diversity

MDPI

Article

Identification of *Pseudo-nitzschia* Cryptic Species Collected in the Gulf of Naples Using Whole-Cell Fluorescent In Situ Hybridization: From Cultured Sample to Field Test

Michele Ferrari [1], Lucia Barra [2,*], Luisa Ruffolo [1], Antonella Muto [1], Christian Galasso [2], Isabella Percopo [3], Silvestro Greco [4] and Radiana Cozza [1,*]

[1] Department of Biology, Ecology and Earth Science, University of Calabria, Ponte P. Bucci, Arcavacata di Rende, 87036 Cosenza, Italy
[2] Department of Ecosustainable Marine Biotechnology, Stazione Zoologica Anton Dohrn, C. da Torre Spaccata, 87071 Amendolara, Italy
[3] Research Infrastructures for Marine Biological Resources Department, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy
[4] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, C. da Torre Spaccata, 87071 Amendolara, Italy
* Correspondence: lucia.barra@szn.it (L.B.); radiana.cozza@unical.it (R.C.)

Abstract: The planktonic diatom genus *Pseudo-nitzschia* contains several genetically closely related species that can produce domoic acid, a potent neurotoxin known to cause amnesic shellfish poisoning (ASP). An early identification and an adequate monitoring of the potential toxic *Pseudo-nitzschia* spp. are necessary. However, effective monitoring programs are time consuming due, in some cases, to the cell morphology similarities among species, determined with light microscopy, that can result in insufficient data to give a definitive species and toxins attribution. In this paper, Whole-Cell Fluorescent In Situ Hybridization (WC-FISH) has been evaluated as a powerful tool to detect and enumerate harmful cryptic and/or pseudo-cryptic *Pseudo-nitzschia* spp. collected in the Gulf of Naples. Fluorescently labelled probes directed against the ribosomal RNA (rRNA) of the 28S large subunit (LSU) were used. In particular, five probes detecting four cryptic species of *Pseudo-nitzschia* delicatissima complex and one specific for *Pseudo-nitzschia multistriata* gave good results for the molecular identification of potentially toxic target species in natural samples. Finally, we can state that the WC-FISH method, to identify *Pseudo-nitzschia* species, is faster and more cost-effective if compared with other rDNA-based methods.

Keywords: fluorescent probes; molecular identification; harmful algae; hybridization; *Pseudo-nitzschia pseudodelicatissima/delicatissima* complex; ribosomal RNA

Citation: Ferrari, M.; Barra, L.; Ruffolo, L.; Muto, A.; Galasso, C.; Percopo, I.; Greco, S.; Cozza, R. Identification of *Pseudo-nitzschia* Cryptic Species Collected in the Gulf of Naples Using Whole-Cell Fluorescent In Situ Hybridization: From Cultured Sample to Field Test. *Diversity* **2023**, *15*, 521. https://doi.org/10.3390/d15040521

Academic Editor: Bert W. Hoeksema

Received: 24 February 2023
Revised: 30 March 2023
Accepted: 2 April 2023
Published: 4 April 2023

1. Introduction

The monitoring of the *Pseudo-nitzschia* spp. is of fundamental importance because of the worldwide distribution of these marine planktonic diatoms producing harmful compounds. Among toxins, domoic acid (DA) is produced by the *Pseudo-nitzschia* spp. under certain conditions [1] and it can bioaccumulate along the food chain causing damage to mammals and human health with symptoms of different nature, such as ataxia, head weaving, muscle tremor, titanic convulsions, rubbing, and lethargy [2–6].

Multiple toxigenic *Pseudo-nitzschia* species frequently coexist in the same environment, even during bloom events that appear to be dominated by a single species [7–9].

Since the genus *Pseudo-nitzschia* includes many species, their accurate taxonomic identification is important since they can be associated with domoic acid production [10].

Unfortunately, all *Pseudo-nitzschia* species have a very similar gross morphology, which makes them difficult and often impossible to identify at the species level with light microscopy. Although species determination, in some cases, can be carried out with

electron microscopy, which allows for the observation of the main diagnostic characters, such as the presence/absence of the central larger interspace or the number and structure of fibulae, striae, and poroids, molecular approaches over time have become an increasingly important supplement, constituting as an essential tool for the identification of the several cryptic/pseudocryptic species now belonging to this genus [11,12].

Indeed, new cryptic and pseudo-cryptic species have recently been described within the *P. pseudodelicatissima* [10,13,14] and *P. delicatissima* complex [15–17].

More recent molecular approaches, such as qPCR, ARISA, microarray, and dot blot hybridization systems have been used for specific and sensitive *Pseudo-nitzschia* species identification and/or quantification from clonal cultures [18–21]. Molecular methods are essentially based on the evaluation of the sequence variation of oligonucleotide primers and/or probes in target nucleotide regions and allow the accurate identification of various phytoplanktonic toxic species [22–24].

In this paper, Fluorescence In Situ Hybridisation (FISH) has been evaluated as a powerful tool to detect and enumerate harmful microorganisms in the marine environment. Different FISH methods are available and, especially in combination with automated counting techniques and the development and updating of oligonucleotide probes able to discriminate toxic species, can be attainable for routine monitoring of harmful marine microalgae [25–32]. However, FISH-based methods are not yet regularly included in monitoring programs tracking the presence of harmful marine microalgae. A limitation factor of the FISH technique is the currently available number of suited fluorochromes attached to the FISH probes to detect various harmful species in one environmental sample at the same time. However, coupled automated techniques, like biosensors, microarrays, and quantitative polymerase chain reaction (qPCR) can facilitate the analysis of numerous field samples and help to overcome this drawback [33]. A great benefit of FISH compared to other molecular detection methods of harmful algal blooms is the direct visualisation of the hybridised target cells (whole cell FISH), which are not allowed in cell free formats, as DNA-dependent analysis methods [34].

In this study, we applied the Whole-Cell Fluorescent In Situ Hybridization (WC-FISH) on cryptic and/or pseudo-cryptic species of the genus *Pseudo-nitzschia* collected in the Gulf of Naples. We used fluorescently labelled probes directed against the ribosomal RNA (rRNA) of the 28S large subunit (LSU). Primers designed on DNA ribosomal large subunit (LSU), targeted on its D1 hyper-variable region, identified the most *Pseudo-nitzschia* species [35]. Oligoprobes, designed on LSU hypervariable region of 28S, of rDNA developed to build up the microarray for toxic *Pseudo-nitzschia* spp. detection [21] have been used in this study to reveal *Pseudo-nitzschia* spp. in natural samples through WC-FISH. The work was carried out to obtain a semi-quantitative estimation of the presence of potentially toxic *Pseudo-nitzschia* cryptic/pseudo-cryptic species in environmental samples by applying the WC-FISH.

2. Materials and Methods

2.1. Pseudo-nitzschia Cultivation

Experiments were carried out with selected strains of *Pseudo-nitzschia* species established from single chains of cells, isolated at the Long Term Ecological Research Station MareChiara (LTER-MC, 40°48.5′ N, 14°15′ E) in the Gulf of Naples. Nine different species of *Pseudo-nitzschia* were used: four belonging to the *P. delicatissima* complex (*P. allochrona*, *P. arenysensis*, *P. delicatissima*, *P. dolorosa*) and two belonging to the *P. pseudodelicatissima* complex (*P. calliantha*, *P. pseudodelicatissima*) together with non-cryptic *P. fraudulenta*, *P. galaxiae*, and *P. multistriata*. The reason for choosing the strains selected is to develop a method for discriminating between toxic and non-toxic species for a person with no training in the genus *Pseudo-nitzschia*. In addition, the reason was to also identify among the species belonging to the same complex the so-called cryptic or pseudo-cryptic ones, which often include both toxic and non-toxic species (as in the case of *P. delicatissima* complex, in which *P. delicatissima* is toxic while the cryptic *P. arenysensis* and *P. allochrona* are not toxic).

All *Pseudo-nitzschia* species were grown in 50 mL flasks containing 35 mL Guillard's f/2 medium [36] at 22 °C on a 16:8 h light:dark cycle at an irradiance of 100 µmol photons m^{-2} s^{-1}. Growth of each species was monitored every 24 h by sedimentation of 1 mL of culture in Sedgewick Rafter Counting Chamber [37] and counted using a Leica DMLD inverted microscope. *Pseudo-nitzschia* cells at the mid/end of the exponential phase were used for further in situ hybridization experiments.

