Environ Sci Pollut Res
DOI 10.1007/s11356-012-1299-9

Introduction to project MIDTAL: its methods and samples from Arcachon Bay, France

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Received: 24 July 2012 / Accepted: 5 November 2012

Abstract Microalgae worldwide regularly cause harmful effects, considered from the human perspective, in that they cause health problems and economic damage to fisheries and tourism. Cyanobacteria cause similar problems in freshwaters. These episodes encompass a broad range of phenomena collectively referred to as "harmful algal blooms" (HABs). For adequate management of these phenomena, monitoring of microalgae is required. However, effective monitoring is time-consuming because cell morphology as determined by light microscopy may be insufficient to give definitive species and toxin attribution. In the European Union FP7 project MIDTAL (Microarrays for the Detection of Toxic Algae), we achieved rapid species identification using rRNA genes as the target. These regions can be targeted for probe design to recognise species or even strains. We also included antibody reactions to specific toxins produced by these microalgae because, even when cell numbers are low, toxins can be present and can accumulate in the shellfish. Microarrays are the state-of-the-art technology in molecular biology for the processing of bulk samples for detection of target RNA/DNA sequences. After 36 months, we have completed RNA-cell number–signal intensity calibration curves for 18 HAB species and the analysis of monthly field samples from five locations from year 1. Results from one location, Arcachon Bay (France), are reported here and compared favourably with cell counts in most cases. In general, the microarray was more sensitive than the cell counts, and this is likely a reflection in the difference in water volume analysed with the volume filtered for the microarray an order of magnitude greater.

Keywords MIDTAL · Harmful/toxic algae · Oligonucleotide microarrays · Molecular monitoring · 18S ribosomal RNA · RNA hybridisation · Phylochips · Environmental water samples

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-012-1299-9) contains supplementary material, which is available to authorized users.

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Introduction

Microalgae (photo-autotrophic and mixotrophic microorganisms) in marine, brackish and freshwaters worldwide regularly bloom and are the essential basis for aquatic trophic networks. Yet, some species can cause harmful effects to human and animal health. From a human perspective, they can also cause economic damages to fisheries, fish- and shell-fish farming, and tourism (Hallegraeff 2003). In freshwaters, some cyanobacteria can cause similar problems. These harmful blooms encompass a broad range of phenomena (i.e., water discoloration, foam, smell nuisances, biogenic toxin production, mechanical damages to gill …), which are collectively called harmful algal blooms (HABs). Only a few hundred toxic cells per litre of some taxa can make shellfish unsuitable for human consumption (Yasumoto et al. 1985), but toxin content is highly variable among strains. Not only can fish and shellfish production be destroyed through stock reduction and consumer mistrust, but there are also ramifications for tourism (Hallegraeff 2003). Tourists do not like to swim in visible algal blooms,

Published online: 22 November 2012

and some toxic species cause skin and lung irritations (Hallegraeff 2003). Monitoring of HAB microalgae is required by all countries with a marine coastline or freshwater reservoirs. However, present-day monitoring is time-consuming and is based on species identification using their cell morphology as determined by light microscopy. Species determination by light microscopy may sometimes be insufficient to give a definitive answer as to whether the cell in question is toxic and to quantify this toxicity. Molecular tools offer a faster and more reliable means of identifying species (Anderson et al. 2012). DNA microarrays for the identification of marine organisms is a relatively new and innovative field of research. It provides the possibility to analyse a large number of targets (species or taxa) in one experiment (Ye et al. 2001) and was the molecular tool of choice for the European Union (EU) seventh Framework Program project MIDTAL (microarrays for the detection of toxic algae).

MIDTAL started on 1 September 2008 and was funded under THEME 6 ENVIRONMENT (including climate change) of Framework 7 of the European Commission for 45 months. Ten partners from seven European countries and the USA include Marine Biological Association (co-ordinator), Stazione Zoologica Anton Dohrn, Linneaus University, Instituto Español de Oceanografía, National University of Ireland, University of Oslo, University of Westminster, Instituto Tecnolóxico para o Control do Medio Mariño de Galicia, University of Rhode Island and Queen's University Belfast. The goal of the research consortium was to make a universal microarray for the detection of toxic algal species and another universal microarray for the detection of the toxins produced by these algae. The purpose of MIDTAL was to support the common fisheries policy, aid the national monitoring agencies by providing new rapid tools for the detection and identification of toxic algae and their toxins so they can comply with EC directive 15/201 (EC 2011) and to replace the mouse bioassay, which has been recently replaced by chemical methods as the standard reference analysis throughout the European Community. We hypothesised that our toxic algal species microarray would be more sensitive than routine cell counting methods and require taxonomic expertise and that our toxin microarray would be more rapid than routine chemical analysis. In the EU FP7 project MIDTAL, we used SSU and LSU rRNA genes as the target for the microarray for species identification. These genes can contain regions that are specific for species or even strains. A toxin-based microarray includes antibody reactions to specific toxins produced by these microalgae because, even when cell numbers are low, toxins can be present and can accumulate in the shellfish (please refer to McNamee et al. (2012) in this volume for more detail on the toxin microarray). Microarrays are the state-of-the-art technology in molecular biology for the processing of bulk samples for detection of target RNA/DNA sequences. Existing rRNA fluorescent in situ probes for toxic algal species/strains and antibodies for their toxins were first adapted and optimised for microarray use. New probes were designed where needed, and these are presently under patent application.

The introduction of DNA microarray technology in 1995 is one of the latest and most powerful innovations in microbiology. Because of true parallelism and miniaturisation, the acquisition of many data with reduced consumption of reagents and time is accomplished using microarrays. This technique offers the possibility to analyse many samples to different probes in parallel under a diverse spectrum of applications (Ye et al. 2001). DNA microarrays consist of glass microscope slides with particular surface properties that allow the probes to bind covalently to their surfaces (Metfies and Medlin 2004). Probes are immobilised as spots on the glass slide in a defined pattern. Each spot consists of many copies of oligonucleotide probes that are complementary to a specific target DNA sequence (Graves 1999), and the targets (RNAs or DNAs) hybridise to the capture oligonucleotide probes on the microarray. The hybridisation is detected using a fluorescent label that is attached to the target (in our case, the rRNA) (Metfies and Medlin 2004). Microarray technology was initially used in studies of gene expression (Schena et al. 1995). Many functional genomic methods benefit from microarrays, such as genome expression profiling, single-nucleotide polymorphism detection and DNA re-sequencing (Ji and Tan 2004; Lipshutz et al. 1999; Kauppinen et al. 2003; Yap et al. 2004; Al-Shahrour et al. 2005; Broet et al. 2006; Gamberoni et al. 2006). Thus, DNA microarrays are a powerful and innovative tool that can facilitate surveying and monitoring of any organism, especially those in the marine environment, tracking changes in biodiversity and ecosystem functioning, and we have used it in MIDTAL to monitor for toxic algae.

The application of DNA microarrays for the identification of marine organisms (the so-called phylochip) is a relatively new and innovative field of research. There are few applications of phylochips in marine biodiversity and ecosystem science, most of them being applied to bacterial species (Peplies et al. 2003; Ye et al. 2001), although a few microarrays have been introduced for toxic eukaryotic algae (Ahn et al. 2010; Galluzi et al. 2011; Gescher et al. 2008a, b; Ki and Han 2006) but not in the format that we have developed and for assessing biodiversity of microalgae difficult to identify microscopically (Gescher et al. 2008a; Metfies and Medlin 2005; Metfies et al. 2010).

