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Phytochip: Development of a DNA-microarray for rapid and accurate identification of *Pseudo-nitzschia* spp and other harmful algal species

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Abstract :

Detection of harmful algal blooms has become a challenging concern because of to the direct impacts on public health and economy. The identification of toxic dinoflagellates and diatoms in monitoring programs requires an extensive taxonomic expertise and is time consuming. Advances in molecular biology have allowed the development of new approaches, more rapid, accurate and cost-effective for detecting these microorganisms. In this context, we developed a new DNA microarray (called, Phytochip) for the simultaneous detection of multiple HAB species with a particular emphasis on *Pseudo-nitzschia* species. Oligonucleotide probes were designed along the rRNA operon. After DNA extraction, the target rDNA genes were amplified and labeled using an asymmetric PCR; then, the amplicons were hybridized to the oligonucleotide probes present on the chips. The total assay from sea water sampling to data acquisition can be performed within a working day. Specificity and sensitivity were assessed by using monoclonal cultures, mixtures of species and field samples spiked with a known amount of cultured cells. The Phytochip with its 81 validated oligonucleotide probes was able to detect 12 species of *Pseudo-nitzschia* and 11 species of dinoflagellates among which were 3 species of *Karenia* and 3 species of *Alexandrium*. The Phytochip was applied to environmental samples already characterized by light microscopy and cloned into DNA libraries. The hybridizations on the Phytochip were in good agreement with the sequences retrieved from the clone libraries and the microscopic observations. The Phytochip enables a reliable multiplex detection of phytoplankton and can assist a water quality monitoring program as well as more general ecological research.

Highlights

► We developed a microarray for HAB species identification with a particular emphasis on *Pseudo-nitzschia*. ► *Pseudo-nitzschia* species identification by microarray was compared to cloning and sequence analysis. ► The DNA microarray enables accurate multiplex identification at low concentration. ► The DNA microarray will improve the performance of the monitoring program

Keywords : HAB, Pseudo-nitzschia, microarray, ribosomal oligonucleotide probes, environmental monitoring

1. Introduction

Monitoring Harmful Algal Blooms (HAB) has become a major challenge in managing coastal areas. It has been motivated by both economic and health impacts (Trainer et al., 2012). Thus, characterizing phytoplankton communities is essential and has become an obligation for different coastal regions in the world. In the European Union, member states have to clearly monitor shellfish production areas and detect the presence of toxins produced by phytoplankton species (Karlson et al., 2010). Along French coasts and in the English Channel, recurrent toxic events were attributed to *Pseudo-nitzschia* blooms and impacted significantly the fishery and shellfish economy. In this diatom genus, toxigenic and non-toxigenic species can co-occur, therefore, it is crucial to discriminate the various *Pseudo-nitzschia* taxa co-occurring within a phytoplankton assemblage to clearly evaluate a potential toxic event and to understand also the dynamics of the toxic bloom. Whereas the total phytoplankton biomass is relatively easy to evaluate (*i.e.* by the estimation of chlorophyll concentrations or by counting using microscopy), the difficulties, which face monitoring programs are related to the identification and quantification of individual species (Galluzzi et al., 2011).

Traditionally, phytoplankton monitoring has been performed by identification and enumeration using optical microscopy; however a precise identification is not always possible (Kaczmarska et al., 2007; Karlson et al., 2010; Lelong et al., 2012). Given the morphological similarities between different species and the morphological variability within a single species (Lelong et al., 2012; Orr et al., 2011; Brand et al., 2012), light microscopy alone is often insufficient to assess the potential toxicity of coastal water blooms (McCoy et al., 2013). For example, it is nearly impossible to assess the species-specific identification especially for cryptic species in the *Pseudo-nitzschia* genus (Penna and Galluzzi, 2013). Moreover, these

practices require highly trained taxonomists and are labor and time consuming. They are inappropriate when a large number of samples has to be routinely examined. Because of the weaknesses and limitations of the classical methods, several molecular biological methods based on the DNA sequences have been developed in the last decade for the accurate identification of phytoplankton (Ebenezer et al., 2011). These alternative methods are in general based on DNA probe hybridization (*i.e* fluorescence *in situ* hybridization (FISH) on fixed cells (Scholin et al., 1997) or sandwich hybridization assay (SHA) on lysed cells, on q-PCR techniques (McDonald et al., 2007; Andree et al., 2011; Penna and Galluzzi, 2013), nucleic acid sequence-based amplification (NASBA) (Ulrich et al., 2010; Delaney et al., 2011), isothermal amplification (Zhang et al., 2014), Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Hubbard et al., 2008), on microarrays (Gescher et al., 2008a and 2008b; Galluzzi, et al., 2011; Smith et al., 2012; Edvardsen et al., 2013; McCoy et al., 2013, Wollschlager et al., 2014) and recently next generation sequencing (Cooper et al., 2014)

For routine monitoring purposes, a method should be user-friendly, high-throughput, rapid and present multiplexing capabilities. Real-time quantitative PCR has been developed to identify and quantify some toxic dinoflagellates (Zhang et al., 2014; Smith et al., 2014; Hariganeya et al., 2013; Kavanagh et al., 2010; Touzet et al., 2009) and a few diatoms (Penna et al., 2013; Andree et al., 2011). Even if q-PCR methods appear to be the gold standard in terms of sensitivity, this approach alone may not be well adapted for monitoring rapidly a large panel of toxic algae, in a single experiment. One q-PCR reaction allows handling only single or a few target species at the same time, in the case of a multiplex PCR. But for genetically close species, multiplex PCR is difficult to develop. As a consequence, several distinct assays would thus be needed in order to obtain a complete view of the phytoplankton

composition in a single sample. This will drastically increase the costs and the time required for the analysis.

In addition to miniaturization, the real key advantage that microarray technology has over PCR–based technologies is their high multiplexing capability (Scheler et al., 2014). DNA microarrays are believed to have the potential of identifying hundreds of species in parallel and to differentiate them among a large number of related species. Over the last few years, the phylochips, microarrays dedicated to taxonomic investigation, have been highly developed to detect and identify various organisms such as cereals (Rønning et al., 2005), microbes (Gentry et al., 2006; Warsen et al., 2004; Franke-whittle et al., 2009; Janse et al., 2012), fishes (Kochzius et al., 2010) and phytoplankton (Gescher et al., 2008a and 2008b; Scorzetti et al., 2009; Metfies et al., 2010; Galluzzi et al., 2011; Smith et al., 2012; Barra et al., 2013; Edvarsen et al., 2013; McCoy et al., 2013); all in highly complex samples.

For microorganism diagnostics, the use of DNA shows several advantages over RNA. DNA is much more stable than RNA, and can easily be isolated without any degradation from frozen, fresh or preserved samples. It is important to be able to analyze seawater samples fixed with lugol solution, the standard process in toxic phytoplankton monitoring programs. Another advantage is that also metabolically inactive organisms can also be detected. Within the MIDTAL project (http://www.midtal.com), several microarrays were set up to detect at the transcript level the main toxic microalgae. These microarrays, which are patented, target quantitatively rRNA, but the approach seems to be difficult to set up for identifying *Pseudo-nitzchia* and *Dinophysis* species because of the lack of probe specificity (Barra et al., 2013; Edvardsen et al., 2013).

The objectives of the present study were to develop and validate a user-friendly DNA-based microarray for the identification of the main harmful marine microalgae species

detected along the French European coasts. This biochip requires only basic hybridization and scanning equipment and new probe designs can be introduced easily and quickly when compared to the manufactured high density microarrays. The Phytochip was developed on Dendrislides; the advantages of these G4-dendrimer-coated slides are their high binding capacity because of the increased number of reactive sites to which probes can be covalently bound, their better accessibility to targets as well as their good quality, accuracy and reliability with low background (Le Berre et al., 2003; Trevisiol et al., 2003, 2009). This study describes the development and optimization of the "Phytochip" targeting various toxic microalgae with strong emphasis on *Pseudo-nitzschia* diatom species, responsible for recurrent shellfish poisoning in France. The Phytochip was subsequently used to monitor the temporal distribution of the *Pseudo-nitzschia* species in the Bay of Concarneau. Clone libraries were also constructed and sequenced. The observed pattern by the microarray was then compared to those given by the molecular sequences of the clone libraries and by the traditional standard optical microscopy procedure.

2. Materials and methods

2.1 Probe design

Oligonucleotide probes were designed using the ARB software (Ludwig et al., 2004) on aligned rRNA sequences found in the SILVA databases (Pruesse et al., 2007); the Small Subunit, 18S (SSU Ref_108_SILVA_09_09_11) and the Large Subunit, 28S (LSU Ref_108_SILVA_16_08_11). The rDNA ITS sequences were retrieved from the public databases (http://www.ncbi.nlm.nih.gov/guide/dna-rna/). The probe-design-function implemented in ARB was used with minimal (or none) non-target hits (number of non-target

species liable to be detected by the probe) and maximal target hits (>95%) in order to design highly specific probes. Additional probes were designed with the FastPCR software (Kalendar et al., 2011). We aimed at selecting oligonucleotide probes with homogenous behavior; therefore, probes were designed according to the following 6 criteria: 1) length 25 ± 2bp; 2) melting temperature (Tm) 55°C based on SantaLucia's table of thermodynamic parameters from Primer3Plus (Untergasser et al., 2007); 3) GC content "around" 50%; 4) limited secondary structures checked with Primer3Plus and Oligo Analyzer (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/); 5) If mismatches with non-specific targets occurred, these were located in the center of the probes (Letowski et al., 2004; 6) General oligonucleotide "quality" was over 80% according to FastPCR. Potential probes were tested *in silico* 1) using the probe match tool from ARB against their respective ARB databases (SILVA databases for the 18S and 28S designed probes, and our databases containing over 2500 ITS phytoplankton sequences from various genera retrieved form public databases (EMBL/GenBank/ /DDBJ); 635 of them belonging to *Pseudo-nitzschia* species) 2) using the probe-match function against the Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp), and 3) using BLAST searches against the GenBank nucleotide database (Altschul et al., 1990). The most specific and thermodynamically stable oligonucleotide probes were selected or modified to complete the requirements defined above. For this purpose, length or position of the probe was adjusted by the addition or removal of nucleotides at either end. Then, probes were checked again by a Primer-Blast to guarantee target specificity (Ye et al., 2012). A hierarchical probe set targeting different taxonomic levels was constructed. (Metfies and Medlin, 2008). Several probes (up to 4) per species were selected in order to limit false positive or negative signals.

