FINAL REPORT

DEVELOPMENT AND ASSESSMENT OF SPECIFIC PROBES FOR THE DETECTION AND MONITORING OF TOXIN-PRODUCING PHYTOPLANKTON SPECIES IN SCOTTISH WATERS

FS241058

AUGUST 2013

SCOTTISH ASSOCIATION FOR MARINE SCIENCE
MARINE SCOTLAND SCIENCE
ALFRED WEGENER INSTITUTE

Summary

Molecular methods to identify and enumerate two harmful algal species of importance in Scottish waters were assessed.

The target species were *Alexandrium tamarense* and *Azadinium spinosum*. In Scottish waters *A. tamarense* exists as two strains: the highly toxic North American (Group I) ribotype and the non-toxic Western-European (Group III) ribotype. These are morphologically identical preventing their discrimination using the light microscopy methods currently employed in regulatory monitoring. *Azadinium spinosum* has only recently (in 2009) been identified as a biotoxin (azaspiracids - AZA) producer and is too small and morphologically indistinct to be routinely identified by light microscopy using standard regulatory monitoring techniques.

A previously developed fluorescence *in situ* hybridisation (FISH) method for the identification of group I and group III *A. tamarense* (Touzet et al. 2010) was optimised in this study. Cross-reactivity with other organisms was verified to be low.

Methods of cell fixation post sample collection were assessed. No suitable fixative was found that would allow FISH methodology to be used routinely within a regulatory monitoring programme. Transport of unfixed water samples is therefore recommended. It is suggested that the FISH methodology is more suitable for identification of key bloom events rather than routine use on all samples.

Three methods of detecting hybridised *A. tamarense* cells were assessed: fluorescence microscopy, flow cytometry and FlowCam. Of these, the fluorescence microscopy technique was found to be the most robust and time/cost effective.

The development of a FISH probe for A*. spinosum* was completed in this study. Given that a routine method of *A. spinosum* detection is required for all samples collected in the Official Control monitoring programme, the issue of a lack of a suitable fixative for use within the programme were was regarded as an insurmountable for this method/species. An alternative catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) method was considered, but this would be markedly more time consuming and expensive to implement.

Given the difficulties in implementing a FISH based method for *A. spinosum*, effort turned to the development of a quantitative polymerase chain reaction (qPCR) method for cell detection. This was successfully tested on cells from laboratory cultures of *A. spinosum*.

Field testing was hampered by relatively low *A. spinosum* densities at test sites, with AZA concentrations determined during regulatory monitoring being below the limit of detection. However, finite AZA concentrations were found in solid phase absorption toxin tracking (SPATT)

bags deployments. These coincided with finite *A. spinosum* abundances as assessed by qPCR, suggesting the method was operating successfully and with high sensitivity.

However, given all field *A. spinosum* cell concentrations were low during this study, further method testing on higher density *A. spinosum* blooms coordinated with SPATT bag deployment is recommended for complete method testing.

SPATT bag deployments were made at two contrasting sites (in Shetland and the Western Isles) to assess the concentration and profile of AZA toxins in the water column over a fourteen month cycle. AZA1 and AZA2 were the two main analogues detected at the two monitored sites. AZA3 was also detected when concentrations of AZA1 and AZA2 were significantly higher in the SPATTs. AZA4 and AZA5 were not detected in the passive samplers even when significant levels of AZA1 and AZA2 were detected, which seems to confirm that these toxins are not produced by A*. spinosum*.

Index

Glossary

AFC: analytical flow cytometry

AZA: azaspiracids

BEH: Ethylene Bridged Hybrid

BV: Basta Voe

CARD-FISH: catalyzed reporter deposition fluorescence *in situ* hybridization

CCAP: Culture Collection of Algae and Protozoa

Cy3: fluorescent cyanine dye

DAPI: 4',6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

DSP: diarrhetic shellfish poisoning

EOL: East of Linga

FISH: fluorescence *in situ* hybridisation

FITC: Fluorescein isothiocyanate

FL1: AFC 1st Fluorescence detector (515-545 nm)

FSC: AFC forward light scatter

IFREMER: French Research Institute for Exploitation of the Sea

IGEPAL: octylphenoxypolyethoxyethanol

LC-MS: liquid chromatography-mass spectrometry

LSU rRNA: large subunit ribosomal RNA

MRM: multiple reaction monitoring

MS: mass spectrometry

OC: Official control

NERC: Natural Environment Research Council

NTC: No template controls

NUIG: National University of Ireland, Galway

PSP: paralytic shellfish poisoning

PVDF: centrifugal filter: polyvinylidene difluoride

qPCR: quantitative polymerase chain reaction

RMP: representative monitoring point

RNA: Ribonucleic acid

SET: Saline, EDTA, Tris

SPATT: solid phase absorption toxin tracking

SS: Stream Sound

SSC: AFC side light scatter

STX: saxitoxins

SV: Sandsound Voe

TamA: Oligonucleotide probe for group I *A. tamarense*

TamToxC: Oligonucleotide probe for group III *A. tamarense*

TaqMan: hydrolysis probe

UHPLC: Ultra high pressure liquid chromatography

Background

The enumeration of biotoxin producing phytoplankton in classified shellfish production waters is a regulatory requirement (EC 854/2004 as amended).

In Scottish waters two of the biotoxin producing genera of greatest concern are *Alexandrium* and *Azadinium*. Both organisms produce biotoxins that can accumulate with shellfish flesh, posing a health risk to human consumers.

Alexandrium in Scotland was historically thought to be dominated by the highly toxic North American (group I) ribotype of *Alexandrium tamarense.* Its presence is a significant threat to human health. However, recent studies have demonstrated the co-occurrence of the non-toxic Western European (group III) ribotype (Touzet et al. 2010). As these strains are morphologically identical it is no longer possible to relate *Alexandrium* blooms to subsequent shellfish toxicity with confidence within the current FSA Official Control (OC) phytoplankton monitoring programme

The toxicity generated by *Azadinium* is less severe than that generated by *Alexandrium*. However, negative human health effects are still potentially significant. This organism is recently discovered and too small and morphologically indistinct to be monitored by light microscopy. This organism is therefore not enumerated within the FSA OC phytoplankton monitoring programme.

The methodological difficulties highlighted above prevent effective monitoring of both organisms. This project therefore sought to develop and apply molecular approaches to allow better detection and enumeration of key species within both genera.

Alexandrium

Through their production of saxitoxins (STXs) and related compounds, dinoflagellates of the genus *Alexandrium* are a significant threat to human health. While a range of *Alexandrium* species are present in Scottish waters, by far the most abundant is *A. tamarense* (Figure 1). The threat posed by *Alexandrium* is therefore particularly acute in Scottish waters due to the high toxicity of some strains of *A. tamarense*. This has led to a phytoplankton alert trigger level (the density of cells that is through to present a risk of a shellfish toxicity event) of the presence of a single *Alexandrium* cell in a sample. Although operationally, a density greater than ~250 cells L^{-1} is likely to be required to cause significant concerns of impending shellfish paralytic shellfish poisoning (PSP) positive shellfish flesh samples.

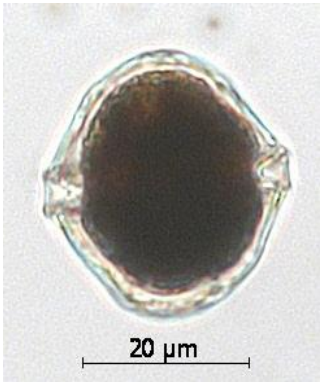


Figure 1: *Alexandrium tamarense*

Analysis of phytoplankton and shellfish flesh toxicity data over the seven years of SAMS' operation of the OC phytoplankton monitoring programme in Scottish waters has often indicated good relationships between *Alexandrium* cell counts and determination of STX within the shellfish flesh (Davidson et al. 2008), potentially allowing *Alexandrium* abundance to be used as a predictor of future toxicity (Figure 2). However, on a significant number of occasions high *Alexandrium* counts did not correspond to elevated shellfish toxicity.

The most plausible explanation for these discrepancies from the expected pattern of elevated *Alexandrium* abundance leading to elevated toxicity, is related to the relative abundance of the different toxic and non-toxic strains of *A. tamarense* that are now thought to be present within Scottish waters.

The *A. tamarense* species complex is made up of five different evolutionary lineages, or ribotypes, indicative of the geographic origin of populations (Lilly et al. 2007). Of these, two forms are present in Scottish waters, the North American (NA) group I and the Western European (WE) group III ribotypes (Collin et al. 2009, Touzet et al. 2010). The group I ribotype is known to produce PSP toxins, whereas the group III ribotype does not.

As these two strains are indistinguishable by light microscopy, their co-occurrence makes it difficult to use overall *Alexandrium* abundance measured by light microscopy as an early warning of future toxicity.

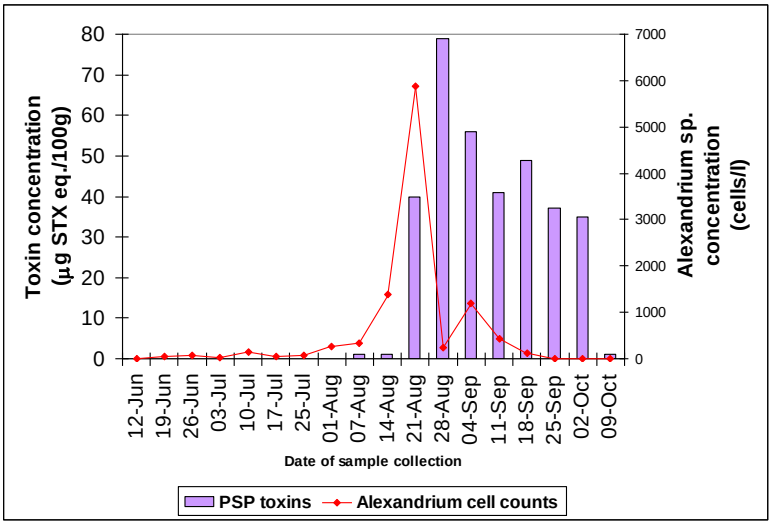


Figure 2: increase in *Alexandrium* preceding an increase in shellfish toxicity

Azadinium

In the Netherlands in 1995 cases of human poisoning resulted from the consumption of cultivated blue mussels from Killary Harbour, Ireland (McMahon & Silke 1996). The symptoms were typical of diarrhetic shellfish poisoning (DSP), caused mainly by lipophilic compounds produced by the dinoflagellate genus *Dinophysis*. However, chemical analysis of contaminated mussels found only insignificant levels of DSP toxins. The causative toxin was later isolated and structurally characterised and named azaspiracid (AZA, Frederick et al. 2007). Since its discovery, AZA and its analogues have been detected in shellfish from a number of European countries including Scotland.

Initially, the causative phytoplankter was thought to be the dinoflagellate *Protoperidinium crassipes* (James et al. 2003). However, Tillman et al. (2009) subsequently demonstrated the small dinoflagellate *Azadinium spinosum* (Figure 3) to be the AZA producing organism, with *P. crassipes* (and potentially other heterotrophic dinoflagellates) thought to vector the toxin through ingestion of *A. spinosum*.

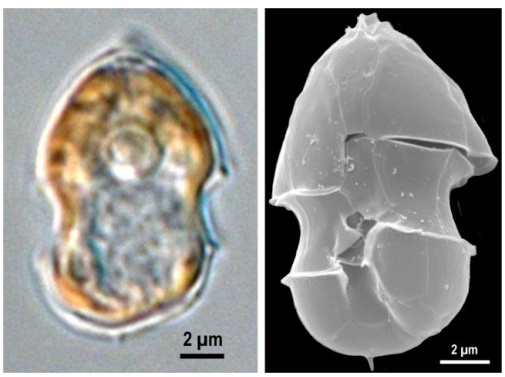


Figure 3: *Azadinium spinosum* (photo U. Tillman)

The discovery that *A. spinosum* is an AZA producer presents a challenge for regulatory monitoring as this species is too small and morphologically indistinct to be routinely identified and enumerated using the light microscopy techniques and magnifications that are typical of most monitoring programmes.

Project aims:

- To further develop and trial existing fluorescent in-situ hybridisation (FISH) probes for the detection of PSP producing strains of *Alexandrium tamarense.*
- To develop and assess auditability for use within OC monitoring of specific molecular methods (FISH or quantitative Polymerase Chain Reaction (qPCR) based) for detection and monitoring of azaspiracid producing *Azadinium spinosum.*
- To determine the seasonal abundance of Azaspiracid toxins at selected sites in Scottish coastal water.

Project structure

Given the difficulties in enumerating the harmful group I *A. tamarense* and *A. spinosum* by light microscopy, molecular methods are considered as potential alternative. In the current OC phytoplankton monitoring programme, approaches to operationalize these methods are not yet fully developed. The project therefore aimed to develop and test molecular methods for the detection and enumeration of *A. tamarense* ribotypes and of the species *A. Spinosum*.

The work undertaken in the project was divided into 7 objectives:

Objectives 1-3 related to the refinement and testing of *A. tamarense* fluorescence *in situ* hybridization (FISH) probes (previously developed as part of the Food Standards Agency in Scotland (FSAS) funded project S14044) and associated methods of enumeration of the hybridized fluorescently labeled cells.

Objectives 4-5 initially related to the development of a FISH-probe base method for *A. spinosum* enumeration. However, early in the project it became clear that a qPCR method would be more appropriate and effort was directed to development of that approach.

Objective 6 related to trial application of both the *A. tamarense* and the *A. spinosum* enumeration methods on field samples.

Objective 7 related to the analytical determination of temporal changes in AZA concentrations in the water column at two contrasting test site, using SPATT bags and subsequent liquid chromatography-mass spectrometry (LC-MS) detection.

Verification of the lack of cross-reactivity of fluorescence *in situ* hybridisation (FISH) probes for toxic and non-toxic *Alexandrium tamarense* (objective 1).

Fluorescence *in situ* hybridization (FISH) methodology has previously been developed for *A. tamarense* at SAMS in Scotland, through a FSAS funded project (S14044, Davidson et al. 2009) that was conducted in conjunction with the National University of Ireland Galway (NUIG) on this organism.

The taxa specific oligonucleotide probes for identification of toxic (North American, Group I) and non-toxic (Western European, Group III) *Alexandrium tamarense* were TamA and TamToxC, respectively (MWG-Biotech) (Touzet et al. 2010).

Application of these probes in Scottish waters was conducted during a field study in the Shetland and Orkney Isles by SAMS/NUIG (and IFREMER), funded through the EU Interreg IIIB project "FINAL" (Touzet et al. 2010) and conducted in 2007. That study demonstrated the probes' suitability for use in Scottish waters. However, it was critical to establish that these probes do not cross-react and detect non target cells, producing in false positive results. The work carried out under objective 1 sought to confirm this.

METHOD

The sample preparation and analysis method used in the previous study (FSA project S14044), was published by Touzet et al. (2010). In this method, cells within water samples were collected on 5 µm mesh, backwashed into 50 ml centrifuge tubes and fixed with 1% formalin. Samples were then centrifuged, discarding the supernatant, and 100% ice cold methanol was added to the pellets. At this stage samples can be stored frozen in centrifuge tubes for in excess of 3 years.

1.1 Preparation of methanol fixed samples for analysis

Samples were centrifuged (4000*g* for 5 min) and the methanol supernatant aspirated off. Hybridisation buffer (5X SET and 0.1% IGEPAL) was added (500 µl) to the cell pellet and the cells are re-suspended to rinse off the methanol. Subsequently, samples were centrifuged (4000*g* for 5 min) and the supernatant removed. The process was then repeated to ensure that all of the methanol was removed from the sample. A 500 µl aliquot of hybridisation buffer containing 1 µl of either TamToxC or TamA was added to each sample. After this time the samples were kept in the dark by wrapping them in aluminium foil and the cells were re-suspended and incubated in the dark (55 °C for 60 min).

