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

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32 *Abstract*

33 The dinoflagellate genus *Alexandrium* contains several toxin producing species and  
34 strains, which can cause major economic losses to the shell fish industry. It is therefore  
35 important to be able to detect these toxin producers and also distinguish toxic strains from  
36 some of the morphologically identical non-toxic strains. To facilitate this DNA probes to  
37 be used in a microarray format were designed *in silico* or developed from existing  
38 published probes. These probes targeted either the 18S or 28S ribosomal ribonucleic acid  
39 (rRNA) gene in *Alexandrium tamarense* Group I, Group III and Group IV, *A. ostenfeldii*  
40 and *A. minutum*. Three strains of *A. tamarense* Group I (UoW 717, UoW 718, UoW 719),  
41 *A. tamarense* Group III (UoW 700, UoW 702, VGO927), *A. minutum* (AMAD06, AL3T,  
42 AMIA5) and two strains of *A. ostenfeldii* (AONOR4, NCH85) were grown at optimal  
43 conditions and transferred into new environmental conditions changing either the light  
44 intensity, salinity, temperature or nutrient concentrations, to check if any of these  
45 environmental conditions induced changes in the cellular ribonucleic acid (RNA)  
46 concentration or growth rate. The aim of this experiment was the calibration of several  
47 species-specific probes for the quantification of the toxic *Alexandrium* strains. Growth  
48 rates were highly variable but only elevated or lowered salinity significantly lowered  
49 growth rate for *A. tamarense* Group I and Group III, differences in RNA content were  
50 not significant ( $p < 0.05$ ) for the majority of the treatments. Only light intensity seemed to  
51 significantly affect the RNA content in *A. tamarense* Group I and Group III, but this was  
52 still within the same range as for the other treatments meaning that a back calibration  
53 from RNA to cell numbers is possible. The designed probes allow the production of  
54 quantitative information for *Alexandrium* species for the microarray chip.

55 *Keywords:*

56     Microarray; Phylochip; rRNA probes; Toxic algae; Alexandrium; MIDTAL; RNA

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78 *Abbreviations*

79 BSA= Bovine serum albumen

80 FISH =Fluorescence in-situ hybridisation

81 DNA =Deoxyribonucleic acid

82 MIDTAL= Microarrays for the detection of toxic algae

83 RNA =Ribonucleic acid

84 rRNA =Ribosomal ribonucleic acid

85 STT =Sodium chloride-Tris-Triton

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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 can 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 distribution (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.



170 Previous culture based studies have shown that *Alexandrium spp* tolerate a wide range of  
171 environmental conditions and in some cases growth rate may be affected by changes in  
172 light, temperature (Hwang & Lu 2000; Grzebyk et al 2003, Jensen & Moestrup 1997;  
173 Hansen et al. 2004) and salinity (Lim & Ogata 2005). Although no previous studies have  
174 looked at RNA content under varying environmental conditions.

175 The MIDTAL (Microarrays for the Detection of Toxic ALgae) project has developed  
176 a microarray using rRNA based detection based on 136 probes at various taxonomic  
177 hierarchies to determine all major species of harmful algae from north western Europe.  
178 This technology can be used in monitoring harmful algae and unlike many previous  
179 molecular techniques would be used to quantify the numbers of cells present not just  
180 detect their presence/absence. Thus, for quantification, it is necessary to understand the  
181 variability of the rRNA pool within cells. The aim of this study was to investigate the  
182 variation of RNA yield per cell within *Alexandrium* species and strain in response to  
183 environmentally relevant conditions to allow calibration of the microarray chip to cell  
184 counts. In order to address this we assessed the relationship between RNA and cell  
185 numbers for each between species or strain. Signal intensity of species specific probes  
186 against amount of RNA hybridized to the chip the probes on the microarray was then  
187 investigated. This was done to investigate the efficiency of back calibration from signal  
188 on the microarray to cell number of a particular *Alexandrium* species or strain and that  
189 there were no cross relativity between the probes for each species. Based on previous  
190 complementary studies using the MIDTAL chip Dittami & Edvardsen (2012a) for  
191 *Pseudochatonella* and Blanco et al. (2013) for *Heterosigma akashiwo Prynensium*,  
192 *Karlodinium veneficum* and cf. *Chatonella sp.* (McCoy et al. 2014a) and unpublished

193 work for four species of *Pseudo-nitzschia* (Medlin et al.) we hypothesised there would be  
194 a positive linear relationship between target RNA amount and target specific probe signal  
195 on the chip and a positive correlation between cell numbers and total RNA.