All probes on the microarray have to work specifically under identical experimental conditions, which is the biggest challenge for a successful microarray. To apply microarray technology as a standard tool with fast and simple routine handling, methodical optimisations need to be done

at each step in the procedure (Peplies et al. 2003). We proceeded through several steps in the optimisation of our protocol. In the initial step, we took published probes used for fluorescence in situ hybridisation (FISH) and converted them to a microarray format by adding a six carbon spacer to the 5' end, and these were tested in a microarray format and in general were not useful. In the second step, we lengthened the probes from 18 to 25 nt with approximately the same (ca 50%) guanine cytosine (GC) content because the increased length provides a stronger and more specific signal, and with similar GC content, the hybridisation temperature is almost identical for all probes (Metfies et al. 2008). An amended and detailed hybridisation protocol is available for purchase from Koeltz (Lewis et al. 2012). Several partners were taxonomic specialists in several toxic algae species, and these partners had the responsibility of testing probe specificity against cultures prior to field testing. Once probe specificity was ascertained (see various papers in this issue that also deal with probe specificity), we proceeded to field testing. We used the second-generation microarray to analyse 1 year of field data, which we present here. Each partner in the consortium will present their microarray analysis in a separate paper in this special issue (seven phylochip papers and one toxin paper). The results of Partner 1 regarding field testing are presented here.

Materials and methods

Field sampling

In 2010, sub-surface (1 m) water samples for both cell count and microarray analysis were collected at Arcachon Bay in France between July and September, during REPHY (IFREMER) and SOMLIT (INSU) monitoring and survey networks, respectively. The sampling site for microarray analysis, termed Tès (1°10′00 W, 44°40′ 00 N), is located directly in front of the town of Arcachon inside the bay and is influenced by both continental runoff and marine waters that enter the bay. Because of logistic problems inherent to the SOMLIT service, only three samples were taken for microarray analysis during this period (06.07, 02.09 and 16.09.2010; Table 1). The sampling site for phytoplankton identification, termed Teychan (1°09.51′W, 44°40.42′N) is located 1.5 km from Tès station. Both stations belong to the same water masses, i.e. the internal neretic waters as described by Robert et al. (1987), and sampling was always performed during high tide. Indeed, Arcachon Bay is a well-mixed system highly influenced by tidal mixing. Spatial patchiness is minimum for a same tide moment in terms of hydrological parameters of the water column as well as of phytoplankton populations (Glé et al. 2007, 2008). Cell counts were done as previously described by Medlin and Schmidt (2010) and provided by IFREMER (results Ifremer/Quadrige^2/Rephy). Briefly: a 1-L sample was collected from a 4-L Niskin bottle and immediately fixed with Lugol's fixative. Ten millilitres was allowed to settle overnight and used for counting by the Utermöhl method (von Utermöhl 1931); for sparse species, an additional settlement of 100 mL is undertaken. The full settling chamber was counted at 20× and across one diameter at 40×. The detection limit for those conditions is estimated to 100 cells/L for the 10-mL chamber and 10 cells/L for the 100-mL chamber (Belin and Raffin 1998). Counts varied from 200 to 1,600 cells per chamber leading to an accuracy range of 5 to 16 % expressed as percent of total (Lund et al. 1958).

For the microarray analysis, around 2 L of water were filtered onto 3-μm nitrocellulose filters (25 mm) until they clogged (Table 1). Two filters were transferred into cryogenic vials containing 1 mL of TRI Reagent (Sigma-Aldrich). The samples were frozen and stored at −80 °C until further processing. One filter was transferred into cryogenic vials without TRI reagent for toxin analysis and sent frozen to Partner 11 (Queens University Belfast, UK) using their toxin microarray.

Toxin extraction

The toxin filters were extracted according to the protocol presented by McNamee et al. (2012; this volume), and their analysis is discussed in detail in that paper. We present here only the correlation between toxin and the species present either in the cell counts or in the microarray (Table 2).

RNA extraction

Acid-washed glass beads (300 μm) were added to the samples and were placed in a bead beater twice for 20 s at 4,800 oscillations/min. Cell+Tri Reagent mixture was transferred into a new microcentrifuge tube, vortexed for 15 s and let stand at room temperature (RT) for 10 min. After vortexing for another 15 s, samples were incubated at 60 °C for 10 min in a Thermoshaker vortexing at maximum speed. After the addition of 100 μL of 1-bromo-3-chloropropane to the samples, the tubes were vortexed for 15 s. Samples were then transferred into pre-spun phase-lock gel heavy 2-mL tubes (5 Prime; 12,000×*g* for 30 s) and were shaken thoroughly for 15 s. Samples were incubated at RT for 5 min and centrifuged (12,000×*g*) for 15 min at 4 °C. The upper phase was mixed gently with 200 μL of chloroform and centrifuged (12,000×*g*) for 2 min at 4 °C. The aqueous phase was then transferred to a fresh 2-mL RNase-free tube. Five hundred microlitres of isopropanol was added, vortexed for 15 s and incubated for 1 h at −20 °C.

Table 1 Information about field samples taken at Arcachon Bay like sample name, sample date, filtered volume, and total extracted RNA

Sample name	Sample date	Volume filtered (L)	Total RNA extracted (ng)
1A	06 July 2010	2.25	6,680
5A	02 September 2010	1.75	5,750
6A	16 September 2010	2.00	5,980

Supernatant was quickly removed and washed with 1 mL ethanol (75 %), vortexed for 5 s and centrifuged (12,000×*g*) for 10 min at 4 °C. The supernatant was carefully removed, and the pellet was air-dried for 5 min. The pellet was dissolved in 100 μL of RNase-free water by flicking the tube. Concentration and integrity of RNA were measured by a Nanodrop (PerkinElmer) and an Agilent Bioanalyzer 2100 (Agilent Biotechnologies). Samples were shock-frozen in liquid nitrogen and stored at −80 °C until further use.

RNA labelling and fragmentation

One microgram RNA of field sample was labelled using the PlatinumBright Infrared Labelling Kit from KREATECH according to the manufacturer's instructions. The total volume was 20 μL containing 2 μL dye and 2 μL 10× labelling solution. Concentration and incorporation of the dye was measured by a Nanodrop (PerkinElmer). The degree of labelling (DOL) was calculated and was between 1.4 and 2.5 %. RNA was fragmented by adding 1/10 volume of RNA fragmentation buffer (100 mM ZnCl2 in 100 mM Tris–HCL, pH 7.0) and an incubation of 15 min at 70 °C. The reaction was stopped with the addition of 1/10 volume of 0.5 M EDTA (pH 8.0), and the samples were placed on ice.

Preparation of controls

The MIDTAL chip currently comprises one spotting control 'Poly-T-Cy5,' two hybridisation controls (Positive_25 and 'Positive_25_dT) and several probes for RNA of algae of the genus *Dunaliella* ('DunGS02_25', 'DunGS02_25_dT' and 'DunGS05_25_dT'), 50,000 cells of which are added as a control before the RNA extraction. Negative controls are 'NEGATIVE1_dT', 'NEGATIVE2_25_dT', 'NEGATIVE3 _25_dT' and '3×SCC'; the first two are nonsense oligonucleotides with no similarity to any sequence in Genbank.

