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Strengths and weaknesses of microarray approaches to detect *Pseudo-nitzschia* species in the field

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Abstract The planktonic diatom genus *Pseudo-nitzschia* contains several genetically closely related species. Some of these can produce domoic acid, a potent neurotoxin. Thus, monitoring programs are needed to screen for the presence of these toxic species. Unfortunately, many are impossible to distinguish using light microscopy. Therefore, we assessed the applicability of microarray technology for detection of toxic and non-toxic *Pseudo-nitzschia* species in the Gulf of Naples (Mediterranean Sea). Here, 11 species have been detected, of which at least 5 are potentially toxic. A total of 49 genus- and species-specific DNA probes were designed in silico against the nuclear LSU and SSU rRNA of 19 species, and spotted on the microarray. The microarray was tested against total RNA of monoclonal cultures of eight species. Only three of the probes designed to be species-specific were indeed so within the limits of our experimental design. To assess the effectiveness of the microarray in detecting *Pseudo-nitzschia* species in environmental samples, we hybridized total RNA extracted from 11 seasonal plankton samples against microarray slides and compared the observed pattern with plankton counts in light microscopy and with expected hybridization patterns obtained with monoclonal cultures of the observed species. Presence of species in field samples generally resulted in signal patterns on the microarray as observed with RNA extracted from cultures of these species, but many a-specific signals appeared as well. Possible reasons for the numerous cross reactions are discussed. Calibration curves for *Pseudo-nitzschia multistriata* showed linear relationship between signal strength and cell number.

Keywords Microarray · *Pseudo-nitzschia* · LSU probes · rRNA · Gulf of Naples · Cryptic species

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Introduction

Pseudo-nitzschia (Heterokonta, Bacillariophyceae) is a cosmopolitan genus of chain-forming, pennate, planktonic diatoms that are often common in coastal marine habitats. The genus is highly diverse, and is composed of several groups (clades) of genetically closely related species that are often indistinguishable in light microscopy (e.g. Amato et al. 2007; Lundholm et al. 2006). Unfortunately, many of these species are notorious for their ability to produce domoic acid, a potent neurotoxin and causing agent of Amnesic Shellfish Poisoning in humans, marine mammals and birds (Bates et al. 1989; Trainer et al. 2012).

To mitigate the risks of food poisoning for humans, monitoring programs need to screen plankton samples for the presence of potentially toxic *Pseudo-nitzschia* species. Traditionally, this is carried out using light microscopy (LM). However, in the case of *Pseudo-nitzschia*, LM is inadequate because potentially toxic species are often morphologically similar to species for which toxicity has never

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been observed (Douglas et al. 1994; Trainer et al. 2012). Failure to detect a potentially toxic species is intolerable because of the possible consequences for human health, but considering a priori all species as potentially toxic is not an option either because of the socioeconomic consequences of closing shellfish industry each time *Pseudo-nitzschia* species appear in the plankton. Screening for toxicity instead of morphology is also inadequate because toxin production depends strongly on the physiological state of the diatom. For instance, a bloom of a potentially toxic species may show different levels of toxicity depending on the available N source (Thessen et al. 2009) and may suddenly become toxic because of silica or phosphate limitation (Amato et al. 2009; Pan et al. 1996a, b, c).

All currently known *Pseudo-nitzschia* species can be identified with molecular markers. Such markers could be used for the development of reliable, rapid and cost-effective tools for the detection of a range of potentially toxic species in phytoplankton samples (e.g. Boenigk et al. 2006; Medlin and Kooistra 2010; Parsons et al. 1999). Several molecular methods permit investigation of species diversity in plankton samples without the need to isolate individual cells from field samples and to grow them in culture (Ebenezer et al. 2012; Kudela et al. 2010; Not et al. 2009). These methods include screening of DNA clones generated from PCR products of environmental DNA (McDonald et al. 2007; Penna et al. 2008), qPCR (Andree et al. 2011), dot blot hybridization (Ayers et al. 2005; Diercks et al. 2008; John 2004; Simon et al. 1997; Scholin et al. 1996, 1999), fluorescent in situ hybridization (Cho et al. 2001; Miller and Scholin 1998, 2000; Simon et al. 2000; Scholin et al. 1996) and microarray hybridization (Ahn et al. 2006; Galluzzi et al. 2011; Gescher et al. 2008; Ki and Han 2006; Metfies and Medlin 2005, 2008; Smith et al. 2012). In particular, microarray hybridization enables rapid, high-throughput and cost-effective monitoring of a large number of species, that is, if a number of quality and accuracy criteria is met.

In the present study, we assessed the applicability of the microarray technology for the accurate detection of *Pseudo-nitzschia* species in our study area, the Gulf of Naples (Mediterranean Sea). The microarray used in this study was designed in the framework of the project MIDTAL (Microarrays for the Detection of Toxic Algae, EU-FP7), aimed to build an universal microarray encompassing a large number of harmful microalgal species belonging to dinoflagellates, raphidophites, prymnesiophytes and diatoms (Kegel et al. 2012). Protocols and data analyses were thus standardized for different classes of organisms studied in the laboratories involved in the project.

At least 11 described *Pseudo-nitzschia* species have been detected locally: *Pseudo-nitzschia arenysensis*, *Pseudo-nitzschia caciantha*, *Pseudo-nitzschia calliantha*, *Pseudo-nitzschia cuspidata*, *Pseudo-nitzschia delicatissima*, *Pseudo-nitzschia dolorosa*, *Pseudo-nitzschia fraudulenta*, *Pseudo-nitzschia galaxiae*, *Pseudo-nitzschia mannii*, *Pseudo-nitzschia multistriata* and *Pseudo-nitzschia pseudo-delicatissima* (Amato et al. 2007; Amato and Montresor 2008; D'Alelio et al. 2009; Lundholm et al. 2006; McDonald et al. 2007; Orsini et al. 2004; Tesson et al. 2011) as well as a species new to science (*Pseudo-nitzschia delicatissima* new clade, in prep). Many of these species are closely related (e.g. Amato et al. 2007; Lundholm et al. 2006, 2012), rendering probe design no sinecure.

