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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 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 seawater 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.

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## 1. Introduction

Monitoring harmful algal blooms (HABs) 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 are faced by 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 (Kaczmarek 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 have to be routinely examined. Because of

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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, 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 and Galluzzi, 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 phyochips, 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, 2008b; Scorzetti et al., 2009; Metfies et al., 2010; Galluzzi et al., 2011; Smith et al., 2012; Barra et al., 2013; Edvardsen 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-nitzschia* 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 Dendriscides; 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  $25 \pm 2$  bp; 2) melting temperature ( $T_m$ )  $55^\circ\text{C}$  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; and 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 from public databases (EMBL/GenBank/DBJ); 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  $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  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  $-20^\circ\text{C}$ . The DNA from the cultures (10 to 30 ng/reaction)

**Table 1**  
Primers for labeled amplification.

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

<sup>a</sup> Labeled Cy5. Ta: annealing temperature.

was amplified by an asymmetric PCR reaction (Janse et al., 2012). Fifty microliter reaction mixtures consisted of 1X Flexi-Promega Buffer, 200  $\mu$ M of dNTPs, 3.5 mM of MgCl<sub>2</sub>, 1.25  $\mu$ l of GoTaq® DNA polymerase (Promega, USA) with 0.5  $\mu$ M 5'-labeled Cy5 forward primer, and 0.1  $\mu$ M 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 (1 $\times$ ); 35 cycles consisting of (30 s 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 was 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-silanized glass surface onto which NH<sub>2</sub>-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  $\mu$ M in 0.15 M 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'-GCCACA GTTGAGTAGATAGCCAGAATC-3' and 5'-AGATAGCCAGAATCCGATAGAC CCC-3') and for which a complementary sequence fragment was mixed into the sample before the PCR step. An anchoring Cy3-labeled 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<sup>-1</sup> 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 4 $\times$ , SDS 0.2%, 0.1 mg/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 2 $\times$  SSC/0.2% (V/

V) SDS and for 1 min. in 0.2 $\times$  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 *Alexandrium minutum* and *Pseudo-nitzschia 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 seawater was spiked with approximately 500, 1000, 5000, 10,000, and 25,000 *A. minutum* cells/L, and 5000, 10,000, 25,000, 50,000 and 100,000 *P. delicatissima* cells/L. These samples were filtered onto Isopore membranes with a pore size of 1.2  $\mu$ m (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 asymmetric PCR using the 5'-labeled 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 8 L 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 seawater 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 Sections 2.2 and 2.3).

In order to test the specificity of the *Pseudo-nitzschia* 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 × 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, 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-nitzschia* 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\_serjata ITS\_8 with its closest relative, the *Pseudo-nitzschia australis* species, was observed, but FS was lower for *P. australis* than for *Pseudo-nitzschia 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 spinosum*, *Lingulodinium polyedrum*, *Ostreopsis ovata*, *Karenia brevis*, *Karenia mikimotoi*, *Karenia selliformis*, *Prorocentrum ovicans* (Table S2B) and 3 *Alexandrium* species

(*Alexandrium catenella*, *Alexandrium tamarense* and *Alexandrium minutum*; Table S2C). *A. tamarense* and *A. catenella* are closely related species that were only discriminated by the probes designed on the 28S rDNA gene sequence. *A. minutum* can be identified with the probes designed on the 18S, ITS region and 28S rDNA gene sequences (Tables 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\_serjataGrp\_f10 and PSN\_serjataGrp\_f4) and one species probe (PSN\_serjata ITS\_8) was barely detected whereas the 2 clade probes reached saturation in simplex (Tables 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 *Vulcanodinium* oligonucleotide probes excluded their presence in the mixtures.

