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INITIAL ASSESSMENT OF TRIPLEX PCR ASSAY APPLICATION FOR DETECTION OF TOXIC DINOFLAGELLATES, *ALEXANDRIUM* SPECIES (DINOPHYCEAE), IN ENVIRONMENTAL SAMPLES

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Condensed Title:

Triplex PCR detection of *Alexandrium*

Abstract

The frequency and geographical range of harmful algal blooms (HABs) are believed to be on the increase, with adverse affects on marine and human health making the implementation of stringent controls governing monitoring programmes commonplace. The South African monitoring programme was established in 1989 and relies upon microscopic identification of HAB species. Microscopic identification is labour-intensive, requiring a high level of taxonomic expertise, and could be considered impractical for routine monitoring where analysis of large numbers of samples is required. Novel monitoring techniques, focusing mainly on probe technology, are being developed for rapid, unequivocal identification and enumeration of HAB species. In this study, a triplex PCR assay, incorporating a genus-specific ribosomal DNA primer designed from phylogenetic studies on local *Alexandrium* populations, was optimised for application to environmental samples and tested against natural assemblages containing *Alexandrium minutum*. Specific positive results were consistently generated for samples containing *A. minutum*. Samples absent of *A. minutum* cells did not generate the *Alexandrium*-specific amplicon. The absolute detection limit of 440 *A. minutum* cells l^{-1} for this assay was established. Effects of non-target cells on the sensitivity of the assay were also investigated: although a decrease in sensitivity was found, *A. minutum* cells could still be detected in the presence of 100 times more non-target cells. This assay has been shown to be a useful tool for unequivocal identification of *A. minutum* cells within local environmental samples.

Introduction

Harmful algal blooms (HABs) can cause severe economic losses within local fisheries, aquaculture, tourism and recreational industries, as well as negatively impacting human and other consumers' health (Anderson *et al.*, 2000; van den Bergh *et al.*, 2002; Shumway *et al.*, 2003). The frequency and geographical range of HABs is believed to be increasing (Anderson, 1994; Hallegraeff, 1995), and the majority of countries regularly encountering these blooms have implemented some form of monitoring programme (Anderson *et al.*, 2001). Fundamentally, these programmes are used as an early warning device and information source to local marine industries for deployment of mitigating procedures. Countries exporting seafood are required, by increasingly more strict import controls, to conduct extensive monitoring, thereby preventing toxic seafood entering international trade (Fernández, 2000). To this end, the number of studies conducted on HABs has increased rapidly over the last decade (Hallegraeff, 1995), with the development of novel techniques for the advancement of these monitoring programmes (Scholin and Anderson, 1998).

Traditionally, identification of algal bloom species relies on microscopy. However, this may not be the most effective technique: phenotypic plasticity may occur within individual species, where expression of phenotypic characteristics can vary in response to environmental conditions as well as growth stages (Adachi *et al.*, 1996), and closely related species can be morphologically indistinguishable, for example the *Alexandrium tamarense* 'species complex' (Dinophyceae) (Scholin *et al.*, 1995; Daugbjerg *et al.*, 2000; Hansen *et al.*, 2000). Not surprisingly, to avoid taxonomic ambiguities and thereby produce reliable data, microscopic identification has become labour intensive, requiring a high level of expertise. For monitoring programmes,

microscopy could be considered impractical (Anderson *et al.*, 1999). Large numbers of samples are collected regularly, for analysis in as near real-time as possible in order to provide up-to-the-minute information to local industries (Tyrrell *et al.*, 2002). Until recently, routine monitoring worldwide has been severely hampered by the lack of simple, sensitive, rapid diagnostic tests for unequivocal identification of HAB species (Scholin and Anderson, 1998).

Molecular and immunological techniques have allowed for an alternative approach to HAB species analysis. Even very low numbers of HAB organisms can, theoretically, be detected within a field sample of mixed background (Scholin and Anderson, 1998). Development of these techniques has primarily focused on whole cell and cell-free probe technology with the objective of constructing automated systems for cell identification and enumeration, with possible remote sensing applications (Environmental Sample Processor, Tyrrell *et al.*, 2002). Antibody, nucleic acid and lectin probes have been designed, mainly for implementation in fluorescent hybridisation procedures. There has been much debate as to the potential of the different types of probes (Costas *et al.*, 1995; Adachi *et al.*, 1996; Scholin *et al.*, 1996; Scholin *et al.*, 1997; Anderson *et al.*, 1999). However, for all documented probe techniques, no one procedure has been shown to be better than the rest and the technique of choice would ultimately depend on the needs and capabilities of the end-user (Scholin *et al.*, 1996).

While the development of probes has been relatively rapid, it is only in recent years that work has been conducted to explore probe application methodology for routine analyses of field samples (Vrieling *et al.*, 1994; Miller and Scholin, 1998; Tyrrell *et*

al., 2002; John *et al.*, 2003). When advancing from laboratory to natural conditions, a regional approach for the application of these techniques needs to be conducted for "fine-tuning" of the procedures - optimising for local conditions and local populations of HAB species (Scholin *et al.*, 1995; Scholin and Anderson, 1998). For some regions of the world, Scholin and Anderson (1998) remark, considerable work remains on a molecular level to characterise local species where rapid identification is desired.