We applied the microarray technology as follows. Species-specific DNA probes were developed against target regions within the ca. 700 base pairs at the 5′-end of the nuclear-encoded large subunit (LSU) rRNA (Supplemental Table 1). Multiple species-specific probes were developed, whenever possible, to improve accuracy by having the possibility to eliminate probes that were found to be a-specific. A set of hierarchical probes was developed to allow identification of false positives (Groben et al. 2004; Lange et al. 1996; Metfies et al. 2008). The LSU region was chosen because it has been sequenced for all known *Pseudo-nitzschia* species, permitting in silico testing for species specificity. This region cannot discriminate *P. pseudodelicatissima* from its close relative *P. cuspidata* (Amato et al. 2007), but both species have been reported to be potentially toxic (Trainer et al. 2012). LSU was also shown to have discrimination power in a low-density microarray application in other harmful algal genera (Ki and Han 2006). Most studies, however, used probes designed against targets in the nuclear encoded small subunit (SSU) rRNA, but this marker was too conserved to discriminate among some of the closely related *Pseudo-nitzschia* species. The internal transcribed spacer region (ITS-1 and ITS-2) in between the nuclear rRNA encoding regions is far less conserved, but the region does not produce stable ribosomal RNA, and therefore, PCR products need to be generated to hybridize against the microarray probes (see e.g. Galluzzi et al. 2011 for selected species of dinoflagellates and Smith et al. 2012 for *Pseudo-nitzschia*).

The majority of the published works up to now used to hybridize PCR products on the microarray (Galluzzi et al. 2011; Gescher et al. 2008; Metfies and Medlin 2008; Smith et al. 2012). The MIDTAL approach was instead to hybridize total RNA for several reasons. To begin with, ribosomal RNA can be used as a proxy for bioactive plankton cytoplasm as it degrades easily in the environment. The use of PCR products has several drawbacks: (1) the so-called universal primers used to produce the PCR fragments are most likely not universal, and a large part of the community could go undetected (Hong et al. 2009); (2) PCR can amplify different targets at different rates thus impairing quantification efforts; (3) the PCR process on a multi-species template could produce chimeric sequences, thus generating confusing fake diversity. Thus, since one of the

Table 1 Seawater samples collected at the LTER_MC station in the Gulf of Naples and quantity and quality of the RNA extracted

Sample code	Sampling date	Sample volume (L)[a]	Extracted RNA (μg)[b]	Hybridized RNA (μg)	Degree of labelling (DoL %)
MT1	08 September 2009	5	3	1	1.2
MT2	06 October 2009	5	2.5	0.9	0.9
MT3	04 November 2009	5	3.5	0.8	3.1
MT4	02 December 2009	4	10	0.9	2.9
MT5	12 January 2010	5	1.5	0.7	2.4
MT7	09 March 2010	2.5	3.5	0.8	2.2
MT8	13 April 2010	2	4.4	1	2.5
MT9	18 May 2010	4	1.7	0.8	2.9
MT10	15 June 2010	1	1.3	0.7	1.6
MT11	13 July 2010	2	4.8	1	2.4
MT12	03 August 2010	1.5	2	0.9	2.1

[a]The standard amount of seawater filtered was 5 L. However, filtering was terminated earlier if a filter became clogged

[b]The extracted RNA contains about 0.1 μg of *D. tertiolecta* RNA

objectives of the project MIDTAL is to obtain at least semi-quantitative results, we used an rRNA-based approach.

All probes that passed the in silico test were spotted on the microarray slides. The microarray (Kegel et al. 2012) included 49 probes designed to distinguish about 19 species of *Pseudo-nitzschia*, 46 of them designed against the D1–D3 large ribosomal subunit (LSU), the three remaining ones were designed against the small subunit ribosomal RNA (SSU rRNA). Two genus-specific probes were designed against the SSU region as well (see Supplemental Table 1).

To test the specificity of the probes, total RNA was isolated from monoclonal cultures of a series of potentially toxic species (Trainer et al. 2012) and incubated on the microarray. A false negative response, i.e. no hybridization between the RNA and its specific probe, implies that that probe does not function properly, whereas a false positive indicates cross-reactivity with the intended region in the rRNA of other species than the target species, or with regions in the rRNA other than the target region. The latter is possible because the whole LSU rRNA is markedly larger than the regions tested, and several additional rRNAs are present in eukaryotic cells. These rRNAs could exhibit regions similar to the intended target region. Even if a few species show cross reactions, i.e. bind also to other probes than the expected ones, then the cross-reacting probes can still be of value as long as each species' rRNA generates specific reaction patterns on the microarray.

To assess the capability of the method to detect the presence of *Pseudo-nitzschia* species in environmental samples, total RNA was isolated from 11 plankton samples taken over a 1-year period to cover the annual *Pseudo-nitzschia* diversity in the Gulf of Naples. The environmental RNA was incubated on the microarray, and the observed pattern was compared with the expected pattern as deduced from plankton counts in light microscopy and the microarray results obtained with RNA from monoclonal cultures of species observed in the counts. To explore the potential of microarray technology to quantify cell concentration, we constructed calibration curves for *P. multistriata*. We selected *P. multistriata* because of its documented toxicity (Amato et al. 2009; Orsini et al. 2002) and its easy identification in light microscopy.

Materials and methods

Study site and sample collection

Seawater samples were collected monthly at a Long-Term Ecological Research station MareChiara in the Gulf of Naples (40°48.5′N, 14°15′E), from September 2009 to August 2010 (Table 1). The station is located two nautical miles from the coast, and the bottom is at ca 70 m. Within the scope of this project, the LTER was sampled on a monthly basis (Fig. 1) along the surface (0–1 m). Five litres

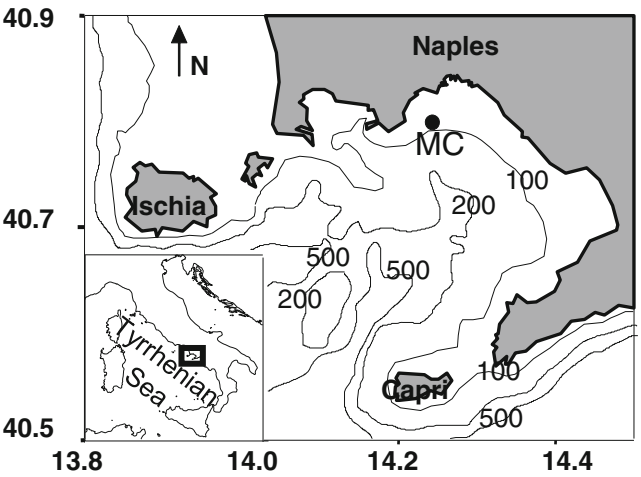

Fig. 1 The sampling site LTER-MareChiara in the Gulf of Naples (Mediterranean Sea)

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, where it was pre-filtered immediately on arrival through a net (160 μm mesh size) to eliminate large zooplankton and debris. Volumes of pre-filtered seawater (see Table 1) were then filtered with aid of mild pressure on cellulose ester filters (47 mm Ø, 1.2 μm pore size, EMD Millipore, USA). The filters were cut in two halves and transferred into cryogenic vials containing 0.8 mL of TRI Reagent (Roche Diagnostics, Mannheim, Germany). The samples were frozen in liquid nitrogen and stored at −80 °C until further processing. The procedure was carried out without delays to minimize RNA degradation. 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 Uthermöhl chamber for counting and enumeration (Utermöhl 1931).