The internal positive control (TBP-Cy5=Positive_25 _dT' and Positive_25_dT) is prepared as follows: DNA from bread yeast powder (*Saccharomyces cerevisiae*) was extracted by using the Plant DNA Extraction Kit from QIAGEN according to the manufacturer's protocol. A 250-bp fragment of the TATA-box binding protein gene (TBP) was amplified with the primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R labeled with CY5 (5'-TTT TCA GAT CTA ACC TGC ACC C-3'). The TBP polymerase chain reaction (PCR) conditions were: (1) predenaturation step for 5 min at 95 °C, 40 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C and elongation for 2 min at 72 °C, with a final extension step of

Table 2 Comparison of species present in the cell counts and on the microarray with two methods of toxin determination

	PSP toxins (STX)		Okadaic acid+DTXS		Domoic acid			
	Multi-SPR	ELISA	Multi-SPR	ELISA	Multi-SPR	ELISA	Species present in cell counts	Species present on microarray
6 Jul 10	−	+	−	−	−	−	*Alexandrium minutum*, *P. micans, K. brevis* and *K. mikimotoi*, *P. rotundatum*	*Alexandrium* genus, *Dinophysis* class level, family and genus, *Haptophytes* class level, clade and genus level for *Prymnesium*, species *C. polylepis*
2 Sep 10	−	+	−	−	−	−	*D. caudata, P. micans*	*Dinophysis* class level, family and genus, *Alexandrium* genus, *Azadinium* genus, Haptophytes class level and clade level for *Prymnesium*
16 Sep 10	−	+	−	−	−	−	*Pseudo-nitzschia*, sigmoid group (*P. multistriata*), *D. caudata, P. micans*	*Pseudo-nitzschia* genus and *P. multistriata*, *Dinophysis* class level and genus, *Alexandrium* genus, *Azadinium* genus, Haptophytes class level and clade level for *Prymnesium*

Multi-SPR is the MIDTAL toxin microarray and the ELISA is a standard antibody test, which is slightly more sensitive to lower amounts of toxin

5 min at 72 °C. The PCR product was purified with the PCR MinElute Cleanup Kit (QIAGEN) and quantified with a Nanodrop using the microarray setting. The TBP fragment was diluted to 50-ng/μL aliquot and stored at −80 °C.

Microarray design

Probe design was done with the open software package ARB (Ludwig et al. 2004). A list of the probes and their target can be found in Table 3; sequences for the probes are not provided because they are patent-pending. A commercial kit with the chip and all reagents will soon be commercially available from Kreatech (Amsterdam, The Netherlands). Probes were made first for a toxic taxon at the species level, and then probes for higher taxonomic levels were designed. The higher taxonomic level probes were either at the genus or family level. Clade-level probes were designed when groups of species were determined to be phylogenetically closely related in the ARB database but were not recognised at a formal taxonomic level. All oligonucleotides including the positive and negative controls were synthesised by Thermo Fisher Scientific (Ulm, Germany) with a C6/MMT aminolink at the 5' end of the molecule. The probe length was 18 or 25 nt. Probes were spotted onto epoxy-coated Genetix or Schott slides using a pin printer VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, Munich, Germany) and split pins (Point Technologies, Inc., CO). The probes had a final concentration of 20 pmol/μL in 3× saline sodium citrate buffer (SSC) and a spot size between 70 and 110 μm. Two arrays were printed on each slide containing 162 different probes (see Table 3) with four to eight replicates, as well as two negative and two positive controls, Poly-T and Poly-T-Cy5 (MIDTAL ver2.52). After spotting, slides were incubated for 30 min at 37 °C and then stored at −20 °C.

Hybridisation

Just prior to use, slides were pre-hybridised in a slide box containing 20 mL pre-hybridisation buffer (1 mg/mL BSA and 1× STT buffer (2× STT/2 M NaCl; 20 mM Tris–Cl, pH 8.0; 0.01 % Triton 100)) for 60 min at 60 °C. The slides were washed in ddH_2O and were dried by centrifugation in a 50-mL Falcon tube for 2 min at 2,000 rpm.

Labeled samples (1 μg RNA) were mixed with 35 μL of 2× hybridisation buffer (1 mg/mL BSA, 0.2 μg/μL Herring sperm DNA, 2 M NaCl, 20 mM Tris–Cl, pH 8.0, 0.01 % Triton 100), 14 μL of formamide, 100 ng TBP-control, and the volume was adjusted with nuclease-free water to 70 μL. Slides were placed into an array holder; coverslips (LifterSlips, Erie Scientific) were cleaned and placed onto the microarrays. Half of the hybridisation mixture (35 μL) was added to one microarray. Prior to hybridisation, a hot start was performed for 10 min at 84 °C. Hybridisation was carried out for 1 h at 50 °C in a 50-mL Falcon tube containing a wet Whatman paper. The DNA chips were washed three times, shaking on a belly dancer (~25 rpm) with increasing stringency (Table 4).

After centrifugation for 2 min at 2,000 rpm in a 50-mL Falcon tube, microarrays were scanned with a GenePix 4000B scanner (Axon Instruments) using the GenePix 6.0 software. Settings for scanning were 600 PMT gain and 100 % of laser power for the 635 nm wavelength, and the pixel size was 10 μm.

Detection limits for probes on the microarray

Each partner was responsible for growing various species under different culture conditions to assess the effect of the selected environmental variables on rRNA content in order to establish calibration curves and to determine detection limits. Details of these experiments will be published elsewhere (Taylor et al. submitted), but a brief description follows below. All calibration curves were conducted in a similar fashion. Three strains for each species were selected and exposed to optimal conditions for nutrients, temperature, light and salinity. During log phase, aliquots of this optimised culture were inoculated into new culture vessels and exposed to stresses of each variable along with controls at the optimum conditions. There was more variation between the strains in response to the stress than the stress itself. Linear responses were obtained in nearly all cases with RNA content increasing with time regardless of the stress (data not shown). Therefore, we can convert RNA content to cell numbers at any stage of the bloom and under any environmental conditions, and from the minimum amount hybridised on the microarray (5 ng), we have determined a corresponding cell number, which varies with each probe because of the strength of the various probes (Table 5).

