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Identification and quantification of toxic and nontoxic strains of the harmful dinoflagellate *Alexandrium tamarensis* using fluorescence in situ hybridization and flow cytometry

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Abstract

The co-occurrence of morphologically identical toxic and nontoxic ribotypes of the biotoxin producing marine dinoflagellate *Alexandrium tamarensis* presents a significant problem for its identification and enumeration, particularly in a regulatory monitoring context. To address this, we have developed a fluorescence in situ hybridization-flow cytometry (FISH-FC)-based method of cell identification and enumeration. This employed the taxa specific oligonucleotide probes TamToxC and TamA to fluorescently label (with the fluorochromes CY.3 and FITC) Group I (toxic) and Group III (nontoxic) *A. tamarensis* ribotypes, respectively. Detection was by fluorescence activated flow cytometric analysis. The FISH-FC method allowed effective discrimination between laboratory cultures of Group I and Group III ribotypes, with toxic and nontoxic cells creating distinct, easily identifiable, clusters in a flow cytometer bi-plot of side scatter (SSC) versus the green (FL1) fluorescence detection channel. Comparison of estimates of cell abundance obtained by the FISH-FC technique with those obtained by microscopy (Sedgwick Rafter technique) showed no statistically significant difference across a range of concentrations. Subsequently, the methodology was successfully applied on natural seawater samples, spiked with known concentrations of toxic and nontoxic *A. tamarensis* cells at environmentally relevant concentrations.

Species within a number of phytoplankton genera are harmful to human health through their production of biotoxins. The species that produce toxins are commonly referred to as 'Harmful Algal Blooms' (HABs). Filter feeding shellfish accumulate algal biotoxins within their flesh, and can pose a risk to human health when consumed (Hallegraeff et al. 1995; Turrell et al. 2006; Gowen et al. 2012).

Within the EU, human health is safeguarded by monitoring programs for the presence of algal toxins within shellfish flesh, as well as the causative phytoplankton in the shellfish-harvesting areas. These areas are closed to harvesting when the algal toxin concentration in the shellfish flesh exceeds the limit prescribed within the EU Directive 91/492/EEC and asso-

ciated recommendations. In some countries, counts of the causative phytoplankton are used to target shellfish flesh sampling. Whereas directive 91/492/EEC has been successful in safeguarding health, the economic implications for the shellfish industry of closures can be significant (Davidson and Bresnan 2009). Particularly damaging can be recalls of product that has been harvested immediately before toxicity levels breaching regulatory threshold levels. Many operators, therefore, use published regulatory harmful phytoplankton counts to plan their harvesting operations to minimize their risk. The health and economic problems highlighted above demonstrate the need by both regulators and shellfish harvesters for rapid and reliable methods of detecting and enumerating biotoxin producing phytoplankton species.

The standard method for phytoplankton enumeration and identification is by light microscopy, typically using the Utermöhl technique (Utermöhl 1958). However, when the species of interest are morphologically similar or identical, it can be impossible to routinely differentiate between species and strains preventing the desired early warning of the presence of harmful organisms.

Dinoflagellates of the genus *Alexandrium* produce potent paralytic shellfish poisoning (PSP) toxins. PSP toxins are highly lethal with an LD₅₀ (median lethal dose) in mice by

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intra-peritoneal injection of 10 mg kg⁻¹; 1000 times more toxic than sodium cyanide (Landsberg 2002). The IOC-UNESCO (Intergovernmental Oceanographic Commission-United Nations Educational, Scientific and Cultural Organisation) Harmful Algal Bloom Programme indicates that there are about 2000 cases of PSP in humans annually with 15% mortality (although not all are related to *Alexandrium*).

Scottish waters experience a range of HAB-based events of relevance to both shellfish and finfish production (Davidson and Bresnan 2009; Davidson et al. 2011). Among these, PSP-based shellfish harvesting closures as a result of the presence of the highly toxic cells of the species *Alexandrium tamarensis* are perhaps the most important given the high risk to human health (Davidson and Bresnan 2009). This organism is routinely found in Scottish waters in spring and summer with 'hot spots' being observed around Shetland, Orkney, the Western Isles, and the East Coast (Bresnan et al. 2008; Töbe et al. 2013). The toxicity of *A. tamarensis* is such that its presence (a single cell in a 50 mL counting chamber, equivalent to 20 cells L⁻¹) is sufficient to breach the regulatory threshold of "presence." Cell fixation is required to allow sample transport before analysis. However, some key identification features are obscured by Lugol's solution, which is used in almost all regulatory monitoring (Collins et al. 2009), identification, and enumeration of *Alexandrium*, even to genus level, is therefore a skilled task. Lugol's fixed *Alexandrium* is often confused with other benign genera including *Scrippsiella* and manual manipulation of individual cells with a pin is often required to allow their identification.