2.2 DNA Extraction and PCR Amplification

The phytoplankton strains were cultured using the f/2 Guillard and Ryther medium under optimal temperatures with 150 µmol photons m^{-2} s^{-1} from a cool-white fluorescent light with a 14:10 LD photoperiod (Andersen, 2005). The cells were harvested by centrifugation (5000 g, 10 min) and then washed three times in PBS (phosphate buffer saline) before the genomic DNA was extracted either using a 10 % CTAB extraction procedure (Murray and Thompson, 1980) or with the DNAeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. All DNA concentrations were measured with a NanoDrop spectrophotometer (ThermoFisher, USA) and DNA was stored at –20°C. The DNA from the cultures (10 to 30 ng/ reaction) was amplified by an asymmetric PCR reaction (Janse et al., 2012). Fifty microliter reaction mixtures consisted of 1X Flexi-Promega Buffer, 200µM of dNTPs, 3.5 mM of $MgCl_2$, 1.25u of GoTaq® DNA polymerase (Promega, USA) with 0.5 µM 5'-labelled Cy5 forward primer, and 0.1 µM reverse primer. We used generic primers previously designed for phytoplankton studies (Lenaers et al., 1989; Metfies and Medlin, 2007, 2008; White et al., 1990) as well as generic primers we developed specifically for the *Pseudo-nitzschia* genus (Table 1). The specificity of the new primers (PSN_F1 and PSN_R1) was tested *in silico* and *in vitro* by PCR using monoclonal cultures or single phytoplankton cells (Table S1). The PCR protocol consisted of 5 min 94°C for 5 min (1x); 35 cycles consisting of (30 sec at 94°C; 1 min at the primer-specific annealing temperature, and 1 min at 72°C) ; and a final elongation of 5 min at 72°C. PCR amplicons were analyzed on 1 % agarose gels and purified with the MinElute PCR Purification Kit (Qiagen, Germany). The quantity of purified products and Cy5 incorporation were measured using a Nanodrop spectrophotometer (ThermoFisher, USA). It should be noted that this PCR step was not only

to amplify the genomic material, but also to eliminate non-specific hybridization due to the non-target sequences of the genomic DNA.

2.3 Microarray Hybridizations

Dendrislides (Dendris™ Diagnosis Designer, France) were used for spotting the microarrays. Dendrimer G4 was employed to generate an amino-silanised glass surface onto which NH_2-modified DNA probes were covalently fixed by their 5'ends (Le Berre et al., 2003, Trevisiol et al., 2003, 2009). A spotting robot Q-Array Mini (Genetix©) was used to spot the oligonucleotide probes with a 5'-amino-C6-modification (Sigma Life Science, France) onto the slides in duplicates at a final concentration of 75 µM in 0.15M sodium phosphate buffer (pH 8.4). Positive and negative controls were spotted onto the slide. Two types of negative controls were used: spotting of pure buffer and an oligonucleotide that does not target phytoplankton sequences (Gescher et al., 2008a). Two positive probes were spotted on the microarray slide, both from the influenza A virus hemagglutinin sequence (5'-GCGACAGTTGAGTAGATAGCCAGAATC-3' and 5'-AGATAGCCAGAATCCGATAGACCCC-3') and for which a complementary sequence fragment was mixed into the sample before the PCR step. An anchoring Cy3-labelled DNA was also spotted on both sides of each block of the microarray to facilitate grid alignment for analysis.

To provide enough time for covalent binding between the oligonucleotide and the dendrislide surface, the microarray slides were maintained overnight in a humid chamber, and then subjected to a reduction of the imine functions by incubating them for 3 h with 3.5 mg. mL^{-1}sodium borohydride solution, rinsed with milliQ-filtered water and dried by cyto-centrifugation. The dendrislides were then stored at 4°C until use. Fluorescently labeled

amplicons (250 ng) were diluted in the hybridization mix (SSC 4X, SDS 0.2 %, 0.1mg/mL salmon sperm DNA), denatured at 92°C for 2 min and then loaded into an Agilent gasket with 8-microarray positions per slide. Hybridization was performed at 65°C for 30 min in an Agilent microarray chamber. The slides were subsequently washed for 1 min in 2X SSC/0.2% (V/V) SDS and for 1 min. in 0.2X SSC, and finally dried with a cyto-centrifuge.

The hybridization signals were quantified as arbitrary units on an Innoscan 900 laser scanner at 635 nm (Cy5) as well as 535 nm (Cy3) (*i.e.* grid alignment spots). The fluorescence signal was analyzed with the MAPIX software (Innopsys, France). The fluorescent signal (FS) of a probe is obtained by calculating the arithmetical means of the intensity median after subtraction of the background noise (directly obtained from the MAPIX software) (He and Zhou, 2008). A minimal fluorescence threshold value was determined as 1% of the highest oligonucleotide probe signal of the chip validated with the DNA samples extracted from monoclonal phytoplankton cultures. A signal was considered positive when the fluorescence was higher than this arbitrary threshold. Furthermore, two positive controls were set up to assess the PCR amplification, labeling and hybridization process. DNA fragments corresponding to the influenza A virus hemagglutinin gene sequence (1730 bp) were constructed with at their 5' and 3' end the complementary sequences of two primer couples (ITS1/ITS4 and PSN-F1/PSN-R1). These were mixed into the samples before the PCR step. The specificity of these control probes was assessed on different microalgal rRNA amplicons and no cross-hybridization could be observed.

2.4 Microarray Performance

Probe specificity was first investigated by hybridization of PCR products from the monoclonal algal culture DNA (Table S1). Each species was tested at least in triplicate using either 3 different strains or with 3 independent PCR experiments, in the case that only one

strain of a species was available. Preliminary results revealed a much higher specificity with the ITS probes, and a lot of cross-hybridization with the 18S rRNA probes for *Pseudo-nitzschia*. For this reason, we decided to focus our efforts on ITS probes for the identification of *Pseudo-nitzschia* species. Once the best probes identified, a hierarchical identification key was established from the genus up to the species. We can use this simple way of scoring as all cross-hybridizing probes were eliminated during the development of our test. A species is thus present if there are signals with the probes from its genus, clade and species level. Then, other validations were investigated by using mixed genomic DNA of *Pseudo-nitzschia* and other dinoflagellate species (species multiplex).

To get insight in the Phytochip sensitivity, cultures of *A. minutum* and *P. delicatissima* were used to spike seawater samples collected at Saint Anne du Porzic (48°21'N; 4°33'W; Brittany, France) 100 ml of environmental sea water was spiked with approximately 500, 1000, 5000, 10000, and 25000 *A. minutum* cells/L, and 5000, 10000, 25000, 50000 and 100000 *P. delicatissima* cells/L. These samples were filtered onto Isopore membranes with a pore size of 1.2 µm (Millipore, Germany). Filters were shock-frozen in liquid nitrogen and stored at -80°C. Subsequently, DNA was extracted from the filters and amplified by an a-symmetric PCR using the 5'-labelled Cy5 forward (28S or ITS) and reverse (28S or ITS) primers (for *A. minutum* and *P. delicatissima* respectively). Amplicons were then purified and Phytochip-tested.

2.5 Phytochip Validation with Environmental Samples

Six samples were collected monthly in the Bay of Concarneau (47°49'N, 3°57'W; Brittany, France) from March to July 2012. An additional sample was taken at the end of June because the highest abundances of *Pseudo-nitzschia* occurred at this time. Water samples

were taken from the sea surface using an 8L Niskin bottle. 100 mL of this seawater was used for microscopic cell counts and preserved within acidic lugol's iodine solution. Fixed phytoplankton samples were counted according to the method of Utermöhl (Karlson et al., 2010). Additionally, 100 mL sea water was vacuum filtered through an Isopore membrane (Millipore, Germany) with a pore size of 1.2 µm. Filters were shock-frozen in liquid nitrogen and stored at -80°C. Sample extraction and preparation followed the protocol described above (see § 2.2 and 2.3).

In order to test the specificity of the *Pseudo-nitzchia* genus primers and validate the microarray *in vivo,* clone libraries were constructed from these environmental samples and the individual clones sequenced. These results were compared to those obtained by the Phytochip and light microscopy identification. In short, the ITS rDNA gene was amplified using the PSN_F1 x PSN_R1 primers. The purified amplicons were then cloned into the pCR2.1 TOPO vector and transformed into Top10 chemo-competent *Escherichia coli* cells (Invitrogen, USA). The clones were Sanger sequenced by MilleGen (France) and GATC Biotech AG (Germany). Sequences were checked and aligned using BioEdit v7.1.3.0 (Hall et al., 1999). Affiliations were confirmed by BLAST and ARB analyses. Sequences were added in our ARB database and inserted into the tree using the maximum parsimony criterion.

Regarding the microarray results, one probe per species was defined as the species-reference probe, which is the probe showing the highest signal. We calculated the ratio between the FS from the species reference probes and the FS from the PSN_genus_Hf20 probe to standardize values and to be able to compare the evolution of relative proportions of *Pseudo-nitzchia* species between samples,

3. Results

3.1 Oligonucleotide Probe Design and Specificity of the Phytochip

284 oligonucleotide probes were initially designed (18S rDNA: 107; ITS rDNA: 164 and 28S rDNA: 13) to target several harmful algal genera and species with a particular interest in detecting and identifying *Pseudo-nitzschia* species. Thus, 109 oligonucleotide probes were dedicated to 18 *Pseudo-nitzschia* species. In our study, a series of 81 probes was finally validated to unambiguously and hierarchically identify 12 *Pseudo-nitzschia* species and 11 Dinoflagellate species (Table S2). Except for the *Alexandrium* genus, the oligonucleotide probes designed on the 18S rRNA gene showed a great deal of cross-hybridization and were not specific in comparison with the ITS or 28S probes. A panel of strains was used to validate the oligonucleotide probes from the phytochip; although not all species could be tested, because of the lack of monoclonal cultures or genomic DNA from these species. The principal genera of HAB species such as *Alexandrium*, *Karenia* and *Pseudo-nitzschia* could be detected with 28S and ITS probes The use of a hierarchical design as well as the design of several different probes per species (a set of probes) allowed the correct identification of 12 species of *Pseudo-nitzschia* (Fig. 1 and Table S2A). A cross-hybridization of the probes PSN_seriata_ITS _8 with its closest relative, the *P. australis* species, was observed, but FS was lower for *P. australis* than for *P. seriata*. Moreover, when *P. australis* was present, the 2 oligonucleotide probes designed for it (i.e PSN_austr5-2m and PSN_australis5m) give a high and specific signal.