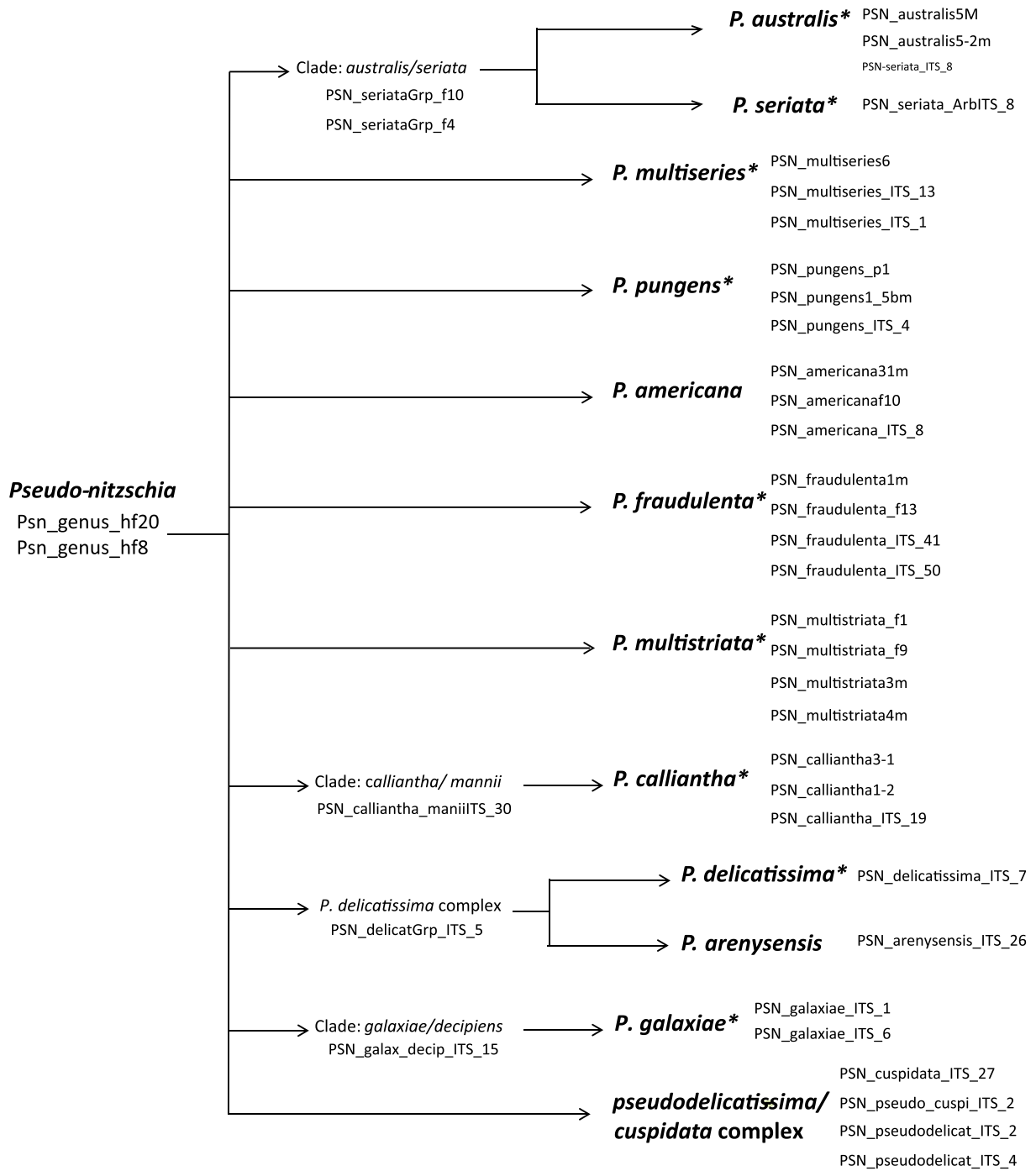
### 3.2. Sensitivity of the Phytochip

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 *serjata* 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 *A. 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-nitzschia* cells in natural sampled seawater. 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, Tables 4 and S3). Nevertheless, a few disparities were observed between microarray and clone library 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-nitzschia* 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 *Minutocella* 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



**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-nitzschia*) 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.