HABs are common within the Benguela upwelling system and are mainly attributed to dinoflagellates (Dinophyceae) (Pitcher and Calder, 2000). Following massive marine mortalities and a number of food poisoning cases in the 1970's, Marine and Coastal Management (MCM), Department of Environmental Affairs and Tourism, South Africa, implemented a monitoring programme within the southern Benguela (Pitcher and Calder, 2000). This programme, established in 1989, has remained simple, still relying on morphological identification. Since its inception, it has identified several toxic and cryptic dinoflagellate taxa, including *Alexandrium* and *Dinophysis* species - locally responsible for paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP) respectively (Pitcher and Calder, 2000). Although these species are frequently found within South African waters, poisoning events are actually rare events (Popkiss *et al.*, 1979; Pitcher *et al.*, 1993).

Targeting local species at the genus- and species-level, a triplex polymerase chain reaction (PCR) assay has been designed by Ruiz Sebastián (2002) in order to aid rapid identification of Southern African HAB species. Through a regional molecular assessment of HAB species, Ruiz Sebastián has genetically characterised several local species and analysed their phylogenetic relationships with conspecifics (Ruiz

Sebastián, unpublished data). Subsequently, specific ribosomal DNA (rDNA) signature sequences for these species were identified, based on which taxon-specific PCR primers were designed to be used in a molecular diagnostic assay. Application of multiplex assays are showing many benefits within both clinical and research laboratories as rapid and convenient screening assays (Henegariu *et al.*, 1997) and a triplex PCR assay has previously been successful in identifying marine algae (Raphidophyceae) in cultured and fixed field samples (Connell, 2002). Theoretically, triplex PCR assays have the benefit of an internal control; in the presence of specific target DNA, both a species-specific amplicon and a generic amplicon will be generated, thereby controlling for false results. Ruiz Sebastián has designed forward primers for the *Alexandrium* and *Dinophysis* genera, as well as species-specific primers for several other local species: *Protoceratium reticulatum* (Claparède et Lachmann) Bütschli, *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup and *Karlodinium micrum* (Leadbeater et Dodge) J. Larsen. These primers, through application of this triplex PCR assay with generic forward and reverse dinoflagellate primers (Hansen *et al.*, 2000), have already completed successful trials under laboratory conditions using cultured samples, and are now at the stage to be applied to field samples (Ruiz Sebastián, unpublished data).

The capabilities of the triplex PCR assay to detect the presence of target HAB species in fresh environmental HAB samples was assessed in this study. The aims were four-fold: (1) To test the *Alexandrium* primers under environmental conditions; (2) To "fine-tune" the triplex PCR assay for optimal reaction conditions; (3) To determine the sensitivity levels of the assay; (4) To compare the application and sensitivity with a duplex PCR assay – a two-primer PCR assay generating the *Alexandrium*-specific

amplicon only. The robustness and sensitivity of the *Alexandrium* primers and the triplex PCR assay were critically assessed, with regard to future research into the development of a quantitative molecular assay for potential implementation in the field.

Material and Methods

Sampling sites and method Surface seawater samples, of approximately 500 ml, were collected weekly from the marina at the Victoria and Alfred (V&A) Waterfront, Table Bay (Figure 1), from 21st November 2003 to 22nd January 2004, following the appearance of a dense algal bloom in that area. All samples were collected at the same location within the marina. Additional surface seawater samples (50 ml) were collected on the 25th January 2004 at ten locations along the southwestern Cape coast (Figure 1). All field samples were collected between 12:00 and 17:00 h. Eight 1 ml aliquots per sample were frozen for archival purposes.

Microscopic identification and enumeration methods For all samples, dominant phytoplankton species were identified to genus-level using a light microscope (Zeiss Inc., Oberkochen, Germany). Estimations of cell numbers were made using a Neubauer® improved bright-line haemocytometer (Throndsen, 1995). Ten counts of the Neubauer® double-chamber were made for each sample and averaged to estimate cell numbers. Estimates for dominant species and total algal cell numbers were determined.

Extraction and quantification of genomic DNA For each V&A sample collected, eight 1 ml aliquots were harvested by centrifugation at 15 000 x *g* for 5 min. With the

regional samples, for each location a single 1 ml aliquot was harvested by the same procedure. From these aliquots, 500 µl of the supernatant was removed and replaced with an equal volume of lysis buffer (50 mM Tris, pH 8.0; 0.75 M Sucrose; 40 mM EDTA) and vortexed to resuspend the algal cell pellets. Lysosyme was added to a final concentration of 1 mg ml^{-1}, and the aliquots were incubated at 37ºC for 45 min. After incubation, sodium dodecyl sulphate (SDS, final concentration 1%) and proteinase K (Boehringer Mannheim GmbH, final concentration 0.2 mg ml^{-1}) were added. The aliquots were further incubated at 55ºC for 1 h and then split approximately equally into two 1.5 ml eppendorf tubes. An equal volume of 25: 24: 1 phenol: chloroform: isoamyl alcohol was added to each, shaken and then centrifuged at 15 000 x *g* for 20 min. The supernatants were transferred to new tubes and the phenol: chloroform: isoamyl alcohol step, including shaking the tubes and centrifugation, was repeated. The supernatants were again transferred to new tubes and an equal volume of 24: 1 chloroform: isoamyl alcohol was added, shaken and centrifuged at 15 000 x *g* for 5 min. After the supernatants were further transferred to new tubes, 3M sodium acetate (10% v/v) and 1 ml of ice-cold 100% ethanol was added to each. The tubes were then placed in either a -20ºC freezer overnight or in a -70ºC freezer for 1 h. Nucleic acids were subsequently pelleted by centrifugation at 15 000 x *g* for 20 min, washed with 70% ethanol, and further centrifuged for 5 min at 15 000 x *g*. The DNA pellets were dried on a hot-block, at 70ºC for approximately 10 min, and each was resuspended in 20 µl of sterile Milli-Q (Millipore, Bedford, MA, USA) distilled water (dH_2O). Extracted DNA concentrations were spectrophotometrically determined using a GeneQuant RNA/DNA Calculator™ (Biochrom Ltd, Cambridge, UK).