We defined up to 5 specific probes for the specific detection of the various dinoflagellates such as *Azadinium spinozum, Lingulodinium polyedrum, Ostreopsis ovata, Karenia brevis, Karenia mikimotoi, Karenia selliformis, Prorocentrum micans* (Table S2B) and 3 *Alexandrium* species (*A. catenella, A. tamarense* and *A. minutum*; Table S2C). *Alexandrium tamarense* and *A. catenella* are closely related species that were only

discriminated by the probes designed on the 28S rDNA gene sequence. *Alexandrium minutum* can be identified with the probes designed on the 18S, ITS region and 28S rDNA gene sequences (Table 2 and S2C).

The main interest of using DNA microarrays lies in multiplex detection when identifying and classifying accurately and straightforwardly species in mixtures. The results obtained from the 10 mixture samples (6 *Pseudo-nitzschia* mixtures and 4 dinoflagellates mixtures) were in agreement with their respective composition (Table 3A and B). None or very little FS below threshold was observed with probes from the absent species (Table 3A and B). In our experiments, we co-amplified and detected successfully up to 7 species simultaneously (we did not test more species in mixture) with the Phytochip. Nonetheless, some differences in probe FS appeared when comparing species in simplex and multiplex assays. For example, when we removed *P. australis* from the *Pseudo-nitzschia* mixture, *P. seriata* specified by 2 clade-probes (PSN_seriataGrp_f10 and PSN_seriataGrp_f4) and one species probe (PSN_seriata_ITS_8) was barely detected whereas the 2 clade probes reached saturation in simplex (Table 3A and S2A). In the dinoflagellate mixtures, 3 probes (Ostreopsis_sp_ITS, Vulcanodinium_ITS_4 and Alex28S) presented less specificity by showing weak FS in the 4 mixtures. However, the absence of FS from the other *Ostreopsis* and *Vulcanodinum* oligonucleotide probes excluded their presence in the mixtures.

3.2 Sensitivity of the Phytochip

To test the Phytochip hybridization process and efficiency on a natural matrix, natural seawater was spiked with *A. minutum* and *P. delicatissima* cells. A prior inspection of the natural seawater under the light microscope revealed the presence of a few *Pseudo-nitzschia* cells from the *seriata* complex. On the phytochip, *A. minutum* was detected in all samples

with the 28S rDNA probes as revealed by high and specific FS for both the genus and species probes. The detection limit for *Alexandrium minutum* is lower than 50 cells because only 50 cells were enough to give a strong FS, for the Amin2-1 probe. The generic Alex28S probe reached directly saturation with the range of cells we tested (Fig. 2A). A positive FS for PSN_28S is observed indicating the presence of *Pseudo-nitzchia* cells in natural sampled sea water. This result confirmed the microscopic observations. However the identification of *Pseudo-nitzschia* species failed probably because of their too low concentration. For samples spiked with *P. delicatissima* culture, FS for clade and species probes were also found specific and allowed to detect 500 *P. delicatissima* cells (Fig. 2B).

3.3 Detection in Environmental Samples

The phytochip was used to identify *Pseudo-nitzschia* species in natural samples from the Bay of Concarneau. A good agreement was observed between the molecular methods (i.e microarray and clone libraries) and light microscopy observations (Fig. 3, Table 4 and S3). Nevertheless, a few disparities were observed between microarray and clone libraries results. In a few cases we obtained a positive microarray signal and their lack of the corresponding sequences in the libraries. Even if the coverage of the library is high, it is not maximal; the coverage ranges from 81.5 to 92.7%. The presence of target species could thus be missed in the library because the number of clones sequenced was too low.

Using the *Pseudo-nitzchia* genus primers on environmental samples, only 9 out of 178 reliable sequences did not belong to *Pseudo-nitzschia* species but to other diatoms: 8 were affiliated to the *Cylindrotheca* genus and 1 sequence showed 89% identity with a *Minutocellus* sp. species. Thus, the primers and probes developed for the Phytochip seemed perfectly adequate to enrich and clearly detect *Pseudo-nitzschia* species in environmental

samples. The microarray hybridization showed that the *delicatissima* complex dominated the *Pseudo-nitzschia* assemblage with a striking dominance of *Pseudo-nitzschia* species from the *cuspidata/pseudodelicatissima* complex which bloomed at sampling time-point BC21 (Fig. 3). Three species from the *delicatissima* complex were detected in these samples. *P. fraudulenta* and *P. pungens* were the most dominant species from the *seriata* complex and were detected in 6 and 3 samples, respectively (Table S3). *P. australis* was also detected in low numbers in BC2, whereas it was not identified in the clone libraries (Fig. 3). It is also interesting to notice that oligonucleotide probes from two species, *P. dolorosa* and *P. subpacifica* which were not validated because of the absence of monoclonal cultures from these species, presented a clearly positive FS in this sample and were also detected in the BC6 and BC26 gene libraries respectively (Table 4 and S3). Oligonucleotide probes of species from the *seriata* complex showed higher sensitivity; especially because of the high FS of the *P. fraudulenta* and *P. pungens* probes.

4. Discussion

Future diagnostic methods will rely on approaches that allow the immediate screening of multiple targets. These methods should be able to be performed quickly and with high specificity, but at the same time with minimal effort and materials to reduce costs. With this perspective in mind, we have developed the Phytochip for the identification of HAB species and monitoring of phytoplankton communities. The detection of phytoplankton species is achieved after the direct hybridization of labeled PCR products, which allows the protocol to remain simple and rapid. By using DNA as a target rather than RNA, it is possible to overcome some practical constraints: DNA is much more stable and it can be extracted easily from fixed samples. Even if DNA microarrays require an additional step for targeting rDNA

genes (amplification and labeling), this step allows gaining sensitivity and specificity. For design purposes, we developed probes according to their thermodynamics and specificity regardless of the secondary structures they can adopt. The initial PCR amplification of target DNA has a major effect on microarray performance (Janse et al., 2012; Scheler et al., 2014). Using fluorochrome labeled primers, the PCR allows sample amplification and labeling in a single step. Furthermore it leads to a labeling efficiency that is theoretically similar for all oligo-pairs, making results easier to compare, which is in contrast to a post-PCR DNA labeling step where the incorporation of the fluorescence may vary (Russell et al., 2009). The asymmetric PCR performed with labeled primers also presents some other benefits. It increases the yield of target ssDNA and thus general fluorescence, and prevents hybridization competition between the target probe and the complementary strand (Szilvási et al., 2005). Moreover, the current asymmetric PCR could successfully co-amplify DNA from up to 7 target species or DNA from field samples without losing individual PCR products. The optimized assay protocol described in this paper allows identification of specific taxa within a single working day. With the microarray format chosen, eight samples can be analyzed in parallel on a single slide, allowing relatively high throughput and further automation. Even if the phytochip is not a quantitative assay, it can save time and reduce the cost of monitoring by screening and selecting only samples containing toxic target microalgae for additional analysis at the single species level to quantify them by microscope count or a specific Q-PCR. If the cell concentration exceeds the sanitary alert thresholds defined for each microalgae, biotoxin analysis will be performed in shellfish. In France, the official control system is based on both phytoplankton monitoring (Utermöhl) and analysis of marine biotoxins in shellfish. Recent reviews outline the requirement of most legislative systems to monitor for toxic phytoplankton species in shellfish production areas (de Grasse and Martinez-Diaz, 2012;

Hess, 2012; Suzuki and Watanabe, 2012). Hence, our microarray, which has a high specificity and allows for simultaneously testing of several microalgae in parallel, could be used as part of an optimized early warning system.

Nonetheless, we observed a discrepancy between the number of *in silico* designed "potentially functional oligonucleotide probes" and the actual number of validated probes (in terms of specificity and FS). It had already been shown from bioinformatics computations that most probes do not perform as expected suggesting that the dynamics and processes of hybridization are not yet fully understood (Kochzius et al., 2010; Chen et al., 2009). In our experimental conditions, several probes did not display any FS, whereas others presented cross-hybridization because of lack of *in situ* specificity. The performance of probes can thus be unpredictable, especially when environmental samples containing numerous unknown organisms are analyzed (Peytavi et al., 2005). Therefore, to try to avoid these complications, we opted for a hierarchical design and developed several probes per species (Metfies and Medlin, 2008), on different positions along the rRNA operon. Thus, a set of probes was used instead of a single one to discriminate the species. The 81 functional probes we designed were tested for their specificity in simplex and multiplex experiments with 32 different phytoplankton species and various strains when possible. Regarding the microalgae tested in this study, we also found a large difference in term of specificity between probes designed from18S and ITS rDNA. Most of the 18S rDNA probes showed a great deal of cross-hybridization and thus a lack of discrimination. Similar results had been obtained with Barra et al. (2013) with 49 genus- and species-specific probes designed against the nuclear SSU and LSU rRNA for 19 *Pseudo-nitzschia* species where only three of them showed specificity. Thus the 18S rDNA is apparently too conserved between the species and these probes were discarded from our Phytochip for the *Pseudo-nitzschia* identification. Most of the probes

designed from the nuclear encoded ITS regions showed high specificity and FS, confirming that the ITS1-5.8S-ITS2 region offers a better, if not, the best resolution to discriminate HAB species. The ITS1 region had already been proven to be useful in differentiating *Pseudo-nitzschia* species in ARISA, quantitative PCR or microarrays (Hubbard et al., 2008; Andree et al., 2011; Smith et al., 2012). The ITS2 with its secondary structure had been found to be useful for taxonomic assignment at various levels (Coleman, 2003), especially the 5.8S-ITS2 region has been proposed as a successful DNA-barcoding region for diatoms (Moniz and Kaczmarska, 2010). However, given the high level of polymorphism in this region, designing probes for higher taxonomic levels (above genera) was more complicated. Higher taxonomic level probes should display roughly the same level of FS among and between the different genera avoiding bias towards some species. We failed to design higher taxonomic oligonucleotide probes from this region; they showed either no FS or irregular FS patterns. As they did not improve identification, they were discarded. Nevertheless, the specificity of molecular probes should be continuously re-evaluated because it is strongly dependent on the amount and the quality of sequences in the ribosomal data bases and accurate assignment at the time of the probe design (Wollschlager et al., 2014). The phytochip design offers a good possibility to introduce and set up new probes quickly and easily. In this study as in others, the absolute FS intensities were heterogeneous, which appeared to be a common feature in DNA microarray hybridization experiments (Rønning et al., 2005; Warsen et al., 2004; Kochzius et al., 2010; Peplies et al., 2003). Considering the principles of DNA duplex stability, FS intensities should be primarily connected to the nucleotide sequence of a probe. Peplies et al. (2003) have highlighted that the FS of a mismatched target for particular probes can be significantly higher than the perfect matched signals of other probes. Similar results were obtained in simplex assays using DNA from cultures. The FS of *P. australis* DNA with

the probes PSN_seriataGrp_f4 and PSN_seriataGrp_f10 (originally designed for *P. seriata*) were higher than for *P. seriata* DNA with the seriata "specific" PSN_seriata_ITS_8 probe. However these two species can be considered as sister species (Lundholm et al., 2002) and are known to be difficult to distinguish on a molecular basis (Hubbard et al., 2008). The Position Of Label (POL) effect of the hybridization probe sequence may also explain some variations in fluorescence intensities (Zhang et al., 2005). Previous studies had already reported a decrease in the hybridization signal intensity as the distance between the binding site and the fluorescent label in the DNA-probe duplex increases (Kochzius et al., 2010). Looking at the *Pseudo-nitzschia* species for which we had various functional probes targeting the same marker, a significant POL effect was also observed; oligonucleotide probes binding with the sequence part with the shortest distance from the fluorescent label presented the highest FS (Fig. S1).

