# 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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# 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

101 *1. Introduction*

102 Harmful algal blooms and coastal eutrophication are subjects of growing interest
103 worldwide due to the pressure of increased exploitation of coastal resources (Van Dolah
104 2000, Ribero et al.,2012, Karydis & Kitsiou 2012). Such blooms can affect fisheries and
105 aquaculture as well as having an impact on the tourism potential of an area (e.g.
106 Hoagland et al.,2002, Smaal 2002). There are a wide range of microalgal species
107 involved in such events. Amongst the most notorious are species of the genus
108 *Alexandrium,* some of which produce potent neurotoxins from the saxitoxin family, the
109 causative agents of paralytic shellfish poisoning (Clark et al., 1999, Anderson et al.,
110 2012). Recent research has shown that *Alexandrium* species are directly responsible for
111 saxitoxin production as several of members of the genus contain the gene specific for
112 saxitoxin production (Murray et al., 2011; Stüken et al., 2011). Hence the monitoring of
113 coastal waters for these species is an important element of health protection programmes
114 as well as being vital for aquaculture (particularly shellfish) producers to manage their
115 enterprises. Such monitoring is typically carried out by light microscopy (Humbert et al.,
116 2010).

117 The genus *Alexandrium* contains more than 25 species (Balech, 1995, Anderson et al
118 2012), which are largely separated by morphological variations in the cellulosic plates of
119 the motile stage. To discriminate species requires the determination of the shape and
120 conformation of the first apical plate (including presence/absence of a pore), shape and
121 conformation of the apical pore complex, distribution and shape of precingular plates and
122 the plate structure of the sulcal region (Steidinger, 2010). Full characterization an take a
123 great deal of time and skill by light microscopy. Furthermore molecular studies have

124 revealed a number of species 'complexes' whose strains can be distinguished by genetic
125 identification but not by light microscopy. One of these complexes is *Alexandrium*
126 *tamarense,* which includes five groups, of which three (I, IV V) are currently thought to
127 contain toxic strains (Lilly et al., 2007; Murray et al. 2012) with some proposing that
128 these groups should become separate species (Wang et al. 2014). Similarly *A. ostenfeldii*
129 and *A.minutum* have both toxic and non-toxic strains although data on distribution of
130 toxic and non-toxic strains is much more limited than for the *A.tamarense* (Cembella and
131 Krock 2007; Touzet et al 2008). Certain strains of *A.ostenfeldii* also produce spirolides
132 which are fast acting potent neuro toxins (Cembella et al 2001). Therefore there is a clear
133 to need to be able to distinguish *A.ostenfeldii* from the both *A.tamarense* complex and
134 *A.minutum*. Additionally it has been shown that both toxic and non-toxic groups of
135 *A.tamarense* can co-occur (Higman et al., 2001, John et al.,2003, Touzet et al.,2010) and
136 so to avoid false positives or negatives in monitoring, faster and more efficient counting
137 methodologies are sought.

138 Previous studies have used a variety of molecular techniques, which can distinguish
139 between toxic species and strains, to detect harmful algae such as quantitative PCR (e.g.,
140 Galluzzi et al. 2004, Handy et al. 2006), iso-thermal amplification (Fengying et al. 2012)
141 fluorescent in situ hybridizations (FISH, Scholin et al. 1997 Not et al. 2002, Groben and
142 Medlin 2005), sandwich hybridizations (e.g., Scholin and Anderson, 1998 Diercks et al.
143 2008), , microarrays (Metfies and Medlin 2005, Gescher et al. 2008, Wollschläger et al
144 2014) and recently next generation sequencing (Egge et al 2013). The majority of these
145 methods rely on species or strain specific RNA or DNA sequences with the most of these
146 targeting ribosomal RNA genes which have highly conserved regions and also highly

147 variable regions which vary between strain or species of microalga. Probes may target
148 ribosomal RNA (rRNA) directly, rRNA-derived cDNA, or the gene in the DNA coding
149 for rRNA (rDNA). The use of RNA or cDNA has several advantages compared to DNA.
150 Within cells RNA is much less stable and is rapidly degraded compared with DNA,
151 therefore this approach means detection of only active cells. Total RNA is constituted
152 mainly of rRNA resulting in a high ratio of target to non- target sequences where as
153 genomic DNA is made up of large majority of non-target sequences. It can therefore be
154 used directly in microarray assays without prior amplification of the target region which
155 can lead to PCR bias for certain sequences (Peplies et al. 2006 and can also be calibrated
156 for (Ayers et al., 2005). Microarrays are one molecular technique that has the potential to
157 be quantitative and previous studies have shown that rRNA content and cell numbers
158 correlate well in some algal species under laboratory conditions (Ayers et al. 2005;
159 Galuzzi et al. 2008) However, very little information is available about how
160 environmental conditions and growth rate affect rRNA content in eukaryotes. It has been
161 shown that for some microalgae the pool of RNA within a cell can vary (Dortch et al.,
162 1984, Berdelet et al., 1994) and that rRNA content may vary with growth phase (Galuzzi
163 et al. 2008). Similarly in bacteria, the per-cell rRNA content has been shown to depend
164 strongly on growth rate and nutrient availability, varying over 10-fold between starved
165 cells in stationary phase and nutrient-replete cells in logarithmic growth phase (Fegatella
166 et al. 1998). As *Alexandrium spp* have a wide geographical distribtution (Gribble et al
167 2005, Lilly et al 2005, Lilly et al., 2007;Anderson et al 2012) and as primarily coastal
168 species can be subjected to a variety of varying environmental conditions such as light,
169 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 invesitgate the effciciency 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 *Pseudochatonella* 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$^{o}$C, 100 µE for *A. minutum* and *A. ostenfieldii* and at 16$^{o}$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

239 NCH85) or 90 mL (strain AONOR4, a slow growing strain) of culture was filtered onto 3
240 μm nitrocellulose or polycarbonate filters (Whatman, U.K), transferred into cryogenic
241 vials containing acid washed glass beads (213-300 μm), shock-frozen in liquid nitrogen,
242 and stored at -80°C until further processing.

243 *2.3 Cell counts*

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

251 Growth rate was defined as divisions per day according to:

252 $K' = \ln(N2 / N1) / (t2 - t1)$

253 Where N1 and N2 = biomass at time1 (t1) and time2 (t2), respectively (Levasseur et al.,
254 1993).

255 *2.4 RNA extraction*

256 RNA extraction was carried out as previously described by Kegel et al. (2013). This
257 protocol was developed, optimised and standardised during the MIDTAL project (Lewis
258 et al 2013) to extract total RNA from multi-species environmental samples. Briefly,
259 RNA was extracted by using a TRI Reagent (Sigma-Aldrich) approach. To remove any
260 remaining TRI Reagent residuals, samples were precipitated with 0.5 volume of 7.5 M
261 $NH_4Ac$ 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 $NH_4Ac$ 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

284 (Thermo Scientific, UK). The degree of labelling (DoL) was calculated and ranged
285 between 1.5-2.8.

286 The MIDTAL microarray slides generation 2 (SCHOTT Nexterion or Genetix)
287 containing the specific probes were run with 4 different amounts of CY5-labelled
288 (cyanine-5) *Alexandrium spp* RNA (1 ng, 5 ng, 25 ng and 100 ng). *A. ostenfeldii* and *A.*
289 *minutum* were hybridised to generation 3. Another calibration curve using generation 3
290 with 25 and 100 ng culture RNA was done with the addition of 10 ng *Dunalliella*
291 *tertiolecta* RNA before labelling. The calibration curves completed with four different
292 RNA amounts showed a linear response. The calibration curves using *Dunaliella* for
293 normalisation were performed only with two data points because of a limited amount of
294 RNA and number of chips. The resulting slopes of the calibration data were implemented
295 in the GPR-Analyzer (Dittami & Edvardsen 2012b) to infer cell numbers per liter.