Identification of *Alexandrium* cells to species level using light microscopy is more difficult still (Collins et al. 2009). Identification relies on the observation of a number of morphological features, such as cell size, thecal plate, the size and shape of the apical pore plates, sulcal list, posterior sulcal plate and the presence or absence and position of the posterior attachment pores (Hosoi-Tanabe and Sako 2006). Such features cannot be identified in Lugol's solution fixed samples, and in regulatory monitoring, there is insufficient time to study samples using calcofluor staining or electron microscopy techniques that can resolve such morphological features. Nor do these techniques allow easy quantification of cell abundance.

A further difficulty is that molecular analysis has distinguished five different ribotypes of *Alexandrium tamarensis* (Groups I–V) with varying potential for toxin production (Lilly et al. 2007). The highly toxic Group I *A. tamarensis* ribotype has historically been thought to dominate in Scottish waters (Bresnan et al. 2008). More recently, it has been shown that the morphologically identical nontoxin-producing Group III ribotype, which had previously only been identified in more southerly waters, can now co-occur with the toxic strain in Scottish waters (Collins et al. 2009; Brown et al. 2010; Touzet et al. 2010; Töbe et al. 2013). This makes it impossible to use *A. tamarensis* abundance as a robust early warning of subsequent

shellfish toxicity. These observations also raise questions over the factors governing the biogeography of this important organism that require laboratory study. However, the inability to discriminate between the different strains currently makes their enumeration in co-culture experiments impossible.

New detection methods for harmful phytoplankton in general, and most pressingly for toxic and nontoxic *A. tamarensis* strains, are therefore required to facilitate better understanding of HAB competitive dynamics in both the laboratory and the field, and to allow monitoring programs to enumerate the different strains for the benefit of both regulators and harvesters. Such methods require both a high sample throughput and a low detection limit.

Developments in molecular biology are increasingly allowing the genetic characterization of HAB populations (Anderson et al. 2012). Nucleic acids on ribosomal genes are commonly used genetic markers that have allowed for taxa specific identification from a number of molecular assays. This includes techniques such as fluorescent in situ hybridization (FISH), which involves intracellular hybridization of fluorochrome-conjugated oligonucleotide probes that are specific to RNA or DNA regions. Application of FISH probes to field material followed by fluorescence microscopy based enumeration has been successfully used by Touzet et al. (2010) and Davidson et al. (2010) to identify and enumerate Group I and Group III *A. tamarensis* ribotypes in Scottish waters. However, the time for an analyst to count the fluorescent cells by microscopy makes this methodology too costly to be used routinely within a monitoring program. Whereas other methods have also been developed that use FISH probe technology, including the ChemsanRDI system (Töbe and Medlin 2006) and microarray techniques (Metfies and Medlin 2008), these are also not yet operational in a monitoring context.

A potentially much more rapid alternative to microscope detection/enumeration of fluorescently labeled cells is flow cytometry (FC). FC 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 focused 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. FC 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 (Cucci et al. 1989). 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).

Here, we describe how a combination of FC and FISH (FISH-FC) allows the rapid discrimination and enumeration of toxic and nontoxic *A. tamarensis* hybridized with fluorochrome-conjugated oligonucleotide probes. As well as its potential appli-

cation to field populations, the method is suitable for enumerating cells in laboratory culture, hence allowing the study of competition between different ribotypes of the same species, an important topic in the study of the biogeography of this key species.

Methods

Alexandrium cultures

Group I (CCAP 1119/28) (toxic) and Group III (CCAP 1119/31) (nontoxic) 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 in this study. The cultures were grown separately in 1 L Erlenmeyer flasks in L1 media at 15°C under a light intensity of 100 $\mu\text{mol } \mu\text{E m}^{-2} \text{ s}^{-1}$ (12h:12h light:dark cycle).

A. tamarense dilution series and light microscopy enumeration

A 50 mL aliquot of each culture was removed aseptically from exponentially growing *A. tamarense* Group I and III cultures. A 15 mL aliquot of this culture was kept as the 100% reference with the remaining culture 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. This 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 (LeGresley and McDermott 2010) at 100 \times magnification by microscope (Leitz Wetzlar Ortholux). This cell number was used to estimate the number of *Alexandrium* cells within the serial dilutions.

