Validation of the detection of *Alexandrium* species using specific RNA probes tested in a microarray format: Calibration of signal using variability of RNA content with environmental conditions

Joe D. Taylor a,*, Jessica U. Kegel b, Jane M. Lewis a, Linda K. Medlin b

a Faculty of Science and Technology, University of Westminster, 115 New Cavendish Street, London W1W 6JW, UK
b Marine Biological Association of the UK, The Laboratory, The Citadel, Plymouth PL1 2PB, UK

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**A B S T R A C T**

The dinoflagellate genus *Alexandrium* contains several toxin producing species and strains, which can cause major economic losses to the shell fish industry. It is therefore important to be able to detect these toxin producers and also distinguish toxic strains from some of the morphologically identical non-toxic strains. To facilitate this DNA probes to be used in a microarray format were designed in silico or developed from existing published probes. These probes targeted either the 18S or 28S ribosomal ribonucleic acid (rRNA) gene in *Alexandrium tamarense* Group I, Group III and Group IV, *Alexandrium ostencdfeldii* and *Alexandrium minutum*. Three strains of *A. tamarense* Group I, *A. tamarense* Group III, *A. minutum* and two strains of *A. ostencdfeldii* were grown at optimal conditions and transferred into new environmental conditions changing either the light intensity, salinity, temperature or nutrient concentrations, to check if any of these environmental conditions induced changes in the cellular ribonucleic acid (RNA) concentration or growth rate. The aim of this experiment was the calibration of several species-specific probes for the quantification of the toxic *Alexandrium* strains. Growth rates were highly variable but only elevated or lowered salinity significantly lowered growth rate for *A. tamarense* Group I and Group III; differences in RNA content were not significant for the majority of the treatments. Only light intensity seemed to affect significantly the RNA content in *A. tamarense* Group I and Group III, but this was still within the same range as for the other treatments meaning that a back calibration from RNA to cell numbers was possible. The designed probes allow the production of quantitative information for *Alexandrium* species for the microarray chip.

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1. Introduction

Harmful algal blooms and coastal eutrophication are subjects of growing interest worldwide because of the pressure of increased exploitation of coastal resources (Van Dolah, 2000; Ribeiro et al., 2012; Karydis and Kitsiou, 2012). Such blooms can affect fisheries and aquaculture as well as have an impact on the tourism potential of an area (e.g. Hoagland et al., 2002; Smaal, 2002). There are a wide range of microalgal species involved in such events. Amongst the most notorious are species of the genus *Alexandrium*, some of which produce potent neurotoxins from the saxitoxin family, the causative agents of paralytic shellfish poisoning (Clark et al., 1999; Anderson et al., 2012). Recent research has shown that *Alexandrium* species are directly responsible for saxitoxin production because several members of the genus contain the gene specific for saxitoxin production (Murray et al., 2011; Stuken et al., 2011). Hence the monitoring of coastal waters for these species is an important element of health protection programmes as well as being vital for aquaculture (particularly shellfish) producers to manage their enterprises. Such monitoring is typically carried out by light microscopy (Humbert et al., 2010).

The genus *Alexandrium* contains more than 25 species (Balech, 1995; Anderson et al., 2012), which are largely separated by morphological variations in the cellulosic plates of the motile stage. To discriminate species requires the determination of the shape and conformation of the first apical plate (including

**Abbreviations:** BSA, bovine serum albumen; FISH, fluorescence in-situ hybridisation; DNA, deoxyribonucleic acid; MIDTAL, microarrays for the detection of toxic algae; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; STT, sodium chloride–tris–triton.

* Corresponding author. Present address: Marine Biological Association of the UK, The Laboratory, The Citadel, Plymouth PL1 2PB, UK.
E-mail address: joetay@mba.ac.uk (J.D. Taylor).

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presence/absence of a pore), shape and conformation of the apical pore complex, distribution and shape of precingular plates and the plate structure of the sulcal region (Steidinger, 2010). Full characterisation can take a great deal of time and skill by light microscopy. Furthermore molecular studies have revealed a number of species ‘complexes’ whose strains can be distinguished by genetic identification but not by light microscopy. One of these complexes is *Alexandrium tamarense*, which includes five groups, of which three (I, IV and V) are currently thought to contain toxic strains (Lily et al., 2007; Murray et al., 2012) with some proposing that these groups should become separate species (Wang et al., 2014). Similarly *Alexandrium ostenfeldii* and *Alexandrium minutum* have both toxic and non-toxic strains, although data on distribution of toxic and non-toxic strains is much more limited than for *A. tamarense* (Cembella and Krock, 2007; Touzet et al., 2008; Brown et al., 2010). Certain strains of *A. ostenfeldii* also produce spiriloïdes, which are fast acting potent neuro toxins (Cembella et al., 2001). Therefore there is a clear need to be able to distinguish *A. ostenfeldii* from the both *A. tamarense* complex and *A. minutum*. Additionally, it has been shown that both toxic and non-toxic groups of *A. tamarense* can co-occur (Higman et al., 2001; John et al., 2003; Touzet et al., 2010) and so to avoid false positives or negatives in monitoring, faster and more efficient counting methodologies are sought.

