NUCLEIC ACID TESTS FOR TOXIC PHYTOPLANKTON IN IRISH WATERS-*PHYTOTEST*

Marine Strategic RTDI project AT/04/02/02-Research Update

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Rationale for the project:
The Phytotest project is a 3 year collaborative project funded through the Marine Strategic Programme in Advanced Technologies as part of the National Development plan 2000-2006. The project partners include the National Diagnostics Centre at NUI Galway and MI. The overall objective of the project is the development of nucleic acid tests (molecular methods) for the identification of key toxic phytoplankton species in Irish waters. In the final year of the programme the aim is to transfer the molecular methods developed in the project into MI to support their monitoring service. Currently, the monitoring for phytoplankton species in Irish waters is performed by light microscopy which can easily identify some plankton species based on distinctive morphological traits. Other species in particular, *Pseudo-nitzschia* spp. and *Alexandrium* spp. cannot be identified to species level by light microscopy. Identification of these species requires more sophisticated microscopic techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM). These techniques cannot easily be integrated into a routine testing environment. Molecular methods utilise unique information contained within an organism's genome in order to identify it. This genetic information can be exploited in a range of molecular test platforms enabling microorganisms to be identified to species level. Additionally, there has been a major drive towards the development of highly automated platforms to support molecular tests for high-throughput testing in routine laboratory settings.

Summary of Project Tasks
The Phytotest project is organised as a number of discrete tasks which include:

1. Sourcing, isolation and culturing of *Dinophysis* and *Pseudo-nitzschia* species. The MI phytoplankton monitoring team are responsible for this task and are proactive in sourcing strains and wild samples for the project.
2. Generation of DNA sequence information for selected genomic targets in *Dinophysis* spp. and *Pseudo-nitzschia* spp. For *Dinophysis* species, *D. acuta* and *D. acuminata*, key DSP producers in Irish waters, the genomic targets selected for DNA sequencing included the small and large rDNA ribosomal sub-units (SSU and LSU). For *Pseudo-nitzschia* spp., the LSU and internally transcribed spacer regions (ITS1+2) were sequenced.
3. Design of the building blocks for molecular tests, DNA probes and PCR primers from the sequence information. To enable the design of PCR primers and DNA probes, sequence information generated in the project is compared with sequence information available in public databases (GenBank). Using selected bioinformatics tools, DNA

sequence regions suitable for the design of PCR primers and probes for the target organism are identified. Usually several candidate PCR primers and DNA probes are designed for each species of interest. They are tested for their ability to detect the species of interest and distinguish it from closely related species (specificity). The performance of the PCR primers and DNA probes for detecting different cellular concentrations of the species of interest is also performed (sensitivity).

4. Compilation of the *Dinophysis* spp. and *Pseudo-nitzschia* spp. tests onto specific test platforms. In Phytotest, real-time PCR (RT-PCR) and fluorescent *in situ* hybridisation (FISH) are the platforms being employed.
5. Performance testing is carried out using well characterised and wild samples.
6. The final task of the project due to commence in mid-2007 is the transfer of the molecular tests to the MI.

Overview of test methods:

FISH is a DNA probe based technique that has been applied to identify plankton cells (Adachi *et al.,* 1996; Scholin *et al.,* 1999; Touzet and Raine, 2006). The plankton cells are chemically fixed on a solid support and treated to make the cell wall permeable. A species-specific DNA probe labelled with a fluorescent reporter is added in a specific buffer (hybridisation buffer) to the cells. The probe binds or hybridises to a specific genomic target usually rRNA at a pre-determined temperature. Unbound probe is removed by a series of washing steps and the results of the DNA probe binding can be viewed by microscopy and the selected species identified.