296 RNA fragmentation and hybridisation was carried out for *A. minutum* and *A.*
297 *ostenfeldii* according to Kegel et al. (2013), and a detailed protocol for all steps in the
298 hybridization and analysis can be found in Lewis et al. (2012). Hybridisation for *A.*
299 *tamarense* was carried out with some modifications, which included a pre-hybridisation
300 at 65°C in pre-hybridization buffer (Final conc., 1 x STT-Buffer, 1mg/mL BSA),
301 hybridisation was run for 10 minutes at 94°C and continued for 60 minutes at 65°C. After
302 three washing steps with increasing stringency, slides were scanned (GenePix 4000B,
303 Molecular Devices), and total signals were calculated as the average of the feature-
304 background ratio of all 8 spots for each probe. Further analysis was carried out with the
305 GPR-Analyzer ver. 1.24 (Dittami and Edvardsen, 2012b) Signals were normalized to one
306 of the positive controls (Positive_25_dT = TATA-box probe or DunGS02_25_dT =

307 specific for *Dunaliella tertiolecta*) also spotted on the slides, to allow comparison of
308 signal strength between slides.

309 *2.6 Statistical analysis*

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

316

317 3.Results

318 *3.1 Effects of environmental stress on growth rate of Alexandrium spp.*

319 There was high variability in growth rates between all species and treatments.
320 However, for *A. tamarense* Group I (Fig. 1a) and Group III (Fig. 1b) only the effect of
321 salinity significantly changed the growth rate (Tukey, $p \leq 0.05$) with lower growth rates at
322 elevated and lower salinities than the ambient. No experimental treatment had a
323 significant effect on the growth of *A. ostenfeldii* (Fig.1c) Growth rates of *A.minutum*
324 showed more of a response to the differing treatments (Fig. 1d) and both high and low
325 light conditions lowered the growth rate significantly when compared to the ambient light
326 conditions (Tukey, $p \leq 0.001$). Also higher and lower temperatures significantly lowered
327 the growth rate when compared to the control (Tukey, $p \leq 0.05$). Average growth rates
328 were similar for most species and strains to published data (Table 3.) No significant
329 correlation was found between growth rate and RNA content in any of the species tested.

330

331

332 *3.2 Total RNA against Cell numbers*

333 Calibrations of RNA content against cell numbers for raw data (Supplementary figure 1)
334 showed positive linear correlations for all species (*A. tamarense* Group I, $r^2$=0.39, r=0.51
335 $p$<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),
336 *A.minutum* 0.16 $r^2$ $r$= 0.41). Despite a weak correlations for *A.minutum* data for
337 individual strains showed stronger calibration curves AL3T which is a slow growing
338 strain showed only a weak positive correlation between RNA and cell numbers (*r*=0.33,
339 p<0.05) AMAD06 (*r*=0.50 p<0.05) however strain AMA5I showed a strong positive
340 correlation (*r*=0.61, p<0.05). For calculations of cell number to RNA the data was
341 averaged between strains and values for each day of sampling. For *A.tamarense* group I
342 an average of the strains and days showed a stronger positive calibration (Figure 3a, $r^2$
343 =0.44 *r*=0.72 p<0.001) as was the case for *A.tamarense* Group III (Figure 3b, $r^2$=0.63,
344 *r*=0.88, *p*<0.001), *A.ostenfeldii* (Figure 3c, $r^2$=0.56, *r*=0.77, *p*<0.001) and A.*minutum*
345 (Figure 3d, $r^2$=0.30, r=0.60 *p*<0.01).

346

347 3.2 *Effects of environmental stress on RNA content of cells of Alexandrium spp.*

348 For *A. tamarense* Group I and *A. tamarense* NA Group III, there were no significant
349 effects on RNA content per cell either in the nutrient experiments or in changing
350 temperatures either between treatments or over time. However, for the light conditions
351 there were significant changes in RNA content per cell for *A. tamarense* Group I in both
352 time for elevated light and lowered light and also between the treatments and the

353 controls. (Treatment $F= 8.14$, $p \leq 0.01$, Time $F=24.467$, $p< 0.0001$, Treatment*Time
354 F=7.23, $p< 0.0001$) and *A. tamarense* Group III (Time $F=7.830$, $p \leq 0.0001$ and
355 Treatment*Time $F=5.822$ $p \leq 0.0001$).

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

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

368 *A. ostenfeldii* showed no significant change in RNA content cell$^{-1}$ under any of the
369 experimental conditions tested (Figure 2). Overall its RNA content was 42.68±3.07 pg
370 cell$^{-1}$ ($n$=72).

371 *A. minutum* showed significant changes in RNA content per cell both over time and
372 between treatments in all of the experimental conditions run. There was no significant
373 difference in RNA content in the light experiment over time in the controls (100 µE), but
374 both the low light (15 µE), and the high light (200 µE) treatment did show a significant
375 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 amsount 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

399 to 240 cells was deemed give a positive signal. Calibrations performed with the
400 generation 3.1 chip showed similar results (Figure 5). However, after normalization to the
401 *Dunaliella* probe, the signal values were higher by a factor of ~ 10. The probe signals for
402 the Group I *A. tamarense* specific probe (ATNA_D02_25) were comparable between all
403 generations of the chip for probes normalized to POSITIVE_25, which was the internal
404 control with TATA box specific groups and showed similar signals ~5 for 100 ng RNA.
405 Overall for the generation 3.1 chip, the Group I specific probes showed a higher affinity
406 for the target RNA, whereas the genus and the species complex probes showed lower
407 affinity for the target RNA.

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

415 The probe AostS02_25_dT (*A. ostenfeldii*) gave a signal (signal to noise ratio above 2)
416 with only 1 ng RNA (corresponding to ~31 cells), whereas the second species level probe
417 AostD01_25_dT (also *A.ostenfeldii*) gave a signal with 5 ng RNA (corresponding to
418 ~154 cells). The species level probe AminuS01_25_dT (*A. minutum*) had a good signal
419 with only 1 ng RNA (corresponding to 270 cells). The regression analysis of probe signal
420 vs. cell numbers showed a positive linear relationship ($r^2$=0.98) for the four-point
421 calibration curves normalized against Positive_25_dT.

422 The two-point calibration curve with *Dunaliella tertiolecta* in the sample and
423 normalised against DunGS02_25_dT showed a positive linear relationship ($R^2 = 0.87$) for
424 28S probe of the *A. ostenfeldii* and the *A. minutum* probe. In the case of the 18S *A.*
425 *ostenfeldii* probe a negative $r^2$ (Figures 4 & 5) was calculated.
426 One final ribotype, the temperate Asian or Group IV, (Figure 8) was hybridised with
427 three amounts of RNA to provide a calibration curve for these strains and showed a linear
428 relationship ($R^2$=0.93) with RNA for the probe signal for Group IV specific probe
429 (ATTA_D01_25_Dt)
430
431 *4. Discussion*
432 There is a need for molecular techniques to quantitatively monitor harmful algae
433 (Kudela et al 2010, Bourlat et al. 2013, Medlin 2013), microarrays are one way in which
434 this can be done, several other studies have calibrated probes for other species on the
435 MIDTAL microarray (Dittami, & Edvardsen 2012a, Perez Blanco et al 2013, McCoy et
436 al 2014). The advantage of the MIDTAL microarray is that it is one technique that can be
437 used to identify the majority of western European toxic species in a sample. There is
438 potential to expand the chip to include new species and also functional genes (i.e
439 saxitoxin) (Medlin et al 2013). However, the main challenge with molecular techniques,
440 particularly in the case of the MIDTAL microarray has been to make them quantitative.
441 The results of the environmental stress experiments looking at RNA variation showed
442 primarily that all the *Alexandrium spp* are able to tolerate a wide range of environmental
443 conditions, at not only environmentally relevant range but also extremes that may be
444 encountered rarely. For example, *A. minutum*, growth was affected by light and

445 temperature, and although this has been previously documented (Hwang & Lu 2000;
446 Grzebyk et al 2003), it surprisingly still grew at 40ºC, although it may not have been able
447 to survive prolonged periods at this temperature. As *A.minutum* may bloom regularly in
448 harbours (Garcés et al. 2004; Pitcher et al. 2007) and is found thoroughout the tropics
449 where coastal temperatures may be several degrees above open seawater, it is clear that
450 its tolerance to conditions which many algae would not survive may give it a selective
451 edge.

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

462 *A. ostenfeldii* showed no changes in growth rate throughout the environmental
463 conditions, as this species often has quite slow growing but can tolerate a wide range of
464 environmental conditions (Jensen & Moestrup 1997; Hansen et al. 2004). Short term
465 effects of nutrients are necessarily stressful even though some nutrients will have been
466 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).

490 RNA content of single cells may change because of a number of factors, such as
491 metabolic activity (Cornelius et al., 1985) or time of day (Waltz et al.,1983). This study is
492 the first to compare RNA content per cell of *Alexandrium* species under differing
493 environmental stress conditions.