Establishment of monoclonal cultures

Monoclonal cultures were established from single cells or chains of *Pseudo-nitzschia* isolated from net samples. Samples were collected by means of pulling a standard phytoplankton net (maze diameter, 20 μm) through the surface layer (0–2 m). An aliquot of the sample was fixed with neutralized formaldehyde and kept for cell counting in LM. Single cells or short chains were isolated with a glass micropipette, sequentially washed in 2–3 drops of sterile seawater, transferred in single wells of a culture plate containing 2 mL of diluted (1:20) f/2 culture medium (Guillard 1975) and placed in a growth chamber at 20 °C at an irradiance of about 80 μmol photons $m^{-2}s^{-2}$ and a 12:12-h light/dark photocycle. After about 10 days, cultures were transferred into culture flasks and transferred periodically. All strains of *Pseudo-nitzschia* used in this study were monoclonal and non-axenic; species were identified based on their morphology as well as on their LSU sequences (Table 2).

RNA extraction from field samples

Prior to RNA extraction, 5×10^5 cells of the chlorophyte *Dunaliella tertiolecta* were added to the cryogenic vials containing the filters in TRI Reagent to serve as an internal control on RNA extraction efficiency. This amount of *Dunaliella* cells gives around 100 ng of RNA as calibrated by McCoy et al. (2012). The *Dunaliella* cells were processed as described in the MIDTAL manual (Lewis et al. 2012). The cell–TRI reagent mixture was transferred into a new microcentrifuge tube to which acid-washed glass beads (ø 300 μm) were added. The content was vortexed for 15 s and left at room temperature (RT) for 10 min. Samples were then incubated at 60 °C for 10 min in a Thermomixer (Eppendorf, Hamburg, Germany) at maximum speed. After the addition of 160 μL of chloroform to the samples, the tubes were vortexed for 15 s. Samples were then transferred into pre-spinned Phase Lock Gel Heavy 2-mL tubes (5 Prime GmbH, Hamburg, Germany) and shaken thoroughly for 15 s. After incubation at RT for 5 min, samples were centrifuged at 12,000×*g* at 4 °C for 15 min. The aqueous phase was transferred to a fresh 2-mL RNAase-free tube and incubated at −20 °C for 1 h after the addition of 500 μL isopropanol. Samples were centrifuged at 12,000×*g* at 4 °C for 15 min. The supernatant was removed quickly, and the pellet was washed with 1 mL ethanol (75 %) and centrifuged again (12,000×*g*) at 4 °C for 10 min. The supernatant was removed carefully, and the pellet was air-dried for 15 min. The pellet was than dissolved in 50 μL of RNase-free water by flicking the tube. Concentration of RNA was measured by Nanodrop (Agilent Technologies, Santa Clara, CA, USA).

RNA extraction from *Pseudo-nitzschia* cultures

For RNA extraction, 50 ml of culture was centrifuged twice. The first centrifugation was carried out in Falcon tubes (BD Biosciences, San Jose, CA, USA) at 4,100 rpm for 30 min (DR15P centrifuge, B. Braun Biotech International, Melsungen, Germany). The pellets were transferred to 2-mL Eppendorf tubes and compacted further at 14,000 rpm for 15 min (5417R centrifuge, Eppendorf, Hamburg, Germany) to eliminate most of the culture medium. The RNA extraction, labelling, fragmentation and hybridization have been performed as for field samples as described above except that no *D. tertiolecta* cells were added.

RNA labelling and fragmentation

About 1 μg of RNA from field samples (Table 1) and monoclonal strains (Table 2) was labelled using the Platinum Bright Infrared Labelling Kit (Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's instructions. Concentration and incorporation of the dye was measured through Nanodrop (Agilent Technologies, Santa Clara, CA, USA). RNA was fragmented by adding 1/10 volume of RNA fragmentation buffer (100 mM $ZnCl_2$ in 100 mM Tris–HCl, pH 7.0) followed by an incubation at 70 °C for 15 min. The reaction was terminated with the addition of 1/10 volume of 0.5 M EDTA (pH 8.0), and the samples were placed on ice.

Preparation of internal control (TBP-Cy5)

In order to have an internal control for microarray hybridization, a PCR fragment of the TATA-box binding protein (TBP-Cy5) gene was produced from DNA of bread yeast

Table 2 List of the *Pseudo-nitzschia* strains used for the probe specificity tests—strain code, species name, collection site and date, strain provider, means of species identification. GoN denotes Gulf of Naples

Strain code	Species	Collection site	Collection date	Provided by	Identified through:
B569	*P. arenysensis*	GoN. Italy	10 February 2010	This study	LSU: as DQ813811
Vigo-1045	*P. australis*	Ria de Muros, Spain	21 May 2010	F. Rodríguez Hernández	SEM
B579	*P. calliantha*	GoN. Italy	09 March 2010	This study	LSU: as DQ813815
B583	*P. delicatissima*	GoN. Italy	09 March 2010	This study	LSU: as DQ813810
B570	*P. fraudulenta*	GoN. Italy	09 February 2010	This study	LSU: as EF522111
B510	*P. galaxiae*	GoN. Italy	04 August 2009	This study	LSU: as AY544792
NWSFC 316	*P. multiseries*	Puget Sound, USA	29 April 2009	J. Chen	LSU: as AF440772
SY575	*P. multistriata*	GoN, Italy	27 October 2009	S.V. Tesson	LSU: as AF416757

powder (*Saccharomyces cerevisiae*) and labelled. A probe on the microarray specific for TBP-Cy5 will allow verifying the efficiency of hybridization. Details of the TBP preparation can be found in MIDTAL manual (Lewis et al. 2012).