Real-time PCR is a rapid and integrated *in-vitro* amplification and detection technology. The incorporation of specialised fluorescent dyes or labelled probes into the PCR reaction enables PCR product formation to be monitored in real-time. This provides the added advantage of eliminating the need for post-PCR processing while adding specificity to the PCR by including probes and minimizing the risk of contamination by performing PCR amplification and detection in a single closed tube system (Bustin, 2000). In this project, the LightCycler® and hybridisation probe technologies are being employed. Hybridisation probes also referred to as FRET probes or HybProbes comprise two specially designed sequence-specific oligonucleotide probes labelled with fluorescent dyes. The sequences of the two probes are designed to hybridise to the amplified DNA fragment in a head to tail arrangement. When the probes hybridise in this orientation, the two fluorescent dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited by the LightCycler™ LED (Light Emitting Diode) filtered light source, and emits green fluorescence at a slightly longer wavelength. This emitted energy excites the second dye (LC Red 640) attached to the second hybridisation probe which emits red fluorescence at an even longer wavelength. This energy transfer, referred to as FRET (Fluorescence Resonance Energy Transfer) is proportional to the increasing amount of PCR product generated. By combining hybridisation probe technology with an analysis function of the LightCycler™ software, melt peak analysis, it is possible to generate temperature specific melt peaks which can distinguish PCR products generated from the target species from PCR products generated from non-target species. For Phytotest, we have designed the species real-time PCR tests to have a common thermocycling regime providing the potential to identify a range of different *Dinophysis* spp. and *Pseudo-nitzschia* spp. in the same analytical run.

In addition to RT-PCR and FISH techniques, other molecular platforms including DNA probe sandwich hybridisation, molecular biosensors and microarray technology have been employed as diagnostic platforms for the identification of toxic phytoplankton species (Simon *et al.,* 2000; Metfies *et al.,* 2005; Medlin *et al.,* 2006). Another important consideration in the application of molecular tests for phytoplankton species identification is the optimisation of suitable sample preparation and nucleic acid extraction procedures. In Phytotest, the samples for testing are filtered onto a membrane and nucleic acids are extracted from phytoplankton cells using a commercially available kit, Qiagen Plant kit or an in-house method.

Results

Nucleic acid tests for Dinophysis spp.-D. acuta and D. acuminata:

The LSU D1-D2 region was PCR amplified from single *Dinophysis* species cells and the resulting PCR products were sequenced. Sequence information was generated for eighteen LSU D1-D2 regions and eight partial SSU regions of *Dinophysis acuta* and *Dinophysis acuminata* isolated from preserved wild samples from around the Irish coast. Sampling sites included Sheephaven Bay, Drumcliff Bay, Killary Harbour and Bantry Bay. Sequence alignments were performed with *Dinophysis* species sequences available from GenBank. Phylogenetic analysis revealed that Irish *D. acuta* clustered with *D. acuta* from the Scottish coastline. Irish *D. acuminata* clustered with *D. acuminata* from the Scottish coastline and isolates from USA, Sweden and Australia.

An internal D1-D2 region primer set was designed based on *Dinophysis* species LSU sequence alignments. DNA HybProbes were designed based on greatest sequence variation between species within this region, following analysis of the sequence alignments. A real-time PCR test was designed, incorporating the HybProbes, for the LightCycler™ for the specific detection and discrimination of *D. acuta* and *D. acuminata*.

The real-time PCR test yielded species-specific melt peaks at 48 ^{0}C for *D. acuminata* and at 61 ^{0}C for *D. acuta* (Figure 1). The specificity of the test for detection and discrimination of *D. acuta* and *D. acuminata* was confirmed using *D. acuta* and *D. acuminata* single cells and also using clones containing target LSU regions from *D. acuta* and *D. acuminata* (Figure 2). The specificity of the test for *D. acuta* and *D. acuminata* was also tested using a panel of phytoplankton species found in Irish waters (Table 1). The test only detected *D. acuta* and *D. acuminata* and did not cross-react with other species tested to date. Twenty-five preserved wild samples from the phytoplankton monitoring program were tested and there was good correlation between *Dinophysis* species cells detected by light microscopy and by the HybProbes (Table 2). The real-time PCR test detected *D. acuta* in all samples identified to contain *D. acuta* and in 90% of samples determined to contain *D. acuminata* by light microscopy.

