (or

Nonidet-P40) to maintain the morphology of the cells and to allow, at the same time, a good penetration of the probe.

Finally, very important is the type of wash used. To reduce the background fluorescence caused by nonspecific binding of the probe we extended the time washings from 5 'to 15'.

Another problem could be the autofluorescence of residual chlorophyll in cells, that can sometimes masks the probe signal. By bleaching the cells in dimetilformamide 50% (DMF) for one hour, we obtained a stronger signal-to-noise ratio for most species, making it easier to distinguish target from non-target cells.

5.2 Specificity assessment of the probes on the target species: calibration of hybridization conditions

Table 7 lists the probes tested, the target species, the probes sequences, the target sites (Large SubUnit rRNA- LSU) and their theoretical temperature of melting. This list included also a positive and negative control probes. The positive control probe (UNIC) is targeted toward a universally eukaryotic conserved sequence of the SSU rRNA; the negative control (UNIR) is targeted toward SSU rRNA and it is specific for prokaryotic organism (Miller and Scholin 2000). The positive control probe (UNIC) repeatedly gave a bright and uniform label intensity for all species examined, while cell treated by negative probe (UniR) and no-probe (black control), appeared uniformly dark. The control probes UniC and UniR were also used to define, for each target species, a range of labeling intensities assuming that cells labeled as the positive controls were scored “+ +” (excellent; fig.21 A,B); otherwise, the cells labeled with an intensity less

than the positive controls, but greater than the negative controls (--) (fig.21 C,D) were scored as "+-" (fig.21 E,F).

Tab.7 List of the probes, their target species, sequences, target genes (SSU or LSU rRNA) and melting temperatures.

Complex	Name probes	Target species	Sequence (5'-3')	Target gene	Melting T
P.delicatissima	Pdel1D01_25	*P.dolorosa*	TTGACAACGACTCACTCCACCAGG	28S	71.5°
	Pdel2D01_25	*P.delicatissima*	TCCAACCACTGTTACTTTCATTACG	28S	65.6°
	Pdel3A_25	*P.arenysensis*	GACAAAAACTCACTCTACCAGGCGG	28S	69.5°
	Pdel3B_25	*P.arenysensis*	TAATGTTAAAGTCTATAGACCACAA	28S	55.5°
	Pdel4D02_25	*P.autumnalis*	GATTGTGCAAATATCCAACCACTGT	28S	66.6°
	Pdel4D03_25	*P.autumnalis*	TGACAACGACTCACTCTACCAGGC	28S	69.4°
P.pseudodelicatissima	PgalaD02_25	*P.galaxiae*	CCAAAGGAATCAACCAAAGCAAACC	28S	71.8°
	PfrauD04_25	*P.fraudulenta*	ACGGGAGTTTCACCCTCTCAGCTGTC	28S	66,3°
	Pman2D03_25	*P.calliantha*	CTTCAGACCACAATTCGGCGCTTAAA	28S	65,6°
	PpdelD02_25	*P.pseudodelicatissima*	CCCGGCAGATAACGTCAAGGTCTAT	28S	70.4°
Controls	PNFRAGA	*Pseudo-nitzschia* spp	ATT CCA CCC AAA CAT GGC	18S	63,3°
	PmulaD03_25	*P.multistriata*	AACCCAAACTCACGAAAGCTCACAG	28S	69.8°
	UniC	Eucarioti	GWATTACCGCGGKGCTG	18S	64.6°
	UniR	Procarioti	CAGCMGCCGCGGUAAUWC	16S	60.6°

Length of probes was 18-26 bp and they were labeled with fluorescein isothiocyanate (FITC) at the 5' terminus. The sequences chance slightly in the amount of CG bases that influence the melting temperature and then their subsequent hybridization temperature. The specificity of each probe was also verified by dot blot hybridization, assays with DNA extracted as reported in Barra et al., 2012.

Pure cultures of *Pseudo-nitzschia* species were hybridized with all oligonucleotides listed in Table 7 and temperature hybridization, wash conditions were accurately calibrated in order to obtain a good cells label intensity. An optimal balance between probe sensitivity and specificity was determined by adding concentrations of formamide (FA) 40% to the hybridization buffer, keeping temperature (45°C) constant (tab.8) (fig.22).

Cells labeled with an intensity similar to that of positive controls were scored "+ +" (excellent; fig.21 A,B). Cells labeled with an intensity less than the positive controls, but greater than the negative controls, were scored as "+-" (fair to poor; fig.21 E,F). Those cells not labeled, similar to the negative control, were scored as "- - " (no reaction). The combination of a hybridization temperature of 45 °C , wash temperature of 45-58 °C and wash buffer concentration of 0,5-2X showed higher probe reactivity without nonspecific signal and was thus used as the optimized protocol for the subsequent work.

Tab. 8. Calibration of the WC-FISH of the probes on their own target.

Probe	Target species	T Hybridization	% FA	T Washing	Conc. Buffer	Epifluorescent Signal
Pdel1D01_25	*P.dolorosa*	45	40	55	0,5X	++
Pdel2D01_25	*P.delicatissima*	45	40	55	0,5X	++
Pdel3A_25	*P.arenysensis*	45	40	55	0,5X	++
Pdel3B_25	*P.arenysensis*	45	40	55	0,5X	++
Pdel4D02_25	*P.autumnalis*	45	40	55	0,5X	++
Pdel4D03_25	*P.autumnalis*	45	40	55	0,5X	++
PgalaD02_25	*P.galaxiae*	45	40	55	2X	++
PmulaD03_25	*P.multistriata*	45	40	55	0,5X	++
Pman2D03_25	*P.calliantha*	45	40	55	0,5X	++
PpdelD02_25	*P.pseudodelicatissima*	45	40	50	0,5X	++
PfrauD04_25	*P.fraudolenta*	45	40	55	2X	++

Of the 10 putative species-specific probes evaluated, all labeled their intended targets specifically with an intensity similar to that of the positive control.

The figure 23 shows that the probe is localized overall towards the ends of the cell and in the peripheral cytoplasm because in the centre of the cells are placed two large chloroplasts.

The intensity of the fluorescent signal is also depends on the growth stage of the culture. Figure 24 shows *P. delicatissima* cells at different growth phases. In particular the label of the cells at stationary growth phase (plateau) is very weak and not spreaded on the all cytoplasm because of their low rRNA content that produces a weak signal.

5.3 Cross-reactivity test of probes

Once confirmed the specificity of all probes on their own target species, the probes were tested against other monoculture of *Pseudo-nitzschia* species, to test the cross-reactivity of the screened probes. In fact, the small size of the probes (24-26 nucleotides) and their high sequence similarity, facilitate the events of cross-hybridization between the probes and species or clades genetically similar, thus making possible the emission of nonspecific signal. The results of the cross-reactivity test are reported in Tab.9.

Tab. 9. Cross-hybridization trials. Specificity and sensitivity of probes against different *Pseudonitzschia* species.

Probe name / *Pseudo-nitzschia* monoculture	Pdel3 AD01	Pdel2 D01	Pdel1D 01	Pdel4D 03	PpdelD 02	PgalaD 02	PmulaD 03	Pman2 D03	PfrauD04
P. arenysensis	++	--	--	--	--	--	--	--	++
P.delicatissima	--	++	--	--	--	--	--	--	--
P.dolorosa	--	--	++	--	--	--	--	--	++
P.autumnalis	--	--	--	++	--	--	--	--	--
P.pseudodeli catissima	--	--	--	--	++	++	--	++	--
P. galaxia	--	--	--	--	++	--	--	++	--
P. multistriata	--	--	--	--	--	--	++	--	--
P. calliantha	--	--	--	--	++	++	--	++	--
P.fraudolenta	--	--	--	--	--	--	--	--	--

The inconveniences caused by cross-hybridization have been resolved, after several tests, by re-setting new experimental conditions, overall in the post-hybridization washing (tab.10).

Tab. 10. Cross- hybridization trials. Re-setting of the parameters to avoid the cross-hybridization.