494 Although linear correlations between RNA were shown in some cases they were quite
495 variable suggested (e.g *A.minutum*) there was quite a high degree of varability between
496 strains one solution to this problem may be to have regional specific calibration, which
497 could be easily performed as most *Alexandrium* strains are easily cultivated (Anderson et
498 al 2012). This biological variation between strains has been observed previously such as
499 Galuzzi et al 2004 and Galuzzi et al 2008 who have shown that rRNA gene content may
500 vary between strains of each species and McCoy et al 2014a who carried out similar
501 experiments for *Karlodinium venerficum* showed similar levels of variability between
502 strains tested.

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

512 *Dunaliella* probes can be compared to the optima for that cell number and the rest of the
513 probes normalised to the *Dunaliella* probes.

514 Linear calibration curves for all *Alexandrium* spp. species and strains on both
515 generation chips mean that back calculation to cell numbers from microarray signal is a
516 real possibility. Also the saturation profile of the spots, as depicted in the linear
517 relationships of the curve, shows that even under relatively high cell numbers >3000 cells
518 $L^{-1}$, the probes will not be saturated. Certainly, the chip operates in the range required for
519 detection of *Alexandrium* spp. and can detect cells at the current limit of detection
520 (presence in the counts within England, Wales and Scotland) for many monitoring
521 programs, and it is likely this would be the limit of detection in natural samples. We
522 assume that minor changes in the hybridization methodology (i.e., increased temperature,
523 the addition of kreablock, and higher stringency in wash buffers) account for any
524 differences in probe performance across the two generations of the microarray. Field
525 studies have further demonstrated the ability of the MIDTAL chip to quantitatively detect
526 *Alexandrium spp* (Taylor et al. 2013, Dittami et al. 2013a Dittami et al 2013b McCoy et
527 al 2013) showing a clear correlation between signal strength and cell number. A recent
528 study by McCoy et al 2014b has also characterised *Alexandrium minutum* in a field study
529 looking at a bloom over a number of months a found a clear relationship between cell
530 number and microarray signal, although detection limits were higher than those reported
531 here they conclude that the microarray chip would still be useful in monitoring.

532 Importantly where the microarray chip has been evaluated under field conditions it has
533 shown that the chip can distinguish between Group I strains and Group III and similarly
534 between the species which matches the results of this study. In addition, the microarray

535 has a detection threshold that is equivalent to the threshold imposed by many monitoring
536 programs. However, further work is needed to make it truly quantitative, especially with
537 other dinoflagellates, such as *Dinophysis* and *Prorocentrum,* for which RNA extraction
538 or RNA quantification can be difficult.

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

547 *5. Conclusions*

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

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

558 between toxic and non-toxic strains. It is clear that signal intensity can be used to
559 quantify cell concentration of one particular species, so this result is very promising for a
560 final universal microarray to detect and quantify this and many other toxic species. But
561 further field testing is needed to fully validate the chip. It suggests that the level of
562 variation would not significantly influence a relationship between RNA content and cell
563 number and allows us to provide quantitative data for more species on the MIDTAL
564 microarray.
565
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573 References

574 Anderson, D.M., Kulis, D.M., Keafer, B.A., Berdalet, E. 1999. Detection of the toxic
575 dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and
576 antibody probes, variability in labelling intensity with physiological condition. J.
577 Phycol. 35, 870-883
578 Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor,
579 M. 2012. The globally distributed genus *Alexandrium*, multifaceted roles in marine
580 eco - systems and impacts on human health. Harmful Algae 14, 10−35.

581 Ayers, K., Rhodes, L. L., Tyrrell, J., Gladstone, M. Scholin, C. 2005. International
582 accreditation of sandwich hybridisation assay format DNA probes for micro-algae.
583 New Zea. J. Marine Freshw. Res. 39, 1225–31.
584 Bartram, A. K., Poon, C., & Neufeld, J. D. (2009). Short Technical Reports.
585 Biotechniques, 47, 1019-1022.
586 Balech, E. 1995. The Genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine
587 Station Publication, Sherkin Island, Co. Cork, Ireland.
588 Berdelet, E, Latasa, M, Estrada, M. 1994. Effects of nitrogen and phosphorus starvation
589 on nucleic acid and protein content of *Heterocapsa* sp. J. Plank. Res. 16, 303-316.
590 Bourlat, S. J., Borja, A., Gilbert, J., Taylor, M. I., Davies, N., Weisberg, S. B., ... & Obst,
591 M. (2013). Genomics in marine monitoring: New opportunities for assessing marine
592 health status. Marine pollution bulletin, 74(1), 19-31.
593 Blanco, E.P., Hagström, J., Salomon, P.S., Graneli, E. 2013. Detection of *Heterosigma*
594 *akashiwo* (Hada) using specific RNA probes, Variability of RNA content with
595 environmental conditions. *Harmful Algae*, Harmful Algae 24, 80–88
596 Brown, L., Bresnan, E., Graham, J., Lacaze, J.P., Turrell, E., et al. 2010 Distribution,
597 diversity and toxin composition of the genus *Alexandrium* (Dinophyceae) in Scottish
598 waters. Eur. J. Phycol., 45, 375–393.
599 Cembella, A.D., Bauder, A.G., Lewis, N.I., Quilliam, M.A., 2001. Association of the
600 gonyaulacoid dinoflagellate Alexandrium ostenfeldii with spirolide toxins in size
601 fractionated plankton. J. Plankton Res. 23, 1413–1419#
602 Cembella, A., Krock, B., 2007. Cyclic imine toxins: chemistry, biogeography,
603 biosynthesis and pharmacology. In: Botana, L.M. (Ed.), Seafood and Freshwater

604 Toxins: Pharmacology, Physiology, and Detection. CRC Press, Boca Raton, FL, pp.
605 561–580
606 Clark, R.F., Williams, S.R., Nordt, S.P., Manoguerra, A.S. 1999. A review of selected
607 seafood poisonings. Undersea Hyperb. Med. 26, 175–184.
608 Collins, C., Graham, J., Brown, L., Bresnan, E., Lacaze, J-P.,Turrell, E.A. 2009.
609 Identification and toxicity of *Alexandrium tamarense* (Dinophyceae) in Scottish
610 waters. J. Phycol., 45, 692–703.
611 Cornelius, G., Schroeder-Lorenz, A, Rensing, L. 1985. Circadian-clock control of protein
612 synthesis and degradation in *Gonyaulax polyhedra*. Planta 166, 365-370.
613 DeSantis, T.Z., Brodie, E.L., Moberg, J.P., Zubieta, I.X., Piceno, Y.M., Andersen, G.L.
614 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity
615 than typical clone library when sampling the environment. Micro. Ecol. 53, 371–383.
616 Diercks, S, Metfies, K, Medlin, L.K. 2008. Colorimetric detection of the toxic
617 dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate
618 assay, Harmful Algae, 7, 137-145.
619 Dittami, S.M., Edvardsen, B., 2012a. Culture conditions influence cellular RNA content
620 in ichthyotoxic flagellates of the genus *Pseudochattonella* (Dictyochophyceae) J.
621 Phycol. 48, 1050–1055.
622 Dittami, S.M., Edvardsen, B. 2012b GPR-Analyzer, a simple tool for quantitative
623 analysis of hierarchical multispecies microarrays. Environ. Sci. Pollut. Res. doi,
624 10.1007/s11356-012-1051-5.
625 Dortch, Q., Clayton, J.R., Thoresen, S.S., Ahmed S.I. 1984 Species differences in
626 accumulation of nitrogen pools in phytoplankton. Mar. Biol. 81,237-250.