2.2. Whole-Cell Fluorescent In Situ Hybridization (WC-FISH) on Pseudo-nitzschia Species Monocultures—Probes Testing and Cross-Reactivity Assays

The WC-FISH protocol used in this work was optimised starting from the methods of Miller and Scholin (1998, 2000) [25,38] and Groben and Medlin (2005) [39] with some minor modifications. An aliquot (25 mL) of each culture at mid-exponential phase was filtered on 0.8 µm isopore polycarbonate membrane filters (Millipore™, Darmstadt, Germany) using a vacuum filtration apparatus. The filters were incubated at 4 °C for 2 h in a modified saline–ethanol fixative freshly prepared by mixing 22 mL 95% ethanol, 5 mL deionized H_2O, and 3 mL 25× SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl, at pH 7.8) [38]. Then, the fixative solution was gently vacuum removed (~100 mmHg), and filters were immediately processed for WC-FISH; alternatively, they were stored at −20 °C [38]. For the pre-hybridization step, the filters were incubated in 5× SET buffer for 5 min at RT and treated with dimetil-formamide (DMF, 50%) for 1 h at RT to remove the chlorophyll autofluorescence and washed with 5× SET buffer for 5 min at RT. Then, each filter was cut into 12 sections, and placed on a 24-well plate minibasket (Thermo Scientific™, Rockford, IL, USA) in hybridization buffer (5× SET buffer, 0.1% *v*/*v* IGEPAL-CA630-octylphenoxypolyethoxyethanol, 30 µg mL^{-1} poly(A), 40% formamide) containing 50 ng μL^{-1} of the specific probes; then, the filters were incubated at 45 °C for 2 h. During initial testing, cultures were hybridized in separate reactions by using their probes labelled with fluorescein 5-isothiocyanate (FITC) at the 5′ end (Thermo Scientific™, Rockford, IL, USA). The probes tested and the targeted species are reported in Table 1.

Table 1. Oligonucleotide probes used for *Pseudo-nitzschia* WC-FISH. The table shows the complex species, name probe, sequence, target species, target genes, and melting temperature (Tm).

Complex	Probe [a]	Sequence (5′-3′)	Target Species	Target Gene	Tm (°C)
P. delicatissima	Pdel4D02_25	GATTGTGCAAATATCCAACCACTGT	*P. allochrona*	28S	66.6
	Pdel4D03_25	TGACAACGACTCACTCTACCAGGC	*P. allochrona*	28S	69.4
	Pdel3D01_25	GACAAAAACTCACTCTACCAGGCGG	*P. arenysensis*	28S	69.5
	Pdel3D02_25	TAATGTTAAAGTCTATAGACCACAA	*P. arenysensis*	28S	55.5
	Pdel2D01_25	TCCAACCACTGTTACTTTCATTACG	*P. delicatissima*	28S	65.6
	Pdel1D01_25	TTGACAACGACTCACTCCACCAGG	*P. dolorosa*	28S	71.5
P. pseudodelicatissima	Pman2D03_25	CTTCAGACCACAATTCGGCGCTTAAA	*P. calliantha*	28S	65.6
	PpdelD02_25	CCCGGCAGATAACGTCAAGGTCTAT	*P. pseudodelicatissima*	28S	70.4
	PNFRAGA	ATTCCACCCAAACATGGC	*Pseudo-nitzschia* spp.	18S	63.3
	PfrauD04_25	ACGGGAGTTTCACCCTCTCAGCTGTC	*P. fraudulenta*	28S	66.3
	PgalaD02_25	CCAAAGGAATCAACCAAAGCAAACC	*P. galaxiae*	28S	71.8
	PmulaD03_25	AACCCAAACTCACGAAAGCTCACAG	*P. multistriata*	28S	69.8
Controls [a]	Uni-C	GWATTACCGCGGKGCTG	Eukaryotic	18S	64.6
	Uni-R	CAGCMGCCGCGGUAAUWC	Prokaryotic	16S	60.6

[a] Uni-C is the positive control, SSU-targeted universally conserved sequence (519r [40]); Uni-R is the complement of Uni-C.

Nine probes tested in dot blot assays for their specificity species were designed to target the large subunit (LSU, 28S) ribosomal DNA of *Pseudo-nitzschia* species [41]. The ARB (from 2005), now SILVA, database alignment was screened for signature positions for the nine species using the "probe design" function of the program package ARB [42].

The specificity of the potential probes was then tested in silico in ARB and by BLAST searches. The probes were examined for hairpin loops and primer dimer formation using the software Oligo 5 (http://www.oligo.net (accessed on 15 May 2012)). The position of probes in the RNA's secondary structure was checked within ARB [41]. The samples were also probed with the positive control probe Uni-C (directed toward universally conserved eukaryotic sequences of 18S rRNA), the negative control probe Uni-R (directed toward universally conserved prokaryotic sequences of 16S rRNA) [38], and hybridization without probe. After the hybridization, the unbound probe was removed with post-hybridization washes with 5× SET buffer; concentration of washing buffer, temperature, and washing time were optimised to obtain the most intense and specific fluorescence signal of the probe compared with positive control (Uni-C). Images of cells post-hybridization were captured using a Leica DMRB fluorescence microscopy and Leica camera system at 100× after mounting the filter pieces on slides with Citifluor Antifade Mountant (Thermo Fisher Rockford, IL, USA)/DAPI (1 μg mL^{-1}) 2:1 (*v*/*v*). In order to identify probes' cross-reactivity, each probe was also hybridised against all non-target species in the same condition established in the preliminary probe tests. After the hybridization, the stringency of the post-hybridization washing (i.e., concentration of 5× SET washing buffer and washing temperature) were strictly optimised in order to remove the nonspecific bounds and to avoid the cross-reaction problems.

2.3. WC-FISH on Artificial Samples

WC-FISH test was applied to artificial samples consisting of both unialgal cultures and mixed cultures (simulated field samples). For the unialgal culture samples, 25 mL of monoculture at mid-exponential phase of the cryptic species belonging to the *P. delicatissima* complex (*P. allochrona*, *P. arenysensis*, *P. delicatissima)* and *P. multistriata* were respectively filtered and hybridised using the above established protocol. The simulated field samples were prepared by adding 25 mL of the cryptic monoculture *P. arenysensis* to 25 mL of *P. multistriata* and also by adding 25 mL of *P. allochrona* at the previous strain mix. All the monocultures were utilised at a mid-exponential phase. Then, the two different simulated field samples were filtered and hybridised as the above established protocol. The relative cell number of both single monocultures and the simulated field samples was estimated with the Utermöhl method in light microscopy. Counting was carried out at 400× magnification in random visual fields to count a minimum of 500 cells, and the cell density was expressed as mean ± standard error (cells mL^{-1}) (n = 3). After the WC-FISH, the labelled fluorescent cells were counted with a Leica DMRB fluorescence microscopy viewing the entire surface of the filter, and the value (cells mL^{-1}) was expressed as mean ± standard error (n = 3). For the different samples, the detection efficiency (%) of the WC-FISH test was evaluated as the ratio between the cell density detected by the epifluorescence microscope after WC-FISH vs. Utermöhl counting method by light microscope before WC-FISH.

2.4. WC-FISH on Environmental Samples

The screened probes were then tested using field samples from the site Long-Term Ecological Research station MareChiara (40°48.5′ N, 14°15′ E) in the Gulf of Naples (Tyrrhenian Sea) that were routinely collected as part of a long-term ongoing study of phytoplankton monitoring. The samples used were those collected from May 2011 to April 2012. Cell counting was done by using light microscopy on the samples fixed in 0.6% formaldehyde and then 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 at 400× magnification along transects (minimum of 200 cells); cell density was expressed as mean ± standard error, cells mL^{-1} (n = 3). For WC-FISH, volume samples of 50 or 75 mL were filtered on 0.8 μm isopore polycarbonate membrane filters (Millipore™, Darmstadt, Germany) fixed in a modified saline–ethanol at 4 °C for 2 h as reported above and hybridised as established by protocol. The probes against the cryptic species *P. allochrona*, *P. arenysensis*, *P. delicatissima*, *P. dolorosa* labelled with fluorescein isothiocyanate -FITC (Thermo Scientific™, Rockford, IL, USA)

were hybridised at the same time but on different filters sections; simultaneously, the probe against *P. multistriata* was labelled with Cyanine-Cy3 (Thermo Scientific™, Rockford, IL, USA) and used.

3. Results

3.1. Probes Testing and Cross-Reactivity Trials

Species-specific candidate probes labelled with FITC fluorophore were tested on *Pseudo-nitzschia* target species monocultures (Table 1). Cells of each pure culture were hybridised with their species-specific probes, and the best WC-FISH hybridization conditions for each probe were established (Table 2). Washing temperature and washing buffer concentration were optimised to obtain a good epifluorescent signal as the visual intensity of the probe fluorescence of each target species in comparison with the positive control (Uni-C) (Figure 1). Essentially, in order to use multiplex probes for the subsequent field sample analysis and to ensure the specificity of the probe, even if at a slightly different melting temperature, we decided to maintain a fixed hybridization temperature and FA concentration. For the same reason, we tested different stringency conditions by regulating both the washing and temperature buffer concentration (Table 2). As a result of protocol optimization, the cells showed a uniform distribution of bright fluorescence throughout the cell except for the nuclear region, where fluorescence was confined to nucleoli (Figure 1). Treatments with a negative control probe (Uni-R) showed samples consistently dark, demonstrating no nonspecific binding or autofluorescence (Figure 1).

