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The competitive dynamics of toxic *Alexandrium fundyense* and non-toxic *Alexandrium tamarense*: the role of temperature

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Highlights

- Yield, growth and toxin production of *A. fundyense* and *A. tamarense* were studied.
- Observed growth rates varied with temperature.
- Cell/biomass yield was greater for *A. tamarense* at all temperatures.
- Species interactions were temperature dependent.
- *A. fundyense* toxin production was inhibited in co-culture with *A. tamarense*.

Key words:

Alexandrium fundyense, Alexandrium tamarense, biogeography, competitive interactions, toxicity, Scotland, FC-FISH

Abstract

The dinoflagellate *Alexandrium* produces paralytic shellfish poisoning toxins. The genus is globally distributed, with Scottish waters being of particular interest due to the co-occurrence of different species and strains. In Scottish waters, *Alexandrium* was historically thought to be dominated by the highly toxic (Group I) *A. fundyense*. However, the morphologically indistinguishable (Group III) *A. tamarense* has recently also been found to co-occur, raising important questions in relation to *Alexandrium* biogeography. To begin to address these, we investigated *Alexandrium* growth, yield and toxin production in a range of temperature conditions characteristics of present and potential future conditions, using a recently developed flow cytometry method that allowed, for the first time, simultaneous enumeration of the cryptic species in co-culture. Experiments were undertaken in a range of temperatures (12, 15, 18 and 21 ^{o}C) in the phosphate (P) limiting conditions that promotes *A. fundyense* toxicity. Cell/biomass yield was greater for *A. tamarense* at all temperatures, with observed growth rates varying with temperature. Growth rather and yield were different in mono- and co-culture with the outcome of these interactions also being temperature dependent. For toxic *A. fundyense*, GTX-3, STX and NEO were the dominant analogues, but total toxicity, toxicity per cell and the number of, and relative proportion of, toxin analogues changed in relation to the onset of P limitation and also as a function of temperature, with the highest toxin concentrations per cell being observed at 12 °C. Toxin concentrations were approximately double in P limited stationary phase compared to exponential growth. Toxin concentrations were lower in the co-cultures, indicating inhibition of production in the presence of non-toxic *A. tamarense*. The strong performance of *A. tamarense* is in co-culture at odds with the historical understanding that Scottish waters were dominated by *A. fundyense* and indicates that changes in water

temperatures, and also potentially alleopathic interactions, will influence *Alexandrium* populations and hence the PSP toxicity threat to humans from shellfish.

Introduction

The observed frequency, intensity, and geographical distribution of observed harmful algal bloom (HAB) events have increased over the last few decades (Gowen et al., 2012; Hallegraeff, 1993; Lilly et al., 2007) with the genus *Alexandrium* being of global importance due to its widespread distribution and synthesis of potent neurotoxins that are associated with Paralytic Shellfish Poisoning (PSP). Humans, birds, sea mammals and fish can all be affected by PSP toxins, with the usual exposure route for humans being through the consumption of contaminated shellfish (Smayda., 2004).

The majority of *Alexandrium* based toxicity events are thought to have been caused by the morphospecies *A. tamarense, A. catanella* and *A. fundyense*, which make up the *A. tamarense* species complex (Scholin et al., 1994). The taxonomy of this complex has been questioned (Anderson et al., 1994; Scholin et al., 1995). Initially Scholin et al., (1994) demonstrated a correlation between strains and their geographical origin with five main phylogenetic clades defined: North American, Temperate Asian, Western European, Tropical Asian and Tasmanian. Subsequently Lilly et al. (2007) demonstrated these clades to have genetically distinct lineages and named them Group I-V. Previously in North West Europe, *A. tamarense* has been reported to occur as either the biotoxin producing Group I (North American) ribotype or the non-toxin producing Group III (Western European) ribotype, with their occurrences being geographically separated. Recently John et al. (2014) have reclassified Group I *A. tamarense* as *A. fundyense*, it is this reassignment that we use within this paper.

In Scottish (and northern English) waters, monitoring programmes have demonstrated that the genus *Alexandrium* is a common constituent of phytoplankton communities in spring and summer (Stubbs et al., 2014). Regional hotspots include: Orkney, Shetland, the Western Isles, the west coast and Northern England (Collins et al., 2009), however information on the biogeography of specific *Alexandrium* spp. in the region remains limited. Medlin et al. (1998) analysed the D1/D2 region of the LSU rRNA gene from number of isolates from the Orkney Islands and found them all to be *A. tamarense* (using the taxonomy of the time) rather than *A. fundyense*. The periodic high toxicity levels in Scottish shellfish, the lack of records of the non-toxic Group III *Alexandrium* above 55 ^{o}N (Töebe et al., 2013), and that UK monitoring programs only identify *Alexandrium* to genus level, led to the assumption that *Alexandrium* populations in Scottish waters were exclusively (toxin producing) Group I. Recent observations question this, as Group III cysts and vegetative cells have now been shown to have a wide geographical distribution in Scottish waters (Brown et al., 2010; Collins et al., 2009) and Group I and Group III cells have been co-observed in the water column, both in the Shetland Isles at 60 ^{o}N by Touzet et al. (2010), and in the North Sea at ~ 57 ^{o}N by Töebe et al. (2013). The spatially extensive distribution of Group III cells in the latter study clearly indicates that these observations are more than transient events, and coincide with the decline in the occurrence of PSP toxins in Scottish shellfish in recent years (Bresnan et al., 2008).