Microarray design and hybridization

The microarray design in terms of probe design, geometry of the array, spotting procedure, and replicates present has been described in Kegel et al. (2012). Slides were pre-hybridized in a slide box containing 20 mL pre-hybridization buffer (2 M NaCl; 20 mM Tris-Cl, pH 8.0; 0.01 % Triton 100) at 65 °C for 1 h. The slides were then washed in ddH_2O and dried by centrifugation in a 50-mL Falcon tube at 2,000 rpm for 2 min.

Labelled samples (around 1 μg RNA, Table 1) were mixed with 35 μL of 2× hybridization buffer (1 $mg mL^{-1}$ BSA, 0.2 $\mu g \mu L^{-1}$ Herring sperm DNA, 2 M NaCl, 20 mM Tris-Cl, pH 8.0, 0.01 % Triton 100), 14 μL of formamide, 100 ng TBP-Cy5), and the volume was adjusted with nuclease-free water to 70 μL. Slides were placed into an array holder, a special coverslip, the Lifter Slip 25×25 (Thomas Scientific, Swedesboro, NJ, USA) were placed onto the microarrays. Prior hybridization, a hot start was performed at 94 °C for 10 min. A volume of 30 μL hybridization solution was pipetted under the coverslip, and capillary action ensured even dispersal of the hybridization solution between the chip and the coverslip. Hybridization was carried out at 65 °C for 1 h (50 °C when deionized formamide 30 % was added in the hybridization mix) in a 50-mL Falcon tube containing a wet Whatman paper. The DNA chips were washed three times shaking on a duomax 1030 shaker (~25 rpm; Cole Parmer, Vernon Hills, IL, USA), with 2× SSC, 10 mM EDTA, 0.05 % SDS, 0.5× SSC, 10 mM EDTA, and then with 0.2× SSC, 10 mM EDTA, each step for 10 min, the last washing was carried out at 50 °C whereupon the slide was dried by centrifugation.

After centrifugation at 2,000 rpm for 2 min, microarrays were scanned with a GenePix 4100B scanner (Molecular Devices, Sunnyvale, CA, USA) using the GenePix 6.0 software. Settings for scanning were 600 PMT gain, 100 % of laser power for the 635 nm wavelength, and the pixel size was 10 μm.

Data analysis

Obtained fluorescent signals and the surrounding background intensity were calculated by superimposing a grid of circles ("gal" file midtal_ver252_20100423, Lewis et al. 2012) onto the scanned image using the GenePix 6.0 software. Results were processed with the program Phylochip Analyzer (http://www.awi.de/en/go/phylochipanalyzer). A signal to noise ratio (s/n ratio) above the threshold of 2 was considered a positive signal (Metfies and Medlin 2008).

Calibration curves

In order to investigate the possibility of obtaining quantitative values for the microarray signals in terms of cell concentration in the field sample, we constructed calibration curves for two strains (SY799 and SY800) of *P. multistriata*: we first extracted RNA from varying culture dilutions (1:1, 1:10 and 1:50) and regressed the obtained RNA quantities against cell numbers. Before RNA extraction, the concentration of each strain and dilution was determined. A total of 1 mL of mother culture and its dilutions at 1:10 and 1:50 were collected, fixed with formaldehyde at a final concentration of 0.6 %. One millilitre of fixed sample was placed in a Sedgewick-Rafter counting chamber (Cole-Parmer, USA) and cell concentration was estimated using a ZEISS Axiophot (Carl Zeiss, Oberkochen, Germany) light microscope.

Four different RNA quantities (1–5–25–100 ng) from *P. multistriata* strain SY373 were hybridized on the microarray and signals from three species-specific probes: PmulaD02_25, PmulaD03_25 and PaustD01_25 (the latter has been designed to be specific against both *P. multistriata* and *Pseudo-nitzschia australis*), were analysed. The final outcome was a calibration curve of signal intensity against cell number.

Results and discussion

Probe specificity

The results of the specificity tests performed on *Pseudo-nitzschia* probes using RNA extracted from monoclonal cultures of eight species are shown in Table 3. The RNA of *P. multistriata*, *P. calliantha* and *P. galaxiae* hybridized with the majority of the probes. The overall high signals and the exceptionally high specific signals suggest that the signal threshold, at least for *P. multistriata* and *P. calliantha*, has been set too low.

Only four probes, designed on *Pseudo-nitzschia multiseries* (PmultD02_25), *P. multistriata* (PmulaD03_25) and *P. galaxiae* (PgalaD01_25; PgalaD04_25), can be considered strictly species-specific as they only responded to their target RNA probes, even if probes for *P. galaxiae* gave weak signals compared with other stronger a-specific signals. However, if the exceptional cross-reactivity shown by the RNAs of *P. calliantha*, *P. multistriata* and *P. galaxiae* is not considered, the number of probes showing an acceptable level of specificity increased. For example, the probe against *P. arenysensis* was observed to be specific, as did all probes against *P. multiseries* (also the probe PmultD01_25, considering the tenfold higher value of the specific versus the a-specific signal of *P. arenysensis*) and the probe PfrauD02_25 against *P. fraudulenta*. The probe PmulaD02_25 was switched on by the RNA of *P. multistriata* and *P. multiseries* with similar s/n values, and could be used with confidence, considering that both species are reported to be toxic. The cross-reactivity between *P. multiseries* and *P.*

