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Field testing for toxic algae with a microarray: initial results from the MIDTAL project

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Abstract
One of the key tasks in the project MIDTAL (MIcroarrays for the Detection of Toxic ALgae) is to demonstrate the applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events. Water samples are collected from a series of sites used in national phytoplankton and biotoxin monitoring programmes across Europe. The samples are filtered; the rRNA is extracted, labelled with a fluorescent dye and applied to a microarray chip. The signal intensity from >120 probes previously spotted on the chip is measured and analysed. Preliminary results comparing microarray signal intensities with actual field counts are presented.

Introduction

Blooms of toxic or harmful microalgae referred to as harmful algal blooms (HABs), represent a significant threat to fisheries resources and human health throughout the world. These phenomena manifest themselves in many ways, ranging from high phytoplankton biomass, which discolour seawater with impact on water quality to low density, yet highly toxic, populations which can contaminate shellfish (GEOHAB 2001). Since many HABs have significant economic impacts, and the danger to human health posed by the consumption of contaminated seafood, monitoring programmes which measure toxins that have accumulated in shellfish flesh has become a necessity. In Europe, this requirement for monitoring is established in a series of directives in which monitoring of coastal waters for potentially harmful phytoplankton is also mandatory. Traditionally phytoplankton monitoring (identification and enumeration) is carried out using light microscopy. It has been recognised for some time that this technique requires a high degree of skill of operator, and is time-consuming. Furthermore, the morphological similarity between different species within or even across phytoplankton genera has meant that light microscopy alone is at times insufficient to assess the potential toxicity of a water sample. A variety of methods based on the sequencing of nucleic acids have been developed over the past decade or so which have considerably improved our ability to accurately identify organisms to the species level. These have been outlined recently in a new manual for phytoplankton analysis (Karlson *et al*. 2010). Microarrays are the state of the art technology in molecular biology for the processing of bulk samples for the detection of target RNA/DNA sequences. In the project MIDTAL, existing rRNA (18S, 28S) probes and antibodies for toxic algal species and their toxins have been adapted for use in a microarray format. This paper presents the first field trial results from the programme.

Materials and Methods

Water samples are taken and a measured volume is filtered through nitrocellulose filters (pore size 1-3 µm). The volume of sample filtered depends on the turbidity of the water: 0.5-2 l is usually filtered up to a point when the filter starts to clog. The filter is then

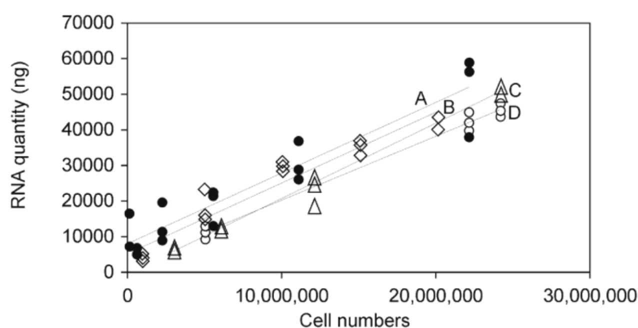

Figure 1. Comparison of RNA extraction efficiencies carried out on cultures of *Dunaliella tertiolecta* between four MIDTAL partners A (R^2 = 0.8836), B (R^2 = 0.9243), C (R^2 = 0.9848) and D (R^2 = 0.9912).

immediately submersed in 1 ml of Tri-Reagent (Ambion, UK) and an aliquot of *Dunaliella tertiolecta* (5 x 10^6 cells) is added as an internal control for the RNA extraction process. The material is then stored at -80 °C. RNA extraction is carried out through cell lysis, sequential extraction with 1-Bromo-3-chloro-propane (BCP) and isopropanol, followed by an ethanol wash. After the final centrifugation step, the pellet is suspended in RNase free water and stored at -80 °C. The RNA is then labelled using a Platinum Bright 647 Infrared Nucleic Acid kit, fragmented and hybridised to a pre-activated epoxysilane-coated microarray chip at a temperature of 65 °C. Unlabelled RNA is removed from the chip surface using 3 washing steps, with different stringency involving EDTA, thereby minimising background noise. The chip, pre-spotted with over 120 oligonucleotide probes corresponding to a taxonomic hierarchy (kingdom, class, genus and species) for harmful algal species, is scanned (Genepix 4000B Axon Inc.) and the fluorescence signal intensity from each probe is measured. Results are then compared with microscopic examination of the original water sample. This ongoing process will be carried out over two years. Preliminary results comparing microarray signal intensities with actual field counts are presented.

