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

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Abstract

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, *A. ostenfeldii* and *A. minutum*. Three strains of *A. tamarense* Group I (UoW 717, UoW 718, UoW 719), *A. tamarense* Group III (UoW 700, UoW 702, VGO927), *A. minutum* (AMAD06, AL3T, AMIA5) and two strains of *A. ostenfeldii* (AONOR4, NCH85) 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 (*p* < 0.05) for the majority of the treatments. Only light intensity seemed to significantly affect 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 is possible. The designed probes allow the production of quantitative information for *Alexandrium* species for the microarray chip.
Keywords:

Microarray; Phylochip; rRNA probes; Toxic algae; Alexandrium; MIDTAL; RNA content
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
Harmful algal blooms and coastal eutrophication are subjects of growing interest worldwide due to the pressure of increased exploitation of coastal resources (Van Dolah 2000, Ribero et al., 2012, Karydis & Kitsiou 2012). Such blooms can affect fisheries and aquaculture as well as having 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 as several of members of the genus contain the gene specific for saxitoxin production (Murray et al., 2011; Stüken 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 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 characterization 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 V) are currently thought to contain toxic strains (Lilly et al., 2007; Murray et al. 2012) with some proposing that these groups should become separate species (Wang et al. 2014). Similarly *A. ostenfeldii* and *A. minutum* have both toxic and non-toxic strains although data on distribution of toxic and non-toxic strains is much more limited than for the *A. tamarense* (Cembella and Krock 2007; Touzet et al. 2008). Certain strains of *A. ostenfeldii* also produce spirolides which are fast-acting potent neurotoxins (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., Galluzzi et al. 2004, Handy et al. 2006), isothermal amplification (Fengying et al. 2012) fluorescent in situ hybridizations (FISH, Scholin et al. 1997 Not et al. 2002, Groben and Medlin 2005), sandwich hybridizations (e.g., Scholin and Anderson, 1998 Diercks et al. 2008), microarrays (Metties and Medlin 2005, Gescher et al. 2008, Wollschläger et al 2014) and recently next generation sequencing (Egge 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 which vary between strain or species of microalga. Probes may target ribosomal RNA (rRNA) directly, rRNA-derived cDNA, or the gene in the DNA coding for rRNA (rDNA). 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 whereas genomic DNA is made up of a 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 and can also be calibrated for (Ayers et al., 2005). 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; Galuzzi et al. 2008) 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 (Galuzzi et al. 2008). 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). As *Alexandrium spp* have a wide geographical distribution (Gribble et al. 2005, Lilly et al 2005, Lilly et al., 2007;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 & Lu 2000; Grzebyk et al 2003, Jensen & Moestrup 1997; Hansen et al. 2004) and salinity (Lim & 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 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 strain 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 between species or strain. Signal intensity of species specific probes against amount of RNA hybridized to the chip the probes on the microarray 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 were no cross relativity between the probes for each species. Based on previous complementary studies using the MIDTAL chip Dittami & Edvardsen (2012a) for *Pseudochnatella* and Blanco et al. (2013) for *Heterosigma akashiwo Prynensium, Karlodinium veneficum* and cf. *Chatonella sp.* (McCoy et al. 2014a) and unpublished
work for four species of *Pseudo-nitzschia* (Medlin et al.) 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 1s, with the exception of *Alexandrium ostenfeldii* where only two cultures were available and the *Alexandrium* clade of 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 & Ryther 1962) media in seawater salinity 30-34 and at 15°C, 100 µE for *A. minutum* and *A. 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 & Edvardsen (2012a). 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, 2, 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 3 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 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 hours of inoculation, after 48 hours, and after 72 hours 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 (Figure 1). For *A. ostenfeldii* 45 (strain
NCH85) or 90 mL (strain AONOR4, 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).

Growth rate was defined as divisions per day according to:

$$K' = \frac{\ln (N2 / N1)}{(t2 - t1)}$$

Where N1 and N2 = biomass at time1 (t1) and time2 (t2), 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 2013) 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.