The detection limit of this test was determined using serial dilutions (10^{8} to 10^{1} copies) of the *D. acuta* and *D. acuminata* clones as templates in the real-time PCR test (Figure 3). Detection limits of 1-10 copies were consistently achieved for both targets indicating that the test can detect 1 cell of these species.

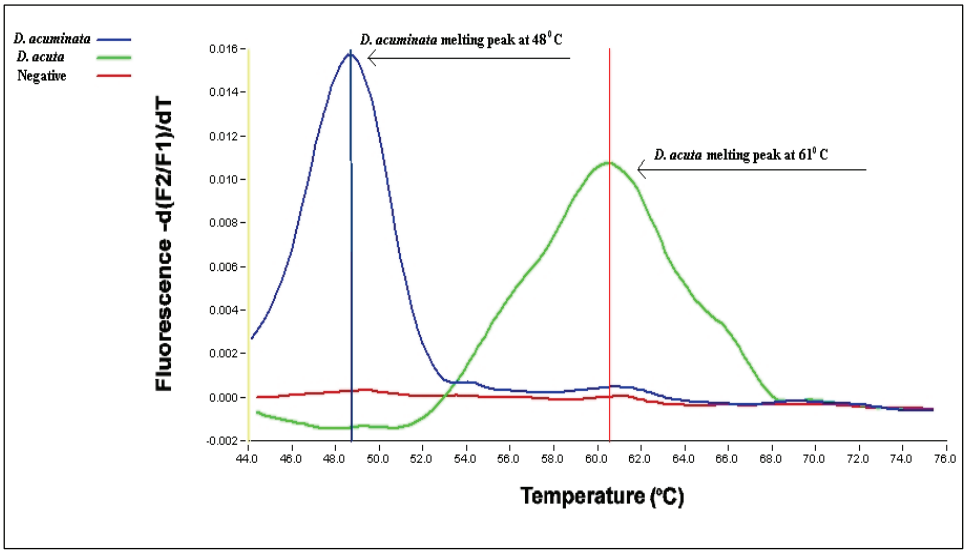

Figure 1. Real-time PCR (LSU) test for the detection of *D. acuta* and *D. acuminata* using HybProbes (FRET probes). *D. acuta* and *D. acuminata* are simultaneously identified in one test. The test target is the LSU D1-D2 region.