Töebe et al. (2013) and Touzet. (2010) have observed Group I A. *fundyense* and Group III *A. tamarense* co-occurring in the water column, with hybrid cysts having also been identified in Scottish sediment samples (Eckford-Soper, 2013), indicating that there is no *a priori* reason to assume that blooms of Group I and Group III cells will occur in isolation, and hence that both mono- and multi-species blooms might be

expected in Scottish waters. Lacking molecular studies of the sediment record, we can only speculate if these new observations relate to a change in the biogeography of the *A. tamarense* species complex, a change in their relative composition, a historical misunderstanding of community composition, or are simply related to historical genus based monitoring. However, given the Group I *A. fundyense* / Group III *A. tamarense* co-occurrence it is clear that laboratory studies of their physiology should consider their co-occurrence, competition and interactions.

The aim of our study was therefore to investigate cell yield, growth rate, and toxin production of Group I *A. fundyense* and Group III *A. tamarense* obtained from Scottish waters in isolation and under co-culture. Experiments were conducted with strains maintained at a range of environmentally relevant temperatures and the nutrient conditions likely to promote toxicity of the *A. fundyense*.

Materials and Methods

2.1. *Culture methods and cell acclimation*

Two Group I *A. fundyense* (CCAP 1119/24 and 1119/28) and two Group III *A. tamarense* (CCAP 1119/31 and 1119/33) strains that had previously been isolated by Marine Scotland Science from Scottish waters (Scapa, Orkney) and subsequently deposited in the Culture Collection of Algae and Protozoa (CCAP) at SAMS, were initially chosen for our study. Cultures were grown in 1litre Erlenmeyer flasks in batch mode, acclimated to a modified phosphate (3 µM) L1 medium (Guillard and Hargraves., 1993) in which all other nutrients were available in excess. Experiments were conducted at four different temperatures: 12 ºC, 15 ºC, 18 ºC and 21 ºC. The three lower temperatures were chosen to represent the range of conditions that an *Alexandrium* cell might experience in Scotland presently or in future scenarios due to climate warming:

12 ºC reflects the current sea surface temperatures of a typical Scottish spring; 15 ºC the current sea surface temperatures of a typical Scottish summer; and 18 ^{o}C was chosen as a hypothetical summer temperature in the coming decades (Harrison et al., 2001; Hughes, 2007). It is unlikely Scottish seas will reach a temperature of 21 ^{o}C and hence only temporal changes in cell yields were determined at this temperature. A light intensity of 100 µmol photons m^{-2} s^{-1} and 12h:12h light:dark cycle was used in all experiments. Stock cultures were maintained in exponential growth by regular sub-culturing every 10 days. During acclimatisation to the different temperature the growth of the cultures was monitored using *in vivo* fluorescence (RFU) as a proxy for cell numbers (Turner Trilogy, UK). Experiments were only conducted when stock cultures exhibited a consistent and reproducible exponential growth rate when assessed using a General linear Model (GLM). Acclimation took between 4-7 re-inoculations depending on the temperature.

2.2. *Mono- and co-culture experiments*

Initial experiments were undertaken to evaluate any influence of agitation associated with sampling and the similarity of growth rates between different *A. fundyense* and *A. tamarense* strains. Given the results of these experiments (detailed below in section 3) and logistical constraints of incubator space, subsequent mono-culture and co-culture experiments were conducted in triplicate on one toxic *A. fundyense* (1119/28) and one non-toxic *A. tamarense* (1119/33) strain.

In the mono-cultures experiments *A. fundyense* and *A. tamarense* were studied separately, and in the co-culture experiments they were grown in the same flasks. Inoculums for all experiments were taken from acclimated late exponential phase stock cultures. The inoculum cell density in the mono-cultures was ≈ 400 cells ml^{-1}, while in

the co-cultures each strain was added at an initial concentration of ≈ 200 cell ml^{-1}. The same modified L1 medium described in section 2.1 was used. Cells were allowed to grow in batch mode until the post stationary phase of the culture had become established.

2.3. *Enumeration of total cell abundance & growth rate determination*

Daily sub-samples were removed aseptically from each flask. Cells were preserved with Lugol's iodine solution (1% final concentration) and enumerated using a 1 ml Sedgewick-Rafter counting chamber. Growth rate was calculated as divisions per day. Using the logistic growth equation (Verhulst, 1838) we were able to take into account the impact of the carrying capacity on growth, Equation 1:

$$\frac{dN}{dt} = \mu N\left(\frac{K-N}{K}\right) \qquad (1)$$

Where dN/dt is the expected rate of change in the population size at any point in time given the population size (N), the carrying capacity of the environment (K), and the intrinsic growth rate (μ). The intrinsic rate of growth is the rate of growth when individuals are not constrained by environmental limits. The integrated form of the logistic equation, Equation 2 (Ferris and Wilson, 1987) was fitted (using a least squares approach):

$$N_{(t)} = \left(\frac{N_0 K}{N_0 + (K-N_0)e^{-\mu t}}\right) \qquad (2)$$

2.4. *Discrimination and enumeration of A. fundyense and A. tamarense in co-culture*

Daily sub-samples were collected for fluorescent *in situ* hybridisation based enumeration of the different cryptic species (Eckford-Soper et al., 2013). A subsample of culture (10 ml) was removed and made up to 40 ml using autoclaved filtered

seawater. This sample was fixed with formalin (1 % final concentration) and left to rest for one hour before being centrifuged (4000 g, 10 mins). The supernatant was then discarded. Ice cold methanol (10 ml) was added to the cell pellet to extract the pigments and nucleic acids (Touzet et al., 2010). The sample was stored at -20 °C until analysis.