After hybridisation the cells were pelleted by centrifugation (4000*g* for 5 min) and the supernatant discarded by aspiration. The samples were then washed with 500 µl of preheated (55 °C) 0.2X SET buffer to remove the excess unbound probes. The 0.2X SET buffer was heated along with the samples in an incubator. The samples were then centrifuged (4000*g* for 5 min) a final time and the supernatant removed. The cells were then re-suspended in 2.5 ml of autoclaved filtered seawater for subsequent enumeration (in the Touzet et al. 2010 method by fluorescence microscopy).

Improvements to the current FISH method

Prior to the cross-reactivity check, two improvements were made to the sample processing and hybridization protocols to allow more rapid sample processing and easier subsequent cell detection/identification.

Improvements to the cell collection protocol

The sample collection and backwash protocol in the Touzet et al. (2010) method is time consuming, particularly when a large bloom of non-target cells occur, this clogs the filter and markedly slows down filtering rates. Typically samples can take 30-40 minutes to filter, with some that contained a particularly high phytoplankton biomass taking up to two hours.

To speed up the sample processing step, we took advantage of developments made within a parallel National Environmental Research Council (NERC) funded PhD project at SAMS (Lisa Eckford-Soper) which developed an alternative cell collection method. In this revised method, water cells were collected by centrifugation rather than filtration. In the revised method, samples were fixed with formalin (1% final concentration) for one hour before being centrifuged (4000*g* for 10 min). The supernatant was then discarded. Ice cold methanol (10 ml) was then added to the cell pellet to extract the pigments. The sample could then be stored at -20 °C if necessary.

Improvements to hybridization protocol

Initial hybridizations of the two *Alexandrium* probes to positive control *A. tamarense* cells (group I and group III *A. tamarense*) resulted in poor resolution between positive and negative hybridizations (data not shown). This was found to be due to incomplete removal of the cell fixative (formaldehyde).

To overcome this problem the standard published hybridization protocol (Touzet et. al. 2010) was amended to include two additional methanol washes immediately post the sample fixation step. This has resulted in clear resolution of positive and negative hybridization when viewed by fluorescence microscope.

ASSESSMENT OF THE CROSS-REACTIVITY OF *A. tamarense* FISH PROBES

Two methods were applied to assess the degree of cross-reactivity of the FISH probes with other species that could co-occur with *A. tamarense* in Scottish waters.

In silico analysis

The publically available computer software suite ARB (Ludwig et al. 2004) was loaded with a pre-compiled large subunit ribosomal RNA (LSU rRNA) data base (available at: http://www.arb-silva.de/download/arb-files/), which contains all publically available LSU rRNA sequences. ARB's 'Probe Match' module was used to query each of the two *A. tamarense* probes against all other LSU rRNAs based on varying stringency (~accuracy) (Figure 4).

Using a low stringency criteria of 3 mis-matches, the TamToxC probe (toxic *A. tamarense*) did not detect any other *Alexandrium* species, dinoflagellate or other algal sequences (Supplementary Information (SI) Figure 1A). The non-toxic probe (TamA) showed some cross-reactivity with some toxic *A. tamarense* (SI Figure 1B), potentially resulting in some false positive non-toxic *A. tamarense* signals. However, the false positive results were not observed in practice during this study nor by Touzet et al. (2010). The TamA probe did not show any cross-reactivity to any other dinoflagellate or other algal LSU rRNA sequence.

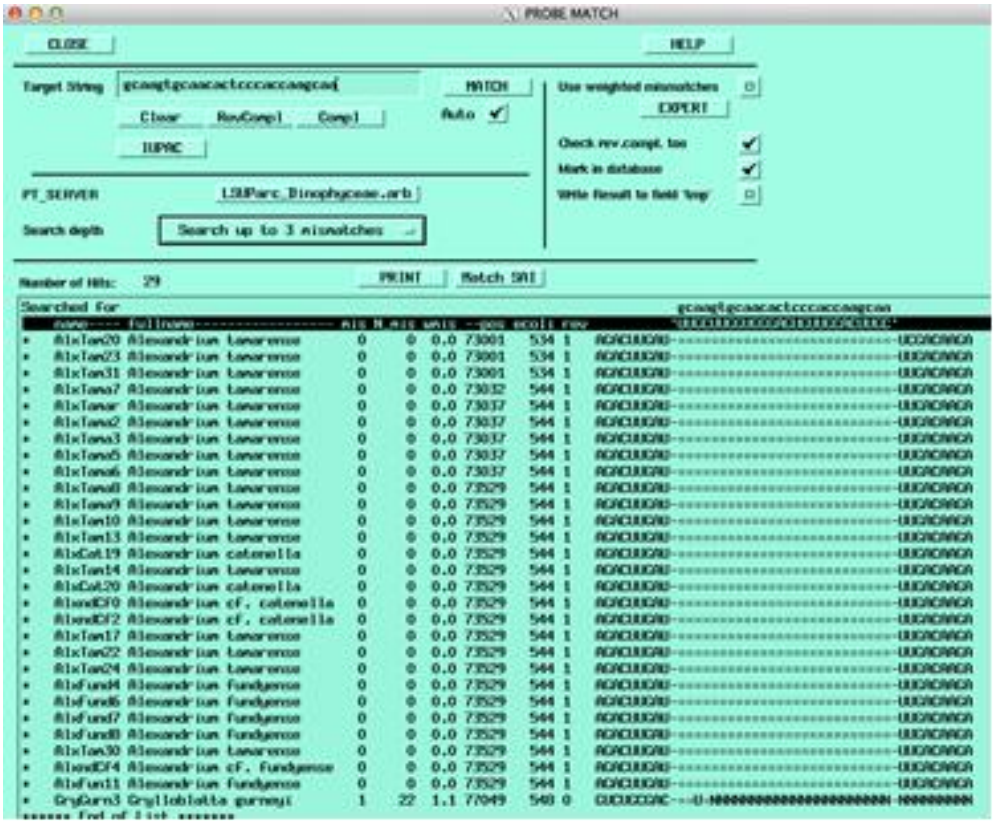


Figure 4: *In silico* analysis of cross-reactivity of *A. tamarense* FISH probes.

FISH analysis

Toxic and non-toxic probes were combined and used to test probe cross-reactivity against a range of dinoflagellates that were available at SAMS and that represent the types of dinoflagellate groups encountered in Scottish waters. Toxic and non-toxic strains of *A. tamarense* were used as controls. The FISH protocol used was as described above.

The toxic *A. tamarense* probe (ToxTamC) did not cross-react with any of the dinoflagellates, corroborating the lack of cross-reactivity of this probe by *in silico* analysis (above).

The non-toxic probe (TamA) gave a faint signal with some of the dinoflagellates (Table 2). Increasing the stringency of the washing step(s) post hybridization may correct what appears to be a low level of cross-reactivity. However, given that cross-reactivity was weak and would not result in the (mis) identification of toxic cells when viewed by microscope such steps are not thought to be required. Moreover, the shape and size differences of any cross-reacting species, with the exception of *A. ostenfeldii*, would be clearly identifiable evident to the observer during microscopy.

Table 2: Summary of FISH cross-reactivity

Dinoflagellate	Strain[a]	ToxTamC	TamA
Amphidinium carterae	1102/2	-	w[b]
Amphidinium carterae	1102/3	-	w
Ceratium horridum	1110/6	-	-
Alexandrium ostenfeldii	1119/45	-	w
Alexandrium minutum	1119/47	-	-
Alexandrium minutum	1119/48	-	-
Alexandrium sp.	1119/50	-	-
Scrippsiella trochoidea	1134/1	-	-
Scrippsiella sp.	1134/8	-	-
Prorocentrum minimum	1136/16	-	-
Prorocentrum sp.	1136/17	-	w
Prorocentrum micans	1136/20	-	-
Gymnodinium sp.	CCAP (unclassified)	-	-
Gymnodinium catenatum	GCHU11	-	-
Gymnodinium catenatum	YC499B15	-	-

[a] Strain numbers relate to Culture Collection of Algae and Protozoa (CCAP) strains or are part of a SAMS research culture collection.
[b] w, weak hybridization signal.

Diatom species cross-reactivity was not tested because the shape and size of diatoms is significantly different to that of *A. tamarense*, and any cross-reactivity, if it did occur, would be clearly evident to the observer during microscopy. Furthermore, *A. tamarense* FISH analysis of field samples has shown no evidence of cross-reactivity to diatoms.

Summary

In silico analysis predicted no cross-reactivity of the TamToxC probe against any other known organisms, and only low-stringency cross-reactivity of the TamA probe against some toxic *A. tamarense*. The actual use both probes in FISH assay against toxic and non-toxic *A. tamarense* showed no cross-reactivity. No cross-reactivity of the TamToxC probe was observed with any other dinoflagellate. A weak signal of the TamA was observed, but was sufficiently weak as to not be scored as positive.

Recommendation

The modified Touzet et al. (2010) FISH probe method was found to have minimal cross-reactivity to other organisms and hence to be suitable for the enumeration of, and discrimination between, group I and group III *A. tamarense* in Scottish waters using fluorescence microscope based detection.

Evaluation of the effectiveness of different fixatives and their use to facilitate *A. tamarense* sample storage during transport to the monitoring laboratory for routine use of FISH probe methodology (objective 2)

The principal fixative used with FISH is formaldehyde because it causes minimal background fluorescence that could interfere with the FISH signal. However, formaldehyde or similar fixatives (e.g. glutaraldehyde) are highly toxic and unsuitable for handling by untrained workers and use in field conditions, or to be posted out using mail or courier delivery services. Therefore an alternative fixative is required if the method is to be used within a regulatory monitoring context.

Suitable alternatives to fix and preserve biological material for FISH analysis could be ethanol and methanol. Due to their flammability, toxicity and cost (due to the volume of alcohol required per sample ≥50 % by volume), they are not suitable for routine field sample collection.

Another alternative fixative available is acidified Lugol's iodine solution which is the least toxic of fixatives and is currently used for field sample collection and preservation in the current OC phytoplankton monitoring programme. However, the presence of acetic acid in the Lugol's iodine solution causes a chemical modification (acetylation) of the rRNA which affects the binding of FISH probes and results in a poor fluorescent signal. Consequently, acidified Lugol's iodine solution is also unsuitable for use as a fixative for samples prepared for FISH analysis.

Subsequently, a non-acidic Lugol's iodine method of cell fixation was trialed, using Lugol's iodine solution not acidified with acetic acid.

To test the suitability of non-acidified Lugol's iodine solution for application in FISH, both toxic and non-toxic *A. tamarense* were fixed with non-acidified solution (using formaldehyde as a control) as per the standard *A. tamarense* FISH protocol.

Fixation of cell with non-acidified Lugol's solution resulted in a strong red fluorescent signal, when viewed for Cy3 labeled probes, and an orange fluorescent signal when viewed for fluorescein isothiocyanate (FITC) (Figure 5). Thus, non-acidified Lugol's fixation resulted in false-positive signals for toxic *A. tamarense*, as toxic cell detected by hybridised FISH probe also emit red fluorescence.

Emission of the orange signal by cells fixed by non-acidified Lugol's solution is less of a problem in the case of the non-toxic *A. tamarense*, where green signal is emitted by positive probe hybridization would not be easily confused with the orange signal emitted by non-target cells fixed with non- acidified Lugol's solution.

It was thought that removal of non-acidified Lugol's iodine solution from the fixed material, could improve the hybridisation. Currently, there is no standard procedure for removing Lugol's iodine solution from fixed cellular material. The only procedure previously used has been in conjunction

with polymerase chain reaction amplification of dinoflagellate DNA (Auinger et al. 2008). This procedure used a dilute solution of sodium thiosulfate to wash the dinoflagellate cells prior to DNA amplification.

We have tried using sodium thiosulfate solutions to wash Lugol's fixed *A. tamarense* cells prior to hybridization, using several different wash durations. In all cases, the thiosulfate solution was not effective in reducing the background signal caused by Lugol's iodine fixation.

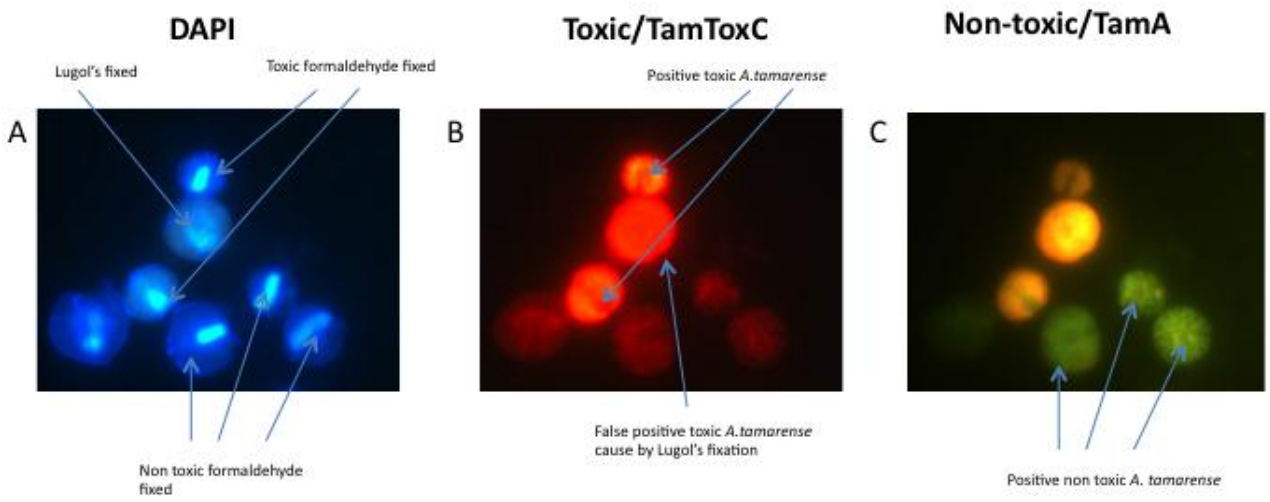


Figure 5: Lugol's iodine interference of *A. tamarense* FISH analysis. Individual populations of toxic and non-toxic *A. tamarense* were fixed with formaldehyde or Lugol's and sub-samples of each cell population mixed together and hybridized with both probes; to identify individual cell signals, individual fixed cell populations were also hybridised. Panels A-C show an example of an interpreted analysis of the mixed samples. **(A)** DAPI staining of DNA and identification of toxic and non-toxic cells fixed with Lugol's iodine or formaldehyde; **(B)** Cy3 fluorescence imaging of the same cells as panel A showing positive FISH for toxic *A. tamarense*, but also very strong false-positive caused by Lugol's fixation; **(C)** FITC fluorescence imaging of the same cells as panel A, showing characteristic green fluorescence of positive non-toxic *A. tamarense*, as well as strong orange fluorescence caused by Lugol's fixation. As the signal is not green, it is easy to not confuse it as a false-positive signal.

Given the difficulties with sample fixation in the field, an alternative approach is the transport of live, unfixed samples. These would first be pre-screened through a 100 µm mesh to remove grazers and then posted unfixed in volumes of 1L to the laboratory for analysis. However, the on-site screening of samples as well as the costs associated with transportation of large volumes (1L) of water could lead to operation difficulties if this method was to be applied in the routine monitoring.

Another issues associated with transport of unfixed samples relates to the possibility of loss of cell material due to grazing. However, previous FSAS funded study (S14044, Davidson et al. 2009) demonstrated that loss of unfixed *Alexandrium* in transit was minor for those samples tested.

