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RESEARCH ARTICLE

Specificity of LSU rRNA-targeted oligonucleotide probes for *Pseudo-nitzschia* species tested through dot-blot hybridisation

Lucia Barra · Maria Valeria Ruggiero · Jixin Chen · Wiebe H. C. F. Kooistra

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Abstract In the scope of the development of a microarray PhyloChip for the detection of toxic phytoplankton species, we designed a large series of probes specific against targets in the nuclear large subunit (LSU) rRNA of a range of *Pseudo-nitzschia* species and spotted these onto the microarray. Hybridisation with rRNA extracted from monoclonal cultures and from plankton samples revealed many cross-reactions. In the present work, we tested the functionality and specificity of 23 of these probes designed against ten of the species, using a dot-blot procedure. In this case, probe specificity is tested against the target region in PCR products of the LSU rRNA gene marker region blotted on nitrocellulose filters. Each filter was incubated with a species-specific oligoprobe. Eleven of the tested probes showed specific responses, identifying seven *Pseudo-nitzschia* species. The other probes showed non-specific responses or did not respond at all. Results of dot-blot hybridisations are more specific than those obtained with the microarray approach and the possible reasons for this are discussed.

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L. Barra · M. V. Ruggiero · W. H. C. F. Kooistra (✉)
Stazione Zoologica Anton Dohrn, Villa Comunale,
80121 Naples, Italy
e-mail: kooistra@szn.it

J. Chen
The Laboratory, Marine Biological Association, Citadel Hill,
Plymouth PL1 2 PB, UK

Present Address:
J. Chen
The State Key Laboratory of Marine Environmental Science,
College of the Environment and Ecology, Xiamen University,
Xiamen, Fujian, People's Republic of China

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Introduction

The marine planktonic diatom genus *Pseudo-nitzschia* includes a large number of species and new ones are described frequently (Amato et al. 2007; Lelong et al. 2012; Lim et al. 2012; Lundholm et al. 2012). Accurate identification of these species is important because several of them can produce domoic acid (DA), a potent neurotoxin responsible for amnesic shellfish poisoning in humans, other mammals and birds (Bates et al. 1989; Lelong et al. 2012; Trainer et al. 2012). Detection approaches for toxic *Pseudo-nitzschia* species need to be affordable, simple to execute and rapid, and they need to be accurate because false-negatives are dangerous to human health whereas false-positives affect the shellfish industry. Traditionally, phytoplankton in environmental samples is identified and enumerated by means of light microscopy (LM). However, for *Pseudo-nitzschia*, LM identification is insufficient because several toxic species are indistinguishable in LM from non-toxic ones (e.g. Amato et al. 2007; Bates et al. 1993). Monitoring for DA, instead, will not do either because production of the toxin depends strongly on the physiological state of the cells (Amato et al. 2009; Lelong et al. 2012; Pan et al. 1996a, b, c; Thessen et al. 2009). Several DNA- or RNA-based methods have been, or are being, developed to monitor harmful algal blooms (Ebenezer et al. 2012; Kudela et al. 2010; Medlin and Kooistra 2010). The methods utilise sequence differences on specific marker DNA or ribosomal RNA regions among species of interest and include, amongst others, fluorescent

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in situ hybridisation (Cho et al. 2001; Miller and Scholin 1996; Simon et al. 2000), dot-blot (Miller and Scholin 1998, 2000; Simon et al. 2000), quantitative and real-time PCR (Andree et al. 2011; Perini et al. 2011), sandwich assay (Ayers et al. 2005; Diercks et al. 2008; Scholin et al. 1996, 1999) and microarray (Barra et al. 2013; Galluzzi et al. 2011; Gescher et al. 2008; Metfies and Medlin 2005, 2008; Smith et al. 2012).

Barra et al. (2013) explored the feasibility of the microarray approach to detect *Pseudo-nitzschia* species in environmental samples. Therefore, 47 species-specific oligonucleotide probes were developed against target regions within the ca. 700 bases at the 5′-end of the nuclear-encoded large subunit (LSU) ribosomal RNA of a series of *Pseudo-nitzschia* species (Kegel et al. 2013). The probes were spotted on a microscope slide and incubated with total RNA extracted from environmental samples or from monoclonal cultures of *Pseudo-nitzschia* species to allow the probes to hybridise with their target regions (Lewis et al. 2012). Results with RNA from monoclonal cultures of *Pseudo-nitzschia* species showed several false-positives and false-negatives. The microarray design included nested sets of hierarchical probes permitting proper identification of many such false-positives, but this safeguard does not explain what caused these unexpected results.