627 Egge, E., Bittner, L., Andersen, T., Audic, S., de Vargas, C., & Edvardsen, B. (2013).
628 454 Pyrosequencing to Describe Microbial Eukaryotic Community Composition,
629 Diversity and Relative Abundance: A Test for Marine Haptophytes. PloS one, 8(9),
630 e74371.
631 Fauchot, J., Saucier, F.J., Levasseur, M., Roy, S., Zakardjian, B., 2008. Wind-driven
632 river plume dynamics and toxic *Alexandrium tamarense* blooms in the St.
633 Lawrence estuary (Canada): A modeling study. Harmful Algae 7, 214–227
634 Galluzi, L., Cegna, A., Casabianca, S., Penna, A., Sunder, N., Magnnai, M. 2011
635 Development of an oligonucleotide microarray for the detection and monitoring of
636 marine dinoflagellates. J. Microbiol. Meth. 84, 234–242.
637 Garcés, E., Bravo, I., Vila, M., Figueroa, R. I., Masó, M., & Sampedro, N. 2004.
638 Relationship between vegetative cells and cyst production during Alexandrium
639 minutum bloom in Arenys de Mar harbour (NW Mediterranean). Journal of Plankton
640 Research, 26(6), 637-645.
641 Gribble, K.E., Keafer, B.A., Quilliam, M.A., Cembella, A.D., Kulis, D.M., Manahan, A.,
642 Anderson, D.M., 2005. Distribution and toxicity of *Alexandrium ostenfeldii*
643 (Dinophyceae) in the Gulf of Maine, USA. Deep-Sea Res. Pt. II 52, 2745–2763.
644 Grzebyk, D., Bechemin, C., Ward, C.J, Verite, C., Codd, G.A., Maestrini, S.Y. 2003.
645 Effects of salinity and two coastal waters on the growth and toxin content of the
646 dinoflagellate *Alexandrium minutum*. J. Plankton Res. 25, 1185–1199.
647 Guillou, L., Nézan, E., Cueff, V., Erard, L., Denn, E., Cambon-Bonavita, M.A., Gentien,
648 P., Barbier, G. 2002. Genetic diversity and molecular detection of three toxic

649 dinoflagellate genera (*Alexandrium*, *Dinophysis*, and *Karenia*) from French
650 coasts. Protist 153, 223-38.
651 Guillard, R.R.L., Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella*
652 *nana* Hustedt and *Detonula confervacea* Cleve. Can. J. Microbiol. 8, 229-239.
653 Higman, W.A., Stone, D.M., Lewis, J.M. 2001 Sequence comparison of toxic and non-
654 toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. Phycologia 40,
655 256–262
656 Hoagland, P., Anderson, D.M., Kaoru, Y., White, A.W. 2002. The economic effects of
657 harmful algal blooms in the United States, estimates, assessment issues, and
658 informational needs. Estuaries, 25, 819-837.
659 Humbert, J. F., Quiblier, C. Gugger, M. 2010. Molecular approaches for monitoring
660 potentially toxic marine and freshwater phytoplankton species. Anal. Bioanalyt.
661 Chem. 397, 1723–32.
662 Jensen, M., Moestrup Ø. 1997 Autoecology of the toxic dinoflagellate *Alexandrium*
663 *ostenfeldii*, life history and growth at different temperature and salinities Eur. J.
664 Phycol., 32, 9–18.
665 John, U., Cembella, A.D., Hummert. C., Elbrachter, M., Groben, R., Medlin, L. 2003.
666 Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A.*
667 *ostenfeldii* in co- occurring natural populations from Scottish coastal waters. Eur. J.
668 Phycol. 38, 25–40.
669 Karydis, M., Kitsiou, D. 2012. Eutrophication and environmental policy in the
670 Mediterranean Sea, a review Environ. Monit. Assess 184, 4931–4984.

671 Kegel, J.U. Del Amo, Y., Medlin, L.K. 2012. Introduction to project MIDTAL, its
672 methods and samples from Arcachon Bay, France. Env. Sci. Pollut. Res. DOI
673 10.1007/s11356-012-1299-9.
674 Kegel, J.U., Del Amo, Y., Costes, L., Medlin, L,K. 2013. Monitoring toxic microalgae in
675 Arcachon Bay in France by microarrays. Microarrays 2, 1-23.
676 Leonardos N., Geider R.J. 2004 Responses of elemental and biochemical composition of
677 *Chaetoceros muelleri* to growth under varying light and nitrate: phosphate supply
678 ratios and their influence on critical N:P. Limnol. Oceanogr. 49, 2105–2114.
679 Lewis, J., Medlin, L.K., Raine, R. 2012. MIDTAL (Microarrays for the Detection of
680 Toxic Algae), A Protocol for a Successful Microarray Hybridisation and Analysis.
681 Koeltz Publishing.
682 Lilly, E.L., Halanych, K.M., Anderson, D.M., 2005. Phylogeny biogeography and
683 species boundaries within the *Alexandrium minutum* group. Harmful Algae 4,
684 1004–1020.
685 Lilly E., Halanych K.M., Anderson, D.M. 2007. Species boundaries and global
686 biogeography of the *Alexandrium tamarense* complex (Dinophyceae). J. Phycol. 43,
687 1329–1338.
688 Lim, P.T., Ogata, T., 2005. Salinity effect on growth and toxin production of four tropical
689 Alexandrium species (Dinophyceae). Toxicon 45, 699–710.
690 McCoy, G. R., Touzet, N., Fleming, G. T., & Raine, R. (2013). An evaluation of the
691 applicability of microarrays for monitoring toxic algae in Irish coastal
692 waters.*Environmental Science and Pollution Research*, *20*(10), 6751-6764.
693 McCoy, G. R., Kegel, J. U., Touzet, N., Fleming, G. T., Medlin, L. K., & Raine, R.
694 (2014a). An assessment of RNA content in *Prymnesium parvum*, *Prymnesium*

695 *polylepis*, cf. *Chattonella* sp. and *Karlodinium veneficum* under varying environmental
696 conditions for calibrating an RNA microarray for species detection. FEMS
697 Microbiology Ecology. doi: 10.1111/1574-6941.12277
698 McCoy, G. R., McNamee, S., Campbell, K., Elliott, C. T., Fleming, G. T., & Raine, R.
699 (2014b). Monitoring a toxic bloom of< i> Alexandrium minutum</i> using novel
700 microarray and multiplex surface plasmon resonance biosensor technology. Harmful
701 Algae, 32, 40-48.
702 Metfies, K., Hujic, S., Lange, M., Medlin, L.K. (2005) Electrochemical detection of the
703 toxic dinoflagellate *A. ostenfeldii* with a DNA Biosensor. Bio. Bioelectr. 20, 1349-
704 1357.
705 Metfies, K., Berzano, M., Mayer, C., Roosken, P., Gualerzi, C., Medlin L., Muyzer, G.
706 2007. An optimised protocol for the identification of diatoms, flagellated algae and
707 pathogenic protozoa with phylochips. Mol. Ecol. Notes, 7, 925-936.
708 Miller, P.E., Scholin C.A. 1998. Identification and enumeration of cultured and wild
709 *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted
710 fluorescent probes and filter-based whole cell hybridization. J. Phycol. 34, 371-382.
711 Murray, S.A., Stuken, A., Neilan, B.A. 2011SxtA-based quantitative molecular assay to
712 identify saxitoxin-producing harmful algal blooms in marine waters. Appl. Environ.
713 Micro.. 77, 7050-7057.
714 Murray, S. A., Wiese, M., Neilan, B. A., Orr, R. J., de Salas, M., Brett, S., & Hallegraeff,
715 G. (2012). A reinvestigation of saxitoxin production and *sxtA* in the 'non-toxic'
716 *Alexandrium tamarense* Group V clade. Harmful Algae, 18, 96-104.

717 Niyogi K. 1999. Photoprotection revisited: genetic and molecular approaches. Annu.
718 Rev. Plant Physiol. Plant Mol. Biol. 50, 333–59.
719 Ribeiro, S., Amorim, A., Thorbjørn J., Andersen, D.M., Abrantes F., Ellegaard, M. 2012.
720 Reconstructing the history of an invasion, the toxic phytoplankton species
721 *Gymnodinium catenatum* in the Northeast Atlantic. Biol. Invasions 14, 969–985.
722 Smaal, A.C. 2002. European mussel cultivation along the Atlantic coast, production
723 status, problems and perspectives. Hydrobiologia 484,89–98
724 Pitcher, G. C., Cembella, A. D., Joyce, L. B., Larsen, J., Probyn, T. A., & Ruiz Sebastián,
725 C. 2007. The dinoflagellate *Alexandrium minutum* in Cape Town harbour (South
726 Africa): Bloom characteristics, phylogenetic analysis and toxin composition. Harmful
727 Algae, 6 , 823-836.
728 Steidinger, K.A. 2010. Research on the life cycles of harmful algae, a commentary.
729 Deep-Sea Research II, 57, 162–165.
730 Stuken, A., Orr, R.J.S., Kellmann, R., Murray, S.A., Neilan, B.A., Jakobsen, K.S. 2011.
731 Discovery of nuclear-encoded genes for the neurotoxin saxitoxin in dinoflagellates.
732 PLoS One. 6(5), e20096.
733 Taroncher-Oldenburg, G., Kulis, D.M., Anderson D.M. 1999. Coupling of saxitoxin
734 biosynthesis to the G1 phase of the cell cycle in the dinoflagellate *Alexandrium*
735 *fundyense*, Temperature and nutrient effects. Nat. Toxins 7, 207–219.
736 Taylor, J.D., Berzano, M., Percy, L., Lewis, J.M. 2013. Evaluation of the MIDTAL
737 microarray chip for monitoring toxic microalgae in the Orkney Islands, U.K. Environ.
738 Sci. Pollut. R DOI, 10.1007/s11356-012-1393-z.