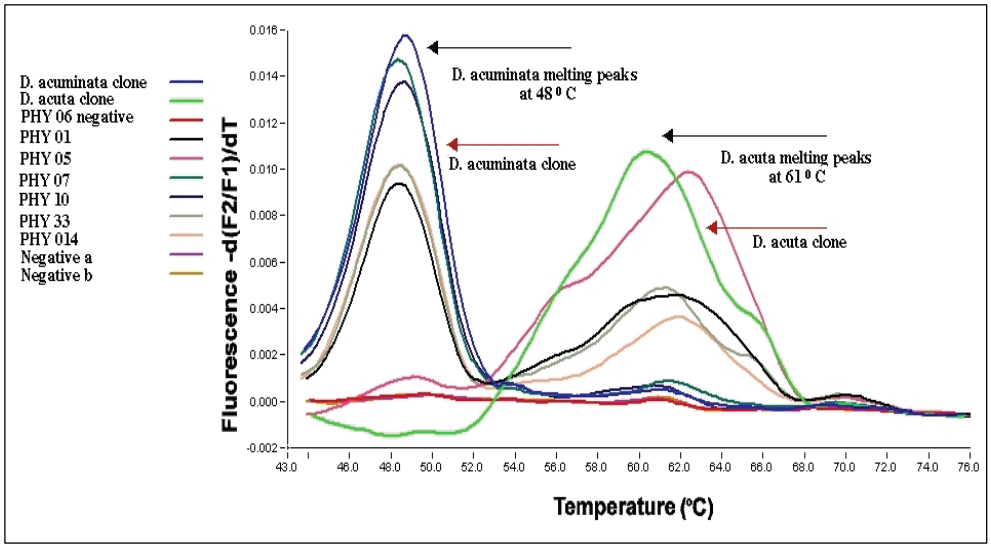

Figure 2: Real-time PCR (LSU) test designed for the detection of *D. acuta* and *D. acuminata* using HybProbes (FRET probes). Melt peak analysis of the HybProbes yielded melt peaks at 48 ^{0}C for *D. acuminata* cells and 61 ^{0}C for *D. acuta* cells. Two positive clones and samples obtained from the phytoplankton monitoring program were included in this run. Field samples tested were: PHY 01: 2 *D. acuminata* cells, 1 *D. acuta* cell, PHY 05: 2 *D. acuta* cells, PHY 07: 1 *D. acuminata* cell, PHY 10: 2 *D. acuminata* cells, PHY 33: 2 *D. acuminata* cells, PHY 014: 2 *D. acuminata* cells, 1 *D. acuta* cell, PHY 06: Negative - No *Dinophysis* species.

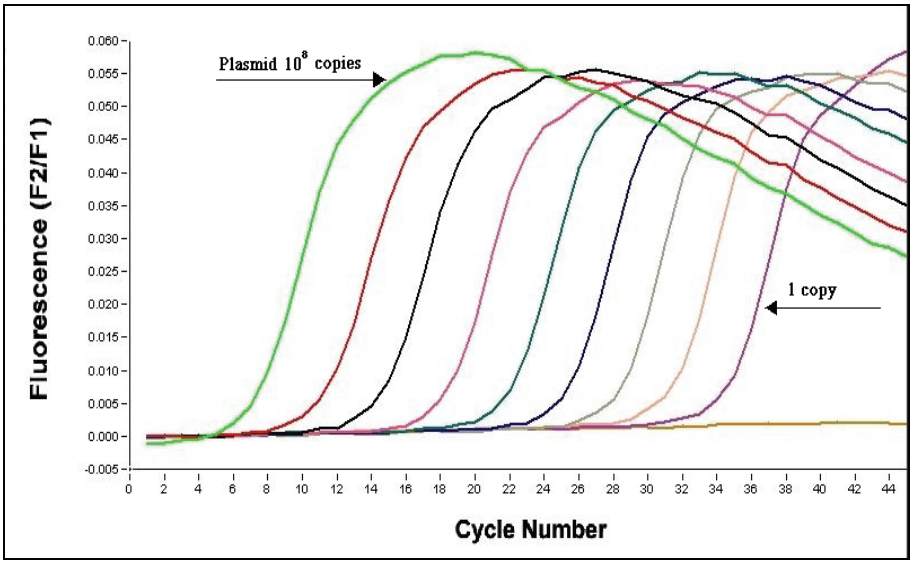

Figure 3. Quantification curves generated from serial dilutions of the *D. acuta* positive control clone in the *D. acuta/D. acuminata* real-time PCR test.

Table 1: Panel of species tested in the *D. acuta/D. acuminata* real-time PCR test.

Phytoplankton species tested with HybProbe probes	Melting peaks observed
Dinophysis acuta	61 ^{0}C
Dinophysis acuminata	48 ^{0}C
Pseudo-nitzschia australis	No melting peak observed
Pseudo-nitzschia fraudulenta	No melting peak observed
Pseudo-nitzschia delicatissima	No melting peak observed
Alexandrium minumum	No melting peak observed
Alexandrium tamarense	No melting peak observed
Alexandrium fundyense	No melting peak observed
Alexandrium catenella	No melting peak observed
Prorocentrum dentatum	No melting peak observed
Chaetoceros debilis	No melting peak observed
Asterionellopsis glacialis	No melting peak observed
Gymnodinium cf species	No melting peak observed

Table 2: Wild Samples tested in the *D. acuta/D. acuminata* real-time PCR test.

