Validation of the detection of *Pseudo-nitzschia* spp. using specific RNA probes tested in a microarray format: Calibration of signal based on variability of RNA content with environmental conditions

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Harmful algal blooms (HAB) occur worldwide and cause health problems and economic damage to fisheries and tourism. Monitoring for toxic algae is therefore essential but is based primarily on light microscopy, which is time consuming and can be limited by insufficient morphological characters such that more time is needed to examine critical features with electron microscopy. Monitoring with molecular tools is done primarily in only a few places world-wide. EU FP7 MIDTAL (Microarray Detection of Toxic Algae) used SSU and LSU rRNA genes as targets on microarrays to identify toxic species. In order to comply with current monitoring requirements to report cell numbers as the relevant threshold measurement to trigger closure of fisheries, it was necessary to calibrate our microarray to convert the hybridisation signal obtained to cell numbers. Calibration curves for two species of *Pseudo-nitzschia* for use with the MIDTAL microarray were presented to obtain cell numbers following hybridisation. It complements work presented by Barra et al. (2012b). Environ. Sci. Pollut. Res. doi: 10.1007/s11356-012-1330-1v for two other *Pseudo-nitzschia* spp., Dittami and Edvardsen (2012a). J. Phycol. 48, 1050 for *Pseudo-chattonella*, Blanco et al. (2013). Harmful Algae 24, 80 for *Heterosigma*, McCoy et al. (2013). FEMS. doi: 10.1111/1574-6941.12277 for *Pyrzenium* spp., *Karlodinium* *veneficium*, and cf. *Chattonella* spp. and Taylor et al. (2014). Harmful Algae, in press) for *Alexandrium*.

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**1. Introduction**

Among the planktonic diatoms, the raphid pennate genus, *Pseudo-nitzschia* is easy to recognise with its needle-shaped cells that overlap end to end to make a slender stepped colony (Hasle and Syvertsen, 1996). Only a few species do not form this characteristic colonial habit. Its species, however, can be difficult to identify and morphometric measurements and observations made at the light microscope level, such as frustule shape, width of apical and transapical axes in valve view, morphology of the valve apices and the degree of overlap of the cells in a colony results in groupings of species into categories based on gross cell morphologies. Based on these morphological features, only a crude identification of ‘morphotypes’ or ‘species complexes’ can be done. Cells < 3 μm are attributed to *Pseudo-nitzschia delicatissima* complex, whereas cells > 3 μm are attributed to *Pseudo-nitzschia seriata* complex (Hasle and Syvertsen, 1996). In Arcachon Bay, France, IFREMER counts *Pseudo-nitzschia* in four morphospecies categories: the “slender” (seriata complex, viz., *Pseudo-nitzschia multiseries* *pungens*), the “thin” (valves < 3 μm, delicatissima complex, viz., *Pseudo-nitzschia calliantha* + *delicatissima* + *pseudodelicatissima*), the “wide” (valve > 3 μm, seriata complex, viz., *Pseudo-nitzschia australis* + *fraudulenta* + *seriata* + *subpacifica*), and the “sigmoid” (*Pseudo-nitzschia multistriata*). In Galicia, Spain, INTECMAR counts two categories > 3 μm and < 3 μm. At Stazione Zoologica Anton Dohrn, in Naples, five groups/species of *Pseudo-nitzschia* spp. are recognised through light microscopy: *Pseudo-nitzschia pseudepeliticissima-group*, *Pseudo-nitzschia delicatissim-group*, *P. multistriata*, *Pseudo-nitzschia galaxiae*, and *Pseudo-nitzschia fraudulenta/subfraudulenta*. An accurate identification of species must rely on examination with transmission electron microscopy (TEM) or scanning electron microscopy (SEM) to reveal ultrastructural features that distinguish the different species: the number of striae and fibulae, in 10 μm, type and arrangement of pores in the valve striae and cingular bands. Molecular examinations have detected even more species, some cryptic and some semi-cryptic (reviewed in Trainer et al., 2012; Lelong et al., 2012). A correct identification is needed because
several species of *Pseudo-nitzschia* produce domoic acid (Trainer et al., 2012), the causative agent for amnesic shellfish poisoning. Identification of toxic species can only be made for certain with electron microscopy.