To find explanations for these false-positives and false-negatives, in the microarray results of Barra et al. (2013), we tested the probes for their specificity making use of a dot-blot approach. The principal difference between the microarray- and dot-blot assays is that in the microarray procedure an immobilised probe on the array hybridises with its target in the ribosomal RNA in solution whereas in the dot-blot procedure the probe in solution links with a target within the sense DNA strand of a PCR product, which together with the antisense strand has been blotted on nitrocellulose filters. On the dot-blot, false-positives can be generated only due to alternative binding sites in the sense and antisense strands of the PCR product, which can be checked in silico. In the microarray procedure, instead, false-positives can be due to alternative binding sites anywhere in the various ribosomal RNAs from the cytoplasm, mitochondria and plastids, most of which are not yet known for the various *Pseudo-nitzschia* species. Thus, the dot-blot procedure excludes the possibility of false-positives arising from probes binding with possible targets elsewhere on the ribosomal RNAs.

In the dot-blot procedure applied in this study, PCR products of the ca 700 bp at the 5′-end of the nuclear LSU rRNA gene cistron, generated from monoclonal strains of ten species across the phylogenetic diversity of *Pseudo-nitzschia*, were blotted onto nitrocellulose filters. Each filter was then incubated with a single species-specific probe (see Miller and Scholin 1998, 2000). Species-specific responses with the dot-blot and negative results with the microarray or vice versa indicate conditions too stringent for hybridisation or probe failure to access target region because of secondary structure formation. False-positives on the microarray and/or dot-blot could result from a too low hybridisation temperature or from the presence of minute amounts of non-specific nucleotide sequences, but the latter is checked by means of blank controls. The same weak false-positives on both could be due to intraspecific variation among the multiple copies of the LSU marker (reviewed by Alverson 2008). Yet, this explanation usually holds only for very closely related species that may still hybridise or share sequence because of incomplete lineage sorting. False-positives on the microarray, but not on the dot-blot, could be due to identical target sequences elsewhere in the nuclear-, plastid- and mitochondrial-encoded rRNA molecules of the *Pseudo-nitzschia* species considered, whereas false-positives on the dot-blot, but not on the microarray, could result from a non-intended target on the antisense strand of the PCR product.

Materials and methods

Culture conditions, DNA extraction and PCR amplification

Ten monoclonal, non-axenic strains used in this study (Table 1) were maintained and prepared for DNA extraction as described

Table 1 List of strains used in this study

Strain code	Species	Collection site	Collection date
B569	*P. arenysensis*	Gulf of Naples, Italy	12 Jan 2010
B513	*P. calliantha*	Gulf of Naples, Italy	04 Aug 2009
B549	*P. delicatissima*	Gulf of Naples, Italy	12 Jan 2010
B498	*P. delicatissima new clade*	Gulf of Naples, Italy	28 July 2009
B570	*P. fraudulenta*	Gulf of Naples, Italy	09 Feb 2010
B510	*P. galaxiae*	Gulf of Naples, Italy	04 Aug 2009
B528	*P. hasleana*	Ria de Muros, Spain	30 Sept 2009
B512	*P. mannii*	Gulf of Naples, Italy	04 Aug 2009
B531	*P. multistriata*	Gulf of Naples, Italy	22 Sept 2009
B545	*P. pseudodelicatissima*	Gulf of Naples, Italy	21 Dec 2009

in Barra et al. (2013). DNA was extracted by incubating cell material in CTAB extraction buffer (2 % CTAB, 200 mM Tris–HCl pH 8.0, 50 mM EDTA, 1.4 M NaCl and 2.5 % PVP; modified from Doyle and Doyle 1987) for 45 min at 65 °C. Proteins and debris were removed using two washes with chloroform–isoamyl alcohol (24:1). DNA was precipitated by mixing the supernatant with an equal volume of isopropanol, a storage step at −20 °C for 1 h, and centrifugation at 4 °C for 30 min at 14,000 rpm. Obtained DNA pellets were rinsed with 75 % ethanol in water (*v*/*v*), dried on air and dissolved in sterile bidistilled water. Eukaryote-specific primers DIR (forward, 5′-ACC CGC TGA ATT TAA GCA TA-3′; Scholin et al. 1994) and D3Ca (reverse, 5′- ACG AAC GAT TTG CAC GTC AG-3′; Lenaers et al. 1989) were used to amplify a ca. 700-bp fragment at the 5′-end of the nuclear-encoded ribosomal LSU RNA gene region according to Amato et al. (2007). Amplification conditions were as follows: 40 cycles (35 s at 94 °C, 35 s at 46.2 °C and 60 s at 72 °C) were performed, with an initial step of 120 s at 94 °C and a final one of 300 s at 72 °C. Small fractions of the PCR products were quality checked for length and distinctness by means of agarose gel electrophoresis (1 % agarose) using ethidium bromide as a dye. The remainder of the PCR products was purified using the QIAquick PCR Purification Kit from Qiagen (Qiagen Ltd, Crawley, West Sussex, UK) following manufacturer's instructions. DNA concentration was measured with a Nanodrop® ND-1000 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