Triplex PCR assay Following optimisation for application to environmental samples, PCR reagent concentrations for amplification reactions were established. PCR reactions were carried out in 25 µl volumes containing 4mM $MgCl_2$, 1x PCR Buffer (16mM $(NH_4)_2SO_4$; 67mM Tris-HCl pH 8.8; 0.01% Tween-20; Bioline), 200µM of each dNTP, 500µM of the generic dinoflagellate forward primer (D1R), 2mM each for the generic dinoflagellate reverse primer (D3B) and the *Alexandrium* forward primer (ALEX533), and 1 unit of *Taq* polymerase (Bioline). Undiluted DNA extract (2 µl) was used as template. A Hybaid PCR Sprint temperature cycling system (Teddington, Middlesex, U.K.) was used to run the reactions with the following thermal profile: denaturation at 94°C for 3 min, 35 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s) and extension (72°C for 1 min 30 s), completed with a final extension at 72°C for 7 min. Standard laboratory procedures for preventing contamination were followed, including positive and negative controls in all PCR reactions. An aliquot of 12 µl of PCR-generated product was visualised on a 1% agarose gel with ethidium bromide (final concentration 0.5 µg ml^{-1}) under ultraviolet light. Duplex PCR assay conditions were the same as for the triplex PCR assay, but omitting the D1R primer from the amplification reactions.

Sensitivity analysis The absolute sensitivity of the triplex PCR assay was defined as the minimum number of target cells detected in a mono-specific sample. The relative sensitivity of the assay was defined as the minimum target cells: non-target cells ratio detectable within a mixed assemblage. Absolute and relative detectable limits were determined by testing the assay with a set of serial dilutions of an *Alexandrium* culture of known concentration. *Alexandrium* cultures (*A. minutum*; Bigelow laboratory, reference 532316), kindly provided by MCM, were used as reference, with the sample

collected at Strandfontein used as the non-target assemblage for relative sensitivity analysis. The application of the duplex PCR assay was also conducted, in both instances, for comparative sensitivity.

Results

The algal bloom located at the V&A Waterfront remained relatively stable throughout this study; although migratory vertically, the bloom stayed confined to the marina. Due to its vertical migration, the ten V&A samples were collected within afternoon hours, when the bloom was closest to the surface. Except for the final sample collected, *Alexandrium* cells were microscopically identified and enumerated within all V&A samples, cell concentrations ranging from 1.0×10^5 to 1.95×10^7 cells l^{-1} (Table I). With very few background cells enumerated, *Alexandrium* was the dominant genus for the first seven weeks of this investigation. In the latter samples, diatoms became dominant. The *Alexandrium* species has been identified as *A. minutum* Halim (Ruiz Sebastián, unpublished data), a species not previously documented in South African waters.

A wide range of cell concentrations, up to 2.12×10^7 cells l^{-1}, were microscopically enumerated for the ten regional samples collected, five along the West coast and five within the False Bay/South coast area (Table I). The greatest cell count was calculated for the Strandfontein sample where a brown water discolouration was observed, identified as a diatom bloom. For locations where the highest cell concentrations were observed, diatoms were the dominant species, although relatively high concentrations of unidentified dinoflagellates were also found, 2.20×10^6 cells l^{-1} within the Strandfontein sample.

PCR amplifications for DNA extracted from samples collected from the 18th December 2003 onwards repeatedly failed. It was hypothesised that this variability in success was due to an environmental inhibitor, carried through the extraction process, causing inhibition within PCR reaction conditions. This was tested by mixing failed extract with extra positive controls, and subsequently confirmed by the production of a weaker amplicon. The DNA extraction protocol was modified, with additional washes with 70% ethanol, thereby diluting the inhibitor to a negligible concentration, to tackle this problem. An additional three washes was found to produce successful PCR amplifications. All samples collected from the 18th December 2003 were subjected to this modified extraction protocol.

Quantification of the extracted DNA revealed that DNA concentrations did not relate to cell concentrations microscopically enumerated within the respective samples (Table I). The sample collected on the 2nd January 2004 had the highest concentration of cells collected for all V&A samples (1.95×10^7 *A. minutum* cells l^{-1}, plus 1.40×10^6 background cells l^{-1}) yet the extraction produced the second lowest DNA concentration for this set of samples. The highest concentration of extracted DNA for any V&A sample was quantified for the sample collected on the 18th December 2003 (4.10×10^6 *A. minutum* cells l^{-1}; 4.00×10^5 background cells l^{-1}).

