Molecular Methods For Monitoring Harmful Algal Bloom Species.

Evelyn Keady[1] and Majella Maher[1*]

Molecular Diagnostics Research Group, National Centre for Biomedical Engineering Science, NUI Galway.

***Phytotest project collaborators** : Siobhán Kavanagh[1], Claire Brennan[1], Josephine Lyons[2], Tara Chamberlain[2], Rafael Salas[2], Siobhán Moran[2], Joe Silke[2] & Majella Maher[1]

[1] NUI, Galway, [2] Marine Institute.

EU SPIES DETOX project collaborators : Nicolas Touzet[1], Robin Raine[1], Elizabeth Turrell[3], Catherine Collins[3] & Jennifer Graham[3] [1] NUI, Galway, [3] Marine Scotland.

Introduction:

Shellfish production worth approximately €60 million to the Irish economy in 2006 (Browne et al., 2007) and several €100 millions to other European countries can be adversely affected by the presence of harmful microalgae (HABs). Toxins produced by *Dinophysis, Alexandrium and Pseudo-nitzschia* species can accumulate in shellfish and have the potential to cause serious human illness. In order to satisfy EU legislative requirements pertaining to the production and export of shellfish (EC Hygiene Regulations 2004, No. 853/2004 and No. 854/2004, which replaced the EU Shellfish Hygiene Directive 91/492/EEC in January 2006), monitoring the presence of harmful algal species and biotoxins in coastal waters is performed by EU member states.

Routine microscopic monitoring methods are unable to identify certain toxic species, in particular, *Alexandrium and Pseudo-nitzschia spp.* Electron microscopy is required for species identification and this technique cannot be integrated into a routine monitoring programme.

Molecular techniques utilise unique sequence signatures within microorganism genomes for species specific identification. Molecular methods applied for the identification and quantification of HAB species include Fluorescent in-situ hybridisation (FISH) and in-vitro amplification based methods, in particular, real-time PCR.

Rationale and benefit to the aquaculture industry:

Molecular methods identify toxic HAB species based on unique sequence signatures found mainly in ribosomal genes (rRNA) and internally transcribed spacer regions (ITS) in ribosomal gene operons. These methods can compliment or replace existing monitoring programme methods by providing species identification rapidly for large numbers of samples. The results of these analyses have the potential to be combined with information obtained from "at site" monitoring of shellfish waters for biotoxins providing an "early warning" to shellfish producers. Additionally, the methods can be applied as research tools to help understand bloom dynamics.
In the Phytotest project, a collaboration between the National Diagnostics Centre at NUI, Galway and the Marine Institute, a panel of real-time PCR tests were designed to detect and identify *Dinophysis spp.* and selected *Pseudo-nitzschia spp*. found in Irish waters. This project, the tests developed and their application in monitoring were described by Kavanagh et al. (2008) in the "Proceedings of the 7th Shellfish Safety Workshop". The tests developed in this project are being used by the Marine Institute to support the current monitoring programme.

The EU SPIES DETOX project is a 3-year EU funded project which commenced in 2006 where research performers are working with the shellfish industry in Ireland, Spain, Scotland and Norway to develop methods to improve monitoring programmes and improve the detoxification of shellfish. In the EU SPIES project, researchers in the Molecular Diagnostics Research group at NUI, Galway are working with researchers at Marine Scotland in Aberdeen, Scotland to develop real-time PCR tests for other important *Pseudo-nitzschia species* and FISH probes and real-time PCR tests for *Alexandrium spp.* In parallel, workpackages 1-2 are investigating the feasibility of using selected resins deployed as "solid phase adsorption toxin tracking" (SPATT) devices to provide an early warning system for biotoxins in shellfish production waters. An overview of the EU SPIES DETOX project has been provided in these proceedings by Dr. Elizabeth Turrell, Marine Scotland.

Design and application of molecular methods:

The development of species-specific FISH probes or real-time PCR tests begins with culturing of the target HAB species and closely related species, extraction of nucleic acid from these species and DNA sequencing of the rDNA or ITS regions. For FISH, a DNA probe targeting a unique region of the rRNA is designed and coupled with a fluorescent tag that acts as a reporter for the probe. The water sample containing HABs is tested with the FISH probe by fixing the water sample to a membrane and treating it to allow the probe pass through the cell wall and hybridise to the specific rRNA sequence identifier within the HAB species cell. After washing to remove unbound probe, the sample is viewed under a fluorescence microscope and samples which are positive for the HAB species will fluoresce (Figure 1). For real-time PCR, PCR primers and a DNA probe are designed and configured into a real-time PCR test. Water samples are tested for HABs species following filtration of the water sample (25ml) and nucleic acid (DNA) extraction. DNA is added to the real-time PCR reaction and a characteristic fluorescent diagnostic signal is obtained for positive samples.

