Fibre optic microarrays for the detection and enumeration of harmful algal bloom species

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Harmful algal blooms (HABs) are a serious threat to coastal resources, causing impacts ranging from the contamination of seafood products with potent toxins to mortalities of wild and farmed fish and other marine animals. As the threat from HABs has expanded, new approaches have become necessary, including monitoring the plankton for HAB species. Preliminary data are presented on an innovative approach to HAB cell enumeration — fibre optic genosensors. Oligonucleotide probes specific for ribosomal RNA (rRNA) of target HAB species are coupled to microspheres (~3µm) placed in wells etched in the ends of optical fibre bundles of 6 000 individual fibres. This bead-based microarray is dipped into a plankton or culture sample that has been lysed to release nucleic acids. The target rRNA is 'captured' on the functionalised beads using a probe complementary to a different region of the molecule. Probe-target hybrids are detected using a second 'signal' probe and visualised via the fibre using epifluorescence and image analysis. Preliminary results show the method to be highly sensitive, with detection limits of approximately 5–10 cells sample^{-1} for *Alexandrium fundyense* and *Pseudo-nitzschia australis*, and about 50 cells sample^{-1} for *A. ostenfeldii*. A multiplexed microarray has also been developed to detect these species simultaneously. In theory, hundreds of species can be detected using a single optical fibre array. The simplicity and the ability to re-use the sensor array without loss of sensitivity makes this a promising procedure for further development, including deployment on moored instruments capable of detecting HAB species in an early warning system.

Keywords: *Alexandrium fundyense, Alexandrium ostenfeldii*, array, HAB, *Pseudo-nitzschia*, rRNA, sandwich hybridisation, toxic algae

Introduction

As the threat of harmful algal blooms (HABs) has expanded (Hallegraeff 1993), new approaches to monitoring have become increasingly important. Traditionally, monitoring focussed on detection of algal toxins in shellfish (e.g. Shumway *et al.* 1988), but a complementary approach has emerged in recent years that involves monitoring the plankton for HAB species (reviewed by Anderson *et al.* 2001). The reasons for this are several, including the proliferation of toxic or harmful algal species and toxins, as well as an increase in the number of commercial fisheries products or resources affected. Overall, the analysis of plankton samples for HAB species has expanded dramatically in scale, often involving collection of hundreds — and sometimes thousands — of samples that must be counted every year for both research (e.g. Townsend *et al.* 2001) and monitoring purposes (e.g. Anderson *et al.* 2001). This focus on plankton analysis will undoubtedly expand in the future, given these needs and the impetus to deploy automated instruments that can detect HAB species remotely in the expanding Ocean Observing System (HABSOS 2000, Scholin *et al.* in press).

HAB monitoring programmes are increasingly reliant on plankton analysis, yet the number of samples involved and the diversity of potentially toxic or harmful algal species pose a significant logistical constraint. In recent years, the identification and enumeration of HAB species have been greatly facilitated by the development of species- or strain-specific molecular probes (Anderson 1995, Litaker and Tester 2002, Scholin *et al.* 2003). The most commonly used are short segments of synthetic DNA designed to bind to complementary sequences on target nucleic acids (i.e. RNA, DNA) in HAB species. They can be used in a variety of formats to detect and enumerate cells, and show great promise as tools that can simplify and expedite the cell counting needs of research and monitoring programmes. Here we describe an approach in which molecular probes are combined with a simple and sensitive fibre optic technology to yield a specific rapid detection and enumeration assay with components that can be re-used hundreds of times without loss of sensitivity. The simplicity of the procedure makes this a promising technology for laboratory, shipboard, or automated use including deployment on

remote moored instruments capable of detecting HAB species in an early warning system. A more detailed presentation of this technology and additional results will be provided by Ahn *et al.* (in press).

Material and Methods

Cultures

Alexandrium fundyense (clone GTCA28 from the Gulf of Maine), *Pseudo-nitzschia australis* (clone 1BA from Santa Cruz, California, USA) and *A. ostenfeldii* (clones Ht 240 D2 and Ht 120 D6 from the Gulf of Maine) were grown in f/2 modified medium under a 14:10h light:dark cycle at 15°C (*A. fundyense, P. australis*) or 10°C (*A. ostenfeldii*). At the mid-exponential phase of growth, cell concentrations were established and dilutions of the cultures were made with fresh f/2 medium to yield a range of cell densities from 5 cells ml^{-1} to 5 000 cells ml^{-1}. Once diluted, several aliquots of each culture dilution were filtered onto hydrophilic Millipore Durapore filters, which were placed into 1.2ml cryovials, flash frozen and stored in liquid nitrogen until shipment for sample analysis. After receipt of the samples, they were stored at –80°C until needed.