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. *Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia 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, *Pseudo-nitzschia dolorosa* and *Pseudo-nitzschia 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 (Tables 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

**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')	
<i>Pseudo-nitzschia</i>			PSN_28S	28S	GACATCAACTCTGACTGCGCTCTTCC	
			PSN_genus_hf20	ITS	AGTGGATCCACAGACACTCAGACAAG	
			PSN_genus_hf8	ITS	TCCCCAAAGCGCAATGTGCGTTCAAAA	
	<i>delicatissima complex</i>	<i>delicatissima*</i>		PSN_delicatGrp ITS_5	ITS	AGAGGCAGTCAAGGCCAAAGCAACC
				PSN_delicat ITS_7	ITS	GCCTACAGAATAGACCAGTGTCTGAG
				PSN_arenys ITS_26	ITS	TGAAACGATGCCGAAGCAGAGGTCG
	<i>cuspidata/pseudodelicatissima</i>			PSN_cuspidata ITS_27	ITS	CGCTCCTGAATAGTAAGATCCAGGCAG
				PSN_pseudo_cuspi ITS_2	ITS	ACAGTCAGTTCAGACCCGTCAAAGCCA
				PSN_pseudodelicat ITS_20	ITS	AGAGATAGACGAGAATGTGACGACCGT
	<i>seriata/australis</i>			PSN_pseudodelicat ITS_4	ITS	AGTGAGAAATCACCAGTGTGAGTGGG
				PSN_seriataGrp_f10	ITS	CGACAGCGCAGGAGAGCTTTAAAGCA
				PSN_seriataGrp_f4	ITS	GTTTGACGACAGCGCAGGAGAGCTT
		<i>seriata*</i>		PSN_seriata ITS_8	ITS	CAACCAGCGACCCGCTAAACCAG
			<i>australis*</i>	PSN_austr5-2 m	ITS	GACAAGACAGGTTGAGGTCTCTAAATC
		<i>americana</i>		PSN_australis5M	ITS	ACAGGTTGAGGTCTCTAAATCTATGCAC
				PSN_americana31m	ITS	CCTAGCTGGCACCCGAGCTGAAATC
				PSN_americana_f10	ITS	GAGACAGCCAAGCGCAGTCAAAGCC
		<i>calliantha*</i>		PSN_americana ITS_8	ITS	ACAAGAGTGCCAACCGCTTCTTTC
				PSN_calliantha_mannii ITS_30	ITS	TAGTACAGCAGTCAAGCTGTCTGCC
				PSN_calliantha1-2	ITS	GGACGACATAGTACAGCAGTCAAGCTG
				PSN_calliantha3-1 m	ITS	GAATTTAAGCAAAGACAGCAGCGGCTG
				PSN_calliantha ITS_19	ITS	GCTACTGGAGCAGCAACCACCGTC
			<i>fraudulenta*</i>	PSN_fraudulenta1m	ITS	CCGAAGCCAGAGTGGCCAGCTGAAATC
				PSN_fraudulenta_f13	ITS	GTTGCCGACCACTGCACITTAAGC
				PSN_fraudulenta ITS_41	ITS	CTTAATGCCACTGGTGGATGTCCG
				PSN_fraudulenta ITS_50	ITS	AGTTTTATGTACAGGGCGGCTCTCTG
	<i>galaxiae*</i>		PSN_galax_decip ITS_15	ITS	GTTTTGTACAGGGCACAGGTGTCT	
			PSN_galaxiae ITS_6	ITS	CTGCACCCGATGAAGTTTGACGAC	
	<i>multiseries*</i>		PSN_galaxiae ITS_1	ITS	CCGTCTTCGGACAAGCCAAGACTT	
			PSN_multiseries6	ITS	GCAAAGTATCAGTGGCAAAGCTCTGC	
			PSN_multiseries ITS_13	ITS	CAAAACAACCAGCAGCCAGCAGCAG	
	<i>multistriata*</i>		PSN_multiseries ITS_1	ITS	TCAAGCCTTCCACGTCTATGCACGT	
			PSN_multistriata_f1	ITS	GAGCCAGTTGCCACTGCAATCGAATC	