The taxa specific oligonucleotide probes TamA (for *A. tamarense*) and TamToxC (for *A. fundyense*) (Touzet et al., 2010) were used to fluorescently label the different strains. Samples were removed from the -20 °C freezer and centrifuged (4000 *g*, 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 re-suspended to rinse off the methanol. The samples were centrifuged (4000 *g*, 5 min) and the supernatant removed. This process was repeated to ensure that the methanol was removed from the sample. Cells were re-suspended in 500 µl of hybridisation buffer containing 1 µl of each of the taxa specific probes. After this time the samples were kept in the dark by wrapping the tubes in aluminium foil and incubated in a dark incubator (55 °C, 60 min). After hybridisation, the cells were pelleted by centrifugation (4000 *g*, 5 min) and the supernatant discarded. Samples were washed with 500 µl of preheated (55 °C) 0.2X SET buffer to remove the excess unbound probes. Samples were then centrifuged (4000 *g*, 5 min) one final time and the supernatant removed. Finally, the cells were re-suspended in 2.5 ml of autoclaved filtered seawater for subsequent enumeration.

Flow cytometric identification and enumeration of the labelled cells was conducted using a FACSort (Beckton Dickinson) instrument fitted with a blue argon (488 nm) laser and a 150 µm instrument aperture. Discrimination and enumeration of the two ribotypes was achieved based on side scatter (SSC) and fluorescence detection of the TamToxC and TamA probes within the FL1 (515-545 nm) channel (Eckford-Soper et al., 2013). Aliquots (1 ml) were analysed with fluorescent acquisition, gated by

light scatter parameters. The sample was analysed rapidly (within two minutes) as the probes are quickly degraded by light.

2.5. *Toxin analysis*

Toxin analysis was only carried out on samples incubated at 12, 15 and 18˚C. Samples were collected during the exponential and stationary phases of growth. Aliquots were harvested aseptically containing a known concentration (typically ~ 250,000 cells) and centrifuged (3000 g, 20 min). The supernatant was removed and the cell pellet was stored at – 20 °C. Subsequent analysis was conducted by the post-column oxidation (PCOX) that uses reversed phase liquid-chromatography (LC) with post-column oxidation and fluorescent detection (Van De Riet et al., 2009). Two analyses were performed per sample, firstly to analyse GTX and STX toxins and secondly to analyse the C-toxins. The samples were tested for GTX-1, GTX-2, GTX-3, GTX-4, de-GTX-3, dc-GTX-2, dc-STX, STX, C-1 and C-2.

Prior to analysis cell pellets were thawed and 1ml of autoclaved filtered seawater was added to the sample and vortexed. The solution was transferred to an Eppendorf micro-centrifuge tube. To ensure all cells were transferred the process was repeated using an additional 200 µl of filtered seawater. The cell solution was centrifuged (3000 g, 20 min), the supernatant discarded, and acetic acid (500 µl of 0.5 M) was added to the cell pellet. Toxin extraction was carried out by freezing at -80 °C (30 min) and thawing at room temperature (30 min) three times. The samples were vortexed and centrifuged (8000 g, 20 min). Aliquots (200 µl) of the toxin extracts were transferred to a Millipore Ultrafree MC 0.2 µm filter unit and centrifuged (6000 g, 3 min) (Van De Riet et al., 2009). The contents were then transferred to glass reaction vials.

The working solutions were prepared from individual PSP standards purchased from NRC (Canada). The stock standard mixture and serial dilutions for the instruments linear calibration were prepared as described in Van De Riet et al. (2009). All calibration curves demonstrated good linearity with the r^2 value being greater than 0.99. To ensure instrument reliability a previously quantified sample was analysed before the culture samples.

The analysis system consisted of: the LC system, post column reaction system, reaction coil, fluorescent detector and LC columns. The mixed working solution and serial dilutions (10 µL for GTX and STX toxins and 5 µL for C-toxins) were injected into the system and separated chromographically. Part of the extract was chromatographed with a step gradient using a heptane sulfonic acid/phosphoric acid buffer system for the analysis of GTX and STX analogues. The extract was also chromatographed for the C-toxins using an isocratic tetrabutylammonium phosphate buffer for the C-toxin analogues. The toxins were detected by PCOX of the analytes at 85 °C with a phosphoric acid periodic acid buffer solution followed by fluorescence detection (excitation: 330 nm, emission: 390 nm). Integration and data analysis was conducted using ChromQuest 2.6.1 software. To calculate the concentration of the toxins as STX equivalents, the concentrations of toxins (in nM) were converted to µmoles and multiplied by the relative toxicities of each individual toxin (Van De Riet et al., 2009).

