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

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11 Highlights

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

18

19 Key words:

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

1 **Abstract**

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

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

3

4 **Introduction**

5 The observed frequency, intensity, and geographical distribution of observed harmful
6 algal bloom (HAB) events have increased over the last few decades (Gowen et al.,
7 2012; Hallegraeff, 1993; Lilly et al., 2007) with the genus *Alexandrium* being of global
8 importance due to its widespread distribution and synthesis of potent neurotoxins that
9 are associated with Paralytic Shellfish Poisoning (PSP). Humans, birds, sea mammals
10 and fish can all be affected by PSP toxins, with the usual exposure route for humans
11 being through the consumption of contaminated shellfish (Smayda., 2004).

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

1 In Scottish (and northern English) waters, monitoring programmes have
2 demonstrated that the genus *Alexandrium* is a common constituent of phytoplankton
3 communities in spring and summer (Stubbs et al., 2014). Regional hotspots include:
4 Orkney, Shetland, the Western Isles, the west coast and Northern England (Collins et al.,
5 2009), however information on the biogeography of specific *Alexandrium* spp. in the
6 region remains limited. Medlin et al. (1998) analysed the D1/D2 region of the LSU
7 rRNA gene from number of isolates from the Orkney Islands and found them all to be *A.*
8 *tamarense* (using the taxonomy of the time) rather than *A. fundyense*. The periodic high
9 toxicity levels in Scottish shellfish, the lack of records of the non-toxic Group III
10 *Alexandrium* above 55 °N (Töebe et al., 2013), and that UK monitoring programs only
11 identify *Alexandrium* to genus level, led to the assumption that *Alexandrium* populations
12 in Scottish waters were exclusively (toxin producing) Group I. Recent observations
13 question this, as Group III cysts and vegetative cells have now been shown to have a
14 wide geographical distribution in Scottish waters (Brown et al., 2010; Collins et al.,
15 2009) and Group I and Group III cells have been co-observed in the water column, both
16 in the Shetland Isles at 60 °N by Touzet et al. (2010), and in the North Sea at ~ 57 °N by
17 Töebe et al. (2013). The spatially extensive distribution of Group III cells in the latter
18 study clearly indicates that these observations are more than transient events, and
19 coincide with the decline in the occurrence of PSP toxins in Scottish shellfish in recent
20 years (Bresnan et al., 2008).

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

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

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

13

14 **Materials and Methods**

15 *2.1. Culture methods and cell acclimation*

16 Two Group I *A. fundyensis* (CCAP 1119/24 and 1119/28) and two Group III *A.*
17 *tamarensis* (CCAP 1119/31 and 1119/33) strains that had previously been isolated by
18 Marine Scotland Science from Scottish waters (Scapa, Orkney) and subsequently
19 deposited in the Culture Collection of Algae and Protozoa (CCAP) at SAMS, were
20 initially chosen for our study. Cultures were grown in 1litre Erlenmeyer flasks in batch
21 mode, acclimated to a modified phosphate (3 μ M) L1 medium (Guillard and Hargraves.,
22 1993) in which all other nutrients were available in excess. Experiments were conducted
23 at four different temperatures: 12 °C, 15 °C, 18 °C and 21 °C. The three lower
24 temperatures were chosen to represent the range of conditions that an *Alexandrium* cell
25 might experience in Scotland presently or in future scenarios due to climate warming:

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

14

15 2.2. *Mono- and co-culture experiments*

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

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

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

5 2.3. Enumeration of total cell abundance & growth rate determination

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

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

11
 12 Where dN/dt is the expected rate of change in the population size at any point in time
 13 given the population size (N), the carrying capacity of the environment (K), and the
 14 intrinsic growth rate (μ). The intrinsic rate of growth is the rate of growth when
 15 individuals are not constrained by environmental limits. The integrated form of the
 16 logistic equation, Equation 2 (Ferris and Wilson, 1987) was fitted (using a least
 17 squares approach):

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

19

20 2.4. Discrimination and enumeration of *A. fundyense* and *A. tamarensis* in co-culture