			PSN_multistriata_f9	ITS	GCAGCGCCTAACCTCTGCATTTTTGC	
			PSN_multistriata3m	ITS	CACCTAGCAGAAAGTTGACGACACTGA	
			PSN_multistriata4m	ITS	TACTATGCAAGCTCGTCACTAGCAGA	
		<i>pungens*</i>	PSN_pungens_p1	ITS	TCAGCGCTCCGAAACACTGCATCA	
			PSN_pungens1-5bm	ITS	CGTTGCAAGTTAAGTTTGACGGCAGC	
			PSN_pungens ITS_4	ITS	CAGTGCAGCAATAGAGTCCGTTTGGT	
			Azadinium ITS_1	ITS	AGAACCACAGCAGCAAGTGAAGGCT	
	<i>Azadinium</i>	<i>spinosum*</i>		Azadinium ITS_11	ITS	GCTACTGGCATTAGAAGTGAAGGCAC
				Azad_spinosum ITS_47	ITS	GAGCCACTCACAGAAAGCATGGAAGC
				Azad_spinosum ITS_19	ITS	GCACITGGTTGTTGAGGCCACITTC
	<i>Lingulodinium</i>	<i>polyedrum*</i>		Azad_spinosum ITS_27	ITS	AAGGTTCCCAACAAGCTCAATGGCT
				Lingu_polyedrum ITS_22	ITS	ACGGCATGCACATGTGGAACAGAC
				Lingu_polyedrum ITS_30	ITS	TACGGAAAAGCCTGCTGCATGGTC
				Lingu_polyedrum ITS_47	ITS	CCAGCAACCACGCGCAGATTTTACGG
<i>Ostreopsis</i>	<i>ovata*</i>		Lingu_polyedrum ITS_52	ITS	GGCGTATCCCAAAGGCACAGCAAAC	
			Ostreopsis_sp ITS	ITS	CAAGAAAAATGACTCACGGAATTTCTG	
			Ostreopsis_ovata ITS	ITS	GGCCCAAGAATGCTCATTTCAAG	
<i>Vulcanodinium</i>	<i>rugosum*</i>		Ostreopsis_ovata ITS_2	ITS	AAATGATGTCCTTATGGGTGGCCCA	
			Vulcano_ARB ITS2_3	ITS	CACAATGCTTCTCAGTTCGCTGC	
<i>Prorocentrum</i>	<i>micans</i>		CAGGCATGCTCCAGGATATCCCG	ITS	GGAAAGGAAGCAGCTCAATCTGGCT	
			Vulcano ITS_34	ITS	GCAGGGACAGGAAAGGACAGTCC	
			Pmicans ITS_2	ITS	CAGTATCGATCCAGATCAAACCT	
<i>Karenia</i>	<i>brevis/mikimotoi/selliformis</i>		Pmicans ITS_5	ITS	GGAAGACGGTCCAATATCAACCAGAC	
			Kare28S	28S	TGTCTCAGCCAAGACACAACCTCA	
			KareITS_49	ITS	GAACTACCCGCGAGATTCTGCACAAG	
	<i>brevis/mikimotoi</i>	<i>mikimotoi*</i>		Kmiki_brev ITS_38	ITS	CATCAGGGGCGAGGAGACACCTTA
				Kmiki_brev ITS_47	ITS	ACAGACACACACTGCTGTGAGTTGC
				Kmiki ITS_23	ITS	GGGCAGGAAGAGCACCTTAATGCAC
	<i>brevis/selliformis</i>	<i>brevis*</i>		Kmiki ITS_6	ITS	TCACCACGTCACAGGAAGATTGA
				Kmiki ITS_8	ITS	AGCACGAAACCCATGCCTGTATGC
				Kbrevis ITS_16	ITS	GGCACAATCAGCAGGTGTTCA
				Kbrevis ITS_20	ITS	CAGGATCAGAGGCAAGTTGTGAGTGC
<i>Alexandrium</i>	<i>selliformis*</i>		Kseli_brev ITS_46	ITS	CACACCACACAGTCAAGTGCAGTTGT	
			Kseli ITS_29	ITS	ACCACCCACTTTGCAITTCATGCC	
			Alex_SILVA	18S	TGACCACAACCTTCCCAGAAGTCA	
	<i>minutum/insuetum</i>	<i>minutum*</i>		Alex28S	28S	CCCAGAAGTCAGGTTTGGATGCATG
				Aminutum_insuet_SILV_5	18S	TGCAACAGACTTGACACACAGCTCA
				Aminutum_SILV_9	18S	CCCACCACAGTCTCAAAAGTCATGC
				Amin ITS_8	ITS	GCAGAACCTAAAGCCTAGGAACCCAC
				Amin ITS_18	ITS	TGCCAGCACTGATGTGAAGGCTT
				Amin ITS_23	ITS	AACCAACGCCACAACCTTTCCCTC
				Amin2-1	28S	
	Acat-tam_SILV_8	18S				

