Real Time PCR methodologies as a routine monitoring tool for the detection of *Pseudo-nitzschia* & *Azadinium* species in Irish waters

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Summary

In Ireland, there are annual intoxifications of marine bivalve shellfish species of the two toxin groups Amnesic Shellfish Poisoning and Azaspiracid Shellfish Poisoning which can cause prolonged closures and economic losses to the shellfish industry. The causative toxin producing phytoplanktonic species of these 2 groups, *Pseudo-nitzschia* genus and the recently described *Azadinium* genus are known to occur regularly in Irish waters. Molecular methods, in particular real-time PCR, are powerful and sensitive techniques which rely on the use of DNA probes and primers which are specific for the detection of DNA sequences of the target species. The Irish monitoring programme has assessed and implemented the use of hydrolysis probes for detection of three *Azadinium* species and hybridisation probes for six *Pseudo-nitzschia* species. This approach allows for confirmatory analysis of a species presence / absence in routine phytoplankton samples, whilst supporting the existing routine microscopic methods. This presentation describes how these probes were developed, assessed for specificity and cross reactivity, method development and validation. The results obtained from field samples from the monitoring programme and coastal survey samples will be presented with particular emphasis on spatial and temporal species composition and distribution.

Introduction

There are a number of difficulties encountered when observing species of the *Pseudo*-nitzschia and *Azadinium* genera under light microscopy, as taxonomic identification at best may only be described with certainty to genus level, and as both genera have non-toxin and toxic producing species, it can be difficult to differentiate between species within the same genus and to predict the potential onset of a toxic event. Molecular methods such as real time PCR and CARD-FISH techniques have been previously shown to be able to identify and distinguish between the varying species of each species. These methods have been assessed, validated and implemented into the National Monitoring Programme for phytoplankton analysis in Ireland as a supplementary method to routine light microscopy.

Materials and Methods

A real time qPCR method using TaqMan Minor Groove Binding Molecular hydrolysis probes and primers for the detection of 3 *Azadinium* species; *A. spinosum, A. poporum* and *A. obesum* were designed and tested by Alfred Wegner Institute, Bremerhaven Germany.

A method incorporating melt peak analysis (T_m) using hybridisation probes and primers for the detection of 6 *Pseudo-nitzschia* species; *P. australis, P. pungens, P. multiseries, P. delicatissma, P. fraudalenta* and *P. seriata* were designed and tested by the collaboration project PHYTOTEST (National Diagnostics Centre, National University of Ireland Galway and the Marine Institute).

Survey samples were collected by Niskin bottles mounted on a CTD, triggered at varying depths, filtered and fractionated into different size fragments from > 120µm through to 3µm TSTP filter papers.

Routine samples taken as part of the National Monitoring programme for phytoplankton were collected as surface or depth samples (via lund tube) in 30ml sterilin bottles, submitted as either preserved with Lugol's Iodine or as live samples.
Genomic DNA extractions were conducted via Qiagen Dneasy Plant Mini kits and the resulting elutants analysed with a Roche LightCycler 480 realtime PCR instrument.

Results

DNA extracts from phytoplankton samples taken at the time of an ASP event during Apr – May 2014 in the SouthWest of Ireland analysed were observed to contain the presence of the Domoic Acid toxin producing causative organism *P. australis*. Other know toxin producing species, *P. seriata* and *P. multiseries* were not present in any of the extracts analysed.
A number of Azaspiracid events were observed during 2013 to 2014 to date, where Azaspiracids were observed to be present in samples of shellfish. Corresponding phytoplankton samples taken and analysed from these affected sites revealed the presence of the Azaspiracid toxin producing organism *A. spinosum* in nearly all of the DNA samples analysed. *A. obesum* (non –toxin producing species) & *A. poporum* (unconfirmed toxin producing species) were only sporadically observed, usually at very low concentrations
Since 2012, 2 surveys have been conducted looking at the presence and distribution of *Azadinium spp.*. In 2012 offshore phytoplankton samples were collected around the coast of Ireland, and in 2014 a transatlantic survey from Newfoundland to Ireland was conducted. It was observed that in nearly all of the 3µm TSTP filter samples collected from around the coast of Ireland showed the presence of *A. spinosum*. It was also observed to be present at lower concentrations in larger size fractions in a smaller number of stations. The transatlantic survey also revealed the presence of *A. spinosum* on 3µm TSTP filters, and only occasionally observed in other size fractions.

Discussion

The presence and distribution of *A. spinosum* will be discussed during the presentation, as well as a breakdown of the *Pseudo-nitzschia spp.* present in Irish Coastal waters.