739 Touzet, N., Davidson, K, Pete, R. Flanagan, K, McCoy, G.R. Amzil, Z Maher, M
740 Chapelle, A., Raine, R. 2010. Co-Occurrence of the West European (Gr.III) and
741 North American (Gr.I) Ribotypes of *Alexandrium tamarense* (Dinophyceae) in
742 Shetland, Scotland Protist, 161, 370–384.
743 Van Dolah, F.M. 2000. Marine algal toxins, origins, health effects, and their increased
744 occurrence. Environ. Health Perspect.108, 133–41.
745 Wallen, D.G, Geen G.H. 1971 Light quality and concentration of proteins, RNA, DNA
746 and photosynthetic pigments in two species of marine plankton algae. Marine Biology
747 10, 44-51.
748
749 Walz, B., Walz A., Sweeney, B. M. 1983. A circadian rhythm in RNA in the
750 dinoflagellate *Gonyaulax polyedra*. J. Comp. Physiol. B. 151, 207-213.
751 Watras, C.J., Chisholm, S.W., Anderson, D.M. 1982. Regulation of growth in an
752 estuarine clone of *Gonyaulax tamarensis* Lebour, salinity-dependent temperature
753 responses . J. Exp. Mar. Biol. Ecol. 62, 25-37.
754 Wolfgang, L., Strunk, O., Westram, R., Richter, L. Meier, H., Yadhukumar, K., Buchner,
755 A., Lai, T., Susanne Steppi, S. Jobb, G., Förster, W., Brettske, I., Gerber, S., Anton W.
756 Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, S., König, A., Liss, T.,
757 Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A.,
758 Wollschläger, J., Nicolaus, A., Wiltshire, K. H., & Metfies, K. (2014). Assessment of
759 North Sea phytoplankton via molecular sensing: a method evaluation. Journal of
760 Plankton Research, fbu003.

761 Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Arndt Bode, A., and Schleifer, K.H.
762 2004. ARB, a software environment for sequence data. Nuc. Acids Res., 32, 1363-
763 1371

Table 1. Summary of strains used in the study, counting methodology employed and experimental conditions tested

| Species | Strain Name | Isolation Location | Cells counted | Salinity range (psu) | Temperature Range (°C) | Light Intensity Range (μmol photons $m^{-2}$ $s^{-1}$) |
|---|---|---|---|---|---|---|
| *Alexandrium minutum* | AMAD06 | Port River, Australia | Coulter Counter | Not tested[1] | 15-30-40 | 15-100-200 |
| | AL3T | Ria de Vigo, Spain | Coulter Counter | Not tested | 15-30-40 | 15-100-200 |
| | AMIA5 | Syracuse, Ionian Sea, Sicily, Italy | Coulter Counter | Not tested | 15-30-40 | 15-100-200 |
| *Alexandrium ostenfeldii* | AONOR4 | 01. 2010, inner Oslofjord, Norway | Flow Cytometer | 17-34-42 | 10-15-18 | 25-100-200 |
| | NCH85 | North Sea | Flow Cytometer | 17-34-42 | 10-15-18 | 25-100-200 |
| *Alexandrium tamarense* North American Clade Group I | UoW 717 | Water column, Stromness pier, Orkney Islands, U.K | Sedgewick rafter | 28-33-38 | 12-16-20 | 26-160-430 |
| | UoW 718 | Water column, Stromness pier, Orkney Islands, U.K | Sedgewick rafter | 28-33-38 | 12-16-20 | 26-160-430 |
| | UoW 719 | Water column, Stromness Orkney Islands, U.K | Sedgewick rafter | 28-33-38 | 12-16-20 | 26-160-430 |
| *Alexandrium tamarense* Western European Clade Group III | UoW 700 | Sediments, Weymouth Harbour, U.K | Sedgewick rafter | 28-33-38 | 12-16-20 | 26-160-430 |
| | UoW 702 | Sediments, Weymouth Harbour, U.K | Sedgewick rafter | 28-33-38 | 12-16-20 | 26-160-430 |
| | VG0927 | Carnota Beach, NW Spain (Atlantic) | Sedgewick rafter | 28-33-38 | 12-16-20 | 26-160-430 |
| *Alexandrium tamarense (*catenella *morphotype)* Temperate Asian Group IV | VGO 598 | Tarragona harbour (Mediterranean Sea) | Not counted | Not tested | Not tested | Not tested |

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.

| **Probe name** | **Targeted species** | **Gene** | **Source/Designer** |
|---|---|---|---|
| AlexGD01_25_dT | Genus *Alexandrium* | 28S | Kegel et al., 2012 |
| AminuS01_25_dT | *Alexandrium minutum* | 18S | Miller and Scholin, 1998 |
| AostS02_25_dT | *Alexandrium ostenfeldii* | 18S | John et al., 2003 |
| AostD01_25_dT | *Alexandrium ostenfeldii* | 28S | John et al., 2003 |
| AtamaS01_25_dT | *Alexandrium* species complex | 18S | John et al.,, 2003 |
| ATNA_D01_25_dT | *Alexandrium tamarense* (North America) | 28S | John et al., 2003 |
| ATNA_D02_25_dT | *Alexandrium tamarense* (North America) | 28S | Guillou et al., 2002 |
| ATTA_D01_25_dT | *Alexandrium tamarense* (Temperate Asian) | 28S | Kegel et al., 2012 |

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.

| Species | **Mean RNA content (pg cell$^{-1}$)** | **Reference** | **Mean growth rate (d$^{-1}$)** | **Reference** |
|---|---|---|---|---|
| *A. tamarense* Group I | 54.66 ± 3.02 | This study | 0.20± 0.22 | **This study** |
| | 16.6 | Carter et al.,unpublished | 0.30-0.4-0 | Lim & Ogata (2005) |
| *A. tamarense* Group III | 40.93 ± 2.74 | **This study** | 0.24 ± 0.03 | **This study** |
| *A. ostenfeldii* | 42.86 ± 3.13 | **This study** | 0.41± 0.05 | **This study** |
| | 20 | Metfies et al., (2005) | 0.30 | Jensen & Moestrup (1997) |
| *A. minutum* | 3.86 ± 0.29 | **This study** | 0.12 ± 0.015 | **This study** |
| | 28.00 ± 0.30 | Diercks et. al (2008) | 0.5 | Grzebyk et al., (2003) |
| *A. fundeyese* (Group I) | 20-60 | Anderson et al., (1999) | 0.031-0.227 | Taroncher-Oldenburg et al., (1999) |
| *Gonyaulax polyedra* | 100 | Walz et al., (1983) | | |