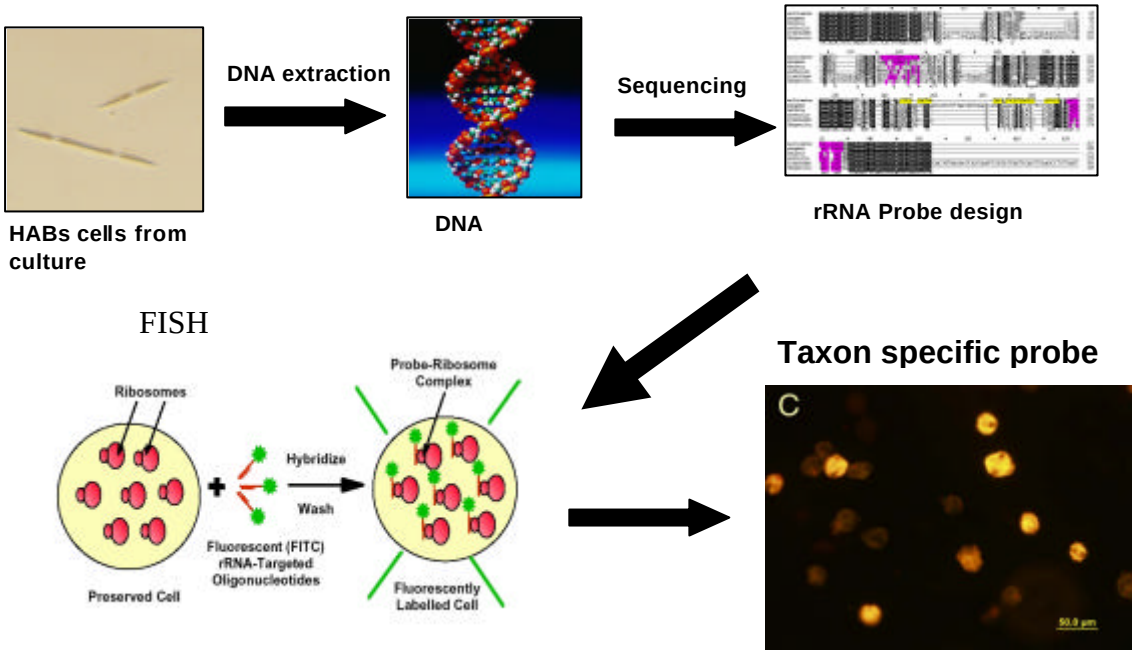

Figure 1: **Schematic representation of the steps involved in design and application of Fluorescent *in-situ* hybridisation (FISH) probes.**

Molecular methods for Alexandrium spp. identification:

At NUI, Galway, FISH probes have been developed for 7 Alexandrium species based on the ribosomal gene targets (rRNA), large ribosomal subunit (LSU) and small ribosomal subunit (SSU). Whole cell FISH (WC-FISH) has been optimised to enable 2 probes labelled with different fluorescent reporters to be used in combination for the simultaneous identification of 2 *Alexandrium* species in environmental samples (Figure 2). Additionally, the method has been optimised so that calcofluor is added to the test enabling the genotypic identification of the *Alexandrium* to species level with the FISH probes and the phenotypic confirmation of the species based on morphology using calcofluor (Touzet and Raine, 2007). WC-FISH probes for *A. minutum GC, A. tamarense* WE *and A. tamarense* NA have been tested in field samples collected from Cork Harbour, Ireland and from Shetland and Orkney, Scotland in 2007 and Cork Harbour 2008. Samples from Cork Harbour were tested with the dual WC-FISH test (*A. minutum GC and A. tamarense* WE) by morphotaxonomy using calcofluor and by qPCR with the *A. minutum* GC real-time PCR test. WC-FISH identified high numbers of *A. minutum* GC in the samples compared to *A. tamarense* WE. When compared with morphotaxonomy using calcofluor (standard method) for enumeration of the target species, WC-FISH under-estimated the *A. minutum GC and A. tamarense* WE cells by a factor of 1.2.

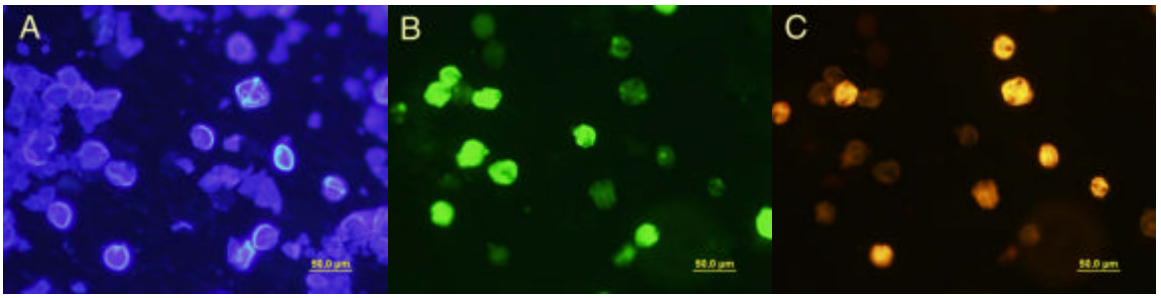

Figure 2**: Dual WC-FISH for *A. tamarense* WE and NA. A. Calcofluor staining; B. *A. tamarense* WE (FITC labeled probe); C. *A. tamarense* NA (CY3 labeled probe).**