Sample #	Cell number/type observed in Phytoplankton Monitoring Program	Melt Peak 48 ^{0}C	Melt Peak 61 ^{0}C
PHY0(62800)1	2 *D. acuminata*, 2 *D. acuta,* 1 *D. dens*	+	+
PHY0(62800)5	2 *D. acuta*	-	+
PHY0(62700)6	4 *D. acuminata*	+	+
PHY0(62700)7	1 *D. acuminata*	+	-
PHY0(62701)0	2 *D. acuminata*, 1 *D. acuta*	+	+
PHY0(62003)3	2 *D. acuminata*, 1 *D. acuta*, 1 *Phalachroma rotundatum*	+	+
PHY0(62701)1	8 *D. acuminata*, 1 *D. dens*, 1 *Phalachroma rotundatum*	+	-
PHY0(62700)8	1 *D. acuminata*, 1 *Phalachroma rotundatum*	+	-
PHY0(62401)4	2 *D. acuminata*, 1 *D. acuta*	+	+
PHY0(62701)3	7 *D. acuminata*	+	-
PHY0(62402)8	1 *D. acuminata*	+	+
PHY0(61903)3	5 *D. acuminata*	+	-
PHY0(62700)2	1 *D. acuminata*	+	-
PHY0(62501)1	2 *D. acuminata*, 1 *Phalachroma rotundatum*	+	+
PHY0(61801)4	1 *D. acuminata*	+	-
PHY0(62001)6	1 *D. acuminata*, 1 *Phalachroma rotundatum*	+	-
PHY0(62402)7	1 *D. acuminata*	+	-
PHY0(62503)3	2 *D. acuminata*	+	-
PHY0(62004)5	3 *D. acuminata*	+	-
PHY0(62600)8	4 *D. acuminata*	+	-
PHY0(62001)7	5 *D. acuminata*	+	+
PHY0(62703)3	6 *D. acuminata*	+	-
PHY0(62404)6	1 *D. acuminata*	-	+
PHY0(62405)7	1 *D. acuminata*	-	+
PHY0(62405)8	1 *D. acuminata*	+	+

Nucleic acid tests for Pseudo-nitzschia spp.:

Cultures of indigenous *Pseudo-nitzschia* spp. and species donated by Dunstaffnage Marine Lab, Oban, Scotland were set-up by the Phytoplankton Monitoring Laboratory at MI. The LSU D1-D2 region was PCR amplified from a DNA extract generated from the indigenous *Pseudo-nitzschia* culture and the resulting PCR products were sequenced. Sequence analysis revealed that the culture organism was *P. fraudulenta*. Sequence alignments were performed using ITS1-ITS2 region sequences for a range of *Pseudo-nitzschia* species downloaded from GenBank and PCR primers were designed to amplify the ITS1 and ITS2 rDNA regions. ITS regions 1 and 2 were PCR amplified from DNA extracts generated from all of the *Pseudo-nitzschia* spp. cultures and the resulting PCR products were sent for sequencing. The ITS

region 1 primers were used to generate PCR products for sequencing from preserved samples taken from the MI phytoplankton monitoring program. *P. delicatissima* was identified in preserved samples from Selax, Invern, Hawks Nest and *P. australis* was identified in a preserved sample from Red Flag. Phylogenetic analysis of the sequence information revealed that Irish *P. fraudulenta* and Scottish *P. australis*, and *P. delicatissima* species sequences formed clusters with corresponding *Pseudo-nitzchia* species from the USA and Denmark.