Probes	Target species	Temp.Hybridization (°C)	% FA	Temp.washing (°C)	Conc. buffer Washing
Pdel1D01	*P. dolorosa*	45	40	58	0,5X
Pdel2D01	*P. delicatissima*	45	40	45	5X
Pdel3AD01	*P. arenysensys*	45	40	58	0,5X
Pdel4D02	*P. autumnalis*	45	40	55	2X
PgalaD02	*P. galaxiae*	45	40	58	5X
Pman2D03	*P. callantha*	45	40	55	0,5X
PpdelD02	*P.pseudodelicatissima*	45	40	55	2X
PfrauD04	*P.fraudolenta*	45	40	55	2X
PmulaD03	*P. multistriata*	45	40	58	2X

In particular, we eliminated the problems of cross-hybridization for all the probes of the *P. delicatissima* complex (Pdel1D01, Pdel2D01, Pdel4D02 and Pdel4D03) which continues to retain a strong signal (+ +) (tab. 11). On the contrary, it was very difficult to eliminate the cross-hybridization of *P. pseudodelicatissima* complex probes (PpdelD02, PgalaD02 and Pman) which, although the new hybridization conditions (tab.10) they continue to cross-hybridize (tab.11). In many cases, by using too stringent post-hybridization washing, it causes the loss of the signal-specific clone (--) (tab.11).

Tab.11 Cross-hybridization trials. Specificity and sensitivity of probes against all *Pseudonitzschia* species after re-setting of the hybridization conditions.

Probes / Cultures	Pdel3AD01	Pdel2D01	Pdel1D01	Pdel4D03	PpdelD02	PgalaD02	PmulaD03	Pman2D03	PfrauD04
P. arenysensis	++	--	--	--	--	--	--	--	--
P. delicatissima	--	++	--	--	--	--	--	--	--
P.dolorosa	--	--	++	--	--	--	--	--	--
P. autumnalis	--	--	--	++	--	--	--	--	--
P. multistriata	--	--	--	--	--	--	++	--	--

5.4 WC-FISH test on simulated natural samples

In order to further verify the accuracy and specificity of the screened species-specific *P.delicatissima* complex probes, our WC-FISH protocol was performed on the simulated field samples. To prepare the test material, field seawater was sampled along Calabrian coast (Northern Tyrrhenian Sea). The seawater was directly mixed evenly with the exponential cultures of *P. arenisensys*, *P. delicatissima*, *P. autumnalis* e *P. multistriata.* The target cells counting in the mixture was performed with methodology Utermohl under the light microscope (LM). The results show that the probes hybridized exclusively with respective target cells well, and the labeled cells could be easily distinguished from other algae in the seawater sample (fig.25). The algal densities of the simulated samples were determined by LM analyzing the discernable morphological characters; after WC-FISH, enumerating the fluorescent cells in epifluoresecent microscope. The two different counting were compared by express as % of agreement between the two methods (tab.12) The two methods produced comparable results for all the algal samples. In particular, comparing the LM counting vs epifluorescence counting (WC-FISH), the two enumeration methods yielded counts for each sample that were not significantly different (range of 77% - 100%) (tab.12), except for *P. delicatissima* (22%).This result may relate to the fact that *P. delicatissima* specie was in a state of culture senescence, emphasizing how the WC-FISH could be sensitive to metabolic state of the cell. The data relating to PmulaD03 probe, that show an agreement between the two countings of +148% (tab.12), was probably due to the natural presence of cells of *P. multistriata* in seawater of Tyrrhenian Sea used to produce the simulated natural samples (fig. 26)

Tab.12. Cell density of simulated natural sample determined by light microscopy (LM) and by epifluorescence microscopy after WC-FISH.

Probe	Target species	Epifluorescence cell/ml	LM(Utermöhl) cell/ml	Agreement (%)
Pdel3D01	*P. arenysensis*	9496	9620	99%
Pdel2D01	*P. delicatissima*	945	4295	22%
Pdel4D03	*P. autumnalis*	6000	7808	77%
PmulaD03	*P. multistriata*	7667	5192	+148%

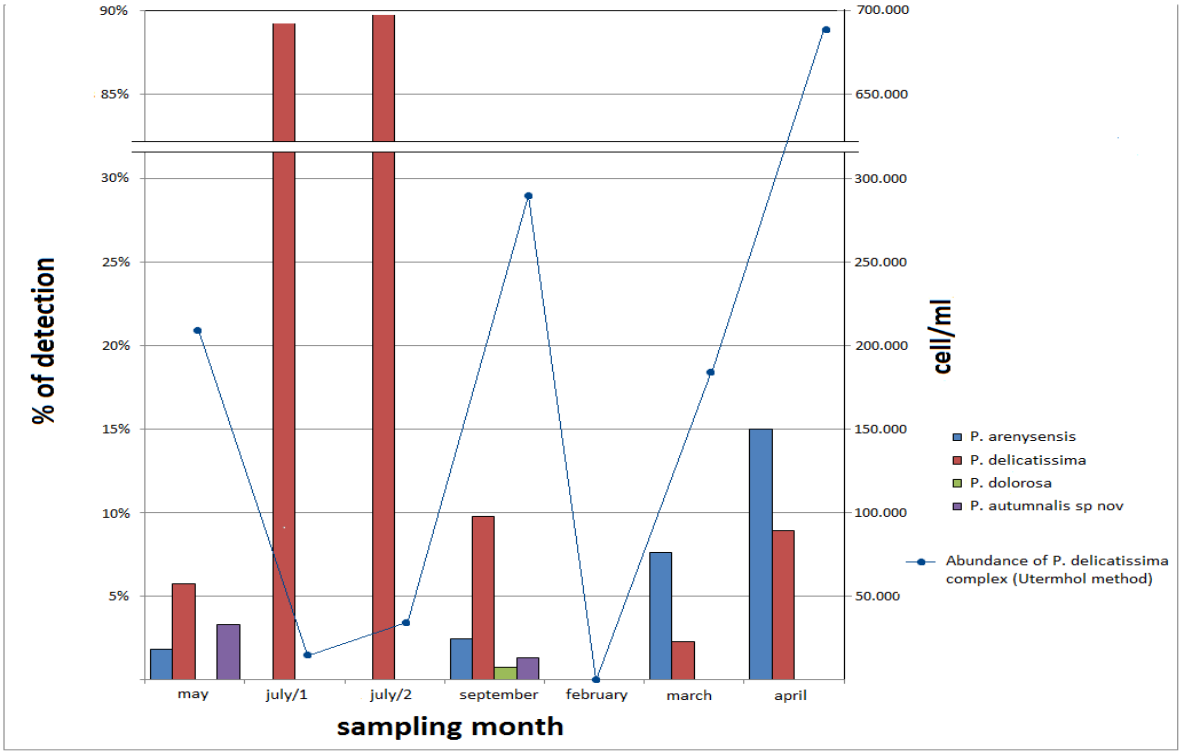
5.5 WC-FISH test on natural samples

Our second step was to try to detect *Pseudo-nitzschia* species from natural communities and to determine their density by WC-FISH using our specie-specific *P. delicatissima* complex probes. The phytoplankton samples employed in this section of work, were collected in the Gulf of Naples in different periods of the year and in corrispondence of *Psudonitzschia* blooms. An aliquot of sample was fixed and *Pseudo-nitzschia* spp. cells were counted using the Utermohl method in light microscopy (LM). By LM it was possible only identify the whole *P. delicatissima* complex (tab. 13).

After the WC-FISH, it was possible to identify the cryptic specie (fig.27) and the counting was performed in epifluorescence microscopy (tab.13). These results were summarized in graph 1.

Tab.13 Cell density of naturals samples determined by light (LM) and and epifluorescence microscopy. Ratio between epifluorescence counting and light microscopy counting was called "detection efficiency" (%).