Table 2. Probes testing and WC-FISH optimisation. The table shows hybridization temperature, formamide concentration (FA), temperature, and concentration of post-hybridization buffer and epifluorescent signal. Cells with signal intensity similar to the positive control were scored as '++'.

Probe	Target Species	Hybridization T (°C)	FA (%)	Washing T (°C)	Washing Buffer Concentration	Epifluorescent Signal
Pdel4D02_25	*P. allochrona*	45	40	55	0.5×	++
Pdel4D03_25	*P. allochrona*	45	40	55	0.5×	++
Pdel3D01_25	*P. arenysensis*	45	40	55	0.5×	++
Pdel3D02_25	*P. arenysensis*	45	40	55	0.5×	++
Pdel2D01_25	*P. delicatissima*	45	40	55	0.5×	++
Pdel1D01_25	*P. dolorosa*	45	40	55	0.5×	++
Pman2D03_25	*P. calliantha*	45	40	55	0.5×	++
PpdelD02_25	*P. pseudodelicatissima*	45	40	50	0.5×	++
PfrauD04_25	*P. fraudulenta*	45	40	55	2×	++
PgalaD02_25	*P. galaxiae*	45	40	55	2×	++
PmulaD03_25	*P. multistriata*	45	40	55	0.5×	++

To identify any cross-reactivity at this calibrated condition, each probe was hybridised against the different *Pseudo-nitzschia* species. Determination of cross-hybridizations was based on visual intensity of fluorescence in comparison with non-target cells; the positive (Uni-C) and negative (Uni-R) control treatments defined a range of labelling intensities providing a visual reference to assess the reactivity of specific probes (Table 3). The results showed that probes Pdel4D03_25, Pdel2D01_25, and PmulaD03_25 exclusively detected their target species *P. allochrona*, *P. delicatissima*, and *P. multistriata*, respectively (Table 3); instead, the probes Pdel3D01_25 and Pdel1D01_25 also weakly detected *P. fraudulenta*; PpdelD02_25 also detected *P. galaxiae* and *P. calliantha*; PgalaD02_25 detected both *P. pseudodelicatissima* and *P. calliantha*; Pman2D03_25 detected both *P. pseudodelicatissima* and *P. galaxiae;* and PfrauD04_25 hybridised with both *P. arenysensis* and *P. dolorosa* species (Table 3).

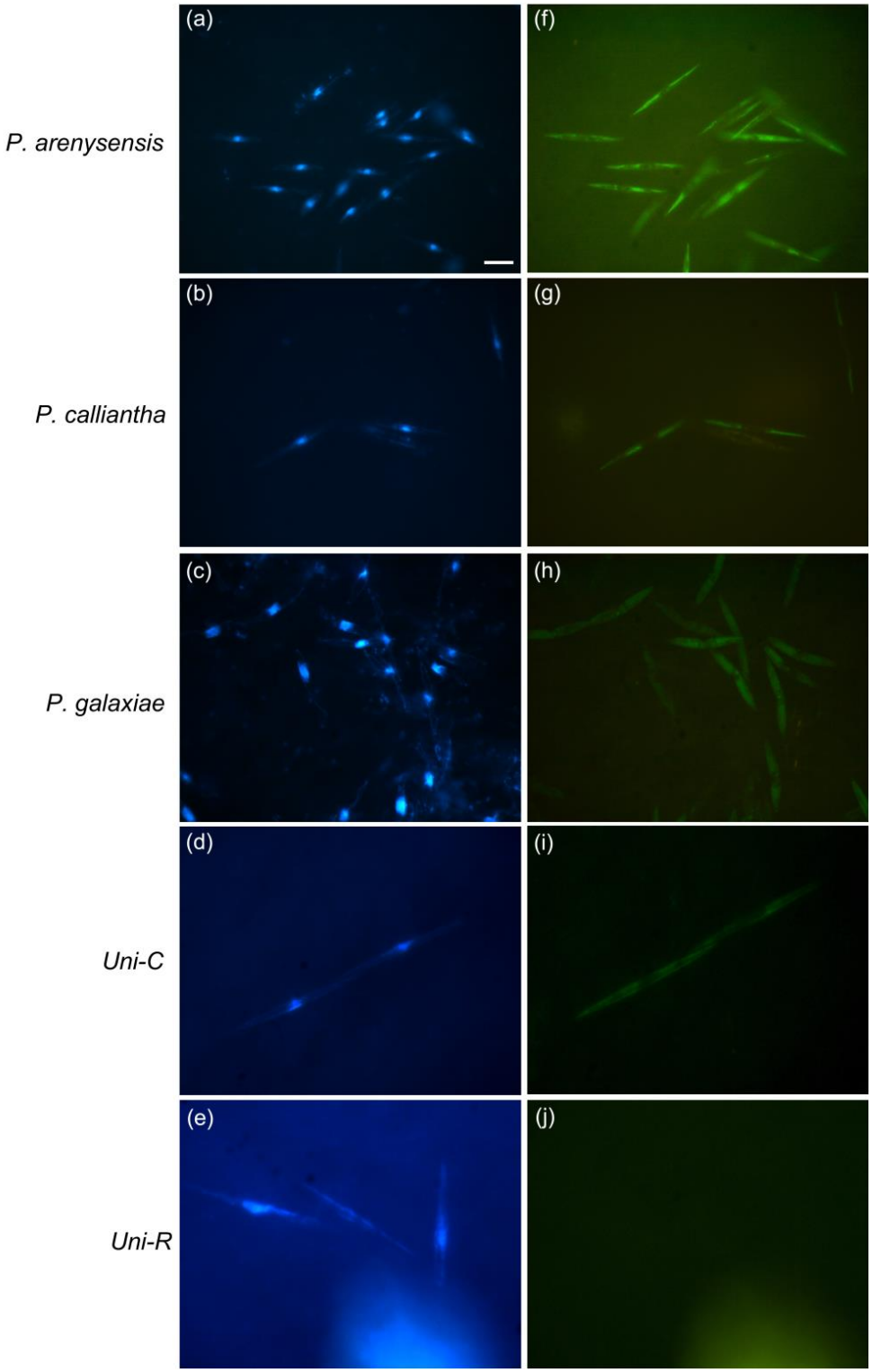

Figure 1. Images of WC-FISH assays of pure culture of some cryptic *Pseudo-nitzschia* species hybridised to their species-specific probes. Each row of micrographs displays cells of pure cultures under epifluorescence microscopy in DAPI (**a**–**e**) and FITC (**f**–**j**) filters. The last two rows of micrographs display the hybridization with Uni-C (positive control) (**d**,**i**) and Uni-R (negative control) (**e**,**j**) probes. Scale = 10 µm.

Table 3. Cross-hybridization trials. Specificity of probes against different *Pseudo-nitzschia* species. Probes that successfully hybridised were scored as positive "++" (highly visible) and "+" (weakly visible) under the epifluorescent microscope; probes that failed to detect a culture were scored as negative "–".

Species	Probe										
	Pdel4D03_25	Pdel3D01_25	Pdel2D01_25	Pdel1D01_25	Pman2D03_25	PpdelD02_25	PfrauD04_25	PgalaD02_25	PmulaD03_25	Uni-C	Uni-R
P. allochrona	++	–	–	–	–	–	–	–	–	++	–
P. arenysensis	–	++	–	–	–	–	+	–	–	++	–
P. delicatissima	–	–	++	–	–	–	–	–	–	++	–
P. dolorosa	–	–	–	++	–	–	+	–	–	++	–
P. calliantha	–	–	–	–	++	+	–	+	–	++	–
P. pseudodelicatissima	–	–	–	–	+	++	–	+	–	++	–
P. fraudulenta	–	+	–	+	–	–	++	–	–	++	–
P. galaxiae	–	–	–	–	+	+	–	++	–	++	–
P. multistriata	–	–	–	–	–	–	–	–	++	++	–

After several tests, the recalibration of the WC-FISH condition by increasing post-hybridization temperature and/or buffer washing stringency allowed it to successfully overcome some cross hybridizations (Tables 4 and 5). In particular, we eliminated the cross-hybridization for all the probes of the *P. delicatissima* complex (Pdel1D01_25, Pdel2D01_25, Pdel4D02_25, and Pdel4D03_25) which continues to retain a strong signal (++) against their target cells (Table 5); on the contrary, it was very difficult to completely remove the cross-hybridization of *P. pseudodelicatissima* complex probes (PpdelD02_25, PgalaD02_25, and Pman_25) despite the new hybridization conditions (Table 5). Therefore, for these probes, no other attempts were made to optimise the whole-cell hybridization conditions, and they were excluded from the subsequent trials.

Table 4. WC-FISH optimization. Hybridization temperature, formamide concentration (FA), temperature, and concentration of post-hybridization buffer to avoid cross-hybridization of the selected probes.