196

## 197 2. Material and methods

### 198 2.1. Algal strains

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

208

### 209 2.2 Experimental design

210 A stock culture of each strain was grown under the control conditions above, with  
211 fresh media added regularly to maintain exponential growth. Experiments were done in  
212 triplicate. Four different treatments (salinity, light intensity, temperature, and nutrient  
213 depletion) were tested in parallel, changing one parameter per set of cultures (Table 1) as  
214 described by Dittami & Edvardsen (2012a). Briefly, the three strains of each species were  
215 inoculated separately in 200 mL tissue culture flasks with vented caps or 250-500 mL

216 bottles. Initial volume in each flask was between 150 and 300 mL, with 20 mL or one-  
217 third of initial strain 1, 2, 3 cultures, respectively, and f/2 modified according to the  
218 conditions applied making up the rest of the volume.

219 This stock culture was then split into 3 replicates of 40 mL for each individual  
220 treatment. For salinity stress, the strains were inoculated in flasks containing f/2 at lower  
221 and higher salinity than the control conditions without accommodation in order to test  
222 immediate stress response, and this varied with each species (Table 1). Low light  
223 intensity was 15-25  $\mu\text{E}$  and high light intensity varied with each species (Table 1).  
224 Temperatures were set at 10-15°C for low temperature and the higher one also varied  
225 with each species (Table 1). Higher temperatures and light intensities were selected based  
226 on both equipment available to carry out the experiments but also aimed use levels at the  
227 maximum tolerance ranges of each of the *Alexandrium* species. Nutrient depletion was  
228 carried out by using modified f/2 medium without either phosphate or nitrate. It should be  
229 noted that the nutrient depleted treatments did contain some N or P at the beginning of  
230 each experiment because a 10% to one-third (by volume) of culture with f/2 medium was  
231 used for inoculation.

232 All sets of conditions were run at the same time so as to use inocula from the same  
233 starting cultures. The day of cell inoculation was considered as time zero (T0).  
234 Subsamples (13 mL) of the cultures for cell counts, and RNA extraction at each different  
235 condition were taken at the same time daily after 24 hours of inoculation, after 48 hours ,  
236 and after 72 hours with 10 mL being used for RNA extraction and 3 mL for cell counts.  
237 For *A. minutum* 10 mL was taken from each flask and mixed in sets of three, to have 3  
238 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  $\mu\text{m}$  nitrocellulose or polycarbonate filters (Whatman, U.K), transferred into cryogenic  
241 vials containing acid washed glass beads (213-300  $\mu\text{m}$ ), shock-frozen in liquid nitrogen,  
242 and stored at  $-80^{\circ}\text{C}$  until further processing.

### 243 2.3 Cell counts

244 Cells for counts (3 mL) for both *A. tamarensis* 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. ostentfeldii*  
248 were kept at  $4^{\circ}\text{C}$  after adding 20  $\mu\text{L}$  of 25 % glutaraldehyde to 500  $\mu\text{L}$  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:

$$252 K' = \text{Ln} (N2 / N1) / (t2 - t1)$$

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  $\text{NH}_4\text{Ac}$  and 2 volumes of ice-cold ethanol (absolute, stored at  $-20^{\circ}\text{C}$ ). Because of low

262 amounts of RNA, triplicates of each time point of *A. ostenfeldii* were mixed before  
263 NH<sub>4</sub>Ac precipitation. The RNA was re-suspended in 20 or 50 µL nuclease-free water and  
264 its concentration and integrity was measured by NanoVue spectrophotometer (GE  
265 Healthcare) or Nanodrop (Thermo-scientific, U.K) and Agilent Bioanalyzer 2100  
266 (Agilent Biotechnologies). Samples were shock-frozen in liquid nitrogen and stored at -  
267 80°C until further use.