During the present study, we attempted to repeat this experiment to verify the results. Pre-screened, unfixed samples of 1L volume were requested from monitoring sites Shetland in August 2012. Unfortunately, microscopy (on parallel Lugol's fixed samples) indicated that most of the samples contained no *Alexandrium* and those that did, contained only 20-40 cells L^{-1}. These densities equate to only 1 or 2 cells in a settling chamber and hence were too low to evaluate cell loss during sample transport.

Summary

No suitable fixatives that would allow safe on site fixation and postal transport of cells, was identified for routine, in the field, application by sampling officers.

Transporting screened but unfixed cells was found to be a viable alternative. Although, some cell loss during sample transport is to be expected. Previous results suggest that cell loss in transport was minor. Hence, although results would be qualitative rather than quantitative it would be possible to process unfixed water samples received at the laboratory and characterise the bloom as potentially toxic or not depending on the composition of group I and group III cells contained within it.

Recommendation

Given the operational difficulties of on-site screening and the costs of transporting relatively large volumes of water, with the possibility of some cells loss during transport, the *Alexandrium* FISH method in not recommended for routine use to characterise the *Alexandrium* community at all phytoplankton monitoring sites.

Rather, we recommend that the method is used opportunistically, triggered by results obtained in standard light microscopy based regulatory monitoring counts exceeding some pre-determined threshold and/or a specific rate of increase. Such an approach would allow important developing blooms to be categorized as group I or group III when thought necessary. An inspection of OC phytoplankton and shellfish biotoxin data sets suggests a concentration threshold of ~ 250 cells L^{-1} might be suitable. However, a more robust estimate would require a more detailed data analysis that was possible within the scope of this project.

Evaluation of the relative speed, accuracy and cost of different methods of cell enumeration of FISH probe labelled *A. tamarense* (fluorescence microscopy, flow cytometry, FlowCam) (objective 3)

Fluorescence Microscopy

Following the refinements in the hybridization protocols described above (objective 1) the ease of analysis of probed samples by fluorescence microscopy has been much improved. Cells hybridized with both the toxic *A. tamarense* probe (ToxTamC) and the non-toxic probe (TamA) are now clearly distinguishable by fluorescence microscopy (Figure 6), reducing the time required for analysis per sample.

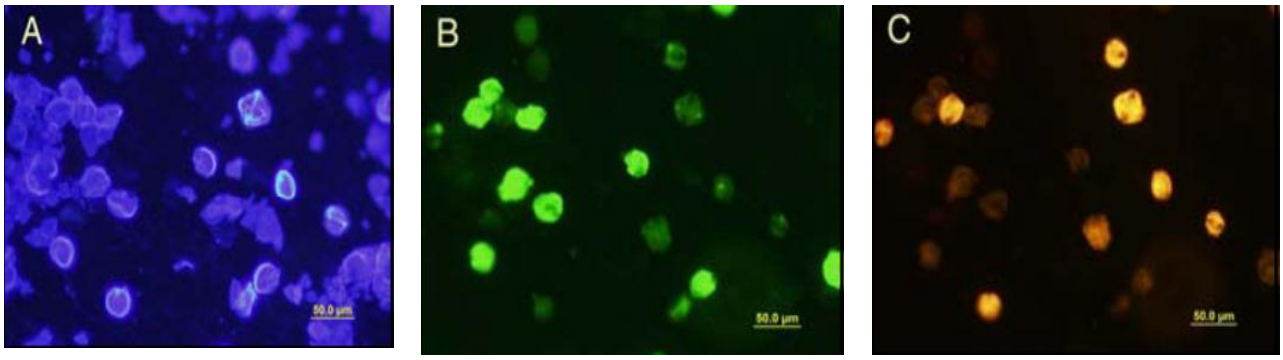


Figure 6: FISH identification of Group I and III *A. tamarense* cells within a mixed species sample. (**A**) Calcofluor fluorescence of whole *A. tamarense* cells; (**B**) FITC fluorescence signal identifying non-toxic A. tamarense and; (**C**) Cy3 fluorescence signal identifying toxic *A. tamarense*.

While analysis time depends on the number of *Alexandrium* cells in a sample, it is typically 30-40 minutes per sample. This method has the added advantage that any cross-reactivity to non *Alexandrium* cells can be identified visually by the microscopist.

Flow Cytometry

Analytical flow cytometry (AFC) is a popular tool for the rapid identification and enumeration of different populations in mixed microbial communities (Sekar et al. 2004). It involves the direction a beam of laser light onto a hydro-dynamically focussed stream of liquid containing the cells of interest. Multi parameter discrimination and enumeration of cells is then based on the forward angle scatter (FSC) (0.5-5°), side angle light scatter (SSC) (15-150°), and fluorescence at a range of wavelengths. AFC provides the user with the ability to distinguish between particles of a similar size which have differing optical properties allowing for several groups to be simultaneously detected. Detection and discrimination is typically based on bi-plots of light scatter,

autofluorescence or laser light excitation of fluorescent stains or probes (Sekar et al. 2004; Kalyuzhnaya et al. 2006).

The use of flow cytometry in combination with FISH based fluorescent labeling offers the potential for more rapid detection and enumeration of cells than can be achieved by light microscopy.

To evaluate the potential of this methodology, hybridized samples of both group I and group III *A. tamarense* (CCAP 1119/24 and CCAP 1119/28) were analysed on a FACSort (Beckton Dickinson) AFC at SAMS. This instrument contains a 488 nm laser and detectors for green, orange and red fluorescence.

The sheath fluid (that allows hydrodynamic focussing of cells in the laser beam) used was FACSFlow (BD Biosciences). Detection and discrimination of group I and group III *A. tamarense* was achieved based on side scatter (SSC) and fluorescence detection of the FITC labelled TamToxC (R2) and Cy3 labelled TamA (R3) probes within the FL1 channel (Figure 7).

The fluorochromes (FITC and Cy3) used were chosen as they are suitable for fluorescent microscope detection. FITC (excitation 490 nm emission 525 nm) is suitable for use with a 488 nm laser. While the main excitation and emission wavelengths of Cy3 are not suitable for our AFC set up, secondary excitation and emission at 512 nm and 615 nm respectively allows for excitation and detection. This set up was therefore found to be capable of exciting, detecting, and discriminating between group I and group III *A. tamarense* cells, with the added advantage of allowing the operator to easily change to the fluorescence microscopy method if necessary.

The AFC flow rate was calculated on each day of use using a 1 µm Fluoresbrite latex bead solution (Polysciences, UK). The bead solution was made up by adding 4 µl of 1 µm beads to 20 ml of FacsFlow solution, filtering it through a 2 µm, 25 mm membrane filter (Poretics, USA) into a further 380 ml of FacsFlow solution and mixing by carefully inverting. A 3 ml aliquot of this solution was then removed via a 5 ml syringe and the syringe placed in a syringe pump (WPI, USA) connected to the flow cytometer. The flow rate of the syringe pump was increased from 0.54 ml h^{-1} to 3.28 ml h^{-1} and the number of bead incidences counted for 30s, 60s and 90s at each flow rate.

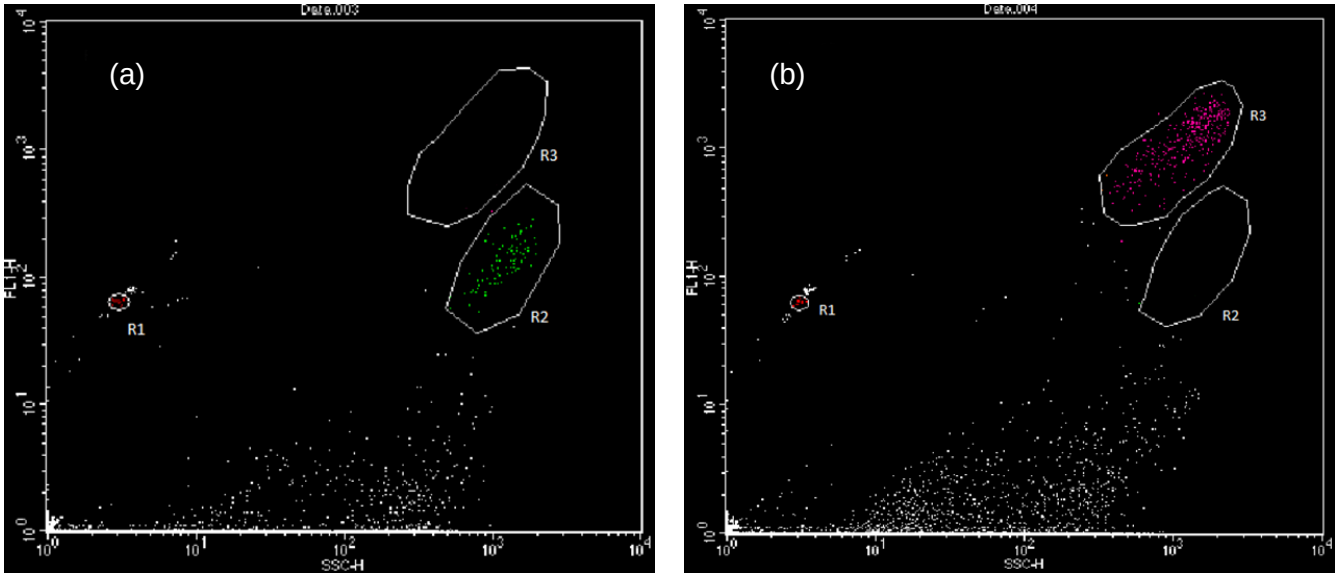


Figure 7: AFC dot-plot from cultured cells demonstrating the enumeration within gated regions of group I and group III *A. tamarense* (a) toxic strain with Cy3 labelled TamToxC probe (R2), and (b) non-toxic strain with FITC labelled TamA probe (R3). R1 is a gated region for the fluorescent latex beads of known concentration that are used to calibrate the instrument.

Comparison of cell counts obtained by AFC and those from microscopy were initially conducted using laboratory cultures. Group I (CCAP 1119/28) (Toxic) and Group III (CCAP 1119/31) (non-toxic) strains of *A. tamarense* that had been isolated by Marine Scotland Science from Scottish waters, and subsequently deposited in the Culture Collection of Algae and Protozoa (CCAP) at SAMS, were used. The cultures were grown separately in 1 litre Erlenmeyer flasks in L1 media at 15 °C under a light intensity of 100 µmol µE m^{-2} s^{-1} (12h:12h light:dark cycle).

A 50 ml aliquot of each culture was removed aseptically from exponentially growing *A. tamarense* Group I and III cultures. A 15 ml aliquot was kept as the 100% reference material with the remainder being serially diluted (to give a final volume of 15 ml) with cell-free culture media to achieve concentrations of 80, 50, 20, 10 and 5% of the initial concentration that spanned the range of typical densities of *Alexandrium* blooms in Scottish waters. The 100% reference sample was counted using light microscopy. A 1 ml aliquot from each dilution was removed aseptically and preserved with Lugol's iodine solution (1% final concentration) and counted using a 1 ml Sedgewick-Rafter counting chamber at 100x magnification by microscope (Leitz Wetzlar Orthoulux). This cell number was used to estimate the number of *Alexandrium* cells within the serial dilutions.

For AFC, three 1 ml aliquots were removed and analysed in triplicate with analysis being conducted rapidly (within two minutes) as the probes are quickly degraded by light. This could sometimes be problematic as clogging of the AFC was frequent and clearing of the orifice can sometimes be time consuming.

Comparison of cell counts achieved by microscopy and AFC at the different cell concentrations is presented in Figure 8. These results showed good agreement between the two methods for both group I and group III cells indicating the suitability of the AFC to detect FISH probed *A. tamarense* cells.

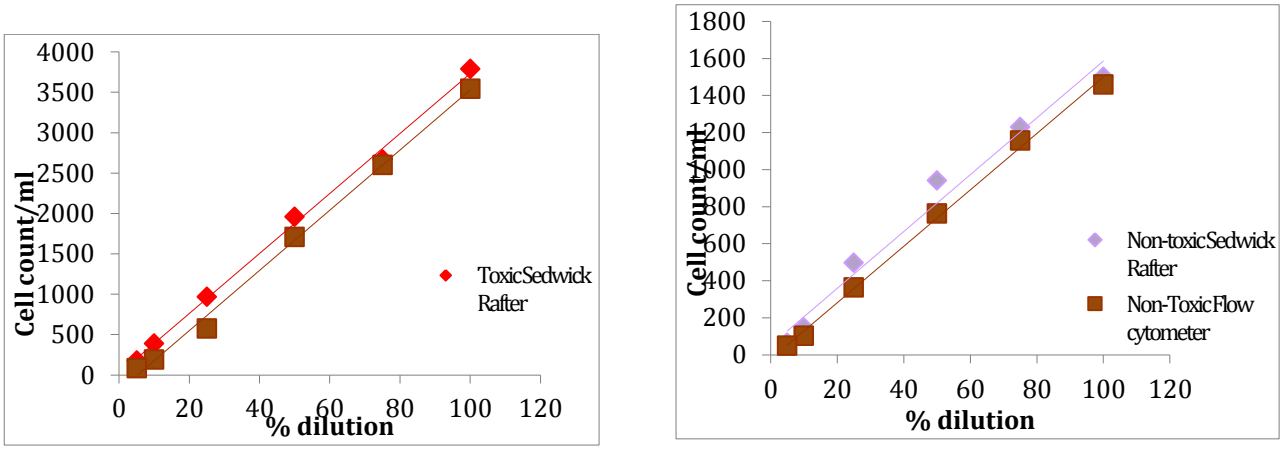


Figure 8: Comparison of cell counts by light microscopy (Sedgwick Rafter) and flow cytometry for both group I and group III cells.

FlowCam

A FlowCam (Fluid Imaging) is an automated particle analysis instrument capable of detecting laser excited fluorescence and which uses digital imaging for measuring size and shape of microscopic particles in a fluid medium. The FlowCam therefore operates in a similar manner to the AFC with the addition of digital imaging. FlowCam offers the potential to use a range of flow cell diameters (with associated magnifications), potentially reducing issues relating to sample clogging that are a concern in relation to the AFC. However, operation does not involve hydrodynamic focusing, thus raising questions over the ability to image the whole sample.

Initial study indicated that quantification of cell abundance on a standard FlowCam was difficult as it was not possible to calibrate the volume of liquid analysed in a similar way to the AFC. While it was possible to collect the analysed water and weigh it to determine the volume analysed, such an approach was not suitable for routine application. A syringe pump was therefore purchased that allowed a precise known volume of liquid to be processed by the instrument.

Initial analysis was conducted using fluorescent beads of a range of different flow cell/magnification combinations as listed in Table 2. During this process, a collaborative visit was made by SAMS' staff to the Fluid Imaging laboratories in Maine USA to compare SAMS's protocols to those used "in house" by the manufactures.

Table 2 Performance of the FlowCam with different flow cells.

Flow cell diameter (µm)	Magnification	Operational effectiveness
50	20x	Instrument triggered well based on fluorescence. Unable to generate accurate counts as only imaging ~ 25% of sample
100	10x	Instrument triggered well based on fluorescence. Counts of beads accurate at higher concentrations (~3000 particles per ml), but poor at lower concentrations
300 (Field of view)	4x	Instrument triggered well based on fluorescence. High density sample requires dilution to achieve densities of 1 particle per image. Counts of beads accurate at higher concentrations (~3000 particles per ml), but poor at lower concentrations.

Subsequent analysis was conducted using hybridized group I and group III *Alexandrium* cells. Again, it was possible to get the instrument to trigger based on fluorescence, but enumeration was poor when densities fell below ~ 3000 cells ml^{-1}.

To verify the above, results were related to abundance rather than signal detection, a range (20 fold difference) of probe concentration was used to prepare samples. This allowed investigation of any influence of the strength of labeling, and achieved similar results as above.

We conclude that the instrument is potentially suitable for detection of dense FISH probe labeled blooms of *A. tamarense*, but only at densities that far exceed those found in Scottish waters.