Probe	Target Species	Hybridization T (°C)	FA (%)	Washing T (°C)	Washing Buffer Concentration
Pdel4D02_25	*P. allochrona*	45	40	55	2×
Pdel3D01_25	*P. arenysensis*	45	40	58	0.5×
Pdel2D01_25	*P. delicatissima*	45	40	45	5×
Pdel1D01_25	*P. dolorosa*	45	40	58	0.5×
Pman2D03_25	*P. calliantha*	45	40	55	0.5×
PpdelD02_25	*P. pseudodelicatissima*	45	40	55	2×
PfrauD04_25	*P. fraudulenta*	45	40	55	2×
PgalaD02_25	*P. galaxiae*	45	40	58	5×
PmulaD03_25	*P. multistriata*	45	40	58	2×

Based on these results, it may be speculated that by using the new calibrated conditions for WC-FISH experiments, the screened probes could be useful for the molecular identification of the target species and also in natural samples containing many different microalgae. To this aim, we proceeded to their validation as reported below.

Table 5. Cross-hybridization trials. Cross-reactivity of the screened probes after WC-FISH optimization conditions. Probes that successfully hybridised were scored as positive "++" (highly visible) and "+" (weakly visible) under the epifluorescent microscope; probes that failed to detect a culture were scored as negative "–". The species/probes still retaining cross-hybridization signals are in bold.

Species	Probe										
	Pdel4D03_25	**Pdel3D01_25**	**Pdel2D01_25**	**Pdel1D01_25**	**Pman2D03_25**	**PpdelD02_25**	**PfrauD04_25**	**PgalaD02_25**	**PmulaD03_25**	**Uni-C**	**Uni-R**
P. allochrona	++	–	–	–	–	–	–	–	–	++	–
P. arenysensis	–	++	–	–	–	–	–	–	–	++	–
P. delicatissima	–	–	++	–	–	–	–	–	–	++	–
P. dolorosa	–	–	–	++	–	–	–	–	–	++	–
P. calliantha	**–**	**–**	**–**	**–**	**++**	**+**	**–**	**+**	–	++	–
P. pseudodelicatissima	–	–	–	–	+	++	–	+	–	++	–
P. fraudulenta	–	–	–	–	–	–	++	–	–	++	–
P. galaxiae	–	–	–	–	+	+	–	++	–	++	–
P. multistriata	–	–	–	–	–	–	–	–	++	++	–

3.2. WC-FISH on Artificial Samples

In order to verify if the screened probes would be useful for qualitative and/or quantitative evaluation, the probes Pdel4D02_25, Pdel3D01_25, Pdel2D01_25, and PmulaD03_25, targeted, respectively, on *P. allochrona*, *P. arenysensis*, *P. delicatissima*, and *P. multistriata*, were hybridised on artificial samples composed of a single and/or mixed *Pseudo-nitzschia* monoculture species. Specifically, when the Pdel4D02_25, Pdel3D01_25, Pdel2D01_25, and PmulaD03_25 probes were hybridised on samples containing only a single species, a positive hybridization with a well-defined signal was detectable (Figure 2).

For quantitative analyses, the cell density of these samples was evaluated by the Utermöhl method in light microscopy before the WC-FISH test, and the value was compared to that obtained by epifluorescence microscopy after WC-FISH [43,44]. The results, expressed as the detection efficiency (%) of the WC-FISH vs. Utermöhl counting method, showed a good agreement between the two methods in the sample of *P. arenysensis* and *P. multistriata* (99% and 89% respectively), slightly an underestimation for *P. allochrona* (78%), and a very low detection efficiency for *P. delicatissima* (23%) (Figure 3). This evidence suggests that only the probes Pdel3D01_25 and PmulaD03_25 can also quantitatively reveal their target species *P. arenysensis* and *P. multistriata* while the signal from probe Pdel4D03_25 for *P. allochrona* is weaker and Pdel2D01_25 for *P. delicatissima* not properly detectable (Figure 3). For this reason, excluding the probe Pdel2D01_25, we evaluated the efficiency of the probes Pdel3D01_25, PmulaD03_25, and Pdel4D03_25 in the artificial mixed samples (simulated field samples).

The results showed that the detection efficiency (%) decreased significantly when mixing only two species (i.e., *P. arenysensis* and *P. multistriata* (from 99% to 59.1% for *P. arenysensis*; from 89% to 59.5% for *P. multistriata*)) (Figures 3 and 4) and dropped when mixing all the three species (i.e., *P. arenysensis*, *P. multistriata*, *P. allochrona*) (Figures 4 and 5). These results confirmed that the detection efficiency of WC-FISH decreases with the increase in the number of species in the sample, probably due to the probe competition at the target sites, even if the probes have been used at saturating concentrations.

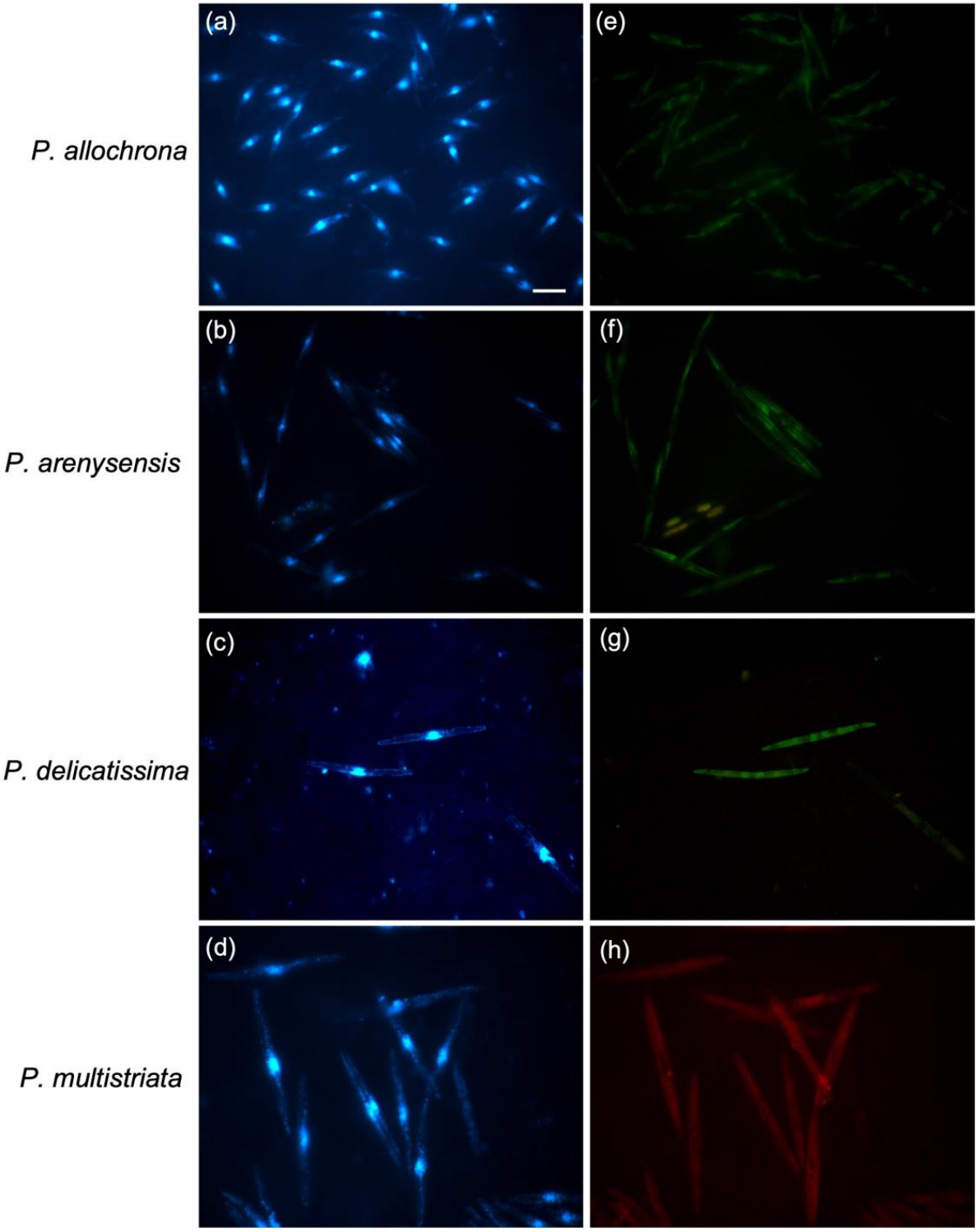

Figure 2. WC-FISH assays the pure culture of some *Pseudo-nitzschia* cryptic species (**a**–**c**,**e**–**g**) and *P. multistriata* hybridised to their species-specific probes. Each row of micrographs displays cells of pure cultures under fluorescence microscopy in DAPI (**a**–**d**) and FITC (**e**–**h**) filters. Scale = 10 µm.