A more accurate identification of *Pseudo-nitzschia* spp. without having to resort to TEM/SEM or to a molecular characterisation of strains isolated from field material into culture is to use molecular probes, which can be used directly with field material without the

### Table 1

Summary of hierarchical probes for the genus *Pseudo-nitzschia*.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Targeted species</th>
<th>Gene</th>
<th>Source/designer</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>EukS_328_25</td>
<td>Eukaryotes</td>
<td>18S</td>
<td>Moon-Van Der (2007)</td>
</tr>
<tr>
<td>EukS_1209_25</td>
<td>Eukaryotes</td>
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<td>Stazy et al. (2001)</td>
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<td>Heterokonta</td>
<td>18S</td>
<td>Eller et al. (2007)</td>
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<tr>
<td><strong>Genus level probes</strong></td>
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<td>18S</td>
<td>Eller et al. (2007)</td>
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<tr>
<td>PpartgenD04_25_dT</td>
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<td><strong>Species level probes</strong></td>
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<td>Diercks et al. (2008)</td>
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<td>*P. delicatissima clade 4 + *P. galaxiae clade2 + <em>P. australis</em></td>
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<td>*P. delicatissima clade 3 + <em>P. micropora</em></td>
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</tbody>
</table>
need to culture. A number of probes for *Pseudo-nitzschia* exist using a whole cell FISH format (Miller and Scholin, 1996). Many of these FISH probes will only work on local populations because they were not designed from a broad enough database. The suite of probes available for *Pseudo-nitzschia* spp. has been expanded and, where necessary, more universal probes were designed for groups of species (clades), the genus and its toxic species (Table 1). These probes have been tested in a microarray format (Barra et al., 2012b; Dittami et al., 2013; Kegel et al., 2012, 2013). Microarrays (as phylochips) detect multiple species simultaneously using species-specific probes and were initially applied for the detection of bacteria (Yergeau et al., 2009). At present, 136 probes for various toxic algal species at various taxonomic levels are spotted onto the current generation of the MIDTAL microarray. As part of MIDTAL project, one goal was to be able to infer cell numbers from the molecular signal. To achieve this goal, it was necessary to assess the degree of variation in RNA content under varying environmental conditions. Variation in RNA content of two species of *Pseudo-nitzschia* is documented and used to construct a calibration curve to infer cell numbers for these species from the microarray signal on the phylochip.

2. Material and methods

2.1. Algal strains

We have used three strains for *Pseudo-nitzschia multiseries* and only one strain of *Pseudo-nitzschia australis* because only one of the four strains obtained from culture collections survived under our culture conditions (Table 2). Before experimental testing, all strains were grown under optimal conditions in f/2 (Guillard, 1983) culture medium, with a reduced amount of nitrogen (580 μM NaNO₃) to reach a Redfield ratio N:P of 6:1 Si-modified 30, 15 °C and 75 μmol photons m⁻² s⁻¹.

Because the results presented here are from the FP7 MIDTAL project, different species were assigned to different partners in the consortium. *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis* experiments were performed at the Marine Biological Association of the UK (MBA). Those for *Pseudo-nitzschia multisiriata* and *Pseudo-nitzschia calliantha* were performed at the Stazione Zoologica Anton Dohrn in Naples (SZN) and are presented elsewhere (Barra et al., 2012b and unpubl.). Standard/optional conditions were identical for these two species of *Pseudo-nitzschia* but different from the species tested in Naples because species were isolated from different geographic sites (Table 2, Barra et al., 2012a,b).

2.2. Experimental design

A stock culture of each strain was grown under optimal conditions, with fresh media added regularly to maintain exponential growth. The three strains of each species were inoculated in triplicate in 200 or 500 ml culture flasks with cotton plugs to test the effect of different stress conditions. An initial volume of 200–500 ml was in each flask, to which 20 or 250 ml of the stock culture of strain 1, 2, 3, respectively, and f/2 modified according to the conditions added to make up the rest of the volume. For the salinity experiments, the initial inoculum at 2000 cells ml⁻¹ was carried out at 25–30 and 35 psu and salinity was progressively increased or lowered every 2 days until salinity of 25 and 35 was reached. At this point, the experiment started.