Data analysis

Obtained fluorescent signals and the surrounding background intensity were calculated by superimposing a grid of circles (midtal_ver252_20100423.gal) onto the scanned image using the GenePix 6.0 software. Results were processed first with the phylochip analyser (Metfies et al. 2008) to construct a hierarchy file. A hierarchy file places probes for species below probes of a higher taxonomic designation, e.g. kingdom, phylum, class, order, family, genus, and species. Table 3 shows the taxonomic hierarchy of the probes we have developed. The use of the hierarchy file ensures that false-positives can be eliminated because, for a species to be present, the entire taxonomic hierarchy leading to that species must also be

Table 3 Summary of probes designed or modified from those published for FISH hybridization and used to form the second generation of the MDITAL microarray

Probe name	Targeted species	Gene	Tm (GC% method)	Source/designer	Toxin reported for the species
Higher group probes					
EukS_328_25	Eukaryotes	18S	79	Moon-Van Der Staay et al. 2001	
EukS_1209_25	Eukaryotes	18S	79	Lim et al. 1993	
HeteroS01_25	Heterokonta	18S	77.3	Eller et al. 2007	
PrymS01_25	Prymnesiophyta	18S	77.3	Lange et al. 1996	
PrymS02_25	Prymnesiophyta	18S	80.6	Simon et al. 2000	
PrymS03_25	Prymnesiophyceae	18S	77.3	Eller et al. 2007	
Class-level probes					
DinoB_25	Dinophyceae (incl. Apicomplexa)	18S	75.7	John et al. 2003	
DinoE12_25	Dinophyceae (incl. Apicomplexa)	18S	77.3	Groben, John and Medlin, unpublished	
Clade-level probes					
ProroFD01	*Prorocentrum* clade	28S	77.3	Groben, Lange and Medlin, unpublished	
DphyFS01_25	Dinophysiaceae (Dinophysis+*Phalacroma*)	18S	77.3	Edvardsen, Groben, Brubak and Medlin, unpublished	
DphyFS02_25	Dinophysiaceae (Dinophysis+*Phalacroma*)	18S	79	Edvardsen, Groben, Brubak and Medlin, unpublished	
Genus-level probes					
PrymGS01_25	*Prymnesium*	18S	79	Eller et al. 2007	
PrymGS02_25	*Prymnesium*	18S	79	Eller et al. 2007	
PsnGS01_25	*Pseudo-nitzschia*	18S	77.3	Eller et al. 2007	
PsnGS02_25	*Pseudo-nitzschia*	18S	79	Eller et al. 2007	
KareGD01_25	*Karenia*	28S	77.4	This study	
AlexGD01_25	*Alexandrium*	28S	75.7	This study	
DphyGD01	*Dinophysis* in part	28S	77.3	Guillou et al. 2002	
DphyGD02	*Dinophysis*	28S	75.6	Guillou et al. 2002	
PschGS01_25	*Pseudochattonella* (genus)	18S	77.3	Riisberg and Edvardsen, unpublished	
PschGS02_25	*Pseudochattonella* (genus)	18S		Riisberg and Edvardsen, unpublished	
PschGS03_25	*Pseudochattonella* (genus)	18S		Riisberg and Edvardsen, unpublished	
PschGS04_25	*Pseudochattonella* (genus)	18S	77.3	Riisberg and Edvardsen, unpublished	
PschG05_25	*Pseudochattonella* (genus)	18S	79	Riisberg and Edvardsen, unpublished	
DphyGS01_25	*Dinophysis* genus sensu stricto	18S	75.9	Edvardsen, Groben, Brubak and Medlin, unpublished	
DphyGS02_25	*Dinophysis* genus sensu stricto	18S	79	Edvardsen, Groben, Brubak and Medlin, unpublished	
DphyGS03_25	All *Dinophysis* and *Phalacroma*	18S	80.6	Edvardsen, Groben, Brubak and Medlin, unpublished	
DphyGS04_25	All *Dinophysis*	18 s	77.3	Edvardsen, Groben, Brubak and Medlin, unpublished	
PrymGS01_25	*Prymnesium*	18S		This study	
KargeD01_25	*Karlodinium*	28S	75.6	This study	
AzaGD01	*Azadinium*	28S	75.9	This study	
AzaGD03	*Azadinium*	28S	75.7	This study	
AzaGS01	*Azadinium*	18S	79	This study	
AzaGS02	*Azadinium*	18S	79	This study	

Table 3 (continued)

Probe name	Targeted species	Gene	Tm (GC% method)	Source/designer	Toxin reported for the species
L*Kare0308A25	*Karenia*	28S	80.6	This study	
ProtuS01_25	*Phalacroma*	18S	79	Edvardsen, Groben, Brubak and Medlin, unpublished	
Species-level probes					
AtamaS01_25	*Alexandrium tamarense* species complex (North America=NA), (Western European=WE), (temperate Asian=TA),	18S	77.3	John et al. 2003	Saxitoxin
AminuS01_25	*A. minutum*	18S	79	Miller and Scholin 1998	Saxitoxin
ATNA_D01_25	*A. tamarense* (NA)	28S	79	John et al. 2003	Saxitoxin
ATNA_D02_25	*A. tamarense* (NA)	28S	77.3	Guillou et al. 2002	Saxitoxin
ATTA _D01_25	*A. tamarense* (TA)	28S	77.3	This study	Saxitoxin
AostD01 _25	*Alexandrium ostenfeldii*	28S	75.7	John et al. 2003	Saxitoxin, spirolides
AostS02 _25	*A. ostenfeldii*	18S	79	John et al. 2003	Saxitoxin, spirolides
CpolyS01_25	*C. polylepis*	18S	77.3	Simon et al. 1997	Prymnesins
PparvD01_25	*Prymnesium parvum*	28S		Töbe et al. 2006	Prymnesins
KbreD03_25	*K. mikimotoi* and *K. brevis*	28S		Milkulski et al. 2005	Brevetoxins
KbreD04_25	*K. mikimotoi* and *K. brevis*	28S	79	Milkulski et al. 2005	Brevetoxins
KmikiD01_25	*K. mikimotoi*	28S	79	Guillou et al. 2002	Brevetoxin s
KbreD05_25	*K. brevis*	28S	80.6	Milkulski et al. 2005	Brevetoxins
SSKbre1448A25	*K. brevis*	18S	80.6	This study	Brevetoxins
LSKbre0548A25	*K. brevis*	28S	82.3	This study	Brevetoxins
KveneD01_25	*Karlodinium veneficum*	28S	77.3	This study	Brevetoxins
KveneD02_25	*K. veneficum*	28S	72.4	This study	Brevetoxins
KveneD03_25	*K. veneficum*	28S	74.1	This study	Brevetoxins
KveneD04_25	*K. veneficum*	28S	80.6	This study	Brevetoxins
KveneD05_25	*K. veneficum*	28S	79	This study	Brevetoxins
KveneD06_25	*K. veneficum*	28	75.7	This study	Brevetoxins
PlimaS01_25	*Prorocentrum lima*	18S	77.3	Groben, Lange and Medlin, unpublished	Okadaic acid
PlimaD01_25	*Prorocentrum lima*	28S	80.6	Groben, Lange and Medlin, unpublished	Okadaic acid
PmicaD02_25	*P. micans*	28S	80.6	Groben, Lange and Medlin, unpublished	Okadaic acid
PminiD01_25	*Prorocentrum minimum*	28S	79	Groben, Lange and Medlin, unpublished	Okadaic acid
GcateS01_25	*Gymnodonium catenatum*	18S	76	Diercks et al. 2008	Saxitoxin
DacumiD02_25	*Dinophysis acuminata* and *Dinophysis dens* and *Dinophysis sacculus*	28S	79	Guillou et al. 2002	Okadaic acid
DacutaD02_25	*D. acuta* and *Dinophysis fortii*	28S	79	Guillou et al. 2002	Okadaic acid
DacumiS01_25	*D. acuminata*	18S	80.6	Edvardsen, Groben, Brubak and Medlin, unpublished	Okadaic acid
DacutaS01_25	*D. acuta*	18S	77.3	Edvardsen, Groben, Brubak and Medlin, unpublished	Okadaic acid
DnorvS01_25	*Dinophysis norvegica*	18S	77.3		Okadaic acid

Table 3 (continued)