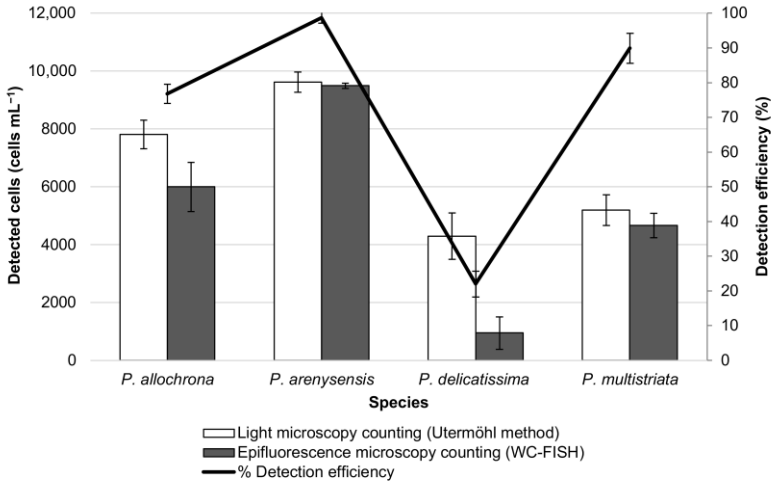

Figure 3. Artificial samples composed of a single monoculture *Pseudo-nitzschia* species. Cell density (cells mL^{-1}) was estimated before and after WC-FISH. Detection efficiency was calculated as FISH detectable cells (%) by the ratio between cells detected with rRNA FISH targeted probes vs. cultured cells before hybridisation.

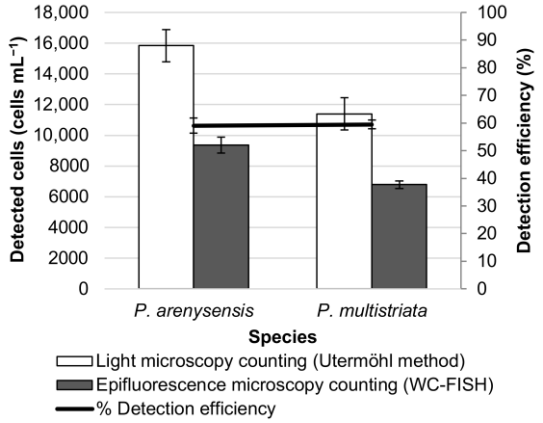

Figure 4. Simulated natural sample composed of two *Pseudo-nitzschia* species. Cell density (cells mL^{-1}) was estimated before and after WC-FISH. Detection efficiency was calculated as FISH detectable cells (%) by the ratio between cells detected with rRNA FISH targeted probes vs. cells in culture before hybridization.

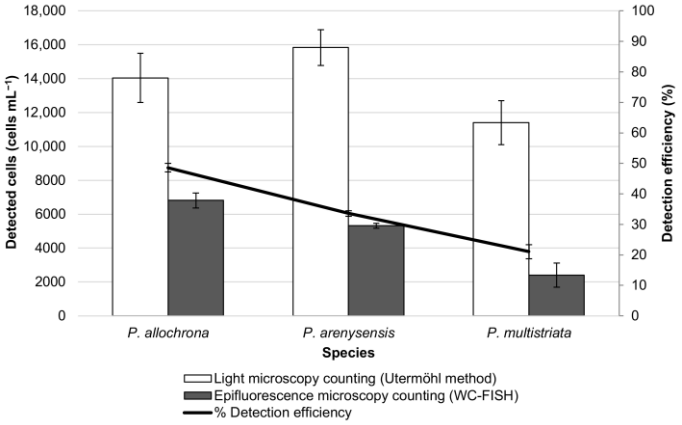

Figure 5. Simulated natural sample composed of three *Pseudo-nitzschia* species. Cell density (cells mL^{-1}) was estimated before and after WC-FISH. The detection efficiency (%) of the WC-FISH is calculated by the ratio between epifluorescence counting vs. light microscopy counting.

3.3. WC-FISH on Environmental Samples

To determine if WC-FISH would be able to detect the targeted cells in natural seawater qualitatively and/or quantitatively, we applied the screened probes for the *P. delicatissima* complex to natural samples (field-test) collected monthly at the LTER station in the Gulf of Naples. After the WC-FISH, it was possible to identify the cryptic species belonging to the *P. delicatissima* complex; otherwise, it would be impossible to identify the species with light microscopy (LM) (Figure 6). Further, the probe against *P. multistriata* (non-cryptic species) labelled with a different fluorochrome (Cy3) was used to verify if it would be possible to simultaneously detect almost two target species in the same sample.

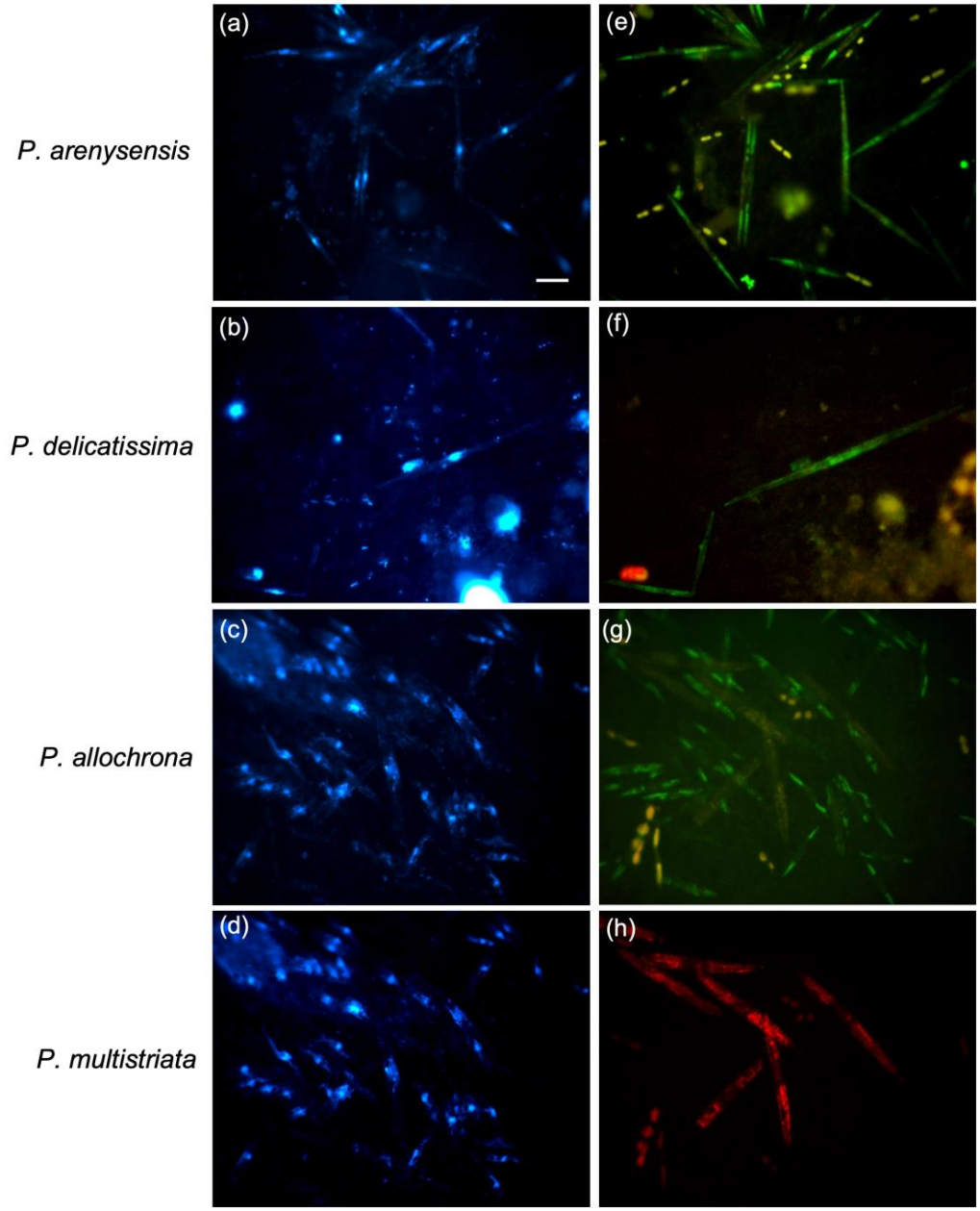

Figure 6. WC-FISH assays on natural samples targeted to species-specific cryptic *Pseudo-nitzschia* probes. Each row of micrographs displays cells of natural samples under fluorescence microscopy in DAPI (**a**–**d**) and FITC (**e**–**h**) filters. The last two rows of micrographs display the same sample processed as multiplex WC-FISH by using the two different probes labelled with FITC and Cy3 respectively. Scale = 10 μm.

The cell density of the different samples was estimated before and after WC-FISH, and the detection efficiency of the WC-FISH was summarised in the Table 6. The highest density of the *P. delicatissima* complex was detected in April (686,861 cells mL^{-1}) when WC-FISH detected only the cryptic species *P. arenysensis* and *P. delicatissima* (Table 6); a conspicuous density has been found in September (288,038 cells mL^{-1}) when the screened probes for the *P. delicatissima* targeted to all four of the species *P. arenysensis*, *P. delicatissima*, P. dolorosa, and *P. allochrona* (Table 6). As already shown by the test on the artificial samples, while the number of cryptic species increased in the sample, the detection efficiency decreased. Indeed, in the samples where only one cryptic species was detected (June and July), the detection efficiency rose up to the 89%, whereas it decreased up to 14.3% in the samples where all four of the cryptic species were detected (Table 6). In the February sample, the total absence of the *P. delicatissima* complex corresponded to the total absence of a signal after WC-FISH, confirming the specificity of the method (Table 6).