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

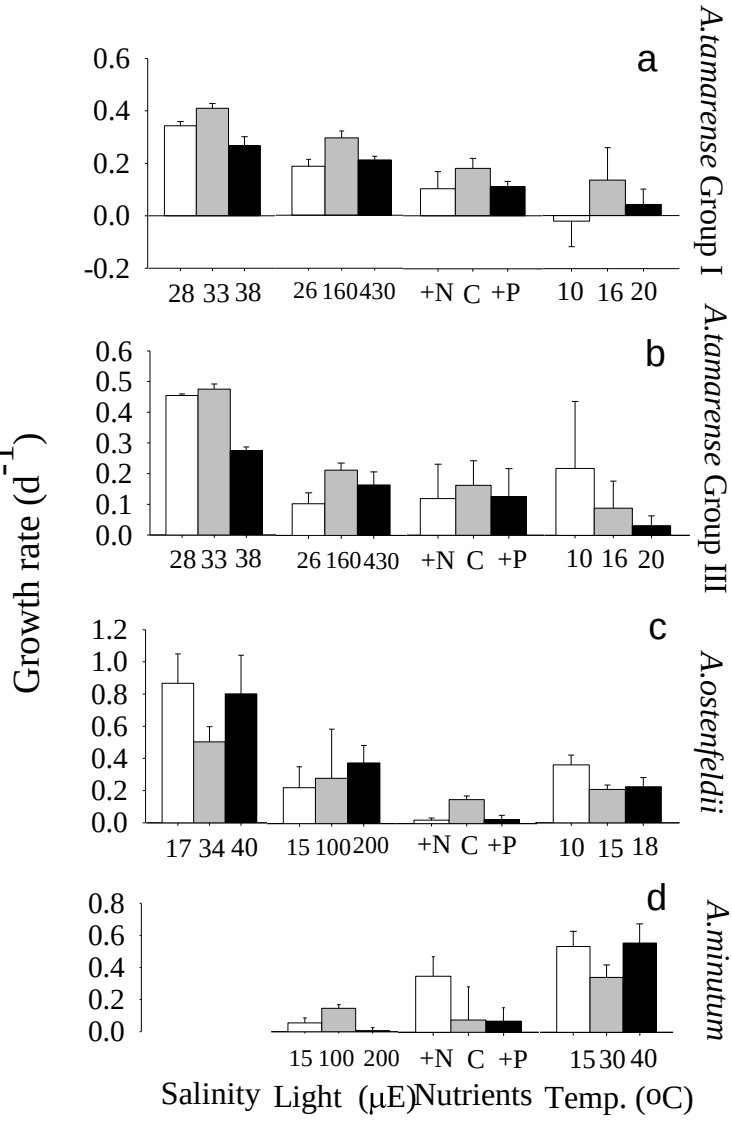
Group III *Alexandrium* genus probe (AlexGD01_25_dT), *Alexandrium tamarense* complex probe (AtamaS01_25) and Group I ribotype specific probes (ATNA_D01_25_dT, ATNA_D02_25_dT) showing normalisation to C) POSITIVE_25_dT and D) the *Dunaliella* specific probe DunGS02_dT. Hybridisation was done on generation 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
b
c
d
A.tamarense Group I
A.tamarense Group III
A.ostenfeldii
A.minutum
Growth rate (d-1)
0.6
0.4
0.2
0.0
-0.2
0.5
0.3
0.1
1.2
1.0
0.8
28 33 38
26 160430
+N C +P
10 16 20
17 34 40
15 100200
10 15 18
15 100 200
15 30 40
Salinity
Light (μE)
Nutrients
Temp. (oC)
Treatment

# Figure 2

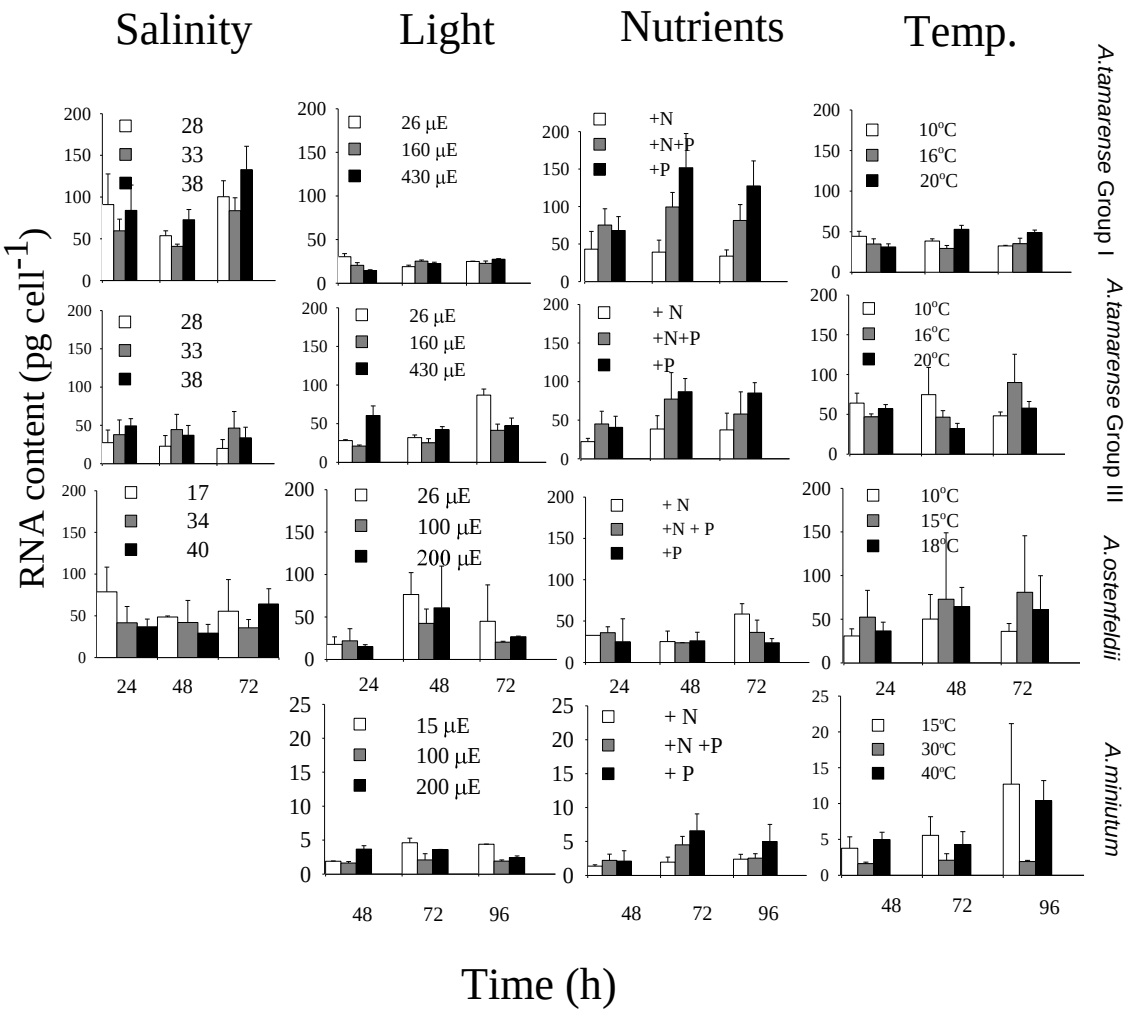

Salinity
Light
Nutrients
Temp.
A.tamarense Group I
A.tamarense Group III
A.ostenfeldii
A.miniutum
RNA content (pg cell-1)
Time (h)
28
33
38
17
34
40
26 µE
160 µE
430 µE
26 µE
100 µE
200 µE
15 µE
100 µE
200 µE
+N
+N+P
+P
+ N
+N+P
+P
+ N
+N + P
+P
+ N
+N +P
+ P
10°C
16°C
20°C
10°C
16°C
20°C
10°C
15°C
18°C
15°C
30°C
40°C
24
48
72
48
72
96