With our Phytovhip, we can respectively detect 50 and 500 cells of *Alexandrium minutum* and *Pseudonitzschia delicatissima.* This detection limit depends on several factors linked to the microarray assay procedure, but mostly on the target species DNAs. For monitoring purposes, it is crucial to assess the sensitivity for each target species to be sure that the detection limit is lower than the sanitary threshold. The sensitivity of a DNA microarray is higher than those obtained using RNA microarrays (Taylor et al., 2014). These authors recently calibrated the microarray signal to the RNA content and cell concentration for several *Alexandrium* species. The detection limit was found to be comprised between 700 and 7000 cells depending on the *Alexandrium* species. The detection limit for the Phytochip is in agreement with those obtained for other DNA microarrays (Galluzzi et al., 2011). Using a RNA microarray, Barra et al. (2013) and Medlin and Kegel (2014) found a detection threshold ranging between 1200 to 50 000 cells for *Pseudo-nitzschia* spp,

Even if a probe may seem promising from a bioinformatics perspective, cross hybridization can occur with closely related species. Considering that probe behavior cannot be predicted and in order to validate our Phytochip, probes were intensively tested with hybridization experiments run in simplex and multiplex to assess their application in species identification with several species, especially with *Pseudo-nitzschia* species. It is crucial to test closely-related species belonging to the same genus. For example, the two probes PSN_seriataGrp_f10 and PSN_seriataGrp_f4 first designed for *P. seriata* showed strong FS with the most closely related *P. australis* species in simplex; for this reason they were then considered to target both species (the *P. seriata/ P. australis* clade). However, it is worth to note the fluorescence decrease when these species was analyzed in multiplex. Designing a hierarchical set of probes proved absolutely essential; it limits false-positives (increase of specificity) and strengthens detection (increase of sensitivity).

The Phytochip was applied to field samples from the French Brittany region. A good congruence was observed between the Phytochip, microscopy and clone library results at the complex level (*delicatissima/seriata*) (Fig. 3). In contrast to the microscopy method, the Phytochip also gives information about the species level and presents the highest sensitivity. A larger diversity of *Pseudo-nitzschia* was estimated with the phytochip than with the clone libraries. Our clone libraries probably underestimated the occurrence of taxons, because the coverage ranged only between 81.5 and 92.7 percent. The number of sequenced clones was probably insufficient to detect all *Pseudo-nitzschia* species in the environmental samples. The microarray signal obtained for the taxa that were not detected in the libraries was also low, probably because of the low abundances of the species underlying once more the sensitivity of our phytochip. It is unlikely that this detection was because of nonspecific binding of

probes to non-target species because the probes were previously validated using natural seawater spiked with various amount of cells of these specific species.

In the *delicatissima* group, we observed that *P. cuspidata/pseudodelicatissima* was present in all samples and represented the dominant species in the observation series on this site. *P. delicatissima* and *P. arenysensis* were also detected in most of the samples but in lower quantities. Moreover, probes of two untested species *P. dolorosa* and *P. subpacifica* showed weak FS in BC6 and BC26 samples, respectively. We know that *P. subpacifica* appeared on the French coast for the first time in 2004 and has since been observed on the South West coast of Brittany (Nezan et al., 2007). One sequence of this species was also retrieved from our clone libraries in one of these same samples, suggesting that this species may indeed be present at very low concentrations in natural sea water. Diversity was maximal in BC26 (Mid-July). Such type of distribution has already been reported in summer, the species of the *seriata*-group blooming after the *delicatissima*-group (Fehling et al., 2006; Downes-Tettmar et al., 2013).

5. Conclusion

The Phytochip enables accurate multiplex identification of toxic phytoplankton even at low concentrations. Multiple species can be detected even if present at different ratios, which is often the case during bloom periods. Comparing to the standard method i.e., light microscopic observation, the strengths of the microarray technique is its ease of use, independence from the trained observer and rapidity. Moreover, it allows higher taxonomic resolution, successfully demonstrated by our diversity study of *Pseudo-nitzschia* species. For a routine monitoring program, the Phytochip could thus represent a useful tool as an early

warning system when used in tandem with microscopic cell counts for screening a large data set targeted on toxic or associated taxa.

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Data Accessibility

Sequences were deposited in GenBank under the Accession numbers KM245341 to KM245518.

Author Contributions

C.D., V.L.B designed research; C.N., C.D., A. A. and L.T performed research; C.N., C.D and V.L.B. analyzed data and wrote the paper.

Figure legends

Fig. 1 Hierarchical key for the identification of *Pseudo-nitzschia* species on the Phytochip. For example, if *P. delicatissima* is present in the sample, it must have a positive signal with probes from the species (*P. delicatissima*), its genus (*Pseudo-nitzchia*) and clade (*P. delicatissima* complex). If there is only a positive signal with the probe for the *P. delicatissima* species, this will be considered as a false positive.

Fig. 2 Seawater spiked with an increasing number of cells and tested on the phytochip (A) spiked with *Alexandrium minutum* cells. Please note that most of the *Alexandrium* sample signals were saturated as we have indicated by dashing of the bars; (B) spiked with *Pseudo-nitzschia delicatissima* cells.

Fig. 3 Evolution of the relative proportions of *Pseudo-nitzschia* from environmental samples collected in the Bay of Concarneau. (A) light microscopy; (B) clone libraries, (C) Phytochip. BC: Bay of Concarneau sample

Table legends

Table 1 Primers for labeled amplification

Table 2 Oligonucleotide probes for the hierarchical identification of HAB species.

Table 3 Detection of species in mixtures using the Phytochip. Genomic DNA was mixed in equal ratio consisting of (A) *Pseudo-nitzschia* species and (B) dinoflagellate species.

Table 4 Characteristics and diversity of clone libraries obtained from environmental samples collected in the Bay of Concarneau

Table 1 Primers for labeled amplification

Primer name	Marker	Target	Direction	Sequence (5'-3')	Ta (C°)	References
82F*	18S (SSU)	Eukaryotes	Forward	GAAACTGCGAATGAATGGCTC	48	Metfies and Medlin 2007, 2008
1528R	18S (SSU)	Eukaryotes	Reverse	TGATCCTTCTGCAGGTTCACCTAC	48	
D1R*	28S (LSU)	Eukaryotes	Forward	ACCCGCTGAATTTAAGCATA	55	Lenaers et al., 1989
D2C	28S (LSU)	Eukaryotes	Reverse	CCTTGGTCCGTGTTTCAAGA	55	
ITS1*	ITS1-5,8S-ITS2	Eukaryotes	Forward	TCCGTAGGTGAACCTGCGG	48	White et al., 1990
ITS4	ITS1-5,8S-ITS2	Eukaryotes	Reverse	TCCTCCGCTTATTGATATGC	48	
PSN_F1*	ITS1-5,8S-ITS2	*Pseudo-nitzschia*	Forward	GGATCATTACCACACCGATCC	58	designed for this study
PSN_R1	ITS1-5,8S-ITS2	*Pseudo-nitzschia*	Reverse	CCTCTTGCTTGATCTGAGATCC	58	

* labeled Cy5. Ta: annealing temperature.

Table 2 Oligonucleotide probes for the hierarchical identification of HABs species. Toxigenic species are marked by an asterisk.

Genus	Clade	Species	Probe name	Marker	Sequence (5'-3')
Pseudo-nitzschia			PSN_28S	28S	GACATCAACTCTGACTGCGCTCTTCC
			PSN_genus_hf20	ITS	AGTGGGATCCACAGACACTCAGACAAG
			PSN_genus_hf8	ITS	TCCCGAAAGCGCAATGTGCGTTCAAAA
	delicatissima complex		PSN_delicatGrp_ITS_5	ITS	AGAGGCAGTCAAGGCCAAAGCAACC
		*delicatissima**	PSN_delicat_ITS_7	ITS	GCCTACAGAATAGACCAGTGCTGAG
		arenysensis	PSN_arenys_ITS_26	ITS	TGAAACGATGCCGAAGCAGAGGTCG
	cuspidata/pseudodelicatissima		PSN_cuspidata_ITS_27	ITS	CGCTCCTGAATAGTAAGATCCAGGCAG
			PSN_pseudo_cuspi_ITS_2	ITS	ACAGTCAGTTCAGACCGTCAAAGCCA
			PSN_pseudodelicat_ITS_20	ITS	AGAGATAGACGAGAATGTCAGCACCGT
			PSN_pseudodelicat_ITS_4	ITS	AGTGAGAAATCACCAGTGCTGAGTGGG
	seriata/australis		PSN_seriataGrp_f10	ITS	CGACAGCGACGGAGAGCTTTAAAAGCA
			PSN_seriataGrp_f4	ITS	GTTTGACGACAGCGACGGAGAGCTT
		*seriata**	PSN_seriata_ITS_8	ITS	CAACCAGCGACCGGCCTAAACCAG
		*australis**	PSN_austr5-2m	ITS	GACAAGACAGGTTGAGGTCTCTAAATC
			PSN_australis5M	ITS	ACAGGTTGAGGTCTCTAAATCTATGCAC
		americana	PSN_americana31m	ITS	CCTAGCTGGCACCGAGCCTGAAATC
			PSN_americana_f10	ITS	GAGACAGCGAAACGCAGTCAAAGCC
			PSN_americana_ITS_8	ITS	ACAAGAGTGCCAACGCCGTCTTTC
		*calliantha**	PSN_calliantha_mannii_ITS_30	ITS	TAGTACAGCAGTCAAGCTGCTGCCC
			PSN_calliantha1-2	ITS	GGACGACATAGTACAGCAGTCAAGCTG
			PSN_calliantha3-1m	ITS	GAATTTAAGCAAAGACAGCAGCGGCTG
			PSN_calliantha_ITS_19	ITS	GCTACTGGAGCAGCAACCACCGTC
		*fraudulenta**	PSN_fraudulenta1m	ITS	CCGAAGCCAGAGTGCCACGCAAATC
			PSN_fraudulenta_f13	ITS	GTTGCCGGACCACTGCACTTTAAGC
			PSN_fraudulenta_ITS_41	ITS	CTTAATGCCACTGGTGCGATGTCGC
			PSN_fraudulenta_ITS_50	ITS	AGGTTTTATGTACAGGGCGGCTCCTG
		*galaxiae**	PSN_galax_decip_ITS_15	ITS	GTTTTTGTACAGGGCACACGGTGCT
			PSN_galaxiae_ITS_6	ITS	CTGCACCGCGATGAAGTTTGACGAC
			PSN_galaxiae_ITS_1	ITS	CCGTCCTTCGGACAAGCCAAGACTT
		*multiseries**	PSN_multiseries6	ITS	GCAAAGTATCAGTGCCAAGCCTCTGC