Table 6. Cell density (cell mL^{-1}) in natural samples before WC-FISH was estimated as a *P. delicatissima* complex and cell density of some *Pseudo-nitzschia* delicatissima cryptic species after WC-FISH. The detection efficiency (%) of the WC-FISH was calculated by the ratio between epifluorescence counting vs. light microscopy counting.

Sampling Date	Light Microscopy Counting (Utermöhl Method) (Cells mL^{-1})	Epifluorescence Microscopy Counting (WC-FISH) (Cells mL^{-1})					% Detection Efficiency
	P. delicatissima complex	*P. allochrona*	*P. arenysensis*	*P. delicatissima*	*P. dolorosa*	Total cryptic species	
May	210,490 ± 12,735	6900 ± 434	3900 ± 273	12,100 ± 726	-	22,900 ± 1433	10.9
June	16,498 ± 989	-	-	14,400 ± 864	-	14,400 ± 864	87.3
July	33,235 ± 2126	-	-	29,600 ± 1776	-	29,600 ± 1776	89.1
September	288,038 ± 17,162	3800 ± 252	7000 ± 470	28,200 ± 1792	2200 ± 153	41,200 ± 2667	14.3
February	-	-	-	-	-	-	-
March	177,855 ± 10,749	-	13,517 ± 821	4091 ± 275	-	17,608 ± 1096	10
April	686,861 ± 41,680	-	103,029 ± 6181	61,130 ± 3767	-	164,159 ± 9948	23.9

4. Discussion

In this study, for in situ hybridization, we applied the protocols from Miller and Scholin (1998, 2000) [25,38] and Groben and Medlin (2005) [39] with some minor modifications, using specific fluorescently labelled probes to *Pseudo-nitzschia* species as tested on a microarray rRNA based phylochip [21]. Our results demonstrate that 28S rRNA targeted oligonucleotides are promising tools that can make the identification of *Pseudo-nitzschia* cryptic and pseudo-cryptic quickly. By using positive and negative control treatments, we defined a range of possible labelling intensities for any sample. Of the nine species-specific probes designed and tested, only six showed a species-specific response with an intensity comparable to that of the positive controls. Unfortunately, the cross-test and the optimization of the WC-FISH hybridization did not eliminate the cross-reaction among the target species of the *P. pseudodelicatissima* complex, probably due to highly similar target regions on the rRNA of these species because of phylogenetic proximity [2,11]. The presence of a-specific signals is attributed to the relatively conserved 28S region rRNA of ca. 700 bp where the probes were designed to discriminate among and within the groups of closely related species of *Pseudo-nitzschia* genus; even if in the 28S region of the genus *Pseudo-nitzschia*, species often differ in single base-pair changes, as reported in the materials and methods [41].

Optimal hybridization conditions of the probes could represent another problem. In particular, the hybridization temperature of probes tested against the *P. pseudodelicatissima* complex was 45 °C. The high stringency of the hybridization condition cannot be suitable and the best choice for all the probes tested.

Moreover, to verify the accuracy and specificity of the screened probes for *P. delicatissima* complex and our method developed above, WC-FISH tests were performed on artificial samples composed of both monoculture samples and the mix of two and/or three different monoculture samples (simulated field samples). As far as the monoculture

samples, the algal densities determined by LM were in agreement with WC-FISH analysis (Figures 2 and 3). The results were rather similar except for probe Pdel2D01_25, for which the counting by the epifluorescence microscopy after WC-FISH detected only 22% of the counted cells in LM (Figure 3). This discrepancy was probably due to the physiological state of the culture. In fact, the labelling intensity could be caused by an rRNA amount, culture age, and metabolic state of cells that influence the abundance of ribosomes and the target nucleic acid; it has been reported that the fluorescence intensity in cells declined sharply in the late stationary stage of batch culture [45,46]. Indeed, although *P. delicatissima* cells were analysed at the mid-exponential phase, as in the other samples, the cellular physiologic state likely influences the label intensity, which in turn could significantly wane, not allowing the probes to be visualized. In the natural population, it has been well established that rRNA varies systematically with growth rate; fast growing cells have more RNA per cell than the cells growing at slow rates [45]. Slow growth, associated with phosphorus and nitrogen limitation, resulted in up to a 400% decrease of rRNA intensity of labelled probes compared to nutrient-replete levels with the rRNA probe [46,47].

Even if one of the advantages of using single, fluorochrome labelled oligonucleotides is that labelling and detection is simple and rapid, our results suggest that WC-FISH is not reliable for quantitative analyses. As shown in simulated natural samples and/or in the field samples collected in the Gulf of Naples, the Utermöhl counting and the epifluorescence signal after whole-cell hybridization did not agree well. A quantitative comparison between the species estimates by WC-FISH and microscopic methods revealed that the whole-cell hybridization underestimated the relative abundance of *Pseudo-nitzschia* species compared to the microscopic results, as the number of species in the simulated natural samples was increasing (Figures 3–5). In fact, the WC-FISH detected up to 98.7% of the cells when the simulated samples contained only one species, whereas the detection efficiency decreased to 21.1% when three different cryptic species were mixed (Figures 4 and 5). In the natural field samples, we observed that the WC-FISH method detected between 10% and 85% of the cells, depending on the species richness of the field sample (Table 6) even if the cells with green fluorescence intensity could be clearly and rapidly detected under the epifluorescence microscope (Figure 6). The results on the field-test showed that the efficiency of the WC-FISH depends on the homogeneity of the sample. Detection efficiency decreased when the number of the cryptic species in the sample increased (e.g., in summer samples), but WC-FISH provided a good quantitative estimate when the *P. delicatissima* complex is likely to be homogeneous in species composition; otherwise, in autumn and early spring, when the *P. delicatissima* complex increased in biodiversity, the WC-FISH showed a lower percentage of species detection. In order to explain these data, we hypothesise that a competition occurred among different probes simultaneously used at the target sites due to the high percentage of similarity in the nucleotide sequences of the different probes; the weak cross-hybridizations were removed during post-hybridization washing, causing sequestration of the probe to their specific target sites and a partial loss of signal. This process is higher when more target species are in the sample. However, different factors can affect hybridization of cells in environmental natural populations. The high affinity of the probes for closely related species that can cause competition between short and similar sequence probes could result in a-specific hybridization [48,49]. 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 [50,51]. The strength with which probes label cells is due to the accessibility of the target sequence in the context of the three-dimensional structure of the ribosome [52]. However, analysing low or undetectable fluorescence in natural samples might occur when cells are unhealthy, resulting in fewer ribosomes and, therefore, in a reduced fluorescence or due to the presence of dead *Pseudo-nitzschia* cells, empty frustules that were free in the sample or hidden within fecal pellets or sediment matrices.

Despite these weaknesses, the WC-FISH method is able to qualitatively detect the different cryptic species present in the samples, confirming that it is a good method for

qualitative analyses of a *Pseudo-nitzschia delicatissima* species complex. In particular, in our study, the probes Pdel1D01_25, Pdel2D01_25, Pdel3D01_25, and Pdel4D03_25 may be useful for molecular identification of the target species in natural samples containing many different potentially toxic microalgae, identifying the specific genus *Pseudo-nitzschia* and the toxin producing species at their bloom. The limit of WC-FISH for quantitative analysis has not yet been completely overcome due to the effort to design and use probes with similar melting temperatures (Tms) to aid in multiplexing; on the other hand, it could be designed and used in groups of riboprobes with different Tms to be employed in parallel experiments (multiprobing) but with different denaturation and washing temperatures. However, this would make the method longer and perhaps not advantageous compared to other molecular methods (i.e., PCR). Nevertheless, WC-FISH could be a useful tool for a very early qualitative screening method to be combined with classical microscopic analysis or to combine it with other very stable environmental processors. Further, this technique will be most useful for the early detection of single species blooms, especially for HAB, when microscopic analyses are not practical (i.e., cryptic species). That issue becomes particularly prominent when considering that HAB species pose human health and ecological threats often at very low cell densities (~10^2 cells L^{-1}).

Finally, it should be noted that the WC-FISH is faster and more cost-effective compared with other rDNA-based methods for the identification of *Pseudo-nitzschia* species. A concrete example of a specific probe hybridization technique used in the environmental field is represented by the company Microbia Environment that patented CARLA technology for Cellular Activity RNA-based eLisA. The assay is designed to detect cyanobacteria and microalgae in environmental water in less than 3 h using sequence-specific hybridization biosensors for a target of microalgae ribosomal RNA (rRNA) (https://www.microbia-environnement.com (accessed on 30 September 2022)).

