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Microarray for the Detection and Quantification of Toxin-Producing Phytoplankton Species in Scottish Coastal Waters

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Executive Summary

A novel technique using the microarray technology was tested using water samples collected offshore from Stonehaven (East coast of Scotland) in 2015/2016. The results of the phytoplankton population were compared with the light microscopy and qPCR results. There was no strong positive correlation that could be established between the microarray and the other two techniques.

Introduction

A small number of phytoplankton species have the ability to produce algal toxins which can accumulate in filter feeding bivalves such as oysters, scallops and mussels. The bioaccumulation of algal toxins can potentially cause serious health issues to shellfish consumers. Countries with a shellfish aquaculture industry (such as Scotland) have set up for their classified production areas a phytoplankton monitoring programme as part of their legal obligations (EC 854/2004 and amendments). This involves the regular collection of water samples to assess the phytoplankton community using light microscopy, with a particular emphasis on the toxin producing species. However, this technique lacks the ability to identify some key phytoplankton to a species level (e.g. *Pseudo-nitzschia spp.*, *Alexandrium spp.*), which is critical to appropriately assess the potential toxicity of a phytoplankton bloom event. The objective of this project was to evaluate a microarray technique which was developed during the MIDTAL project (Microarrays For The Detection of Toxic Algae: http://www.midtal.com/) to identify phytoplankton to species level. Microarrays are modified glass supports on which are printed RNA probes that are species-specific. Each targeted phytoplankton species is defined by a set of probes that are statistically unique to each one.

For the purpose of this project, water samples were collected offshore from Stonehaven (East coast of Scotland) over a two years period (starting in 2015) and processed using the microarray to assess its specificity and sensitivity in relation to the identification and potential semi-quantification of toxic strains of phytoplankton.

To achieve this, we collaborated with the University of Aberdeen (UoA) to access their equipment and laboratory facilities as well as use their expertise with this new technique.

Methods

Sample Collection

Integrated water samples were collected weekly (25/03/2015 - 19/09/2016) at Stonehaven (about three miles offshore) using a 10 m tube sampler. The contents of each integrated water sample were mixed into a 10 L carboy before 1 L aliquots were taken for light microscopy analysis and microarray processing. For the microarray, each aliquoted sample was filtered through a 1 µm, 25 mm nitrocellulose filter. The filter was then soaked in 1 mL of Tri-reagent and stored at -80°C until RNA extraction. In total, 72 phytoplankton samples were processed using that protocol. Another water sample aliquot was also processed for light microscopy analysis using the Utermöhl method (Utermöhl, 1931) to estimate the phytoplankton community.

RNA Extraction

A selection of samples were chosen for RNA extraction based on the concentration of key planktonic species as determined in-house by light microscopy. Whenever possible, sample time-series were selected for microarray testing based on spikes in abundance of the following phytoplankton species: *Alexandrium spp.*, *Dinophysis spp.*, *Pseudo-nitszchia spp.* and *Karenia spp.* A total of 56 samples were extracted for RNA prior to microarray testing. The method involved thermal and physical cell lysing, followed by an extraction step using 1-bromo-3-chloropropane (Sigma, UK) and Phase Lock Gel (5 PRIME, UK) tubes to separate aqueous and organic phases. This was followed by a clean-up step of the obtained aqueous extract using isopropanol and 75% ethanol which was subsequently dried and re-suspended in 50 µL nuclease free water. The final RNA concentration and quality was measured using a Nanodrop spectrophotometer (Nanodrop 1000, Thermo Scientific, www.nanodrop.com).

Sample Labelling and Hybridization

A number of samples (34) were then chosen for labelling depending on the quantity and the quality of the extracted RNA. During this step, a dye (Cy5) was attached to the extracted RNA strands to enable quantification. Once labelled, the samples

were cleaned-up once more before hybridization was performed. The labelled RNA samples were incubated with the microarray at 65°C for one hour, allowing the sequences printed on the microchip to bind. In total, 32 samples were hybridized. The microarrays (Figure 1) were then read using a Microarray Scanner (GenePix 4100A) at the University of Aberdeen (Scotland). The intensity of each bonded RNA sequence gives an indication of the phytoplankton concentration in the sample.