Date	LM counting	Epifluorescent counting					Detection efficency
	P.delicatissima complex	*P.arenysensis*	*P.delicatissima*	*P.dolorosa*	*P.autumnalis*	Total	
25 May	210490 cell/l	3900 cell/l	12100 cell/l	0	6900 cell/l	22900 cell/l	10,87%
19 July	16468 cell/l	0	14400 cell/l	0	0	14400 cell/l	87,44%
26 July	33235 cell/l	0	29600 cell/l	0	0	29600 cell/l	89,06%
27 September	288038 cell/l	7000 cell/l	28200 cell/l	2200 cell/l	3800 cell/l	41200 cell/l	14,30%
7 February	0	0	0	0	0	0	0
7 March	177855 cell/l	13517 cell/l	4091 cell/l	0	0	17608 cell/l	9,90%
7 April	686861 cell/l	103029 cell/l	61130 cell/l	0	0	164159 cell/l	23,9%



Graph.1 Cell density estimated by light microscopy and by epifluorescence after WC-FISH. The ratio between epifluorescence counting and light microscopy counting was called " % of detection " or detection efficiency.

In the sample of May 2011, the LM counting revealed a density of 210490 cells / l for the group *P. delicatissima* complex; after WC-FISH, three cryptic species belonging to *P. delicatissima* complex were detected: *P.arenysensis, P.delicatissima, P. autunnalis* (tab.13, graph 1). The total abundance of this three species correspond to 10,87% of the *P. delicatissima* complex estimated in LM.

In the summer, in the sample of 19th July 2011, we observed a strong decrease in the density of the *P. delicatissima* complex and an appreciable increase in the density of the *P. pseudodelicatissima* complex; unfortunately for these group we have not yet provided effective probes. For the *P. delicatissima* complex, after WC-FISH, we detected only onecryptic species (*P. delicatissima*) that represents 87,44% of the *P. delicatissima* complex estimated in LM (tab.13, graph 1).

In the second sample of July (26th July 2011) the abundance of the *P. delicatissima* complex was two fold the previous sample (33235 cell/l) and also the WC-FISH detected a density of *P. delicatissima* about twice respect to the previous sample (29600 cell/l) which correspond to (89%) of the *P. delicatissima* complex estimated in LM (tab.13, graph 1).

In the sample of September, the *P. delicatissima* complex abundance greatly increased (288038 cell/l) (tab.13, graph 1); the WC-FISH was able to detect the presence of all 4 cryptic species (*P.arenysensis, P.dolorosa, Pdelicatissima* and *P.autumnalis*) which represent 14% of the *P. delicatissima* complex estimated in LM (fig.27).

In February, the total absence of *P. delicatissima* complex (tab.13, graph 1) corresponds to the total absence of signal after processing this sample for the WC-FISH, to confirming the specificity of the method.

In March, the WC-FISH detects only two species (*P.delicatissima and P.arenysensis*) which represent, in total, about 10% of the estimated population of the *P. delicatissima* complex in this sample (tab.13, graph 1).

Finally, in April when the density of the *P. delicatissima* complex increased greatly reaching a value four fold respect March sample, also there was a direct increase in the abundance of the two species detected by WC-FISH (*P.arenysensis and P.delicatissima*), representing 24% of the *P. delicatissima* complex estimated in LM (tab.13, graph 1).

The results of the fied-test show that the efficiency of the WC-FISH depends on the homogeneity of the sample. The efficiency of the detection decreases when the number of the cryptic in the sample was higher: e.g. in summer samples, when *P. delicatissima* complex is likely to be homogeneous in species composition, the WC-FISH provides a good quantitative estimate (tab 13, graph1) otherwise, in autumn and early spring, when the group of our interest increased in biodiversity, the WC-FISH show much lower percentages of detection (tab.13, graph 1). To explain these data we hypotize that events of competition among similar target sites occurred, due to the high percentage of similarity in the nucleotide sequences of the different probes. These weak cross-hybridization were then removed during post-hybridization washing, causing sequestering of the probe to their specific target sites and loss of signal.

To verify this hypothesis we performed some test in which we have: i) evaluated the correlation between the detection efficiency of WC-FISH and number of cryptic species present in simulated natural samples; ii) evaluated the correlation between the detection efficiency of WC-FISH and concentration of the specific probe.

Tables 14-16 show the correlation between detection efficiency and number of cryptic species in the sample. In fact, the detection efficiency is higher in the sample with only two species (tab.14) respect to the sample with three (tab.15) and four species, (tab.16); in this last sample the value reach its minimum (3.95%)

Tab. 14. Correlation between number of the species and detection efficiency. Artificial sample composed of two species .

Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	15841000	9367333	59,13%
P. multistriata	PmulaD03_25	11402000	6747667	59,18%

Tab. 15. Correlation between number of the species and detection efficiency. Artificial sample composed of three species

Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	15841000	5321000	33,59%
P. multistriata	PmulaD03_25	11402000	2409667	21,13%
P. delicatissima	Pdel2D01_25	14042000	6821000	48,58%

Tab.16 Correlation between number of the species and detection efficiency. Artificial sample composed of four species .

Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	15841000	1205333	7,61%
P. multistriata	PmulaD03_25	11402000	514000	4,51%
P. delicatissima	Pdel2D01_25	14042000	4193333	29,86%
P. dolorosa	Pdel1D01_25	9082000	358667	3,95%

Progressive increase in the concentration of the specific probe, increase also, the detection efficiency, showing direct correlation between the two parameters (tab.17-20) (graph 2) .

Tab. 17. Artificial samples composed of four species hybridized with different concentrations of probes. Test with 6 µl of probe.

Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	141800000	4112000	2,90%
P. delicatissima	Pdel2D01_25	134600000	24202667	17,98%
P. dolorosa	Pdel1D01_25	52000000	1332000	2,56%
P. multistriata	PmulaD03_25	68400000	2836000	4,15%

Tab 18. Artificial samples composed of four species hybridized with different concentrations of probes. Test with 12 µl of probe.

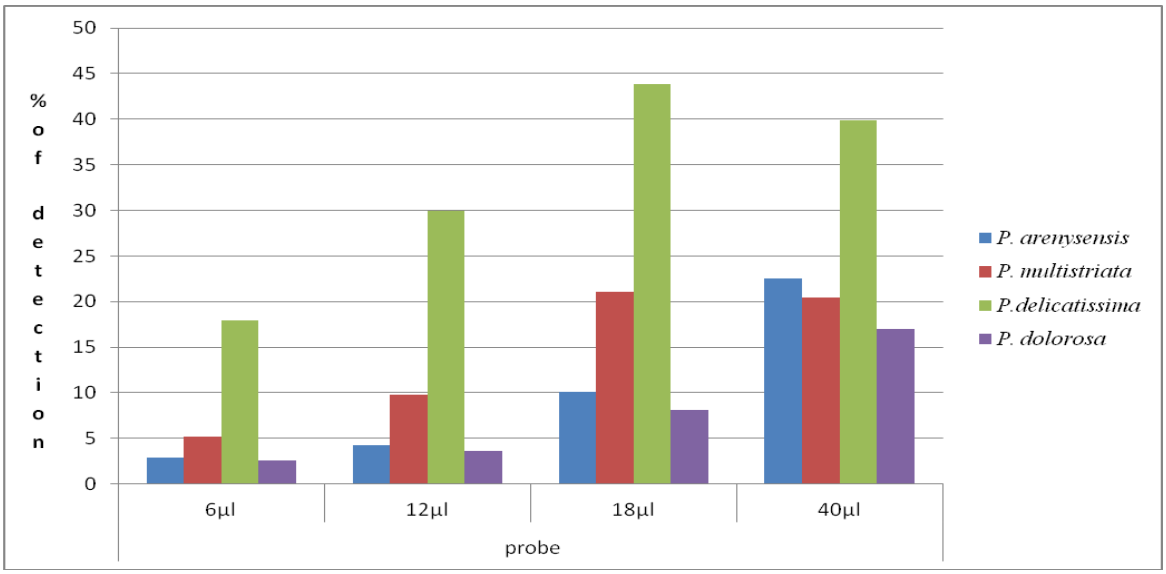
Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	141800000	6060333,333	4,27%
P. delicatissima	Pdel2D01_25	134600000	40352000	29,98%
P. dolorosa	Pdel1D01_25	52000000	1900000	3,65%
P. multistriata	PmulaD03_25	68400000	5093000	7,45%

Tab.19 Artificial samples composed of four species hybridized with different concentrations of probes. Test with 18 µl of probe.

Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	141800000	14379333	10,14%
P. delicatissima	Pdel2D01_25	134600000	59096000	43,90%
P. dolorosa	Pdel1D01_25	52000000	4232000	8,14%
P. multistriata	PmulaD03_25	68400000	11016333	16,11%

Tab. 20 Artificial samples composed of four species hybridized with different concentrations of probes. Test with 49 µl of probe.

Clade	Probe	LM counting cell/L	Epifluorescent counting cell/L	Detection efficency
P. arenysensis	Pdel3_25	141800000	31920000	23%
P. delicatissima	Pdel2D01_25	134600000	53688000	40%
P. dolorosa	Pdel1D01_25	52000000	8832000	19%
P. multistriata	PmulaD03_25	68400000	14016000	20%



Graph .2 Artificial samples composed of four species hybridized with different concentrations of probes.

5.6 Application of the probes on Calabrian field samples.

In order to verify the applicability of the isolated probes and the calibrated WC-FISH technique to detect *Pseudo-nitzschia* species in Calabrian sea water, we collected environmental samples in coastal water in the Northern Ionian Sea. In the sample of June 2012, the analysis in LM revealed the presence of species belonging to *Pseudo-nitzschia* spp. at a density of 10900 × 10^3 cells / l; unfortunately, due the conspicuous organic material in suspension and high density of planktonic cells, the WC-FISH did not work well and the test showed a high fluorescent background and lot of a-specific signal (fig.28). In the sample of October 2012,we estimated, in LM, a density of *Pseudo-nitzschia* species of 222 × 10^3 cells / l. The application of our probes on this sample detected the only two cryptic species *P. dolorosa* and *P. delicatissima* (tab.21) (fig.28).

Tab. 21 Natural Calabrian seawater sample. Detection of cryptic species by WC-FISH.

Date	LM counting	Epifluorescence detection			
	Pseudonitzschia spp	*P.arenysensis*	*P.delicatissima*	*P.dolorosa*	*P.autumnalis*
June 2012	10900×10^3 cell/l	Not detected	Not detected	Not detected	Not detected
October 2012	222×10^3 cell/l	Detected	Not detected	Detected	Not detected

6 Discussion

The results of this study show that the probes directed to the LSU rRNAs are a useful tool for the molecular identification of potentially toxic species in the genus *Pseudo-nitzschia,* although there are some limits to use the WC-FISH for quantitative estimates. The broad diversity of the phytoplankton makes it difficult to develop an *in situ* protocol capable of analyzing all kinds of algal cell due also to the different types of cell walls, membranes that may require different condition for probe penetration. Moreover, pigments autofluorescence, especially from chlorophyll, may be a problem when it is very strong and, therefore, masks the probe signal. Taking these problems into account, we adapted an existing protocols (Scholin et al. 1996,1997; Medlin e Simon, 1998; Groben and Medlin, 2005; Scholin et al., 1999; Metfies et al., 2006) for *in situ* hybridization with specific, fluorescent-labeled probes to *Pseudo-nitzschia* species.

In our work we tested different collecting samples methods. Filter-based method reduces the work associated with sample processing compared to the centrifuge-based method (Miller and Scholin 1996, Scholin et al. 1996) by eliminating the multiple centrifugation steps and the repetitive transfers of samples to and from centrifuge tubes (Miller and Sholin 1998). In addition, the samples collected and fixed on a polycarbonate filter were easier to manipulate than the centrifuge-based method and also reduce sample loss; finally it is easier to enumerate the target species.

For the fixation and hybridization of algal cells using fluorescent labeled probes, various protocols are used (Scholin et al., 1996; Simon et al.,2000) that are mainly derived from those developed for bacteria and often use paraformaldehyde (PFA) as the fixative (Amann, 1995). Comparing different protocols, we found that the approach of Scholin et al. (1996,1997) gave the best results. This method eliminates PFA and

replaces it with a saline-ethanol fixative. Thus, we modified the hybridization condition to increase stringency by addition of formamide (FA) to the hybridization buffer. FA concentrations must be established empirically for every probe and normally range between 0% and 50%.

During the testing of different fixation/hybridization protocols, we found that one of the most important components was the type of detergent used. Sodium dodecyl sulfate (SDS), for example, which is often used in hybridization buffers (Simon et al., 2000), destroys the more fragile cells like unarmored Dinoflagellates, whereas IGEPAL-CA630 maintains cells stability and permit efficient probe penetration into the cell (Groben and Medlin, 2005).

In the *in situ* hybridization experiment with phytoplankton the autofluorescence can be problematic. The saline ethanol (Scholin et al., 1996, 1997) aids to extracts the chlorophyll from the cells and bleaches them, thus enabling good visualization of probe signal. Nevertheless, sometimes the autofluorescence of some species is too strong and persistent; in these cases an additional treatment with 50% dimethylformamide (DMF) can help bleaching the chlorophyll from the cells.

Using this modified protocol, it was possible to analyze a range of laboratory cultures of cryptic and pseudo-cryptic species of *Pseudo-nitzschia* and field sample with LSU rRNA targeted oligonucleotides. Results of this study demonstrate that LSU rRNA targeted oligonucleotides are promising tools that could speed the identification of *Pseudo-nitzschia* cryptic and pseudo-cryptic. By using a positive and negative control treatments we defined a range of labeling intensities possible for any given sample.

Given these responses, we were able to determine whether or not our probes can discriminated *Pseudo-nitzschia* species as predicted and also determined the relative intensity with which they did so.

Of the ten putative species-specific probes designed and tested, all yielded a species-specific response with an intensity similar to that of the positive controls. But, the probes specific to *P.pseudodelicatissima* complex showed cross-reactions with non-target species. These results demonstrate that empirical tests are required to determine whether or not a probe binds its intended target specifically.

The cross-reactivity between *P.pseudodelicatissima* complex probes with non-target species might be due to highly similar target regions on the rRNA of these species because of phylogenetic proximity (Lundholm et al. 2006). To explain the large number of a-specific signals is that the region used to design the probes is relatively conserved in order to discriminate among and within the groups of closely related species in this genus (Barra et al 2012). By using probes longer than the ones used in the present study, we might to allow stronger and more specific signals. However, in the LSU region of the genus *Pseudo-nitzschia*, species often differ in single base-pair changes, and a longer probe is in fact not necessarily an option because the effect of the single mismatch could be hidden in a longer, highly conserved stretch of nucleotides, decreasing instead of increasing the discrimination power of the probe (Barra et al.2012).

Another potential problem is that each probe requires its own optimal hybridization conditions. In particular, the hybridization temperatures of the probes tested here are little variable, a 45 °C, making the high stringency of the hybridization impossible to accommodate for all probes.

In order to further verify the accuracy and specificity of the screened probes for *P.delicatissima* complex and our method developed above, FISH tests were performed on the simulated field samples. The algal densities of

the simulated samples determined by LM were agreement with FISH analysis (tab.12). The results were rather similar except for probe *pdel2*, for which counts with the LM were 22% higher than the fluorescence microscope. This discrepancy was probably due to physiological state of the cultured *P. delicatissima* cells causing a weaker labeling intensity. The variability in labeling intensity is caused rRNA amount and the culture age and the metabolic state influences the abundance of ribosomes and the target nucleic acid. For RNA, it has been well established that rRNA varies systematically with growth rate; faster growing cells have more RNA per cell than do cells growing at slower rates (De Long et al. 1989). Slow growth accompanying phosphorus and nitrogen limitation resulted in up to a 400% decrease in labeling intensity with the rRNA probe compared to nutrient-replete levels (Anderson et al. 1999).