Previous studies have used a variety of molecular techniques, which can distinguish between toxic species and strains, to detect harmful algae, such as quantitative PCR (e.g. Galluzzo et al., 2004; Handy et al., 2006), iso-thermal amplification (Zhang et al., 2013) fluorescent in situ hybridisations (FISH, Scholin et al., 1997; Not et al., 2002; Groben and Medlin, 2005), sandwich hybridisations (e.g. Scholin and Anderson, 1998; Anderson et al., 2005; Diercks et al., 2008), microarrays (Metties and Medlin, 2005; Gescher et al., 2008; Wollschlager et al., 2014) and recently next generation sequencing (Ege et al., 2013). The majority of these methods rely on species or strain specific RNA or DNA sequences with the most of these targeting ribosomal RNA genes, which have highly conserved regions and also highly variable regions that vary between strain or species of microalga. The use of RNA or cDNA has several advantages compared to DNA. Within cells RNA is much less stable and is rapidly degraded compared with DNA; therefore this approach means detection of only active cells. Total RNA is constituted mainly of rRNA resulting in a high ratio of target to non-target sequences where as genomic DNA is made up of large majority of non-target sequences. It can therefore be used directly in microarray assays without prior amplification of the target region, which can lead to PCR bias for certain sequences (Peplies et al., 2006).

Microarrays are one molecular technique that has the potential to be quantitative and previous studies have shown that rRNA content and cell numbers correlate well in some algal species under laboratory conditions (Ayers et al., 2005; Galluzzo et al., 2010) However, very little information is available about how environmental conditions and growth rate affect rRNA content in eukaryotes. It has been shown that for some microalgae the pool of RNA within a cell can vary (Dortch et al., 1984; Berdelet et al., 1994) and that rRNA content may vary with growth phase (Galluzzo et al., 2010). Similarly in bacteria, the per-cell rRNA content has been shown to depend strongly on growth rate and nutrient availability, varying over 10-fold between starved cells in stationary phase and nutrient-replete cells in logarithmic growth phase (Fegatella et al., 1998). Because *Alexandrium* spp. have a wide geographical distribution (Gribble et al., 2005; Lilly et al., 2005, 2007; Collins et al., 2009; Anderson et al., 2012) and as primarily coastal species can be subjected to a variety of varying environmental conditions, such as light, temperature, salinity and nutrients, which may cause variations in RNA content. Previous culture based studies have shown that *Alexandrium* spp. tolerate a wide range of environmental conditions and in some cases growth rate may be affected by changes in light, temperature (Hwang and Lu, 2000; Grzebyk et al., 2003; Jensen and Moeustrup, 1997; Hansen et al., 2003) and salinity (Lim and Ogata, 2005), although no previous studies have looked at RNA content under varying environmental conditions.

The MIDTAL (Microarrays for the Detection of Toxic Algae) project has developed a microarray using rRNA based detection based on 136 probes at various taxonomic hierarchies to determine all major species of harmful algae from North-Western Europe. This technology can be used in monitoring harmful algae and unlike many previous molecular techniques would be used to

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary of strains used in the study, counting methodology employed and experimental conditions tested.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain name</th>
<th>Isolation location</th>
<th>Cells counted</th>
<th>Salinity range (psu)</th>
<th>Temperature range (°C)</th>
<th>Light intensity range (μmol photons/m²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alexandrium minutum</em></td>
<td>AMAD06</td>
<td>Port River, Australia</td>
<td>Coulter counter</td>
<td>Not tested</td>
<td>15–30–40</td>
<td>15–100–200</td>
</tr>
<tr>
<td></td>
<td>AL3T</td>
<td>Ria de Vigo, Spain</td>
<td>Coulter counter</td>
<td>Not tested</td>
<td>15–30–40</td>
<td>15–100–200</td>
</tr>
<tr>
<td></td>
<td>AMIA5</td>
<td>Syracuse, Ionian Sea, Sicily, Italy</td>
<td>Coulter counter</td>
<td>Not tested</td>
<td>15–30–40</td>
<td>15–100–200</td>
</tr>
<tr>
<td></td>
<td>VG0927</td>
<td>Carnota Beach, NW Spain (Atlantic)</td>
<td>Sedgewick rafter</td>
<td>28–33–38</td>
<td>12–16–20</td>
<td>26–160–430</td>
</tr>
<tr>
<td><em>Alexandrium tamarense</em> (catenella morphotype) Temperate Asian Group IV</td>
<td>VGO 598</td>
<td>Tarragona harbour (Mediterranean Sea)</td>
<td>Not counted</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>
quantify the numbers of cells present not just detect their presence/absence. Thus, for quantification, it is necessary to understand the variability of the rRNA pool within cells. The aim of this study was to investigate the variation of RNA yield per cell within *Alexandrium* species and strains in response to environmentally relevant conditions to allow calibration of the microarray chip to cell counts. In order to address this, we assessed the relationship between RNA and cell numbers for each species or strain. Signal intensity of species specific probes against amount of RNA hybridised to the chip was then investigated. This was done to investigate the efficiency of back calibration from signal on the microarray to cell number of a particular *Alexandrium* species or strain and that there was no cross relativity between the probes for each species. Based on previous complementary studies using the MIDTAL chip by Dittami and Edvardsen (2012) for *Pseudochattonella*, Blanco et al. (2013) for *Heterosigma akashiwo*, McCoy et al. (2014a) for *Prymnesium*, Karnoldinum veneicum and cf. *Chatonella* sp. and unpublished work for two species of *Pseudo-nitzszia* (Medlin et al. submitted), we hypothesised there would be a positive linear relationship between target RNA amount and target specific probe signal on the chip and a positive correlation between cell numbers and total RNA.