FISH sample preparation

Triplicate subsamples (10 mL) were removed from each culture dilution and made up to 40 mL using cell free medium. This was then fixed with formalin (1% final concentration) for 1 h before being centrifuged (4000 rpm for 10 min). The supernatant was then discarded. Ice cold methanol (10 mL) was then added to the cell pellet to extract the pigments (Touzet et al. 2010) The sample was then stored at -20°C overnight before analysis using FC.

The taxa specific oligonucleotide probes TamA and Tam-

ToxC (MWG-Biotech) (Touzet et al. 2010) were used to label the Group III and Group I *A. tamarense* ribotypes, respectively. The probes were synthesized and labeled with either CY.3 (TamToxC) or fluorescein isothiocyanate (FITC) (TamA) (Table 1), with binding positions differing between the two probes. The probes were diluted to a final concentration of 80 ng μL^{-1} and stored at -20°C .

The cell samples were removed from the -20°C freezer and centrifuged (4000g for 5 min) and the methanol supernatant aspirated off. Hybridization buffer was added (500 μL) to the cell pellet and the cells re-suspended to rinse off the methanol. Hybridization buffer consisted of 10 mL 25 \times SET and 500 μL of 10 % IGEPAL (Sigma Aldrich). 25 \times SET included 54.8 g NaCl, 124 mL Tris (1 M, pH 7.8), 12.5 mL EDTA (0.5 M), and 57.7 mL di- H_2O . The samples were then centrifuged (4000g for 5 min) and the supernatant removed. The process was then repeated to ensure that all methanol was removed from the sample. A 500 μL aliquot of hybridization 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. The cells were then re-suspended and incubated in the dark (55°C for 60 min).

After hybridization the cells were pelleted by centrifugation (4000g for 5 min) and the supernatant discarded by aspiration. The samples were then washed with 500 μL of preheated (55°C) 0.2 \times SET buffer to remove the excess unbound probes. The 0.2 \times SET buffer was heated along with the samples in an incubator. The samples were then centrifuged (4000g for 5 min) a final time and the supernatant removed. The cells were then resuspended in 2.5 mL autoclaved filtered seawater for subsequent enumeration.

To assess whether the signals detected subsequently by FC could be attributed to the FISH probes, and not residual pigment autofluorescence, negative control samples were also assessed. These control samples consisted of a mixed *A. tamarense* population containing an equal number of Group I and Group III cells that had undergone identical fixation to the probed samples but did not contain either probe.

FC enumeration of ribotypes

To identify and enumerate the fluorescently labeled *A. tamarense* cells a FACSsort (Becton Dickinson) flow cytometer fitted with a blue argon laser (488 nm) and a 150 μm instrument aperture was used. The sheath fluid used was FACSFLOW (BD Biosciences). Detection and discrimination of toxic and

Table 1. Taxa-specific oligonucleotide probes TamA (Group III) and TamToxC (Group I) targeting sites in the D1-D2 domain of LSU rDNA (Touzet et al. 2010).

Target taxon	Probe sequence [5'-3']	Melting temp. ($^{\circ}\text{C}$)	Fluorochrome
<i>A. tamarense</i> (Group III)	TamA TCACCCACAGCCAAAACCTA	63.3	FITC (5' + 3')
<i>A. tamarense</i> (Group I)	TamToxC GCAAGTGCAACTCCCACCA	67.7	CY.3 (5')

nontoxic *A. tamarense* was achieved based on side scatter (SSC) and fluorescence detection of the FITC labeled TamToxC and CY.3 labeled TamA probes within the FL1 channel.

A 1 mL aliquot was removed and analyzed. In all cases, samples were analyzed in triplicate with analysis being conducted rapidly (within 2 min) as the probes are quickly degraded by light.

A. tamarense concentrations, X (cells mL⁻¹), were calculated from:

$$X = (1/V) \times C$$

where C is the cell incidences count within the gated regions (R2 and R3) of Fig. 1, and V is the volume (mL) of the sample analyzed and calculated by

$$X = \left(1 / \frac{F}{D}\right) \times B$$

where F is the flow rate of the flow cytometer, D is the dilution factor of calibration beads to sample, and B is the bead incidences within the gated region R1 (Fig. 1).

The FC flow rate was calculated by using a 1 μ m Fluoresbrite latex bead solution (Polysciences). The bead solution was made up by adding 4 μ L of 1 μ m beads to 20 mL FACSFlow solution and filtering it through a 2 μ m, 25 mm membrane filter (Poretics) into a further 380 mL FACSFlow solution then 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) 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 30 s, 60 s, and 90 s at each flow rate. The bead incidences were then used to calculate the flow rate daily.