2.6. *Other parameters*

The pH of each flask could be measured using a SevenEasy pH meter and calibrated using two solutions with known pH values. Every second day samples were removed aseptically for the determination of intracellular biomass and extracellular

nutrient concentrations. For particulate carbon and nitrogen (POC, PON), samples were collected by filtering 60-100 ml of culture (depending on the cell density) through a 25 mm diameter pre-combusted (450 ºC for 4h) GF/F filter (Fehling et al., 2005), which was then frozen at -20 °C for subsequent analysis. Aliquots (50 ml) of the filtrate were removed and stored at -20 °C for extracellular nutrient analysis (total phosphorus (TP) and total nitrogen (TN)). After defrosting TP and TN were analysed using a Lachat QuikChem auto analyser with Omnion 3.0 software (Davidson et al., 2007). POC measurements were made as described in (Flynn and Davidson, 1993) using a 20-20 stable isotope mass spectrometer (PDZ Europa) with an ANCA-NT prep system calibrated with isoleucine.

3. Results

3.1 *Preliminary experiments*

Initial monoculture experiments allowed comparison of the growth rate of the two *A. fundyense* and two *A. tamarense* strains. Growth rates of both *A. fundyense* and both *A. tamarense* strains were found to not be statistically significantly different (GLM $P>0.05$), with no influence of agitation on the calculated rates. Hence, only one *A. fundyense* strain (CCAP 1119/28) and one *A. tamarense* strain (CCAP 1119/31) were used for all further experiments.

3.2. *Mono-culture: temporal dynamics*

A. fundyense and *A. tamarense* were studied in the triplicated mono-culture experiments at the four experimental temperatures, and both exhibited net positive growth at all the temperatures studied (Fig. 1). The linear phase of logarithmic plots of cell abundance were examined, and in all cases exponential growth ceased a few days after the concentrations of

P (the yield limiting nutrient) reached its minimum value (data not shown). However, differences in response were evident between *A. fundyense* and *A. tamarense* at different temperatures. In general, the length of the exponential phase increased with temperature until 18 °C, and at all temperatures the duration of the exponential phase of cell division was longer for *A. tamarense*.

3.3. *Mono-culture: cell yields and growth rates*

Spearman Rank correlation analysis found statistically significant linear correlations between cell abundance and POC for both ribotypes at all temperatures evaluated (12 °C: *A. fundyense*, r = 0.954, p= 0; *A. tamarense* r = 0.939, p =0. 15 °C . *A. fundyense* r = 0.939, p = 0; *A. tamarense* r = 0.973, p = 0. 18 °C, *A. fundyense*, r = 0.918, p = 0, *A. tamarense*, r = 0.923, p = 0), demonstrating that cell numbers were correlated with biomass.

Mean peak cell density of the replicate *A. tamarense* cultures exceeded that of *A. fundyense* at all temperatures (Fig. 1). This difference was significant at all temperatures except 15 ^{o}C (Mann-Whitney, $p < 0.05$). While the extracellular liming P decreased through uptake to a low concentration, it was not always completely eliminated from the cultures, and between 0.01 – 0.22 µM remained (data not shown). Fig. 2 therefore displays peak cell yields normalised per µM of P utilised. This confirms that the *A. tamarense* strain was able to create a greater cell yield (and biomass) per unit of resource ($p < 0.05$).

Mean cell specific growth rate calculated is plotted as a function of temperature in Fig. 3, and is tabulated in Table 1. Growth rates exhibited a generally “humped” response for both strains, with highest rates at the intermediate temperatures of 15 and 18 ^{o}C and lowest at 12 ^{o}C. The bell shaped curve was more pronounced for *A. fundyense*, which had temperature optima of 18 °C with a sharp decline in growth rate either side of this. *A. tamarense* had a broader tolerance range at all temperatures except 12 °C. *A. tamarense* had a maximum SGR

that was statistically significantly higher (GLM $p > 0.05$) than that of *A. fundyense* at 12 °C, but at 18 °C the reverse was true (Fig. 3).

3.4 *Mono-culture: PSP toxins*

The amount and composition of STX produced by *A. fundyense* varied with both growth phase and temperature (Fig. 4). At all temperatures, cellular toxin concentrations increased considerably once cells entered stationary phase, with a ≥2-fold increase in comparison to the exponential phase of growth. This increase was due to an increase in individual analogue concentrations, and at 12 °C and 18 °C an increase in the total number of analogues detected. STX equivalents per cell were greatest at 12 °C (Fig. 4a), with concentrations in the exponential phase being approximately one to two thirds higher than at 15 °C (Fig. 4b) and 18 °C (Fig. 4c).

While STX was the main toxin analogue found at all temperatures (Fig. 4), a range of others were present. In exponential growth at 12 ^{o}C the analogues GTX-1, GTX-3, GTX-2, GTX-5 dc-GTX-3, NEO, STX, C1 and C2 were found. GTX-1, GTX-2, dc-GTX-3, GTX-3, NEO, STX and C-1, C-2 were all present at 15 °C. Fewer analogues were present at 18 ^{o}C with: dc-GTX-3 GTX-3, NEO, STX, C-1 and C-2 being observed. On entering stationary growth the number of analogues remained unchanged at 15 ^{o}C but increased at both 12^{o}C (to include GTX-2) and 18 ^{o}C with the number of analogues now including: GTX-1, GTX-3, dc-GTX-3, NEO, STX, C-1 and C2 (Fig. 4). GTX-2 and GTX-5 were not found at this temperature and the analogues GTX-4, dc-GTX-2 and dc-STX were not found at any temperature.