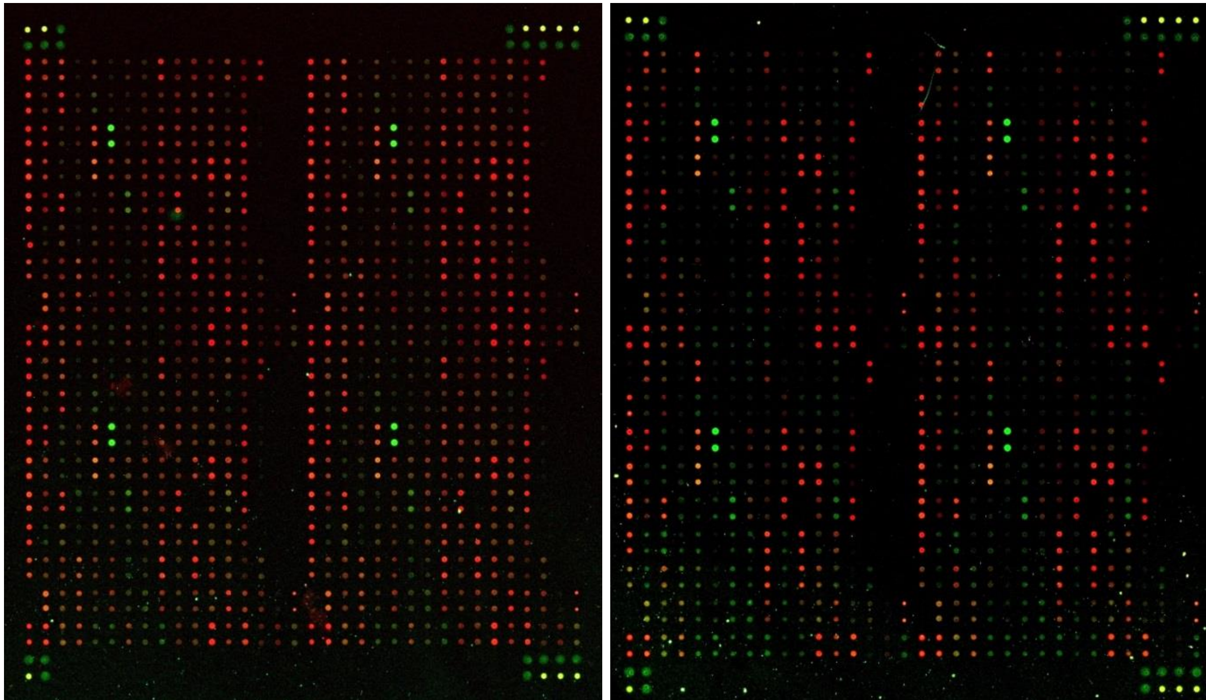
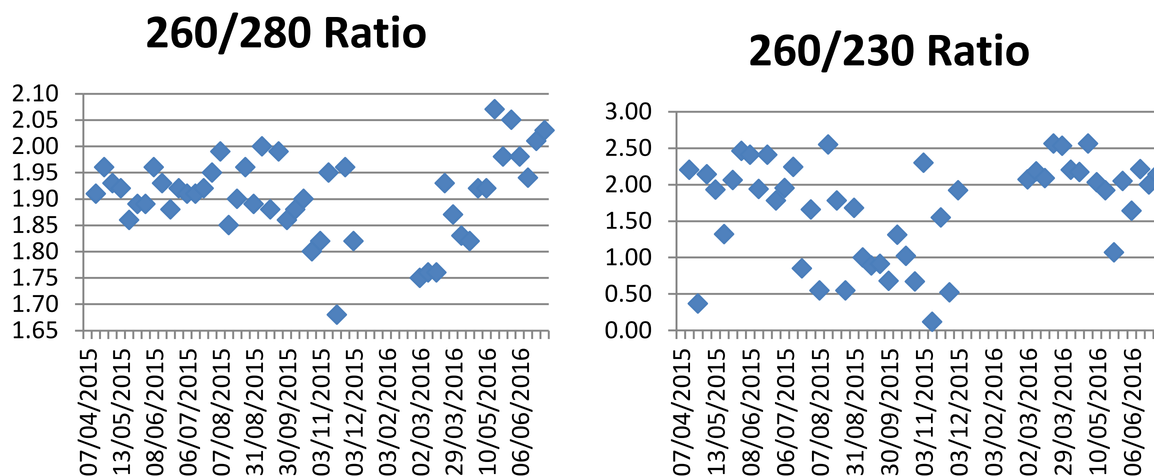


Figure 1: Example of microarray slide images.

Results and Discussion

RNA Extraction

The quantity and quality of the RNA extracted was measured using a Nanodrop spectrophotometer, which quantifies the absorbance of the phytoplankton sample extract at various wavelengths (230 nm, 260 nm and 280 nm). Both RNA and DNA have a maximum absorbance at 260 nm while various contaminants such as phenol or proteins mainly absorb at 280 nm. Measurement of the 260/280 ratio, therefore, gives an indication of the purity of the extracted RNA. Another ratio (260/230) was also monitored for nucleic acid purity. Values lower than 1.8 could indicate the presence of co-purified contaminants. The two measured ratios are plotted in Figure 2.



Note: water samples collected between the 13/01/2016 and the 22/02/2016 were not extracted due to the low abundance of the phytoplankton species of interest in those samples.

Figure 2: 260/280 and 260/230 ratios.