Table 3 Results of the probe specificity tests

		P. australis	*P. calliantha*	*P. arenysensis*	*P. delicatissima*	*P. multiseries*	*P. multistriata*	*P. fraudulenta*	*P. galaxiae*
P. australis-P. multistriata	PaustD01_25	3.11	36.17	2.06			26.41		
P. australis-P. seriata	PaustD02_25	3.53	40.34			14.96	12.24	3.14	11.21
	PaustD03_25	2.31					2.24	3.24	
P. pseudodelicatissima-P. cuspidata	PpdeD02_25								2.88
P. caciantha	PcaciD04_25		26.56						
P. manni	Pcal2D01_25		4.43						
	Pcal2D02_25		20.22						
	Pcal2D03_25						2.02		
P. arenysensis	Pdel3D01_25			4.31			6.92		
P. delicatissima	Pdel1D01_25		11.74				4.02		3.64
	Pdel1D03_25	2.09	12.76		2.02		2.60		6.51
P. delicatissima new clade	Pdel2D01_25		2.02				2.17		4.84
P. multiseries	PmultD01_25	2.20	18.32			20.45			
	PmultD02_25					16.79			
	PmultD03_25		16.21			13.18	5.02		
	PmultD04_25		9.64			17.18	7.67		6.08
P. multistriata	PmulaD02_25		13.51			11.91	11.52		
	PmulaD03_25						21.55		
P. fraudulenta	PfrauD02_25		21.50				5.55	2.75	3.20
	PfrauD04_25	4.70	109.89	3.41	2.30	25.35	40.09	9.60	18.37
P. galaxiae	PgalaD01_25								5.60
	PgalaD04_25								5.56
P. hemeii	PhemeD2_25	3.27	49.80			15.30	34.86	4.28	17.02
P. pungens	PpungD02_25		35.59			25.61			5.09
	PpungD04_25		8.23				2.40		2.76
P. subpacifica	PsubpD01_25		29.06				5.25	2.25	8.56
P. seriata	PseriD01_25		11.63			2.42	13.43		6.81
P. brasiliana	PbrasD03_25						6.22		

In rows, the probes clustered by species; in columns, the species from which the tested RNA was extracted. Numbers in cells indicate signal to noise ratio (s/n). Probes not reaching the threshold (s/n=2) in any of the samples are not listed

multistriata RNAs might be due to highly similar target regions on the rRNA of these species because of phylogenetic proximity (Lundholm et al. 2012). The use of an internal control for standardization of the signals between hybridizations would allow a better description of differences in probe behaviour among different experiments. Unfortunately, the signal of the TBP-Cy5, used in the hybridization mix as a control for labelling efficiency, was too low. In future studies, the quantity of this internal control should be adjusted to obtain a consistent signal (Lewis et al. 2012).

All other probes showed unacceptable levels of cross-reactivity. The probe PfrauD04_25, in particular, exhibited cross-reactivity with all *Pseudo-nitzschia* species tested in this study. This probe also hybridized with the RNA of its target species, but the resulting signal was lower than the signal of this probe with the RNA of *P. multiseries*. A possible explanation is that this probe reacts with a conserved region elsewhere in the ribosomal RNA sequences of these species. The high cross-reactivity of this probe could be a result of its melting temperature, that was more than 15 °C higher than the hybridization temperature (Supplemental Table 1), but this was probably not the case, as other probes with the same T_m behave differently. Interestingly, in a study by Lundholm et al. (2006), their probe frD1, designed also on the *P. fraudulenta* LSU sequence, but located circa 40 bp further to the 3′-end, with respect to our probe, was found to work a-specifically as well, with the brightest signal coming from hybridization with *P. delicatissima* and *P. dolorosa* RNAs. Probe PfrauD04_25 could have the potential to become a *Pseudo-nitzschia* genus-specific probe, but this needs to be tested by means of hybridization with RNA from all other species.

A whole set of probes, listed in Supplemental Table 1, but omitted from Table 3, failed to give a signal above the threshold with the RNA of any of the tested species. Notably, the RNA of *P. calliantha* cross-reacted with many different probes, but failed to switch on its own specific probes (Supplemental Table 1) above the threshold. The remaining probes (Supplemental Table 1), designed against the *P. delicatissima* complex, *P. delicatissima* new clade, *P. caciantha*, *P. mannii*, *P. pseudodelicatissima*, *P. americana*, *P. turgiduloides* and *P. brasiliana* never produced a signal. Strangely enough, none of the genus-specific probes showed positive signals with the RNAs of any of the tested species. Failure of these probes could depend on their binding site position on the rRNA, due to possible hindrance because of rRNA secondary structure, as previously shown for probes designed against the SSU-18S (Metfies and Medlin 2008). The specificity of the probes for some of those species, namely *P. americana*, *P. turgiduloides* and *P. brasiliana*, was not tested, and we cannot assume that those probes would not work specifically with their own target RNAs.

A fundamental problem to explain the large number of observed a-specific signals is that the region used to design the probes is relatively conserved to discriminate among and within the groups of closely related species in this genus. In other works (e.g. Galluzzi et al. 2011; Smith et al. 2012), the probes used were generally longer than the ones used in the present study to allow stronger and more specific signals. In the LSU region of the genus *Pseudo-nitzschia*, however, species often differ in single base-pair changes, and this means that one is forced to develop a probe against that target region. 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.

Another potential problem is that each probe requires its own optimal hybridization conditions. In particular, the melting temperatures of the probes tested here are highly variable (Supplemental Table 1), going from 74 to 84 °C, making the stringency of the hybridization impossible to accommodate for all probes (see Gresham et al. 2010). However, our data show no clear relationship between probe melting temperature and lack of species-specific response.

Several studies have already investigated the use of microarrays to detect HAB species (Ahn et al. 2006; Barlaan et al. 2007; Gescher et al. 2008; Metfies and Medlin 2008; Smith et al. 2012). Our approach differs from the approaches in most of these studies in three aspects. We used RNA instead of PCR products to hybridize against the probes; we used nuclear LSU instead of nuclear and plastidial SSU or ITS; we used species-specific probes instead of higher taxonomic level probes and, in addition, our species were very closely related. That is why future work will need to focus on completing the matrix in Table 3 with RNA from all known *Pseudo-nitzschia* species as well as on the normalization of the signal strength of the probes given standard amounts of RNA. From such a matrix of specific reactions and a-specific cross reactions, a program could be designed to calculate the probability of any known species being present in a sample given the observed pattern of probe responses from the RNA of an environmental sample, as in Smith et al. 2012. The cross-reactivity of the various probes is a setback for the development of probes in *Pseudo-nitzschia*. The composition of this genus of groups of closely related species renders further development of probes to distinguish among all the different species no sinecure. Other authors have struggled with the same problem (e.g. Lundholm et al. 2006; Scholin et al. 1999). Scholin et al. (1999), in particular, reported cross hybridization reactions in their dot-blot approach to distinguish among the species known at that time.