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

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

5 The taxa specific oligonucleotide probes TamA (for *A. tamarensis*) and
6 TamToxC (for *A. fundyensis*) (Touzet et al., 2010) were used to fluorescently label the
7 different strains. Samples were removed from the -20 °C freezer and centrifuged (4000
8 g, 5 min) and the methanol supernatant aspirated off. Hybridisation buffer (5X SET and
9 0.1 % IGEPAL) was added (500 µl) to the cell pellet and the cells re-suspended to rinse
10 off the methanol. The samples were centrifuged (4000 g, 5 min) and the supernatant
11 removed. This process was repeated to ensure that the methanol was removed from the
12 sample. Cells were re-suspended in 500 µl of hybridisation buffer containing 1 µl of
13 each of the taxa specific probes. After this time the samples were kept in the dark by
14 wrapping the tubes in aluminium foil and incubated in a dark incubator (55 °C, 60 min).
15 After hybridisation, the cells were pelleted by centrifugation (4000 g, 5 min) and the
16 supernatant discarded. Samples were washed with 500 µl of preheated (55 °C) 0.2X
17 SET buffer to remove the excess unbound probes. Samples were then centrifuged (4000
18 g, 5 min) one final time and the supernatant removed. Finally, the cells were re-
19 suspended in 2.5 ml of autoclaved filtered seawater for subsequent enumeration.

20 Flow cytometric identification and enumeration of the labelled cells was
21 conducted using a FACSort (Beckton Dickinson) instrument fitted with a blue argon
22 (488 nm) laser and a 150 µm instrument aperture. Discrimination and enumeration of
23 the two ribotypes was achieved based on side scatter (SSC) and fluorescence detection
24 of the TamToxC and TamA probes within the FL1 (515-545 nm) channel (Eckford-
25 Soper et al., 2013). Aliquots (1 ml) were analysed with fluorescent acquisition, gated by

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

3

4

5 *2.5. Toxin analysis*

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

16 Prior to analysis cell pellets were thawed and 1ml of autoclaved filtered seawater
17 was added to the sample and vortexed. The solution was transferred to an Eppendorf
18 micro-centrifuge tube. To ensure all cells were transferred the process was repeated
19 using an additional 200 µl of filtered seawater. The cell solution was centrifuged (3000
20 g, 20 min), the supernatant discarded, and acetic acid (500 µl of 0.5 M) was added to the
21 cell pellet. Toxin extraction was carried out by freezing at -80 °C (30 min) and thawing
22 at room temperature (30 min) three times. The samples were vortexed and centrifuged
23 (8000 g, 20 min). Aliquots (200 µl) of the toxin extracts were transferred to a Millipore
24 Ultrafree MC 0.2 µm filter unit and centrifuged (6000 g, 3 min) (Van De Riet et al.,
25 2009). The contents were then transferred to glass reaction vials.

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

7 The analysis system consisted of: the LC system, post column reaction system,
8 reaction coil, fluorescent detector and LC columns. The mixed working solution and
9 serial dilutions (10 μ L for GTX and STX toxins and 5 μ L for C-toxins) were injected
10 into the system and separated chromatographically. Part of the extract was
11 chromatographed with a step gradient using a heptane sulfonic acid/phosphoric acid
12 buffer system for the analysis of GTX and STX analogues. The extract was also
13 chromatographed for the C-toxins using an isocratic tetrabutylammonium phosphate
14 buffer for the C-toxin analogues. The toxins were detected by PCOX of the analytes at
15 85 °C with a phosphoric acid periodic acid buffer solution followed by fluorescence
16 detection (excitation: 330 nm, emission: 390 nm). Integration and data analysis was
17 conducted using ChromQuest 2.6.1 software. To calculate the concentration of the
18 toxins as STX equivalents, the concentrations of toxins (in nM) were converted to
19 μ moles and multiplied by the relative toxicities of each individual toxin (Van De Riet et
20 al., 2009).