Figure 3

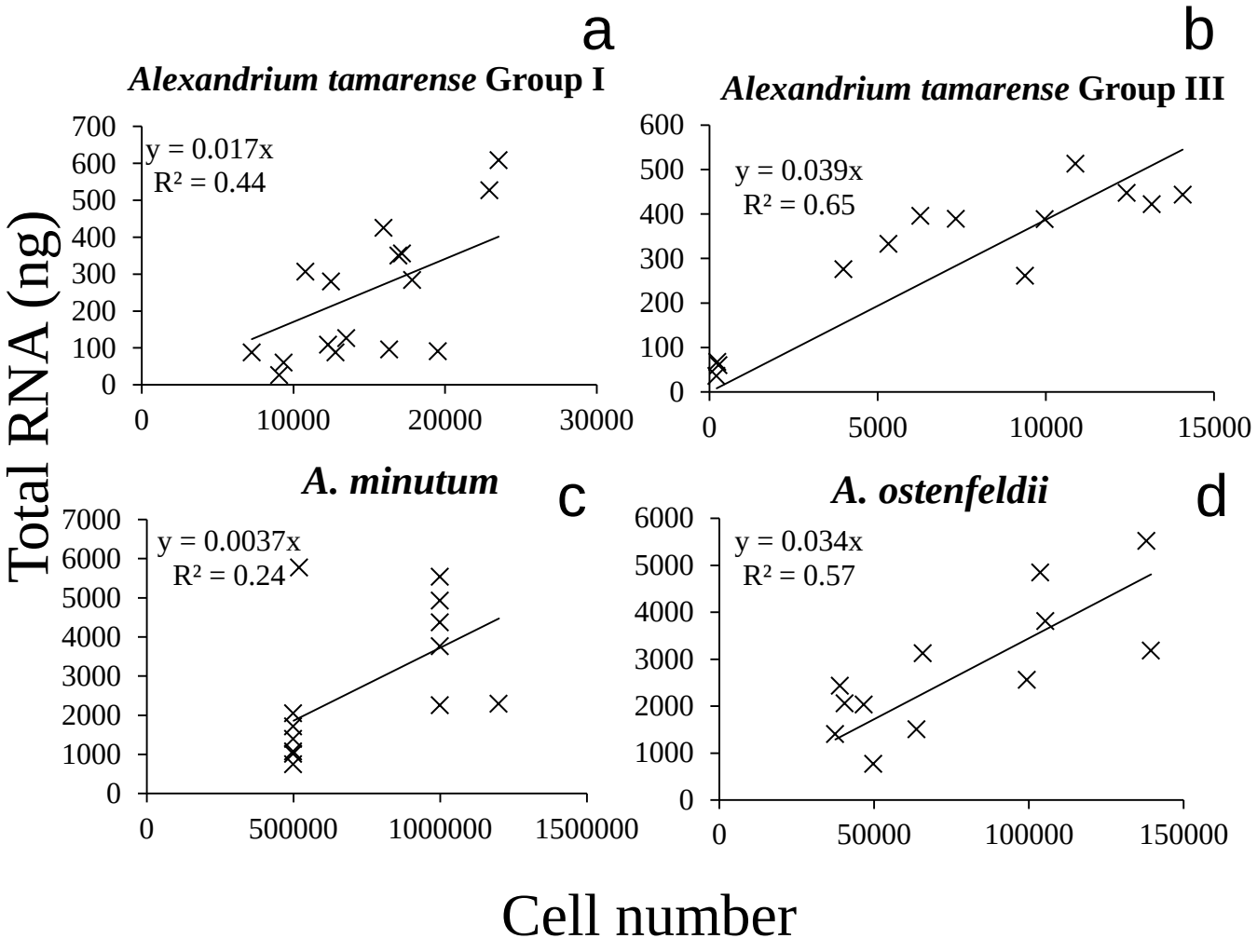
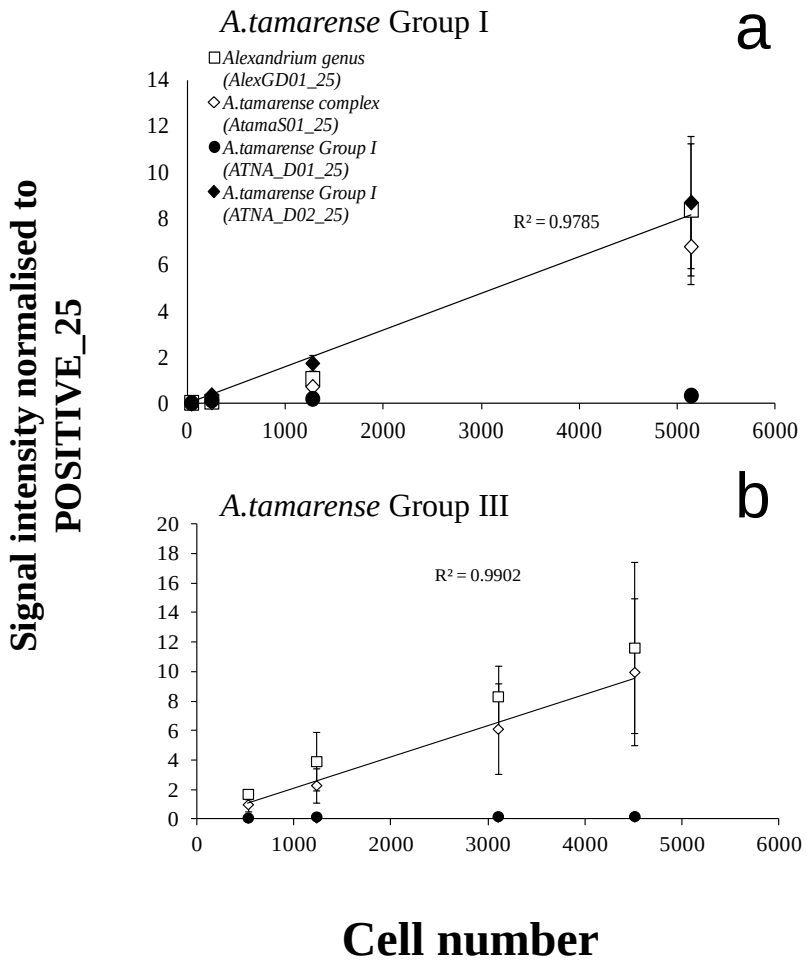
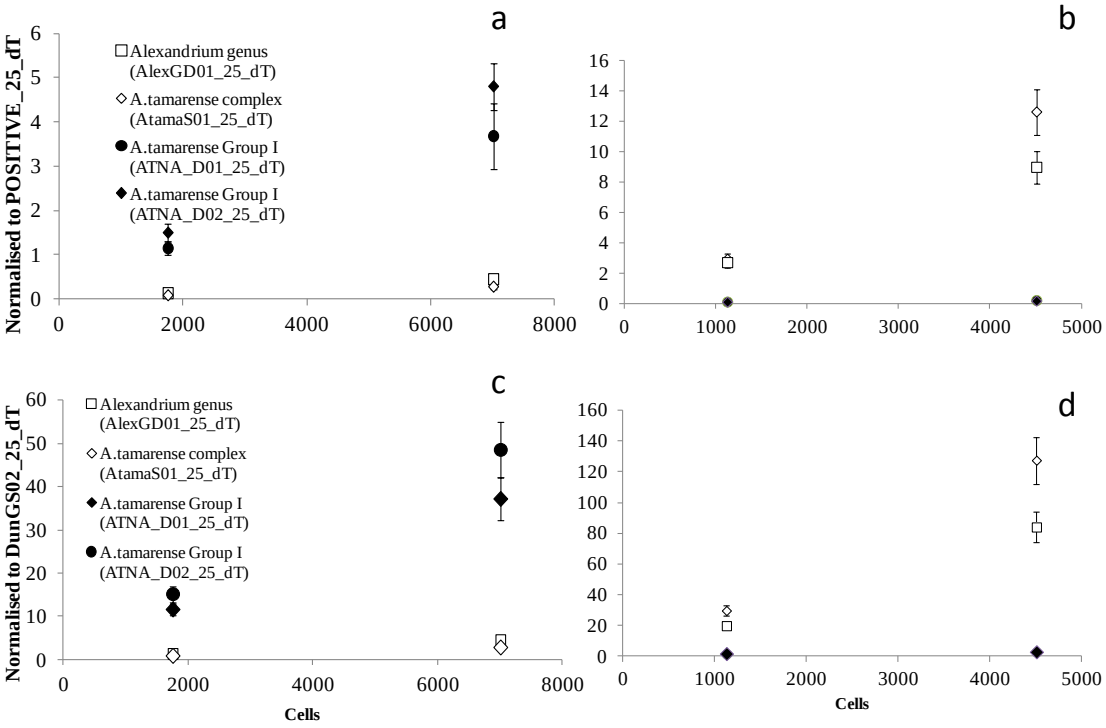

a
Alexandrium tamarense Group I
y = 0.017x
R² = 0.44
b
Alexandrium tamarense Group III
y = 0.039x
R² = 0.65
A. minutum
c
y = 0.0037x
R² = 0.24
A. ostenfeldii
d
y = 0.034x
R² = 0.57
Total RNA (ng)
Cell number

Figure 4


A.tamarense Group I
a
Alexandrium genus (AlexGD01_25)
A.tamarense complex (AtamaS01_25)
A.tamarense Group I (ATNA_D01_25)
A.tamarense Group I (ATNA_D02_25)
R² = 0.9785
A.tamarense Group III
b
R² = 0.9902
Signal intensity normalised to POSITIVE_25
Cell number