Control cultures were grown at the same conditions as the initial stock cultures. Salinity, light intensity and temperature stress and nutrient depletion were tested in parallel, changing one parameter for each experiment; the stress conditions did not vary between *Pseudo-nitzschia australis* and *Pseudo-nitzschia multiseries* strains (Table 2). The general principle was to test lower and higher values as compared to the control/optimal conditions for salinity, irradiance and temperature. For salinity stress, the strains were inoculated in flasks containing f/2 at lower (LS) and higher salinity (HS) than the control conditions. Low light intensity (LL) was 25 μmol photons m⁻² s⁻¹ and high light intensity (HL) was 200 μmol photons m⁻² s⁻¹. Temperatures were set at 10 °C for low temperature (LT) and the higher one (HT) at 20 °C (Table 2). Nutrient depletion tests were carried out by using modified f/2 medium without either phosphorus or nitrogen. All conditions were tested for all three strains of a species in triplicate. It should be noted that the nutrient depleted treatments did contain at the beginning of the experiment, some N or P because ca. 10% (by volume) of culture with full strength f/2 medium was used for inoculation and was carried over into the experimental flask.

Each set of conditions was run at the same time to use inocula from the same starting cultures. The day of cells inoculation was considered as time zero (T0). Subsamples were taken from the initial cultures at T0 for cell counts and RNA extraction. Subsamples of the cultures at each different condition were taken after 24 h of inoculation (T1), after 48 h (T2) and after 72 h (T3). For these, 10–17 ml were taken from each flask and mixed in sets of two-three, to have three replicates of a 30–34 ml mix of the three strains (Fig. 1). Strains were not mixed before, to ensure having cells from all three strains in the final mix. It was assumed that if the three strains were mixed at the beginning, one strain could out-compete the others.

2.3. Cell counts

Subsamples for cell counts were kept at 4 °C after adding 20 μl of 25% glutaraldehyde to 500 μl of the culture mix (MBA). Counts were carried out with a flow-cytometer (Becton Dickinson FACSCalibur, BD) after adding a known amount of beads (4.8 μm, calibriTE Beads BD Biosciences).

2.4. RNA extraction

Subsamples of 30–34 ml of the culture mix were used for RNA extractions according to a protocol developed during the MIDTAL project (chapter 9 in Lewis et al., 2012). Samples were pelleted and kept frozen (−80 °C) in 2 ml screw-cap Eppendorf tubes with

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain name</th>
<th>Isolation location</th>
<th>Cells counted</th>
<th>Salinity range tested (psu)</th>
<th>Temperature range tested (°C)</th>
<th>Light intensity range tested (μmol photons m⁻² s⁻¹)</th>
</tr>
</thead>
</table>
0.8 ml of TriReagent (Sigma) until extraction. Acid washed glass beads (213–300 μm) were added to each sample. This was followed by 10 min in a thermo-shaker (Thermomixer comfort, Eppendorf) at maximum speed and at 60 °C. Afterwards, 160 μl of chloroform were added to each sample and the tubes were vortexed for 15 s. After incubation at room temperature for 5 min, samples were centrifuged at 12,000 × g at 4 °C for 15 min and the upper phase of the supernatant was transferred to a clean Eppendorf tube. An equal volume of isopropanol (500 μl) was added and samples were kept at −20 °C for 1 h. After this time, samples were centrifuged as above and the supernatant was removed. Ethanol 80% (1 ml) was added to the pellet, and removed again after one more centrifugation. The pellet was left to dry and RNA was dissolved in 25 μl of RNase free water. RNA concentrations were measured with a Nanodrop (Nanodrop 2000 spectrophotometer, Thermo Scientific), and the rest of the sample was stored at −80 °C until later use.

2.5. Microarray calibration

Sequences of Pseudo-nitzschia spp. were analysed in silico using ARB (Ludwig et al., 2004) to design specific probes in those instances where published FISH probes were not available. Probes originally designed for FISH format for species or for higher taxonomic levels (Table 1) were lengthened to 25 nts. The probe sequence for all probes designed or modified from FISH probes for the entire project for the MIDTAL microarray is patented but a universal microarray for the detection of toxic algae, and the entire hybridisation kit including the array and all necessary reagents is commercially available from Microbia Environnement (France) and can be ordered by contacting delphine.guillebault@microbiaenvironnement.com. The microarray slides for generation 2 selection of probes (SCHOTT nexerton) containing the specific probes were run with 4 different amounts of Cy5-labelled (cyanine-5) Pseudo-nitzschia spp. RNA (1 ng, 5 ng, 25 ng and 100 ng). Hybridisation was carried out as in chapter 10 in Lewis et al. (2012) with some modifications, which includes a pre-hybridised at 65 °C, hybridisation was run for 10 min at 94 °C and continued for 60 min at 65 °C. After hybridisation, slides were washed with washing buffers (SSC/EDTA/SDS) at room temperature and a final wash at 50 °C. Finally, the slides were scanned (GenePix 4000B, Molecular Devices) and total signals were calculated as the average of the feature-background ratio of all eight spots for each probe. For generation 2 arrays, signals were normalised to the positive control (TATA box gene) added to each hybridisation experiment. Further analysis was carried out with the GPR-analyser ver. 1.24 (Dimatti and Edvardsen, 2012). Linear relationships were obtained for all probes and because of that relationship, a shortage of RNA, and a shortage of available arrays, generation 3 arrays were hybridised with only two amounts of Cy5-labelled RNA (5 ng and 100 ng). These signals were normalised to the total signal of Dunaliella (probe DUNA GSS02.25), also spotted on the slides, to which either 500,000 cells of Dunaliella or 50 μg RNA were added to the extraction to allow comparison of signal strength between slides. Generation 2 and generation 3 differed only in the repertoire of probes on the microarray, and non-specific probes were eliminated in generation 3. In this study, only one Pseudo-nitzschia probe was eliminated for generation 3. Probes for Pseudo-nitzschia spp. had to be used in a combinatorial fashion because of cross reaction within the genus (Table 3).