			PSN_multiseries_ITS_13	ITS	CAAAACAACCAGCAGCCAGCACGAG
			PSN_multiseries_ITS_1	ITS	TCAAGCCTTCCACGTCTATGCACGT
		*multistriata**	PSN_multistriata_f1	ITS	GAGCCAGTTGCCACTGCAATCGAATC
			PSN_multistriata_f9	ITS	GCAGCGCCTAACCTCTGCATTTTTGC
			PSN_multistriata3m	ITS	CACCTAGCAGAAAGTTGACGACACTGA
			PSN_multistriata4m	ITS	TACTATGCAAGCTCGTCACCTAGCAGA
		*pungens**	PSN_pungens_p1	ITS	TCAGCGCTCCGAAACACTGCATCA
			PSN_pungens1-5bm	ITS	CGTTGCAAGTTTAAGTTTGACGGCAGC
			PSN_pungens_ITS_4	ITS	CAGTGCCAGCAATAGAGTCGGTTTGGT
Azadinium			Azadinium_ITS_1	ITS	AGAACCCGACAGCAGGAATGAGGCT
			Azadinium_ITS_11	ITS	GCTACTGGCATTAGAAGGTAGAGGCAC
		*spinosum**	Azad_spinosum_ITS_47	ITS	GAGCCACTCACAAGAAAGCATGGAAGC
			Azad_spinosum_ITS_19	ITS	GCACTTGGTTGTTGAGGCCACCTTC
			Azad_spinosum_ITS_27	ITS	AAGGTTCCCCACAAGCTCAATGCGT
Lingulodinium		*polyedrum**	Lingu_polyedrum_ITS_22	ITS	ACGGCATGCACATGCTGGAACAGAC
			Lingu_polyedrum_ITS_30	ITS	TACGGAAAAGCCTGCCTGCATGGTC
			Lingu_polyedrum_ITS_47	ITS	CCAGCAACCACGGCAGATTTTACGG
			Lingu_polyedrum_ITS_52	ITS	GGCGTATCCCAAAGGCACAGCAAAC
Ostreopsis			Ostreopsis_sp_ITS	ITS	CAAGAAAAATGACTCACGGAATTCTGC
		*ovata**	Ostreopsis_ovata_ITS	ITS	GGCCCAAGAACATGCCTACATTCAAG
			Ostreopsis_ovata_ITS_2	ITS	AAATGATGTCCTTAGGGGTGGCCCA
Vulcanodinium		*rugosum**	Vulcano_ARB_ITS2_3	ITS	CACAATGCTTCTCACAGTTCGCTGC
			Vulcano_ITS_34	ITS	CAGGCATGCTTCCAGGGATATCCCG
Prorocentrum		*micans*	Pmicans_ITS_2	ITS	GGAAAGGAAGACAGTCCAATCCTGGCT
			Pmicans_ITS_5	ITS	GCAGGGACAGGAAAGGAAGACAGTCC
			Kare28S	28S	CAGTATCGCATCCAGATCAAAACCT
Karenia	*brevis/mikimotoi/selliformis*		KareITS_49	ITS	GGAAGACGGTCCAATATCACCCGGAC
	brevis/mikimotoi/selliformis		Kmiki_brev_ITS_38	ITS	TGTCTCCAGCCAAGAGCACAACTCA
	brevis/mikimotoi		Kmiki_brev_ITS_47	ITS	GAACTACCCGCGAGATTCTGCACAAG
		*mikimotoi**	KmikiITS_23	ITS	CATCAGGGGCAGGAAGAGCACCTTA
			KmikiITS_6	ITS	ACAGACACACACTGCTGTCAGTTGC
			KmikiITS_8	ITS	GGGCAGGAAGAGCACCTTAATGCAC
			Kbrevis_ITS_16	ITS	TCACCCACGTCACCAGGAAGATTGA
		*brevis**	Kbrevis_ITS_20	ITS	AGCACGAAACCCATGCACTGTATGC
	brevis/selliformis		Kselli_brev_ITS_46	ITS	GGCACAAATCACAGCAGGTGGTTCA

		*selliformis**	Kselli_ITS_29	ITS	CAGGATCAGAGGCAAGGTTGTCAGTGC
Alexandrium			Alex_SILVA	18S	CACACCACACAGTCAAGTGCAGTTGT
			Alex28S	28S	ACCACCCACTTTGCATTCCAATGCC
			Aminutum_insuet_SILV_5	18S	TGACCACAACCCTTCCCAGAAGTCA
	minutum/insuetum		Aminutum_SILV_9	18S	CCCAGAAGTCAGGTTTGGATGCATG
		*minutum**	Amin_ITS_8	ITS	TGCAACAGCATTGACACACACAGCTCA
			Amin_ITS_18	ITS	CCCACCACAGCTCACAAAGTCATGC
			Amin_ITS_23	ITS	GCAGAACCTAAAGCCTAGGAACCCCAC
			Amin2-1	28S	TGCCAGCACTGATGTGTAAGGGCTT
			Acat-tam_SILV_8	18S	AACCAACGACCACAACCTTTCCCCC
	tamarense/catenella		A_tamarenseGrp_SILV_5	18S	GGCGGACCAGCCATCCTCAGCA
			A_tamarenseGrp_ITS	ITS	CTGTTAGCTCACGGAATTCTGC
			Acatenella_SILV_6	18S	GGTTCTGCAACCAACGACCACAACC
		*catenella**	Alexcat_15	28S	GCACTACAATCTCACTGAGGAAATC
			Alexcat_17	28S	TTATTGCACTTGCAGCCAAAACCCA
		tamarense	Alex_tam11	28S	CATTACACCCACAGCCCAAAGCTCT

Table 3 Detection of species in mixtures using the Phytochip. Genomic DNA was mixed in equal ratio consisting of (A) *Pseudo-nitzschia* species and (B) dinoflagellates species.

A) Composition: Core species	*P. delicatissima; P. cuspidata/pseudodelicatissima; P. australis; P. seriata; P. americana; P. fraudulenta; P. multiseries; P. pungens*					
Species not present from the composition	*P. americana*$^{(-)}$	*P. australis*$^{(-)}$	*P. multiseries*$^{(-)}$	*P. pungens*$^{(-)}$	*P. delicatissima*$^{(-)}$	*P. delicatissima*$^{(-)}$ *P. fraudulenta*$^{(-)}$ *P. arenysensis*$^{(+)}$
PSN_genus_hf20	**65368**	**7555**	**35548**	**8163**	**46516**	**57739**
PSN_genus_hf8	**57683**	**5910**	**12867**	**6689**	**38935**	**24449**
PSN_delicatGrp_ITS_5	**26862**	**3124**	**10198**	**6093**	42	**6728**
PSN_delicatissima_ITS_7	**20397**	**1087**	**2960**	**2323**	-	-
PSN_arenysensis_ITS_26	-	-	-	-	-	**12872**
PSN_cuspidata_ITS_27	**6128**	**347**	**1356**	**1944**	**1042**	**1375**
PSN_pseudodelicat_ITS_4	**376**		3			
PSN_seriataGrp_f10	**1436**	**658**	**881**	**2372**	**5261**	**4210**
PSN_seriataGrp_f4	**783**	**146**	**646**	**460**	**997**	**409**
PSN_seriata_ITS_8	**5465**	119	**1785**	**2219**	**2784**	**1630**
PSN_australis5-2m	**2516**	-	**420**	**401**	**1402**	**985**
PSN_americana31m	-	**33065**	**65415**	**65415**	**65408**	**65409**
PSN_americana_f10	-	**9768**	**7267**	**16014**	**11209**	**15212**
PSN_fraudulenta1m	**65315**	**54248**	**65409**	**65416**	**65408**	12
PSN_fraudulenta_f13	**11902**	**840**	**1579**	**2078**	**8570**	-
PSN_fraudulenta_ITS_41	**1090**	2	516	149	366	-
PSN_fraudulenta_ITS_50	96			41		-
PSN_multiseries6	**20735**	**4256**	65	**16572**	**11161**	**9990**
PSN_multiseries_ITS_13	185	42	-	63		23
PSN_multiseries_ITS_1	361	1	-	190	21	17
PSN_pungens_p1	**65346**	**65394**	**65413**	-	**65403**	**65381**
PSN_pungens1-5bm	**65335**	**62717**	**65408**	-	**65403**	**65402**

B) Species composition	*A. minutum, A. catenella, A. tamarense; K. brevis, K. selliformis*	*A. minutum; K. mikimotoi*	*L. polyedrum; K. mikimotoi, K. brevis, K. selliformis*	*A. minutum; K. mikimotoi, K. selliformis*
PSN_genus_hf8	92			45
Lingu_polyedrum_ITS_22			**2753**	
Lingu_polyedrum_ITS_30			**31046**	
Lingu_polyedrum_ITS_47			**42403**	
Lingu_polyedrum_ITS_52			**8685**	
Ostreopsis_sp_ITS	86	90	152	133
Vulcano_ARB_ITS2_3			130	85
Vulcanodinium_ITS_4	616	194	725	318
Kare28S	158			
KareITS_49	**31350**	**33173**	**55244**	**38705**
Kmiki_brev_ITS_38	**7058**	**65430**	**57271**	**58211**
Kmiki_brev_ITS_47	**29697**	**53993**	**65429**	**51455**
KmikiITS_23		**652**	**1497**	**1220**
KmikiITS_6		**888**	**1198**	**1283**
KmikiITS_8	31	**1172**	**2321**	**1891**
Kbrevis_ITS_16	**27215**		**12751**	
Kbrevis_ITS_20	**2327**		**430**	
Kselli_brev_ITS_46	**23926**		**15474**	**4550**
Kselli_ITS_29			**97**	**159**
Alex28S	77	270	275	232
AminITS_8	**1978**	**519**		**745**
AminITS_18	**1886**	**954**		**1462**
AminITS_23	**4894**	**2561**		**3203**
A_tamarenseGrp_ITS	**6451**	77	88	92
Alexcat_17			6	14

Table 4 Characteristics and diversity of clone libraries obtained from environmental samples collected in the Bay of Concarneau.