2.57 Microarray calibration
Sequences of *Alexandrium spp* were analysed in silico using ARB (Wolfgang 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 nts in length and for MIDTAL array generation 3 (Kegel et al., 2013), a
15 dT- tail was added according to Metfies 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 as 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 Kreatech (UK). Prior to labelling, the different strains of
each species were mixed in equal amounts. In the case of *A. ostenfeldii*, RNA of strain
AOF0940 was added in an equal amount to the other two strains. 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
The degree of labelling (DoL) was calculated and ranged between 1.5-2.8.

The MIDTAL microarray slides generation 2 (SCHOTT Nexterion or Genetix) containing the specific probes were run with 4 different amounts of CY5-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-Analyzer (Dittami & Edvardsen 2012b) to infer cell numbers per liter.

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 hybridization 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-hybridization buffer (Final conc., 1 x STT-Buffer, 1mg/mL BSA), hybridisation was run for 10 minutes at 94°C and continued for 60 minutes 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 8 spots for each probe. Further analysis was carried out with the GPR-Analyzer ver. 1.24 (Dittami and Edvardsen, 2012b) Signals were normalized to one of the positive controls (Positive_25_dT = TATA-box probe or DunGS02_25_dT =
specific for *Dunaliella 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* ≤ 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* ≤ 0.001). Also higher and lower temperatures significantly lowered the growth rate when compared to the control (Tukey, *p* ≤ 0.05). Average growth rates 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 figure 1) showed positive linear correlations for all species (A. tamarense Group I, $r^2=0.39$, $r=0.51$ $p<0.05$) Group III $r^2=0.47, r=0.68$ $p<0.05$, A.ostenfeldii $r^2=0.47$ $r=0.71$, $p<0.05$), A.minutum $0.16$ $r^2= 0.41$). Despite a weak correlations 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 ($r=0.33$, $p<0.05$) AMAD06 ($r=0.50$ $p<0.05$) however strain AMA5I 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.tamarense group I an average of the strains and days showed a stronger positive calibration (Figure 3a, $r^2=0.44$ $r=0.72$ $p<0.001$) as was the case for A.tamarense Group III (Figure 3b, $r^2=0.63$, $r=0.88$, $p<0.001$), A.ostenfeldii (Figure 3c, $r^2=0.56$, $r=0.77$, $p<0.001$) and A.minutum (Figure 3d, $r^2=0.30$, $r=0.60$ $p<0.01$).

3.2 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 time for elevated light and lowered light and also between the treatments and the
controls. (Treatment $F= 8.14, \ p \leq 0.01$, Time $F=24.467, \ p < 0.0001$, Treatment*Time $F=7.23, \ p < 0.0001$) and A. tamarense Group III (Time $F=7.830, \ p \leq 0.0001$ and Treatment*Time $F=5.822 \ p \leq 0.0001$).

In A. tamarense Group I cultures, after 24 h, RNA content per cell was significantly higher (Tukey, $p \leq 0.01$) 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, $p \leq 0.01$) and at 72h the high light treatment was significantly higher than the low light treatment (Tukey, $p \leq 0.01$). 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, $p \leq 0.0001$) 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 treatment had significantly higher RNA content (Tukey, $p \leq 0.05$) than the low light treatment and the controls.

A. ostenfeldii showed no significant change in RNA content cell$^{-1}$ under any of the experimental conditions tested (Figure 2). Overall its RNA content was $42.68\pm 3.07$ 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, $p \leq 0.01$) 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 significant higher than both the control and low light; however at 96 hours the low light was now significantly higher (Tukey, $p \leq 0.01$).

3.4 Microarray calibration

Results from Taylor et al 2013 for calibration curves of signal strength against RNA amount for generation 2 (Figure 4) and 3.1 (Figure 5) showed probes for *Alexandrium 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 ($R^2=0.9785$, $p<0.05$) 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. For *Alexandrium tamarense* Group I showed a strong signal. *Alexandrium* genus level genus probe (AlexG_D01_25) with the former showing a greatly reduced signal (greater than 0.2 but less than 1) when compared to the latter.