Cellular toxicity did not translate into total toxicity per unit of culture volume. For example, although cellular toxicity was greatest at 12 °C, the total toxicity per unit of culture volume was lowest at 12 °C (2845 pg STX diHCleq ml^{-1}) and highest at 15

°C (5583 pg STX diHCleq ml^{-1}) with no significant linear relationship with temperature ($r^2 = 36.9$, $p = 0.584$).

3.5 *Co-culture: cell yields and growth rates*

Growth rate estimates calculated by light microscopy (Sedgwick Rafter) and flow cytometry are compared in Table 1. Growth rates calculated by each method were found to not be statistically significantly different (GLM P>0.05).

Fig. 1 shows the changes with time of the combined abundance of *A. fundyense* and *A. tamarense* enumerated by FISH-FC in the co-culture experiments (in comparison to their growth in monoculture). Fig. 5, explores the dynamics of these culture in more detail by displaying the temporal evolution of cell densities of each of the competing cryptic species.

At all temperatures interaction between the two strains was evident. The effect was most clearly seen at 12 ^{o}C where *A. tamarense* dominated to the near exclusion of *A. fundyense*. In this case, the peak yield of *A. tamarense* was 87 % of the combined peak cell yield. While net growth of the *A. fundyense* did occur, it increased from 200 cells ml $^{-1}$ to a mean peak density of only 599 cells ml^{-1} and did not exhibit clear exponential growth. A competitive dominance of *A. tamarense*, although not to such a great extent, was also evident at 15 ^{o}C and 21 ^{o}C. In these cases, the peak yield of *A. tamarense* was 68% and 67% of the respective totals. However, at 18 ^{o}C these interactions were more balanced with similar peak densities (*A. fundyense* achieving 51% of the total). At all temperatures, except 18 °C where yields were similar, the dominance of the *A. tamarense* peak cell density over *A. fundyense* was statistically significant (Mann-Whitney $p > 0.05$). In co-culture the peak cell densities achieved by *A. tamarense* were relatively constant at all temperatures, varying from 4156 cells ml^{-1}

at 18 °C to 5350 cells ml^{-1} at 21 °C. However, the peak cell density of *A. fundyense* varied much more markedly with temperature from 599 cells ml^{-1} at 12 °C to 2847 cells ml^{-1} at 18 °C.

Maximum specific growth rates of the co-cultures (Fig. 3) exhibited a similar 'humped response' for *A. fundyense* as was evident in monoculture. For *A. tamarense*, the response was similar to monoculture at the three higher temperatures, but a markedly higher growth rate was evident in co-culture at 12 ^{o}C.

3.6. *Co-culture: toxins*

In co-culture, the pattern of increased toxicity from exponential to stationary phase evident in the *A. fundyense* monocultures was conserved (Figs. 4a-c). However the toxin per (*A. fundyense*) cell was markedly lower than in monoculture at all of the temperatures studied. At 15 °C the same toxin analogues were present as in monoculture. However, at 12 ^{o}C only GTX-3, NEO, STX, C-1 and C-2 were present during exponential growth with the addition of GTX-1, GTX-3, GTX-5, dc-GTX-3, STX and C-2 in stationary phase. At 18°C GTX-1 was absent from the mixed culture samples with GTX-3, NEO, STX, C-1 and C-2 being present during exponential growth, plus dc-GTX-3 in the stationary phase.

The observed toxin concentration normalised per unit culture volume was consistently lower in co-culture culture compared to monoculture. Concentrations exhibited a clear linear relationship with temperature (r^2 = 99.8, p = 0.025), increasing from (305 pg STX diHCleq ml^{-1}) at 12 °C to (2517 pg STX diHCleq ml^{-1}) at 18 °C.

4. Discussion

4.1 A. tamarense in Scottish waters

There are no major physical barriers to transport of phytoplankton cells in the waters surrounding the British Isles, as evidenced by examples of large distance transport of *Dinophysis* spp. and *Karenia mikimotoi* (Davidson et al., 2009; Farrell et al., 2012; Whyte et al., 2014). The coastal current of the Scottish west coast (Simpson and Hill., 1986) provides a mechanism of northwards transport of cells, with the fjordic sea lochs that dominate the Western and Northern coastline of Scotland providing an ideal refuge for cyst forming organisms such as the *Alexandrium*. However, biological barriers to range expansion may exist, with temperature tolerance being thought to be one of these (Hinder et al., 2011). For example, we have previously found strains of *A. minutum* to be unable sustain net positive growth at 12 ^{o}C (Davidson et al., 1999), an observation that may in part explain the lack of *A. minutum* in the relatively cold Scottish waters. Temperature is a key driver of ecological processes and a key abiotic driver of ecological systems; yet, the effects of temperature are complex. A review of 688 species by Tylianakis et al. (2008) found that temperature alterations affect a number of species to species interactions, including competition. Community structure is not just affected by the direct impacts of temperature on physiology but also on how these direct impacts affect other processes (Reuman et al., 2014). A better general understanding of how the physiological influences of temperature will give us a better understanding of species to species interactions and ultimately community dynamics. For example, Tilman et al. (1981) studied the competition interactions between two freshwater diatoms *Asterionella formosa* and *Synedra ulna* (Nitszch). They found that that due to their ability to reduce environmental silica at different temperatures *A. formosa* was able to displace *S. ulna* at temperatures below 20 ^{o}C with the reverse being true above 20 ^{o}C.