The efficiency of data acquisition by the FC depends on the coincidence rate. At high event rates (>1500 events s⁻¹), events can be lost if the processor cannot keep up with the number of events, or if more than one cell enters the detector at one time (Campbell 2001). To obtain the greatest accuracy the samples were therefore diluted to ensure that the event rate was < 1000 event s⁻¹.

Spiked field samples

To test the effectiveness of the method on natural samples, seawater samples (2 L) were collected by ship-deployed niskin bottles from Scottish coastal waters at site LY1 in the Firth of Lorn, Scotland (Fehling et al. 2006) in late summer. In the laboratory, the water samples were screened through a 100 μ m filter to remove larger zooplankton. Aliquots (200 mL) were removed and spiked with known concentrations of both toxic and nontoxic *A. tamarense* cells. Spiked concentrations were as follows: 6000 cell L⁻¹, 4000 cell L⁻¹, 2000 cell L⁻¹, 1000 cell L⁻¹, 500 cell L⁻¹, and 100 cell L⁻¹ and were chosen to represent typical natural *A. tamarense* densities that may cause shellfish toxicity. Samples were fixed with formalin (1% final concentration) and left for 1 h before being centrifuged and prepared as

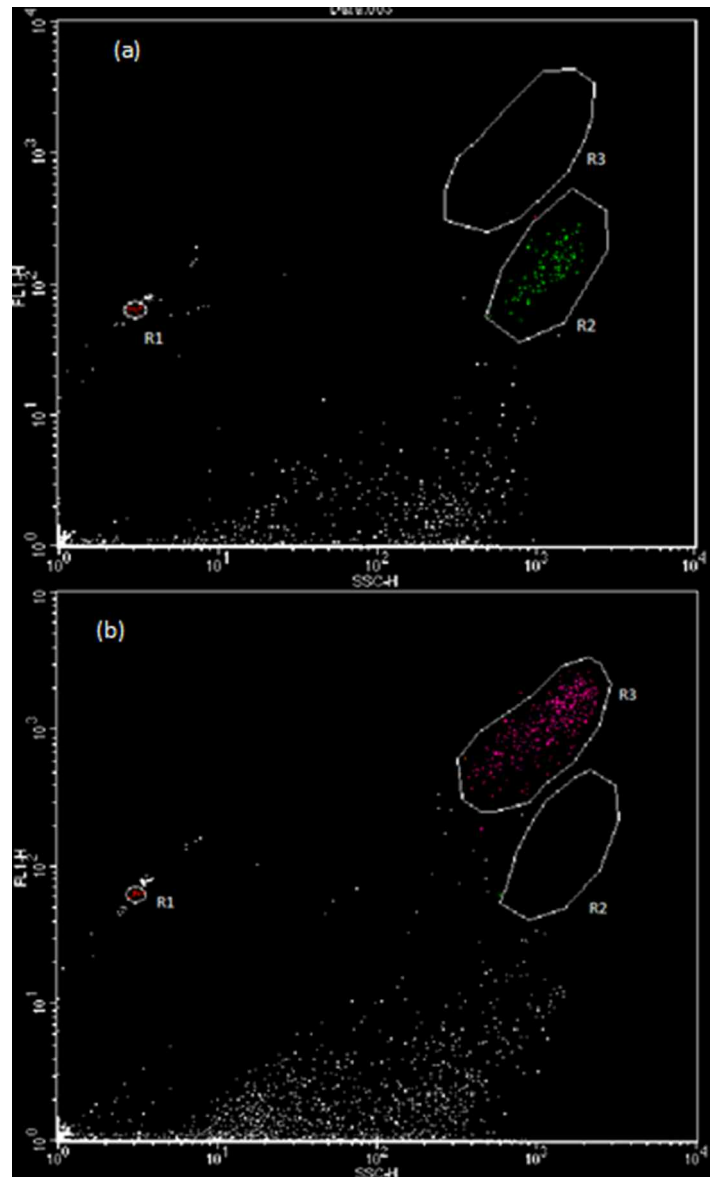


Fig. 1. FC dot-plot from cultured cells demonstrating the enumeration within gated regions of toxic and nontoxic *A. tamarense* (a) toxic strain with CY.3 labeled TamToxC probe, and (b) nontoxic strain with FITC-labeled TamA probe, R2, and R3, respectively. R1 is a gated region for the fluorescent latex beads of known concentration that are used to calibrate the instrument.

described above and enumerated as described previously. Both probes were then added to each sample.

Statistical procedures were carried out in Minitab statistical software. A Mann-Whitney test was used to test the differences between the mean cell densities between counting methods. A general linear model (GLM) was used to compare the linear trends of FISH-FC and microscope enumerations over the full range of dilutions. $P < 0.05$ was considered significant and variability was measured by standard error of the mean (S.E.M.).