Probe name	Targeted species	Gene	Tm (GC% method)	Source/designer	Toxin reported for the species
				Edvardsen, Groben, Brubak and Medlin, unpublished	
PaustS01_25	*P. australis*	18S	80.6	Diercks et al. 2008	Domoic acid
PmultS01_25	*P. multiseries*	18S	80.8	Diercks et al. 2008	Domoic acid
PpungS01_25	*Pseudo-nitzschia pungens*	18S	79	Diercks et al. 2008	Domoic acid
PamerD01_25	*Pseudo-nitzschia americana*	28S	79	This study	Domoic acid
PaustD01_25	*P. australis* and *P. multistriata*	28S	77.3	This study	Domoic acid
PdeliD02_25	*P. delicatissima*	28S	75.7	This study	Domoic acid
PfrauD02_25	*Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia subfraudulenta*	28S	82.3	This study	Domoic acid
PfrauD04_25	*P. fraudulenta*	28S	82.1	This study	Domoic acid
PaustD02_25	*P. australis* and *Pseudo-nitzschia seriata*	28S	77.3	This study	Domoic acid
PaustD03_25	*P. australis* and *P. seriata*	28S	83.9	This study	Domoic acid
PbrasD01_25	*P. brasiliana*	28S	79	This study	Domoic acid
PbrasD02_25	*P. brasiliana*	28S	78.9	This study	Domoic acid
PbrasD03_25	*P. brasiliana*	28S	79	This study	Domoic acid
PcaciD01_25	*Pseudo-nitzschia caciantha*	28S	74.1	This study	Domoic acid
PcaciD02_25	*P. caciantha*	28S	79	This study	Domoic acid
PcaciD04_25	*P. caciantha*	28S	75.7	This study	Domoic acid
Pcal1D01_25	*Pseudo-nitzschia calliantha*	28S	77.3	This study	Domoic acid
Pcal2D01_25	*P. calliantha*	28S	77.3	This study	Domoic acid
Pcal2D02_25	*P. calliantha*	28S	75.7	This study	Domoic acid
Pcal2D03_25	*P. calliantha*	28S	77.4	This study	Domoic acid
Pcal2D05_25	*P. calliantha*	28S	77.4	This study	Domoic acid
Pdel1D01_25	*P. delicatissima*	28S	74.1	This study	Domoic acid
Pdel2D01_25	*P. cf. delicatissima* Clade4	28S	79	This study	Domoic acid
Pdel2D02_25	*P. cf. delicatissima* Clade4	28S	74.1	This study	Domoic acid
Pdel3D01_25	*Pseudo-nitzschia arenysensis*	28S	79	This study	Domoic acid
Pdel1D03_25	*P. delicatissima*	28S	79	This study	Domoic acid
PgalaD01_25	*P. galaxiae*	28S	75.8	This study	Domoic acid
PgalaD02_25	*P. galaxiae*	28S	75.7	This study	Domoic acid
PgalaD04_25	*P. galaxiae*	28S	74.1	This study	Domoic acid
PhemeD2_25	*Pseudo-nitzschia hemeii*	28S	77.3	This study	Domoic acid
PmultD01 _25	*P. multiseries*	28S	80.8	This study	Domoic acid
PmultD02 _25	*P. multiseries*	28S	77.3	This study	Domoic acid
PmultD03 _25	*P. multiseries*	28S	84.2	This study	Domoic acid
PmultD04 _25	*P. multiseries*	28S	80.8	This study	Domoic acid
PmulaD02_25	*P. multistriata*	28S	81	This study	Domoic acid
PmulaD03_25	*P. multistriata*	28S	77.3	This study	Domoic acid
PpdeD01_25	*P. pseudodelicatissima* and *Pseudo-nitzschia cuspidata*	28S	74.1	This study	Domoic acid
PpdeD02_25	*P. pseudodelicatissima* and *P. cuspidata*	28S	79	This study	Domoic acid
PpungD02_25	*P. pungens*	28S	82.7	This study	Domoic acid
PpungD04_25	*P. pungens*	28S	80.8	This study	Domoic acid
PsubpD01_25	*Pseudo-nitzschia subpacifica*	28S	77.3	This study	Domoic acid
PseriD01_25	*P. seriata*	28S	79	This study	Domoic acid

Table 3 (continued)

Probe name	Targeted species	Gene	Tm (GC% method)	Source/designer	Toxin reported for the species
PturgD1_25	*Pseudo-nitzschia turgiduloides*	28S	74.2	This study	Domoic acid
PturgD3_25	*P. turgiduloides*	28S	79	This study	Domoic acid
Pdel4D01_25	*P. cf. delicatissima* Clade4	28S	79	This study	Domoic acid
PvigoD01	*Pseudo-nitzschia hasleana*	28S	79	This study	Domoic acid
PvigoD03	*P. hasleana*	28S	79	This study	Domoic acid
CtoxiS05	*Chloromorum toxicum nom. nud.*	18S	78.8	This study	Brevetoxin
CtoxiS06	*cf. Chattonella* sp.	18S	78.9	This study	Brevetoxin
CtoxiS07	*cf. Chattonella* sp.	18S	80.6	This study	Brevetoxin
CtoxiS09	*cf. Chattonella* sp.	18S	78.8	This study	Brevetoxin
SSGcat0826A27	*G. catenatum*	18S	77.4	This study	Saxitoxin
LSGcat0270A24	*G. catenatum*	28S	80.8	This study	Saxitoxin
LSGcat0544A24	*G. catenatum*	28S	82.5	This study	Saxitoxin
SSHaka0193A25	*Heterosigma akashiwo*	18S	79	This study	Hemolysins
SSHaka0200A25	*H. akashiwo*	18S	77.4	This study	Hemolysins
LSHaka0544A25b	*H. akashiwo*	28S	82.3	Tyrrell et al. 2001	Hemolysins
LSHaka0268A25	*H. akashiwo*	28S	82.3	Tyrrell et al. 2001	Hemolysins
LSHaka0544A25c	*H. akashiwo*	28S	82.3	Tyrrell et al. 2001	Hemolysins
LSHaka0548A25	*H. akashiwo*	28S	82.3	Chen et al. 2008	Hemolysins
LSHaka0329A25	*H. akashiwo*	28S	82.3	This study	Hemolysins
LSHaka0358A24	*H. akashiwo*	28S	82.5	Bowers et al. 2006	Hemolysins
PfarD01_25	*Pseudochattonella farcimen*	28S	78	This study	Hemolysins

Probe sequences are not provided because the microarray is patent pending

present. The hierarchy file and the gpr file from the scanned image were then loaded into the gpr-analyser program ver 1.24 (Dittami and Edvardsen 2012; this volume). A signal-to-noise ratio (S/N ratio) above 2 was taken as a cut-off for a positive signal. To compare values from different hybridisations, we normalised the total signal intensity of features with an S/N ratio above 2 against the positive control POSITIVE_25 (corresponds to TBP). The data are stored at http://www.mba.ac.uk/midtal/login.php?ret_link=%2Fmidtal%2F&type=notLogged. New users of the MIDTAL chip can obtain detailed instructions as to how to log into the database, upload their own microarray and compare it to any experiment in the MIDTAL project from Lewis et al. (2012).