Field samples

The success of RNA extraction from field samples was evaluated by means of the brightness of the *Dunaliella*

probe on the microarray. Signal to noise values were high, ranging from 4 to 15 (data not shown). The lowest values have been obtained for those dates at which the highest amounts of RNA have been extracted (MT4 and MT8). Apparently, the higher the biomass, c.q. RNA present in a sample, the lower the efficiency of harvesting, possibly because of saturation of the Tri-reagent medium.

In Table 4, results of the hybridization of RNAs extracted from field samples are shown. Of the two genus-specific probes, only PsnGS02_25 reacted with the RNA of 5 out of the 12 environmental samples. This result is peculiar for several reasons. First, PsnGS02_25 reacted with none of the RNA extracts obtained from the monoclonal cultures of the *Pseudo-nitzschia* species tested, and therefore, it is peculiar that this probe showed a reaction with field samples. Second, if it does react to *Pseudo-nitzschia*, then it should have given a reaction with all samples because all 11 environmental samples contained *Pseudo-nitzschia* species (Table 4 and Fig. 2). Possible explanations are that this generic *Pseudo-nitzschia* probe reacts only with RNA of a restricted set of *Pseudo-nitzschia* species not included in the culture tests or that the number of *Pseudo-nitzschia* cells in the sample was too low to generate a signal above the threshold, if the probe shows low hybridization efficiency. However, the latter ad hoc explanation does not explain why the probe did not react against the RNA of the cultured species. In any case, the probe PfrauD04_25, which reacted well above the threshold with the RNA of all of the cultured species tested, reacted likewise with the RNA from all of the field samples except for MT9. The latter sample also showed only a response with probes of *Pseudo-nitzschia hemeii* and *P. australis*, two species never recorded in the Gulf of Naples.

The P. delicatissima complex

Figure 2a shows the cell counts within the *P. delicatissima* complex compared with the signal strength for the probes against two of the genetically distinct species in this complex, namely *Pseudo-nitzschia delicatissima sensu stricto* (Pdel1D03_25) and *P. arenysensis* (Pdel3D01_25). Cell counts and probe signals did not corroborate well. The lack of corroboration may be due to the fact that the *P. delicatissima* species complex includes at least four genetically distinct species: *P. arenysensis*, *P. delicatissima sensu stricto*, *P. delicatissima* (new clade) and *P. dolorosa*, all of which have been observed in the Gulf of Naples. Moreover, both probes cross-react not only strongly with RNA of *P. calliantha* (within the *P. pseudodelicatissima* species complex) but also, albeit weakly, with RNA of *P. galaxiae* and *P. multistriata*, which are outside the *P. delicatissima* species complex (Table 3).

Table 4 Results of field samples' hybridizations

		September 2009	October 2009	November 2009	December 2009	January 2010	March 2010	April 2010	May 2010	June 2010	July 2010	August 2010
Pseudo-nitzschia genus	PsnGS02_25	2.13	2.18								2.28	2.78
P. australis	PaustD03_25			2.88			2.15	3.79	2.07			2.36
	PaustS01_25	2.59		2.71		4.24				2.15		
P. caciantha	PcaciD02_25			3.93	2.61			2.66			2.42	2.08
P. mannii	Pcal2D03_25			3.17	3.12			5.22				2.16
P. delicatissima	Pdel1D01_25		2.40	5.64	4.09		3.38					2.48
	Pdel1D03_25		3.52	6.29	3.04		2.17	2.04			2.06	2.41
P. arenysensis	Pdel3D01_25			2.54								
P. fraudulenta	PfrauD04_25	3.97	3.14	18.64	23.04	6.07	11.12	13.81		3.56	4.24	17.36
P. hemeii	PhemeD2_25	39.18	40.46	76.14	43.82	32.52	13.71	9.38	18.48	7.37	15.06	21.71
P. multistriata	PmulaD02_25			2.34								
P. multiseries	PmultD01_25_25			2.28								
	PmultS01_25			2.27				2.56			2.15	2.80
P. pungens	PpungD02_25				3.76							

Numbers indicate signal to noise ratio (s/n). Probes not reaching the threshold (s/n=2) in any of the samples are not listed