2. Material and methods

2.1. Algal strains

Three strains of each *Alexandrium* species (or Group) were used in these experiments (Table 1), with the exception of *A. ostenfeldii* where only two cultures were available and the *A. tamarense* Group IV where only one strain was available. We selected strains from varied locations, where available, to maximize the genetic difference. In each species, the strains are referred as strain 1, strain 2, and strain 3, respectively (Table 1). Before experimental testing, all strains were grown in f/2 (Guillard and Ryther, 1962) media in seawater salinity 30–34 and at 15 °C, 100 μE for *A. minutum* and *Alexandrium ostenfeldii* and at 16 °C, 160 μE for *A. tamarense* Group I and Group III and *A. tamarense* Group IV (catenella morphotype).

2.2. Experimental design

A stock culture of each strain was grown under the control conditions above, with fresh media added regularly to maintain exponential growth. Experiments were done in triplicate. Four different treatments (salinity, light intensity, temperature, and nutrient depletion) were tested in parallel, changing one parameter per set of cultures (Table 1) as described by Dittami and Edvardsen (2012). Briefly, the three strains of each species were inoculated separately in 200 mL tissue culture flasks with vented caps or 250–500 mL bottles. Initial volume in each flask was between 150 and 300 mL, with 20 mL or one-third of initial strain 1–3 cultures, respectively, and f/2 modified according to the conditions applied making up the rest of the volume.

This stock culture was then split into three replicates of 40 mL for each individual treatment. For salinity stress, the strains were inoculated in flasks containing f/2 at lower and higher salinity than the control conditions without accommodation in order to test immediate stress response, and this varied with each species (Table 1). Low light intensity was 15–25 μE and high light intensity varied with each species (Table 1). Temperatures were set at 10–15 °C for low temperature and the higher one also varied with each species (Table 1). Higher temperatures and light intensities were selected based on both equipment available to carry out the experiments but also aimed to use levels at the maximum tolerance ranges of each of the *Alexandrium* species. Nutrient depletion was carried out by using modified f/2 medium without either phosphate or nitrate. It should be noted that the nutrient depleted treatments did contain some N or P at the beginning of each experiment because a 10% to one-third (by volume) of culture with f/2 medium was used for inoculation.

All sets of conditions were run at the same time so as to use inocula from the same starting cultures. The day of cell inoculation was considered as time zero (T0). Subsamples (13 mL) of the cultures for cell counts, and RNA extraction at each different condition were taken at the same time daily after 24 h of inoculation, after 48 h, and after 72 h with 10 mL being used for RNA extraction and 3 mL for cell counts. For *A. minutum* 10 mL was taken from each flask and mixed in sets of three, to have 3 replicates of a 30 mL mix of the three strains (Fig. 1). For *A. ostenfeldii* 45 (strain NCH05) or 90 mL (strain AON004, a slow growing strain) of culture was filtered onto 3 μm nitrocellulose or polycarbonate filters (Whatman, U.K.), transferred into cryogenic vials containing acid washed glass beads (213–300 μm), shock-frozen in liquid nitrogen, and stored at −80 °C until further processing.

2.3. Cell counts

Cells for counts (3 mL) for both *A. tamarense* Group I and Group III were preserved in Lugol’s iodine (0.1%) and cell counts were carried out in duplicate using a Sedgewick rafter counting chamber under light microscopy. Counts for *A. minutum* were carried out with a Coulter Counter (Beckman Coulter). Subsamples for cell counts of *A. ostenfeldii* were kept at 4 °C after adding 20 μL of 25% glutaraldehyde to 500 μL of the culture mix, and were counted with a flow cytometer (accuri C6 Flow Cytometer or Becton Dickinson FACSCalibur, BD).

![Fig. 1. Growth rates (d⁻¹) of (A) A. tamarense Group I (B) A. tamarense Group III (C) A. ostenfeldii (D) A. minutum, under varying conditions of salinity, light (μE), nutrients (+N, control (C), +P) and temperature (°C).](image-url)
Growth rate was defined as divisions per day according to:

\[ K' = \frac{\ln(N_2/N_1)}{(t_2 - t_1)} \]

where \( N_1 \) and \( N_2 \) = biomass at time1 \( (t_1) \) and time2 \( (t_2) \), respectively (Levasseur et al., 1993).