As *Alexandrium 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 *Alexandrium 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 (Figure 5). However, after normalization 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 normalized 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 normalized with one of the positive controls (Positive_25_dT = TATA-box probe; DunGS02_25_dT = specific for *Dunaliella tertiolecta*). Each normalised probe correlated in relation to the RNA concentration hybridised and showed an exponential increase of signal to RNA concentration (Figure 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). The regression analysis of probe signal vs. cell numbers showed a positive linear relationship ($r^2=0.98$) for the four-point calibration curves normalized against Positive_25_dT.
The two-point calibration curve with *Dunaliella 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* and the *A. minutum* probe. In the case of the 18S *A. ostenfeldii* probe a negative $r^2$ (Figures 4 & 5) was calculated.

One final ribotype, the temperate Asian or Group IV, (Figure 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 quantitatively monitor harmful algae (Kudela et al 2010, Bourlat et al. 2013, Medlin 2013), microarrays are one way in which this can be done, several other studies have calibrated probes for other species on the MIDTAL microarray (Dittami, & Edvardsen 2012a, Perez Blanco et al 2013, McCoy et al 2014). 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 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 & 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 & 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, as this species often has quite slow growing but can tolerate a wide range of environmental conditions (Jensen & Moestrup 1997; Hansen et al. 2004). 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 & 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 both between individual treatments and there was no significant difference over time. *Alexandrium spp.* have also been shown to have a wide ranging tolerance to salinities (Lim & 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 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 (Waltz 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 suggested (e.g. *A. minutum*) there was quite a high degree of variability between strains one solution to this problem may be to have regional specific calibration, which could be easily performed as most *Alexandrium* strains are easily cultivated (Anderson et al. 2012). This biological variation between strains has been observed previously such as Galuzzi et al. 2004 and Galuzzi et al. 2008 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 *Karlodinium venerficum* 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 be 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 efficiently 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\(^{-1}\), 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 hybridization methodology (i.e., increased temperature, the addition of krebblock, 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 quantitatively detect Alexandrium spp (Taylor et al. 2013, Dittami et al. 2013a Dittami et al 2013b 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 Alexandrium 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 conclude 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 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 on 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 *Alexandrium 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 colonized 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 independently
of the environmental conditions to which little significant effect was seen.

This study showed that species-specific probes on the MIDTAL microarray are able to
detect all the species tested here and in case of the *A.tamarense* complex distinguish
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 fully validate 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.

6. Acknowledgements

We thank Dennis Gowland from Research Relay for carrying out some of the sampling in the Orkney Islands and other MIDTAL partners for method development and some cultures. Marco Berzano for help with culturing. Jixen Chen spotted the generation 2 slides and Scienion (Berlin, Germany) generation 3. M. Bayer performed the RNA experiments at the AWI. This work was funded by the European Union under the FP7 water framework directive Grant agreement number 201724.

References


Toxins: Pharmacology, Physiology, and Detection. CRC Press, Boca Raton, FL, pp. 561–580


<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⁻¹)</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>NCH85</td>
<td>North Sea</td>
<td>Flow Cytometer</td>
<td>17-34-42</td>
<td>10-15-18</td>
<td>25-100-200</td>
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<tr>
<td><em>Alexandrium tamarense</em></td>
<td>UoW 717</td>
<td>Water column, Stromness pier, Orkney Islands, U.K</td>
<td>Sedgewick rafter</td>
<td>28-33-38</td>
<td>12-16-20</td>
<td>26-160-430</td>
</tr>
<tr>
<td>Group I</td>
<td>UoW 719</td>
<td>Water column, Stromness Orkney Islands, U.K</td>
<td>Sedgewick rafter</td>
<td>28-33-38</td>
<td>12-16-20</td>
<td>26-160-430</td>
</tr>
<tr>
<td>Group III</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></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>
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<tr>
<td>(catenella morphotype)</td>
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<td></td>
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</table>
Table 2: Summary of *Alexandrium* species specific probes designed or modified from those published for FISH hybridization and used for the third generation of the MIDTAL microarray. Details of probe sequences for the microarray are patent pending.