To ensure the results of our study were not an artefact of rapidly changing temperature regimes, cultures were first acclimated to the different temperatures.

Experiments were only conducted when the stock cultures exhibited a consistent and reproducible exponential growth rate. Although Scottish waters do not reach 21 ^{o}C, the inclusion of this temperature allowed us to study the growth and interaction response of these globally important organisms over a wider range of temperatures. Our observations of positive net growth of the different *tamarense* strains at all temperatures studied, and that the growth rate and cell yield of *A. tamarense* exceeded that of *A. fundyense*, at the lowest experimental temperature in our study (12 ^{o}C) demonstrates that temperature should not be an ecophysiological barrier to Group III *A. tamarense* survival in Scottish waters. Results at 12 ^{o}C are consistent with the study of Touzet et al. (2010) who found co-existence of Group I and Group III cells in the water column at this temperature. Both ribotypes were observed at even lower temperatures (10 ^{o}C) by Töebe et al. (2013). The "appearance" of Group III *A. tamarense* in Scottish waters is therefore likely not related to a climate-mediated northward increase in temperature, suggesting that Group III *A. tamarense* blooms may have been much more spatially and temporally extensive than previously recognised.

4.2 Yield and growth rates

Differences in cell yield between the different strains occurred at all temperatures. Cell densities were lower than those that might be expected to be impacted by light limitation through self-shading (Agusti., 1991). Furthermore, while growth in culture can often be inhibited by pH, it did not exceed 8.4 in our experiments (data not shown) and *A. tamarense* can typically tolerate a pH of above 9 (Hansen, 2002; Schmidt and Hansen, 2001).

It is well know that different species of phytoplankton can display a wide range of cellular composition with plasticity in cellular nutrient content and ratios, with

nutrient limiting conditions typically extending the range of C:N:P stoichiometry through increases in C:N or C:P ratios for N and P limited cells respectively (Flynn et al., 1993; Geider and La Roche., 2002). Hence the greater mean cell yield (and given the correlation between cells and C, of biomass yield) that was achieved by *A. tamarense* at all temperatures indicates a more efficient utilisation of P.

The lower yield of *A. fundyense* raises the question of whether the production of toxin has an energetic cost to the cell that diverts resources from biomass production. However, the fraction of cellular N associated with PSP toxins is small, and in a modelling study Flynn (2002) found toxin production to have no significant metabolic cost to the cell. Hence, a direct influence of toxin producing capability on the lower yield of *A. fundyense* seems unlikely. The difference in cell/biomass yield achieved with the same unit of resource (P) is therefore consistent with these being co-existing sympatric cryptic species (Töebe et al., 2013; John et al., 2014).

While the observed increase in maximum cell yield with increasing (monoculture) temperature exhibited by the different strains was not statistically significant, this response was similar to *Alexandrium catenella* isolated from the Thau lagoon in Southern France (Laabir et al., 2011), that exhibited an increase in peak cell yield per unit limiting nutrient over the range of 12 to 24 ^{o}C.

The optimal temperature for *A. fundyense* growth in monoculture was 18 ^{o}C, with a sharp decline in growth rate either side of this optima. *A. tamarense* exhibited a lower maximum but a broader tolerance range, perhaps indicating a different growth strategy. Laabir et al. (2011) summarised published growth rates for different *Alexandrium* species, with the values obtained in our experiments falling within the observed range for *A. tamarense*. Taylor et al. (2014) observed growth rates ranging from 0.1-0.5 div day^{-1} depending on the strain and culture conditions. Average growth

rates for Scottish Group I isolates were 0.2 +/- 0.2 in comparison to 0.24 +/- 0.03 for English Group III isolates. Other studies carried out on some tropical strains of *A. tamarense* exhibited maximum growth rates at considerably higher water temperatures than those in our experiments (Lim and Ogata, 2005). Our observed temperature response, with an optimal of 15-18 ^{o}C for both strains, is consistent with the response one might expect for other dinoflagellates from temperate northern latitudes (Eppley, 1972), including Danish isolates of *A. ostenfeldii* (Jensen and Moestrup, 1997). The preference of both strains for the intermediate water temperatures is also consistent with the observation that most Scottish *Alexandrium* blooms occur in summer when upper water column water temperature is typically 14-16 ^{o}C (Fehling et al., 2006). While *A. fundyense* growth rates in particular were depressed at the lowest temperature (12 °C), growth did occur, consistent with the ability of *Alexandrium* to sometimes bloom during the Scottish spring.