2.4. RNA extraction

RNA extraction was carried out as previously described by Kegel et al. (2013). This protocol was developed, optimised and standardised during the MIDTAL project (Lewis et al., 2012) to extract total RNA from multi-species environmental samples. Briefly, RNA was extracted by using a TRI Reagent (Sigma-Aldrich) approach. To remove any remaining TRI Reagent residuals, samples were precipitated with 0.5 volume of 7.5 M NH₄Ac and 2 volumes of ice-cold ethanol (absolute, stored at –20 °C). Because of low amounts of RNA, triplicates of each time point of A. ostenfeldii were mixed before NH₄Ac precipitation. The RNA was re-suspended in 20 or 50 µL nuclease-free water and its concentration and integrity was measured by NanoVue spectrophotometer (GE Healthcare) or Nanodrop (Thermo-scientific, U.K.) and Agilent Bioanalyzer 2100 (Agilent Biotechnologies). Samples were shock-frozen in liquid nitrogen and stored at –80 °C until further use.

2.5. Microarray calibration

Sequences of Alexandrium spp. were analysed in silico using ARB (Ludwig et al., 2004) to design specific probes in those instances where published FISH probes were not available. Probes originally designed for FISH format for species and or for higher taxonomic levels (Table 2) and for the microarray designed by Gescher et al. (2008) were lengthened to 25 nt in length and for MIDTAL array generation 3 (Kegel et al., 2013), a 15 d-tail was added according to Mettles et al. (2007). The probe sequence for all probes designed or modified from FISH probes for the entire project for the MIDTAL microarray are patent pending for a universal microarray for the detection of toxic algae, and the entire hybridisation kit including the array and all necessary reagents are now commercially available from Microbia Environment (France). Prior to labelling, the different strains of each species were mixed in equal amounts. In the case of A. ostenfeldii, RNA of strain AOP0940 was added in an equal amount to the other RNA. The second RNA was labelled using the PlatinumBright Infrared Labelling Kit from Kreatech and purified with KREApure columns according to the manufacturer’s instructions. Concentration and incorporation of the dye was measured by a NanoVue (GE Healthcare) or Nanodrop (Thermo Scientific, UK). The degree of labelling (DoI) was calculated and ranged between 1.5 and 2.8.

The MIDTAL microarray slides generation 2 (SCHOTT Nexerton or Genetix) containing the specific probes were run with 4 different amounts of CY3-labelled (cyanine-5) Alexandrium spp. RNA (1 ng, 5 ng, 25 ng and 100 ng). A. ostenfeldii and A. minutum were hybridised to generation 3. Another calibration curve using generation 3 with 25 and 100 ng culture RNA was done with the addition of 10 ng Dunaliella tertiolecta RNA before labelling. The calibration curves completed with four different RNA amounts showed a linear response. The calibration curves using Dunaliella for normalisation were performed only with two data points because of a limited amount of RNA and number of chips. The resulting slopes of the calibration data were implemented in the GPR-Analyser (Dittami and Edvardsen, 2013) to infer cell numbers per litre.

RNA fragmentation and hybridisation was carried out for A. minutum and A. ostenfeldii according to Kegel et al. (2013), and a detailed protocol for all steps in the hybridisation and analysis can be found in Lewis et al. (2012). Hybridisation for A. tamarense was carried out with some modifications, which included a pre-hybridisation at 65 °C in pre-hybridisation buffer (Final conc., 1× STT-Buffer, 1 mg/mL BSA), hybridisation was run for 10 min at 94 °C and continued for 60 min at 65 °C. After three washing steps with increasing stringency, slides were scanned (GenePix 4000B, Molecular Devices), and total signals were calculated as the average of the feature-background ratio of all eight spots for each probe. Further analysis was carried out with the GPR-Analyser ver. 1.24 (Dittami and Edvardsen, 2013) Signals were normalised to one of the positive controls (Positive_25_dT = TATA-box probe or DunGS02_25_dT = specific for D. tertiolecta) also spotted on the slides, to allow comparison of signal strength between slides.

2.6. Statistical analysis

Statistical analysis was carried out in XLSTAT (Addinsoft SARL, France). For the RNA stress experiments a two-way ANOVA was used followed by Tukey’s B post-hoc analysis to look at differences between each treatment at each time point. For the analysis of linear relationships between the amount of RNA and cell number and also amount of RNA against the microarray signal, a regression analysis as well as a Pearson’s correlation test.

3. Results

3.1. Effects of environmental stress on growth rate of Alexandrium spp.

There was high variability in growth rates between all species and treatments. However, for A. tamarense Group I (Fig. 1A) and Group III (Fig. 1B) only the effect of salinity significantly changed the growth rate (Tukey, \( p \leq 0.05 \)) with lower growth rates at elevated and lower salinities than the ambient. No experimental treatment had a significant effect on the growth of A. ostenfeldii (Fig. 1C) Growth rates of A. minutum showed more of a response to the differing treatments (Fig. 1D) and both high and low light conditions lowered the growth rate significantly when compared to the ambient light conditions (Tukey, \( p \leq 0.001 \)). Also higher and lower temperatures significantly lowered the growth rate when compared to the control (Tukey, \( p \leq 0.05 \)). Average growth rates