Assessment

Count comparison: Culture

Group I and Group III *A. tamarense* cells labeled by FISH with fluorochrome-conjugated oligonucleotide probes created distinct clusters on the FC bi-plot of SSC versus FL1 (Fig. 1a,b) in the CellQuest software (Becton Dickinson). The clusters formed by the Group I and Group III strains allowed their enclosure in gated regions (R2 and R3 enclosed by the white lines in Fig. 1), and hence enumeration of the number of cells in each region.

To confirm that the signals detected were attributable to the FISH probed cells and not residual pigment autofluorescence, the unprobed blank samples were analyzed using the same FC gain settings as the probed samples. Fig. 2 demonstrates that the unprobed cells created a cluster (R4) on the FC bi-plot of SSC versus FL1 that was distinct from the regions occupied by the probed Group I and Group III cells.

A comparison of cell counts obtained by FISH-FC and light microscopy using a Sedgwick Rafter counting method was performed on the monocultures of the two ribotypes of *A. tamarense* at the six cell densities of each, generated by the dilution series (Fig. 3). A Mann-Whitney test was used to evaluate the differences between the mean cell densities obtained using the different counting methods. For both toxic and nontoxic cells, we found no significant difference between methods at the 5% level ($P > 0.05$). Similarly, if the linear trends in FISH-FC and microscope enumerations were compared over the full

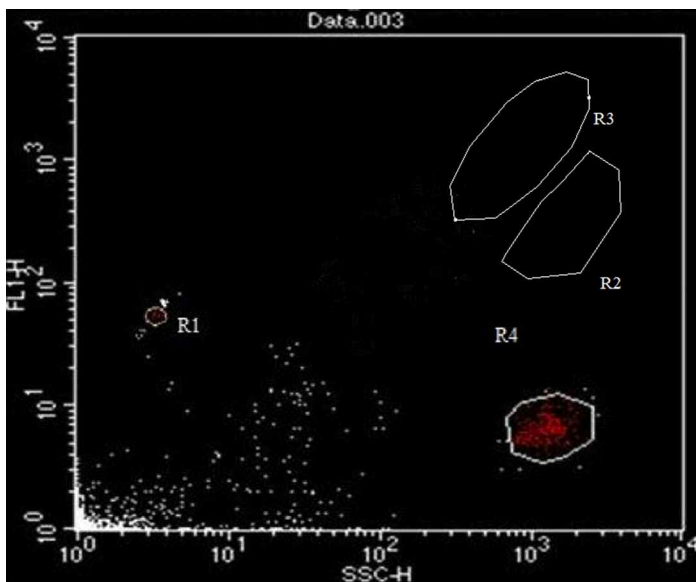


Fig. 2. FC dot-plot from cultured cells demonstrating the enumeration within gated regions of an unprobed toxic and non-toxic *A. tamarense* mix (R4). The gated regions R2 and R3 show the regions where the labeled toxic and non-toxic cells should concentrate. R1 is a gated region for the fluorescent latex beads of known concentration that are used to calibrate the instrument.

range of dilutions, no significant difference was found (GLM $P > 0.05$) for either toxic (Fig. 3a) or nontoxic (Fig. 3b) cells.

Field samples

Alexandrium blooms are temporally and spatially variable in Scottish waters (Davidson et al. 2011, Swan and Davidson 2011), and no blooms of this organism occurred in suitable sampling locations during our study. Moreover, we wished to evaluate the ability of our method to enumerate a range of concentrations of toxic and nontoxic cells in field samples, something that would not be easily achieved when sampling natural *Alexandrium* populations of unknown and rapidly changing density and composition. Hence, natural seawater samples collected in late summer were spiked with known concentrations of Group I (toxic) and Group III (nontoxic) *A. tamarense* strains from laboratory cultures. The seawater samples were collected at time when there were large numbers of