A good RNA extraction is considered when the value of the 260/280 ratio is higher than 1.8 (see Section 5-2, p. 28, V3.7 Nanodrop User's Manual). Of the 56 extracted water samples, 52 presented ratios above 1.8, showing that the clean-up process was overall efficient. Failure to clean the extract properly though leads to the carryover of phenolic compounds present in the Tri-reagent which would increase the absorbtion at 280 nm, leading to a poor 260/280 ratio. However, a 260/230 ratio lower than 1.8 which is the case for 3 samples indicates the probable presence of co-purified contaminants. Those compete with the extracted RNA for binding to the Cy5 dye, thus detrimentally affecting the RNA hybridization and the overall sensitivity of the assay.

RNA Labelling and Hybridisation

The Degree of Labelling (DoL) quantifies the amount of Cy5 dye that successfully binds to the target RNA. The DoL is a ratio between the Cy5 dye concentration and the RNA concentration in the sample after the labelling step. It is recommended by the assay manufacturer (Leica Biosytem) that the DoL should be between 1 and 3% to increase the success rate of a sample hybridization. Of the 36 labelled samples, four samples presented a DoL lower than 1%. Looking at the different quality control measurements, the RNA purity quantified by the 260/280 ratio does not seem to be a crucial factor influencing the DoL (Figure 3). However, the 260/230 ratio correlates much better with the DoL (Figure 3).

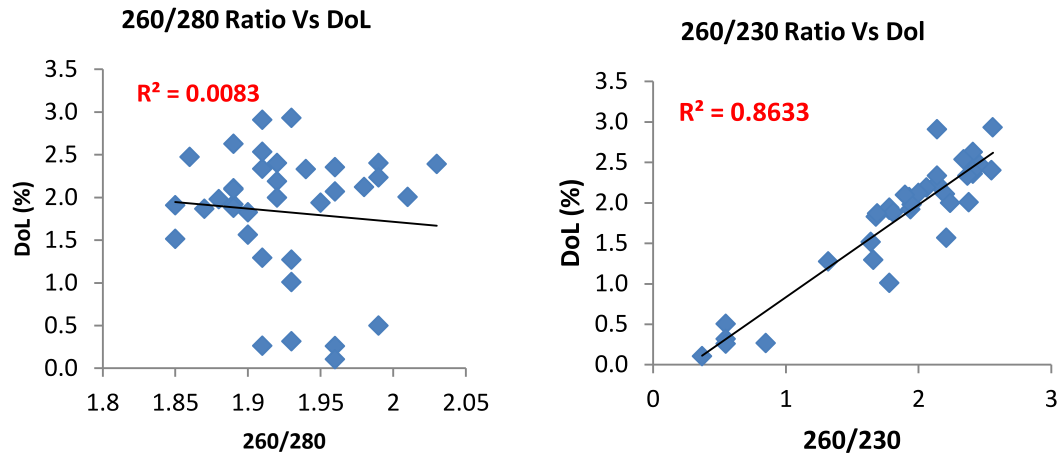


Figure 3: 260/280 Ratio and 260/230 ratio Vs DoL.

Hybridized samples with a low DoL have a weaker signal measured during the scanning process, thus affecting the sensitivity of the assay. Another possible reason for the observation of poor hybridisation, was the evaporation of the hybridisation mixture during the incubation step of the microarray. This could lead to a much higher background noise when scanning (Figure 5), affecting the sensitivity of the method. Different operating conditions of the Omnislide in-situ Hybrisidation System were assessed to try to minimise this evaporation issue. Tissue soaked with water were placed inside the instrument to increase the moisture content which seem to improve the issue slightly. The volume of reagents and sample used for the hybridisation were also increased (10%) to counteract the loss of fluid through evaporation.