<p>| Table 2 | Summary of Alexandrium species specific probes designed or modified from those published for FISH hybridisation and used for the third generation of the MIDTAL microarray. Details of probe sequences for the microarray are patent pending. |</p>
<table>
<thead>
<tr>
<th>Probe name</th>
<th>Targeted species</th>
<th>Gene</th>
<th>Source/designer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlexGD01_25_dT</td>
<td>Genus Alexandrium</td>
<td>25S</td>
<td>Kegel et al. (2012)</td>
</tr>
<tr>
<td>Aminus001_25_dT</td>
<td>Alexandrium minutum</td>
<td>18S</td>
<td>Miller and Scholin (1998)</td>
</tr>
<tr>
<td>AostS0Z_25_dT</td>
<td>Alexandrium ostenfeldii</td>
<td>18S</td>
<td>John et al. (2003)</td>
</tr>
<tr>
<td>Atam0S01_25_dT</td>
<td>Alexandrium species complex</td>
<td>18S</td>
<td>John et al. (2003)</td>
</tr>
<tr>
<td>ATNA_D01_25_dT</td>
<td>Alexandrium tamarense (North America) Group I</td>
<td>28S</td>
<td>Guillou et al. (2002)</td>
</tr>
<tr>
<td>ATTA_D01_25_dT</td>
<td>Alexandrium tamarense (Temperate Asian) Group IV</td>
<td>28S</td>
<td>Kegel et al. (2012)</td>
</tr>
</tbody>
</table>
were similar for most species and strains to published data (Table 3). No significant correlation was found between growth rate and RNA content in any of the species tested.

3.2. Total RNA against cell numbers

Calibrations of RNA content against cell numbers for raw data (Supplementary Fig. 1) showed positive linear correlations for all species (A. tamarensis Group I, R² = 0.39, r = 0.51, p < 0.05, Group III, R² = 0.47, r = 0.68, p < 0.05, A. ostfiedi, R² = 0.47, r = 0.71, p < 0.05), A. minutum, R² = 0.16, r = 0.41, p < 0.05). Despite a weak correlation for A. minutum, data for individual strains showed stronger calibration curves AL3T, which is a slow growing strain showed only a weak positive correlation between RNA and cell numbers (r = 0.33, p < 0.05) and AMAD06 (r = 0.50, p < 0.05). However, strain AMAS1 showed a strong positive correlation (r = 0.61, p < 0.05). For calculations of cell number to RNA, the data was averaged between strains and values for each day of sampling. For A. tamarensis group I an average of the strains and days showed a stronger positive calibration (Fig. 3A, R² = 0.44, r = 0.72, p < 0.001) as was the case for A. tamarensis Group III (Fig. 3B, R² = 0.63, r = 0.88, p < 0.001), A. ostfiedi (Fig. 3C, R² = 0.56, r = 0.77, p < 0.001) and A. minutum (Fig. 3D, R² = 0.30, r = 0.60, p < 0.01).

Table 3
Mean RNA content (pg/cell) and mean growth rate (d⁻¹) of the Alexandrium species/strains used in this study with literature references.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean RNA content (pg/cell)</th>
<th>Reference</th>
<th>Mean growth rate (d⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tamarensis Group I</td>
<td>54.66 ± 3.02</td>
<td>This study</td>
<td>0.20 ± 0.22</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>16.6</td>
<td>Carter et al. (unpublished)</td>
<td>0.30–0.4–0</td>
<td>Lim and Ogata (2005)</td>
</tr>
<tr>
<td>A. tamarensis Group III</td>
<td>40.93 ± 2.74</td>
<td>This study</td>
<td>0.24 ± 0.03</td>
<td>This study</td>
</tr>
<tr>
<td>A. ostfiedi</td>
<td>42.86 ± 3.13</td>
<td>This study</td>
<td>0.41 ± 0.05</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Metfies et al. (2005)</td>
<td>0.30</td>
<td>Jensen and Mostrup (1997)</td>
</tr>
<tr>
<td>A. minutum</td>
<td>3.86 ± 0.29</td>
<td>This study</td>
<td>0.12 ± 0.015</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>28.00 ± 0.30</td>
<td>Diercks et al. (2008)</td>
<td>0.5</td>
<td>Grzebyk et al. (2003)</td>
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<tr>
<td>A. fundeyes (Group I)</td>
<td>20–60</td>
<td>Anderson et al. (1999)</td>
<td>0.031–0.227</td>
<td>Taroncher-Oldenburg et al. (1999)</td>
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<tr>
<td>Gonyaulax polyedra</td>
<td>100</td>
<td>Walz et al. (1983)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Mean RNA yield for Alexandrium species under various culture conditions (n = 9 for A. tamarensis Group I, n = 9 for A. tamarensis Group III, n = 6 for A. ostfiedi and n = 3 for A. minutum; error bars ±SE). The statistical significance of the effects of the treatment (condition) as well as the interaction term (condition × time) as assessed by two-way ANOVA with Tukey post hoc analysis is indicated in the graphs (n.s. = not significant; * p < 0.05).
3.3. Effects of environmental stress on RNA content of cells of *Alexandrium* spp.