Results and Discussion

RNA extraction efficiency. Good yields of high quality RNA were extracted from *D. tertiolecta* cells when a preliminary standard curve was made (Fig. 1). The relationship between cell numbers and RNA content was linear with a satisfactory coefficient of determination obtained from four randomly selected project partners.

*Sensitivity of the hybridisations.*The sensitivity of hybridisations onto the microarray were investigated by testing a range of probes which should be highlighted by a particular organism growing under different environmental conditions. Figure 2 shows results from probes for prymnesiophytes, which were tested on a culture of *Prymnesium parvum.* These probes were adapted for the microarray from those published by Lange *et al*. 1996; Simon *et al*. 1997; 2000; Töbe *et al*. 2006; Eller *et al*. 2007. A NanoDrop Spectrophotometer was used to quantify the RNA after the labelling and RNA clean-up steps to determine the exact labelled RNA amount when approximately 1 ng, 5 ng, 25 ng and 100 ng were hybridised to the chip. A pre-selected signal: noise ratio threshold level was applied so that the limit of quantification was represented by a signal of 2. Thus if the optimum probe for prymnesiophytes (PrymS02_25; Lange *et al*. 1996) is applied, then the microarray can not accurately detect RNA amounts below 5 ng (Fig. 2a). Example of image intensities is also shown in Figure 2b.

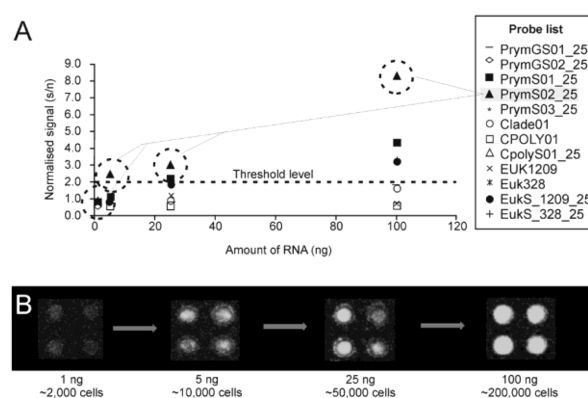

Figure 2. Standardisation of the *Prymnesium parvum* signal. (A) Calibration curve of RNA (1ng, 5ng, 25ng and 100ng) against signal intensity for a range of probes. (B) Images of the optimum probe PrymS02_25 when increasing amounts of RNA are hybridised to the microarray. Increasing signal intensity represents increasing cell numbers.

Development of microarray chip. A first chip designed for a specified range of HAB species produced weak signals for several species-probe combinations. A second generation chip was subsequently designed in which the probes were increased in length to 25 base pairs. This meant that a higher melting point temperature was required and thus the hybridisation temperature was increased from 58 to 65 °C. This temperature was adopted as standard between all project partners. Hybridisation temperature will be further optimised for the next generation of chip.

Light microscopy and microarray field results. Examples of microarray results are shown in Figures 3 and 4. Figure 3 compares data obtained from the 1st