In that instance, variability in labeling intensity is meaningful as an indicator of physiological state, but for other applications, the variability in labeling may lead to ambiguous results.

The results on natural samples show that WC-FISH is not reliable for quantitative analyses. In the natural sample collected in the Gulf of Naples, Utermohl counting and epifluorescence signal, after the WC-FISH, did not corroborate well (10%-85% discrepancy). A quantitative comparison between the species estimates by the whole-cell hybridization and microscopic methods revealed that the whole-cell hybridization underestimated the relative abundance of *Pseudo-nitzschia* species relative to the microscopic results.

Our results also show that the detection efficiency of WC-FISH decreases when the number of species present in the sample is higher. The discrepancies between the probe and microscopic results were caused by cross-hybridization between probes and aspecific sites that were removed subsequently during post-hybridization washing.

Different factors can affect hybridization of cells in mixed natural population. The high affinity of the probes for closely related species, causes competition of probes with short (24-26 bp) and very similar sequences that could result in aspecific hybridization.

The cross-reactivity of the various probes is a setback for the development of probes in *Pseudo-nitzschia* species. In fact, the composition of this genus of groups of closely related species renders further development of probes to distinguish among all the different species very difficult. Other authors have struggled with the same problem (Lundholm et al. 2006; Scholin et al. 1999), Scholin et al. (1999); in particular, Barra et al. (2012), reported cross hybridization reactions in their dot-blot and microarray. Unfortunately, the ca. 700-bp region of the LSU does not contain enough variation to provide enough alternative solutions for probe design.

Another parameter that can influence the specificity and strength of signals is the probe length; finding a compromise between probe length and specificity is still a challenging task (Metfies and Medlin 2008). Increasing probe length is counterproductive in terms of specificity if the increasing of the length does not add mismatches, which is generally the case with the LSU data of *Pseudo-nitzschia* (Barra et al.2012). We presume that the relative strength with which a probe labels a cell is due in part to the accessibility of the target sequence in the context of the three-dimensional structure of the ribosome; other investigators have drawn similar conclusions (Vaheri et al. 1991).

Other false negative discrepancies could have been due to poor probe reactions resulting in low or undetectable fluorescence. Such reactions might occur when cells are unhealthy, resulting in fewer ribosomes and, therefore, reduced fluorescence. False negative results could be due to the presence of dead *Pseudo-nitzschia* cells or empty frustules that were either

free in the sample or hidden within fecal pellets or sediment matrices. Dead cells would not be detected by the probes but would be indistinguishable from the living flora in the cleaned samples used for microscopic analysis. The fluorescent oligonucleotide probes tested in this study show great promise as tools that can facilitate routine monitoring of a *Pseudo-nitzschia* population. Overall, there was qualitative agreement between the whole-cell hybridization and microscopic methods. One of the aims of this study was to test the probes on field samples; our results show some discrepancies between the WC-FISH and the traditional microscopic results; however more effort was spent in focusing on this.

Complessively, by comparing to other rDNA-based methods of identifying *Pseudo-nitzschia* species, whole-cell hybridization is faster and more cost-effective. In particular, in this study we have demonstrated that it is possible to discriminate in near real-time a variety of *Pseudo-nitzschia* species collected from natural populations as shown by the data obtained in field test sampled in Northern Ionian Calabrian Sea. The presence of *Pseudo-nitzschia* spp. were confirmed by traditional LM technique; the application of all species specific probe, detected only the two *P.delicatissima* and *P. arenysensis* cryptic species. The low and/or non-reactivity of the other probes, might depend to small variations within the targeted rRNA sequences, implying that genetic differences might exist between the Gulf of Naples *Pseudo-nitzschia* strains used to develop the probes, and Ionian Sea strains.

So we can confirm that the application of FISH is straightforward and the procedure can be accomplished by taxonomically non-trained operators. The methods is also relatively fast and the results are potentially available after a some hours after field sampling. Further advances in the methodology could combine FISH with automated techniques. Therewith,

FISH coupled with automated analysing methods will be a powerful tool in HAB monitorin purposes.

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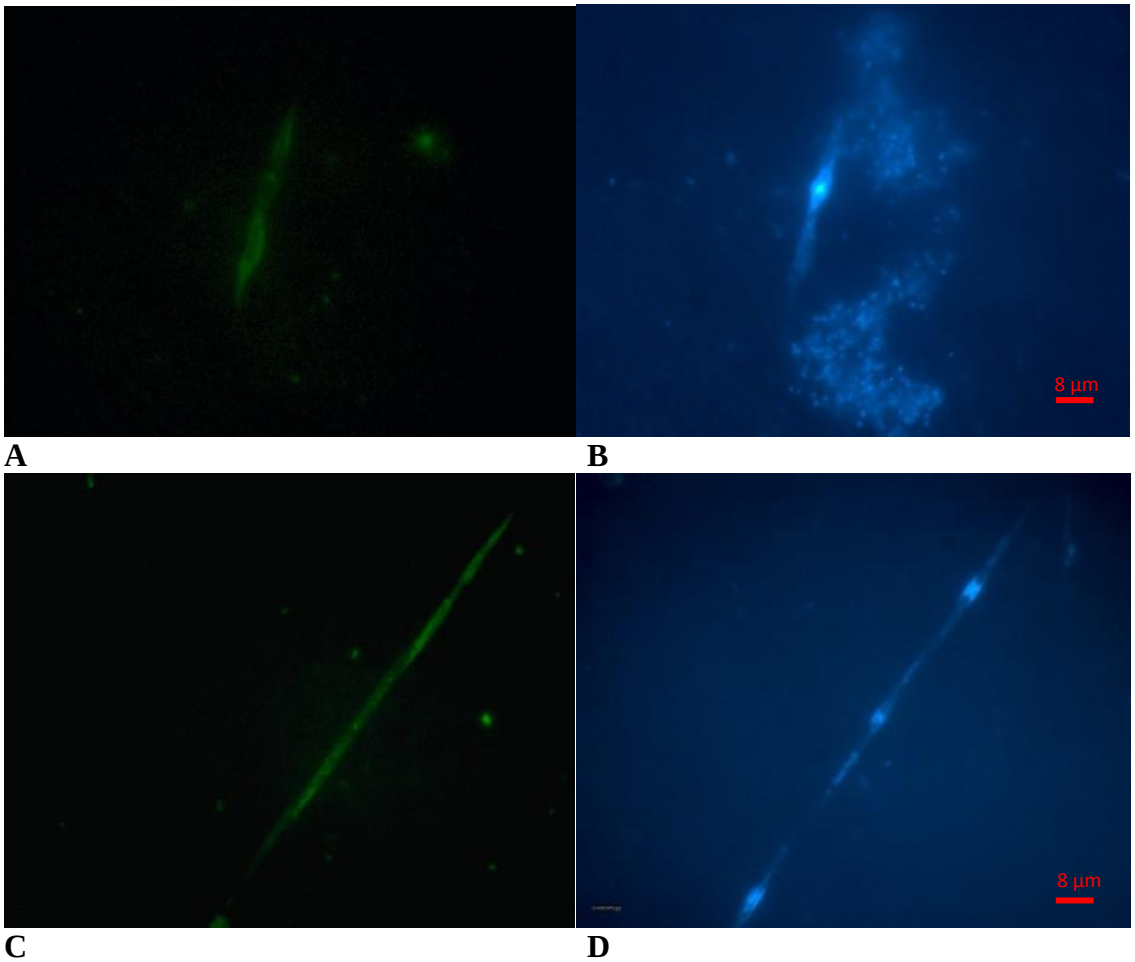
Photographic plates

For many of the plates, the cells of the same optical field, are simultaneously visualized with fluorescein isothiocyanate (FITC) (green), which detects the probe, and with 4',6-diamidino-2 DAPI (blue) for counterstaining the nuclear DNA.