Probe design and DIG labelling

Probes were designed using the ARB software package (http://www.arb-home.de/) and the associated SILVA comprehensive ribosomal database (Pruesse et al. 2007; http://www.arb-silva.de/) for the ca. 700 bp at the 5′-end of the nuclear-encoded LSU rRNA region. The LSU database included all aligned, quality checked rRNA sequences longer than 300 bases. All distinct, nuclear-encoded LSU rRNA gene sequences of *Pseudo-nitzschia* and *Fragilariopsis* were downloaded from Genbank (http://www.ncbi.nlm.nih.gov) and imported in the ARB LSU database. All probes against species, clades within species or groups of species were selected by means of the probe design function of ARB. All were designed to be 25 bases in length.

The Oligo6 Primer Analysis Software (http://www.oligo.net) was used to obtain thermo-kinetics information of the designed probes. Probe–target double-stranded DNA melting temperatures (T_m) were calculated according to the GC% method with conditions set at 50 mM NaCl and 50 μM of probe. Probes exhibiting a T_m close to 75 °C were preferred to permit development of a standard protocol with universal hybridisation- and wash temperatures in the microarray- and dot-blot procedures. To avoid disruption of duplex formation between probe and target, probes with a consecutive length of a dimer or hairpin exceeding 4 bases were excluded. In case multiple probes could be designed against the same species, then those probes were preferred that exhibited a free energy (ΔG, in kilocalorie per mole) closest to zero (Table 2). Probes have been labelled for dot-blot analysis with DIG Oligonucleotide Tailing Kit, Second Generation (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Of the 47 probes against *Pseudo-nitzschia* species designed for the microarray analysis procedure in Barra et al. (2013), 23 were selected for screening by means of dot-blot hybridisation (Table 2). One reason for selecting these was their specificity against one or more of the ten *Pseudo-nitzschia* strains used in this study. Other selection criteria included the following: probes covering various regions along the target sequence, probes exhibiting a range of melting temperatures (T_m) and free energy (ΔG) values, in order to explore the effect of these factors in the obtained results.

Dot-blot hybridisation

One microliter (ca. 20 ng) of purified LSU rRNA gene PCR product of each of the species listed in Table 1 was denatured for 10 min at 97 °C and spotted on a series of nylon membranes to be used in all of the experiments. One microliter of labelled DNA solution provided with the labelling kit (ca. 260 ng linearised pBR328 DNA, labelled with digoxigenin; Roche Diagnostics, Mannheim, Germany) has been spotted in triplicate on the same membrane as the positive control. The DNA was fixed to the nylon membrane by 90–120 s of exposure to UV light. Blots were pre-incubated for 30 min with 20 mL pre-heated hybridisation buffer (Roche Diagnostics) per 100 cm^2 of membrane surface to block the remainder of the membrane surface. Thereupon, 0.01 pmol/μL (10 pmol/mL) of labelled probe was added to the blots and they were agitated gently for 2 h to overnight at the hybridisation temperature. The reaction was stopped by washing the membranes for 2×5 min in 2× SSC and 0.1 % SDS solution at 15–25 °C and 2×15 min in 0.5× SSC and 0.1 % SDS solution at hybridisation temperature under constant agitation. Detection was performed with Chemiluminescent Substrate For Alkaline Phosphatase as substrate (DIG Luminescent Detection Kit, Roche Diagnostics) according to the manufacturer's instructions. Membranes were exposed to X-ray film for 10, 15 or 20 min depending on the strength of the signal.