Sample	Date	N	S	d	Coverage (%)	*Pseudo nitzschia* species
BC2	20/03/2012	41	3	0.54	92.7	*P. sp (cuspidata complex) P. fraudulenta*
BC6	17/04/2012	27	3	0.61	88.9	*P. sp (cuspidata complex) P. delicatissima. P. dolorosa*
BC12	14/05/2012	37	3	0.55	91.9	*P. sp (cuspidata complex) P. fraudulenta. P. pungens*
BC18	12/06/2012	24	4	0.94	83.3	*P. sp (cuspidata complex) P. fraudulenta. P. pungens*
BC21	26/06/2012	22	2	0.32	90.9	*P. sp (cuspidata complex)*
BC26	12/07/2012	27	5	1.21	81.5	*P. sp (cuspidata complex) P. fraudulenta. P. pungens, P. subpacifica*

N: number of used sequences; S: number of OTUs; d: diversity estimated as Margalef species richness (S-1)/log(N); coverage calculated as 1-S/N (McDonald *et al*. 2007).

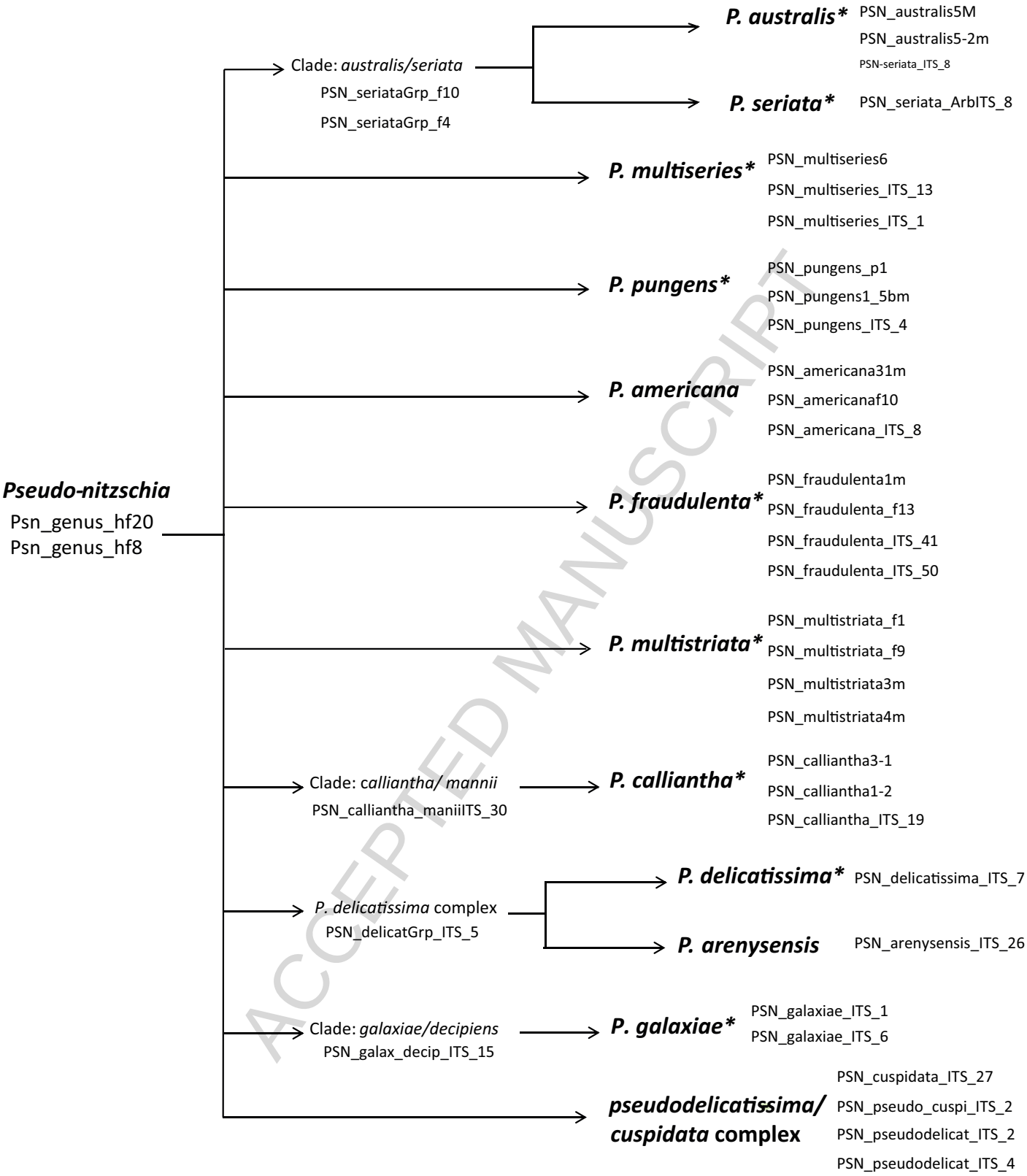

Figure 1

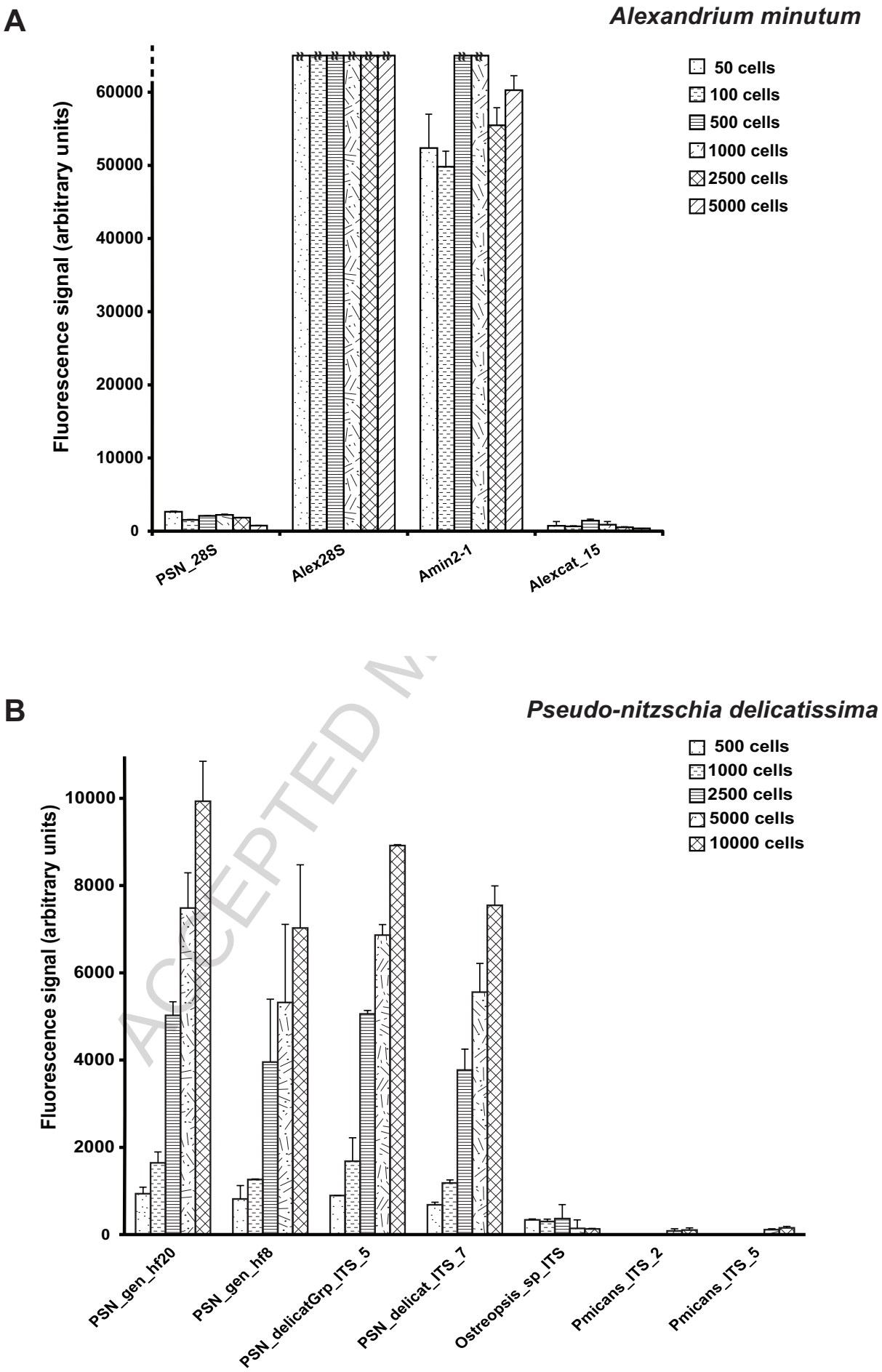

Figure 2

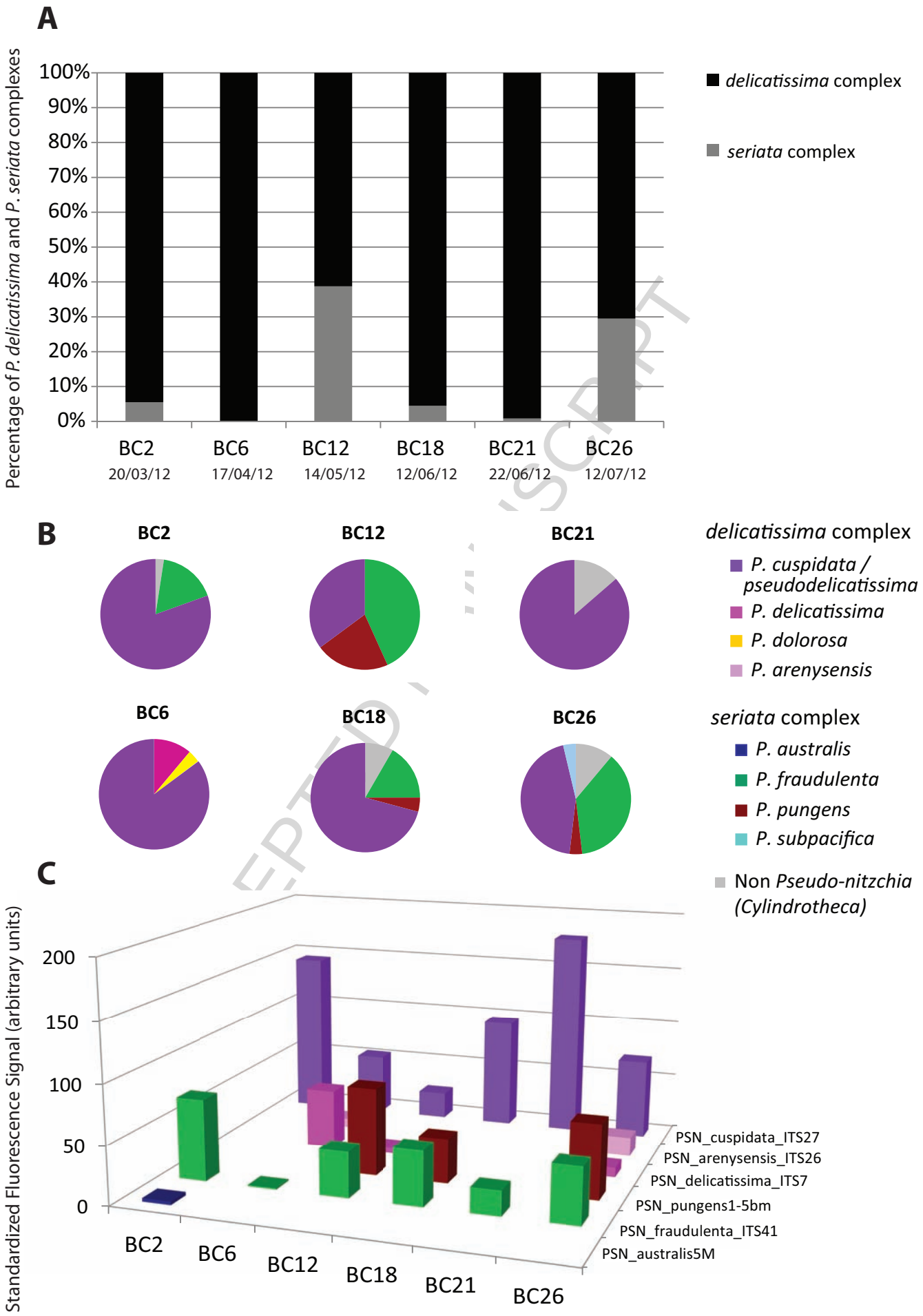

Figure 3

Table S1: Microalgae used to test Phytochip specificity.

Genus	Species	Strains	Collecting site	Location (culture or DNA bank)	Remarks
Pseudo-nitzschia	*australis*	Cabourg (C6)	France, Cabourg	Caen University, France	J. Fauchot, M. Thorel
Pseudo-nitzschia	*australis*	A1	France, Boyard	Ifremer, France	
Pseudo-nitzschia	*australis*	Kervel	France, Kervel	Ifremer, France	
Pseudo-nitzschia	*americana*	Antifer	France, Antifer	Ifremer, France	
Pseudo-nitzschia	*americana*	Cabourg	France, Cabourg	Ifremer, France	
Pseudo-nitzschia	*americana*	Kervel	France, Kervel	Ifremer, France	
Pseudo-nitzschia	*fraudulenta*	Pn427		Gent University, Belgium,	G. Casteleyn
Pseudo-nitzschia	*fraudulenta*	LsM1	France, Luc-sur-Mer	Caen University, France	J. Fauchot, M. Thorel
Pseudo-nitzschia	*fraudulenta*	Cabourg (C4)	France, Cabourg	Caen University, France	J. Fauchot, M. Thorel
Pseudo-nitzschia	*fraudulenta*	B2	France, Baie de Seine	Ifremer, France	
Pseudo-nitzschia	*fraudulenta*	CL192	Canada, Deadman's Hbr	Ifremer, France	
Pseudo-nitzschia	*fraudulenta*	CL194	Canada, Bay of Fundy	Ifremer, France	
Pseudo-nitzschia	*fraudulenta*	IFSA1	France, Baie de Seine	Ifremer, France	
Pseudo-nitzschia	*seriata*	CL160	Canada, Tracadie bay	Ifremer, France	
Pseudo-nitzschia	*seriata*	CL163	Canada, Tracadie Bay	Ifremer, France	
Pseudo-nitzschia	*seriata*	CL166	Canada	Ifremer, France	
Pseudo-nitzschia	*seriata*	CL167	Canada, Rustico bay	Ifremer, France	
Pseudo-nitzschia	*seriata*	CL168	Canada, Rustico Bay	Ifremer, France	
Pseudo-nitzschia	*pungens*	Pn567		Gent University, Belgium	G. Casteleyn
Pseudo-nitzschia	*pungens*	CL200	Canada, Miramichi bay	Ifremer, France	
Pseudo-nitzschia	*pungens*	D10		Ifremer, France	
Pseudo-nitzschia	*pungens*	PE6Kervel	France, Kervel	Ifremer, France	
Pseudo-nitzschia	*calliantha*	D1	France, Men Du	Ifremer, France	
Pseudo-nitzschia	*calliantha*	D2	France, Men Du	Ifremer, France	
Pseudo-nitzschia	*calliantha*	D9	France, Men Du	Ifremer, France	
Pseudo-nitzschia	*multiseries*	CL174	Canada, Cardigan Bay	Ifremer, France	
Pseudo-nitzschia	*multiseries*	CL191	Canada, Deadman's Hbr	Ifremer, France	
Pseudo-nitzschia	*multiseries*	CL195	Canada, Bay of fundy	Ifremer, France	
Pseudo-nitzschia	*multiseries*	CL197	Canada, Mirimachi Bay	Ifremer, France	
Pseudo-nitzschia	*multiseries*	Pn556		Gent University, Belgium	G. Casteleyn

Pseudo-nitzschia	*delicatissima*	Gefosse G3	France, Gefosse	Caen University, France	J. Fauchot, M. Thorel
Pseudo-nitzschia	*delicatissima*	IFSG3	France, St Germain de Varreville	Ifremer, France	
Pseudo-nitzschia	*delicatissima*	IFSG5	France, St Germain de Varreville	Ifremer, France	
Pseudo-nitzschia	*delicatissima*	IFG2	France, Gefosse	Ifremer, France	
Pseudo-nitzschia	*delicatissima*	IFG4	France, Gefosse	Ifremer, France	