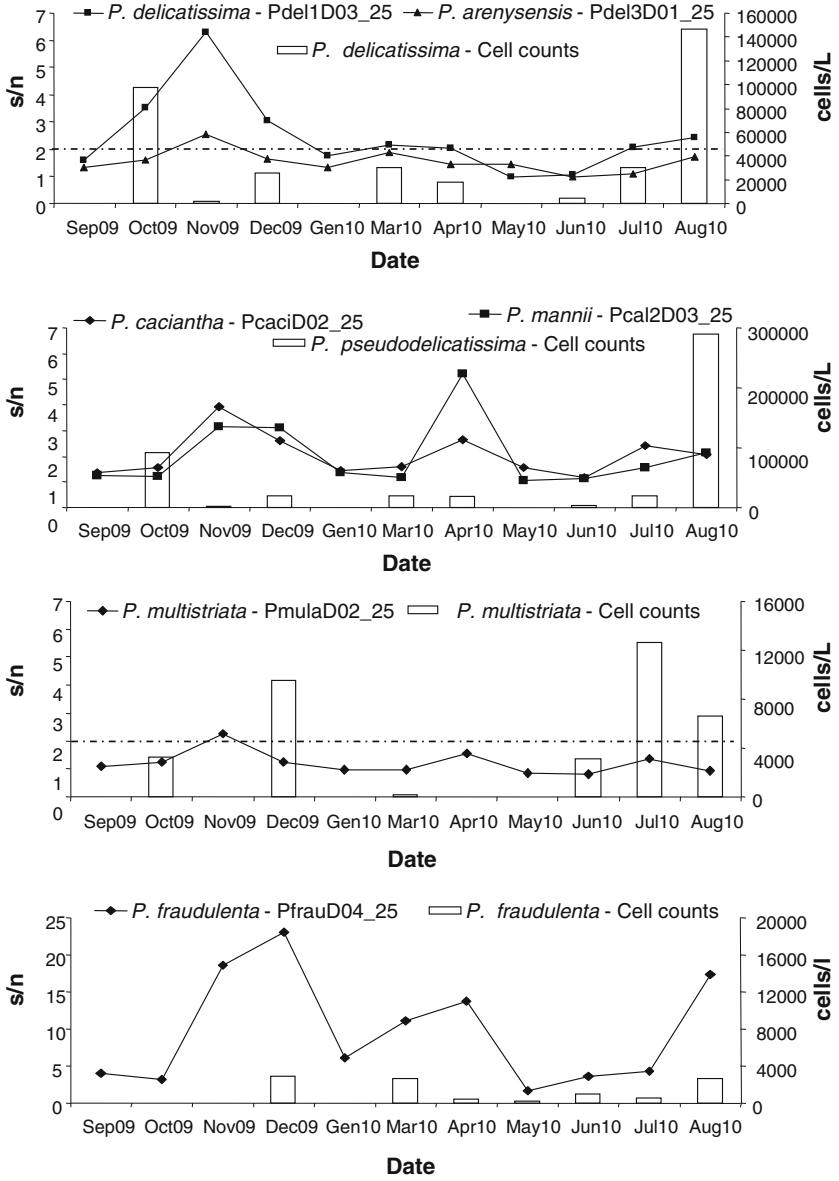

Fig. 2 Temporal pattern of probe responses to field samples for *Pseudo-nitzschia*. The s/n (*lines*) and cell counts (cells per litre, *bars*) are plotted for each sampling date. *Dashed lines* indicate the threshold for positive signals. **a** *P. delicatissima* and *P. arenysensis*; **b** *P. caciantha* and *P. mannii*; **c** *P. multistriata*; **d** *P. fraudulenta*

Probe Pdel3D01_25 (to *P. arenysensis*) also showed the cross-reactivity with RNA of *P. multistriata* (see Table 3), but since the probe specific for *P. multistriata* did not respond positively, the positive response of probe Pdel3D01_25 probably signals the presence of *P. arenysensis*. Probes Pdel1D01_25 and Pdel1D03_25 reacted positively with several of the field samples. The first of these probes showed a-specific reactions (Table 3), but the latter reacted with RNA of *P. delicatissima*. These results suggest that this species is present in several of the samples, which is confirmed with the cell counts of members of this species complex.

The P. pseudodelicatissima complex

Figure 2b shows the number of cells within the *P. pseudodelicatissima* species complex compared with the signal strength for the probes against two of the genetically distinct species in this complex, namely *Pseudo-nitzschia caciantha* (PcaciD02_25) and *P. mannii* (Pcal2D03_25). Only one of the three probes designed against *P. caciantha* (PcaciD01_25, PcaciD02_25 and PcaciD04_25) was switched on in the field sample, although the probe PcaciD04_25 was the only one that rose up in the specificity tests (Table 3). Also in this case, the microscopy counts and the probe signals did not corroborate well. This lack may be due to the fact that the *P. pseudodelicatissima* species complex includes at least five genetically distinct species: *P. caciantha*, *P. calliantha*, *P. mannii*, *P. cuspidata* and *P. pseudodelicatissima sensu stricto*, all of which have been observed in the Gulf of Naples, whereas the working probes PcaciD02_25 and Pcal2D03_25 are supposed to detect only two of these.

Table 5 Correlation between numbers of cells and total RNA for *P. multistriata*

Strain	Dilution factor	Cells	Total RNA	RNA pg/cell	R^2
SY799	1:1	3,237,000	3,509	0.0023	0.997
	1:10	259,800	251	0.0023	
	1:50	35,100	167	0.0023	
SY800	1:1	3,158,700	2,485	0.0025	0.986
	1:10	346,450	414	0.0025	
	1:50	38,700	209	0.0025	

The amount of RNA per cell (in picograms) and the correlation coefficient (R^2) of the calibration are also shown

Unfortunately, we were unable to test the specificity of the probes for *P. mannii* and *P. caciantha* because we lacked cultures of these species. Therefore, we cannot exclude that the signals may result from cross-reactions with unknown species.

P. multistriata

The *P. multistriata*-specific probe PmulaD03_25 did not react above the threshold with any of the field samples, suggesting that this species was absent, or that there were not enough cells to reach the signal threshold. The probe PmulaD02_25 to *P. multistriata* reacted positively with sample MT3 (Fig. 2c; Table 4), but this probe reacted also to the RNA of other species. The reason for the low signal may be the relatively low cell numbers in comparison with the counts in Fig. 2a, b. Apparently, not enough RNA is provided by these few cells to generate strong enough signals.

P. fraudulenta

Microscopy counts and the probe signals for cells of *P. fraudulenta* did not corroborate well (Fig. 2d). Probe signals are very strong, whereas cell numbers are low. Notably, the probe reacted not only strongly with the RNA of this species but also strongly to weakly with that of all the other species with which the probe specificity tests were carried out. Due to this apparent cross-reactivity, the signals have little bearing on the cell counts.

P. galaxiae

From the results in Table 3, we concluded that the probes PgalaD01_25 and PgalaD04_25 to *P. galaxiae* are species specific. The probe did not react with the RNA of any of the field samples (are therefore not shown in Table 4), in spite of the fact that cell counts revealed the presence of the species in many of the samples. The lack of reaction does not result from the fact that *P. galaxiae* is composed of a species complex and that the LSU rRNA sequences of the particular groups of cryptic species present in the GoN do not react with the probes designed for *P. galaxiae* because the probe target region is the same in all the LSU variants known to us to occur in the GoN (McDonald et al. 2007). Alternatively, cell density in the samples may not have been high enough to generate a signal strong enough to reach the threshold.

Other species

The absence of *P. multiseries* in the cell counts and the fact that PmultD02_25 probe, considered specific to *P. multiseries*, did not react positively with any of the field samples are consistent with the absence of these species in the Gulf of Naples. Therefore, positive responses of PmultD01_25 and PmultS01_25 with RNA from samples MT3, MT8, MT11 and MT12 must represent cross-reactions with RNA of other species.

Also, *Pseudo-nitzschia pungens*, *P. hemeii* and *P. australis* have never been observed in cell counts or recorded in the Gulf of Naples. This means that the positive reaction of probes PpungD02_25, PhemeD02_25, PaustD03_25 and PaustS01_with RNA from several of the samples represents possibly an a-specific response.

Calibration curves and trials for signal quantification

Correlation between the RNA extracted from different numbers of cells of *P. multistriata* was high (Table 5).