With the initial conditions of the triplex PCR optimised for laboratory testing, optimisation for environmental application was empirically established. This was conducted progressively; determining optimal conditions for the most influential parameters first, annealing temperature and magnesium concentration, followed by "fine-tuning" template and primer concentrations. The targeted generic and

Alexandrium-specific fragments were approximately 1 400 base pairs (bp) and 900 bp in length, respectively. At annealing temperatures of 60ºC and 65ºC, priming was inhibited and both the generic and specific amplicons were either very weak or absent. Stronger amplicons were observed at lower annealing temperatures (50ºC and 55ºC) although false priming was encouraged at the lower of the two. As with annealing temperature, a range of amplicon strength was observed with increasing magnesium concentration. A low concentration of magnesium chloride (2mM) resulted in weak amplification – insufficient magnesium resulting in primers not able to bind to template DNA. A concentration of 4mM magnesium chloride was sufficient to produce clean, strong amplicons. Following optimal laboratory conditions, the concentration of template DNA added to the amplification reactions produced amplification product that was not always seen or was relatively weak; doubling the concentration of template DNA produced strong amplicons that could be replicated. With application of the initial concentrations, the generic D1R primer was seen to be stronger at priming with template DNA than could the specific ALEX533 primer; the resultant generic amplicon was strong compared to a very weak specific amplicon. Proportions of primer concentrations were modified to encourage equal signal strength in both the generic and specific amplicons: D1R concentration decreased and ALEX533 concentration increased. The generic D3B primer concentration was also increased for sufficient amplification of the targeted nucleotide fragment.

The optimised triplex PCR assay was tested on all samples collected, for molecular determination of the presence of *Alexandrium*. In addition, a duplex PCR assay was conducted in order to assess ALEX533 primer application, in terms of robustness and sensitivity, within a simpler assay compared to the triplex PCR assay. All V&A

samples tested positive for *Alexandrium* using the triplex PCR assay, unlike detection by microscopy (Figure 2a). From the results generated, there are few discrepancies between triplex and duplex application (Figure 2a & 2b); replicates of each amplification reaction would be advised for confirmation of results for standard operating procedures.

Under triplex conditions, the DNA extracted from the V&A sample collected on the 4th December 2003, generated a single *Alexandrium*-specific amplicon only: a false positive (Figure 2a, lane 3). In fact, this result is most likely to be a true positive. Probe failure was not observed for any other amplification reaction, demonstrating its unlikelihood to be the cause for the generic amplicon not generated for this sample. Also, the size of the amplicon is correct for amplification of the targeted fragment (900 bp). Apart from relatively low numbers of *A. minutum* (2.0 x 10^5 cells l^{-1}), no other dinoflagellates were identified microscopically within this sample. Under optimal triplex conditions, if the *A. minutum* cells are the only dinoflagellates in the sample and present in low numbers, the specific ALEX533 primer concentration could very well have saturated the extracted dinoflagellate DNA, out-competing the generic D1R primer, with the generation of the specific amplicon only. For the sample collected on the 15th January 2004, a very weak specific amplicon was produced in the triplex assay, which was not replicated under duplex conditions (Figure 2a & 2b, lane 9). Although detection of lower concentrations of *A. minutum* has been successful (samples collected on 4th December 2003, Figure 2a, lane 3, and 9th January 2004, lane 8), the background cell count in this sample is relatively high (8.0 x 10^5 cells l^{-1}) for this bloom and the apparent discrepancy may be a sensitivity issue:

the proportion of *A. minutum* cells to other background cells within this sample could be at the limits of primer detection.

A. minutum was not detected microscopically within the last V&A sample collected. Application of the triplex PCR assay, however, produced positive results (Figure 2a, lane 10). The possibility of this result being a false positive is small: application of the ALEX533 primer, within the triplex PCR assay, to a range of environmental samples has produced specific results consistently. For this sample, the result produced for the triplex PCR assay is replicated in the duplex PCR assay (Figure 2b, lane 10), thereby demonstrating greater confidence in this positive result.

All amplifications for V&A samples showed great specificity. Amplicons of equal size to the targeted fragments were generated only; no extra amplification product resulting from cross-reactions of the generic primers and the ALEX533 primer with non-target-species template was generated. For many of the samples, more than one generic amplicon, signalling detection of different dinoflagellate species, were generated. Primer specificity was further confirmed with application to extractions from the regional samples (Figure 3). Clean generic amplicons were generated only; *Alexandrium* was not detected in any of the regional samples, in accordance with microscopic detection.

The *A. minutum* culture was microscopically enumerated at the time of harvest for sensitivity analyses; a total *A. minutum* concentration of approximately 4.40 x 10^7 cells l^{-1} was calculated. For absolute sensitivity analysis, weak amplicons were visualised for detection limits corresponding to approximately 440 cells l^{-1} for the

triplex assay and 40 cells l^{-1} for the duplex (Figure 4a & 4b). Stronger signals were seen for *A. minutum* concentrations at a factor of ten above each of these limits for both assays. In investigating relative sensitivity, an initial 1:1 ratio of *A. minutum* cells to background cells was approximated by diluting the culture by half. The triplex and duplex PCR assays were capable of detecting approximately 2.15×10^5 *A. minutum* cells l^{-1} and 2.15×10^4 *A. minutum* cells l^{-1} respectively, within a non-target cell concentration of 2.12×10^7 cells l^{-1} (Figure 4c & 4d).