Results and discussion

Phylochips have been used successfully and designed mainly for bacterial diversity research (Ye et al. 2001; Rudi et al. 2000; Peplies et al. 2003) and the characterisation of ectomicrorhizal fungal communities (Reich et al. 2009). Only a few studies have used 18S rDNA to identify eukaryotes, such as waterborne protozoan pathogens (Lee et al. 2010) or to assess the community composition of microalgae difficult to identify with light microscopy, such as prasinophytes (Gescher et al. 2008a) or cryptophytes (Metfies et al. 2010). A microarray for a selection of potentially toxic species has been developed by Ahn et al. (2010), Galluzi et al (2011), Gescher et al. (2008a, b) and Ki and Han

Table 4 Washing buffers used in this study and their final concentration, washing temperature, and incubation time

Wash buffer	Final concentration	Temperature (°C)	Time (min)
W1	2× SSC/10 mM EDTA/0.05 % SDS	RT	10
W2	0.5× SSC/10 mM EDTA	RT	10
W3	0.2× SSC/10 mM EDTA	45	10

(2006). All of these studies, except that by Ahn et al. (2010), are based on DNA-PCR products of the ribosomal RNA operon (16S, 18S, 28S or ITS). By introducing a PCR step into the protocol, a bias can be potentially introduced into the analysis. We have used total RNA from the entire plankton community at the time of sample filtration. The study by Ahn et al. uses a sandwich hybridisation protocol (capture and signal probe) on the microarray using fibre optic rather than fluorescent detection and was tested only with three species.

The MIDTAL project took advantage of using total RNA to detect potentially toxic algae and not genomic DNA and PCR products. The latter one can have high copy numbers of the 18S rRNA, which influences the signal as more copy numbers result in a higher signal, and because monitoring is based on cell numbers, harvesting sites would be closed even when sanitary thresholds are not reached, if PCR products are used. Our calibration curves generated for each species is based on total RNA content=microarray signal= cell numbers for each probe tested (see papers in this volume and Blanco et al. and Taylor et al. submitted). We developed probes based on sequences from a global database, thus, our probes were designed to construct a universal microarray. The microarray designed by Ahn et al. (2010) contains a probe set for *Pseudo-nitzschia australis* that can only be applied to Pacific Ocean isolates.

Probe specificity was greatly improved by increasing the length of the probe from 18 to 25 nt (Fig. 1a). This also enabled us to use some probes, such as DinoB, which were unusable at the a length of 28 nt because the secondary structure of the 18S rRNA molecule prevented probe access if the probe target region was in the last 900 bases of the molecule (Metfies and Medlin 2008). Fragmentation of the RNA enhanced access to the target site especially for the weakest of probes whose target site was likely blocked by secondary structure of the RNA (Metfies and Medlin 2008, Fig. 1c), but fragmentation inhibited the strongest probes likely because the target site was broken by the fragmentation (Fig. 1d). Both the length of time and temperature at which the fragmentation took place were optimised (Fig. 1b).

Using the gpr-analyser version 1.24 and the hierarchy file for the second-generation slides, we were able to exclude almost all false-positive signals. Only signals with a signal-to-noise ratio above 2 were considered for this analysis. Positive samples that failed because of the taxonomic hierarchy (false-positives) were also excluded. The following points could be ascertained from our microarray analysis:

- The three Arcachon samples showed only a few taxa that could be considered toxic, i.e. six taxa ranging from 10 to 800 cells/L (Electronic supplementary material 1), whereas our microarray revealed more taxa. This is likely smaller volume (10–100 mL) used for counting relative to the volume filtered for the microarray (1–2 L).
- Dinoflagellates (Fig. 2a): Both class-level probes for Dinophyceae (DinoB_25, DinoE12_5) showed a higher signal in sample 1A, where 13,130 dinoflagellates were counted for 1 L, than in sample 5A with 29,570 cells/L. The higher signal means more dinoflagellate cells were present in sample 1A than in sample 5A, but these cells were not counted or identified as dinoflagellates (Fig. 2a). The lower volume analysed and/or the existence of small nano-dinoflagellates <10 to 15 μm could help explain these differences. DinoB_25 had always a higher signal than DinoE12_25, which means that DinoB_25 has a better detection. This is in stark contrast to its non-usability with a length of 18 nt. Each of these probes recognises about 95 % of all dinoflagellates in Genbank, and the same suite of species is not recognised by each probe; thus between the two probes, nearly all dinoflagellates can be detected (data not shown).
- The genus-level probe of *Alexandrium* showed a signal in all three samples, but no *Alexandrium* cells were recorded in the cell counts from September samples (Fig. 2b). The higher signal matched the cell counts in July. However, small amounts of paralytic shellfish poisoning (PSP) toxin were detected using the ELISA tests for all three samples (Table 2).
- *Azadinium* genus-level probes AzaGD01 produced a signal in samples 5A and 6A and AzaGS01 produced a signal in sample 6A. The species probe AzptoxiD05

Table 5 Determination of the number of cells corresponding to a hybridization of 5 ng on the microarray and with a signal-to-noise ratio above background

Species	Cells
K. veneficum	6,000
P. multiseries	2,500
P. australis	1,200
A. ostenfeldii	750
A. minutum	7,000
P. multistriata	3,000
Pseudo-nitzschia calliantha	50,000
H. akashiwo	15,000
K. mikimotoi	1,000
K. brevis	1,000
Prymnesium (=C.) polylepis	20,000
P. parvum	50,000
Pseudochattonella verruculosa	1,000
P. farcimen	5,000
D. acuminata	10,000
D. acuta	2,000

Lower amounts of RNA can be detected by stronger probes and be above S/N ratio of 2