Results and discussion

Probe design

Up to four probes were available for each of the ten strains utilised in this study (Table 2). The T_m values of the probes

Table 2 List of tested probes

Probe	Target region	T_m (°C)	ΔG (kcal/mol)	Targeted species	Strain code	Accession number
Pdel3D01_25	200–300	79.0	−3.8	*P. arenysensis*	B569	as DQ813811
Pcal1D01_25	500–600	77.3	−3.1	*P. calliantha*	B513	as DQ813815
Pdel1D01_25	600–700	74.1	−3.6	*P. delicatissima*	B549	as DQ813810
Pdel1D03_25	600–700	77.3	−3.6	*P. delicatissima*	B549	as DQ813810
Pdel2D01_25	200–300	79.0	−3.8	*P. delicatissima* new clade	B498	as KC801041
Pdel2D02_25	600–700	74.1	−6.9	*P. delicatissima* new clade	B498	as KC801041
Pdel4D01_25	200–300	79.0	−3.6	*P. delicatissima* new clade	B498	as KC801041
PdeliD02_25	400–500	75.7	−4.7	*P. delicatissima* complex		
PfrauD04_25	100–200	82.1	−10.1	*P. fraudulenta*	B570	as EF522111
PfrauD02_25	150–250	82.3	−3.8	*P. fraudulenta*	B570	as EF522111
PgalaD01_25	400–500	75.8	−4.8	*P. galaxiae*	B510	as EF506607
PgalaD02_25	400–500	75.7	−3.1	*P. galaxiae*	B510	as EF506607
PgalaD04_25	500–600	74.1	−8.0	*P. galaxiae*	B510	as EF506607
PvigoD01_25	400–500	79.0	−3.6	*P. hasleana*	B528	as JN050298
PvigoD03_25	500–600	79.0	−6.8	*P. hasleana*	B528	as JN050298
Pcal2D01_25	50–150	77.3	−11.4	*P. mannii*	B512	as DQ813814
Pcal2D02_25	100–200	75.7	−4.5	*P. mannii*	B512	as DQ813814
Pcal2D03_25	100–200	77.4	−9.8	*P. mannii*	B512	as DQ813814
Pcal2D05_25	500–600	77.4	−10.0	*P. mannii*	B512	as DQ813814
PmulaD02_25	400–500	81.0	−9.8	*P. multistriata*	B531	KF241715
PmulaD03_25	400–500	77.3	−6.3	*P. multistriata*	B531	KF241715
PpdeD01_25	400–500	74.1	−4.7	*P. pseudodelicatissima*	B545	KF241716
PpdeD02_25	100–200	79,0	−9,8	*P. pseudodelicatissima*	B545	KF241716

Tested probes under patent. The target region indicates the range of nucleotide positions within which the target sequence is located on the sense strand of the LSU PCR product downstream of the 5′-end of primer D1R. Melting temperature (T_m), free energy (ΔG), targeted species, strain code and GenBank accession numbers are provided

ranged between 74.1 and 82.3 °C with most of them ≤79.0 °C. Their ΔG values ranged from −3.1 down to −11.4 kcal/mol with the large majority being less negative than −9.8 kcal/mol. The probe targets were located all over the variable regions in the PCR products (Table 2).

In silico, all 23 probes matched perfectly with their intended targets in the PCR product of the strains against which they were designed and mismatched at least at 1 base with their target regions in the PCR products of the other strains (Table 3). Probe PdeliD02_25, intended against *Pseudo-nitzschia delicatissima* and *P. delicatissima*-like species (*Pseudo-nitzschia arenysensis* and *P. delicatissima* clade 4) did indeed match with the targets in the PCR products of strains B569, B549 and B498. The antisense strands of the PCR products were checked in silico for potential target regions for any of the 23 probes, but no such (near-) fit was detected.

Dot-blot screening results; possible causes of annealing problems

The experimental results showed that 11 of the 23 probes were specific to their intended target, identifying seven species (Table 4). These probes included the one for *P. arenysensis*, the three for *P. delicatissima* clade 4, the two for *Pseudo-nitzschia fraudulenta*, in spite of their high T_m value and the markedly negative ΔG of one of these probes, two of the three for *Pseudo-nitzschia galaxiae* (PgalaD02_25 and PgalaD04_25), one of the two for *Pseudo-nitzschia hasleana* (PvigoD03_25), one of the two for *Pseudo-nitzschia multistriata* (PmulaD03_25; see Fig. 1) and one of the two for the *Pseudo-nitzschia pseudodelicatissima–cuspidata* complex (PpdeD01_25).

Four probes did hybridise with the PCR product of their intended target species, but in addition with the PCR products of one or more non-target species (Table 4). The *Pseudo-nitzschia calliantha* probe Pcal1D01_25 hybridised also with the PCR products of *P. arenysensis* (B569; four mismatches; see Table 3), *P. fraudulenta* (B570; four mismatches) and *P. pseudodelicatissima* (B545; three mismatches). The *P. hasleana* probe PvigoD01 hybridised also with the PCR products of *P. calliantha* (B513; two mismatches) and *P. pseudodelicatissima* (B545; five mismatches). A weak reaction of this probe was obtained with those of *P. arenysensis* (B569; five mismatches), *P.*