Table 2 (continued)

Genus	Clade	Species	Probe name	Marker	Sequence (5'-3')
	<i>tamarense/catenella</i>		A_tamarenseGrp_SILV_5	18S	GGCGGACCAGCCATCCTCAGCA
			A_tamarenseGrp_ITS	ITS	CTGTTAGCTCACGGAATTCTGC
			Acatenella_SILV_6	18S	GGTTCTGCAACCAACGACCACAACC
		<i>catenella*</i>	Alexcat_15	28S	GCACTACAATCTCACTGAGGAAATC
			Alexcat_17	28S	TTATTGCACCTTGACGCCAAAACCCA
		<i>tamarense</i>	Alex_tam11	28S	CATTACACCCACAGCCAAAGCTCT

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

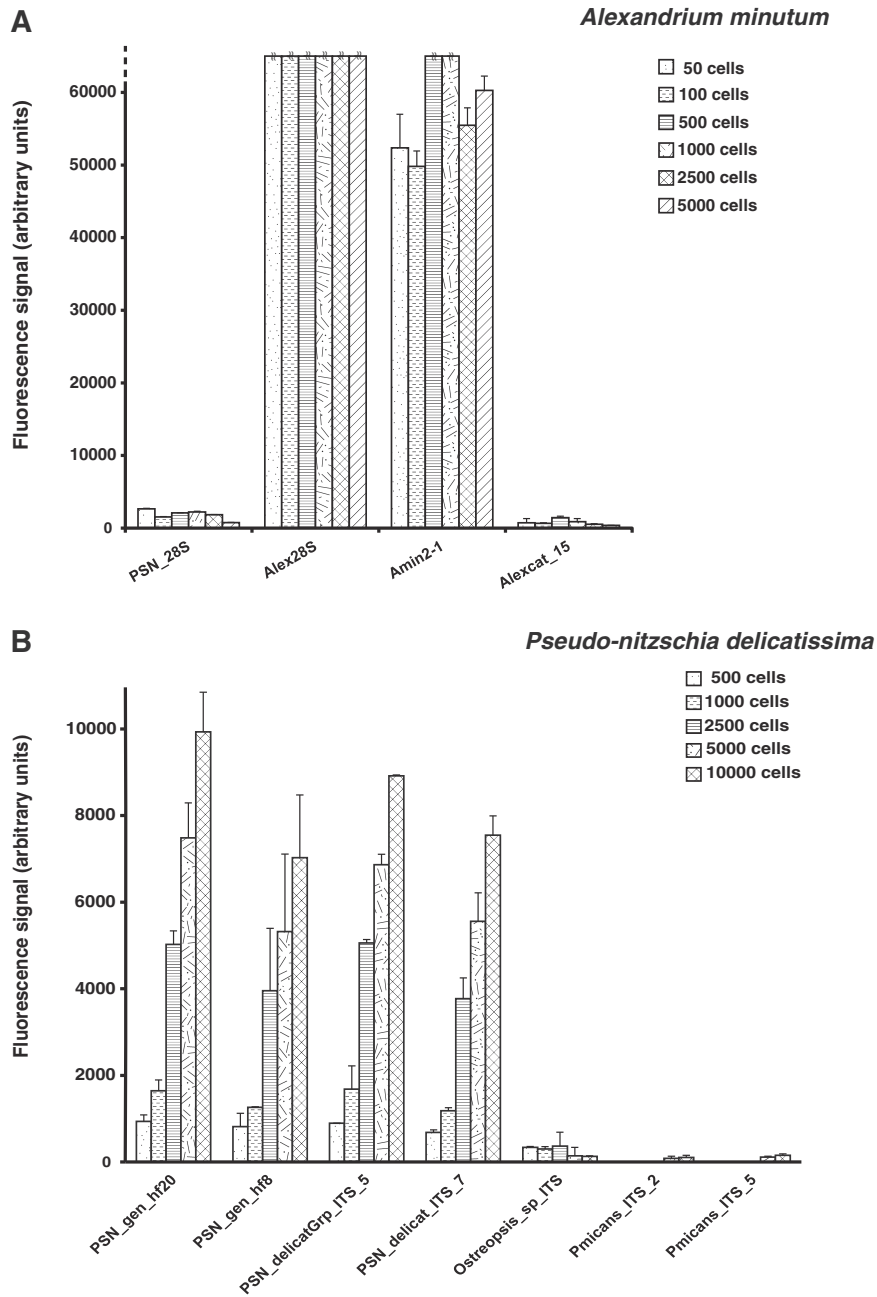
Table 3

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	<i>P. delicatissima</i> ; <i>P. cuspidata/pseudodelicatissima</i> ; <i>P. australis</i> ; <i>P. seriata</i> ; <i>P. americana</i> ; <i>P. fraudulenta</i> ; <i>P. multiseriis</i> ; <i>P. pungens</i>					
Species not present from the composition	<i>P. americana</i> <sup>(-)</sup>	<i>P. australis</i> <sup>(-)</sup>	<i>P. multiseriis</i> <sup>(-)</sup>	<i>P. pungens</i> <sup>(-)</sup>	<i>P. delicatissima</i> <sup>(-)</sup>	<i>P. delicatissima</i> <sup>(-)</sup> <i>P. fraudulenta</i> <sup>(-)</sup> <i>P. arenysensis</i> <sup>(+)</sup>
PSN_genus_hf20	<b>65,368</b>	<b>7555</b>	<b>35,548</b>	<b>8163</b>	<b>46,516</b>	<b>57,739</b>
PSN_genus_hf8	<b>57,683</b>	<b>5910</b>	<b>12,867</b>	<b>6689</b>	<b>38,935</b>	<b>24,449</b>
PSN_delicatGrp_ITS_5	<b>26,862</b>	<b>3124</b>	<b>10,198</b>	<b>6093</b>	42	<b>6728</b>