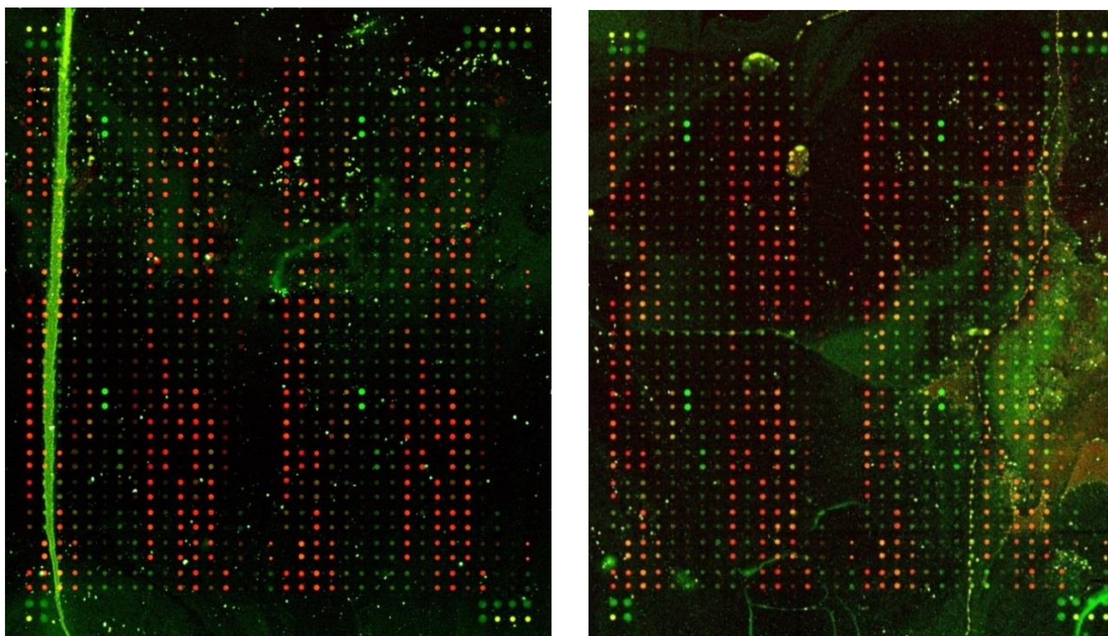


Figure 4: Example of an unsuccessful hybridisation.

The green stains seen on those two microarray slides are directly affecting the normalised absorbance calculated for each dot, therefore contributing to false results.

Data Processing

The software called “GenePix PRO” was used to quantify each dot intensity while another software provided by Microbia Environement called “*MIDTAL GPR analyser*” was used to process the data. A positive control consisting of 500,000 cells of *Dunaliella tertiolecta* was added to each sample before the extraction step, allowing the calculation of a normalised fluorescence signal (NFS) that could be compared between samples. Each sample was analysed in duplicate using two different slides, to assess the repeatability of the hybridisation (Figure 5) and to reduce the risk of a lack of data for a specific sample in case one slide were to be faulty.

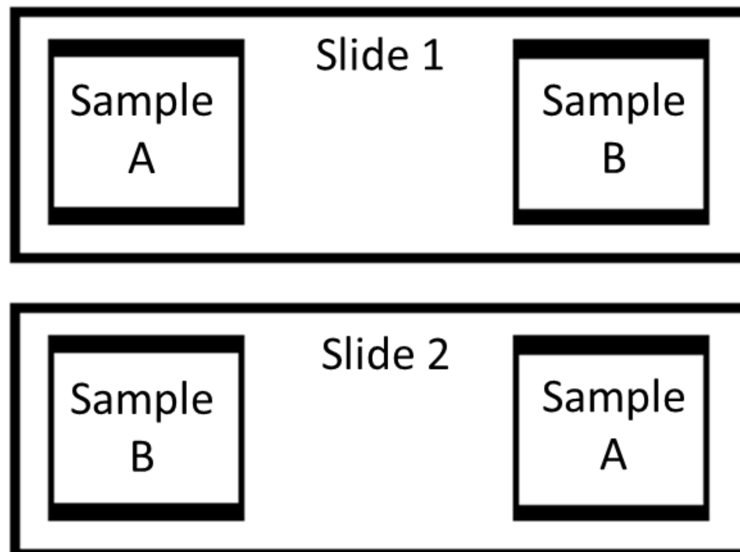


Figure 5: Slide set-up.

For each sample, the intensities of each duplicate probe were plotted against each other, allowing us to calculate a slope and a correlation factor (Figure 6).

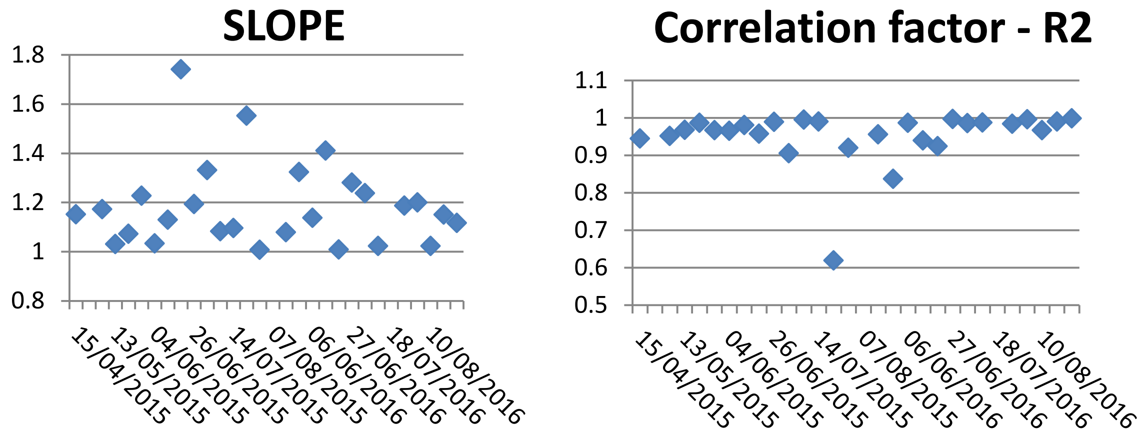


Figure 6: Duplicate analyses.