# Figure 5




*A.tamarense* Group I

*A.tamarense* Group III

Figure 6

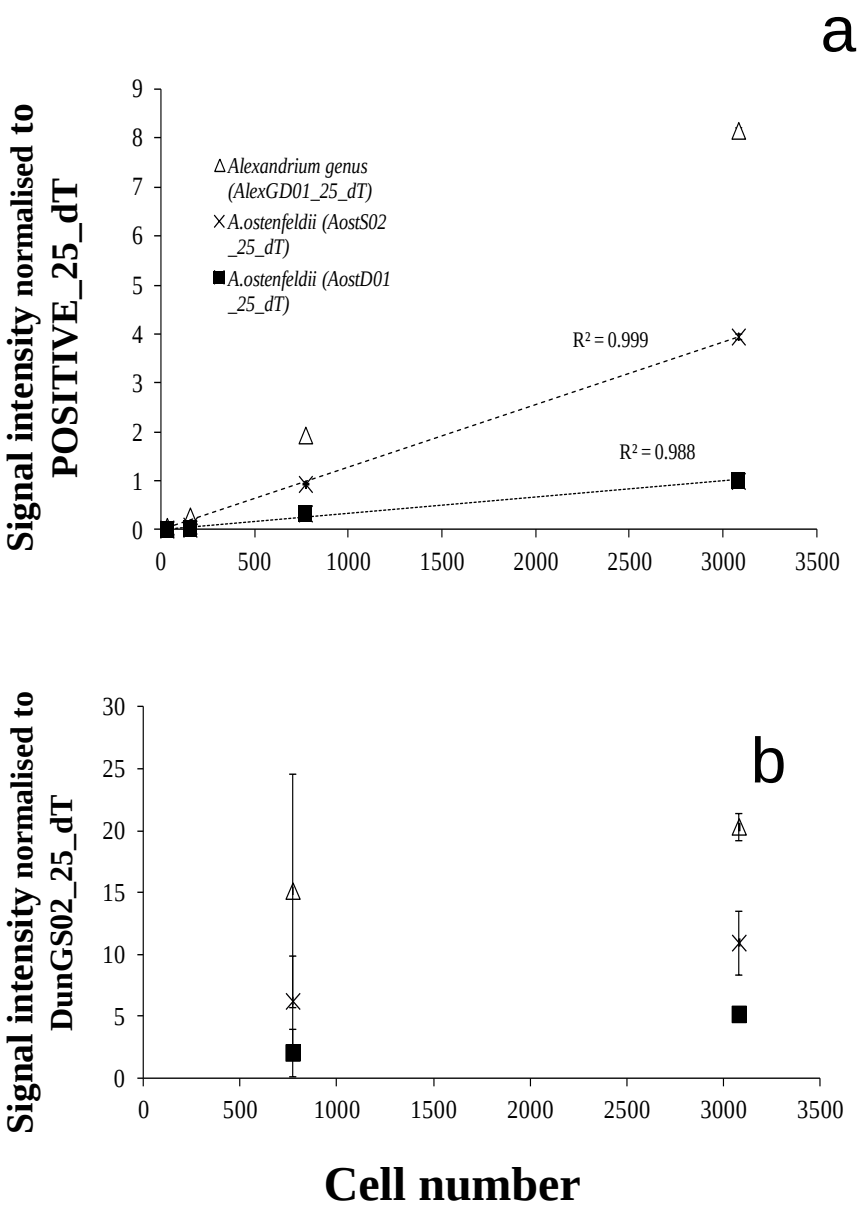
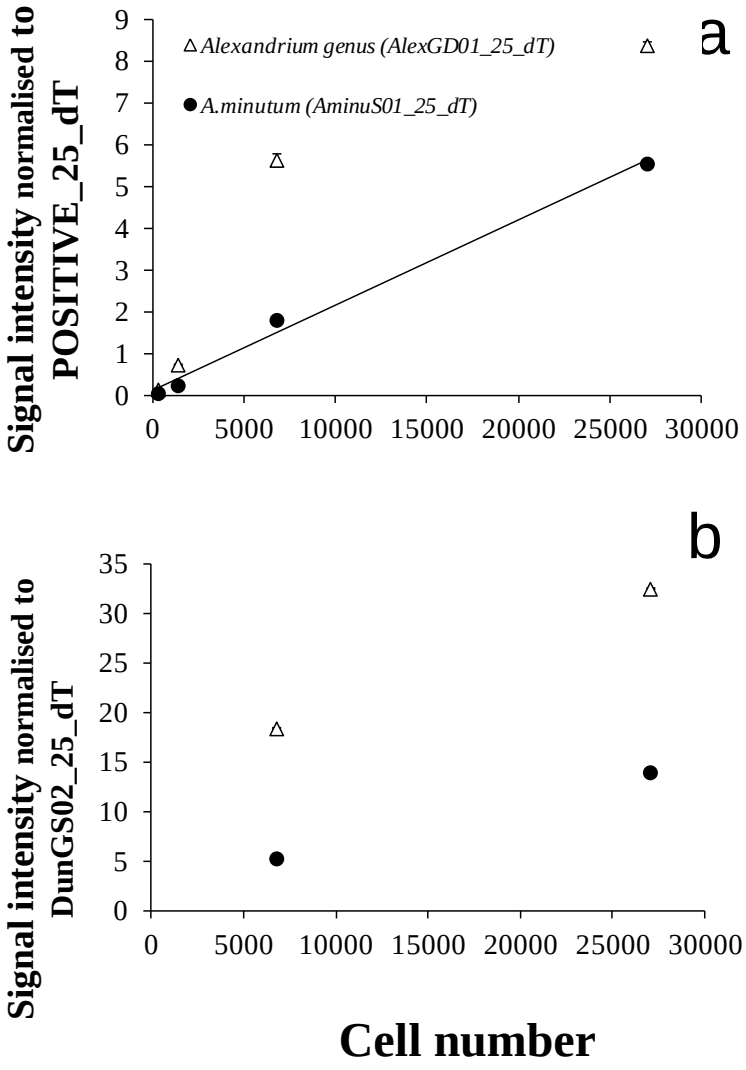
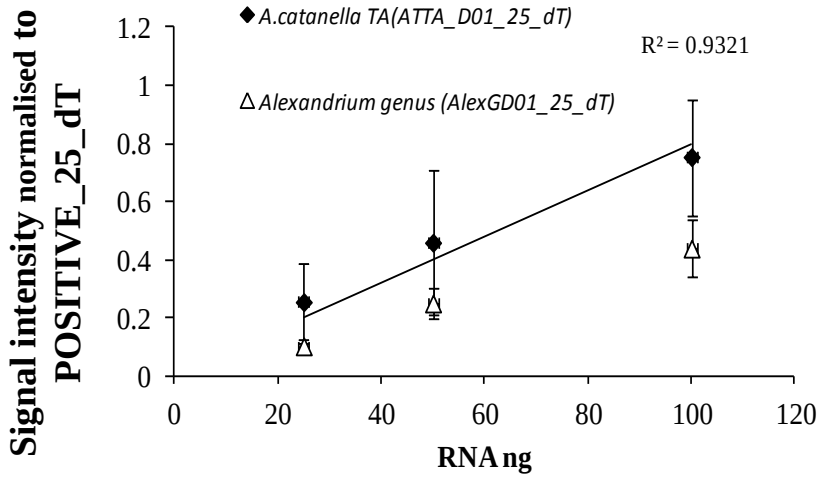

a
Signal intensity normalised to POSITIVE_25_dT
Alexandrium genus (AlexGD01_25_dT)
A.ostenfeldii (AostS02 _25_dT)
A.ostenfeldii (AostD01 _25_dT)
R² = 0.999
R² = 0.988
b
Signal intensity normalised to DunGS02_25_dT
Cell number

Figure 7


a
Alexandrium genus (AlexGD01_25_dT)
A.minutum (AminuS01_25_dT)
Signal intensity normalised to POSITIVE_25_dT
0
1
2
3
4
5
6
7
8
9
0
5000
10000
15000
20000
25000
30000
b
Signal intensity normalised to DunGS02_25_dT
0
5
10
15
20
25
30
35
0
5000
10000
15000
20000
25000
30000
Cell number

# Figure 8


A.catanella TA(ATTA_D01_25_dT)
R² = 0.9321
Alexandrium genus (AlexGD01_25_dT)
Signal intensity normalised to POSITIVE_25_dT
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
RNA ng

# Supplementary Figure 1