A **B**

C **D**

Fig.20- Collection of algal cells. *Pseudo-nitzschia* cell collected by centrifugation - based method (A, B) and filter-based method (C, D). After WC-FISH with labeled FITC probe (A,C); after staining with DAPI (B,D).

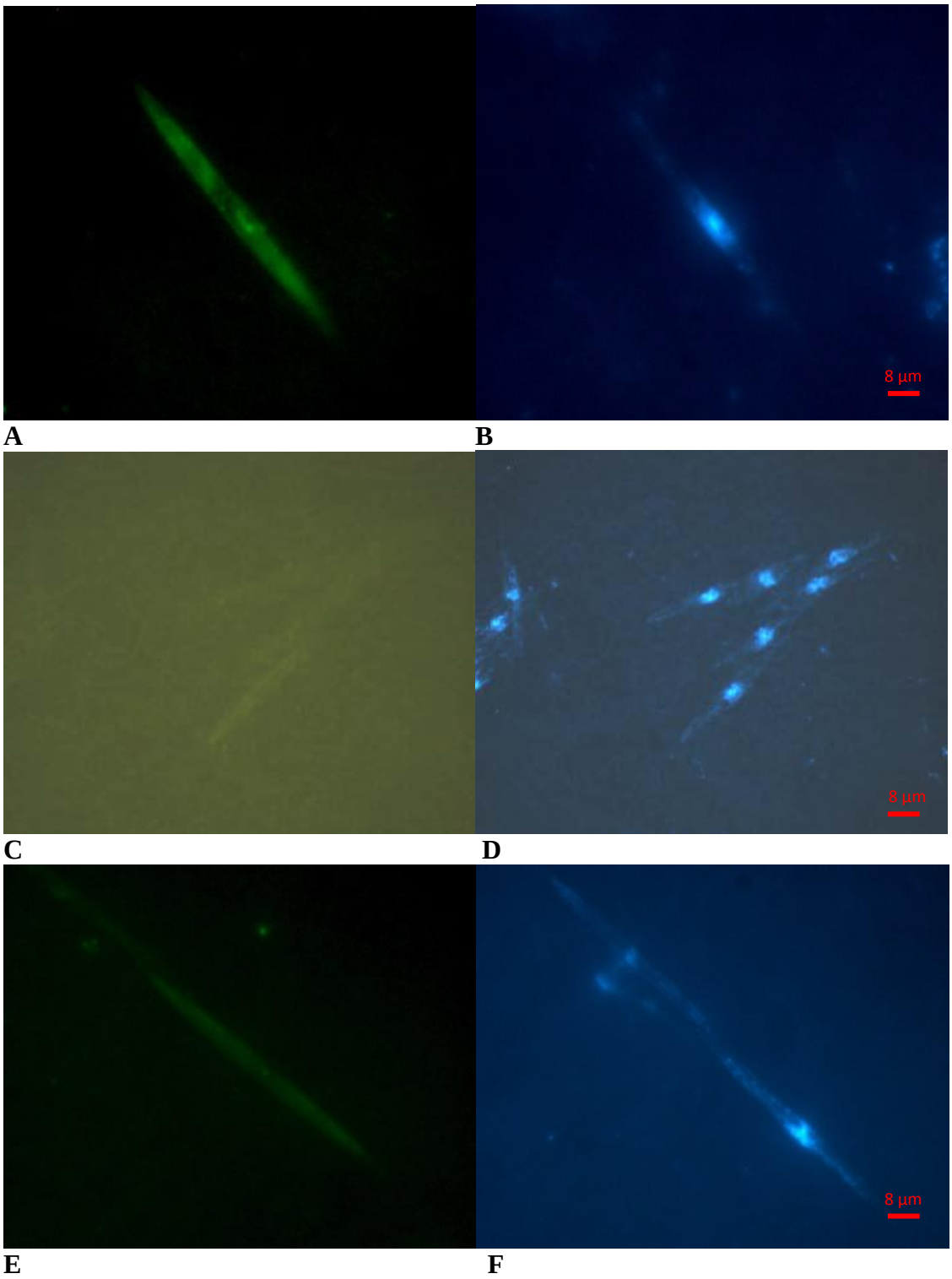
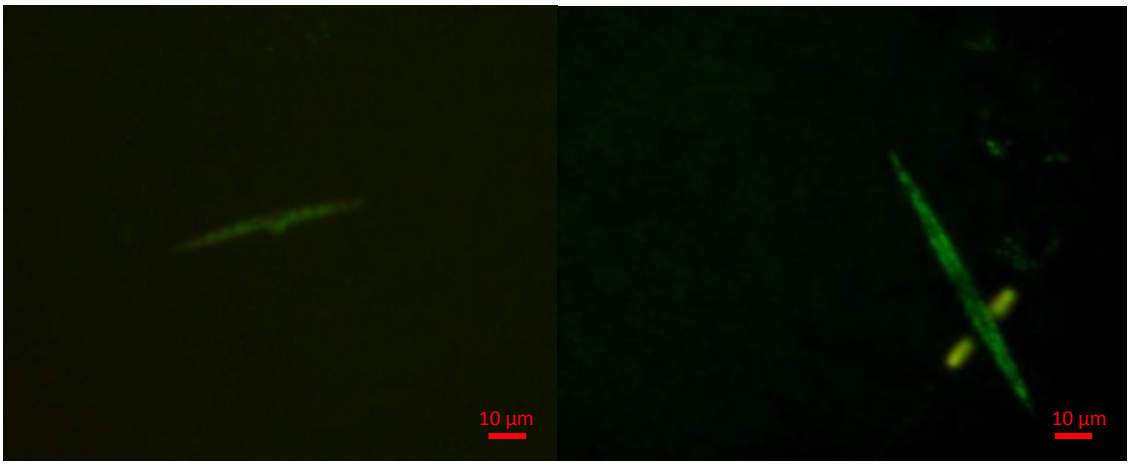
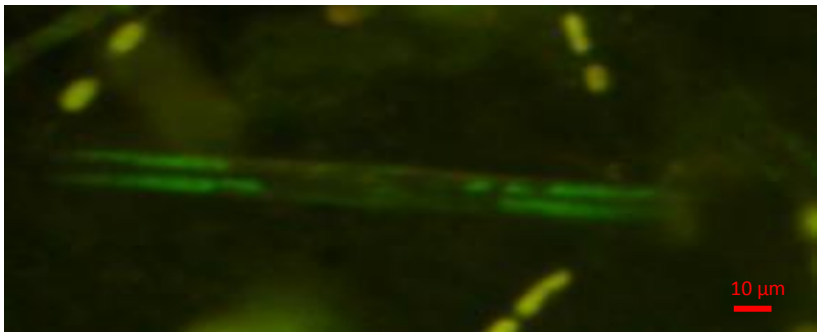


Fig.21-Calibration of hybridization conditions and labeling intensity. The positive control probe UniC with excellent signal"+ +" (A) and after DAPI staining (B); the negative control probe UniR with no signal "--" (C) and after DAPI staining (D); intermediate signal for UniC "+ -" (E) and after DAPI staining (F).

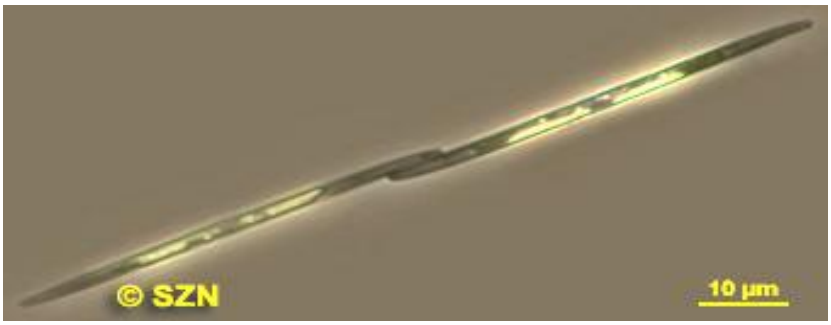


A B

Fig.22 Calibration of the WC-FISH of the probes on their own target: determining concentrations of formamide (FA). *Pseudo-nitzschia* cell hybridized with 0% of FA (A) and 40% of FA (B).