At Marine Scotland and NUIG, real-time qPCR tests have been designed for a range of *Alexandrium spp. (A. tamarense WE, A. tamarense NA, A. minutum NH, A. minutum GC, A. ostenfeldii, Alexandrium genus)* incorporating TaqMan or Hybridization probe (HybProbe) fluorescent detection chemistries and targeting either the rDNA LSU or ITS1-5.8S genomic regions in *Alexandrium*. Figure 3 shows an example of the *A. minutum* GC real-time PCR test incorporating Hybprobes showing the detection of 3 strains of *A. minutum* GC by quantification curves and melt-peak analysis. The tests were determined to be specific with selected specificity panels and the limits of detection of the tests (LODs) were established reproducibly in independent experiments at approximately 1 cell or less equivalents for all tests.

Figure 3:

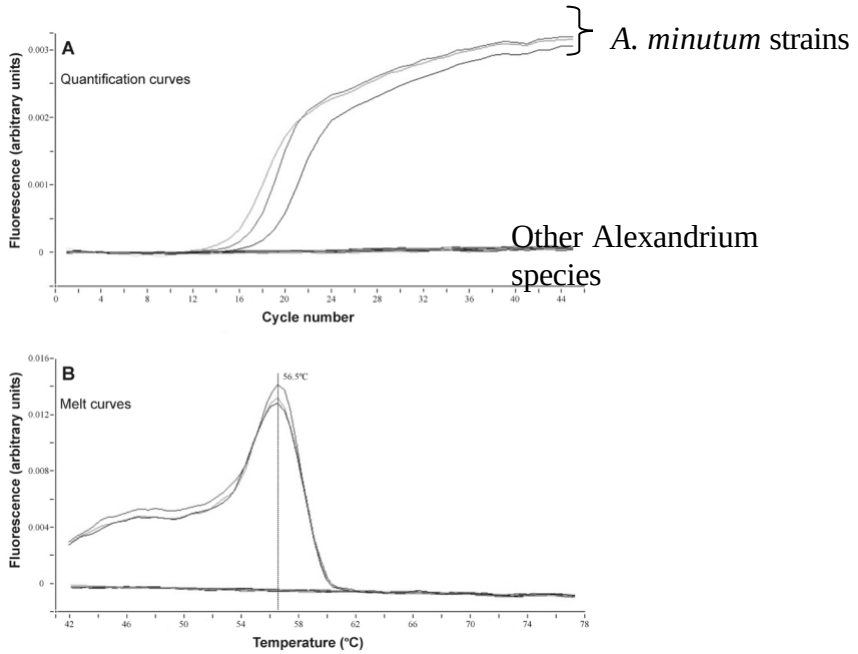

Figure 3: Real-Time PCR (A) quantification curves and (B) melt-curves showing the specificity of the *A. minutum* (GC) test tested against a panel of Alexandrium and dinoflagellate species. In this test run, the 3 *A. minutum* strains tested were detected while a range of *Alexandrium spp.* and other spp. were not detected in the test. \

The per test for *A. minutum* GC was evaluated on wild samples collected from Cork Harbour in 2007 and 2008. Quantification of the *A. minutum* GC by qPCR was compared with enumeration by WC-FISH and morphotaxonomy. Linear regression analyses were carried out to examine the distributions of the *A. minutum* concentrations derived using the three quantification methods. Positive relationships were observed between the methods. In 2007, qPCR overestimated the concentration of *A. minutum* GC compared to morphotaxonomy while in 2008 qPCR underestimated the **concentration of *A. minutum* GC** compared to the reference method (Touzet et al., 2009). Testing of field samples from Scottish waters is ongoing

Molecular Methods for *Pseudo-nitzschia spp.* identification:

As part of the EU SPIES DETOX project real-time PCR tests were developed to identify *P. seriata and P. multiseries.* These tests target the rRNA ITS1 region in *Pseudo-nitzschia spp.* and incorporate hybridization probe technologies. Specificity of each test has been verified using a broad panel of indigenous non-target phytoplankton species. Figure 4 shows the specificity of the *P. seriata* test as demonstrated by a *P. seriata* species-specific melt peak at 56°C.