For *A. tamarense* Group I and *A. tamarense* NA Group III, there were no significant effects on RNA content per cell either in the nutrient experiments or in changing temperatures either between treatments or over time. However, for the light conditions there were significant changes in RNA content per cell for *A. tamarense* Group I in both times for elevated light and lowered light and also between the treatments and the controls (treatment *F* = 8.14, *p* ≤ 0.01, time *F* = 24.467, *p* < 0.0001, treatment × time *F* = 7.23, *p* < 0.0001) and *A. tamarense* Group III (time *F* = 7.830, *p* < 0.0001 and treatment × time *F* = 5.822, *p* = 0.0001).

In *A. tamarense* Group I cultures, after 24 h, RNA content per cell was significantly higher (Tukey, *p* ≤ 0.01) in the low light (26 μE) treatment than both the controls (160 μE) and the high light treatment (430 μE). However, this higher RNA content in the low light treatments had significantly decreased by 72 h (Tukey, *p* ≤ 0.01) and at 72 h the high light treatment was significantly higher than the low light treatment (Tukey, *p* ≤ 0.01). The RNA content in the controls stayed constant throughout the experiment.

In *A. tamarense* Group III cultures RNA content per cell in the low light treatment was significantly lower after 24 h than the high light treatment (Tukey, *p* < 0.0001) with no significant difference between the low light treatment and the controls. There was no difference between treatments after 48 h but at 72 h cells in the low light treatment had significantly higher RNA content (Tukey, *p* < 0.05) than the low light treatment and the controls.

*A. ostenfeldii* showed no significant change in RNA content per cell under any of the experimental conditions tested (Fig. 2). Overall its RNA content was 42.68 ± 3.07 pg cell⁻¹ (*n* = 72).

*A. minutum* showed significant changes in RNA content per cell both over time and between treatments in all of the experimental conditions run. There was no significant difference in RNA content in the light experiment over time in the controls (100 μE), but both the low light (15 μE), and the high light (200 μE) treatment did show a significant change (Tukey, *p* ≤ 0.01) with an increase from 24 h to 48 h in the low light and a decrease from 48 to 96 to the high light. After 48 h, the high light treatment was significantly higher than both the control and low light; however at 96 h the low light was now significantly higher (Tukey, *p* ≤ 0.01).

3.4. Microarray calibration

Results from Taylor et al. (2013) for calibration curves of signal strength against RNA amount for generation 2 (Fig. 4) and 3.1.

![Fig. 3. Average cell number against total amount of RNA extracted (ng) from stress experiments for (A) A. tamarense Group I, (B) A. tamarense Group III, (C) A. ostenfeldii and (D) A. minutum.](image-url)

![Fig. 4. (A) Calibration curves for Alexandrium tamarense Group I from Taylor et al. (2013), showing the Alexandrium genus probe (AlexGD01_25) Alexandrium tamarense species complex probe (ATNA_S01_25) and Group I ribotype specific probes (ATNA_D01_25, ATNA_D02_25) (Taylor et al., 2013). (B) Calibration curves for Alexandrium tamarense NA Group III Alexandrium genus probe (AlexGD01_25), Alexandrium tamarense species complex probe (AtamaS01_25) and Group I ribotype specific probes (ATNA_D01_25, ATNA_D02_25) Hybridisation was done on the 2nd generation MIDTAL chip (Taylor et al., 2013).](image-url)
(Fig. 5) showed probes for *A. tamarense* Group I (ATNA_D01_25, ATNA_D02_25), which have a different sequence and target different regions of the Group I rRNA genes, showed positive linear relationships ($R^2 = 0.98$, $p < 0.05$) for signal against the amount of RNA hybridised to the chip. In both versions the probe ATNA_D02_25_dT was the stronger of the two strain specific probes with the highest signal of all the probes.

Because *A. tamarense* Group III does not have specific probes on the chip, its calibration was based on the single *A. tamarense* complex probe. Both these curves were linear $R^2 = 0.97$ (Fig. 4, Taylor et al., 2013). Importantly *A. tamarense* Group III RNA did not cross-react with any of the Group I *A. tamarense* specific probes. The probes with the highest signal for Group III *A. tamarense* were the *A. tamarense* complex probe (AtamaS01_25_dT) and *Alexandrium* genus probe (AlexGD01_25_dT). RNA equivalent to 35 cells of this group did produce a very weak signal (Fig. 5B) for the Group I *A. tamarense* strain but it was deemed not to be positive <0.2 signal. RNA equivalent to 240 cells was deemed give a positive signal. Calibrations performed with the generation 3.1 chip showed similar results (Fig. 5). However, after normalisation to the *Dunaliella* probe, the signal values were higher by a factor of ~10. The probe signals for the Group I *A. tamarense* specific probe (ATNA_D02_25) were comparable between all generations of the chip for probes normalised to Positive_25, which was the internal control with TATA box specific groups and showed similar signals ~5 for 100 ng RNA. Overall for the generation 3.1 chip, the Group I specific probes showed a higher affinity for the target RNA, whereas the genus and the species complex probes showed lower affinity for the target RNA.