21

22 *2.6. Other parameters*

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

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

11

12 **3. Results**

13 *3.1 Preliminary experiments*

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

20 *3.2. Mono-culture: temporal dynamics*

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

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

6

7 3.3. Mono-culture: cell yields and growth rates

8 Spearman Rank correlation analysis found statistically significant linear correlations
 9 between cell abundance and POC for both ribotypes at all temperatures evaluated (12 °C: *A.*
 10 *fundyense*, $r = 0.954$, $p = 0$; *A. tamarensis* $r = 0.939$, $p = 0$. 15 °C . *A. fundyense* $r = 0.939$, $p =$
 11 0 ; *A. tamarensis* $r = 0.973$, $p = 0$. 18 °C, *A. fundyense*, $r = 0.918$, $p = 0$, *A. tamarensis*, $r =$
 12 0.923 , $p = 0$), demonstrating that cell numbers were correlated with biomass.

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

20 Mean cell specific growth rate calculated is plotted as a function of temperature in
 21 Fig. 3, and is tabulated in Table 1. Growth rates exhibited a generally “humped” response for
 22 both strains, with highest rates at the intermediate temperatures of 15 and 18 °C and lowest at
 23 12 °C. The bell shaped curve was more pronounced for *A. fundyense*, which had temperature
 24 optima of 18 °C with a sharp decline in growth rate either side of this. *A. tamarensis* had a
 25 broader tolerance range at all temperatures except 12 °C. *A. tamarensis* had a maximum SGR

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

3

4 3.4 Mono-culture: PSP toxins

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

13 While STX was the main toxin analogue found at all temperatures (Fig. 4), a
14 range of others were present. In exponential growth at 12 °C the analogues GTX-1,
15 GTX-3, GTX-2, GTX-5 dc-GTX-3, NEO, STX, C1 and C2 were found. GTX-1, GTX-
16 2, dc-GTX-3, GTX-3, NEO, STX and C-1, C-2 were all present at 15 °C. Fewer
17 analogues were present at 18 °C with: dc-GTX-3 GTX-3, NEO, STX, C-1 and C-2
18 being observed. On entering stationary growth the number of analogues remained
19 unchanged at 15 °C but increased at both 12°C (to include GTX-2) and 18 °C with the
20 number of analogues now including: GTX-1, GTX-3, dc-GTX-3, NEO, STX, C-1 and
21 C2 (Fig. 4). GTX-2 and GTX-5 were not found at this temperature and the analogues
22 GTX-4, dc-GTX-2 and dc-STX were not found at any temperature.

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

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

3

4 3.5 Co-culture: cell yields and growth rates

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

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

13 At all temperatures interaction between the two strains was evident. The effect
14 was most clearly seen at 12 °C where *A. tamarensis* dominated to the near exclusion of
15 *A. fundyense*. In this case, the peak yield of *A. tamarensis* was 87 % of the combined
16 peak cell yield. While net growth of the *A. fundyense* did occur, it increased from 200
17 cells ml⁻¹ to a mean peak density of only 599 cells ml⁻¹ and did not exhibit clear
18 exponential growth. A competitive dominance of *A. tamarensis*, although not to such a
19 great extent, was also evident at 15 °C and 21 °C. In these cases, the peak yield of *A.*
20 *tamarensis* was 68% and 67% of the respective totals. However, at 18 °C these
21 interactions were more balanced with similar peak densities (*A. fundyense* achieving
22 51% of the total). At all temperatures, except 18 °C where yields were similar, the
23 dominance of the *A. tamarensis* peak cell density over *A. fundyense* was statistically
24 significant (Mann-Whitney $p > 0.05$). In co-culture the peak cell densities achieved by
25 *A. tamarensis* were relatively constant at all temperatures, varying from 4156 cells ml⁻¹

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

4 Maximum specific growth rates of the co-cultures (Fig. 3) exhibited a similar
5 'humped response' for *A. fundyense* as was evident in monoculture. For *A. tamarensis*,
6 the response was similar to monoculture at the three higher temperatures, but a
7 markedly higher growth rate was evident in co-culture at 12 °C.