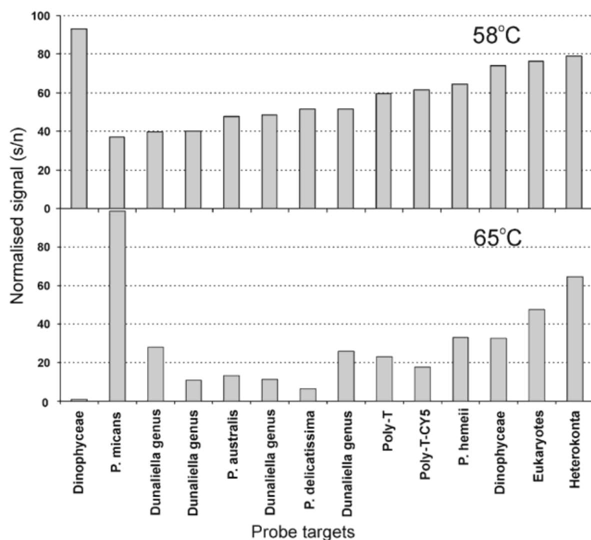

Figure 3. Microarray results of 1st and 2nd generation chips both hybridised with the same Bell Harbour field extract (25/08/09) at a temperature of 58 °C and 65 °C respectively. Note the difference between the Pmica02 and PmicaD02_25 probe species specific for *P. micans*.

and 2nd generation chips. The sample was taken in Bell Harbour on the west coast of Ireland in 2009, when a bloom of *Prorocentrum micans* was occurring at the time. Examination under the light microscope showed this was the dominant species with cell density of 360,000 cells l^{-1}. The *P. micans* probe used on the 2nd generation chip (PmicaD02_25 (98.53 s/n ratio); L.K. Medlin unpubl.) gave a vastly stronger signal to its complement (Pmica02 (37.15 s/n ratio)) on the 1st generation microarray, which was seven base pairs shorter. A general agreement between microarray signal results and cell counts was obtained. There is also an elevated signal from the class level probe for Dinoflagellates. The strongest signals in Fig. 3 signify eukaryotes, heterokonts, dinoflagellates, as well as the chlorophyte *Dunaliella* and Poly-T-CY5 which were used as controls. Cross-reactivity with *Pseudo-nitzschia heimii* will need to be addressed on the 3rd generation chip because it reacts with many target species. A second comparison between light microscopy counts and a selection of 2nd generation microarray results from a sample obtained from Killala Bay in August 2009 is shown in Fig. 4. An assemblage of *Pseudo-nitzschia. seriata* group organisms numerically dominated the sample (112,000 cells l^{-1}) (Fig. 4a). The microarray data could identify these as *P. fraudulenta, P. seriata, australis,* and *P. multiseries.* A variety of *Alexandrium* probe signals were also evident, which could not be resolved by light microscopy (Fig. 4b). Electron microscopy would need to be used to confirm the species.

Conclusions.

The aim of MIDTAL is to provide a new method to support toxic algal monitoring and reduce the need for the mouse bioassay. Demonstration of its capabilities is the first step towards this goal. These first field results indicate that there remains further development work to be done but point towards the potential successful development of a 'universal' HAB microarray.

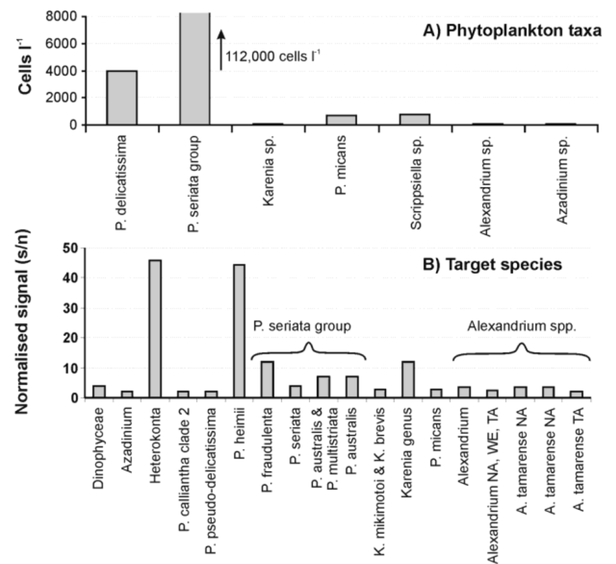

Figure 4. (A) Cell counts and (B) 2nd generation microarray chip hybridised with RNA at a temperature of 65 °C from a Killala Bay field extract taken on 15 August 2009.

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