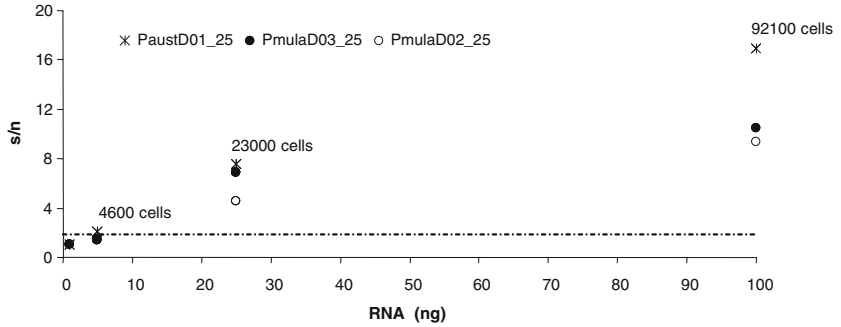

Fig. 3 Calibration curve for *P. multistriata*: s/n vs RNA quantity. Cell numbers corresponding to RNA quantity are also shown

Table 6 Correlation coefficients (R^2) and equations of the regression between s/n and number of cells for *P. multistriata*

Probe	R^2	Equation
PaustD01_25	0.999	Y=0.00029X+0.776
PmulaD02_25	0.998	Y=0.00016X+0.810
PmulaD03_25	0.991	Y=0.00028X+0.504

The strain used in this experiment was SY799

Given the numbers of cells and the obtained quantity of RNA extracted from these cells, the calculated amount of RNA extracted per cell was ca 0.0023 pg for *P. multistriata* strain SY799 and 0.0025 pg for strain SY800 (Table 5). The calibration curves (Fig. 3) revealed a linear relationship between RNA quantity and probe signal strength, which only flattened off at RNA quantities of 100 ng; this point was thus eliminated from following regression analyses. The detection threshold for the RNA of *P. multistriata* cells at s/n=2 was at 5 ng of RNA which corresponds to 4,600 cells (Fig. 3). Thus, at least with RNA obtained from monoclonal cultures, there exists a semi-linear relationship between cell number and probe signal. We calculated the linear regression of the curve correlating cell number and probe signals for the three *P. multistriata*-specific probes reported in Table 6. The number of inferred cells (Table 7), calculated on the probe showing the highest R^2 value (PaustD01_25, Table 6), shows little or no correlation with our field samples and observed cell numbers counted in light microscopy (Table 7). In fact, regardless of the presence or not of *P. multistriata* in light microscopy observations, the s/n ratios never reach the threshold except for one probe (PmulaD02_25) in one sample (MT3), so that a sound comparison between the two data types is not possible. The most likely explanations for this lack of correlation are that (1) probes may be not efficient enough in field samples due to steric hindrance in a rRNA-rich sample or because of competition with other probes; (2) even if the rRNA is more stable (longer turnover) and far more abundant than mRNA, comprising ca. 99 % of the total RNA, the RNA content can also vary according to the physiological state of cells (Berdalet et al. 1994; De Madariaga and Joint 1992; Dittami and Edvardsen 2012b; Dortch et al. 1983). Dittami and Edvardsen (2012a) described how to transform calibration signals into cell numbers for most of the species present on the same microarray used in this study. They have taken into account quality of hybridization, degree of labelling and possible losses of RNA, due to an incomplete cells disruption and different amounts of RNA in species of different size. All these parameters and how they influence the correlation between signal intensity and cell number need to be considered and integrated in the algorithm for cell number calculation in future experiments.

Conclusions

Microarrays are potentially powerful tools, allowing the detection of hundreds of different species in field samples in a cost- and time-effective way. This is particularly important when toxic species bloom as in such cases the time interval between detection and management decision needs to be as short as possible. Microarrays could also be of use in the detection of species when cryptic diversity is present —as is the case of the genus *Pseudo-nitzschia*—where LM is not the proper tool for discerning among morphologically similar species. However, this study has demonstrated that in groups of closely related species it is challenging to design probes that are not only species-specific in silico but also in experiments with real RNA from lab- or field samples, under standardized hybridization conditions.

Table 7 Conversion from s/n to cell number for each *P. multistriata* probe per each sampling date

Sampling date	PmulaD02_25	PmulaD03_25	PaustD01_25	Inferred cell concentration (cells/L)	Observed cell concentration (cells/L)
MT1 08/09/2009	1.07	1.14	1.35	395.4	0
MT2-06/10/2009	1.23	1.05	1.26	333.4	3,294
MT3-04/11/2009	2.24	1.38	1.76	678.2	0
MT4-02/12/2009	1.25	1.25	1.83	908.0	16,468
MT5-12/01/2010	0.99	1.00	1.41	436.8	0
MT7-09/03/2010	0.96	0.94	1.22	611.6	0
MT8-13/04/2010	1.56	1.11	1.85	1,850.5	0
MT9-18/05/2010	0.85	1.23	1.32	468.2	0
MT10-15/06/2010	0.82	0.79	1.17	1356.0	44,314
MT11-13/07/2010	1.38	1.07	1.14	626.5	37,166
MT12-03/08/2010	0.93	1.11	1.39	1,410.0	0

Inferred cell concentrations are calculated from s/n for the probe PaustD01_25 and taking into account the filtered sea-water volumes as from Table 1. Last column shows light microscopy cells counts

A major constraint in the multi-probe approach of the microarray is the fact that hybridization conditions have to be preset, based on an optimum of the requirements of all the probe–target region interactions. A precise control of the hybridization conditions is needed: the probes should be designed to possess similar melting temperatures, to make stringency controls effective. Unfortunately, the ca. 700-bp region of the LSU does not contain enough variation to provide enough alternative solutions for probe design. The designed probes have to do, and therefore, not all probes function perfectly under the given the standard conditions. The risk is a high propensity for a-specificity, i.e. false positives can rise because of a failure to discriminate a single or few mismatches. Or probes fail to work because the hybridization temperature is a little high.

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*. Improvements can be: lowering the background by blocking the slide prior to the hybridization step, calibrating the quantity of the TBP-Cy5 as internal control to avoid saturated or poor signals, spiking of the samples with known numbers of cells, i.e. for species of interest (Galluzzi et al. 2011).

Another problem that needs to be investigated in detail is that RNA concentration differs from species to species and that some species could disintegrate easier than others during extraction. A hierarchical approach, with probes designed from higher taxonomic levels down to the species levels is also desirable as it can help identifying false positives.

Several matters still need to be addressed towards developing a commercially available microarray for the reliable detection of harmful microalgal species of *Pseudo-nitzschia* and discriminating them from non-toxic species.

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