Pseudo-nitzschia	*pseudodelicatissima*	205 PN-JF	France	Ifremer, France	
Pseudo-nitzschia	*calliantha*	120		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*delicatissima*	AR1		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*delicatissima*	171		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*delicatissima*	161		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*arenysensis*			Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*arenysensis*			Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*fraudulenta*	AR3		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*galaxiae*	173		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*multistriata*	150		Tarragona, IRTA	K. Andree
Pseudo-nitzschia	*pungens*	143		Tarragona, IRTA	K. Andree
Alexandrium	*minutum*	AM89BM	France, Baie de Morlaix	Ifremer, France	
Alexandrium	*minutum*	AM99PZ	France, Penzé	Ifremer, France	
Alexandrium	*catenella*	v676	France, Etang de Thau	Ifremer, France	
Alexandrium	*catenella*	v817	France, Etang de Thau	Ifremer, France	
Alexandrium	*catenella*	B9-1		Montpellier University, France	M. Laabir
Alexandrium	*catenella*	C1-1		Montpellier University, France	M. Laabir
Alexandrium	*catenella*	D3-1		Montpellier University, France	M. Laabir
Alexandrium	*catenella*	A12-1		Montpellier University, France	M. Laabir
Alexandrium	*catenella*	D9-1		Montpellier University, France	M. Laabir
Alexandrium	*catenella*	C10-1		Montpellier University, France	M. Laabir
Alexandrium	*tamarense*	ATT07	France, Etang de Thau	Ifremer, France	
Alexandrium	*tamarense*	H2-1		Montpellier University, France	M. Laabir
Alexandrium	*tamarense*	A33		Montpellier University, France	M. Laabir
Alexandrium	*tamarense*	H4-2		Montpellier University, France	M. Laabir
Alexandrium	*tamarense*	B11-2		Montpellier University, France	M. Laabir
Alexandrium	*tamarense*	C12-1		Montpellier University, France	M. Laabir
Alexandrium	*tamarense*	B9-3		Montpellier University, France	M. Laabir

Karenia	*mikimotoi*	GATIN95	France	Ifremer, France	
Karenia	*selliformis*		France	Ifremer, France	
Karenia	*brevis*	sp3		Texas A &M University, dept of Oceanography, USA	L. Campbell
Karenia	*brevis*	wilson		Texas A &M University, dept of Oceanography, USA	L. Campbell
Karenia	*brevis*	sp1		Texas A &M University, dept of Oceanography, USA	L. Campbell
Karenia	*mikimotoi*	C21		Texas A &M University, dept of Oceanography, USA	L. Campbell
Prorocentrum	*micans*		France	Ifremer, France	
Prorocentrum	*triestrum*		France	Ifremer, France	
Prorocentrum	*lima*	PL2000	France	Ifremer, France	
Lingulodinium	*polyedrum*	VGO668	France	Ifremer, France	
Ostreopsis	*ovata*	IFR-OST	France	Ifremer, France	
Vulcanodinium	*rugosum*		France, Etang d'Ingril	Ifremer, France	
Dinophysis	*caudata*			Instituto Español de Oceanografía Vigo, Spain	F. Rodriguez
Dinophysis	*acuminata*			Instituto Español de Oceanografía, Vigo, Spain	F. Rodriguez
Dinophysis	*acuta*			Instituto Español de Oceanografía, Vigo, Spain	F. Rodriguez
Dinophysis	*tripos*			Instituto Español de Oceanografía, Vigo, Spain	F. Rodriguez
Dinophysis	*acuminata*	D1	France, Antifer	Ifremer, France	
Dinophysis	*acuminata*	D2	France, Antifer	Ifremer, France	
Azadinium	*spinozum*			Ifremer, France	
Chaetoceros	*grande*			Ifremer, France	
Scripsiella	*trochoidea*	ST97PZ	France, Penzé	Ifremer, France	
Tetraselmis	*sp.*			Ifremer, France	
Skeletonema	*costatum*			Ifremer, France	

Table S2: Experimental validation of probe specificity**.** Fluorescence signal of single target hybridizations to the 81 validated probes. (A) *Pseudo-nitzschia* species; (B) various dinoflagellate species; (C) *Alexandrium* species. Probes showing no signal are not listed. Bold: values above the threshold.