<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 <em>Alexandrium</em></td>
<td>28S</td>
<td>Kegel et al., 2012</td>
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<tr>
<td>AminuS01_25_dT</td>
<td><em>Alexandrium minutum</em></td>
<td>18S</td>
<td>Miller and Scholin, 1998</td>
</tr>
<tr>
<td>AostS02_25_dT</td>
<td><em>Alexandrium ostenfeldii</em></td>
<td>18S</td>
<td>John et al., 2003</td>
</tr>
<tr>
<td>AostD01_25_dT</td>
<td><em>Alexandrium ostenfeldii</em></td>
<td>28S</td>
<td>John et al., 2003</td>
</tr>
<tr>
<td>AtamaS01_25_dT</td>
<td><em>Alexandrium</em> species</td>
<td>18S</td>
<td>John et al., 2003</td>
</tr>
<tr>
<td></td>
<td>complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATNA_D01_25_dT</td>
<td><em>Alexandrium tamarense</em></td>
<td>28S</td>
<td>John et al., 2003</td>
</tr>
<tr>
<td></td>
<td>(North America)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATNA_D02_25_dT</td>
<td><em>Alexandrium tamarense</em></td>
<td>28S</td>
<td>Guillou et al., 2002</td>
</tr>
<tr>
<td></td>
<td>(North America)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATTA_D01_25_dT</td>
<td><em>Alexandrium tamarense</em></td>
<td>28S</td>
<td>Kegel et al., 2012</td>
</tr>
<tr>
<td></td>
<td>(Temperate Asian)</td>
<td></td>
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Table 3 Mean RNA content (pg cell\(^{-1}\)) 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(^{-1}))</th>
<th>Reference</th>
<th>Mean growth rate (d(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. tamarense</em> Group I</td>
<td>54.66 ± 3.02</td>
<td>This study</td>
<td>0.20 ± 0.22</td>
<td>This study</td>
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<tr>
<td></td>
<td>16.6</td>
<td>Carter et al., unpublished</td>
<td>0.30-0.4-0</td>
<td>Lim &amp; Ogata (2005)</td>
</tr>
<tr>
<td><em>A. tamarense</em> 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><em>A. ostenfeldii</em></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 &amp; Moestrup (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.12 ±</td>
<td></td>
</tr>
<tr>
<td><em>A. minutum</em></td>
<td>3.86 ± 0.29</td>
<td>This study</td>
<td>0.015</td>
<td>This study</td>
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<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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<td>Taroncher-Oldenburg et al., (1999)</td>
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<td><em>A. fundeyese</em> (Group I)</td>
<td>20-60</td>
<td>Anderson et al., (1999)</td>
<td>0.031-0.227</td>
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<td>Walz et al., (1983)</td>
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<tr>
<td><em>Gonyaulax polyedra</em></td>
<td>100</td>
<td></td>
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</tbody>
</table>
Figure Legends

Figure 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).

Figure 2. Mean RNA yield for *Alexandrium* species under various culture conditions (*n* = 9 for *A. tamarense* Group I, *n* = 9 for *A. tamarense* Group III, *n* = 6 for *A. ostenfeldii* 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).

Figure 3. Average cell number against total amount of RNA extracted (ng) from stress experiments for a) *A. tamarense* NA, b) *A. tamarense* WE, c) *A. ostenfeldii* and d) *A. minutum*

Figure 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 (AtamaS01_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) Hybridization was done on the 2nd generation MIDTAL chip. (Taylor et al 2013),

Figure 5. Two point calibration curves for *Alexandrium tamarense* from Taylor et al (2013), using the 3rd generation MIDTAL chip and the addition of 10 ng of *Dunaliella tertiolecta*. Group I showing an *Alexandrium* genus probe (AlexGD01_25_dT), *Alexandrium tamarense* complex probe (AtamaS01_25) and Group I ribotype specific probes (ATNA_D01_25_dT, ATNA_D02_25_dT) showing normalisation to A) POSITIVE_25_dT and B) the *Dunaliella* specific probe DunGS02_dT; and calibration curves for *Alexandrium tamarense* NA
Group III *Alexandrium* genus probe (AlexGD01\_25\_dT), *Alexandrium tamarense* complex probe (AtamaS01\_25) and Group I ribotype specific probes (ATNA\_D01\_25\_dT, ATNA\_D02\_25\_dT) showing normalisation to C) POSITIVE\_25\_dT and D) the *Dunaliella* specific probe DunGS02\_dT. Hybridisation was done on generation 3.1 of the chip.