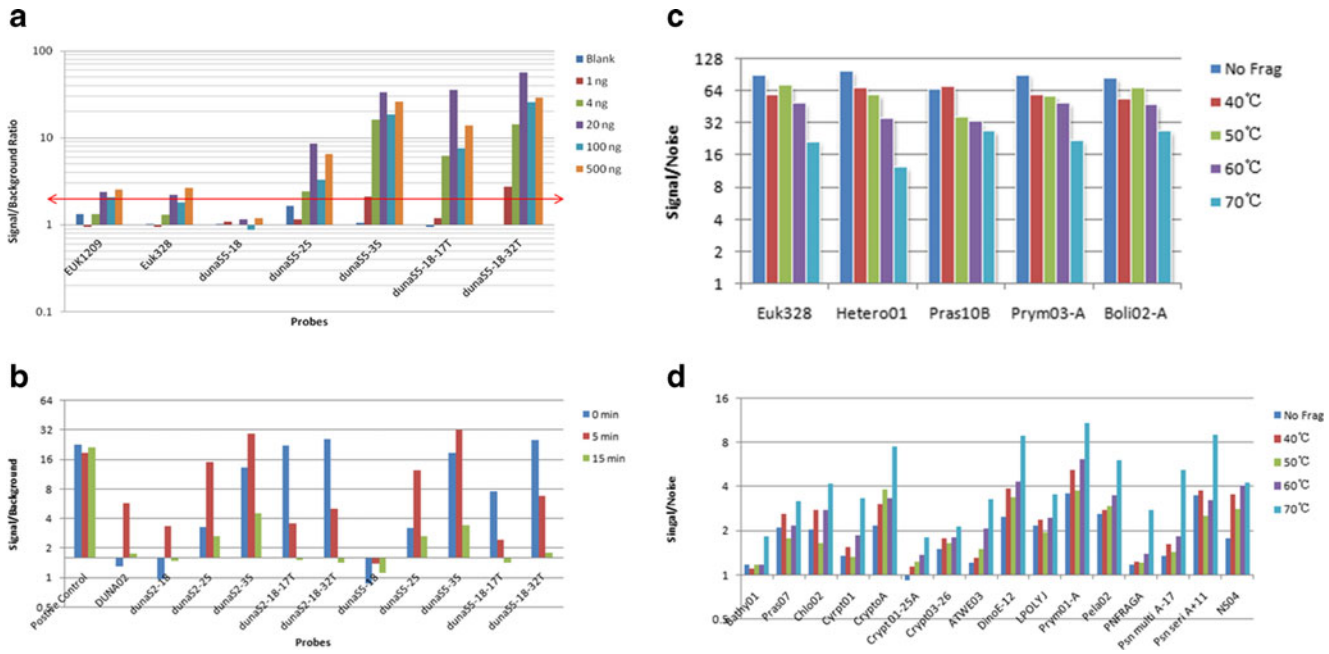

Fig. 1 Summary of the modifications to either the probes or the hybridisation protocols to enhance the microarray signal of the second-generation chip. The detection cut-off signal-to-noise (S/N) ratio of 2 is shown in **a** by the *red arrow*. **a** Comparison of different probe lengths and length of spacer to raise the probe above the surface of the chip. **b** Comparison of the signal proportional to the length of time the RNA was exposed to fragmentation before hybridised to the chip. **c** Comparison of strong probes whose performance decreased by fragmentation. **d** Comparison of weak probes whose performance was enhanced by fragmentation

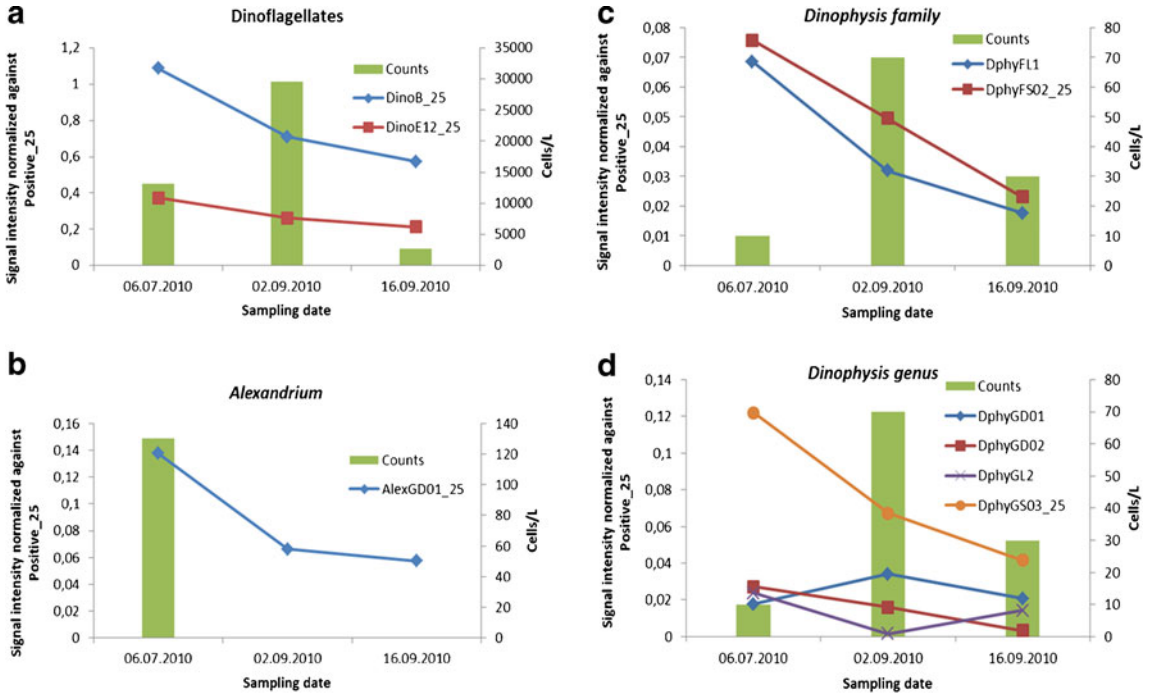

Fig. 2 Microarray signals normalised against the positive control (*Positive_25*) for three field samples taken in Arcachon Bay, France, and compared with cell counts. The *graphs* show only probes that yielded a signal above the detection limit (signal/noise ratio>2) except for *graph* **d**. The sampling dates (06.07.2010, 02.09.2010, and 16.09.2010) correspond to the sampling names: 1A, 5A, and 6A. Cell counts are depicted on the secondary *y*-axis and as columns. **a** Normalised signal of the Dinophyceae class-level probes (*DinoB_25*, *DinoE12_25*) in comparison to the cell numbers of all counted dinoflagellates. **b** Normalised signal of the *Alexandrium* genus-level probe *AlexGD01_25* in comparison to *A. minutum* cell counts. **c** Normalised signal of the Dinophysis family-level probes (*DphyFL1*, *DphyFS02_25*) in comparison to cell counts of *D. caudata* and *D. tripos*. **d** Normalised signal of *Dinophysis* genus-level probes (*DphyGD01, DphyGD02, DphyGL2, DphyGS03_25*) in comparison to cell counts of *D. caudata* and *D. tripos*

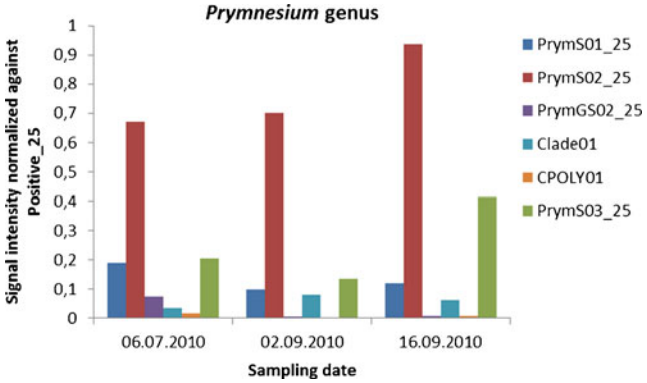

Fig. 3 Normalised signals of haptophyte class-level probes (*PrymS01_25, PrymS02_25, PrymS03_25*) and the clade-level probe for *Prymnesium* (*Clade01*). The genus-level probe of *Prymnesium* (*PrymGS02_25*) and the species-level probe of *C. polylepis* (*CPOLY01*) had only in sample 1A an S/N ratio above 2

for *Azadinium poprum* produced a signal in sample 1A failed the hierarchy test because the two generic probes were not highlighted. This genus was not counted in the Arcachon samples. It is a relatively newly described species (Tillmann et al. 2009), and not all monitoring agencies are adjusting their cell counts to account for this new toxic species.