2.6. Statistical analysis

Statistical analysis was carried out in XLSTAT and http://web.mst.edu/~psyworld/tukeycalculator.htm#1 (Tables 4 and 5). For the RNA stress experiments, a two-way ANOVA was used followed by Tukey’s B post-hoc analysis to look at differences between each treatment at each time point. For the analysis of linear relationships between the amount of RNA and cell number and also amount of RNA against the microarray signal, a regression analysis was used along with a Pearson’s correlation test.

3. Results

3.1. P. multiseries (Figs. 2 and 3)

In all of the conditions tested, the amount of RNA per cell or strain varied over the time course that the cells were exposed to the stress (Fig. 2). Of the four different stress conditions tested for Pseudo-nitzschia multiseries, temperature caused the greatest variation in RNA content, with optimal and lower stress conditions producing the largest amount of RNA after 2 days exposure. For the salinity stress, the lower salinity conditions induced a higher RNA content per cell as compared to the control after 3 days exposure to
Fig. 2. Summary of responses of P. multiseries to various nutrient stresses throughout the entire experimental period. (A) Temperature (B) salinity (C) light (D) nutrient depletion.

Fig. 3. Relationship of RNA to cell number and microarray signals for P. multiseries (A) linear regression of total RNA extracted from each replicate in each stress experiment against cell numbers at the time of sampling, (B) calibration curve relating cell numbers to microarray signal hybridised with four different amounts of RNA on generation 2 array for each probe that targets P. multiseries, using a log 10 plot of cell numbers, (C) calibration curve relating cell numbers to microarray signal with two different amounts of RNA on generation 3 array for each probe that targets P. multiseries.
the stress. Over the 3 day exposure, high light stress induced more RNA production, whereas low light reduced RNA content. In all nutrient stress conditions, RNA content decreased over time with a slight recovery on day three. A regression of all data points from all strains, replicates and time points showed a linear increase in RNA content with cell numbers (Fig. 3A) but the R value is only 0.58. Four quantities of RNA were hybridised to probes spotted on generation 2 microarray to correlate the microarray signal with the cell numbers (Fig. 3B). Signals were normalised to the positive control. These data are plotted using a log 10 transformation of the cell numbers to demonstrate the linear response of the signal to increasing cell numbers. A similar calibration was performed with generation 3 suite of probes with two quantities of RNA, which were normalised to Dunaliella (Fig. 3C). Because there is a linear response, only two data points can be used to draw a regression line (Fig. 3C) and these data are plotted without the log 10 transformation of the cell numbers. In both calibrations, the R value was above 0.9.

An analysis of variance followed by a Tukey post hoc analysis showed that the treatments, the strains and the times were not significantly different (Table 5) except in the temperature experiments where Day 1 at the higher temperature with a mean of 10.5 was significantly different at the 1% level from Day 2 but not from Day 3. A correlation of the cell numbers with RNA content was zero when pooled together over all treatments but were slightly negative when correlated within each treatment indicating that it was slightly easier to extract RNA from less dense cultures.