In this study we have demonstrated that it is possible to discriminate a variety of *Pseudo-nitzschia* species collected from natural populations in situ and in near real-time. Altogether, the fluorescent oligonucleotide probes tested in our study show great promise as tools that can facilitate the monitoring of *Pseudo-nitzschia* species in natural samples.

Author Contributions: Conceptualization, M.F., L.R., I.P. and R.C.; methodology, M.F., L.R., I.P. and L.B.; validation, M.F., L.R., I.P. and L.B.; investigation, M.F. and L.R.; writing—original draft preparation, M.F. and L.R.; writing—review and editing, M.F., L.B., A.M., C.G., S.G. and R.C.; supervision, R.C.; project administration, R.C.; funding acquisition, R.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by University of Calabria (ex 60% University Research Funds 2010–2012).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Moreno, A.R.; Anderson, C.; Kudela, R.M.; Sutula, M.; Edwards, C.; Bianchi, D. Development, Calibration, and Evaluation of a Model of *Pseudo-nitzschia* and Domoic Acid Production for Regional Ocean Modeling Studies. *Harmful Algae* **2022**, *118*, 102296. [CrossRef] [PubMed]
2. Scholin, C.A.; Gulland, F.; Doucette, G.J.; Benson, S.; Busman, M.; Chavez, F.P.; Cordaro, J.; DeLong, R.; De Vogelaere, A.; Harvey, J.; et al. Mortality of Sea Lions along the Central California Coast Linked to a Toxic Diatom Bloom. *Nature* **2000**, *403*, 80–84. [CrossRef] [PubMed]
3. Guillou, L.; Nézan, E.; Cueff, V.; Erard-Le Denn, E.; Cambon-Bonavita, M.-A.; Gentien, P.; Barbier, G. Genetic Diversity and Molecular Detection of Three Toxic Dinoflagellate Genera (Alexandrium, Dinophysis, and Karenia) from French Coasts. *Protist* **2002**, *153*, 223–238. [CrossRef] [PubMed]
4. Zabaglo, K.; Chrapusta, E.; Bober, B.; Kaminski, A.; Adamski, M.; Bialczyk, J. Environmental Roles and Biological Activity of Domoic Acid: A Review. *Algal Res.* **2016**, *13*, 94–101. [CrossRef]

5. Bates, S.S.; Hubbard, K.A.; Lundholm, N.; Montresor, M.; Leaw, C.P. *Pseudo-nitzschia*, *Nitzschia*, and *Domoic Acid*: New Research since 2011. *Harmful Algae* **2018**, *79*, 3–43. [CrossRef]
6. Smodlaka Tanković, M.; Baričević, A.; Gerić, M.; Domijan, A.-M.; Pfannkuchen, D.M.; Kužat, N.; Ujević, I.; Kuralić, M.; Rožman, M.; Matković, K.; et al. Characterisation and Toxicological Activity of Three Different *Pseudo-nitzschia* Species from the Northern Adriatic Sea (Croatia). *Environ. Res.* **2022**, *214*, 114108. [CrossRef]
7. Trainer, V.L.; Hickey, B.M.; Horner, R.A. Biological and Physical Dynamics of Domoic Acid Production off the Washington Coast. *Limnol. Oceanogr.* **2002**, *47*, 1438–1446. [CrossRef]
8. Orsini, L.; Procaccini, G.; Sarno, D.; Montresor, M. Multiple RDNA ITS-Types within the Diatom *Pseudo-nitzschia Delicatissima* (Bacillariophyceae) and Their Relative Abundances across a Spring Bloom in the Gulf of Naples. *Mar. Ecol. Prog. Ser.* **2004**, *271*, 87–98. [CrossRef]
9. Sahraoui, I.; Bates, S.S.; Bouchouicha, D.; Mabrouk, H.H.; Hlaili, A.S. Toxicity of *Pseudo-nitzschia* Populations from Bizerte Lagoon, Tunisia, Southwest Mediterranean, and First Report of Domoic Acid Production by *P. Brasiliana*. *Diatom Res.* **2011**, *26*, 293–303. [CrossRef]
10. Lundholm, N.; Bates, S.S.; Baugh, K.A.; Bill, B.D.; Connell, L.B.; Léger, C.; Trainer, V.L. Cryptic and pseudo-cryptic diversity in diatoms—with descriptions of *Pseudo-nitzschia hasleana* sp. *nov.* and *P. fryxelliana* sp. nov. *J. Phycol.* **2012**, *48*, 436–454. [CrossRef]
11. Lundholm, N.; Moestrup, Ø.; Kotaki, Y.; Hoef-Emden, K.; Scholin, C.; Miller, P. Inter-and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *J. Phycol.* **2006**, *42*, 464–481. [CrossRef]
12. Ruggiero, M.V.; Sarno, D.; Barra, L.; Kooistra, W.H.C.F.; Montresor, M.; Zingone, A. Diversity and Temporal Pattern of *Pseudo-nitzschia* Species (Bacillariophyceae) through the Molecular Lens. *Harmful Algae* **2015**, *42*, 15–24. [CrossRef]
13. Lim, H.C.; Teng, S.T.; Leaw, C.P.; Lim, P.T. Three Novel Species in the *Pseudo-nitzschia Pseudodelicatissima* Complex: *P. Batesiana* Sp. Nov., *P. Lundholmiae* Sp. Nov., and *P. Fukuyoi* Sp. Nov. (Bacillariophyceae) from the Strait of Malacca, Malaysia. *J. Phycol.* **2013**, *49*, 902–916. [CrossRef]
14. Percopo, I.; Ruggiero, M.V.; Balzano, S.; Gourvil, P.; Lundholm, N.; Siano, R.; Tammilehto, A.; Vaulot, D.; Sarno, D. *Pseudo-nitzschia Arctica* Sp. Nov., a New Cold-Water Cryptic *Pseudo-nitzschia* Species within the *P. pseudodelicatissima* Complex. *J. Phycol.* **2016**, *52*, 184–199. [CrossRef]
15. Quijano-Scheggia, S.I.; Garcés, E.; Lundholm, N.; Moestrup, Ø.; Andree, K.; Camp, J. Morphology, Physiology, Molecular Phylogeny and Sexual Compatibility of the Cryptic *Pseudo-nitzschia* Delicatissima Complex (Bacillariophyta), Including the Description of *P. Arenysensis* Sp. Nov. *Phycologia* **2009**, *48*, 492–509. [CrossRef]
16. Ruggiero, M.V.; Kooistra, W.H.C.F.; Piredda, R.; Sarno, D.; Zampicinini, G.; Zingone, A.; Montresor, M. Temporal Changes of Genetic Structure and Diversity in a Marine Diatom Genus Discovered via Metabarcoding. *Environ. DNA* **2022**, *4*, 763–775. [CrossRef]
17. Percopo, I.; Ruggiero, M.V.; Sarno, D.; Longobardi, L.; Rossi, R.; Piredda, R.; Zingone, A. Phenological Segregation Suggests Speciation by Time in the Planktonic Diatom *Pseudo-nitzschia Allochrona* Sp. *Nov. Ecol. Evol.* **2022**, *12*, e9155. [CrossRef] [PubMed]
18. Hubbard, K.A.; Rocap, G.; Armbrust, E.V. Inter-and intraspecific community structure within the diatom genus *Pseudo-nitzschia* (Bacillariophyceae). *J. Phycol.* **2008**, *44*, 637–649. [CrossRef]
19. Galluzzi, L.; Cegna, A.; Casabianca, S.; Penna, A.; Saunders, N.; Magnani, M. Development of an Oligonucleotide Microarray for the Detection and Monitoring of Marine Dinoflagellates. *J. Microbiol. Methods* **2011**, *84*, 234–242. [CrossRef]
20. Andree, K.B.; Fernández-Tejedor, M.; Elandaloussi, L.M.; Quijano-Scheggia, S.; Sampedro, N.; Garcés, E.; Camp, J.; Diogène, J. Quantitative PCR Coupled with Melt Curve Analysis for Detection of Selected *Pseudo-nitzschia* Spp. (Bacillariophyceae) from the Northwestern Mediterranean Sea. *Appl. Environ. Microbiol.* **2011**, *77*, 1651–1659. [CrossRef]
21. Barra, L.; Ruggiero, M.V.; Sarno, D.; Montresor, M.; Kooistra, W.H.C.F. Strengths and Weaknesses of Microarray Approaches to Detect *Pseudo-nitzschia* Species in the Field. *Environ. Sci. Pollut. Res.* **2013**, *20*, 6705–6718. [CrossRef] [PubMed]
22. Edvardsen, B.; Dittami, S.M.; Groben, R.; Brubak, S.; Escalera, L.; Rodríguez, F.; Reguera, B.; Chen, J.; Medlin, L.K. Molecular Probes and Microarrays for the Detection of Toxic Algae in the Genera Dinophysis and Phalacroma (Dinophyta). *Environ. Sci. Pollut. Res.* **2013**, *20*, 6733–6750. [CrossRef]
23. Kegel, J.U.; Del Amo, Y.; Medlin, L.K. Introduction to Project MIDTAL: Its Methods and Samples from Arcachon Bay, France. *Environ. Sci. Pollut. Res.* **2013**, *20*, 6690–6704. [CrossRef]
24. McCoy, G.R.; Touzet, N.; Fleming, G.T.; Raine, R. An Evaluation of the Applicability of Microarrays for Monitoring Toxic Algae in Irish Coastal Waters. *Environ. Sci. Pollut. Res.* **2013**, *20*, 6751–6764. [CrossRef] [PubMed]
25. Miller, P.E.; Scholin, C.A. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. *J. Phycol.* **1998**, *34*, 371–382. [CrossRef]
26. Parsons, M.L.; Scholin, C.A.; Miller, P.E.; Doucette, G.J.; Powell, C.L.; Fryxell, G.A.; Dortch, Q.; Soniat, T.M. *Pseudo-nitzschia* species (Bacillariophyceae) in Louisiana coastal waters: Molecular probe field trials, genetic variability, and domoic acid analyses. *J. Phycol.* **1999**, *35*, 1368–1378. [CrossRef]
27. Sako, Y.; Hosoi-Tanabe, S.; Uchida, A. fluorescence in situ hybridization using rRNA-targeted probes for simple and rapid identification of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella*. *J. Phycol.* **2004**, *40*, 598–605. [CrossRef]
28. Anderson, D.M.; Kulis, D.M.; Keafer, B.A.; Gribble, K.E.; Marin, R.; Scholin, C.A. Identification and Enumeration of *Alexandrium* Spp. from the Gulf of Maine Using Molecular Probes. *Deep. Sea Res. Part II Top. Stud. Oceanogr.* **2005**, *52*, 2467–2490. [CrossRef]