Discussion

The main objectives of this study were to optimise a triplex PCR assay, incorporating *Alexandrium* primers, for application under environmental conditions. Comparative analyses were conducted, for environmental application and sensitivity, between the triplex PCR assay and a duplex PCR assay. Testing the *Alexandrium*-specific ALEX533 primer, using a triplex PCR assay as the application tool, has demonstrated the effectiveness of the primer for specific detection of *A. minutum* cells within the mixed non-target assemblages of environmental samples. The triplex PCR assay has been optimised for environmental application and detection limits of the assay determined. Comparable results were produced with application of the triplex and duplex PCR assays to environmental samples, although greater sensitivity levels for detecting *A. minutum* cells were determined for the duplex PCR assay.

The triplex PCR assay was optimised with application to DNA extractions from V&A samples. The HAB present at the V&A waterfront was fairly mono-specific, especially for the first seven weeks of testing where *A. minutum* was the dominant species (Table I). Optimisation was empirically established for these samples in such

a way that, when *Alexandrium* cells are detected within an environmental sample, reaction conditions are biased towards amplification of the *Alexandrium*-specific fragment rather than the generic dinoflagellate fragment, thereby encouraging target detection. It is not possible to optimise this triplex PCR assay to provide a balanced (amplicons of equal intensity) and measurable response for all possible natural combination of target/non-target cells.

Application of the triplex PCR assay to the V&A samples collected over time and to the regional samples collected over a range of locations along the southwestern Cape coast (Table I; Figure 2a; Figure 3), has demonstrated the triplex PCR assay to be both robust, in applying to a wide variety of environmental conditions, and specific at detecting *A. minutum* cells within a range of non-target cell concentrations - generation of non-target products did not occur for any of the samples collected. Application to the V&A samples generated generic dinoflagellate amplicon, excluding the sample collected on the 4th December 2003, plus the genus-specific amplicon identifying the presence of *Alexandrium* (Figure 2a). The sample collected on the 4th December 2003 (Figure 2a, lane 3), demonstrates the bias of the triplex PCR assay conditions in the presence of low dinoflagellate cell concentrations, with only the *Alexandrium*-specific amplicon generated. In the absence of the target genus, as for the multi-specific regional samples, generic amplicons only were generated. One advantage of applying molecular techniques over microscopic work for phytoplankton identification purposes was shown with the application of the triplex PCR assay to DNA extracted from the V&A sample collected on the 22nd January 2004. *A. minutum* DNA was detected by the assay where, microscopically, the cells were not (Table I; Figure 2a & 2b, lane 10). At this stage of development however, it

is prudent not to dismiss one technique over the other, but to conduct the two concurrently: primer application testing complementing microscopy work. Direct comparisons of this ALEX533 primer application to previous studies is difficult due to differences in methodology: PCR assays targeting the dinoflagellate *Pfiesteria piscicida* Steidinger et Burkholder, have been able to detect as little as 1 cell ml^{-1} of environmental sample (Litaker *et al.*, 2003); a triplex PCR assay developed to target Raphidophyceae can also detect one cell from filtered environmental samples (Connell, 2002).

The absolute limit of detection for the triplex PCR assay was determined, detecting *A. minutum* cell concentrations as low as 440 cells l^{-1} (Figure 4a). Detecting less than 1 cell ml^{-1} in mono-specific assemblages, this PCR assay is extremely sensitive. The addition of high concentrations of non-target cells detrimentally affected the sensitivity of the triplex PCR assay (Figure 4c). Although sensitivity decreased, *A. minutum* cells could still be detected in a sample containing 100 times more non-target cells.

Applications of a duplex PCR assay to the V&A sample extractions generated comparable results to the triplex PCR assay (Figure 2b). The only discrepancy observed concerned application to the sample collected on the 15th January 2004: a negative result was generated with application of the duplex PCR assay yet a positive result was observed for the triplex PCR assay (Figure 2a & 2b, lane 9), emphasising the need for replicates for confirmation of results. Greater sensitivity, for the detection of *A. minutum* cells, was achieved with application of the duplex PCR assay (Figure

4b & 4d): omitting the D1R primer results in less competition within the duplex amplification reactions where only one, genus-specific, DNA fragment is targeted.

The presence of inhibitors within many of the samples may have yielded lower DNA content values from the extraction procedure. Due to the possibility of DNA quantification discrepancies, the triplex and duplex PCR assays cannot be relied upon for semi-quantitative information. It must be emphasised, however, that the objective for the development of probe technology is to design a novel monitoring technique that would replace microscopy work with a simple, rapid quantitative procedure. Application of genus-specific primers here, through PCR-based assays, is just one stage of the development toward this goal. As such, the triplex PCR assay is a fairly intricate protocol to test primer application, relative to the more straightforward duplex PCR assay. Optimising for environmental conditions was time-consuming: multiplex PCR assays are a lot more sensitive to optimal requirements (Henegariu et al., 1997) and establishment of these conditions can be troublesome, especially with the added complexity introduced with the application to environmental, rather than cultured, samples. With the advantage of their built-in control, but under conditions that have been shown to be less sensitive at detecting target cells than the duplex PCR assay, it would have to be considered whether the added effort put into optimising triplex PCR assays is worth the benefits of this additional control.