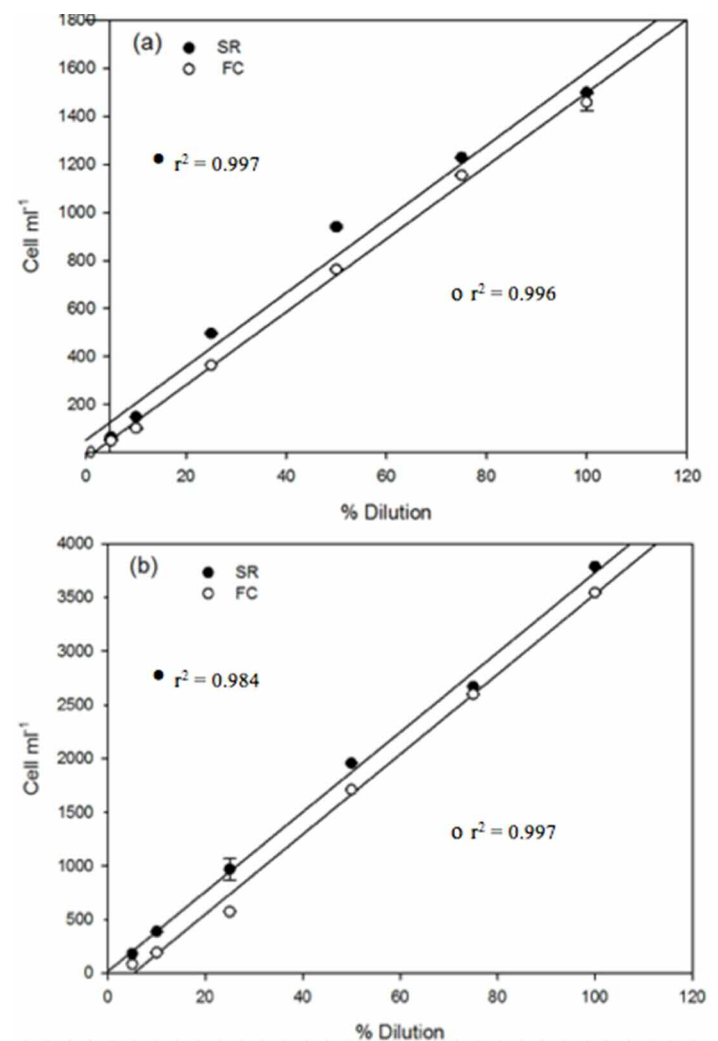


Fig. 3. the relationship between FISH-FC (○FC) derived *A. tamarense* abundance for (a) toxic and (b) non-toxic cells and those obtained using a Sedgwick rafter (●SR) counting chamber. All results are means of triplicate samples. Error bars represent SE.

non-target cells in the water column including a bloom of the toxic diatom *Pseudo-nitzschia* (~1500 cell L⁻¹). This organism is common in Scottish waters (Fehling et al. 2006) and can co-occur with *Alexandrium* (Fehling et al. 2012). A range of *A. tamarense* concentrations were used, and samples were spiked with either Group I or Group III cells or a combination of the two. Fig. 4 is a dot plot that demonstrates the separation and discrimination between toxic (R2) and nontoxic III (R3) cells in a natural seawater sample. The ribotypes again formed distinct clusters that were easily identifiable and showed little to no influence from other phytoplankton cells that were in the sample.

Fig. 5 compares the spiked *A. tamarense* cell concentrations determined by microscopy and FISH-FC in these natural samples. Over the full range studied, FISH-FC estimated cell concentrations closely matched the known (Sedgwick Rafter cell determined) spiked cell concentrations for both the toxic and nontoxic cells, indicating that few cells had been lost during sample processing. There was also no statistically significant difference between toxic or nontoxic cell concentrations in comparison to the expected microscope counts (GLM $P > 0.05$ in both cases).

Discussion

The aim of this study was to assess whether the use of fluorescent in situ hybridization with flow cytometry (FISH-FC) has the capacity to provide regulators and researchers with an

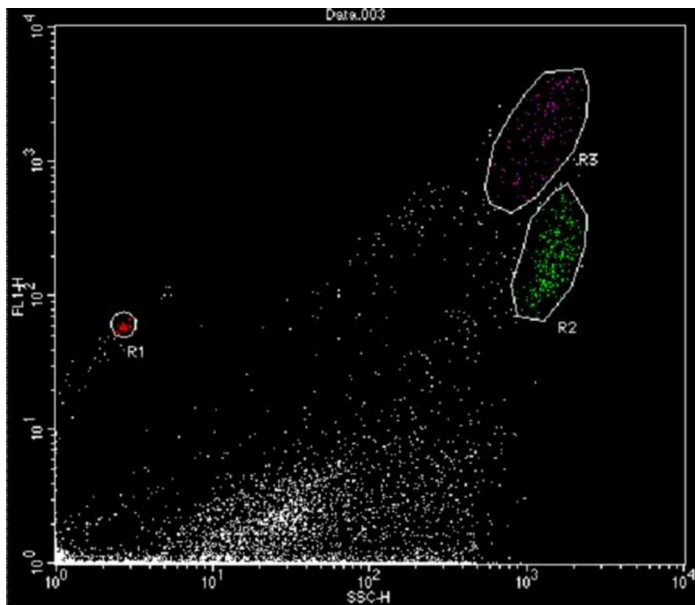


Fig. 4. FC dot-plot of a natural seawater sample spiked with cultured *A. tamarense* cells. This demonstrates the discrimination that could be achieved between toxic (R2) and nontoxic (R3) cells even in the presence of a natural assemblage of nontarget organisms. R1 is a gated region for the fluorescent latex beads of known concentration that are used to calibrate the instrument.