The correlation between each duplicate was good overall, with only six values out of 32 being below a correlation factor value of 0.95. A low correlation value was observed when the probes signal intensities in each duplicate did not correlate well. This could occur when higher background noise affects different areas of the slide replicates (as seen on Figure. 5), which would affect some probes for one slide but not in the other replicate. A deterioration of the probe printed on the microarray over time could also lead to extra variability in the results (Figure 7).

Figure. 7: Evidence of probe deterioration.

A higher slope value highlighted the fact that the intensity of one of the duplicate was lower than the other one, which would lead to an alteration in the sensitivity of the method.

Environmental Sample of Special Interest – 08/05/2015

The highest density of *Alexandrium spp.* measured by light microscopy (1,440 cells/L) occurred in Stonehaven on the 08/05/2015. In addition to the replicate

sample being processed for microarray analysis, the sample was also tested using a qPCR method developed in-house (Collins et al, 2009) to quantify different species and strains of *Alexandrium spp* (Table 1).

Table 1

Analysis results using the Microarray, qPCR and light microscopy methods for sample collected on the 08/05/2015

	MICROARRAY			**qPCR**	**Light microscopy**
Species	RNA Probe	Average Conc. (Cells/L)	STDEV (cells/L)	Conc. (Cells/L)	Conc. (Cells/L)
Alexandrium tamarense (NA)	ATNA_D01_25_dT	6,972	530	5,030	1,440
	ATNA_D02_25_dT	8,301	2,118		
Alexandrium tamarense (WE)	AtamaS01_25_dT	402	42	<LOD*	
Alexandrium minutum	AminuS01_25_dT	3,137	380	110	
Alexandrium ostenfeldii	AostD01 _25_dT	<LOQ	-	580	
Alexandrium ostenfeldii	AostS02 _25_dT	<LOD	-		
TOTAL	**-**	**11,176****	**-**	**5,720**	**1,440**

Note *: LOD is Limit of Detection, LOQ is Limit of Quantification
**: ATNA_D01_25_dT was used *for Alexandrium tamarense (NA)* to allow the calculation of the total average cell concentration

Both molecular techniques gave similar results for *A. tamarense (NA)*, *A. tamarense (WE)* and *A. ostenfeldii*. However, the cell count results obtained by the two techniques were very different for *A. minutum*. The microarray found a concentration of *A. minutum* in the sample more than thirty times higher than the qPCR technique. The total cell count obtained by both methods was higher than the total cell count obtained using light microscopy (four times higher for qPCR and eight times higher for the microarray). The second probe for *A. tamarense (NA)* (ATNA_D02_25_dT) estimated the cell concentration at 8,301 cells/L, which is higher than the first probe indicated in Table 1. However, this second probe was not used for the calculation of the total concentration of *Alexandrium spp*. cells as only species level probe should be considered for quantification (discussion with Microbia Environnement). The RNA

concentration of this sample was the highest recorded from all the samples processed (325.2 ng/µL), and the quality control checks gave satisfactory values (260/280 ratio: 1.93; 260/230 ratio: 2.14, DoL: 2.2 %, R^2: 0.9517 and slope: 1.173) indicating the hybridisation protocol was successfully carried out. One reason which could have led to the difference in the cell abundance lies with the calibration curves used to generate the quantitative results for the microarray and the qPCR. The calibration curves for the microarray were being updated at the time of the project by the microarray provider as part of their continuous development programme. This might have had an impact on the accuracy of the microarray results.

The microarray technique is based on the extraction of RNA which comes from live phytoplankton cells. The RNA in dead cells is not stable in the water column and degrades very rapidly and would less likely to be picked by the microarray assay. As light microscopy and qPCR are less able to differentiate between live and dead cells, some discrepancy between those two methods and the microarray is to be expected.

Environmental Samples – Time series

A time series of 12 samples, from 06/06/2016 to 22/08/2016, was selected due to the presence of potentially toxin-producing phytoplankton in the samples analysed by light microscopy (Table 2). Due to the overall low cell numbers in the analysed samples, the majority of microarray results were below the LOQ, therefore, it was advised to use the NFS results to assess the correlation between the microarray and the light microscopy results. The RNA concentration of those samples varied from 127.4 to 255.8 ng/µL and all quality control checks gave satisfactory values except for one sample (18/07/2016) where one replicate failed. The two samples collected on the 13/06/17 and the 20/06/17 had the lowest correlation factor from the duplicate analysis (0.9395 and 0.9247 respectively) indicating a higher variability in the replicates.