Table 3 Matrix of mismatches between probe (rows) and target LSU sequence from tested strains (columns); for strain codes, see Table 1

Probe name	Strain code	Targeted species	No. of mismatches with PCR product of tested strains									
			B550	B513	B549	B498	B570	B510	B528	B512	B531	B545
Pdel3D01_25	B569	*P. arenysensis*	**0**	3	3	1	3	2	3	4	1	3
Pcal1D01_25	B513	*P. calliantha*	4	**0**	4	4	**4**	3	1	3	**4**	**4**
Pdel1D01_25	B549	*P. delicatissima*	2	3	**0**	1	1	3	2	3	4	1
Pdel1D03_25	B549	*P. delicatissima*	2	1	**0**	1	1	1	1	1	2	1
Pdel2D01_25	B498	*P. delicatissima* new clade	1	2	2	**0**	2	1	2	3	2	2
Pdel2D02_25	B498	*P. delicatissima* new clade	1	2	1	**0**	1	2	4	2	2	1
Pdel4D01_25	B498	*P. delicatissima* new clade	1	2	2	**0**	2	1	2	3	2	2
PdeliD02_25		*P. delicatissima* complex	**0**	2	**0**	**0**	2	1	1	2	3	3
PfrauD04_25	B570	*P. fraudulenta*	1	1	1	1	**0**	1	1	1	1	1
PfrauD02_25	B570	*P. fraudulenta*	2	1	1	1	**0**	1	1	2	2	1
PgalaD01_25	B510	*P. galaxiae*	4	5	4	4	4	**0**	4	4	6	4
PgalaD02_25	B510	*P. galaxiae*	1	3	1	1	3	**0**	2	3	4	4
PgalaD04_25	B510	*P. galaxiae*	3	3	3	3	3	**0**	4	2	3	3
PvigoD01_25	B528	*P. hasleana*	2	2	2	2	4	2	**0**	3	6	5
PvigoD03_25	B528	*P. hasleana*	5	1	5	5	**5**	**4**	**0**	4	**5**	5
Pcal2D01_25	B512	*P. mannii*	4	2	5	4	5	5	3	**0**	5	6
Pcal2D02_25	B512	*P. mannii*	3	2	3	3	2	3	1	**0**	3	4
Pcal2D03_25	B512	*P. mannii*	**5**	3	6	5	**5**	6	2	**0**	5	**7**
Pcal2D05_25	B512	*P. mannii*	4	3	4	4	4	2	4	**0**	4	4
PmulaD02_25	B531	*P. multistriata*	2	3	2	2	**2**	6	2	2	**0**	2
PmulaD03_25	B531	*P. multistriata*	4	6	4	4	2	4	6	5	**0**	4
PpdeD01_25	B545	*P. pseudodelicatissima*	2	3	2	2	2	3	3	3	2	**0**
PpdeD02_25	B545	*P. pseudodelicatissima*	1	4	1	1	2	1	5	4	3	**0**

Boldface '0' entries indicate expected positive reactions; other figures in boldface denote observed false positive reactions.

delicatissima (B549; two mismatches), *P. delicatissima* new clade (B498; two mismatches), *P. galaxiae* (B510; two mismatches) and *P. multistriata* (B531; six mismatches). The *Pseudo-nitzschia mannii* probe Pcal2D03_25 hybridised also with the PCR products of *P. fraudulenta* (B570; five mismatches), *P. multistriata* (B531; five mismatches) and *P. pseudodelicatissima* (B545; seven mismatches), and the *P. multistriata* probe PmulaD02_25 hybridised also with the PCR product of *P. fraudulenta* (B570; two mismatches). The relative intensity of cross-reacting signals versus the specific signal is shown in Table 4.

Possible explanations for the cross-reaction are as follows: (1) secondary structure formation, (2) insufficient quality of the annealing target (the PCR product), (3) intra-individual polymorphism among the LSU rRNA gene copies of non-target strains and (4) contaminant DNA from other species in PCR reaction mixes. Secondary structure formation can render a target region inaccessible to a probe (see e.g. Chandler et al. 2003). However, this phenomenon cannot explain failure of probes to anneal with their target in our experiments. Probes Pcal2D01_25, Pcal2D02_25 and PpdeD02_25 failed to anneal with their intended target region between positions 100 and 200 in our LSU alignment. Yet, probe Pcal2D03_25 annealed properly with its target situated within the same region. The other failing probes were targeted to regions between alignment positions 400 and 700, but some of the functioning probes exhibited target regions at these positions as well. Thus, annealing failure is probably not position dependent.