4.3 Co-culture interactions

Given that *A. fundyense* and *A. tamarense* are morphologically identical, experiments to study their competitive interaction on a temporal basis have previously been impractical. While the development of taxa-specific molecular assays, such as fluorescent *in situ* hybridisation (FISH) probes targeting rDNA regions, has allowed the identification and enumeration of cryptic species in field samples (Touzet et al., 2010), the time intensive nature of the fluorescence microscopy based cell enumeration that forms part of this technique, makes it impractical to apply to time course laboratory culture studies that generate large numbers of samples. However, by combining FISH based labelling of cells with their flow cytometric enumeration (Eckford-Soper et al., 2013) we were able to achieve rapid discrimination and enumeration of the different

strains. Such an approach makes, for the first time, mixed community experiments practicable. It could easily be extended to incorporate other species of *Alexandrium* or to study the dynamics other genera that contain morphologically indistinct species.

A clear finding from our experiments was that the interaction of the different toxic and non-toxic strains had the potential to influence the composition and toxicity of the *Alexandrium* "bloom" as a whole. At all temperatures, the cell yield in co-culture was approximately mid-way between the respective higher and lower values exhibited by *A. fundyense* and *A. tamarense* in monoculture, but as demonstrated by Fig. 5 this mixed community did not contain an equal abundance of the different cryptic species. Nor did it achieve the same ratio of *A. fundyense* and *A. tamarense* cells that would be expected from their relative performance in the monocultures. One possible cause is the production of growth inhibiting substances that was first considered by Harder (1917) in *Nostoc punctiforme*, and has now been shown to be common (Ikawa et al., 1997). These substances can either affect other organisms (hetero-inhibition) or themselves (auto-inhibition) (Stephens et al., 2010). Such alleopathy has been demonstrated for *Alexandrium* (Fistarol et al., 2004), but no attempt was made to evaluate alleopathic chemicals here.

Temperature has also previously been demonstrated to have an important influence on the outcome of competition between phytoplankton species in both the laboratory and outdoor mass culture (Goldman and Mann, 1980; Goldman, 1977) and we found this parameter to influence the competitive yields of *A. fundyense* and *A. tamarense*. The interaction effect was most pronounced at 12 ^{o}C where *A. tamarense* out-competed *A. fundyense* by a large margin. *A. tamarense* was also dominant at 15 ^{o}C and 21 ^{o}C, but at 18 ^{o}C, the different strains had approximately equal maximum yields. In co-culture *A. tamarense* exhibited higher or similar maximum specific growth rate

that *A. fundyense* at three out of the four temperatures, with the exception being 18 ^{o}C. This suggests that changes of just a few degrees could have a large impact on interaction between the two cryptic species.

The results discussed above are consistent with recent field evidence that, should a suitable seed population be present, non-toxic group III *A. tamarense* blooms could develop in Scottish waters to the competitive detriment of toxic group I *A. fundyense* should they co-occur. Given that Brosnahan et al. (2010) found that the zygotes formed from mixing Group I and Group III cells were not viable, and hence that co-existence is likely to persist, the competitive interactions within mixed populations are likely to be important to the toxicity of *Alexandrium* blooms in Scottish waters.

4.4 Toxins

Different *Alexandrium* species/strains produce PSP toxins in differing compositions and concentrations with cellular toxin also varying as a function of nutrient limitation (Boyer et al., 1987; John and Flynn., 2000), growth stage (Proctor et al., 1975; Anderson et al., 1990a; Flynn et al., 1994), water temperature (Ogata et al., 1987) and salinity and senescence (Landsberg, 2002). No saxitoxin or analogues were produced by *A. tamarense* in any phase of growth, consistent with the reported lack of toxicity of these group III cells. In contrast, toxin production by *A. fundyense* occurred during exponential growth, but with a marked increase in total toxicity (approximately double) in the P limited stationary phase. Such observations are consistent with other *Alexandrium* species (*A. minutum* and A. *fundyense*) (Boyer et al., 1987; Anderson et al., 1990b; Flynn et al., 1994; Lippemeier et al., 2003).

Characteristic toxin profiles of individual isolates or strains have been suggested as a phenotypic markers (Cembella and Taylor, 1986; Anderson et al., 1994; Flynn et

al., 1994). A number of studies have observed that *A. tamarense* exhibits a "relatively stable" profile of different STX analogues (Boyer et al., 1987; Cembella et al., 1987). Others have observed significant changes to the toxin profile with life cycle stage, irradiance, temperature and nutrient availability (John and Flynn. 2000), with large changes in toxin composition of individual isolates having been observed in *A. tamarense* and *A. catenalla* (Boczar et al., 1988), *A. fundyense* (Anderson et al., 1990b) and *A. minutum* (Lippemeier et al., 2003; Xu et al., 2012). Our study is consistent with these latter observations, with toxin composition varying with changing nutritional status and, as discussed below, temperature and competition. STX, NEO and GTX-3 were the dominant analogues produced by our cultures, with other analogues found in lower concentrations. STX, NEO, GTX-3, as well as GTX-4 were also the dominant analogues in the toxin profile of two different Group I strains isolated from Scottish waters (Collins et al., 2009). However, the toxin profiles observed by Collins et al. (2009) and Medlin et al. (1998) had more analogues than our study. The roles of individual toxin analogues have not been established so it difficult to hypothesise why, under different conditions, different toxin analogues were synthesised in differing combinations.