Analysis Time

The time for sample analysis was compared for the two potentially operational detection methods: fluorescence microscopy and AFC. Both methods require a similar amount of sample preparation time prior to analysis.

Given that samples are likely to arrive late morning by post it is expected that preparation and analysis will be a two day processes.

On day 1 sample preparation will require approximately 2 hours for centrifugation and fixation. Samples can then be stored in the freezer over-night for analysis next day.

On day 2 further centrifugation occurs before methanol removal and then an 3 hour incubation with the probe (with the potential to process a number of samples in parallel).

Subsequently, AFC allows for a much higher sample analysis throughput, as FISH-FC can quantify cells quickly (~ 60s) in comparison to the 30-40 minutes required per sample for analysis by microscope. However, this has to be set against the time required to set up and clean out the flow cytometer as this is greater than for microscope (~ 1.5 hrs per run). Clogging of the AFC orifice can also occur. This is sporadic and hence difficult to quantify but might be expected to add another 30 minutes to a run.

Summary

The FlowCam is still a developing instrument that has the potential for application in harmful algal detection, using both imaging and fluorescence detection. However, in its current state of development we were unable to produce reliable counts of *A. tamarense* at environmentally relevant concentrations. This instrument is therefore not recommended for use within the monitoring programme at the present time.

For a large number of samples the use of FlowCam may be most efficient, however, as our recommendation is that the *Alexandrium* FISH detection method only be used for occasional samples where group I/group III discrimination is thought necessary, it is most likely that small samples numbers will be analysed.

For these low sample numbers (≤~6), fluorescence microscopy is as time-effective as the analytical methods. It also has the advantage that cross-reactivity can be assessed visually.

Recommendation

Given the expected use of the method for low numbers of analyses, analysis by fluorescence microscopy is recommended as it is more time/cost efficient than automated methods and allows any cross-reactivity with non-target cells to be assessed visually.

Completion of the development of FISH probes for *Azadinium spinosum* and other *Azadinium* species (objective 4)

Azadinium probe development (AWI)

Development of FISH probes for *A. spinosum* and two further *Azadinium* species has been completed (SI Table 1). FISH probes were designed using ARB software and 'probe design' and 'probe match' sub-routines in ARB software. Each FISH probe targets the LSU rRNA gene of *Azadinium*. Protocols for FISH hybridization using standard FISH have been developed by the AWI. However, these suffer from the same issues of cell fixation as were evident for *Alexandrium*. In addition to fixative issues, the rRNA content and/or matrix effects of environmental samples mean that the standard FISH procedure would not be sufficiently sensitive to detect *Azadinium* in field sample material. Given that a routine method of *Azadinium* detection for application to all samples is required, standard *Azadinium* FISH was not pursued.

An alternative would be to use a higher sensitivity Catalyzed Reporter Deposition Fluorescence *In Situ* Hybridization (CARD-FISH) method to identify *Azadinium* from field collected samples. Such a method has been developed at the AWI.

CARD-FISH is able to reliably discriminate *Azadinium* in field material, but the procedure is more time-consuming (ca. double the time) and the reagents significantly more expensive (5-10x). Bearing in mind time and cost limitations, following discussion with the FSAS, the *Azadinium* component of the project was focused on a quantitative polymerase chain reaction (qPCR) method.

Azadinium qPCR development (AWI/SAMS)

Development of qPCR primers/probe combination has been completed (SI Table 2). The qPCR primers and TaqMan minor groove binding (MGB)-probes target the LSU rDNA of the three *Azadinium* species. They were designed with Primer Express software (Ver. 2.0.0 Applied Biosystems, Darmstadt, Germany) and the ARB software packaging and its sub-routine 'probe design'. The TaqMan MGB-probes are labeled with a 5' reporter dye (6FAM; 6-carboxyfluorescein, excitation wavelength 483 nm, emission wavelength 533 nm) and a 3' non-fluorescent quencher to alleviate background fluorescence. Incorporating MGB chemistry into the oligonucleotide probe enabled the probe to be shorter (more discriminatory) than traditional TaqMan probes, whilst maintaining the requisite annealing temperature. 28S rRNA gene PCR amplicons of different *Azadinium* species were used to construct standard curves. Real time qPCR assays were performed using a TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany). The qPCR primer concentration was optimised, each forward and reverse primers were added at a final concentration of 900 nM and probe added at 200 nM. Each primer and probe combination were run individually. The qPCR cycle conditions for the TaqMan MGB approach were: Stage 1,

50 °C for 2 min; Stage 2, 95 °C for 10 min; Stage 3, 40 cycles of 95 °C for 15 sec, 59 °C for 1 min. Instrumentation was an ABI PRISM 7000 SDS real time PCR thermocycler (Applied Biosystems, Darmstadt, Germany).

Azadinium qPCR assay refinement (SAMS)

Application of the above qPCR at SAMS involved a different thermocycler (Quantica, Techne, UK) and a different qPCR master mix supplier (Eurogentec, Belgium). In consequence, the thermocycling profile was altered according to the manufacturer's recommended protocol and experiments showing that the primer annealing temperature should be 62˚C. We identified that using the thermocycling protocol as recommended above could result in false positive detection of *A. spinosum* (AZP-producing species) where non-AZP species of *Azadinium obesium* were present in the sample. To optimize the annealing temperature of the primers and probe, a gradient PCR experiment was run varying the annealing temperature between 55˚C to 65˚C and detecting at what temperature the target species *A. spinosum* was detected compared to the non-target species, *A. obesium*, using the primer and probe set specific for *A. spinosum*. This identified that the optimal *A. spinosum* primer and probe annealing temperature for detection of *A. spinosum* needed to be raised from 59 ˚C to 62 ˚C. Subsequently, the qPCR primer sets for *A. obesium* and *A. poporum* were shown to function correctly at 62 ˚C as well.

Consequently, the final qPCR thermocycling protocol that is recommended is: Stage 1, 50 °C for 2 min; Stage 2, 95°C for 5-10 min (time dependent on master mix manufacturer); Stage 3, 40 cycles of 95°C for 15 sec, 62°C for 1 min.

Recommendation

FISH probe development *for A. spinosum* was successful. However, due to issues relating to fixatives and cost, the further development and implementation of FISH or CARD-FISH methodology for *Azadinium* detection in a regulatory monitoring context is not recommended.

Initial development of qPCR methodology for *A. spinosum* detection was successful and we recommend that this method is pursued.

Refinement of *Azadinium spinosum* FISH probes to account for matrix and concentration effects to allow their application in Scottish waters (objective 5)

This task was modified due to the re-focusing of effort on qPCR development and aimed to identify the limits of detection of *A. spinosum* in the field samples and ensure that commonly identified dinoflagellates around Scotland would not result in false-positive signals.

Cross-reactivity analysis of *A. spinosum* qPCR assay with non-target dinoflagellate species

In silico analysis using ARB software did not identify any potential cross-reactivity of the qPCR primer and probe sets with non-target dinoflagellate species (data not shown).

To test this further, the most abundant dinoflagellate species found in Scottish waters were identified from SAMS monitoring data. DNA from representative species that were available in the CCAP was extracted using standard methods. The DNA was submitted for *Azadinium* qPCR analysis as per the conditions described in the previous section. The DNA was diluted across a range of dilutions to mimic high to low cell abundance and to accentuate the potential for cross-reactivity. None of the four commonly occurring dinoflagellates gave a qPCR signal greater than the bottom standard used as the cut-off for a positive result (Figure 9; Table 3). Thus, even when excess non-target dinoflagellate DNA is present, the non-target DNA will not give a false-positive *A. spinosum* result.

The lack of cross-reactivity with non-target dinoflagellate DNA and the lack of cross-reactivity by *in silico* analysis indicate that the *A. spinosum* qPCR assay is specific for its target species.

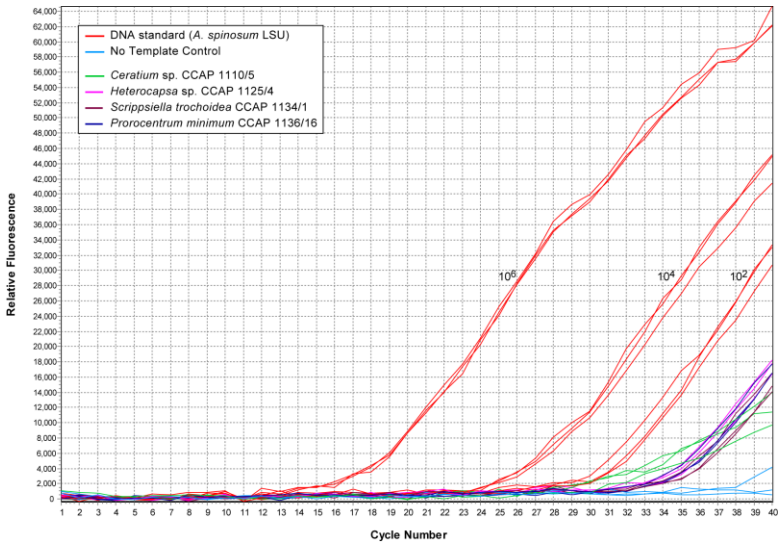


Figure 9: qPCR cross-reactivity analysis of non-target dinoflagellates. This figure shows use of *A. spinosum* qPCR primers and probe to amplify 10 ng total non-target dinoflagellate DNA per reaction. DNA standards ranged between 150 to 1.5 x 10^6 copies/well.

Table 3: Non-target dinoflagellate Azadinium qPCR cross-reactivity.

Dinoflagellate	qPCR Reactivity
Ceratium sp. CCAP 1110/5	Negative
Heterocapsa sp. CCAP 1125/4	Negative
Scrippsiella trochoidea CCAP 1134/1	Negative
Prorocentrum minimum CCAP 1136/16	Negative

A. spinosum 28S rRNA gene copy number calculation

To be able to relate the rRNA copy number per qPCR assay to a number of *A. spinosum* cells present in the water sample, it is necessary to calculate the number of 28S rRNA gene copies a single *A. spinosum* cells contains.

To do this, living cells of *A. spinosum* were placed in PCR reaction tubes using a glass capillary micropipette at concentrations ranging from one cell to 52 cells per reaction tube. The cells in each tube were washed by the addition of 10 mM Tris-HCl (pH 8) and collected by centrifuge. Subsequently, qPCR master mix and *A. spinosum* primer and probes were added to each reaction and thermocycling completed as described in the previous section. The total number of rRNA gene copies detected was calculated from cloned DNA standards and these values plotted as a function of the average number of cells per reaction (Figure 10). The rRNA copy number per single cell was calculated by dividing the rRNA copy number by the number of cells. It was calculated that there are ~3 x 10^4 28S rRNA gene copies per *A. spinosum* cell. This copy number is well within the range of other dinoflagellate species such as *Alexandrium* that can contain copy numbers spanning thousands to hundreds of thousands and even millions per cell (Brosnahan et al 2010; Galluzzi et al 2010).

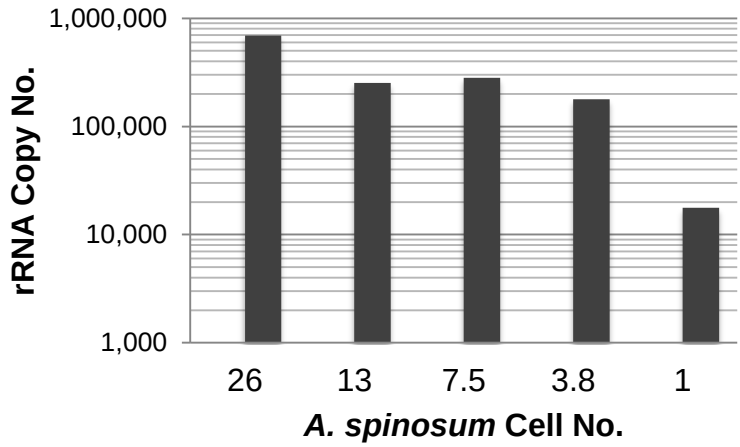


Figure 10: 28S rRNA gene copy number determination by qPCR. A defined number of whole *A. spinosum* cells were placed in replicate PCR tubes and qPCR performed on the whole cells. The rRNA copy number for the average number of *A. spinosum* is reported. Overall, the average rRNA gene copy number per *A. spinosum* cell is 3×10^4 copies.

Recommendation

Cross-reactivity evaluation and gene copy number calculation suggest the *A. spinosum* qPCR methodology is fit for purpose and suitable for implementation within a monitoring programme.

Field trials to optimise the operational application of the FISH probes for *Alexandrium* sp. and *Azadinium* spp. to determine the temporal changes in the abundance of these harmful phytoplankters at selected sites (objective 6)

Alexandrium field trials

Detection of hybridized samples within a natural phytoplankton community

As described above, unfixed samples received during the project contained very low *Alexandrium* densities and were unsuitable for statistical evaluation of the operational application of the *Alexandrium* FISH method or the assessment of cell loss during unfixed sample transport.

As the ability of the fluorescence microscopy FISH based method to enumerate accurately the group I and group III ribotypes of *A. tamarense* in Scottish field collected samples was previously demonstrated (Davidson et al. 2009), the *Alexandrium* component of this workpackage therefore concentrated on evaluation of the ability of the AFC detection method to enumerate *A. tamarense* cells added to natural samples.

Natural seawater samples collected in late summer from coastal waters adjacent to SAMS were spiked with known concentrations of group I and group III A. *tamarense* strains from laboratory cultures. This approach has the added advantage that the concentrations of toxic and non-toxic cells in field samples could be controlled. This allowed evaluation of the method at a range of densities, something that would not be easily achieved when sampling natural *Alexandrium* populations of unknown and rapidly changing density and composition.

The seawater samples were collected at time when there were typical densities of non-target cells in the water column including a bloom of the toxic diatom *Pseudo-nitzschia* (~1500 cell L^{-1}). This organism is common is Scottish waters (Fehling et al. 2006) and can co-occur with *Alexandrium* (Fehling et al. 2012).

A range of *A. tamarense* concentrations were added, and samples were spiked with either Group I or Group III cells or a combination of the two. Figure 11 is a dot plot that demonstrates the separation and discrimination between group I (R2) and group III (R3) cells in a natural sweater samples. The ribotypes formed distinct clusters that were easily identifiable and showed little to no influence from other phytoplankton cells that were in the sample.

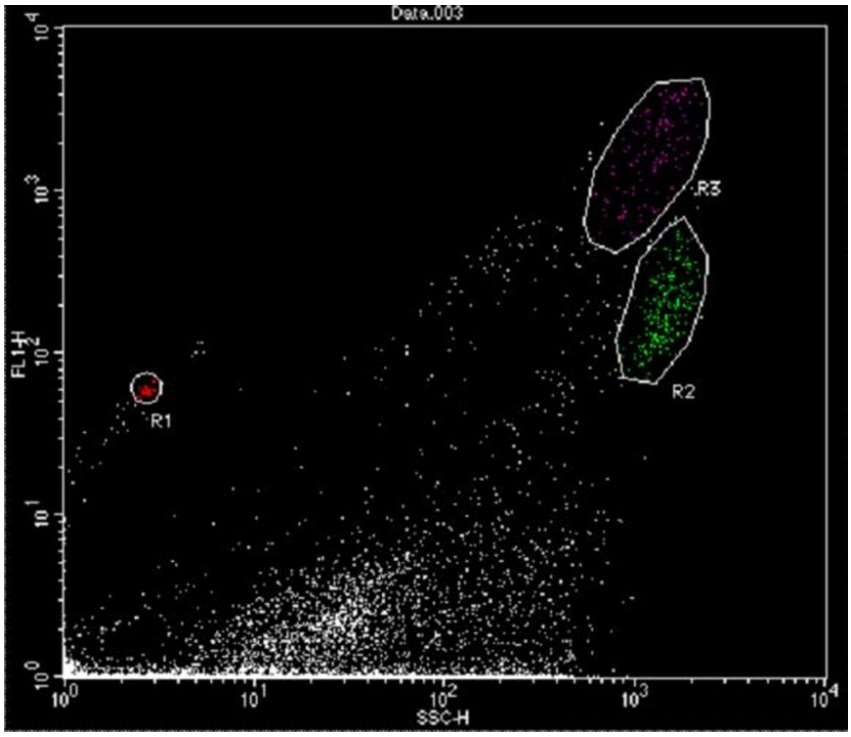


Figure 11: FC dot-plot of a natural seawater samples spiked with cultured *A. tamarense* cells. This demonstrates the discrimination that could be achieved between toxic (R2) and non-toxic (R3) cells even in the presence of a natural assemblage of non-target organisms. R1 is a gated region for the fluorescent latex beads of known concentration that are used to calibrate the instrument.