Table 2

Cell concentration measured by light microscopy of the main toxic phytoplankton species being investigated

Sampling date	Alexandrium spp. (cells/L)	Pseudo-nitzschia spp. (cells/L)	Dinophysis spp. (cells/L)	Karenia spp. (cells/L)
06/06/2016	80	1,440	220	20
13/06/2016	60	82,540	1380	320
20/06/2016	0	17,240	780	40
27/06/2016	80	74,740	2800	880
04/07/2016	300	295,880	1720	1600
12/07/2016	100	1,226,120	560	1200
18/07/2016	80	106,460	1280	2920
26/07/2016	0	116,700	780	800
01/08/2016	40	78,820	620	1580
10/08/2016	20	3,140	580	500
15/08/2016	20	12,260	120	100
22/08/2016	0	8,820	60	40

Both the NFS of the genus probe for each of the four phytoplankton genera being investigated and the phytoplankton cell count were plotted for each sample of the time series (Figures 7, 8, 9 and 10). The microarray probe selected for each phytoplankton group was designed to specifically identify a genus, so those probes would not be able to differentiate between phytoplankton species. Unfortunately, no probes targeting Dinophysis spp. had values over the LOQ across the time-series so a higher hierarchy probe for dinoflagellates was selected. The probes selected were as follow:

- AlexDG01_25_dT for *Alexandrium spp*.
- PsnGS02_25_dT for *Pseudo-nitzschia spp.*
- DinoE12_25_dT for Dinoflagellates.
- KareDG01_25_dT for *Karenia spp.*

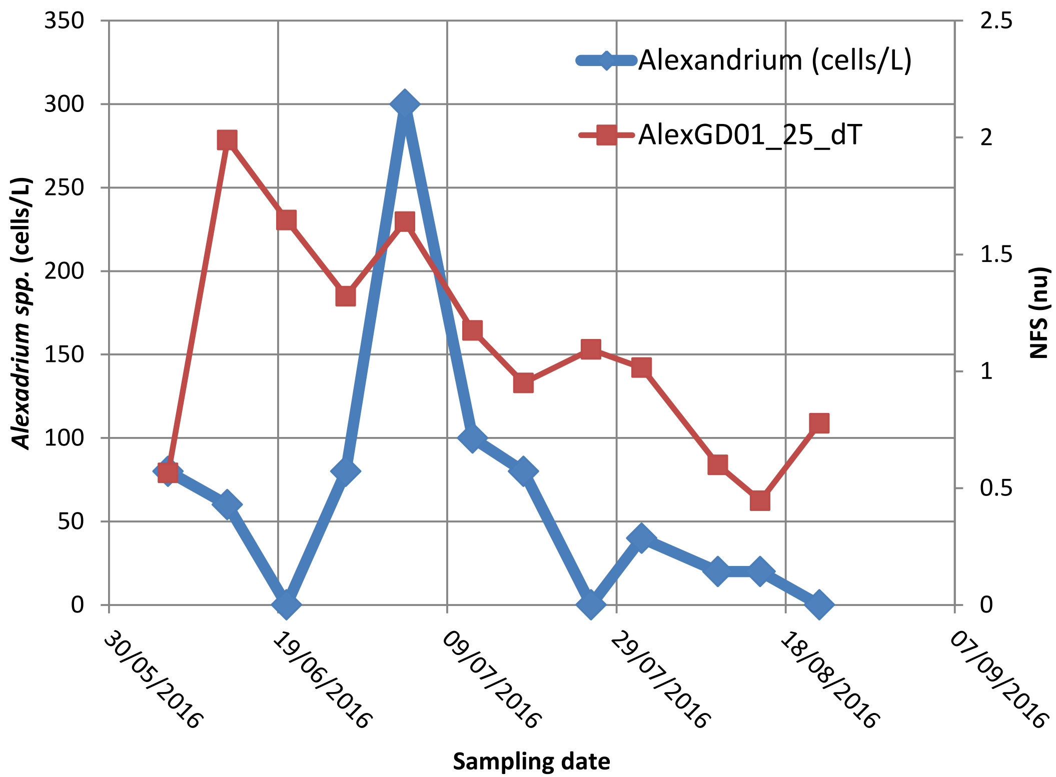


Figure8: *Alexandrium spp*. concentration and Normalised Fluorescence Signal of AlexDG01_25_dT VS time.