- Cells of *Karenia brevis* (20 cells/L) and *Karenia mikimotoi* (50 cells/L) were counted in sample 1A. Slight signals of *Karenia* species-level probes (KB4, KbreD04_25) were registered, but the presence of this species failed when using the hierarchy file because the *Karenia* genus-level probe (KareGD01_25) had an S/N ratio below 2. For this species, the present microarray needs at least 1,000 cells in the filtered sample to get a signal. However, the sanitary threshold of this potentially toxic species is not usually given because its toxins only affect fish and not humans.
- The toxic alga, *Dinophysis caudata,* was identified in sample 5A (70 cells/L) and sample 6A (30 cells/L). The toxic *Dinophysis tripos* and the *Phalacroma rotundatum* were identified in sample 1A (10 and 20 cells/L, respectively). The Dinophysis family probes DphyFL1 and DphyFS02_25 showed signals for all three samples (Fig. 2c) with an S/N ratio above 2. The highest signals were obtained for the sample 1A and were lower in the September samples. These results suggest that cells of *Dinophysis* were missed during counting for sample 1A, and this is likely caused by the lower volume of water used for the cell counts. The genus-level probe DphyGS03_25 showed a consistent signal for all three samples following a similar pattern as the family probes (Fig. 2d). The other four generic-level probes, DphyGD01, DphyGD02, DphyGL2 and ProroFD01 (= all *Dinophysis* except *Dinophysis acuta*), did not have a S/N ratio above 2 in the three samples. The probe DphyGD01 was detected in samples 5A and 6A () and the probe DphyGD02in sample 1A and 5A (). In addition, only sample 1A showed a signal for the two probes DphyGL2 and ProroFD01. No okadaic acid was detected in any of the samples (Table 5)
- Haptophyta/Prymnesiophyceae/phyta were not identified in cell counts, but all three class-level probes (PrymS01_25, PrymS02_25, PrymS03_25) and the clade-level probe for *Prymnesium* (Clade01_25) had a permanent signal in all three samples, especially in the last one (Fig. 3). In addition, sample 1A had a signal for the genus-level probe of *Prymnesium* (PrymGS02_25) and the species-level probe of *Chrysochromulina polylepis* (CPOLY01), both of these species being too small to count accurately by LM. The reason for that is, again, most likely the larger volume used for filtration (~2 L) than for counting (10 or 100 mL) and the definitely small size of this phytoplankton species.

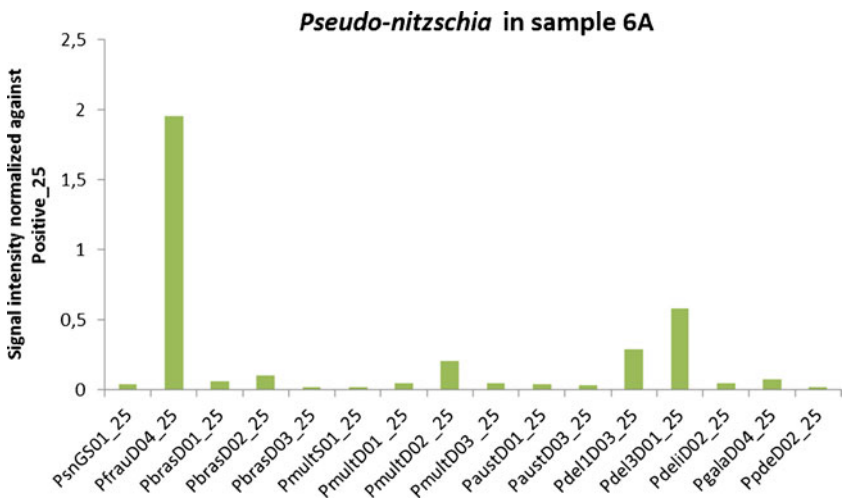

Fig. 4 Signals with an S/N ratio above two for *Pseudo-nitzschia* genus- and species-level probes in sample 6A normalised against *Positive_25*

- *Pseudo-nitzschia* sigmoid species (=*multistriata*) was identified only in sample 6A (Electronic supplementary material 1), which agrees with the microarray signals (Fig. 4). Some other species-level probes gave a signal in sample 1A and 5A, but they failed the hierarchy test because the signal of the genus-level probe failed and were therefore categorised as false-positives. The highest signal in sample 6A was achieved from PfrauD04_25, followed by Pdel1D03_2, Pdel3D01_25 and PmultD02_25 (Fig. 4). However, no domoic acid was detected in any of the samples (Table 5).
- Field sample 6A had also signals for *Pseudo-nitzschia* (genus-level probe) and the species-level probes *Pseudo-nitzschia brasiliana*, *Pseudo-nitzschia multiseries* and *P. australis*. Three *Pseudo-nitzschia delicatissima* probes, one *Pseudo-nitzschia galaxiae* and one *Pseudo-nitzschia pseudodelicatissima* exhibited signals. PfrauD04 had a very high signal. This latter probe has consistently cross-reacted with nearly all *Pseudo-nitzschia* spp., and its hierarchic level has been elevated to a genus-level probe.
- *Prorocentrum micans* was detected in cell counting in all three samples but not with the microarray. The reason for that is probably because the cells were not successfully broken open or the detection limit of *Prorocentrum* probes is higher than 1,000 cells. No calibration curves for these species were performed. This species is generally not considered to be toxic in European waters (but see the related paper by McCoy et al. in this volume).

The results of the first-year samples from MIDTAL show that microscopic cell counts often can underestimate cell numbers and some rare taxa can be undetected because of the smaller volume analysed for cell counts as compared with the volume filtered for the microarray. It is well-known that cell counting when using light microscopy and the classical Utermöhl (von Utermöhl 1931) method has limits when trying to detect rare species with very low densities. Indeed, Maurer et al. (2010) concluded that the sampling strategy and the methodology developed within the REPHY network is not adapted for listing all rare or low abundant taxa and that other strategies and tools would be required in order to be exhaustive (i.e. plankton tow, or molecular probes …). Furthermore, morphological identification of some taxa is not always possible at the species or the genus level, especially for the smallest organisms, and molecular probes are therefore a powerful tool. Thus, because the volume filtered for the microarray is several times more than that counted (>10×), the possibility of detecting rare species of other potentially toxic species with low densities at a pre-bloom stage makes this molecular tool a much more sensitive monitoring tool.

One possible external validation improvement for the microarray would be to settle larger volumes of water for the cell counts and perhaps to perform quantitative PCR (qPCR) when the microarray signal contradicts the cell counts based on a smaller volume. In the few cases where qPCR has been done, the results have confirmed the microarray signals (data not shown). Only in the case of *Prorocentrum* did the microarray not pick up the potentially toxic species, and this is most likely a failure to break open the cells. For other species (e.g. *Karenia* spp.), the detection limit of microarray may not be low enough and needs improvement.

The MIDTAL phylochip is still not fully optimised, but these preliminary results gave us a good indication of which areas we need to optimise in our protocols for the next steps of the project. The RNA extraction protocol has to be adapted so that all cells can be better broken. The hybridisation protocol revealed a high background and often bad smears on the arrays. Our next goals are therefore to reduce the background as well as to enhance the signal. The next-generation chip (version 3) for the second year will be spotted with new probes (non-specific ones from generation 2 will be deleted), and year 2 samples will be extracted with an enhanced RNA extraction protocol and hybridised with an optimised hybridisation protocol.

Acknowledgments This work was supported by EU FP7 project MIDTAL, contract no 201724. Jixin Chen contributed to early stages of the protocol development and spotted the microarray. Delphine Guillebault and Angelica Herrera helped with the RNA extractions and hybridisations. YDA thanks her crew members (Laurent Letort and Francis Prince) and René Parra and Pascal Lebleu for technical support in Arcachon. Thanks are also due to Danièle Maurer from IFREMER/LER Arcachon for cell counts data and comments on the manuscript.

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