PSN_delicatissima_ITS_7	<b>20,397</b>	<b>1087</b>	<b>2960</b>	<b>2323</b>	–	–
PSN_arenysensis_ITS_26	–	–	–	–	–	<b>12,872</b>
PSN_cuspidata_ITS_27	<b>6128</b>	<b>347</b>	<b>1356</b>	<b>1944</b>	<b>1042</b>	<b>1375</b>
PSN_pseudodelicat_ITS_4	<b>376</b>		3			
PSN_seriataGrp_f10	<b>1436</b>	<b>658</b>	<b>881</b>	<b>2372</b>	<b>5261</b>	<b>4210</b>
PSN_seriataGrp_f4	<b>783</b>	<b>146</b>	<b>646</b>	<b>460</b>	<b>997</b>	<b>409</b>
PSN_seriata_ITS_8	<b>5465</b>	119	<b>1785</b>	<b>2219</b>	<b>2784</b>	<b>1630</b>
PSN_australis5-2 m	<b>2516</b>	–	<b>420</b>	<b>401</b>	<b>1402</b>	<b>985</b>
PSN_americana31m	–	<b>33,065</b>	<b>65,415</b>	<b>65,415</b>	<b>65,408</b>	<b>65,409</b>
PSN_americana_f10	–	<b>9768</b>	<b>7267</b>	<b>16,014</b>	<b>11,209</b>	<b>15,212</b>
PSN_fraudulenta1m	<b>65,315</b>	<b>54,248</b>	<b>65,409</b>	<b>65,416</b>	<b>65,408</b>	12
PSN_fraudulenta_f13	<b>11,902</b>	<b>840</b>	<b>1579</b>	<b>2078</b>	<b>8570</b>	–
PSN_fraudulenta_ITS_41	<b>1090</b>	2	516	149	366	–
PSN_fraudulenta_ITS_50	96			41		–
PSN_multiseriis6	<b>20,735</b>	<b>4256</b>	65	<b>16,572</b>	<b>11,161</b>	<b>9990</b>
PSN_multiseriis_ITS_13	185	42	–	63		23
PSN_multiseriis_ITS_1	361	1	–	190	21	17
PSN_pungens_p1	<b>65,346</b>	<b>65,394</b>	<b>65,413</b>	–	<b>65,403</b>	<b>65,381</b>
PSN_pungens1-5bm	<b>65,335</b>	<b>62,717</b>	<b>65,408</b>	–	<b>65,403</b>	<b>65,402</b>