A)	***P. delicatissima***	***P. arenysensis***	***P. sp cuspidata/ pseudodelicatissima***	***P. seriata***	***P. australis***	***P. americana***	***P. calliantha***	***P. fraudulenta***	***P. galaxiae***	***P. multiseries***	***P. multistriata***	***P. pungens***
PSN_gen_hf20	**8914**	**6877**	**1045**	**16926**	**39580**	**19965**	**9168**	**7386**	**6852**	**4708**	**7082**	**39286**
PSN_gen_hf8	**10546**	**7666**	**11624**	**18569**	**40755**	**21099**	**12816**	**6527**	**17038**	132	**5155**	**39132**
PSN_delicatGrp_ITS_5	**4989**	**4613**	39	83	136		**586**				82	
PSN_delicat_ITS_7	**5689**	560						9	37		89	
PSN_arenys_ITS_26		**46016**										
PSN_cuspidata_ITS_27			**60539**								167	
PSN_pseudo_cuspi_ITS_2			**6135**									
PSN_pseudodelicat_ITS_20			159									
PSN_pseudodelicat_ITS_4	19		**2822**									
PSN_seriataGrp_f10				**65364**	**54598**							
PSN_seriataGrp_f4				**65365**	**54663**							
PSN_seriata_ITS_8				**8825**	**1382**							
PSN_austr5-2m					**7946**							
PSN_australis5M					**11848**							
PSN_americana31m						**65295**						
PSN_americana_f10						**6663**						
PSN_americana_ITS_8						451				**422**		
PSN_calliantha_manii_ITS_30							**4836**					
PSN_calliantha1-2							**16586**				347	
PSN_calliantha3-1m							**3908**				13	
PSN_calliantha_ITS_19				25			**5628**				214	
PSN_fraudulenta1m							153	**63778**				57
PSN_fraudulenta_f13							52	**27813**	355			
PSN_fraudulenta_ITS_41							84	**36123**				
PSN_fraudulenta_ITS_50								**9209**				
PSN_galax_decip_ITS_15									**27471**			
PSN_galaxiae_ITS_1		103							**3412**			
PSN_galaxiae_ITS_6					8				**47120**			
PSN_multiseries6								135		**26733**		
PSN_multiseries_ITS_1	71	236								**3136**		
PSN_multiseries_ITS_13	36									**8237**		
PSN_multistriata_f1				22				124		**315**	**35346**	
PSN_multistriata_f9						33					**4869**	

PSN_multistriata3m											**34417**	
PSN_multistriata4m											**33577**	
PSN_pungens_p1												**65177**
PSN_pungens1-5bm		211	9								**1432**	**65133**
PSN_pungens_ITS_4												**65 73**
Vulcanodinium_ITS_4			44									
Ostreop_ovata_ITS_2					64							
KareITS_44												
KareITS_49		102										177
Kmiki_brev_ITS_38		179		16								82
Kmiki_brev_ITS_47	45											172
Kselli_brev_ITS_46		290										
KmikiITS_23				162		14						45
Kbrevis_ITS_16	141											
Amin2-1						435						
AminITS_18			159	345								
AminITS_23												129
A_tamarenseGrp_ITS						59						

B)	*A. spinosum*	*L. polyedrum*	*O. ovata*	*V. rugosum*	*K. mikimotoi*	*K. brevis*	*K. selliformis*	*P. micans*
PSN_gen_hf8			**650**	100				155
PSN_pseudodelicat_ITS_4	28							
PSN_fraudulenta_ITS_41	299							
PSN_multiseries_ITS_1					304			
Azadinium_sp_ITS_1	**65337**							
Azadinium_sp_ITS_11	**25437**							
Azad_spinosum_ITS_47	**48216**				53			
Azad_spinosum_ITS_19	**3249**							
Azad_spinosum_ITS_27	**3629**							
Lingu_polyedrum_ITS_22		**5305**						
Lingu_polyedrum_ITS_30		**40914**						
Lingu_polyedrum_ITS_47		**50000**						
Lingu_polyedrum_ITS_52		**12163**						
Ostreopsis_sp_ITS	473		**28897**		893	27		
Ostreopsis_ovata_ITS			**24781**					
Ostreop_ovata_ITS_2			**10891**					
Vulcano_ARB_ITS2_3		3		**7968**	328	79	50	
Vulcanodinium_ITS_34				**895**	50			
KareITS_49					**59312**	**53378**	**60109**	
Kmiki_brev_ITS_38					**64455**	**12536**	26	
***Kmiki*_brev_ITS_47**					**64142**	**54209**	150	
KmikiITS_23					**13626**			
KmikiITS_6					**12943**			
KmikiITS_8					**17993**	72		
Kbrevis_ITS_16						**37541**		
Kbrevis_ITS_20						**2075**		
Kselli_brev_ITS_46					307	**35432**	**65340**	
Kselli_ITS_29							**1118**	
Pmicans_ITS_2	524							**65322**
Pmicans_ITS_5	543							**65327**
A_tamarenseGrp_ITS	199				44	217		
Alexcat_15					27			
Alexcat_17					80			
Amin2-1					149			
AminITS_8						92		

C)	18S			28S			ITS		
	A. minutum	*A. catenella*	*A. tamarense*	*A. minutum*	*A. catenella*	*A. tamarense*	*A. minutum*	*A. catenella*	*A. tamarense*
PSN_28S					109	45			
PSN_gen_hf20		15					78	51	53
PSN_gen_hf8							19	64	22
PSN_delicatGrp_ITS_5		67							
PSN_pseudodelicat_ITS_4		90	104						
PSN_seriataGrp_f4		53							
PSN_seriata_ITS_8		125							
PSN_americana31m		156					104	28	
PSN_fraudulenta_ITS_41								10	14
PSN_fraudulenta1m								29	61
PSN_multiseries_f8		50							
PSN_multiseries_ITS_1		69							
PSN_multiseries_ITS_13	95	**2940**	**2673**		6				
Ostreopsis_sp_ITS							33	**812**	**319**
Vulcanodinium_ITS_25							53		
Vulcanodinium_ITS_4							57		
KareITS_44								190	
KareITS_49								722	
Kselli_brev_ITS_46								505	
Kselli_ITS_29								82	
Pmicans_ITS_2							45		
Alex_SILVA	**8423**	**17783**	**14052**						
Alex28S		43		**53577**	**65289**	**59391**		2	
Aminutum_insuet_SILV_5	**28673**	66							
Aminutum_SILV_9	**41907**	142							
AminITS_23							**65355**	10	
AminITS_8		57					**10933**		
Amin_ITS_18							**61080**		
Amin2-1				**2073**					
Acat-tam_SILV_8		**60618**	**39343**					19	
A_tamarenseGrp_SILV_5	**1049**	**25667**	**19395**						
A_tamarenseGrp_ITS							**1554**	**33604**	**20820**
Acatenella_SILV_6		**44666**	**1522**						
Alexcat_15					**31551**				
Alexcat_17					**60514**	39			
Alex_tam11					149	**1368**			

Table S3: Identification of *Pseudo-nitzschia* species by the Phytochip in environmental samples collected in the Bay of Concarneau. FS value in bold: values above of the threshold.

Samples		**BC2**	**BC6**	**BC12**	**BC18**	**BC21**	**BC26**
Target Species	**Probes**						
	PSN_genus_hf20	**9509**	**10852**	**3327**	**8605**	**12784**	**9530**
	PSN_genus_hf8	**7554**	**12421**	**4529**	**10455**	**16108**	**12329**
P. delicatissima/ arenysensis/ micropora	PSN_delicatGrp_ITS_5	**155**	**5541**	**155**	87	125	**796**
P. delicatissima	PSN_delicatissima_ITS_7	78	**5212**	**91**	48		**372**
P. arenysensis	PSN_arenysensis_ITS_26		**849**				**1570**
P. cuspidata. pseudodelicatissima	PSN_cuspidata_ITS_27	**13679**	**5751**	**769**	**8263**	**22758**	**6624**
	PSN_pseudo_cuspi_ITS_2	**7118**	**4551**	**1537**	**5634**	**7670**	**3061**
	PSN_pseudodelicat_ITS_20	**1901**	**1528**	**129**	**1624**	**2165**	**838**
	PSN_pseudodelicat_ITS_4	**1925**	**1367**		**1435**	**2523**	**845**
P. seriata/ australis	PSN_seriataGrp_f10	**133**					
	PSN_seriataGrp_f4	10					
P. australis	PSN_australis5-2m	**257**					
	PSN_australis5M	**274**					
P. americana	PSN_americana31m						**175**
	PSN_americana_f10						
P. fraudulenta	PSN_fraudulenta1m	**16455**	**381**	**2341**	**9123**	**6209**	**11196**
	PSN_fraudulenta_f13	**4914**	75	**534**	**2658**	**2364**	**2992**
	PSN_fraudulenta_ITS_41	**6664**	**128**	**1308**	**4057**	**2742**	**4589**
	PSN_fraudulenta_ITS_50	**1530**		**280**	**1115**	**843**	**1307**
P. pungens	PSN_pungens_p1			**1052**	**1505**		**2950**
	PSN_pungens1-5bm			**2492**	**3289**		**6068**
	PSN_pungens_ITS_4			**3843**	**4318**		**7225**
Species not experimentally validated	PSN_dolorosa_ITS_36						
	PSN_dolorosa_ITS_44		**180**				
	PSN_heimii_f18						80
	PSN_heimii_ITS_12	21	33				**264**
	PSN_heimii_ITS_6			28			**169**
	PSN_subpacifiqua_f6	71					**315**

Fig. S1.

Evidence of a “Position Of Label” (POL) effect. A) Schematic representation of hybrid pairs on the chip surface is shown for four hybridization positions. PSN_fraudulenta1m, PSN_fraudulenta_ITS_41, PSN_fraudulenta_f13 and PSN_fraudulenta_ITS_50 probes (in black) are spotted. The fluorescent Cy5 label located at the 5′ end of the DNA target (i.e. *P. fraudulenta* species — in red) is indicated as a star. Four pairs of hybridization spots are shown for the different probes. The distance between the Cy5 label and the oligonucleotide probe, which clearly influences the fluorescent signal, are graphically represented. Larger distances lead to weaker signals. B) Relationship between the FS and the distance between the fluorescent label and the oligonucleotide probes based on 25 *Pseudo-nitzschia* probes from 7 species for which we have at least 3 specific probes. Species probes were separately analyzed to limit the nucleotide composition effect. Relationships were significant except for *Pseudo-nitzschia calliantha*.