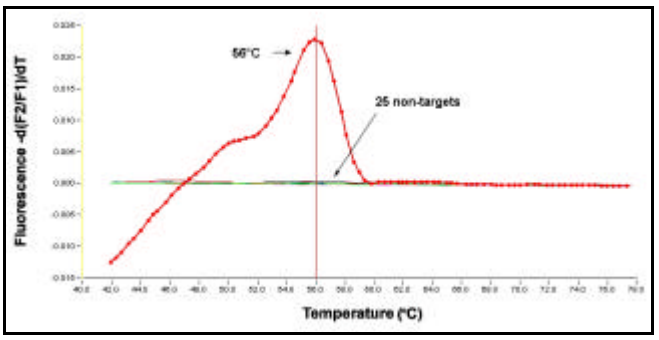

Figure 4: **Real-time PCR melt-peak at 56°C for the *P. seriata* specific test.**

The limits of detection of the P. seriata and P. multiseries real-time PCR tests are 1-10 cell equivalents. Figure 5 shows the quantification curves obtained when testing DNA from P. seriata cells in the range 10,000 – 10 cells.

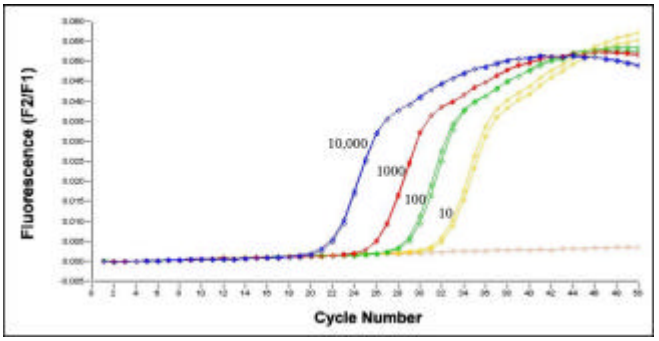

Figure 5: Real-Time PCR quantification curves for the P. seriata specific test testing DNA from cells in the range 10,000 – 10 cells.

Testing of water samples (25ml) is performed on DNA extracted from filtered samples using a combination of freeze-thawing and chemical extraction. Real-time PCR tests for *Pseudo nitzschia spp* -. including *P. australis, P. fraudulenta, P. pungens and P. delicatissima* (Phytotest project) and *P. seriata and P. multiseries* (EU SPIES DETOX project) and *Dinophysis spp. D. acuta and D. acuminata* (Phytotest project) have been evaluated on field samples collected between May and August 2008 from Killary Harbour, Clew Bay and Cork Harbour. Sixty-two weekly water samples (25 ml) were analysed by light microscopy for the presence of *Pseudo-nitzschia spp. –P. seriata and P. delicatissima* type cells and *Dinophysis spp.* cells. *P. seriata* type cells were present in 87% of samples while *P. delicatissima* type cells were observed in 64 % of samples.

D. acuta and D. acuminata were observed in 5 % and 21 % of samples respectively. The real-time PCR tests identified *P. seriata, P. multiseries, P. australis, P. fraudulenta, P. delicatissima and P. pungens* in 24%, 30%, 24%, 32%, 0% and 22% of samples respectively. *D. acuta and D. acuminata* were detected in 19% and 1% of samples respectively. Further evaluation of these tests is planned for 2009.

Conclusions:

Molecular methods have been developed to identify *Dinophysis, Alexandrium and Pseudo-nitzschia* species. Their application for monitoring water samples for these species has been demonstrated. These methods are rapid and can handle large sample numbers. They provide important information to monitoring programmes to help inform the decision making regarding the safety of shellfish production waters.

References:

Browne, R., Deegan, B., O'Carroll, T., Norman, M. and Ó'Cinnéide, M., 2007. Status of Irish Aquaculture, 2006. Marine Institute/Bord Iascaigh Mhara/Taighde Mara Teo: 113pp.

Kavanagh, S., Brennan, C., Touzet, N., Keady, E., Lyons, J., Chamberlain,T., Salas, R., Moran, S., Raine, R., Patching, J., Silke,, J and Maher, M. (2008). Development and implementation of the Phytotest project. Proceedings of the 7th Irish Shellfish Safety Workshop, Galway December 2007. Marine Environment and Health Series, 36pp.

Touzet, N., and Raine, R. (2007). Discrimination of *Alexandrium andersoni and A. minutum (Dinophyceae)* using LSU rRNA-targeted oligonucleotide probes and fluorescent whole cell hybridization. Phycologia 46: 168-177.

Touzet, N., Keady, E., Raine, R. and Maher. M. (2009). Evaluation of taxa-specific real-time PCR, whole cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae *Alexandrium minutum (Dinophyceae*), Global Clade ribotype. FEMS Microbiol Ecol. 67, 329-341.