Figure 12 shows comparison of the spiked *A. tamarense* cell concentrations determined by microscopy and AFC across the range of concentrations added to the natural sweater samples. Over the full range studied, AFC estimated cell concentrations closely matched the known (Sedgwick Rafter cell determined) spiked cell concentrations for both the toxic and non-toxic cells, indicating that few cells had been lost during sample processing. There was also no statistically significant difference between toxic or non-toxic cell concentrations in comparison to the expected microscope counts (GLM, $p > 0.05$ in both cases).

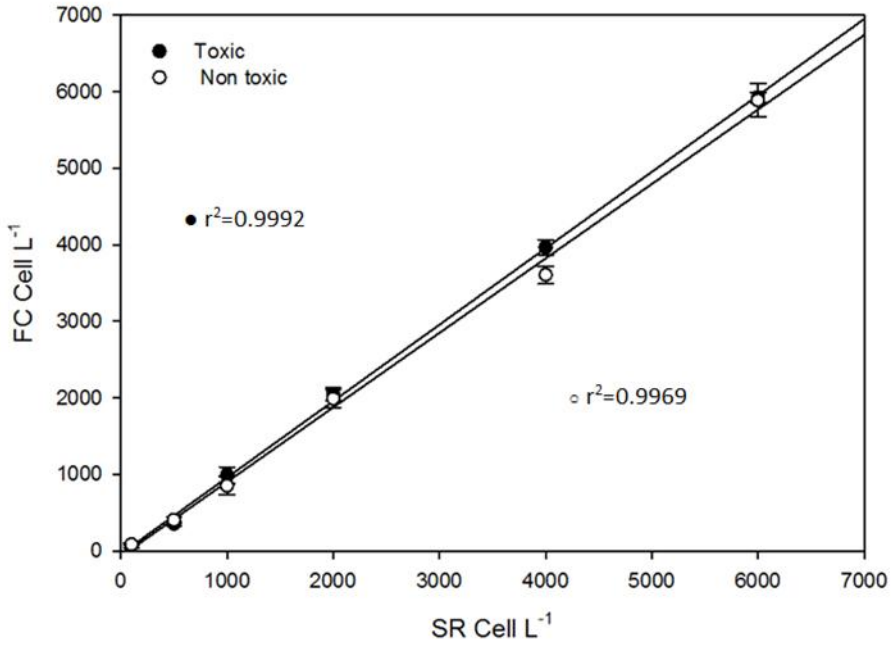


Figure 12: The relationship between AFC derived *A. tamarense* abundance for natural seawater samples spiked with microscope derived (SR) concentrations of toxic (●) and non-toxic (○) *A. tamarense* cells. All results are means of triplicate samples. Error bars represent the standard error**.**

Azadinium **field trials**

Detection limit of *A. spinosum* in field samples

An important issue for the successful use of qPCR to detect DNA in environmental samples is the carry-over of inhibitory material into the qPCR that can cause failures of the qPCR assay. To prevent this, the total amount of material submitted for DNA extraction needs to be determined. A total water sample volume of 50 ml was the recommended volume of field sample to extract DNA from. This sets the threshold of detection to 20 *Azadinium* cells per litre (Dr Joe Silke, Marine Institute Galway, personal communication).

The qPCR assay must have sensitivity to detect one *Azadinium* cell in the DNA extracted from 50 ml. The protocol adopted here was based on our own experiences of DNA extraction from field phytoplankton samples was to use the Qiagen DNeasy Plant extraction kit (cat no. 69104) with the sample suspended in a final volume of 100 µl. Therefore, if a single *Azadinium* cell (3×10^4 rRNA copies per cell) was present in the 50 ml sample, this would mean that 1 µl of the final DNA extract should contain 300 copies of rRNA.

Therefore to achieve a detection limit of ≤300 rRNA copies per qPCR assay, DNA standards in the range from 1.5×10^6 to 150 copies, and the qPCR assay after 40 cycles was shown to easily achieve detection of 150 rRNA gene copies in a 40 cycle assay (see Figure 13).

A total of eight field samples from sites around the Shetlands known to have recorded AZP biotoxins were collected. DNA was extracted from 50 ml sample volumes. Figure 12 illustrates the qPCR quantitation, with all samples proving to be positive (greater than 150 rRNA copy standard) and containing between 20 to 140 cells per litre of sample (Table 4). While these positive values potentially contradicted results obtained from samples of shellfish flesh collected and analysed as part of the OC biotoxin monitoring programme (levels below limit of detection of azaspiracid), they did correspond to finite values obtained from SPATT bag deployments (Objective 7). This indicates the high sensitivity of both qPCR and SPATT methodology.

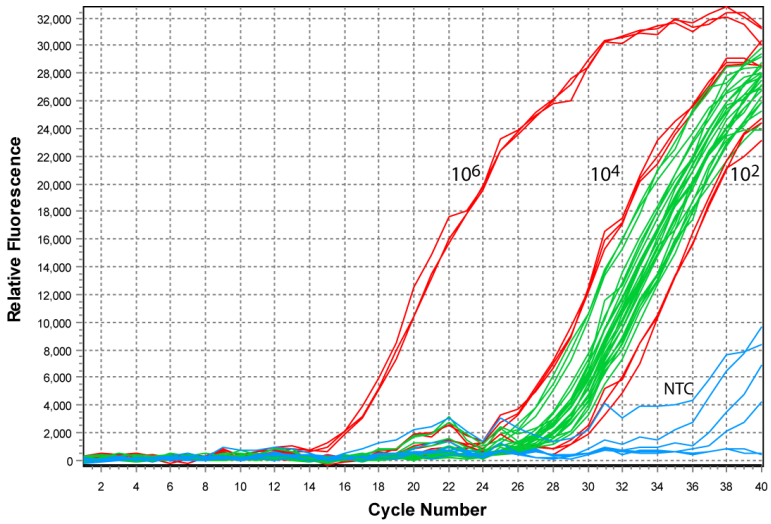


Figure 13: *A. spinosum* qPCR assay of Scottish phytoplankton field samples. To each qPCR assay (20 µl final vol.) was added 1 µl of DNA standard or 1 µl of field sample DNA extract. To a no template controls (NTC) 1 µl sterile water was added. All samples and standards were assayed in triplicate or greater. DNA standards (red lines) used were set at 1.5 x 10^6 (10^6), 1.5 x 10^4 (10^4) and 1.5 x 10^2 (10^2). Field samples analysed are in green.

Table 4: *A. spinosum* detection in Shetland field phytoplankton samples by qPCR.

Sample	rRNA copy No.	Cells (50 ml) [b]	Total *Azadinium* (cell l^{-1})
BV1	1.94×10^2	~1	20
BV2	5.90×10^2	~2	40
BV3	2.94×10^2	~1	20
SV1	2.04×10^3	~7	140
SV2	4.79×10^2	~2	40
SV3	6.80×10^2	~2	40
EOL	4.28×10^2	~1	20
SS	3.56×10^2	~1	20

[a] BV: Basta Voe; SV: Sandsound Voe; EOL: East of Linga; SS: Stream Sound

[b] Assumes 3×10^2 rRNA copies equates to 1 cell.

Recommendation

Alexandrium

The results confirm the potential suitability of the AFC method to detect and enumerate both group I and group III *Alexandrium* cells in the presence of natural blooms of non-target organisms.

Further study using naturally occurring *Alexandrium* blooms (that were not evident at our study sites during this project) would be required prior to any attempt to operationalize this method.

However, we do not recommend that this is pursued as fluorescence microscopy was found to be the preferred method of analysis in objective 3.

Azadinium

The specificity of the *A. spinosum* assay following optimization of annealing temperature and lack of cross-reactivity *in silico* and to other non-target dinoflagellates show this qPCR assay to be potentially useful for the field detection of the AZP-producing *A. spinosum*.

The detection of a single *A. spinosum* cell in field samples collected from the Shetland indicates that the assay can detect at the highest level of sensitivity possible (~1 cell). qPCR assays are highly sensitive and can detect cells at levels which would be missed in standard light microscopy detection. There remains the question as to what number of *A. spinosum* cells represents a threat to public health.

To establish the qPCR assay as a routine monitoring tool, coordinated SPATT bag deployment and phytoplankton sample collection over a period of time is required to enable the appropriate trigger level to be established.

Determination of the temporal changes in concentration and profile of azaspiracid toxins in the water column at two contrasting locations in Scottish waters (objective 7)

SPATT passive sampling technique

It was relatively recently recognised that significant amounts of biotoxins can be found in the water column at the same time as toxin-producing phytoplankton. This led to the creation of a passive sampling monitoring technique named SPATT (Solid Phase Adsorption Toxin Tracking – Mackenzie et al. 2004) which uses the ability of the toxins to adsorb onto synthetic resins from which they can be subsequently simply extracted using a solvent.

A resin (Sepabeads® SP-700, Mitsubishi Chemical Corporation) was adopted by MSS for the passive adsorption of lipophilic toxins after extensive laboratory testing. The resin, which comes as small beads, is placed into small nylon mesh bags (15 x 5 cm) which are then simply attached to a mooring line using a cable tie (Figure 14). These bags, which have the dimensions and appearance of tea bags, were used for the purpose of this project and were generally deployed and replaced on a weekly basis.

Figure 14: SPATT bag containing SP700 resin

SPATT deployment – Basta Voe and Loch Roag

- SPATT bags deployment started at Basta Voe Outer on the 8th of November 2011. The deployment occurred on a weekly basis for the first month, due to bad weather and the Christmas period, a single SPATT was deployed in December. A total of 5 SPATT samplers were deployed at the Basta Voe Outer site.

From January 2012, the SPATT sampling site moved to Basta Voe Inner site 2 to follow the change of biotoxin monitoring site. The new deployment site is situated roughly 2.5 km away from Basta Voe Outer (Figure 15). From January to March 2012, the SPATT bags were generally deployed fortnightly, but the deployment regime moved back to a weekly basis thereafter. There was no SPATT deployment during the first 3 weeks in August 2012, then the deployment resumed, mostly on a weekly basis, until the 8th of January 2013. A total of 36 SPATT samplers were deployed at the Basta Voe Inner site 2.

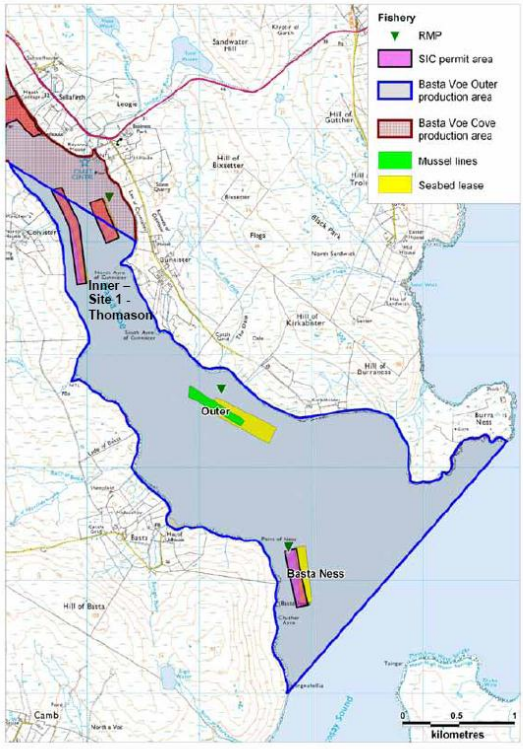


Figure 15: Basta Voe production areas and biotoxin monitoring sites (RMP)

- SPATT bags were also deployed at Loch Roag on the Eilean nam Feannag site (Figure 16). Due to high winds in November and early December 2011, work at the farm was limited and this delayed the deployment of the first SPATT bag to the 16th of December 2011. This first SPATT was deployed and left at the site over a period of one month. Thereafter, SPATT bags were deployed more or less on a weekly basis. SPATT deployment stopped at the beginning of July 2012 for a couple of weeks then resumed mostly on a weekly basis, until the 24th of January 2013. A total of 48 SPATT bags were deployed at Eilean nam Feannag over the length of the project.

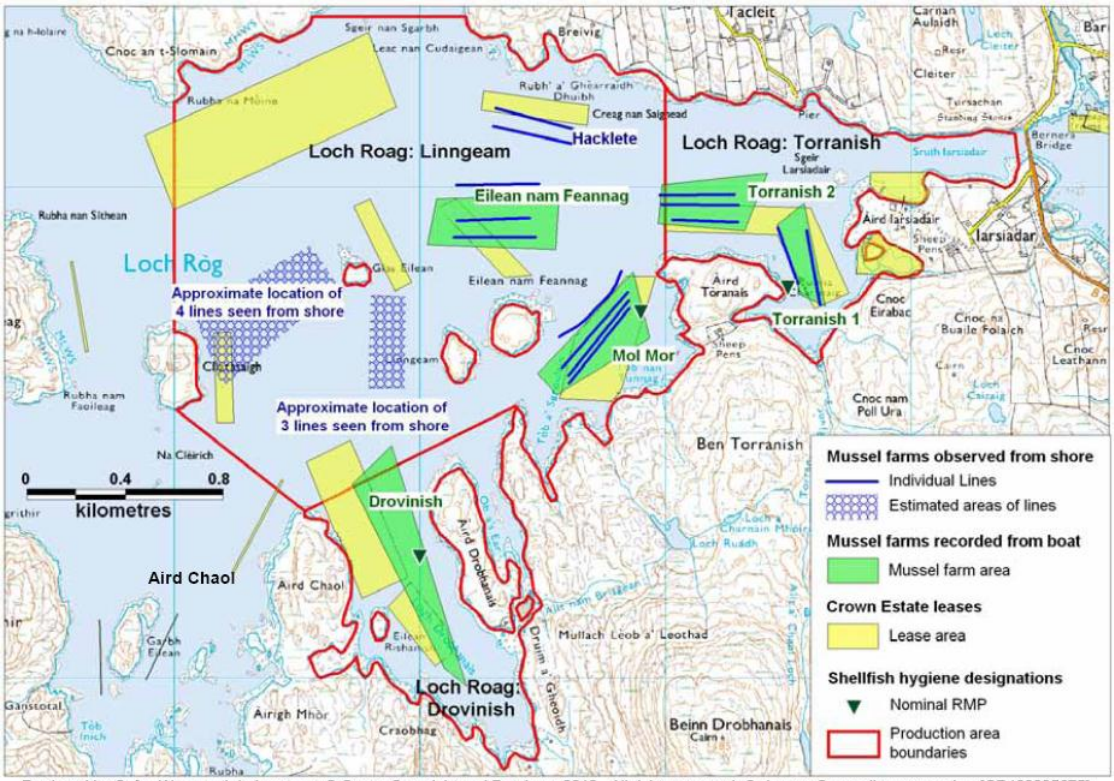


Figure 16: Loch Roag: Linngeam production areas and biotoxin monitoring sites (RMP)

Mussel contamination by AZAs in the vicinity of the SPATT deployments

Samples of shellfish flesh (mussels) are routinely collected and analysed as part of the OC biotoxin monitoring in production areas in the vicinity of the sites where SPATT bags were deployed. Comparison of the levels of AZA detected in the shellfish flesh in the OC programmed and in water column samples in this project is therefore possible.