Two species-specific probes were designed for *A. ostenfeldii*, one from the 18S region and one from the 28S region (Table 2). For *A. minutum*, only one probe from the 18S was designed. Signals with a signal to noise ratio above two were regarded as a positive signal.
and were normalised with one of the positive controls (Positive_25_dT = TATA-box probe; DunGS02_25_dT = specific for D. tertiolecta). Each normalised probe correlated in relation to the RNA concentration hybridised and showed an exponential increase of signal to RNA concentration (Fig. 6).

The probe AostS02_25_dT (A. ostenfeldii) gave a signal (signal to noise ratio above 2) with only 1 ng RNA (corresponding to ~31 cells), whereas the second species level probe AostD01_25_dT (also A. ostenfeldii) gave a signal with 5 ng RNA (corresponding to ~154 cells). The species level probe Aminus01_25_dT (A. minutum) had a good signal with only 1 ng RNA (corresponding to 270 cells) (Fig. 7). The regression analysis of probe signal vs. cell numbers showed a positive linear relationship ($R^2 = 0.98$, $p < 0.05$) for the four-point calibration curves normalised against Positive_25_dT.

The two-point calibration curve with D. tertiolecta in the sample and normalised against DunGS02_25_dT showed a positive linear relationship ($R^2 = 0.87$) for 28S probe of the A. ostenfeldii (Fig. 6) and the A. minutum probe (Fig. 7). In the case of the 18S A. ostenfeldii probe a negative $R^2$ (Figs. 4 and 5) was calculated.

One final ribotype, the temperate Asian or Group IV, (Fig. 8) was hybridised with three amounts of RNA to provide a calibration curve for these strains and showed a linear relationship ($R^2 = 0.93$) with RNA for the probe signal for Group IV specific probe (ATTA_D01_25_dT).

4. Discussion

There is a need for molecular techniques to monitor quantitatively harmful algae (Kudela et al., 2010; Bourlat et al., 2013; Medlin, 2013; Wolfgang et al., 2014), microarrays are one way in which this can be done, several other studies have calibrated probes for other species on the MIDTAL microarray (Dittami and Edvardsen, 2012; Blanco et al., 2013; McCoy et al., 2014a,b). The advantage of the MIDTAL microarray is that it is one technique that can be used to identify the majority of western European toxic species in a single sample. There is potential to expand the chip to include new species and also functional genes (i.e. saxitoxin) (Medlin et al., 2013). However, the main challenge with molecular techniques, particularly in the case of the MIDTAL microarray has been to make them quantitative.

The results of the environmental stress experiments looking at RNA variation showed primarily that all the Alexandrium spp. are able to tolerate a wide range of environmental conditions, at not only environmentally relevant range but also extremes that may be encountered rarely. For example, A. minutum, growth was affected by light and temperature, and although this has been previously documented (Hwang and Lu, 2000; Grzebyk et al., 2003), it surprisingly still grew at 40°C, although it may not have been able to survive prolonged periods at this temperature. As A. minutum may bloom regularly in harbours (Garcés et al., 2004; Pitcher et al., 2007) and is found throughout the tropics where coastal temperatures may be several degrees above open seawater, it is clear that its tolerance to conditions which many algae would not survive may give it a selective edge.

The main factor influencing growth rate change was salinity in the A. tamarense strains and it is well documented that salinity affects the growth of A. tamarense (Watras et al., 1982; Lim and Ogata, 2005). However, the majority of these studies focus on long term effects of salinity on growth. In coastal settings, salinity can be periodically variable and can change quickly. In estuarine zones during heavy rain, salinity can decrease significantly (Fauchot et al., 2008), in contrast to semi-enclosed coastal lagoons or bays where evaporation can take place during long dry summer periods. In this respect, this study shows the effects of environmentally relevant changes in salinity conditions on growth rates and RNA content and certainly when comparing to other species and strains results were comparable with previous results (Table 3).

A. ostenfeldii showed no changes in growth rate throughout the environmental conditions. This species often is quite slow growing but can tolerate a wide range of environmental conditions (Jensen and Moestrup, 1997; Hansen et al., 2003). Short term effects of nutrients are necessarily stressful even though some nutrients will have been carried over in this experimental protocol.

Light appeared to be the most significant factor influencing RNA content within the Alexandrium species and strains tested. Light has been shown in other algae to influence cell activity (Wallen and Geen, 1971); changes in light conditions may result in increased production of chlorophyll a in the case of low light (Leonardos and
Geider, 2004) or the decreased chlorophyll a and increased production of photoprotective pigments in the case of higher light intensities (Niyogi, 1999). These processes require certain enzymes and specific proteins and so it is very likely there would be an upregulation of RNA.

Salinity showed no significant effects on the RNA content per cell in any of the *Alexandrium* strains or species tested between individual treatments or over time. *Alexandrium* spp. have also been shown to have a wide ranging tolerance to salinities (Lim and Ogata, 2005), and certainly within the ranges tested for this experiment, for which there is little effect on RNA content. Although there are significant changes in the responses to light stress, the RNA concentrations per cell are still within the same range and it would have little effect on the overall cell number calculations from the microarray signal.