Sequence alignments were performed using ITS1 and ITS2 sequences and ITS1-2 sequence information for other *Pseudo-nitzschia* species available from GenBank. DNA Hybprobes were designed for *P. australis*, *P. fraudulenta* and *P. delicatissima* based on analysis of sequence information for the ITS region 1. Three independent real-time PCR tests were designed for the identification of *P. australis*, *P. fraudulenta* and *P. delicatissima*. *P. australis* produces a melt peak at 57 ^{o}C with *P. australis* specific probes. *P. fraudulenta* produces a melt peak at 55 ^{o}C, with *P. fraudulenta* specific probes and *P. delicatissima* produces a melt peak at 60 ^{o}C with *P. delicatissima* specific probes. The specificity of the tests for detection and discrimination of individual *Pseudo-nitzschia* species was confirmed using DNA from all available *Pseudo-nitzschia* species. The *P. australis* real-time PCR test detected the presence of *P. australis* in a DNA extract of 173 cells isolated from a preserved seawater sample (Figure 4). This seawater sample was taken at Castlemaine Harbour as part of the routine phytoplankton monitoring program and was associated with a toxic event in April 2005. The identity of the organism as *P. australis* was further confirmed by sequencing of the LSU D1-D2 region and Transmission Electron Microscopy (TEM). Published FISH probes (Miller and Scholin 1996) for the identification of *P. fraudulenta, P. australis and P. delicatissima* are currently being investigated using the whole-cell hybridisation method.

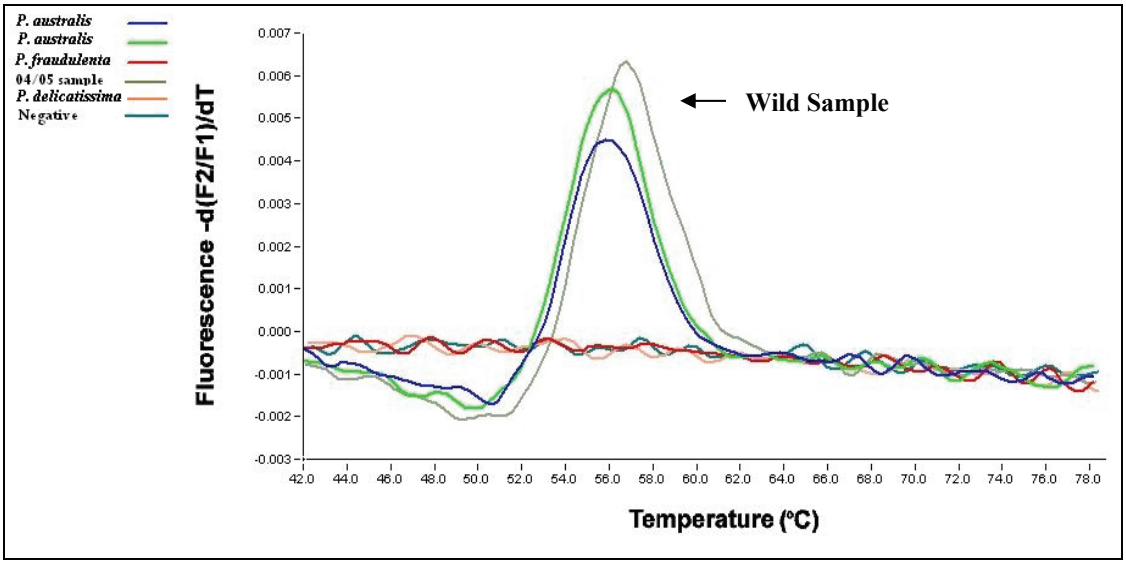

Figure 4. Real-time PCR (ITS-1) test designed for the detection of *P. australis* using FRET probes. Melt peak analysis of the FRET probes yielded a melt-peak at 57 ^{0}C for *P. australis*.

Conclusion and Future Work:

In the Phytotest project to date, real-time PCR tests on the LightCycler incorporating hybridisation probes (HybProbes) have been designed for the identification of *D. acuta*, *D. acuminata*, *P. fraudulenta, P. australis* and *P. delicatissima*. The application of these nucleic acid tests for the identification of these species in wild samples has been demonstrated. Evaluation of the real-time PCR tests is continuing. In parallel, the evaluation of FISH probes for the identification of *Pseudo-nitzschia* spp. is ongoing. The aim for 2007-2008 will be to optimise these real-time species assays as quantitative real-time PCR tests and to transfer the tests to MI.

References

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