Insufficient quality of the annealing target (the PCR product) could in theory result in probe failure (Kegel et al. 2013). This could explain the failure of the two probes specific against *P. delicatissima* to react with the PCR products of strain B549. These probes exhibited T_m values close to 75 °C and moderately negative ΔG values. In any case, the PCR products of all other species annealed with at least one of their specific probes, demonstrating that these PCR products were of adequate quality and quantity.

Intra-individual polymorphism among the LSU rRNA gene copies of non-target strains could, in theory, explain the cross-reactions of four of the probes. The LSU sequence is part of a ribosomal cistron, occurring in tandem repeats in each genome (Alvarez and Wendel 2003). Concerted evolution tends to homogenise differences among these cistrons (Alvarez and Wendel 2003), but other processes introduce

Table 4 Dot-blot results of species-specific- and group-specific probes against PCR product of the LSU rDNA from a series of strains of *Pseudo-nitzschia* species

Strain	Species	Probe	Hybridisation temp. °C	Specific reaction	Cross-reaction with PCR product of strain:							
					B569	B513	B549	B498	B570	B510	B531	B545
B569	*P. arenysensis*	Pdel3D01_25	65	+								
B513	*P. calliantha*	Pcal1D01_25	65	+	S				E			E
B549	*P. delicatissima*	Pdel1D03_25	65	−								
		Pdel1D01_25	62	−								
B498	*P. delicatissima* new clade	Pdel2D02_25	62	+								
		Pdel2D01_25	62	+								
		Pdel4D01_25	65	+								
	P. delicatissima complex	PdeliD02_25	65	−								
B570	*P. fraudulenta*	PfrauD02_25	65	+								
		PfrauD04_25	65	+								
B510	*P. galaxiae*	PgalaD01_25	65	−								
		PgalaD02_25	62	+								
		PgalaD04_25	62	+								
B528	*P. hasleana*	PvigoD01_25	65	+	W	E	W	W		W	W	E
		PvigoD03_25	65	+								
B512	*P. mannii*	Pcal2D01_25	65	−								
		Pcal2D02_25	65	−								
		Pcal2D03_25	65	+					E		E	E
		Pcal2D05_25	65	−								
B531	*P. multistriata*	PmulaD02_25	65	+					S			
		PmulaD03_25	65	+								
B545	*P. pseudodelicatissima*	PpdeD01_25	65	+								
		PpdeD02_25	62	−								

Specific reaction denotes reaction with PCR product of intended target species. Cross-reaction intensity relative to specific signal: S = cross-reaction markedly stronger than the specific one, E = cross-reaction and specific signals of similar intensity and W = cross signal very weak relative to the specific one

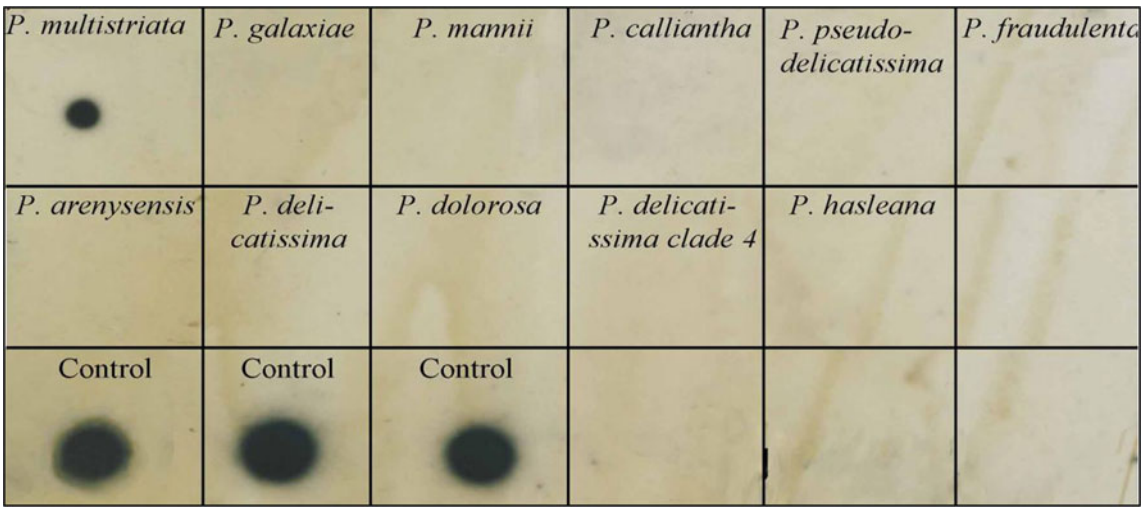

Fig. 1 Example of probe–target response on dot-blot membrane; the LSU PCR product of reported *Pseudo-nitzschia* species has been spotted on nylon membrane. The membrane has been hybridised with labelled PmulaD03_25 probe. On the bottom, the DNA labelled control spotted in triplicate is reported