Sample processing

A lysate of the filtered cells was obtained by adding 0.5ml lysis buffer (Orca Research Inc., Bothell, Washington, USA) to the cryovial, which was then vortexed and heated to 85°C for 5 minutes. The lysate was filtered through a Millipore, Millex®-HV, 0.45µmol l^{-1} syringe filter, and 200µl of lysate were analysed with the optical fibre microarray as described below.

Fibre optic microarrays

Fibre optic bundles consisting of 6 000 individual optical fibres (each 3µm in diameter) were obtained from Galileo Electro-optics Corp. (Sturbridge, Massachusetts, USA). The distal end of each polished fibre bundle was chemically etched to form a microwell at the end of each of the individual optical fibres (Pantano and Walt 1996). Beads were loaded into the wells by applying a small aliquot of bead solution directly onto the distal end of the etched fibre bundle. Upon drying, the beads are held firmly within the wells allowing for each bead to be optically wired to permit individual interrogation (Michael *et al.* 1998). The fibre bundle plus beads is hereafter referred to as the fibre optic microarray.

Sandwich hybridisation

The fibre optic method used herein relies on a 'sandwich hybridisation' procedure using ribosomal RNA (rRNA)-targeted oligonucleotide probes. These probes were modified from existing probes for *A. fundyense, P. australis* and *A. ostenfeldii* (Scholin *et al.* 1996, Anderson *et al.* 2005, Ahn *et al.* in press), with detailed probe sequence information to be provided elsewhere. 'Capture' probes were coupled to microspheres embedded in the etched end of the optical fibre
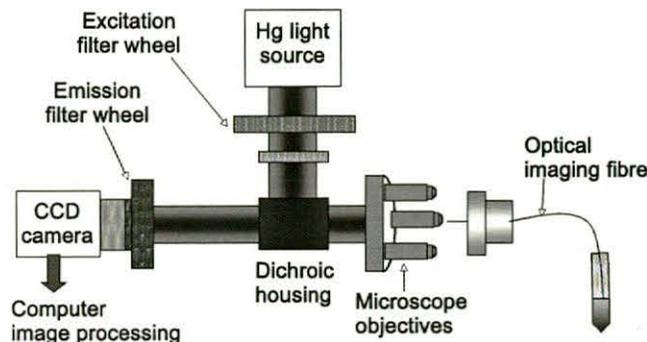

Figure 1: Schematic of the fibre optics instrumentation. Internal reflection allows both excitation and emission light to be carried in a single fibre

bundle, with each bead carrying ≥10^5 copies of the probe sequence. For multispecies detection, each probe bead type was encoded with a different concentration of fluorescent dye such that each type could be uniquely identified (Walt 2000). Individual populations of beads were then mixed together to form a stock solution that was distributed randomly over the array surface. To run the assay, the microarray is dipped into the cell lysate, allowing the rRNA molecules of the target HAB species to bind to their complementary probe sequences on the beads. A second hybridisation is then performed to allow a fluorescently labelled probe — the 'signal' probe — to hybridise to a second binding site on the HAB rRNA, providing a detectable fluorescent signal. This completes the 'sandwich' — a fragment of target rRNA sandwiched between the capture and signal probes. The fluorescence from each signal probe bound to a molecule of target rRNA is transmitted through the optical fibre and quantified. The average light intensity per bead is proportional to the number of HAB cells in the original sample. For multispecies enumeration, a fluorescent image was obtained at the excitation and emission wavelengths of the encoding dye, allowing each bead type and its position in the array to be registered. The hybridisation signal from each bead is then quantified at a single wavelength by image analysis software, and matched to the bead type from which it was derived. To obtain a background signal (i.e. no sample), the average value of the signal intensity from individual bead types (~100 beads per microarray) was calculated from each of three images. The sample hybridisation signal was then obtained with three replicate hybridisations, each producing an image of the array, and the average fluorescence calculated. Results are obtained in relative fluorescence units (RFU). Hereafter, the term 'net fluorescence signal' is used to represent the average sample reading minus background fluorescence, in RFU. After a reading, the microarray is dipped into 90% formamide heated to 85°C to remove bound rRNA, and the bundle is re-used on another sample.