A



B

Fig.23. Localization of the probe on *Pseudo-nitzschia* cell after WC-FISH. The ends of the cell and the peripheral cytoplasm are intensely labelleld (A) whereas the centre of the cells are placed two large chloroplasts (A,B).

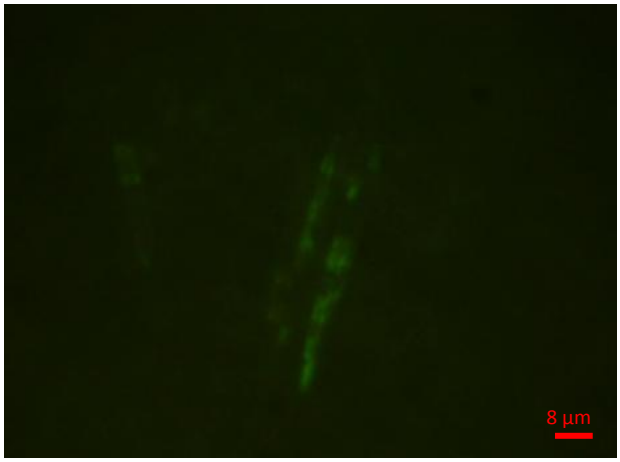


Fig.24. Growth stage and intensity of the labeling. *P.delicatissima* at stationary growth phase hybridized on its specific probe Pdel2. The signal is very weak and spreaded on whole the cell.

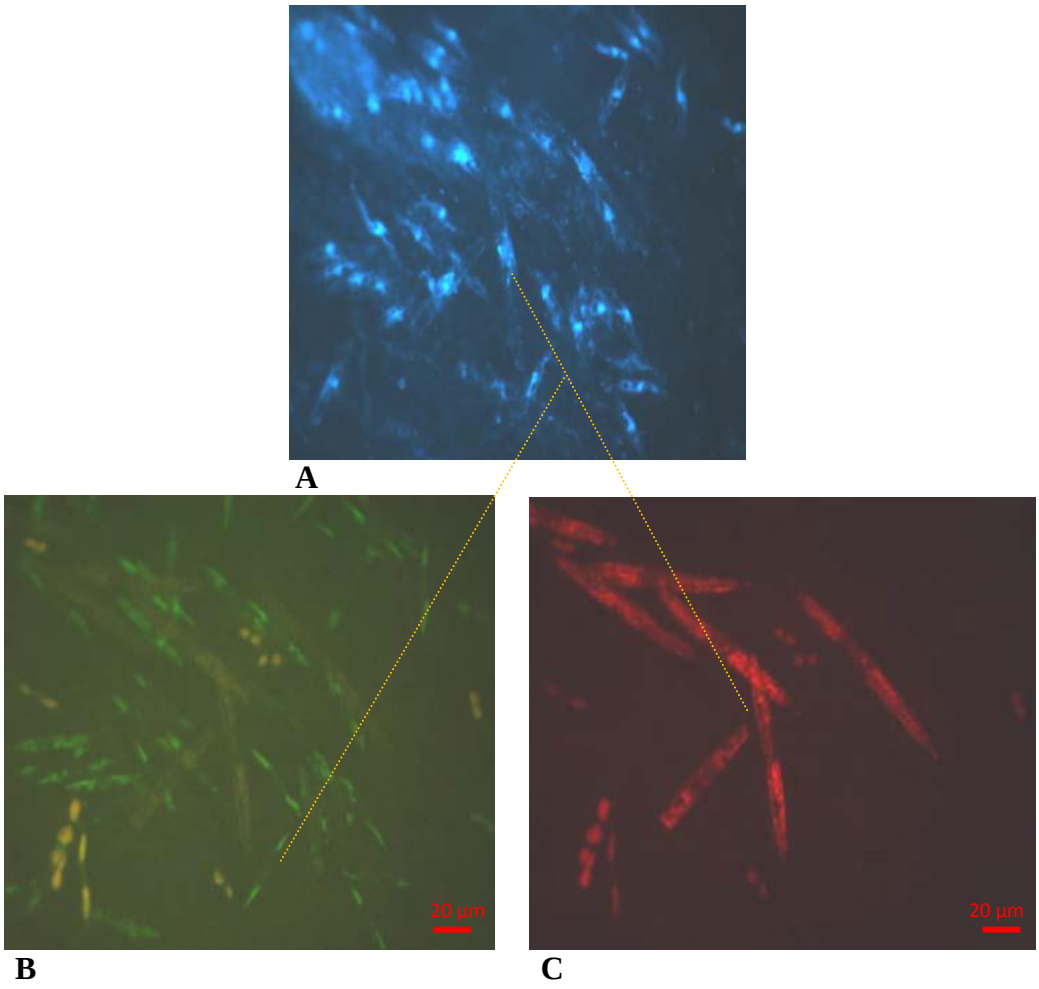


Fig.25 Simulated natural samples. Calabrian seawater sample (Northern Tyrrhenian Sea) after DAPI staining (A) and after WC-FISH FITC with specific probe Pdeli4 (*P. autumnalis)* (B) and PmulaD03(*P.multistriata*) (C).