them just as fast because most diatoms exhibit such polymorphism (Alverson and Kolnick 2005; Beszteri et al. 2005; Kooistra et al. 2008; Lundholm et al. 2003; reviewed in Alverson 2008). Polymorphism may result from introgression or incomplete lineage sorting (Wendel and Doyle 1998). Casteleyn et al. (2009) presented evidence for sexual reproduction (hybridisation) between related species in the *Pseudo-nitzschia pungens* complex. In contrast, crossing experiments by Amato et al. (2007) indicated reproductive isolation among closely related species within the *P. delicatissima* and *P. pseudodelicatissima* complexes. Nonetheless, Lundholm et al. (2006) demonstrated cross-reactivity of probes between species in the *P. delicatissima* complex, which suggests that these species still share LSU copies with identical target regions because of incomplete lineage sorting.

The obtained results show no evidence that intra-individual polymorphism is responsible for the cross-reactions. The marked cross-reaction of the *P. calliantha* probe Pcal1D01_25 with PCR products of B569, B570 and B545 would imply that these strains possess a marked proportion of LSU's typical for *P. calliantha.* Yet, sequenced PCR products of their LSU region reveal no trace of that (results not shown). Cross-reactions of PvigoD01_25 with PCR products of B569, B513, B549, B498, B510, B531 and B545 cannot be explained by the presence of LSU sequences typical for *P. hasleana* in these strains because the other probe for this species (PvigoD03_25) showed no such cross-reactivity. In addition, none of the sequenced LSU PCR products of these strains revealed any trace of *P. hasleana* sequences (results not shown). Similarly, the strong cross-reaction of the *P. multistriata* probe PmulaD02_25 with the PCR product of B570 (*P. fraudulenta*) cannot result from the presence of *P. multistriata* LSU sequences in this strain because the other *P. multistriata* probe, PmulaD03_25, did not show any cross-reactivity and neither did sequence reads of the LSU PCR product of strain B570 show any trace of *P. multistriata* sequences. Cross-reaction of Pcal2D03_25 with the PCR products of B570, B531 and B545 cannot be due to the presence of LSU's typical for *P. mannii* in these strains because sequence reads of their LSU PCR products show no trace of that.

The a-specific reactions do not result from near-matches between probes and their targets because the target regions of all non-target strains exhibited between two and seven mismatches and hybridisation conditions were stringent at 65 or 62 °C (see Table 4) to prevent any non-specific reactions. Moreover, signal strength of probe PvigoD01_25 with PCR products of B569, B513, B549, B498, B510, B531 and B545 was unrelated to the number of nucleotide mismatches between probe and target regions. In a few cases, a markedly high T_m for probes PvigoD01_25 (79 °C) and PmulaD02_25 (81.0 °C) could have resulted in the probe binding a-specifically at the hybridisation condition of 65 °C, but then it remains to be explained why other probes with similarly high T_m values were, instead, specific.

In theory, contaminant DNA from other species in PCR reaction mixes could generate the observed cross-reactions. However, this explanation can be ruled out because results of negative-control PCR reactions showed no trace of any PCR product. In addition, if such contaminant DNA was present, then it should have been detectable also in other PCR products, and it should be traceable in sequence reads. The cross-reactions did not result from cultures being "polyclonal" because all of them were checked and found to be monoclonal. Thus, in short, we lack an explanation for the cross-reactions.

Eight probes did not react at all. These included all probes against *P. delicatissima* (Pdel1D01_25 and Pdel1D03_25); probe PdeliD02_25 against the *P. delicatissima* complex; probes Pcal2D01_25, Pcal2D02_25 and PcalD05_25 against *P. mannii*; probe PgalaD01_25 against *P. galaxiae* and probe PpdeD02_25 against *P. pseudodelicatissima* and *P. cuspidata* (Table 4). Extremely high or low melting temperatures (T_m) and/or a high propensity to form hairpins or probe dimers (very negative ΔG) could explain, in theory, the failure of the probes. However, in the results of the hybridisation experiments presented in the present study, these phenomena do not explain failure of probes to anneal with their targets. Failure of Pcal2D01_25, Pcal2D05_25 and PpdeD02_25 to anneal with their intended target could be due to very negative ΔG values and/or a high T_m, but these factors do not explain the failure of probes Pcal2D02_25, Pdel1D01_25, Pdel1D03_25, Pdeli D02_25 and PgalaD01_25 because the latter exhibit T_m values close to 75 °C and only moderately negative ΔG values. In contrast, PfrauD04_25, PvigoD03_25, Pcal2D03_25 and PmulaD02_25 all annealed in spite of their extreme values for T_m and/or ΔG. Failure was not due to low melting temperatures either because none of the probes exhibited a melting temperature lower than 74.1 °C.