8

9 3.6. Co-culture: toxins

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

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

23

24 4. Discussion

25 4.1 *A. tamarensis* in Scottish waters

1 There are no major physical barriers to transport of phytoplankton cells in the waters
2 surrounding the British Isles, as evidenced by examples of large distance transport of
3 *Dinophysis* spp. and *Karenia mikimotoi* (Davidson et al., 2009; Farrell et al., 2012;
4 Whyte et al., 2014). The coastal current of the Scottish west coast (Simpson and Hill,
5 1986) provides a mechanism of northwards transport of cells, with the fjordic sea lochs
6 that dominate the Western and Northern coastline of Scotland providing an ideal refuge
7 for cyst forming organisms such as the *Alexandrium*. However, biological barriers to
8 range expansion may exist, with temperature tolerance being thought to be one of these
9 (Hinder et al., 2011). For example, we have previously found strains of *A. minutum* to
10 be unable sustain net positive growth at 12 °C (Davidson et al., 1999), an observation
11 that may in part explain the lack of *A. minutum* in the relatively cold Scottish waters.
12 Temperature is a key driver of ecological processes and a key abiotic driver of
13 ecological systems; yet, the effects of temperature are complex. A review of 688 species
14 by Tylianakis et al. (2008) found that temperature alterations affect a number of species
15 to species interactions, including competition. Community structure is not just affected
16 by the direct impacts of temperature on physiology but also on how these direct impacts
17 affect other processes (Reuman et al., 2014). A better general understanding of how the
18 physiological influences of temperature will give us a better understanding of species to
19 species interactions and ultimately community dynamics. For example, Tilman et al.
20 (1981) studied the competition interactions between two freshwater diatoms
21 *Asterionella formosa* and *Synedra ulna* (Nitzsch). They found that that due to their
22 ability to reduce environmental silica at different temperatures *A. formosa* was able to
23 displace *S. ulna* at temperatures below 20 °C with the reverse being true above 20 °C.

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

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

16

17 4.2 Yield and growth rates

18 Differences in cell yield between the different strains occurred at all
19 temperatures. Cell densities were lower than those that might be expected to be
20 impacted by light limitation through self-shading (Agusti., 1991). Furthermore, while
21 growth in culture can often be inhibited by pH, it did not exceed 8.4 in our experiments
22 (data not shown) and *A. tamarensis* can typically tolerate a pH of above 9 (Hansen,
23 2002; Schmidt and Hansen, 2001).

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

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

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

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

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

1 rates for Scottish Group I isolates were 0.2 +/- 0.2 in comparison to 0.24 +/- 0.03 for
2 English Group III isolates. Other studies carried out on some tropical strains of *A.*
3 *tamarensis* exhibited maximum growth rates at considerably higher water temperatures
4 than those in our experiments (Lim and Ogata, 2005). Our observed temperature
5 response, with an optimal of 15-18 °C for both strains, is consistent with the response
6 one might expect for other dinoflagellates from temperate northern latitudes (Eppley,
7 1972), including Danish isolates of *A. ostenfeldii* (Jensen and Moestrup, 1997). The
8 preference of both strains for the intermediate water temperatures is also consistent with
9 the observation that most Scottish *Alexandrium* blooms occur in summer when upper
10 water column water temperature is typically 14-16 °C (Fehling et al., 2006). While *A.*
11 *fundyensis* growth rates in particular were depressed at the lowest temperature (12 °C),
12 growth did occur, consistent with the ability of *Alexandrium* to sometimes bloom during
13 the Scottish spring.

14

15 *4.3 Co-culture interactions*

16 Given that *A. fundyensis* and *A. tamarensis* are morphologically identical, experiments to
17 study their competitive interaction on a temporal basis have previously been
18 impractical. While the development of taxa-specific molecular assays, such as
19 fluorescent *in situ* hybridisation (FISH) probes targeting rDNA regions, has allowed the
20 identification and enumeration of cryptic species in field samples (Touzet et al., 2010),
21 the time intensive nature of the fluorescence microscopy based cell enumeration that
22 forms part of this technique, makes it impractical to apply to time course laboratory
23 culture studies that generate large numbers of samples. However, by combining FISH
24 based labelling of cells with their flow cytometric enumeration (Eckford-Soper et al.,
25 2013) we were able to achieve rapid discrimination and enumeration of the different