Nevertheless, the results from this investigation show that the ALEX533 primer, applied within either PCR assay, is at this stage ready for full-scale testing. Given that much of this study involved optimising the triplex PCR assay for environmental conditions, testing was relatively limited and conducted on a single primer only;

further comprehensive, systematic testing is still required. This assessment should be conducted for several months with dual molecular/microscopic examination. Following successful completion of this testing, the design of an easy-to-use quantitative hybridisation technique could be considered for the next stage of development. Primers designed for PCR assay application require different characteristics than hybridisation probes. Therefore, the ALEX533 primer would need to be adapted for incorporation into one of several quantitative molecular assays available, such as sandwich hybridisation assays (Tyrell *et al.*, 2002) or fluorescent *in situ* hybridisation (John *et al.*, 2003), the choice of which would depend on the specifications of the end-user (Scholin *et al.*, 1996).

Further testing is also necessary for the design and implementation of a simplified DNA extraction technique. In this study, results were available relatively quickly, within 24 h. However, in order to provide results in as near real-time as possible for large numbers of routine samples, simplification of the extraction technique would be desirable. Limited success was achieved during this investigation where the phenol: chloroform: isoamyl alcohol steps were omitted from the extraction procedure (results not shown), but further research would be necessary for design and implementation of such a modified protocol. Testing would also be required for application of the remaining primers designed by Ruiz Sebastián. Here, three other *Alexandrium* primers, plus three primers for each of the *Dinophysis* genus, *P. reticulatum* and *K. micrum* were applied to some of the V&A samples using the optimised triplex PCR assay (results not shown). It was clear, from the amplicons generated, that optimisation of the triplex PCR assay needs to be empirically determined for each primer individually.

In conclusion, it has been demonstrated that the ALEX533 primer, in conjunction with the application techniques tested here, is effective in detecting *A. minutum* cells within natural marine assemblages. Optimisation of the triplex PCR assay generates specific results when applied to a range of target and background cell concentrations and is extremely sensitive to low concentrations of *A. minutum* cells, making it a valuable tool for unequivocal detection of target cells within environmental samples. The triplex PCR assay optimised here has demonstrated its usefulness in testing the ALEX533 primer, and should be applied to the remaining primers for their testing. Application within the research stage would benefit from the internal triplex PCR assay control. Confirmation with replicate, or with the more sensitive duplex PCR assay, would also be of benefit, not forgetting the usefulness of microscopic identification complementing the results of the primer testing. With the success shown here, following further comprehensive testing, the ALEX533 primer would be a good candidate for incorporation into possible quantitative molecular techniques developed within this field of HAB monitoring.

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Table I: Microscopically determined algal assemblages of samples collected, and quantification of genomic DNA extracted from each.

"nd" not detected

Sites	Cell concentrations. l^{-1}						Total DNA quantification. $\mu g\ ml^{-1}$
	A. minutum	other dinoflagellates	flagellates	centroid diatoms	pennate diatoms	ciliates	
V&A – 21.11.03	2.40×10^6	nd	nd	nd	nd	nd	104
25.11.03	7.10×10^6	nd	nd	nd	nd	nd	61
04.12.03	2.00×10^5	nd	nd	2.00×10^5	nd	2.00×10^5	87
10.12.03	3.90×10^6	nd	nd	4.00×10^5	nd	2.00×10^5	70
18.12.03	4.10×10^6	nd	nd	nd	nd	4.00×10^5	164
26.12.03	6.00×10^5	2.00×10^5	nd	nd	nd	nd	87
02.01.04	1.95×10^7	2.00×10^5	nd	nd	nd	1.20×10^6	67
09.01.04	1.00×10^5	nd	nd	nd	nd	2.00×10^5	83
15.01.04	6.00×10^5	nd	nd	8.00×10^5	nd	nd	99
22.01.04	nd	2.00×10^5	nd	2.00×10^6	nd	nd	138
Gordon's Bay	nd	nd	nd	nd	nd	nd	261
Strand	nd	2.00×10^5	4.00×10^5	nd	2.00×10^5	2.00×10^5	22
Strandfontein	nd	2.20×10^6	2.00×10^5	1.72×10^7	1.40×10^6	2.00×10^5	109
Muizenberg	nd	1.20×10^6	nd	2.00×10^6	2.00×10^6	nd	32
Simon's Town	nd	nd	4.00×10^5	nd	nd	nd	16
Kommetjie	nd	nd	nd	nd	nd	nd	48
Hout Bay	nd	6.00×10^5	nd	1.78×10^7	2.40×10^6	nd	39
Llandudno	nd	4.00×10^5	nd	nd	nd	nd	172
Milnerton	nd	1.60×10^6	nd	4.20×10^6	nd	nd	21
Melkbosstrand	nd	nd	nd	nd	nd	nd	22