What do the dot-blot results teach us about the unexpected microarray results?

In the microarray procedure deployed by Barra et al. (2013), total rRNA extracted from eight monoclonal cultures (*P. arenysensis*, *Pseudo-nitzschia australis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. galaxiae*, *Pseudo-nitzschia multiseries* and *P. multistriata*) was hybridised against series of species-specific and generic probes immobilised onto a microscope slide (microarrays).

Their results, when compared with expectations based on cell counts of each of the species—or morphologically highly similar groups of species—as recognisable in light microscopy, showed the presence of several false-positives (cross-reacting probes) and false-negatives (probes that did not give a signal). Here, we focus especially on those probes that generated problematic results on the microarrays (a-

specific or not working), grouping them according to their different behaviour between the two methods. The probes against *P. delicatissima* clade 4, *P. hasleana*, *P. mannii* and *P. pseudodelicatissima* were not tested with their species-specific rRNA in the microarray procedure (Barra et al. 2013). Therefore, results obtained in the present study with these probes have been excluded from the comparison. For the probes that showed cross-reactivity on microarrays, results on the dot-blots were as follows. Three probes (Pdel3D01_25, PfrauD02_25 and PfrauD04_25) were specific; these probes will benefit from fine adjustments of the microarray hybridisation protocols. Two probes (Pdel1D01_25, Pdel1D03_25) did not work and one (PmulaD02_25) was also a-specific rendering their suitability doubtful. The same is true for probes that did not give a signal on the microarray but showed a-specificity (Pcal1D01_25) or no reaction (PdeliD02_25, PgalaD02_25) on the dot-blot. Notably, none of the dot-blot-tested probes that were specific on the microarray cross-reacted on the dot-blot, although two of those probes (PgalaD04_25, PgalaD01_25) did not show any signal on the dot-blot. In general, the dot-blot procedure showed a higher level of probe specificity than the microarrays, as expected from the nature of the method itself.

Results of microarray analyses (Barra et al. 2013) revealed that the rRNA of some species reacted indiscriminately, and at times strongly, with a large number of probes. Such high levels of cross-reactivity could be due to problems with rRNA quality, its labelling efficiency or the choice of a correct internal normalisation standard. Alternatively, these high levels could be due to problems with secondary structure formation of the probes or mismatches to their targets due to genetic variants in natural samples. In the microarray method, a major limiting factor for a successful experiment is the quality and quantity of the ribosomal RNA, which includes a target within a whole range of potentially unstable RNAs. Instead, in the dot-blot method, the quality of the probes (perfect fit to target, no probe dimers or hairpins) seems to be the most important factor; the target is located not on a ribosomal RNA but instead on a PCR product, which consists of more stable DNA.

In cases in which probes cross-reacted on the microarray, but generated a specific result on the dot-blot, we expect that improved RNA quality and/or more fine-tuned hybridisation conditions will improve the specificity of microarray results, though we cannot exclude the existence of alternative target regions elsewhere in the ribosomal RNA's. In any case, probes that cross-reacted also on the dot-blot, or failed to generate a signal, should be omitted from further testing. Interestingly, the probes that failed to generate a signal on the microarray also failed to do so on the dot-blots, or reacted a-specifically, and can be eliminated as well.

A compromise between the two techniques could be the use of PCR products from environmental samples in microarray hybridisations. Smith et al. (2012) explored the usefulness of probes against target sequences in the ITS region of the nuclear rRNA gene cistron for the detection of *Pseudo-nitzschia* species using PCR products. The ITS-1 and ITS-2 regions evolve much faster than the LSU region and, therefore, offer ample opportunities for the design of species-specific probes. However, the ITS regions of diatom species are also notorious for their intra-individual variation (Behnke et al. 2004; Beszteri et al. 2005; D'Alelio et al. 2009; Vanormelingen et al. 2007). Moreover, the ITS regions are not incorporated in the mature ribosomes and, thus, are no good targets for rRNA-based methods. Therefore, Smith et al. (2012) obtained PCR products generated from DNA isolated from environmental samples instead of obtaining RNA isolated directly from these environmental samples. Their results showed species-specific responses for all their species. However, routine PCR-based monitoring methods need to be carried out using rigorous procedures of cleanliness because otherwise they will be prone to generate false-positives.

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