Basta Voe - First indication of AZA contamination in mussels at the Basta Voe production area (Outer: until end of December 2011, then Cove from 2012 onwards) was recorded before the start of the project on the 30th of August 2011 (21 ng AZA1 eq/g). This toxic event spread approximately over a 7 month period until the beginning of April 2012. During this period, closure of the mussel production area occurred in September and October just before the beginning of SPATT deployment, where the concentration of AZA reached a maximum of 626 ng AZA1 eq/g (11th of October). After the re-opening of the production area, the only time the AZA maximum permitted level (MPL, 160 µg AZA1 eq/kg or 160 ng/g) was breached was on the 6th of December 2011, a few weeks after the deployment of the first SPATT bag.

From April 2012, mussel samples collected from Basta Voe Cove were not found to contain any quantifiable amounts of AZAs.

Loch Roag - Over a 3 weeks period (20th August – 3rd September 2012), AZAs were detected in mussels from Loch Roag: Linngeam – Cliatasay). Quantified levels in mussels were below the MPL (98, 112 and 25 µg AZA1 eq/kg). From September 2012 until the end of January 2013, mussels from this area were not found to contain any quantifiable amounts of AZAs.

Ultra High Pressure Liquid Chromatography with tandem mass spectrometry (UHPLC-MS/MS) method development

During the course of the project, MSS purchased a new 1290 Infinity ultra-high pressure liquid chromatography (UHPLC) system from Agilent which was installed in March 2012 at the front end of a mass spectrometer (3200 QTrap from ABSciex). The upgraded LC-MS/MS system was used for the analysis of the SPATT bags and the detection and quantification of AZA analogues.

A method dedicated to the analysis and separation of AZA analogues was then developed, based on a relatively recently published method (Fux *et al*., 2007). Specifically, chromatographic separation was achieved using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm). A binary mobile phase was used for the analysis. Mobile phase A was 100% aqueous and mobile phase B was 95% acetonitrile, both containing 2 mM ammonium formate and 50 mM formic acid. Separation of the AZA analogues was performed through a gradient which was ramped from 30% to 90% B over the first 3 min and then held for 1.5 min. At 4.5 min the composition was reset to 30% B and held for 2 min to re-equilibrate the column before the next injection. Flow rate was set at 0.4 ml/min, column temperature was maintained at 20 ºC throughout the analysis and sample injection was 5 µl.

The 3200 QTrap MS detector was used in multiple reaction monitoring (MRM) mode where specific transitions, one for quantitation and one for confirmation, were monitored for 5 AZA analogues (AZA1-5). The following transitions were selected for MRM analysis: AZA1 (m/z 842.5 → 824.4 and 842.5 → 362.3), AZA2 (m/z 856.6 → 838.6 and 856.6 → 672.6), AZA3 (m/z 828.58 → 810.6 and 828.5 → 362.3), AZA4 (m/z 844.5 → 826.5 and 844.5 → 658.6) and AZA5 (m/z 844.5 → 826.6 and 844.5 → 446.4). The electrospray source was operated in positive mode and other parameters set up were as follow: ionspray voltage: 4.4 kV, source temperature: 700ºC, Gas 1 and Gas 2: 44 psi and 48 psi respectively, curtain gas: 20 psi and CAD gas: 6.

Other parameters, specific to the MRM transitions, are detailed in the Table 5.

Table 5: AZA transitions and corresponding parameters

Analyte		Transition (m/z)		Parameters (V)				
		Precursor	Product	CE	CXP	DP	EP	CEP
AZA1	**T1**	842.5	824.4	43	32	76	6.5	40
	T2	842.5	362.3	67	4			
AZA2	**T1**	856.6	838.6	41	32	86	9	32
	T2	856.6	672.6	53	6			
AZA3	**T1**	828.5	810.6	43	8	81	7	34
	T2	828.5	362.3	61	4			
AZA4	**T1**	844.5	826.5	41	32	81	9.5	36
	T2	844.5	658.6	53	32			
AZA5	**T1**	844.5	826.6	41	8	76	9.5	32
	T2	844.5	446.4	53	6			

CE: Collision Energy, DP: Declustering potential, EP: Entrance Potential, CEP: Collision cell Entrance Potential, CXP: Collision cell eXit Potential

Chromatographic separation of AZA1-5 is illustrated in Figure 17.

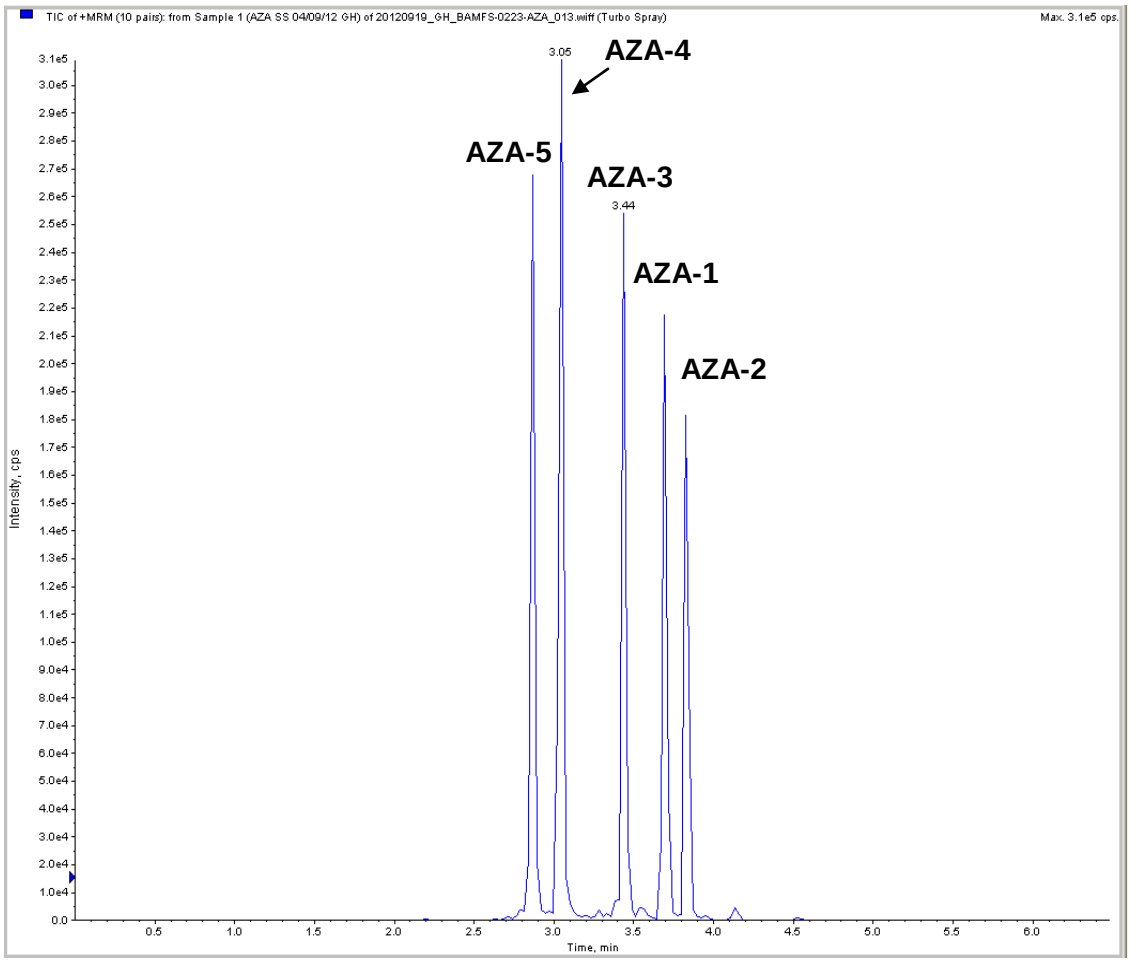


Figure 17: chromatogram of a standard solution of AZAs.

SPATT extraction

The following procedure describes the extraction of AZA from the SP-700 resin held in the SPATT bags.

1. SPATT bags were defrosted at room temperature for 1 hour.
2. The Midi clip was removed from the top of the mesh bag as well as the releasable cable tie.
3. The resin (SP-700) was transferred from the mesh bag to a Duran bottle and deionised water was used to rinse off the resin from the mesh bag into the bottle.
4. The Duran bottle was made up to approximately 200 ml (using the bottle's graduation as a guide) using deionised water.
5. The bottle was closed with a screw cap and shaken by hand for 1 minute.
6. A 25 ml reservoir (with 20 µm frit at the bottom) was installed on a vacuum manifold and the resin was then poured into it to a height equivalent to the bed height of the procedural blank (corresponding to 4.7 g of resin).
7. The remaining resin (~ 11 g) was poured into a second cartridge and stored at -20ºC. This constitutes a spare sample which can be used for the analysis of other toxins (e.g. domoic acid).
8. The resin was rinsed with deionised water (100 ml) and interstitial water was gently removed using a low vacuum.
9. Individual SPATT resin samples were extracted using methanol. Some methanol (10 ml) was added to the resin contained in the reservoir which was then capped at both ends.
10. The cartridge was passed on a vortex mixer (1 min) and the resin was allowed to soak in the methanol (30 min) prior collection of the eluent in a Duran bottle (100 ml).
11. The resin was further eluted with methanol (90 ml) so a total of 100 ml extract per sample was collected in the Duran bottle. The bottle was capped and mixed by hand.
12. An aliquot (~20 ml) of the extract was placed in a 25 ml borosilicate vial and stored in a freezer at -20ºC.
13. Another aliquot (10 ml) was placed in 12 ml borosilicate tubes for evaporation.
14. This aliquot was evaporated to dryness using a Turbovap LV (nitrogen drying gas with water bath at 45ºC) and reconstituted in methanol:water (80% v/v, 500 µL).
15. The extract was filtered (0.2 µm, PVDF centrifugal filter) through centrifugation at 1100 g for 2 min.
16. The extract was transferred to 350 µL insert vials and analysed by UPLC-MS/MS.

SPATT analysis results and comments

AZA results for the SPATT bags deployed during the project are summarised next in Tables 6 and 7. All concentrations for the different azaspiracid analogues are expressed in ng/g resin. AZA results showing <LOQ or <LOD refer to “detected but below limit of quantification” and “below limit of detection” respectively.

Loch Roag - AZA1 and AZA2 were detected in all deployed SPATT samplers (Figure 18). AZA3 was also detected in all the SPATT bags deployed from August 2012 until the end of the deployment in January 2013. The other two AZA analogues investigated, namely AZA4 and 5, which are the 3-hydroxy and 23-hydroxy analogues of AZA3, were not detected in any of the SPATT bags deployed although significant amounts of AZA1 and AZA2 were detected in November 2012 and January 2013. This would seem to support that AZA4 and AZA5 are not produced by *A. spinosum* but are obtained through biotransformation in shellfish (O’Driscoll et al. 2011).

Quantities of AZA1 and AZA2 in the passive samplers started to significantly increase from September 2012 and reached a maximum mid-November (109.8 and 21.8 ng/g resin respectively). Levels of AZA1 and AZA2 decreased slightly thereafter then increased again to reach a maximum mid-January 2013 (116.3 and 25.3 ng/g resin respectively).

Although the project did not cover two complete years, apparent results indicate an increase in the presence of AZA producers during a temporal window stretching from September to the end of January at this specific location.

It is interesting to note that quantifiable AZAs were detected in mussels collected for OC biotoxin monitoring purposes at Loch Roag: Linngeam – Cliatasay during a 3 weeks period stretching from August to early September 2012, but AZA levels were below the MPL. A slight increase in the levels of AZA1 and AZA2 in SPATT bags weekly deployed during the same period was simultaneously observed. However, AZA was not detected in mussels from OC biotoxin monitoring programme during September-January while at the same time, AZA1 and AZA2 levels were at their highest in SPATT bags. The distance between the mussel monitoring location and the SPATT deployment site is thought to be less than a mile apart. It is possible that currents and wind (direction and force) in this part of the loch could potentially influence strongly the localisation of the phytoplankton, resulting in different contamination levels for shellfish from not so distant monitored sites.

Table 6: Concentration (ng/g resin) of AZA analogues in SPATT samplers deployed at Loch Roag (Eilean nam Feannag site)

Sample ID	Sample Description	AZA1	AZA2	AZA3	AZA4	AZA5
S00114-12-O	SPATT - V1 - Loch ROAG - 16/12/11 to 17/01/12	**7.6**	**2.7**	**<LOQ**	<LOD	<LOD
S00115-12-O	SPATT - V2 - Loch ROAG - 17/01/12 to 25/01/12	**2.8**	**0.9**	<LOD	<LOD	<LOD
S00124-12-O	SPATT - V11 - Loch ROAG - 25/01/12 to 31/01/12	**0.9**	**0.3**	<LOD	<LOD	<LOD
S00116-12-O	SPATT - V3 - Loch ROAG - 31/01/12 to 07/02/12	**1.3**	**0.5**	<LOD	<LOD	<LOD
S00117-12-O	SPATT - V4 - Loch ROAG - 07/02/12 to 15/02/12	**1.4**	**0.5**	<LOD	<LOD	<LOD
S00118-12-O	SPATT - V5 - Loch ROAG - 15/02/12 to 09/03/12	**6.6**	**2.0**	<LOD	<LOD	<LOD
S00119-12-O	SPATT - V6 - Loch ROAG - 09/03/12 to 15/03/12	**1.5**	**0.5**	<LOD	<LOD	<LOD
S00120-12-O	SPATT - V7 - Loch ROAG - 16/03/12 to 23/03/12	**1.2**	**0.4**	<LOD	<LOD	<LOD
S00121-12-O	SPATT - V8 - Loch ROAG - 23/03/12 to 30/03/12	**0.7**	**0.2**	<LOD	<LOD	<LOD
S00122-12-O	SPATT - V9 - Loch ROAG - 30/03/12 to 09/04/12	**1.4**	**0.4**	<LOD	<LOD	<LOD
S00123-12-O	SPATT - V10 - Loch ROAG - 09/04/12 to 16/04/12	**0.4**	**<LOQ**	<LOD	<LOD	<LOD
S00125-12-O	SPATT - V12 - Loch ROAG - 16/04/12 to 23/04/12	**1.1**	**0.3**	<LOD	<LOD	<LOD
S00150-12-O	SPATT - V13 - Loch ROAG - 23/04/12 to 30/04/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00151-12-O	SPATT - V14 - Loch ROAG - 30/04/12 to 07/05/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00152-12-O	SPATT - V15- Loch ROAG - 07/05/12 to 14/05/12	**0.5**	**0.2**	<LOD	<LOD	<LOD
S00153-12-O	SPATT - V16 - Loch ROAG - 14/05/12 to 21/05/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00154-12-O	SPATT - V17 - Loch ROAG - 21/05/12 to 28/05/12	**<LOQ**	**<LOQ**	<LOD	<LOD	<LOD
S00155-12-O	SPATT - V18 - Loch ROAG - 28/05/12 to 04/06/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00156-12-O	SPATT - V19 - Loch ROAG - 04/06/12 to 12/06/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00157-12-O	SPATT - V20 - Loch ROAG - 12/06/12 to 20/06/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00158-12-O	SPATT - V21 - Loch ROAG - 21/06/12 to 28/06/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00159-12-O	SPATT - V22 - Loch ROAG - 28/06/12 to 05/07/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00024-13-O	SPATT - V23 - Loch ROAG - 23/07/12 to 30/07/12	**<LOQ**	**<LOQ**	<LOD	<LOD	<LOD
S00025-13-O	SPATT - V24 - Loch ROAG - 30/07/12 to 06/08/12	**<LOQ**	**<LOQ**	**<LOQ**	<LOD	<LOD
S00026-13-O	SPATT - V25 - Loch ROAG - 06/08/12 to 13/08/12	**0.7**	**0.2**	**<LOQ**	<LOD	<LOD
S00027-13-O	SPATT - V26 - Loch ROAG - 13/08/12 to 20/08/12	**1.2**	**0.3**	**<LOQ**	<LOD	<LOD
S00028-13-O	SPATT - V27 - Loch ROAG - 20/08/12 to 28/08/12	**3.5**	**0.7**	**<LOQ**	<LOD	<LOD
S00029-13-O	SPATT - V28 - Loch ROAG - 28/08/12 to 04/09/12	**5.5**	**1.6**	**<LOQ**	<LOD	<LOD
S00030-13-O	SPATT - V29 - Loch ROAG - 04/09/12 to 11/09/12	**13.6**	**3.7**	**<LOQ**	<LOD	<LOD
S00031-13-O	SPATT - V30 - Loch ROAG - 11/09/12 to 18/09/12	**12.9**	**3.1**	**<LOQ**	<LOD	<LOD
S00032-13-O	SPATT - V31 - Loch ROAG - 18/09/12 to 25/09/12	**16.0**	**4.1**	**<LOQ**	<LOD	<LOD
S00033-13-O	SPATT - V32 - Loch ROAG - 25/09/12 to 02/10/12	**27.0**	**8.5**	**0.2**	<LOD	<LOD
S00034-13-O	SPATT – V33 - Loch ROAG - 02/10/12 to 10/10/12	**21.6**	**5.6**	**<LOQ**	<LOD	<LOD