Instrumentation

The microscope-based instrument used for cell enumeration is shown in Figure 1. Excitation light is focussed into the proximal end of the fibre and excites the fluorescent label on the distal end that has been exposed to the
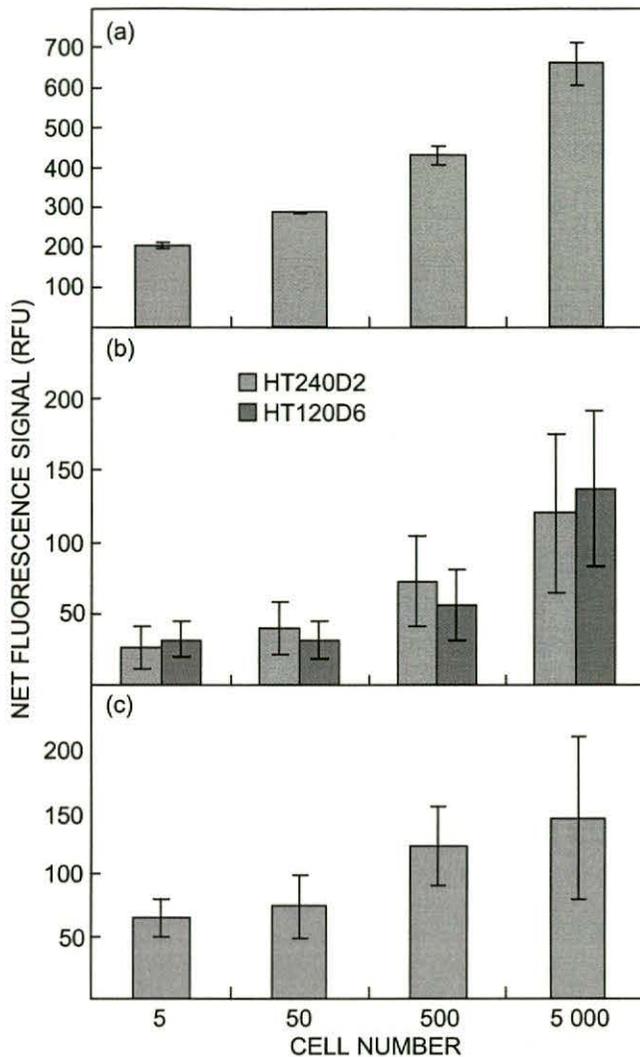
Figure 2: Net fluorescence signal vs cell number per sample for (a) *A. fundyense*, (b) *A.ostenfeldii* and (c) *P. australis*. Error bars denote ±1SD

Figure 3: Net fluorescence signals from a multiplexed array containing probes specific for *A. fundyense* (NA1S), *A. ostenfeldii* (AO) and *P. australis* (PN) treated with cell lysates from each of the three species. Species abbreviations are 'AF' for *Alexandrium fundyense*, 'PN' for *Pseudo-nitzschia australis* and 'AO' for *A. ostenfeldii*

sample. Light emitted isotropically from the fluorophore is captured by the same fibre and sent back through a dichroic filter system that separates excitation and emission light signals. A sensitive CCD camera (Orca ER, Hamamatsu Photonics, Trenton, New Jersey, USA) is used to collect spatially resolved fluorescent images returning through the fibre.

Results and Discussion

Single species analyses

Figure 2 shows the net fluorescence signal obtained from different numbers of cultured cells of *A. fundyense*, *P. australis* and *A. ostenfeldii*. A remarkably low detection limit of approximately 5–10 cells sample^{-1} has been demonstrated for *A. fundyense* and *P. australis*, whereas for *A. ostenfeldii* the limit is about 50 cells. The detection limit is defined as the number of cells for which the net fluorescence signal is greater than three times the standard
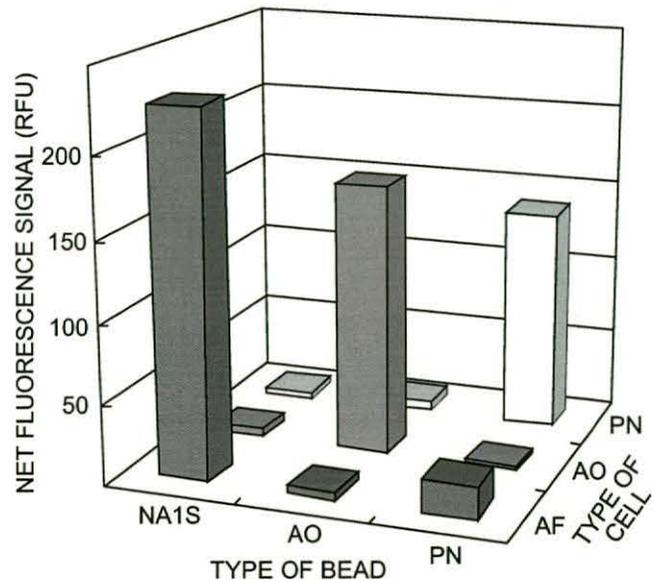
deviation of the background signal. Here, it is given as the total number of cells in the sample that were extracted, because the volume of sample collected onto a filter would vary between different methods. Importantly, signal intensity is unaffected by the number of co-occurring non-target cells and the amount of detritus in the plankton sample, even without RNA purification (data not shown). Whereas the *A. fundyense* probes demonstrate both excellent sensitivity and precision, the *A. ostenfeldii* and *P. australis* probes do not perform as well. These latter two sensors are newer than the *A. fundyense* arrays, and we are currently refining the probe sequences as well as the reaction conditions in order to improve sensitivity and reduce the measurement error.