Diercks et al. (2008) showed that total RNA isolated from three different strains of *A. minutum* at optimum growth conditions and the mean concentration of RNA per cell were within our range of results. This is comparable to results presented by Metfies et al. (2005) for *A. ostenfeldii* with a slightly smaller concentration per cell. *Alexandrium fundyense* (the third morphotype in Group I) showed a wider range of cell concentrations (Anderson et al., 1999). Interestingly, short term stress that may occur under natural conditions had little effect on RNA content per cell (for example the salinity response, which is likely to be the most abrupt environmental change that the cells will encounter). RNA content of single cells may change because of a number of factors, such as metabolic activity (Cornelius et al., 1985) or time of day (Walz et al., 1983). This study is the first to compare RNA content per cell of *Alexandrium* species under differing environmental stress conditions.

Although linear correlations between RNA were shown in some cases, they were quite variable (e.g. *A. minutum*). There was quite a high degree of variability between strains. One solution to this strain variability may be to have regional specific calibration, which could be easily performed because most *Alexandrium* strains are easily cultivated (Anderson et al., 2012). This biological variation between strains has been observed previously, such as Galluzzi et al. (2005) and Galluzzi et al. (2010) who have shown that rRNA gene content may vary between strains of each species and McCoy et al. (2014a) who carried out similar experiments for *Karłodinium veneficum* showed similar levels of variability between strains tested.

The RNA extraction was optimised for the MIDTAL project (Lewis et al., 2012). However there may be a need to optimise the RNA extraction efficiency further where cell numbers/RNA concentrations are low as precipitation in isopropanol may incomplete, full precipitation relies on the number of Na⁺ ions present in the solution, further improvements could be adding Sodium acetate and the addition of glycogen or linear polyacrylamide as a precipitation carrier, which can improve yields by up to 80% for very low RNA amounts (Bartram et al., 2009). Controls for RNA extraction efficiency have been taken into account and the current methodology (Lewis et al., 2012) involves the addition of a known amount of *Dunaliella* cells to samples; signals on the chip for *Dunaliella* probes can be compared to the optima for that cell number and the rest of the probes normalised to the *Dunaliella* probes.

Linear calibration curves for all *Alexandrium* spp. species and strains on both generation chips mean that back calculation to cell numbers from microarray signal is a real possibility. Also the saturation profile of the spots, as depicted in the linear relationships of the curve, shows that even under relatively high cell numbers >3000 cells/L, the probes will not be saturated. Certainly, the chip operates in the range required for detection of *Alexandrium* spp. and can detect cells at the current limit of detection (presence in the counts within England, Wales and Scotland) for many monitoring programs, and it is likely this would be the limit of detection in natural samples. We assume that minor changes in the hybridisation methodology (i.e. increased temperature, the addition of Kreblock, and higher stringency in wash buffers) account for any differences in probe performance across the two generations of the microarray. Field studies have further demonstrated the ability of the MIDTAL chip to detect quantitatively *Alexandrium* spp. (Taylor et al., 2013; Dittami et al., 2013a,b; McCoy et al., 2013) showing a clear correlation between signal strength and cell number. A recent study by McCoy et al. (2014b) has also characterised *A. minutum* in a field study looking at a bloom over a number of months a found a clear relationship between cell number and microarray signal, although detection limits were higher than those reported here they concluded that the microarray chip would still be useful in monitoring.

Importantly where the microarray chip has been evaluated under field conditions it has shown that the chip can distinguish between Group I strains and Group III and similarly between the species, which matches the results of this study. In addition, the microarray has a detection threshold that is equivalent to the threshold imposed by many monitoring programs. However, further work is needed to make it truly quantitative, especially with other dinoflagellates, such as *Dinophysis*and *Prorocentrum*, for which RNA extraction or RNA quantification can be difficult.

The MIDTAL array follows in the footsteps of other microarrays made primarily for the detection of prokaryotic organisms (DeSantis et al., 2007) and is a vast improvement over the array originally designed for *Alexandrium* by Gescher et al. (2008) because it uses longer probes, providing a stronger signal, and also over a second array designed for toxic species by Galluzzi et al. (2011) because it uses RNA and avoids a PCR step. In addition to this, it targets far more species than any chips so far. These improvements over previous chips make the MIDTAL array potentially quantitative, more universal, and less prone to biases.

5. Conclusions

All *Alexandrium* and species had a high tolerance to rapid change in environment conditions and showed a tolerance to those which are considered outside the optimal range particularly *A. minutum*. This can in part explain why they can become a bloom forming species outcompeting other phytoplankton and also why many *Alexandrium* species have increased their or colonised new areas in recent years. Total RNA extracts were positively correlated to cell numbers for all the tested species and strains in this study but there was a good deal of variability between strains independent of the environmental conditions to which little significant effect was seen.

This study showed that species-specific probes on the MIDTAL microarray were able to detect all the species tested here and in case of the *A. tamarensis* complex distinguished between toxic and non-toxic strains. It is clear that signal intensity can be used to quantify cell concentration of one particular species, so this result is very promising for a final universal microarray to detect and quantify this and many other toxic species. But further field testing is needed to validate fully the chip. It suggests that the level of variation would not significantly influence a relationship between RNA content and cell number and allows us to provide quantitative data for more species on the MIDTAL microarray.

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