29. Chen, G.; Zhang, C.; Zhang, B.; Wang, G.; Lu, D.; Xu, Z.; Yan, P. Development of a PNA Probe for Fluorescence In Situ Hybridization Detection of Prorocentrum Donghaiense. *PLoS ONE* **2011**, *6*, e25527. [CrossRef]
30. Caracciolo, A.B.; Dejana, L.; Fajardo, C.; Grenni, P.; Martin, M.; Mengs, G.; Sánchez-Fortún, S.; Lettieri, T.; Saccà, M.L.; Medlin, L.K. A New Fluorescent Oligonucleotide Probe for In-Situ Identification of Microcystis Aeruginosa in Freshwater. *Microchem. J.* **2019**, *148*, 503–513. [CrossRef]
31. Morais, S.L.; Barros, P.; Santos, M.; Delerue-Matos, C.; Gomes, A.C.; Fátima Barroso, M. Electrochemical Genosensor for the Detection of *Alexandrium Minutum* Dinoflagellates. *Talanta* **2021**, *222*, 121416. [CrossRef]
32. Liu, F.; Zhang, C.; Wang, Y.; Chen, G. A Review of the Current and Emerging Detection Methods of Marine Harmful Microalgae. *Sci. Total Environ.* **2022**, *815*, 152913. [CrossRef] [PubMed]
33. Medlin, L.K.; Orozco, J. Molecular Techniques for the Detection of Organisms in Aquatic Environments, with Emphasis on Harmful Algal Bloom Species. *Sensors* **2017**, *17*, 1184. [CrossRef] [PubMed]
34. Toebe, K. Whole Cell Hybridisation for Monitoring Harmful Marine Microalgae. *Environ. Sci. Pollut. Res.* **2013**, *20*, 6816–6823. [CrossRef]
35. McDonald, S.M.; Sarno, D.; Scanlan, D.J.; Zingone, A. Genetic Diversity of Eukaryotic Ultraphytoplankton in the Gulf of Naples during an Annual Cycle. *Aquat. Microb. Ecol.* **2007**, *50*, 75–89. [CrossRef]
36. Guillard, R.R.L. Culture of Phytoplankton for Feeding Marine Invertebrates. In *Culture of Marine Invertebrate Animals: Proceedings—1st Conference on Culture of Marine Invertebrate Animals Greenport*; Smith, W.L., Chanley, M.H., Eds.; Springer: Boston, MA, USA, 1975; pp. 29–60. ISBN 978-1-4615-8714-9.
37. Zingone, A.; Totti, C.; Sarno, D.; Cabrini, M.; Caroppo, C.; Giacobbe, M.; Lugliè, A.; Nuccio, C.; Socal, G. Fitoplancton: Metodiche Di Analisi Quali-Quantitativa. In *Metodologie di Studio del Plancton Marino. Manuali e Linee Guida 56/2010*; ISPRA: Roma, Italy, 2010; pp. 213–237. ISBN 978-88-448-0427-5.
38. Miller, P.E.; Scholin, C.A. On Detection of *Pseudo-nitzschia* (Bacillariophyceae) Species Using Whole Cell Hybridization: Sample Fixation and Stability. *J. Phycol.* **2000**, *36*, 238–250. [CrossRef]
39. Groben, R.; Medlin, L. In Situ Hybridization of Phytoplankton Using Fluorescently Labeled RRNA Probes. In *Methods in Enzymology*; Academic Press: Cambridge, MA, USA, 2005; Volume 395, pp. 299–310. ISBN 0076-6879.
40. Field, C.B.; Behrenfeld, M.J.; Randerson, J.T.; Falkowski, P. Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science* **1998**, *281*, 237–240. [CrossRef] [PubMed]
41. Barra, L.; Ruggiero, M.V.; Chen, J.; Kooistra, W.H.C.F. Specificity of LSU rRNA-Targeted Oligonucleotide Probes for *Pseudo-nitzschia* Species Tested through Dot-Blot Hybridisation. *Environ. Sci. Pollut. Res.* **2013**, *21*, 548–557. [CrossRef]
42. Ludwig, W.; Strunk, O.; Westram, R.; Richter, L.; Meier, H.; Yadhukumar; Buchner, A.; Lai, T.; Steppi, S.; Jobb, G.; et al. ARB: A Software Environment for Sequence Data. *Nucleic Acids Res.* **2004**, *32*, 1363–1371. [CrossRef] [PubMed]
43. Gilbert, J.Y. The Errors of the Sedgwick-Rafter Counting Chamber in the Enumeration of Phytoplankton. *Trans. Am. Microsc. Soc.* **1942**, *61*, 217–226. [CrossRef]
44. Lund, J.W.G.; Kipling, C.; Le Cren, E.D. The Inverted Microscope Method of Estimating Algal Numbers and the Statistical Basis of Estimations by Counting. *Hydrobiologia* **1958**, *11*, 143–170. [CrossRef]
45. DeLong, E.; Wickham, G.; Pace, N. Phylogenetic Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells. *Science* **1989**, *243*, 1360–1363. [CrossRef]
46. Anderson, D.M.; Kulis, D.M.; Keafer, B.A.; Berdalet, E. Detection of the Toxic *Dinoflagellate Alexandrium Fundyense* (Dinophyceae) with Oligonucleotide and Antibody Probes: Variability in Labeling Intensity with Physiological Condition. *J. Phycol.* **1999**, *35*, 870–883. [CrossRef]
47. Dittami, S.M.; Edvardsen, B. Culture conditions influence cellular RNA content in ichthyotoxic flagellates of the genus *Pseudochattonella* (Dictyochophyceae). *J. Phycol.* **2012**, *48*, 1050–1055. [CrossRef] [PubMed]
48. Medlin, L.K.; Kegel, J.U. Validation of the Detection of *Pseudo-nitzschia* Spp. Using Specific RNA Probes Tested in a Microarray Format: Calibration of Signal Based on Variability of RNA Content with Environmental Conditions. *Harmful Algae* **2014**, *37*, 183–193. [CrossRef]
49. Wright, E.S.; Yilmaz, L.S.; Corcoran, A.M.; Ökten, H.E.; Noguera, D.R. Automated Design of Probes for RRNA-Targeted Fluorescence In Situ Hybridization Reveals the Advantages of Using Dual Probes for Accurate Identification. *Appl. Environ. Microbiol.* **2014**, *80*, 5124–5133. [CrossRef] [PubMed]
50. Suzuki, S.; Ono, N.; Furusawa, C.; Kashiwagi, A.; Yomo, T. Experimental Optimization of Probe Length to Increase the Sequence Specificity of High-Density Oligonucleotide Microarrays. *BMC Genom.* **2007**, *8*, 373. [CrossRef] [PubMed]
51. Metfies, K.; Medlin, L.K. Feasibility of Transferring Fluorescent in Situ Hybridization Probes to an 18S RRNA Gene Phylochip and Mapping of Signal Intensities. *Appl. Environ. Microbiol.* **2008**, *74*, 2814–2821. [CrossRef] [PubMed]
52. Behrens, S.; Fuchs, B.M.; Mueller, F.; Amann, R. Is the In Situ Accessibility of the 16S RRNA of *Escherichia Coli* for Cy3-Labeled Oligonucleotide Probes Predicted by a Three-Dimensional Structure Model of the 30S Ribosomal Subunit? *Appl. Environ. Microbiol.* **2003**, *69*, 4935–4941. [CrossRef]