S00035-13-O	SPATT - V34 - Loch ROAG - 10/10/12 to 18/10/12	**5.9**	**1.6**	**<LOQ**	<LOD	<LOD
S00036-13-O	SPATT - V35 - Loch ROAG - 18/10/12 to 25/10/12	**4.4**	**1.4**	**<LOQ**	<LOD	<LOD
S00037-13-O	SPATT - V36 - Loch ROAG - 25/10/12 to 01/11/12	**29.8**	**7.4**	**<LOQ**	<LOD	<LOD
S00038-13-O	SPATT - V37 - Loch ROAG - 01/11/12 to 08/11/12	**62.8**	**12.4**	**<LOQ**	<LOD	<LOD
S00039-13-O	SPATT - V38 - Loch ROAG - 08/11/12 to 15/11/12	**109.8**	**21.8**	**0.2**	<LOD	<LOD
S00040-13-O	SPATT - V39 - Loch ROAG - 15/11/12 to 22/11/12	**34.8**	**12.3**	**<LOQ**	<LOD	<LOD
S00041-13-O	SPATT – V41 - Loch ROAG - 22/11/12 to 29/11/12	**79.4**	**15.5**	**0.2**	<LOD	<LOD
S00042-13-O	SPATT – V40 - Loch ROAG - 29/11/12 to 06/12/12	**58.5**	**13.0**	**<LOQ**	<LOD	<LOD
S00043-13-O	SPATT – V42 - Loch ROAG - 13/12/12 to 20/12/12	**85.6**	**19.2**	**<LOQ**	<LOD	<LOD
S00044-13-O	SPATT – V43 - Loch ROAG - 20/12/12 to 27/12/12	**69.4**	**15.6**	**<LOQ**	<LOD	<LOD
S00045-13-O	SPATT – V44 - Loch ROAG - 27/12/12 to 03/01/13	**86.5**	**19.7**	**<LOQ**	<LOD	<LOD
S00046-13-O	SPATT – V45 - Loch ROAG - 03/01/13 to 10/01/13	**82.1**	**18.7**	**0.2**	<LOD	<LOD
S00047-13-O	SPATT – V46 - Loch ROAG - 10/01/13 to 17/01/13	**116.3**	**25.3**	**0.2**	<LOD	<LOD
S00048-13-O	SPATT – V47 - Loch ROAG - 17/01/13 to 24/01/13	**95.0**	**21.2**	**<LOQ**	<LOD	<LOD
S00049-13-O	SPATT – V48 - Loch ROAG - 24/01/13 to 31/01/13	**10.0**	**2.8**	**<LOQ**	<LOD	<LOD

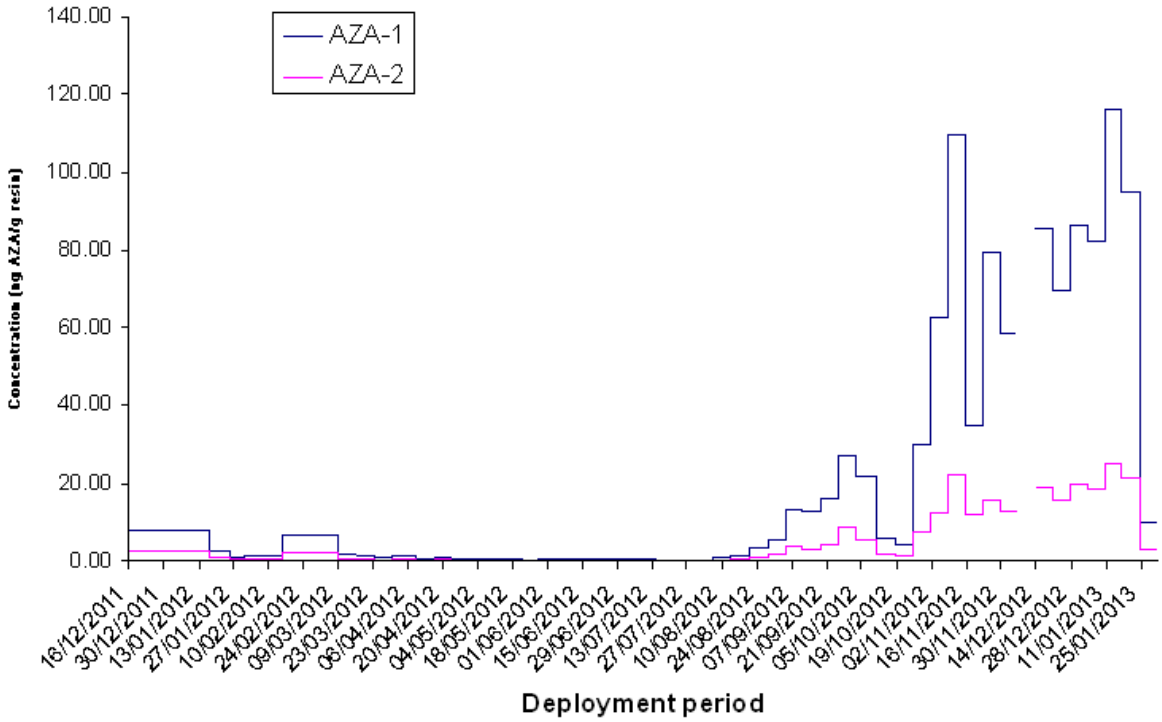


Figure 18: Plot of the concentration of AZA1 and AZA2 recovered from SPATT bags deployed at Loch Roag.

Basta Voe - AZA1 and AZA2 were also the azaspiracid analogues which were mostly detected in SPATT bags deployed at Basta Voe (Figure 19), although the AZA levels were much lower compared to those detected in the SPATT bags deployed at Loch Roag. AZA3 was also detected in the SPATT bags deployed during the November 2011 - March 2012 period.

Similarly to Loch Roag, AZA levels were significantly higher during the autumn-winter months with an increase starting at the end of August. More SPATT deployments would be necessary to confirm this trend.

At the beginning of the deployment period in November 2011, AZA1 and AZA2 levels were particularly high in the SPATT in comparison to levels in SPATT deployed later on. This corresponded to the end of an AZA contamination episode which affected Basta Voe mussels during October and November 2011. It was unfortunate that the deployment of the first SPATT at Basta Voe was done after the peak of AZA contamination in mussels.

It is important to note that the AZA event of autumn 2011 did not occur the following year.

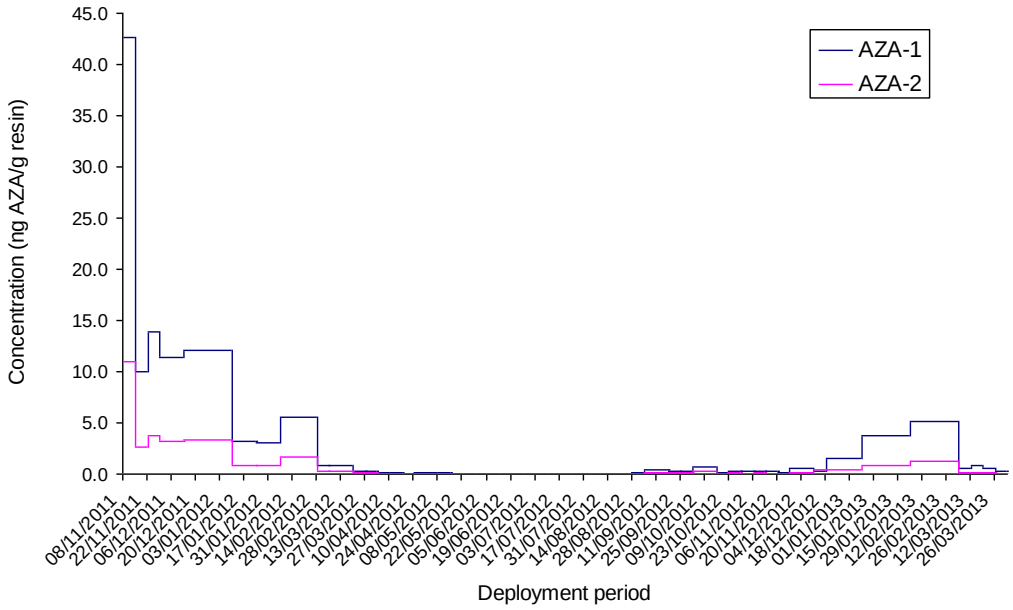


Figure 19: Plot of the concentration of AZA1 and AZA2 recovered from SPATT bags deployed at Basta Voe.

Sample ID	Sample Description	AZA1	AZA2	AZA3	AZA4	AZA5
S00074-12-O	SPATT - T0 - BASTA VOE - 08/11/11 to 15/11/11	**42.7**	**11.0**	**0.2**	<LOD	<LOD
S00075-12-O	SPATT - T1 - BASTA VOE - 15/11/11 to 22/11/11	**10.0**	**2.6**	**<LOQ**	<LOD	<LOD
S00076-12-O	SPATT - T2 - BASTA VOE - 22/11/11 to 29/11/11	**13.9**	**3.8**	**<LOQ**	<LOD	<LOD
S00077-12-O	SPATT - T3 - BASTA VOE - 29/11/11 to 13/12/11	**11.4**	**3.2**	**<LOQ**	<LOD	<LOD
S00078-12-O	SPATT - T4 - BASTA VOE - 13/12/11 to 10/01/12	**12.1**	**3.3**	**<LOQ**	<LOD	<LOD
S00079-12-O	SPATT - T5 - BASTA VOE - 10/01/12 to 24/01/12	**3.2**	**0.8**	**<LOQ**	<LOD	<LOD
S00080-12-O	SPATT - T6 - BASTA VOE - 24/01/12 to 07/02/12	**3.1**	**0.8**	**<LOQ**	<LOD	<LOD
S00081-12-O	SPATT - T7 - BASTA VOE - 07/02/12 to 28/02/12	**5.5**	**1.7**	**<LOQ**	<LOD	<LOD
S00082-12-O	SPATT - T8 - BASTA VOE - 28/02/12 to 06/03/12	**0.8**	**0.2**	**<LOQ**	<LOD	<LOD
S00083-12-O	SPATT - T9 - BASTA VOE - 06/03/12 to 20/03/12	**0.9**	**0.3**	**<LOQ**	<LOD	<LOD
S00237-12-O	SPATT - T10 - BASTA VOE - 20/03/12 to 27/03/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00238-12-O	SPATT - T11 - BASTA VOE - 27/03/12 to 03/04/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00239-12-O	SPATT - T12 - BASTA VOE - 03/04/12 to 10/04/12	**<LOQ**	**<LOQ**	<LOD	<LOD	<LOD
S00240-12-O	SPATT - T13 - BASTA VOE - 10/04/12 to 17/04/12	**<LOQ**	**<LOQ**	<LOD	<LOD	<LOD
S00241-12-O	SPATT - T14 - BASTA VOE - 17/04/12 to 24/04/12	<LOD	<LOD	<LOD	<LOD	<LOD
S00242-12-O	SPATT - T15 - BASTA VOE - 24/04/12 to 08/05/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00243-12-O	SPATT - T16 - BASTA VOE - 08/05/12 to 15/05/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00244-12-O	SPATT - T17 - BASTA VOE - 15/05/12 to 29/05/12	**<LOQ**	**<LOQ**	<LOD	<LOD	<LOD
S00245-12-O	SPATT - T18 - BASTA VOE - 29/05/12 to 05/06/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00246-12-O	SPATT - T19 - BASTA VOE - 05/06/12 to 12/06/12	<LOD	<LOD	<LOD	<LOD	<LOD
S00247-12-O	SPATT - T20 - BASTA VOE - 12/06/12 to 26/06/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00248-12-O	SPATT - T21- BASTA VOE - 26/06/12 to 03/07/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00249-12-O	SPATT - T22 - BASTA VOE - 03/07/12 to 10/07/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00250-12-O	SPATT - T23 - BASTA VOE - 10/07/12 to 17/07/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00251-12-O	SPATT - T24 - BASTA VOE - 17/07/12 to 24/07/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00252-12-O	SPATT - T25 - BASTA VOE - 24/07/12 to 31/07/12	<LOD	<LOD	<LOD	<LOD	<LOD
S00001-13-O	SPATT - T26 - BASTA VOE - 21/08/12 to 28/08/12	**<LOQ**	<LOD	<LOD	<LOD	<LOD
S00002-13-O	SPATT - T27 - BASTA VOE - 28/08/12 to 04/09/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00003-13-O	SPATT - T28 - BASTA VOE - 04/09/12 to 18/09/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00004-13-O	SPATT - T29 - BASTA VOE - 18/09/12 to 25/09/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00005-13-O	SPATT- T30 - BASTA VOE - 25/09/12 to 02/10/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00006-13-O	SPATT - T31 - BASTA VOE - 02/10/12 to 16/10/12	**0.7**	**0.3**	<LOD	<LOD	<LOD
S00007-13-O	SPATT - T32 - BASTA VOE - 16/10/12 to 23/10/12	**<LOQ**	**<LOQ**	<LOD	<LOD	<LOD
S00008-13-O	SPATT - T33 - BASTA VOE - 23/10/12 to 30/10/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00009-13-O	SPATT - T34 - BASTA VOE - 30/10/12 to 06/11/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00010-13-O	SPATT - T35 - BASTA VOE - 06/11/12 to 13/11/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00011-13-O	SPATT - T36 - BASTA VOE - 13/11/12 to 20/11/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00012-13-O	SPATT - T37 - BASTA VOE - 20/11/12 to 27/11/12	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00013-13-O	SPATT - T38 - BASTA VOE - 27/11/12 to 11/12/12	**0.5**	**0.2**	<LOD	<LOD	<LOD
S00014-13-O	SPATT - T39 - BASTA VOE - 11/12/12 to 18/12/12	**0.3**	**<LOQ**	<LOD	<LOD	<LOD
S00015-13-O	SPATT - T40 - BASTA VOE - 18/12/12 to 08/01/13	**1.5**	**0.4**	<LOD	<LOD	<LOD
S00060-13-O	SPATT - T41 - BASTA VOE - 08/01/13 to 05/02/13	**3.8**	**0.9**	<LOD	<LOD	<LOD
S00061-13-O	SPATT - T42 - BASTA VOE - 05/02/13 to 05/03/13	**5.2**	**1.3**	<LOD	<LOD	<LOD
S00062-13-O	SPATT - T43 - BASTA VOE - 05/03/13 to 12/03/13	**0.6**	**<LOQ**	<LOD	<LOD	<LOD
S00063-13-O	SPATT - T44 - BASTA VOE - 12/03/13 to 19/03/13	**0.8**	**<LOQ**	<LOD	<LOD	<LOD
S00064-13-O	SPATT - T45 - BASTA VOE - 19/03/13 to 26/03/13	**0.6**	**<LOQ**	<LOD	<LOD	<LOD
S00065-13-O	SPATT - T46 - BASTA VOE - 26/03/13 to 02/04/13	**0.2**	**<LOQ**	<LOD	<LOD	<LOD
S00066-13-O	SPATT - T47 - BASTA VOE - 02/04/13 to 09/04/13	**0.5**	**<LOQ**	<LOD	<LOD	<LOD

Table 7: Concentration (ng/g resin) of AZA analogues in SPATT samplers deployed at Basta Voe Outer (2011) and Basta Voe Cove (from 2012).