Figure 6. Cell number against microarray signal for probes specific for *A. ostenfeldii* Hybridization was done on the 3rd generation MIDTAL chip.

Figure 7. Cell number against microarray signal for probes specific for *A. minutum*. Hybridization was done on the 3rd generation MIDTAL chip.

Figure 8. Amount RNA extracted from *A. tamarense (catanella morphotype) Group IV* against microarray signal for the probe ATTA\_D01\_25\_dT. Hybridised with the generation 3.1 chip.
Figure 1

A. tamarense Group I
A. tamarense Group III
A. ostreifeldii
A. minutum

Salinity Light (µE) Nutrients Temp. (°C)

Treatment
Figure 2

Salinity

Light

Nutrients

Temp.

RNA content (pg cell\(^{-1}\))

Time (h)

A.tamarense Group I

A.tamarense Group II

A.tamarense Group III

A.ostenfeldii

A.minutum

0
50
100
150
200

0
50
100
150
200

0
50
100
150
200

0
50
100
150
200

0
50
100
150
200

0
50
100
150
200

0
50
100
150
200

0
50
100
150
200

24 48 72

24 48 72

24 48 72

24 48 72

24 48 72

24 48 72

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24 48 72

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24 48 72

24 48 72

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24 48 72

24 48 72
Figure 3

Alexandrium tamarense Group I

Alexandrium tamarense Group III

A. minutum

A. ostenfeldii

Cell number
**Figure 4**

- **A. tamarense Group I**
  - □ Alexandria genus (AlexGD01_25)
  - ◊ A. tamarense complex (AtamaS01_25)
  - ● A. tamarense Group I (ATNA_D01_25)
  - ■ A. tamarense Group I (ATNA_D02_25)

\[ R^2 = 0.9785 \]

- **A. tamarense Group III**

\[ R^2 = 0.9902 \]

**Signal intensity normalised to**

**POSITIVE_25**

**Cell number**
Figure 5

A.tamarense Group I

A.tamarense Group III
Figure 6

- **a**
  - Signal intensity normalised to POSITIVE\_25\_dT
  - 
    - $\Delta$ Alexandrium genus
    - (AlexGD01\_25\_dT)
    - × A. ostenfeldii (AostS02\_25\_dT)
    - ■ A. ostenfeldii (AostD01\_25\_dT)
  - $R^2 = 0.999$

- **b**
  - Signal intensity normalised to DunGS02\_25\_dT
  - $R^2 = 0.988$

- Cell number
Figure 7

(a) Signal intensity normalised to POSITIVE_25_dT
- △ Alexandrium genus (AlexGD01_25_dT)
- • A. minutum (AminuS01_25_dT)

(b) Signal intensity normalised to DunGS02_25_dT
- △ Alexandrium genus (AlexGD01_25_dT)
- • A. minutum (AminuS01_25_dT)
Figure 8

The graph shows the signal intensity normalized to "POSITIVE_25_dT" as a function of RNA ng. The data points are represented by two different markers: "A. catanella TA (ATTA_D01_25_dT)" and "Alexandrium genus (AlexGD01_25_dT)". The graph has a linear trend line with a $R^2 = 0.9321$.

- **A. catanella TA (ATTA_D01_25_dT)**
- **Alexandrium genus (AlexGD01_25_dT)**

The x-axis represents the RNA ng ranging from 0 to 120, and the y-axis represents the signal intensity normalized to "POSITIVE_25_dT" ranging from 0 to 1.2.
Supplementary Figure 1

**Alexandrium tamarense Group I**

- $y = 0.039x$
- $R^2 = 0.39$

**Alexandrium tamarense Group III**

- $y = 0.019x$
- $R^2 = 0.47$

**A. ostenfeldii**

- $y = 0.033$
- $R^2 = 0.47$

**A. minutum**

- $y = 0.0038x$
- $R^2 = 0.16$