Multispecies analyses

Figure 3 shows the results from analyses using a single fibre optic microarray containing three types of beads functionalised with probes for three target HAB species — *A. fundyense*, *P. australis* and *A. ostenfeldii*. In this instance, single cultures of each species were lysed and analysed separately, but the same microarray was used for all measurements. It is evident that a single 'multiplexed' array can readily distinguish among the three species. Studies are now ongoing to make these same measurements in a mixed plankton sample.

General considerations

Images cannot be carried over single optical fibres because the light signals mix and spatial resolution is not preserved. To circumvent this problem, imaging optical fibre bundles have been created containing an array of thousands of

densely packed individual optical fibres fused into a coherent unitary bundle. Each fibre carries its own light signal; consequently such arrays can be used to build up images using a pixel-by-pixel image reconstruction.

The technology described here takes advantage of the multiplicity of individual fibres in an imaging array by placing microspheres at the end of each optical fibre and coupling these to oligonucleotide probes that capture the rRNA of HAB species. As demonstrated, sandwich hybridisation assay coupled to the imaging fibre optic array technology can be used to enumerate HAB cells at very low cell densities. An important aspect of this detection method is the large number of fibres and beads that can be used for each species. Redundancy provides two primary advantages (Walt 2000). Firstly, 'voting' schemes can be employed so that both false positives and false negatives are virtually eliminated. Secondly, redundancy provides enhanced sensitivity. Signal-to-noise ratios scale as the square root of the number of identical sensing elements. Consequently, enhanced sensitivity can be achieved by summing the signals from numerous identical microspheres in the array.

There are a number of benefits of the fibre optic technology compared with other probe-based assays. First, ongoing experiments suggest that this method is not significantly affected by the matrix of nucleic acids from co-occurring plankton and detritus, as has been a problem with some probe-based technologies, particularly when the ratio of detritus to HAB target cells is high (Anderson 1995, Scholin and Anderson 1998). This is an advantage that derives from the sandwich hybridisation assay approach in which target rRNA is captured and remaining material in the sample discarded. Second, by multiplexing an array so that it contains different types of probes, simultaneous detection of multiple HAB species is possible. Microspheres can be labelled with optically distinct dyes, and each dye can be used at several different concentrations. Hence, it is theoretically possible to detect hundreds of species on a single multiplexed array consisting of 50 000 individual fibres. More work is clearly needed to demonstrate adequate resolution among species using multiplexed arrays on mixed plankton samples, but the results presented here are highly encouraging in this regard.

Optical sensor arrays provide a high degree of flexibility with respect to operational issues in the laboratory or in the field. A simple wash in formamide accomplishes dehybridisation, allowing the array to be immediately re-used. Individual fibre arrays have been used for over 200 hybridisation/dehybridisation cycles with <2% degradation (Ferguson et al. 2000, Walt 2000). This confers an extraordinary advantage to this method, as other approaches require new probes or reagents for each analysis. The technology is capable of discriminating single-base mismatches in target sequences, by adjusting stringency either with temperature or solvent. Furthermore, the incremental cost of adding new detection targets is negligible with respect to both materials and time; as new sequences of interest are identified, additional microspheres with probes for new target species are simply added to the sensor library. This re-usability and flexibility offer a significant advantage to the individual researcher or monitoring

programme, and are of particular relevance for remote automated applications. It is also notable that purification requirements (i.e. the need for column-purified RNA) presently constrain several other HAB cell assay methods, limiting their use in the field, especially in the automated moored instruments that many envision as components of the future US Ocean Observing System. The technique described here utilises a crude cell lysate that can readily be accommodated in automated systems.

In summary, a new approach to molecular probe-based detection of HAB cells is described that relies on sandwich hybridisation technology coupled to a fibre optic microarray. More details of this approach and its application to multiple HAB species in cultures and simulated field samples are currently under investigation (Ahn et al. in press).

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