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

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

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

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

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

11

12 4.4 Toxins

13 Different *Alexandrium* species/strains produce PSP toxins in differing
14 compositions and concentrations with cellular toxin also varying as a function of
15 nutrient limitation (Boyer et al., 1987; John and Flynn., 2000), growth stage (Proctor et
16 al., 1975; Anderson et al., 1990a; Flynn et al., 1994), water temperature (Ogata et al.,
17 1987) and salinity and senescence (Landsberg, 2002). No saxitoxin or analogues were
18 produced by *A. tamarense* in any phase of growth, consistent with the reported lack of
19 toxicity of these group III cells. In contrast, toxin production by *A. fundyense* occurred
20 during exponential growth, but with a marked increase in total toxicity (approximately
21 double) in the P limited stationary phase. Such observations are consistent with other
22 *Alexandrium* species (*A. minutum* and *A. fundyense*) (Boyer et al., 1987; Anderson et
23 al., 1990b; Flynn et al., 1994; Lippemeier et al., 2003).

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

1 al., 1994). A number of studies have observed that *A. tamarense* exhibits a “relatively
2 stable” profile of different STX analogues (Boyer et al., 1987; Cembella et al., 1987).
3 Others have observed significant changes to the toxin profile with life cycle stage,
4 irradiance, temperature and nutrient availability (John and Flynn. 2000), with large
5 changes in toxin composition of individual isolates having been observed in *A.*
6 *tamarense* and *A. catenalla* (Boczar et al., 1988), *A. fundyense* (Anderson et al., 1990b)
7 and *A. minutum* (Lippemeier et al., 2003; Xu et al., 2012). Our study is consistent with
8 these latter observations, with toxin composition varying with changing nutritional
9 status and, as discussed below, temperature and competition. STX, NEO and GTX-3
10 were the dominant analogues produced by our cultures, with other analogues found in
11 lower concentrations. STX, NEO, GTX-3, as well as GTX-4 were also the dominant
12 analogues in the toxin profile of two different Group I strains isolated from Scottish
13 waters (Collins et al., 2009). However, the toxin profiles observed by Collins et al.
14 (2009) and Medlin et al. (1998) had more analogues than our study. The roles of
15 individual toxin analogues have not been established so it difficult to hypothesise why,
16 under different conditions, different toxin analogues were synthesised in differing
17 combinations.

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

1 (2001), who demonstrated that toxic dinoflagellates have a higher cellular toxicity at
2 low temperatures. It has been hypothesised that lower temperatures act to reduce protein
3 synthesis, resulting in an excess of arginine which is thought to be a precursor of toxin
4 biosynthesis (Anderson et al., 1990b). Arginine availability and thus toxin
5 concentrations are thought to increase further in low P but high N conditions, such as
6 those used in this study (Flynn et al., 1994). Alternatively, the higher cellular toxin
7 content at low temperature could be an adaptation to lower cell numbers as it has been
8 proposed that PSP toxins may act as a chemical signal, stimulating mating in
9 *Alexandrium* spp., and therefore the higher PSP toxin concentrations in non-optimum
10 conditions may be a strategy to increase the chances of mating to compensate for their
11 lower cell densities (Lim and Ogata., 2005; Wyatt and Jenkinson., 1997). Nevertheless,
12 despite the higher cellular toxin concentrations in our cultures at 12 °C, the total toxin
13 concentration per unit culture volume were higher at the temperatures where higher cell
14 yields were evident (15-21 °C).

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

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

3

4

5 **5. Conclusions**

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

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

18

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6

1 Table 1: Maximum specific growth rates (divisions day⁻¹) for the group I *A. fundyense*
 2 and group III *A. tamarensis* grown in mono and co-culture.

3

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

4

5

6

1 Legends

2

3 Figure 1: Cell densities (ml^{-1}) at 12°C (a), 15°C (b), 18°C (c) and (d) 21°C. Group I *A.*
4 *fundyense* (●), co-culture (◇) and group III *A. tamarensis* (○) strains. All results are
5 means of triplicate flasks. Error bars represent SE.

6

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

9

10 Figure 3: Maximum Specific Growth Rate (SGR) div day^{-1} at 12°C, 15°C, 18°C and
11 21°C for *A. fundyense* (●), *A. tamarensis* (○), co-cultured *A. fundyense* (▼) and *A.*
12 *tamarensis* (▽).

13

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

17

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

23