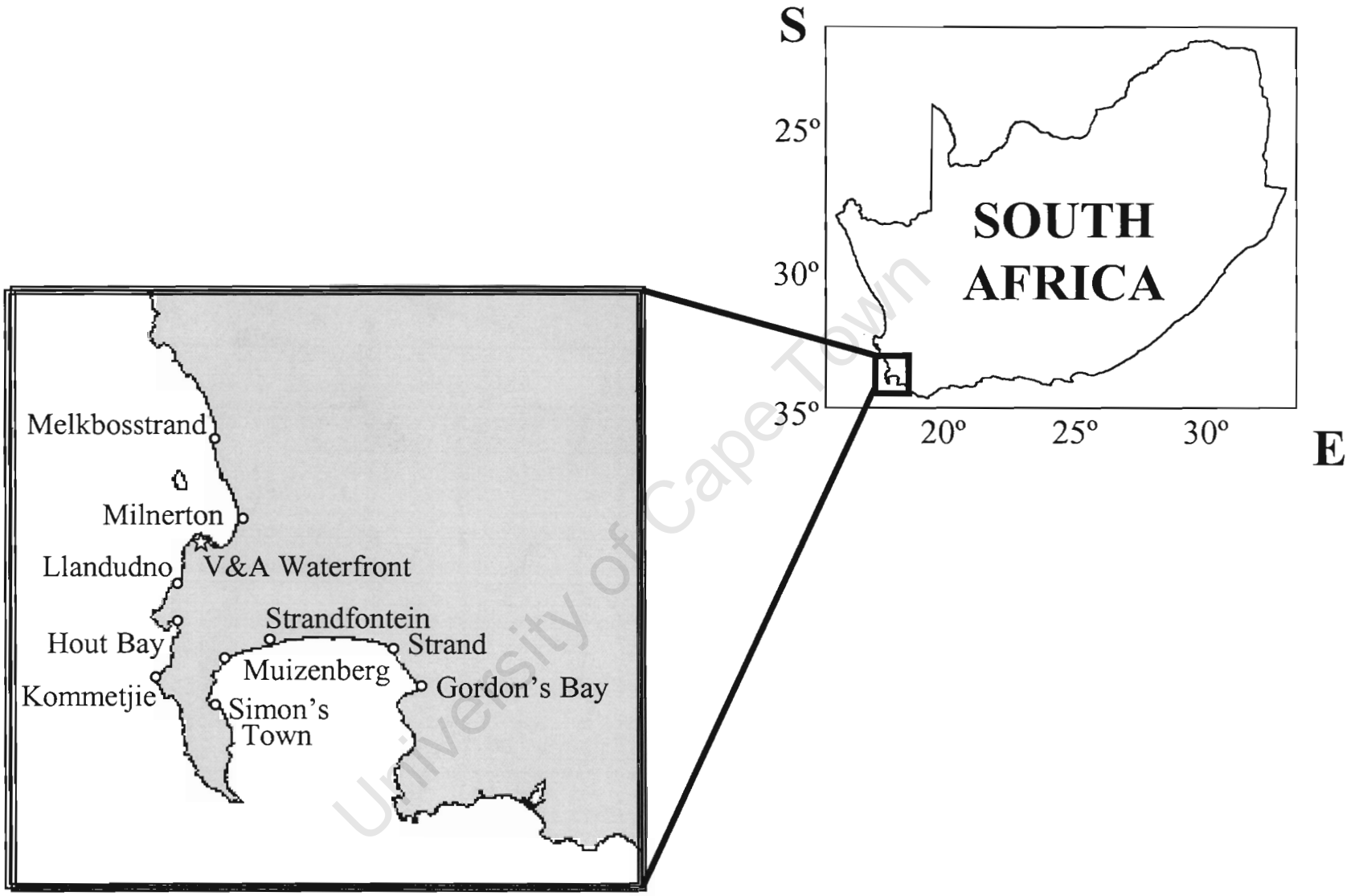

Figure 1: Map of the southwestern cape coast of South Africa indicating the sampling sites. Ten weekly samples were collected from the V&A Waterfront, plus ten regional samples, one from each of the other sites shown

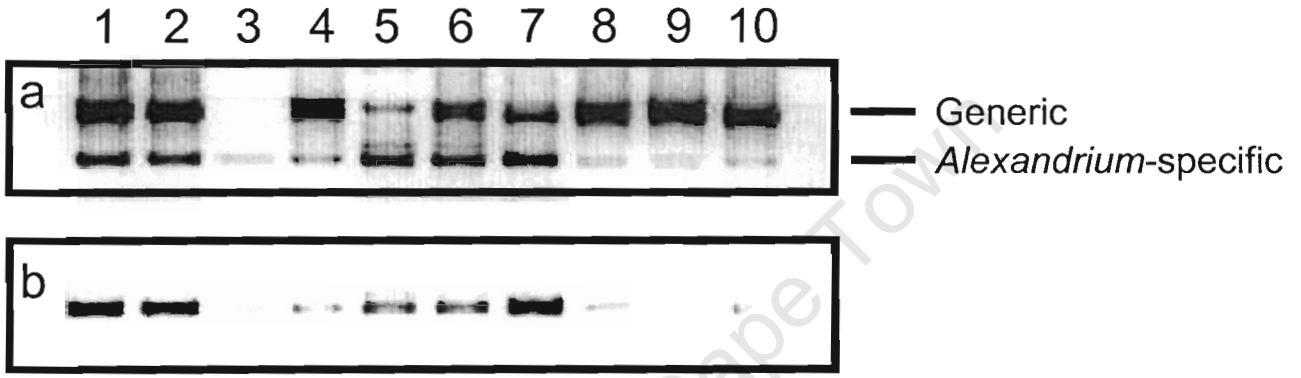

Figure 2: PCR assay amplification products generated with application to DNA extracted from V&A samples. (a) Application of triplex PCR assay. (b) Application of duplex PCR assay, *Alexandrium*-specific amplification only. Ten samples were collected weekly from the 21st November 2003 (lane 1) to 22nd January 2004 (lane 10)