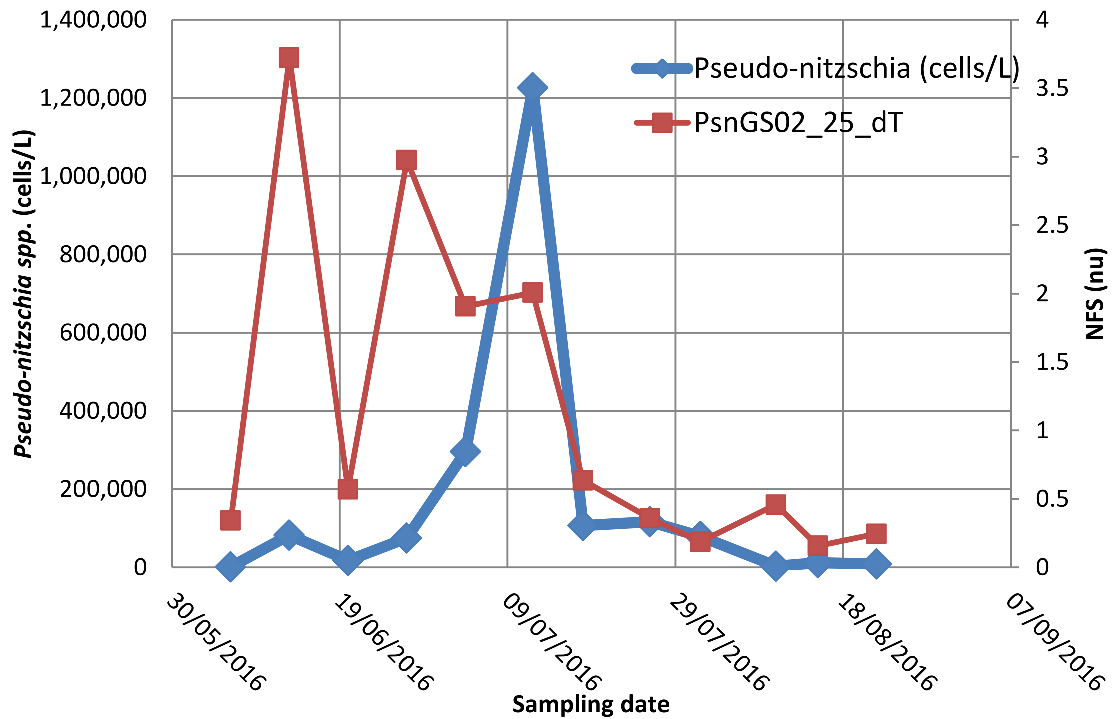


Figure 9: *Pseudo-nitzschia spp.* concentration and Normalised Fluorescence Signal of PsnS02_25_dT Vs Time.

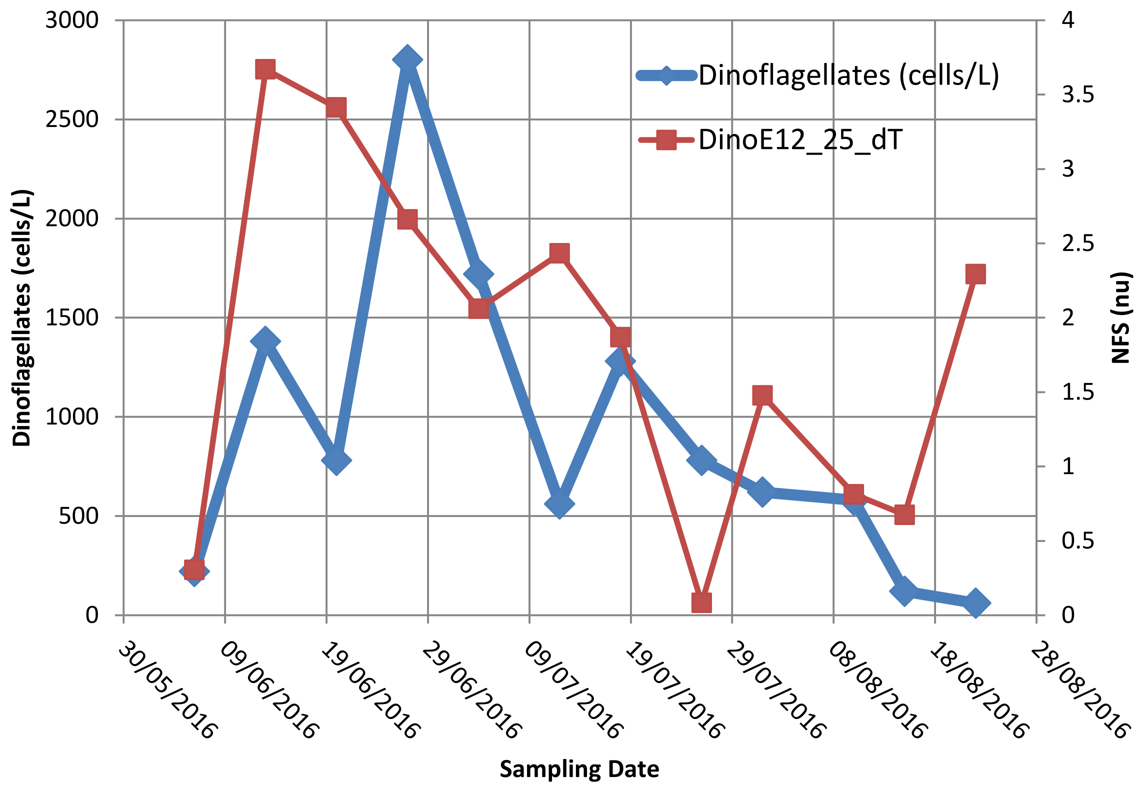


Figure 10: Dinoflagellates concentration and Normalised Fluorescence Signal of DinoE12_25_Vs Time.