3.2. *P. australis* (Figs. 4 and 5)

All stress experiments were performed with one strain of *Pseudo-nitzschia australis* in triplicate (Fig. 4). As can be seen by the error bars, the identical strain tested in triplicate under the same conditions produced variable results. As with *Pseudo-nitzschia multiseries*, temperature stress produced the most variation in RNA content. RNA content increased gradually with salinity stress over the 3 days exposure. There appeared to be a variable response to high light, with an increase coming on the second day, which then decreased on Day 3 in low light stress and the opposite response for high light stress. Responses to nutrient stress were depressed on Day 2 but returned to values near those of Day 1 by Day 3. Responses to phosphate depletion were only slightly stronger than those to nitrogen depletion. A plot of RNA content extracted from all stress conditions is shown in Fig. 5A, where a slight linear increase in RNA content is shown with increasing cell numbers. Despite this somewhat lower correlation, there was a good correlation with microarray signal in both the generation 2 and generation 3 microarrays (Fig. 5B and C, respectively). The data are plotted as described above for *P. multiseries*.

An analysis of variance followed by a Tukey post hoc analysis showed that the treatments, the strains and the times were not significantly different (Table 5). A correlation of the cell numbers with RNA content was slightly negatively correlated (– 0.06) when pooled over all treatments, again indicating that it was slightly easier to extract the RNA from less dense cultures (Table 3).
3.3. Species determination with the microarray

During probe specificity testing, it was found that most of the probes developed for detecting *Pseudo-nitzschia* spp. were not species-specific (Barra et al., 2012b). The probes were initially tested using PCR products in a dot blot format (Barra et al., 2012a) and were specific but when total RNA was extracted and tested in a microarray format, there was extensive cross-reactions among the *Pseudo-nitzschia* spp. Those probes that reacted with taxa outside *Pseudo-nitzschia* were discarded (hence the major difference between generation 2 and 3 arrays). In silico, all probes were specific but they were designed from a database with sequences from the LSU, from which most species have only the D1–D2 region sequenced. It is presumed that the non-specificity stems from homology to other regions of the LSU that have not yet been sequenced in most species or there is messenger RNA that is cross-reacting with the probes. It was found, however, that the probes could be used in a combinatorial fashion to determine which species was present (Table 3) because they did not cross react outside of the genus *Pseudo-nitzschia*. In this manner, the species of *Pseudo-nitzschia* present in field samples could be determined from the microarray. At least one of the probes produced a stronger signal than the others and the presence of the signal from that probe was used as the entry point for that species in the GPR-analyser programme (Dittami and Edvardsen, 2012b) to test the hierarchy (Fig. 6). A strict protocol in designing hierarchical probes was followed and with this approach false positives can be detected because for the species to be present, all other probes in the taxonomic hierarchy must also have a signal for the species to be recorded as being present (Table 1, Fig. 6). The complete taxonomy hierarchy is shown in Table 1. The regression coefficient for each probe (Table 4) was entered into the GPR-analyser programme to infer cell numbers and 5 ng of RNA could be equated to 2500 cells of *Pseudo-nitzschia multiseries* and 1200 cells of *Pseudo-nitzschia australis*.

4. Discussion

The aim of MIDTAL was to investigate the variation of RNA yield per cell within *Pseudo-nitzschia* species at environmentally relevant conditions to allow calibration of the microarray chip to estimate cell counts. From these data, a calibration curve for two species relating RNA amount (=cell numbers) to a microarray signal was constructed. All probes showed a linear response, although some probes produced a stronger signal than others for the same amount of RNA hybridised to the array. Linear calibration curves for all *Pseudo-nitzschia* spp. species and strains on both generation chips mean that back calculation to cell numbers from microarray signal is possible. In addition to this, RNA content appears stable regardless of the stress. The slope of the calibration curve for each probe and whether it was normalised by the positive control or Dunaliella was entered into the GPR analyser programme to infer species numbers. By using the combinatorial approach to species identification, the hierarchical probe approach, and the GPR analyser programme, each species could be identified and quantified. The MIDTAL microarray was successfully applied to field data where *Pseudo-nitzschia* occurs at several European sites (partners in the project www.midtal.com). In Arcachon Bay, France, two years of field data were analysed (Kegel et al., 2012, 2013) and in both years, blooms of *Pseudo-nitzschia multisiria* were successfully identified and quantified. In year 2, where more samples were analysed, signals for this species with the microarray increased as cell numbers increased (Kegel et al., 2013). In the Galician Rias, Spain, several *Pseudo-nitzschia* spp. were detected by the microarray (Dittami et al., 2013) and the numbers inferred from the microarrays were always higher than the counts, bearing
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in mind that the LM counts only tally species by gross morphology. A higher diversity was deemed present in the Rias than previously assumed because the microarray provides a species identification, which is not confirmed by electron microscopy. In Naples, during the field testing, signals always were higher than cell counts (Barra et al., 2012b). In those samples where cells were counted but no microarray signal was obtained, it was assumed that either the RNA extraction was poor or there was not enough RNA to produce a signal. Notably, the microarray has a detection threshold that is lower than the threshold imposed by monitoring programmes so a lack of signal could indicate that the cells are not in sufficient quantity to cause a problem. From our experiments and those from Naples, 5 mg of RNA yields a signal above the cut-off of 2 for a normalised signal to noise ratio and corresponds to the following number of cells of these *Pseudo-nitzschia* spp.: multiseris = 2500; *australis* = 1200; *multistriata* = 3000; and *calliantha* = 50,000. A notable advantage of the microarray is that it provides species identification without having to resort to electron microscopy, which is particularly useful for *Pseudo-nitzschia*.