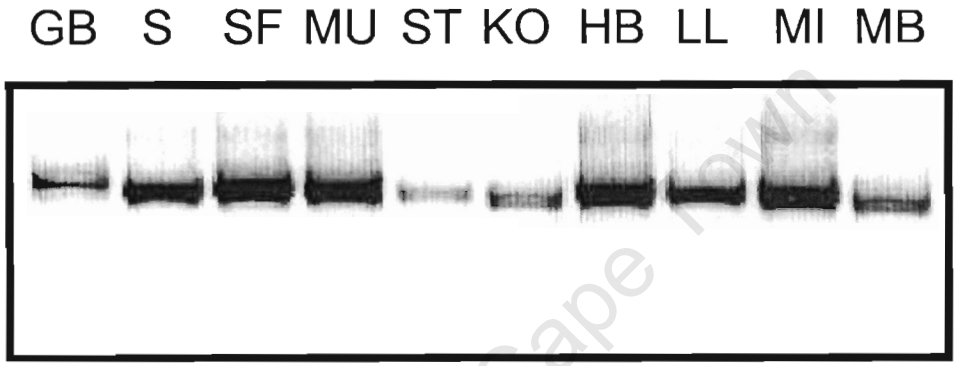

Figure 3: Triplex PCR assay amplification products generated with application to DNA extracted from ten regional samples collected. No *Alexandrium* cells were detected, with only generic bands generated, in line with microscopical analyses. "GB" Gordon's Bay; "S" Strand; "SF" Strandfontein; "MU" Muizenberg; "ST" Simon's Town; "KO" Kommetjie; "HB" Hout Bay; "LL" Llandudno; "MI" Milnerton; "MB" Melkbosstrand

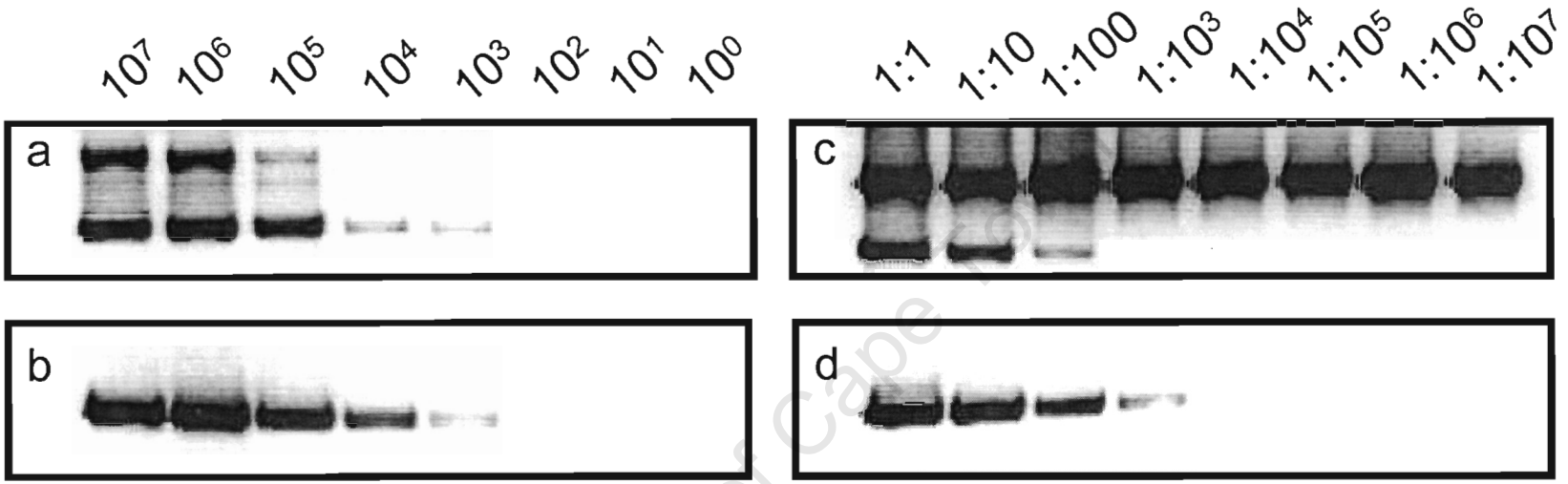

Figure 4: PCR assay amplifications showing the assays' sensitivity for *A. minutum*. Absolute sensitivity, the minimum number of target cells detected in a mono-specific sample, was determined for (a) the triplex PCR assay, (b) the duplex PCR assay. Lane 1, 4.4 x 10^7 *A. minutum* cells l^{-1}; lane 8, 4.4 x 10^0 *A. minutum* cells l^{-1}. Relative sensitivity, the minimum target cells: non-target cells ratio detectable within a mixed assemblage, was also determined for the triplex (c) and duplex (d) PCR assays. Lane 1, 2.1 x 10^7 *A. minutum* cells l^{-1}: 2.1 x 10^7 non-target cells l^{-1}; lane 8, 2.1 x 10^0 *A. minutum* cells l^{-1}: 2.1 x 10^7 non-target cells l^{-1}