B) Species composition	<i>A. minutum</i> , <i>A. catenella</i> , <i>A. tamarense</i> ; <i>K. brevis</i> , <i>K. selliformis</i>		<i>A. minutum</i> ; <i>K. mikimotoi</i>		<i>L. polyedrum</i> ; <i>K. mikimotoi</i> , <i>K. brevis</i> , <i>K. selliformis</i>	
PSN_genus_hf8	92					45
Lingu_polyedrum_ITS_22				<b>2753</b>		
Lingu_polyedrum_ITS_30				<b>31,046</b>		
Lingu_polyedrum_ITS_47				<b>42,403</b>		
Lingu_polyedrum_ITS_52				<b>8685</b>		
Ostreopsis_sp_ITS	86		90	152		133
Vulcano_ARB_ITS2_3				130		85
Vulcanodinium_ITS_4	616		194	725		318
Kare28S	158					
KareITS_49	<b>31,350</b>		<b>33,173</b>	<b>55,244</b>		<b>38,705</b>
Kmiki_brev_ITS_38	<b>7058</b>		<b>65,430</b>	<b>57,271</b>		<b>58,211</b>
Kmiki_brev_ITS_47	<b>29,697</b>		<b>53,993</b>	<b>65,429</b>		<b>51,455</b>
KmikiITS_23			<b>652</b>	<b>1497</b>		<b>1220</b>
KmikiITS_6			<b>888</b>	<b>1198</b>		<b>1283</b>
KmikiITS_8	31		<b>1172</b>	<b>2321</b>		<b>1891</b>
Kbrevis_ITS_16	<b>27,215</b>			<b>12,751</b>		
Kbrevis_ITS_20	<b>2327</b>			<b>430</b>		
Kselli_brev_ITS_46	<b>23,926</b>			<b>15,474</b>		<b>4550</b>
Kselli_ITS_29				<b>97</b>		<b>159</b>
Alex28S	77		270	275		232
AminITS_8	<b>1978</b>		<b>519</b>			<b>745</b>
AminITS_18	<b>1886</b>		<b>954</b>			<b>1462</b>
AminITS_23	<b>4894</b>		<b>2561</b>			<b>3203</b>
A_tamarenseGrp_ITS	<b>6451</b>		77		88	92
Alexcat_17					6	14

Positive FS are indicated in bold.