accurate and rapid method of enumerating Group I (toxic) and Group III (nontoxic) strains of *A. tamarense* in the laboratory and the field. As it is often difficult to distinguish between phytoplankton species or strains of interest and other morphologically similar organisms by light microscopy alone, identification using molecular markers is increasingly common (Touzet et al. 2010). Species or strain specific FISH probes used in combination with methods that characterize cells, such as microscopy or flow cytometry, can therefore be useful tools for evaluating individual phytoplankton species or strains. However, the use of epifluorescent microscopy to characterize individual species or strains, despite its accuracy, is time consuming and labor intensive with each sample taking in the region of 30 min or more to count. Therefore such methods are often impractical for large numbers of samples in laboratory conditions, or for the enumeration of field samples (for example within regulatory monitoring programs).

The FC-based method developed here requires a similar amount of preparation as would be the case if cells were analyzed by fluorescent microscopy. However, it allows for a much higher sample throughput, as FISH-FC can quantify cells quickly (~60 s). This suggests that the FISH-FC method is suitable for routine use in the laboratory. Moreover, when analyzing field samples, the time for analysis also compares favorably to light microscopy where a 20 h settling period is required using the Utermöhl method, prior to analysis.

The fluorochromes used in our previous microscopy-based application of these probes (Touzet et al. 2010) proved to be suitable for this FC-based application. With 488 nm excitation FITC emits at ~ 519 nm within the green FL1 channel of the FC. Whereas the optimal excitation and emission wavelengths of CY.3 are 550 and 570 nm, respectively, its excitation and

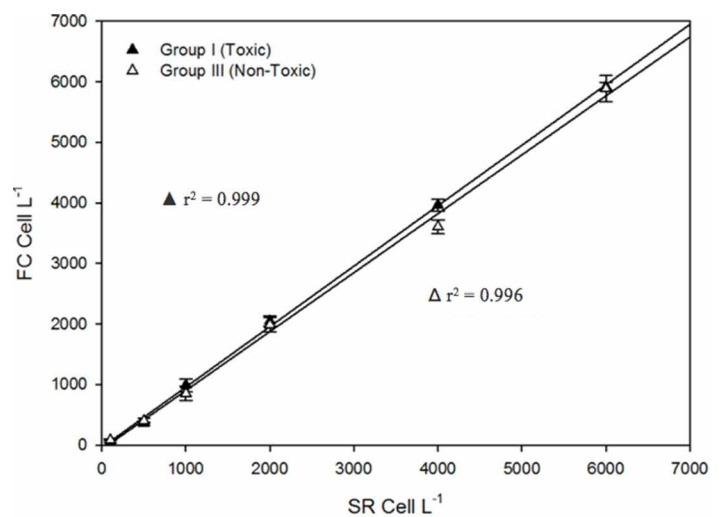


Fig. 5. The relationship between FISH-FC derived *A. tamarense* abundance for natural seawater samples spiked with microscope derived (SR) concentrations of toxic (▲) and nontoxic (△) *A. tamarense* cells. All results are means of triplicate samples. Error bars represent the SE.

emission spectra are very broad, hence allowing its detection in the FL1 channel in our set up. Hence, whereas it would be possible to substitute an alternative fluorochrome, use of CY.3 achieved excellent detection (Figs. 3 and 5). We therefore retained this fluorochrome as it also allows easy examination of the samples by fluorescence microscopy (using mercury lamp excitation) should further direct investigation of the sample be desirable.

There are two main problems when labeling cells for enumeration by FC. First fixation: fixation of cells has traditionally been used to avoid changes in cells numbers and cell characteristics over time. In addition to this, some fixative permeate the cells, allowing molecules such as nucleic acid fluorochromes to penetrate the cells easily (Troussellier et al. 1995). The most widely used fixative, particularly within phytoplankton monitoring programs, is Lugol's solution, an acid iodine solution (Modigh and Castaldo 2005). Lugol's solution is a fixative-preservative stain; however it causes shrinkage and cell distortion as well as masking fluorescence even after washing with sodium thiosulphate (Auinger et al. 2008). On this basis, Lugol's solutions was not used for cell fixation in our study. Glutaraldehyde is another common fixative in microscopy, but cells fixed with glutaraldehyde have a lower permeability causing incomplete staining (Troussellier et al. 1995) and its high auto-fluorescence makes it unsuitable for FISH detection. Hence, the fixative used for this method was formaldehyde. Formaldehyde as a fixative can result in lower or variable fluorescence (Baerlocher et al. 2006), but it was the most appropriate fixative for our method as it increases cell permeability and can give recoveries of 70% to 99% (Peperzak et al. 2000). Subsequent transference to alcohol allows good long-term preservation (Troussellier et al. 1995).