Problems were encountered with RNA extraction with some species, especially the *Pseudo-nitzschia* spp. tested by the Naples group in the project MIDTAL (Barra et al., 2012a,b) and there was a tendency for RNA extraction to be easier with less dense cultures as shown by the Pearson correlation. Because a slight negative correlation with the amount of RNA with increasing cell numbers was obtained, it is advised that if field samples are quite dense, the aliquots taken should be extracted in variable amounts of TriReagent to maximise/optimise the extraction yield. For example, if the colour of the TriReagent turns to brown after addition of this compound to the sample, this likely indicates that the sample is highly concentrated and more TriReagent should be added. Successful extractions with the addition of an additional 4 ml to the original 1 ml added to the concentrated sample (filter) to

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**Table 4**

Linear regression with interception of the slope at 0.0 containing y-intercept and R-squared value for each microarray probe of *Pseudo-nitzschia multiseris* and *P. australis* normalised either to the probe Positive_25 or DunGS02_25_dt.

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**Fig. 6.** GPR analyser results showing how the hierarchy tests works and inferred cell numbers of *P. calliantha* and *P. seriata/australis*, which passed the hierarchy test and *P. pseudodelicatissima*, which did not.
achieve a 1:5 dilution factor have be routinely obtained (data not shown). This may be quite cumbersome at first but a balance between cells present and amount of TRIReagent needed to obtain a good extraction can easily be obtained with little effort and is highly recommended. It is strongly recommend to test of the RNA extraction procedure with cultures of *Pseudo-nitzschia* spp. prior to field testing to ensure a more accurate conversion of the hybridisation signal to cell numbers and to optimise the extraction protocol, which is relatively easy but must be optimised to obtain maximum results from the microarray.

The results shown here are in keeping with those found by (Dittami and Edvardsen, 2012a) for *Pseudochattonella*, Blanco et al. (2013) for *Heterosigma*, McCoy et al. (2013) for *Prymnesium* spp., *Karolinindium veneficum*, and cf. *Chattonella* spp. and Taylor et al. (2014) for *Alexandrium*, all of whom found linear increases of signal intensity with increasing cell numbers and RNA concentrations. RNA concentration was not seriously affected by the environmental stresses exposed to each of the toxic algae tested. The response of each species has not been tested through the stationary phase and later, but the microarray is designed as an early warning system and as such the conditions tested here are appropriate.

It has been shown the effectiveness of using microarrays for the detection of toxic algae and that RNA content per cell is not significantly affected by environmental stress conditions. The microarrays used in this study for final calibration represents the third generation array developed within the EU-MIDTAL project. In generation one, probes (18–22 nt) developed for Fluorescent in-situ Hybridisation (FISH) were used directly; in generation two, these FISH probes, and any newly designed probes were lengthened to 25 or more nt; in generation three, an additional spacer was tested and optimised. At each generation, minor changes in the hybridisation protocol were made and a final optimised protocol (Lewis et al., 2012) has enabled to construct calibration curves which when used within the GPR analyser programme (Dittami and Edvardsen, 2012b) can be used to infer cell numbers in the sample whose detection limit is below regulatory numbers for closure of the fisheries.

### Acknowledgements

Brian D. Bill, NOAA’s Northwest Fisheries Science Center, USA supplied the strains of *P. multiseries* and Dr. B. Reugera, IEO, Vigo Spain supplied the strain of *P. austalis*. Table 3 was provided by the MIDTAL partners at the Stazione Zoologica Aonton Dohrn, Naples.[SS]

### References


### Table 5

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Phyceae) species, domotic acid and amnesic shellfish poisoning: revisiting previous paradigms. Phycologia 51, 168–216.


Miller, P.E., Scholin, C.A., 1996. Identification of cultured Pseudo-nitzschia (Bacillar-