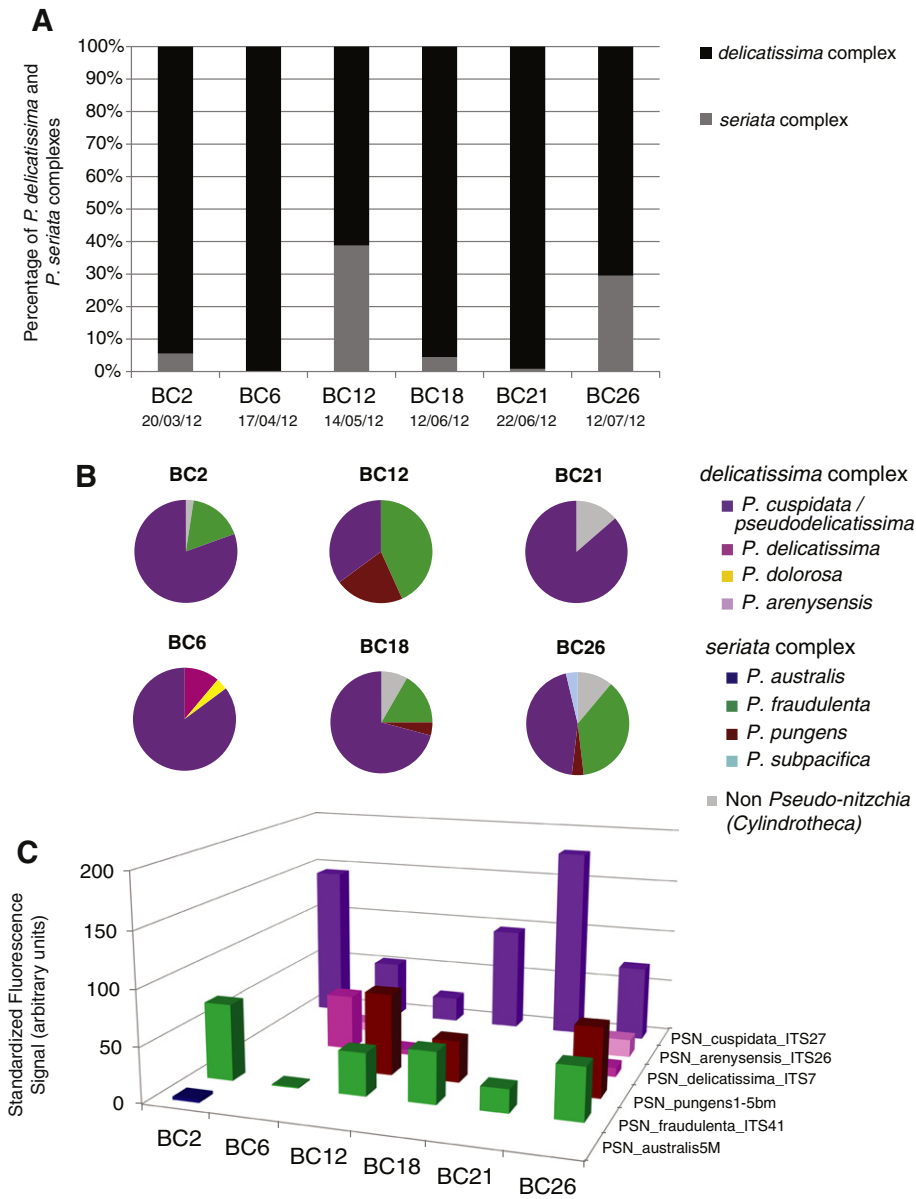




**Fig. 2.** Seawater spiked with an increasing number of cells and tested on the phytochip (A) spiked with *Alexandrium minutum* cells. Please note that most of the *Alexandrium* sample signals were saturated as we have indicated by dashed of the bars; (B) spiked with *Pseudo-nitzschia delicatissima*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

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



**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.

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,

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

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

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).

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 the 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 from 18S 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 Kaczmarek, 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\_seriateGrp\_f4 and PSN\_seriateGrp\_f10 (originally designed for *P. seriata*) was higher than that of *P. seriata* DNA with the *seriate* specific” PSN\_seriate 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 Phytochip, we can respectively detect 50 and 500 cells of *A. minutum* and *P. 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 and 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\_seriateGrp\_f10 and PSN\_seriateGrp\_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 that the fluorescence decreases when these species were 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%. 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 non-specific binding of probes to non-target species because the probes were previously validated using natural seawater spiked with various amounts 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 *Pseudo-nitzschia arenysensis* were also detected in most of the samples but in lower quantities. Moreover, probes of two untested species *P. dolorosa* and *P. subpacific*a showed weak FS in BC6 and BC26 samples, respectively. We know that *P. subpacific*a 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 seawater. 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 are 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.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mimet.2015.03.002>.

## Data accessibility

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

## Author contributions

C.D. and 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.

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