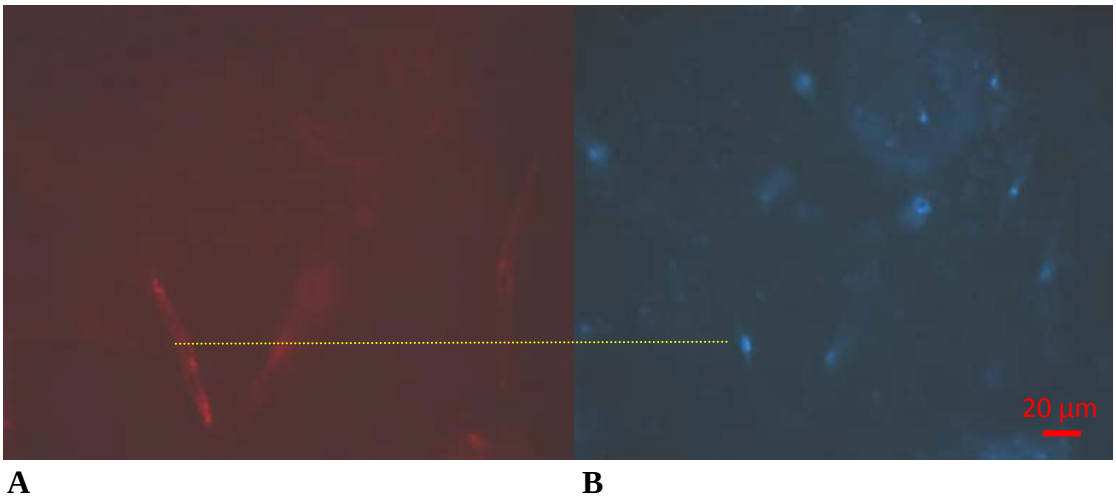
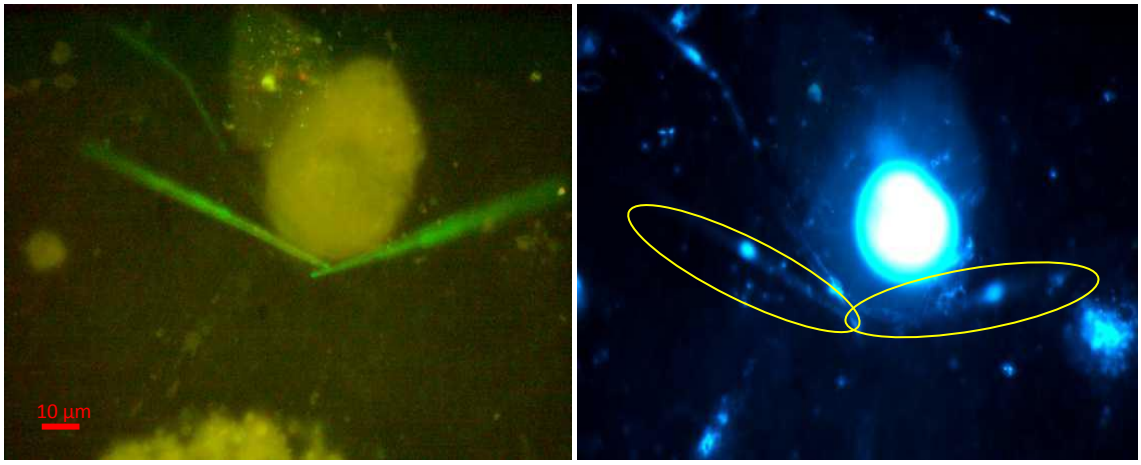
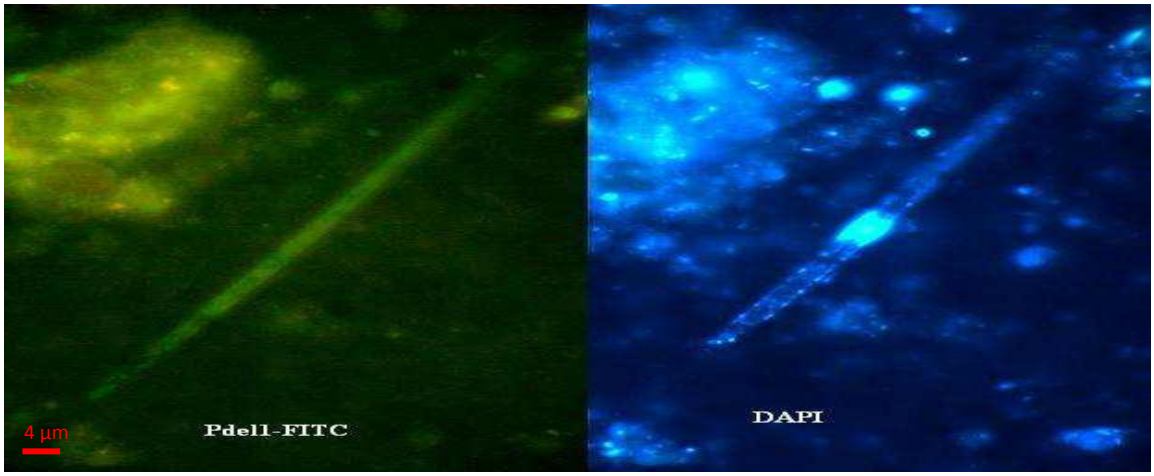


Fig. 26 Natural samples. Calabrian seawater sample (Northern Tyrrhenian Sea) after WC-FISH with specific Cy3 labeled probe PmulaD03 (*P.multistriata*) (A) and after DAPI staining (B).

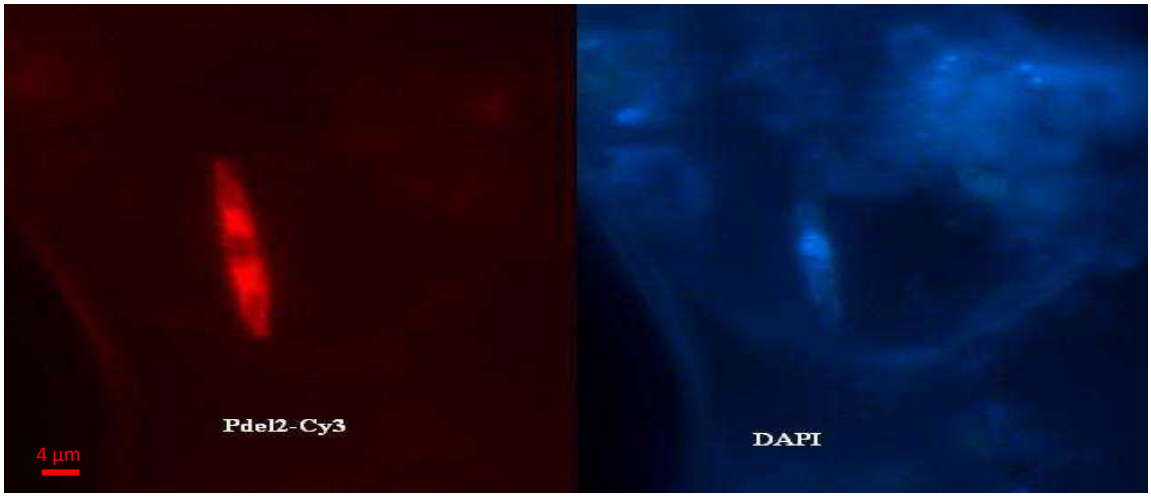


A **B**

Fig.27 Natural samples of the Gulf of Naples after WC-FISH with specific FITC Pdel1D01probe (*P.dolorosa*) (A) and after DAPI staining (B).



A **B**



C **D**

Fig.28 Natural seawater samples of the Northern Ionian Sea after WC-FISH with FITC labeled specific probe Pdel1D01 (*P.dolorosa*) (A) and after DAPI staining (B); after WC-FISH with Cy3 labeled specific probe Pdel2D01(*P.delicatissima*) (C) and after DAPI staining (D).

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Trace metals in *Posidonia oceanica* in a coastal area of the Ionian Sea (Calabria, Italy)

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ABSTRACT

As, Cd, Cu and Pb concentrations were measured in *Posidonia oceanica* sampled from meadows located in two sites along the calabrian coast (Ionian Sea, South-eastern Mediterranean). By dating the scales and the rhizome using retrospective procedures (lepidochronology), a time series over a period of nine years (1995-2004) was analysed. Throughout the whole lepidochronological period, the arsenic content in the scales was tenfold higher than that measured in the rhizome with a peak in the lepidochronological year 1996-1997 and a general decrease in the years after. Pb concentration also showed a tendency to decrease with time, whereas the Cd and Cu concentration were increasing. A comparison of the metal level in dead sheaths (scales) and living tissue (leaves) was also performed. The detailed distribution of the trace metals along the leaf axis and in the leaves at different developmental stages (adult, intermediate and juvenile) showed some differences in the metal content. The results also indicate that the arsenic content measured in the leaf blade reflects the high As content measured at the superficial sediments at one of the two sampled sites, demonstrating, for the first time, that *P. oceanica* would be a good indicator for this element.

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Phytoplankton Communities in a Coastal site of Natural Riserve "Lake of Tarsia-Mouth of river Crati"- Northern Ionian Sea, Calabria (Italy)

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Abstract

Our work was aimed to study the phytoplankton communities in two coastal sites placed in Natural Riserve "Lake of Tarsia-Mouth of river Crati" in Northern Ionian Sea-Calabria (Italy). This zone represents a wetland area of high natural interest populated by different organisms (animals and plants) living in a transitional ecosystem characterized by a great biodiversity. The sampling was performed in two different seasonal periods (summer and autumn 2012) and in two different sites (marine site vs fluvial one). Different algal genera/species were recognized and analyzed by Utermöhl inverted microscope method and also qualitative and quantitative measures of biomass were performed. The results showed that the dominant group was the Diatoms with exclusive species in such periods in both the sampling sites; we also observed the presence of genera and/or species of potentially toxic algae (*Pseudo-nitzschia sp., Alexandrium taylori, Prorocentrum micans, Skeletonema sp.*), mostly in the summer. Complessively, the phytoplankton biomass was always higher in marine site than in the fluvial site. The results were also processed considering the context of the chemical-physical parameters (such as temperature, pH, salinity, nutrients). The high concentration of the nutrients N and P in such periods, indicated a state of meso-eutrophic waters both in the proximity of river site as well as in the marine zone. The data represent the first contribute to the knowledge of the phytoplankton structure in this area which result a very variable environment with a high recovery capacity.