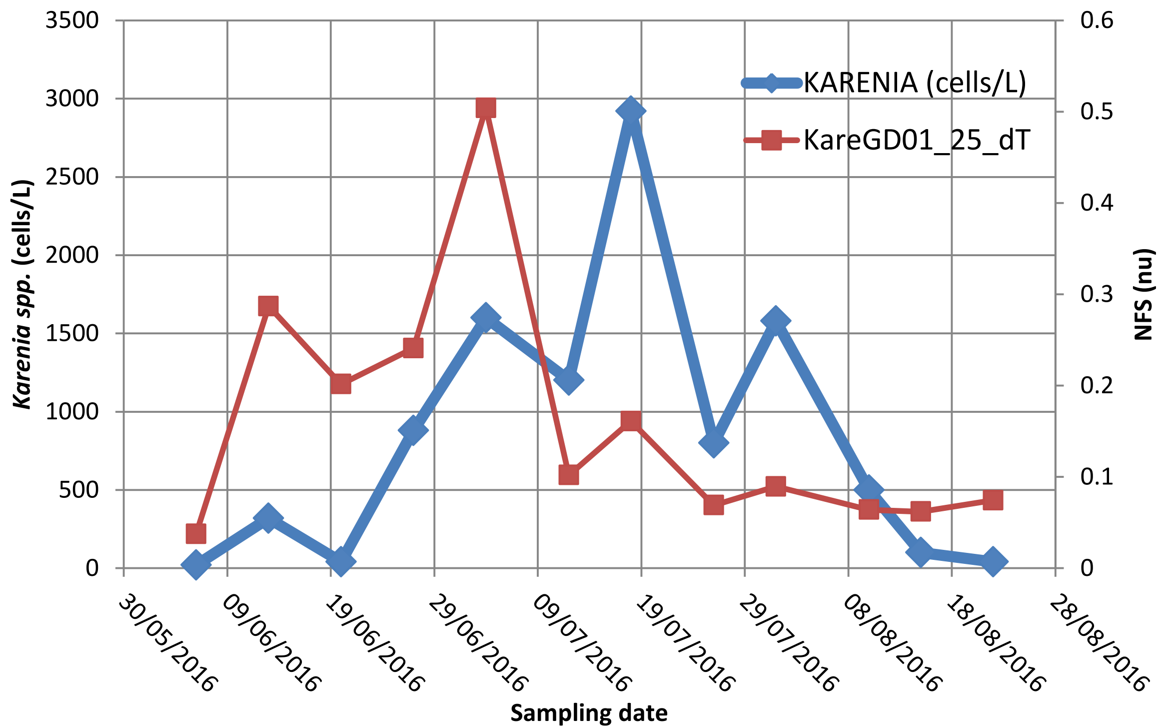


Figure 11: *Karenia spp.* concentration and Normalised Fluorescence Signal of KareDG01_25_dT Vs Time.

Within this time series, the highest microarray concentrations calculated seemed to occur before the light microscopy count (two weeks delay for Karenia spp. and Dinoflagellates, three weeks for Alexandrium spp. and four weeks for Pseudo-nitzschia spp.). This early increase of RNA could be explained because the RNA content doubled before cell division. This could lead to an increase in RNA concentration in the water column without a direct simultaneous increase of cell number measured by light microscopy. The water samples analysed by light microscopy and using the microarray technique were taken from the same 10 L carboy which eliminates the sampling step as a possible source of divergence observed in the results.

Conclusion

The Microarray technique was developed using phytoplankton cultures collected from various locations around western European coastal waters (Kegel, 2013). It is possible that different strains of phytoplankton occur along the Scottish coast making the microarray assay less specific and sensitive. The RNA sequence selected for designing the probes printed on the microarray might not match any sequence present in the phytoplankton sampled in Scotland. Previous studies found strong positive correlation between microarray and light microscopy cell count (Taylor, 2013) but this was not replicated in this study.

Some toxin-producing phytoplankton such as *Azadinium spp.* cannot be easily identified using light microscopy due to their small size while molecular probes targeting this genus are still under development (Touzet, 2010; Smith, 2016). The microarray assay used in this project contained four different probes targeting several *Azadinium spp.* Variation in the normalised fluorescence signal was observed in the time-series studies (data not shown). This suggests a weekly change in the abundance of *Azadinium spp*. cells in the water column.

The range of information provided by the microarray technique is very promising, but failed to provide strong positive correlation with the light microscopy results available. However, better correlations have been observed at locations studied in the MIDTAL project (Orkney, Taylor, 2013 and France, Kegel, 2013). MICROBIA Environnement is still working on the technique to improve its general outcome. MSS is in regular contacts with MICROBIA in a shared effort to progress this technique. Scottish phytoplankton cultures have been shared to allow MICROBIA to develop new RNA probes better suited to recognise and semi-quantify the strains that can be found in Scottish waters. New calibration curves and software are being developed to improve the technique and the user’s interface while processing the

data. Molecular genetics methods could be the way forward to better identify the toxin producing phytoplankton community.

Acknowledgements

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