The second potential problem is cell loss in the number of washing and centrifugation steps involved in the labeling process. We found that cell loss was low in our methodology with no statistically significant loss of cells following the preparation steps. However, we did observe a small (non-statistically significant) percentage cell loss (typically ~3%), when compared to light microscopy, through repeated centrifugations and resuspensions. Similar cell loss is also evident if the FISH samples are analyzed by fluorescent microscopy (Touzet et al. 2010). Cell losses can be reduced with practice and certainly for regulatory monitoring, given the vagaries related to collection of phytoplankton samples (Davidson et al. 2011) are unlikely to be of significance.

For field samples, in addition to the difficulty in discriminating between Group I and Group III cells, a further hurdle in the enumeration of *Alexandrium tamarense* cells (Group I or Group III) is that this species rarely "blooms" to high density, and hence, rarely numerically dominates the phytoplankton assemblage. Cell densities ranging between 20–10,000 cells L⁻¹ are typical in Scottish waters (Swan and Davidson 2011; Davidson et al. 2010). The difficulties encountered by the microscopist are clear given these low densities and the mor-

phological similarity of these cells (particularly when Lugol's solution fixed) to other benign organisms. Whereas microscopy-based enumeration can exhibit a low limit of detection, for example the Utermöhl method allows densities as low as 20 cells L⁻¹ to be enumerated, such methods exhibit reduced precision and reducibility at low target cell concentrations. In contrast, molecular methods can have a standard deviation that is not affected by target cell concentration (Godhe et al. 2007).

A concern in FC analysis is the ability to readily distinguish between target populations and non-target populations of cells (Anderson et al. 1999). It is therefore important when using FC that control samples are used (Hulspas et al. 2009). Fig. 2 demonstrates that natural autofluorescence of *A. tamarense* does not overlap the target gated regions for probed Group I or Group III cells. Moreover, the post fixation methanol extraction reduced autofluorescence by up to 90% (Gard and Kropf 1993). This minimizes any autofluorescence from other cells should this technique be used with a natural community, allowing good *A. tamarense* cell recovery (Figs. 4, 5).

Aperture clogging is a concern with FC of natural planktonic assemblages. The initial filtration step carried out on the field samples meant that large aggregations and chains were removed before examination. Re-suspension of the cells prior to analysis using a desk top vortexer also reduces instrument blockages. Due to this, and the large aperture used (150 µm), the instrument clogged infrequently, blocking only twice while carrying out testing (≈200 samples). However, it is clear that clogging may occur for some samples, but the use of the FITC and CY.3 allows such samples to be enumerated by fluorescence microscopy if necessary.

Molecular methods are also the obvious choice when co-occurring morphologically similar non-target species are present, particularly as microscopic examination of field samples requires a high degree of taxonomic experience. While an individual conducting identification by molecular methods also requires experience, it is faster and easier to become competent in the use of such techniques (Godhe et al. 2007). Fortunately, while the regulatory threshold for *Alexandrium* is "presence," shellfish toxicity is rarely observed at *A. tamarense* cell densities below ~ 500 cells L⁻¹ in Scottish waters (K. Davidson pers. observation) and hence the data of Fig. 5 demonstrates the potential of FISH-FC to both enumerate and categorize as Group I (toxic) and Group III (nontoxic), "blooms" of typical natural concentrations. In particular, no significant cell loss was observed in comparison to light microscopy though sample screening. Neither were the results influenced by the natural fluorescence of other co-occurring organisms in the natural seawater samples despite there being a high *Pseudo-nitzschia* cell abundance in the water column at the time of sampling.

In summary, FISH-FC enumeration of *Alexandrium tamarense* was found to compare well with light microscopy in

terms of cell abundance estimation, with (through the removal of the need for a settlement phase) more rapid analysis being possible. In addition, and critically for regulators and the shellfish industry, the method allows the discrimination and enumeration of toxic and nontoxic ribotypes of this important organism, allowing its potential application within multi-species studies and/or regulatory or environmental monitoring programs where they co-exist.

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