There are a number of theories as to why some harmful algal species produce toxins. One is that toxin production is adaptation by some dinoflagellates to overcome the ecological disadvantage of low nutrient affinity. Frangopulos et al. (2004) observed that there was a negative relationship between toxin content per cell and P-uptake efficiency, suggesting that as toxin production is enhanced under P-limitation, grazing pressure would be redirected towards the Group III cells (Guisande et al., 2002). The elevated toxin concentrations we observed at 12 °C are apparently consistent with the finding of Proctor et al. (1975), Sui et al. (1997), Anderson. et al. (1990) and Hamasaki

(2001), who demonstrated that toxic dinoflagellates have a higher cellular toxicity at low temperatures. It has been hypothesised that lower temperatures act to reduce protein synthesis, resulting in an excess of arginine which is thought to be a precursor of toxin biosynthesis (Anderson et al., 1990b). Arginine availability and thus toxin concentrations are thought to increase further in low P but high N conditions, such as those used in this study (Flynn et al., 1994). Alternatively, the higher cellular toxin content at low temperature could be an adaptation to lower cell numbers as it has been proposed that PSP toxins may act as a chemical signal, stimulating mating in *Alexandrium* spp., and therefore the higher PSP toxin concentrations in non-optimum conditions may be a strategy to increase the chances of mating to compensate for their lower cell densities (Lim and Ogata., 2005; Wyatt and Jenkinson., 1997). Nevertheless, despite the higher cellular toxin concentrations in our cultures at 12 °C, the total toxin concentration per unit culture volume were higher at the temperatures where higher cell yields were evident (15-21 °C).

A surprising finding was that the total toxicity per ml and per cell was lower in the cultures grown in mixed culture, suggesting some form of inhibition of toxin production. This difference was most evident at 12 °C and 15 °C where the total toxicity per ml of co-cultured *A. fundyense* cells was approximately four times less than that in monoculture. Guisande et al. (2002) suggested that toxin production acts to inhibit competing co-occurring species. While direct evidence of this remains limited, as noted above a number *Alexandrium* species are thought to synthesise allelochemicals that may have a number of negative ecological functions. These chemicals appear to not be related to PSP toxicity but rather appear to have other modes of action such as lytic compounds (Ma et al., 2009; Tillmann and John., 2002; Fistarol et al., 2004). Some

form of alleopathic interaction is therefore a potential explanation for the observed suppression of *A. fundyense* toxicity when in co-culture.

5. Conclusions

Using a novel application of FISH and flow cytometry we were able, for the first time, to conduct the first laboratory growth, competition and toxin production study on morphologically identical toxic Group I *A. fundyense* and non-toxic Group III *A. tamarense*.

The influence of temperature on a range of parameters was evident from monoculture experiments. Alterations in temperature had a marked effect on toxicity and toxin profile. Our results therefore suggest that changing water temperatures will influence the growth, competition and hence biogeography of the *A. tamarense* species complex in Scottish waters. Despite this, temperature cannot exclusively explain all the interactions we observed between cells grown together, especially the impact of co-culture on toxicity. This would suggest that there are other interactions occurring between the cells that we are not yet able to quantity.

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Table 1: Maximum specific growth rates (divisions day^{-1}) for the group I *A. fundyense* and group III *A. tamarense* grown in mono and co-culture.

Strain	**12°C**	**15°C**	**18°C**	**21°C**
A. fundyense	0.12	0.22	0.27	0.16
A. tamarense	0.16	0.21	0.22	0.16
Co-culture (combined)	0.16	0.18	0.20	0.15
Co-Culture (*A. fundyense*)	0.09	0.20	0.26	0.16
Co-Culture (*A. tamarense*)	0.23	0.24	0.21	0.16

Legends

Figure 1: Cell densities (ml^{-1}) at 12ºC (a), 15ºC (b), 18ºC (c) and (d) 21ºC. Group I *A. fundyense* (•), co-culture (♢) and group III *A. tamarense* (○) strains. All results are means of triplicate flasks. Error bars represent SE**.**

Figure 2: Maximum cell yields (ml^{-1}) normalised per µm PO_4 consumed for *A. fundyense* (●), and *A. tamarense* (○) strains in monoculture.

Figure 3: Maximum Specific Growth Rate (SGR) div day^{-1} at 12°C,15°C,18°C and 21°C for *A. fundyense* (●), *A. tamarense* (○), co-cultured *A. fundyense* (▼) and *A. tamarense* (▽).

Figure 4: PSP Toxin concentrations in saxitoxin equivalents per cell at 12°C (a), 15°C (b) and 18°C (c) in Exponential (Ex) and Stationary (Sta) phases of growth for the group I strain in mono-culture (T) and in co-culture culture (M).

Figure 5: Cell densities (ml^{-1}) at 12ºC (a), 15ºC (b), 18ºC (c) and (d) 21ºC for the co-culture experiments calculated using a flow cytometer (FC) and (for comparison) a Sedgwick Rafter (SR). *A. fundyense* (■), *A. tamarense* (□), total flow cytometer counts (♦) and Sedgwick rafter counts (♢). All results are means of triplicate flasks. Error bars represent SE.