Summary

SPATT as a passive sampling technique demonstrated its usefulness in tracking the presence of toxins present in the water column. The utilisation of SPATT at Basta voe and Loch Roag allowed the detection and quantification of AZA1 and AZA2 the two main azaspiracid analogues found at these locations thus confirming the presence of organisms capable of producing these toxins.

Quantifiable amounts of AZA3 were also detected a few times in deployed SPATT at both sites, but only when significant concentrations of AZA1 and AZA2 were concurrently found in the passive samplers. AZA4 and AZA5 were however not detected in any of the deployed SPATT bags although significant amounts of AZA1 and AZA2 were detected at Loch Roag in November 2012 and January 2013. This would seem to support that AZA4 and AZA5 are not produced by *A. spinosum* but are obtained through biotransformation in shellfish.

There seems to be a similar temporal pattern for both monitored sites where the occurrence of AZAs in the water column remains low during spring and the first part of the summer. There is a marked increase in the concentration of AZAs especially at Loch Roag from September and during autumn and the first part of the winter. However, further comparison between the two monitored sites remains limited due to the relatively monitoring period (14 months).

Summary and Recommendations

The project demonstrated that molecular methods are capable of detecting and enumerating group I and group III *Alexandrium tamarense*, and *Azadinium spinosum* in Scottish waters.

Alexandrium

FISH based *Alexandrium* detection based on the previously published methods of Touzet et al. (2010) was found to have minimal cross-reactivity to other organisms and hence to be suitable for the enumeration of, and discrimination between, group I and group III *A. tamarense* in Scottish waters.

Three methods of enumeration of the fluorescently labeled *A. tamarense* cells were evaluated. Of these, fluorescence microscopy is recommended as it is more time/cost efficient than automated methods and allows any cross-reactivity with non-target cells to be assessed visually.

A major constraint to the routine use of FISH based methodology was the incompatibility of the method with Lugol's iodine fixed cells that are collected within the OC phytoplankton monitoring programme. No other suitable fixative could be found for routine use within the OC programme. It is therefore necessary to send pre-screened but unfixed water samples to the laboratory for analysis.

Given the operational difficulties of on-site screening and the costs of transporting relatively large volumes of water, with the possibility of some cells loss during transport, the *Alexandrium* FISH method in not recommended for routine use to characterise the *Alexandrium* community at all phytoplankton monitoring sites. Rather, we recommend that the method is used opportunistically allowing important developing blooms to be categorized as group I or group III when thought necessary.

Samples should be requested for FISH analysis when standard light microscopy based regulatory monitoring counts exceeding some pre-determined threshold and/or a specific rate of increase. While a threshold of ~ 250 cells L^{-1} is suggested, determining the value of such a threshold was outside the scope of this study, and would benefit from a more detailed analysis of *Alexandrium* abundance and shellfish toxicity data.

When this project was close to completion, a qPCR method for the detection and enumeration of group I and group III *A. tamarense* was published by Töbe et al. (2013). The method would require some validation to verify lack of cross reactivity with other species. However, given that the method is suitable for us on Lugol's iodine fixed samples it may be more easily be incorporated in OC phytoplankton monitoring that FISH based detection.

Azadinium

FISH probe development *for A. spinosum* was successful. However, due to issues relating to fixatives and cost, the further development and implementation of FISH or CARD-FISH methodology for *Azadinium* detection in a regulatory monitoring context is not recommended.

Development of qPCR methodology for *A. spinosum* detection was undertaken. Cross-reactivity evaluation and gene copy number calculation indicate suggest the *A. spinosum* qPCR methodology is fit for purpose and suitable for implementation within the OC monitoring programme.

The specificity of the *A. spinosum* assay following optimization of annealing temperature and lack of cross-reactivity *in silico* and to other non-target dinoflagellates show this qPCR assay to be potentially useful for the field detection of the AZP-producing *A. spinosum*.

The detection of a single *A. spinosum* cell in field samples collected from the Shetland indicates that the assay can detect at the highest level of sensitivity possible (~1 cell).

To establish the qPCR assay as a routine monitoring tool, coordinated SPATT bag deployment and phytoplankton sample collection over a period of time is required to enable the appropriate trigger level to be established.

The usefulness of SPATT as a passive sampling technique for tracking the presence of toxins present in the water column was demonstrated.

The utilisation of SPATT at Basta Voe and Loch Roag allowed the detection and quantification of AZA1 and AZA2 the two main azaspiracid analogues found at these locations thus confirming the presence of organisms capable of producing these toxins.

Acknowledgements

We thank Callum Iain MacKenzie and Sean Williamson for the deployment of SPATT bags, Joe Silke for discussions related to Azadinium qPCR and Lisa-Eckford Soper for discussions related to Alexandrium FISH methodology.

References

Auinger BM, Pfandl K,; Boenigk J (2008) Improved methodology for identification of protists and microalgae from plankton samples preserved in Lugol's iodine solution: combining microscopic analysis with single-cell PCR. *Appl Environ Microbiol* 74:2505-2510

Brosnahan ML, Kulis DM, Solow AR, Erdner DL, Percy L, Lewis J, Anderson DM (2010) Outbreeding lethality between toxic Group I and nontoxic Group III Alexandrium tamarense spp. isolates: Predominance of heterotypic encystment and implications for mating interactions and biogeography. *Deep Sea Res. II:* 57: 175-189

Collins C, Graham J, Brown L, Bresnan E, Lacaze J.-P, Turrell EA (2009) Identification and toxicity of *Alexandrium tamarense* (Dinophyceae) in Scottish waters. *J. Phycol.* 45: 692-703

Davidson K, McCoy G, Touzet G, Raine R (2009) Characterisation of mixed *Alexandrium* populations in Scottish waters using whole cell fluorescent *in situ* hybridisation. Report to the Food Standards Agency Scotland

Fehling J, Davidson K, Bolch CJ, Tett P (2006) Seasonality of *Pseudo-nitzschia* spp. (Bacillariophyceae) in western Scottish waters. *Mar. Ecol. Prog. Ser.* 323:91-105

Fehling J, Davidson K, Bolch CJ, Brand T, Narayanaswamy BE (2012) The Relationship Between Phytoplankton Distribution and Water Column Characteristics in North West European Shelf Sea Waters. *PLoS ONE* 7(3): e34098. doi:10.1371/journal.pone.0034098

Frederick MO, Cole K.P., Pertovic, G, Loizidou EZ, Nicolaou, KC (2007) Structural assignment and total synthesis of azaspirazid-1. In *Phycotoxins: Chemistry and Biochemistry* (Botana LM, editor), 297–309. Blackwell Publishing, Oxford, UK

Fux E, McMillan D, Bire R, Hess P (2007) Development of an Ultra Performance Liquid Chromatography – Mass Spectrometry method for the detection of lipophilic marine toxins. *J. Chromatogr*. 1157: 273-280

Galluzzi L, Bertozzini E, Penna A, Perini F, Garces E, Magnani, M (2010) Analysis of rRNA gene content in the Mediterranean dinoflagellate Alexandrium catenella and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. *J. Appl. Phycol.* 22: 1-9

James KJ, Moroney C, Roden C, Satake M, Yasumoto T, Lehane M, Furey A (2003) Ubiquitous ‘‘benign’’ alga emerges as the cause of shellfish contamination responsible for the human toxic syndrome, azaspiracid poisoning. *Toxicon* 41: 145–154

Kalyuzhnaya MG, Zabinsky R, Bowerman S, Baker DR, Lidstrom ME, Chistoserdova L (2006) Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. *Appl. Environ. Micro*. 72: 4293–301

Lilly EL, Halanych KM, Anderson DM (2007) Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). *J. Phycol*. 43: 1329-1338

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H (2004) ARB: a software environment for sequence data. *Nucl. Acids Res.* 32:1363-1371

Mackenzie L, Beuzenberg V, Holland P, McNabb P, Selwood A (2004) Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. *Toxicon* 44: 901-918

Mcmahon T, Silke, J (1996) West coast of Ireland; winter toxicity of unknown aetiology in mussels. *Harmful Algae News* 14: 2

O'Driscoll D, Skrabakoya Z, O'Halloran J, van Pelt FN, James KJ (2011) Mussels increase xenobiotic (azaspiracid) toxicity using a unique bioconversion mechanism. *Environ. Sci. Technol.* 45: 3102-3108

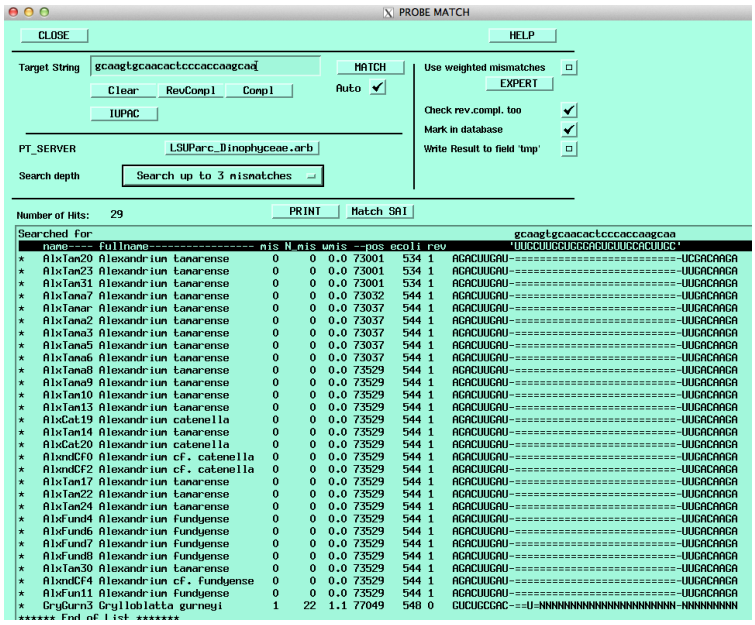
Sekar R, Fuchs BM, Amann R, Pernthaler J (2004) Flow Sorting of Marine Bacterioplankton after Fluorescence *In Situ* Hybridization. *Appl. Environ. Microbiol.* 70: 6210–6219

Töbe K, Alpermann TJ, Tillmann U, Krock B, Cembella A, John U (2013) Molecular discrimination of toxic and non-toxic *Alexandrium* species (Dinophyta) in natural phytoplankton assemblages from the Scottish coast of the North Sea*. E. J. Phycol*. 48: 12-26

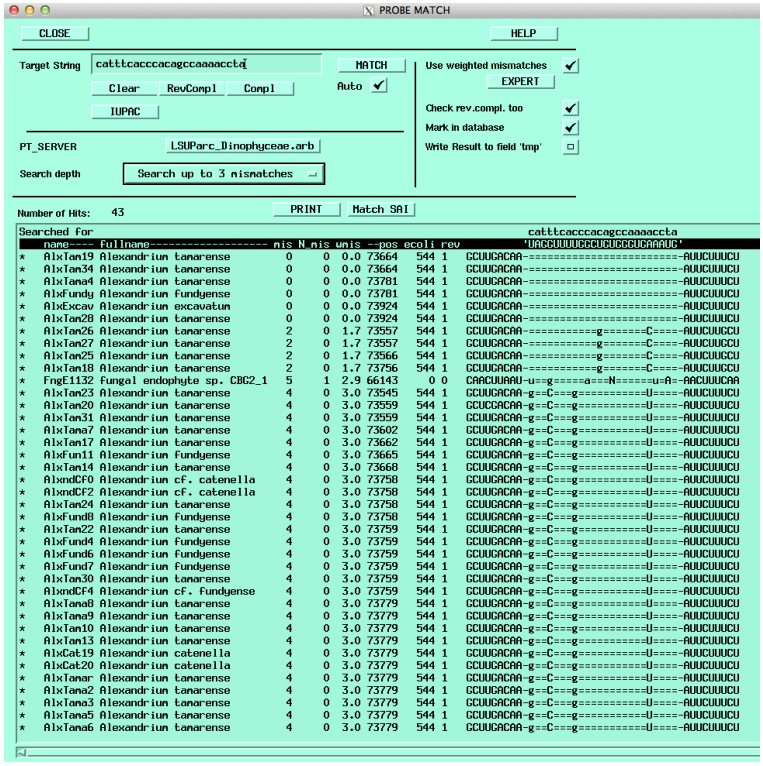
Touzet N, Davidson K, Pete R, Flanagan K, McCoy GR, Amzil Z, Maher M, Chapelle A, Raine R (2010) Co-occurrence of the West European (Gr.III) and North American (Gr.I) ribotypes of *Alexandrium tamarense* (Dinophyceae) in Shetland, Scotland. *Protist* 161:370-384

Supplementary Information

A



B



SI Figure 1. *In silico* FISH probe cross-reactivity analysis using ARB 'Probe Match'. (A) "toxic" *A. tamarense* probe TamToxC with 3 mis-matches; (B) "non-toxic" *A. tamarense* probe TamA with 3 mis-matches.

SI Table 1.Probe sequences specific for *Azadinium* representatives applied in Standard-FISH and CARD-FISH applications

Probe Name	Probe Sequence [5'-3']	Formamide Concentration	
		Standard-FISH	CARD-FISH
A. spinosum 28S / **Aza_544**	TGG TCG AGT TAC CAG CCC	20	40
A.poporum 28S**/ Apop_544**	CGA GTT ACC AGT TCT CCG	20	40
*A.obesum*28S**/ Aob_544**	AAG ACA TTC GAC CTA CCG	20	40

SI Table 2. Primer and TaqMan™ MGB-probes specific for *Azadinium* representatives for qPCR approaches

Target	TaqMan MGB probe [5'-3']	Forward Primer [5'-3']	Reverse Primer [5'-3']	Amplicon Size (bp)	Efficiency qPCR (slope)
A. spinosum 28S	**Aspin77T**	**Asp48F**	**Asp120R**		
	CGC CCA AAA GGA CTC CT	TCG TCT TTG TGT CAG GGA GAT G	GGA AAC TCC TGA AGG GCT TGT	72bp	-3.29
A. poporum 28S	**Apop112**	**Apop62F**	**Apop148R**		
	TTC CAG ACG ACT CAA A	GAT GCT CAA GGT GCC TAG AAA GTC	CCT GCG TGT CTG GTT GCA	68bp	-3.34
*A. obesum*28S	**Aob163**	**Aob134F**	**Aob208R**		
	AAG ACA TTC GAC CTA CCG T	AGG GAT CGA TAC ACA AAT GAG TAC TG	AAA CTC CAG GGA CAT GGT AGT CTT A	74bp	-3.37