#### 268 *2.57 Microarray calibration*

269 Sequences of *Alexandrium spp* were analysed *in silico* using ARB (Wolfgang et al.,  
270 2004) to design specific probes in those instances where published FISH probes were not  
271 available. Probes originally designed for FISH format for species and or for higher  
272 taxonomic levels (Table 2) and for the microarray designed by Gescher et al. (2008) were  
273 lengthened to 25 nts in length and for MIDTAL array generation 3 (Kegel et al., 2013), a  
274 15 dT- tail was added according to Metfies et al. (2007). The probe sequence for all  
275 probes designed or modified from FISH probes for the entire project for the MIDTAL  
276 microarray are patent pending as a universal microarray for the detection of toxic algae,  
277 and the entire hybridisation kit including the array and all necessary reagents are now  
278 commercially available from Kreatech (UK). Prior to labelling, the different strains of  
279 each species were mixed in equal amounts. In the case of *A. ostenfeldii*, RNA of strain  
280 AOF0940 was added in an equal amount to the other two strains. RNA was labelled using  
281 the PlatinumBright Infrared Labelling Kit from KREATECH and purified with  
282 KREApure columns according to the manufacturer's instructions. Concentration and  
283 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  $\mu\text{E}$ ) treatment than both the controls (160  $\mu\text{E}$ )  
358 and the high light treatment (430  $\mu\text{E}$ ). 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  $\text{cell}^{-1}$  under any of the  
369 experimental conditions tested (Figure 2). Overall its RNA content was  $42.68 \pm 3.07$  pg  
370  $\text{cell}^{-1}$  ( $n=72$ ).

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  $\mu\text{E}$ ), but  
374 both the low light (15  $\mu\text{E}$ ), and the high light (200  $\mu\text{E}$ ) 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

376 from 48 to 96 to the high light. After 48 h, the high light treatment was significant higher  
377 than both the control and low light; however at 96 hours the low light was now  
378 significantly higher (Tukey,  $p \leq 0.01$ ).

379

### 380 3.4 Microarray calibration

381 Results from Taylor et al 2013 for calibration curves of signal strength against RNA  
382 amount for generation 2 (Figure 4) and 3.1 (Figure 5) showed probes for *Alexandrium*  
383 *tamarense* Group I (ATNA\_D01\_25, ATNA\_D02\_25), which have a different sequence  
384 and target different regions of the Group I rRNA genes, showed positive linear  
385 relationships ( $R^2=0.9785$ ,  $p < 0.05$ ) for signal against the amount of RNA hybridised to the  
386 chip. In both versions the probe ATNA\_D02\_25\_dT was the stronger of the two strain  
387 specific probes with the highest signal of all the probes. For *Alexandrium tamarense*  
388 Group I showed a strong signal. *Alexandrium* genus level genus probe (AlexG\_D01\_25)  
389 with the former showing a greatly reduced signal (greater than 0.2 but less than 1) when  
390 compared to the latter.

391 As *Alexandrium tamarense* Group III does not have specific probes on the chip, its  
392 calibration was based on the single *A. tamarense* complex probe. Both these curves were  
393 linear  $R^2 = 0.97$  (Fig 4, Taylor et al. 2013). Importantly *A. tamarense* Group III RNA did  
394 not cross-react with any of the Group I *A. tamarense* specific probes. The probes with  
395 the highest signal for Group III *A. tamarense* were the *Alexandrium tamarense* complex  
396 probe (AtamaS01\_25\_dT) and *Alexandrium* genus probe (AlexGD01\_25\_dT). RNA  
397 equivalent to 35 cells of this group did produce a very weak signal (Fig 5b) for the Group  
398 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 40°C, 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 throughout 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.

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

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

482 Diercks et al., (2008) showed that total RNA isolated from three different strains of *A.*  
483 *minutum* at optimum growth conditions and the mean concentration of RNA per cell were  
484 within our range of results. This is comparable to results presented by Metfies et al.,  
485 (2005) for *A. ostenfeldii* with a slightly smaller concentration per cell. *Alexandrium*  
486 *fundyense* (the third morphotype in Group I) showed a wider range of cell concentrations  
487 (Anderson et al., 1999). Interestingly, short term stress that may occur under natural  
488 conditions had little effect on RNA content per cell (for example the salinity response,  
489 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 variability 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<sup>+</sup> 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 prokaryotic 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.tamarensis* 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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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 ( $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ )
<i>Alexandrium minutum</i>	AMAD06	Port River, Australia	Coulter Counter	Not tested <sup>1</sup>	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
<i>Alexandrium ostenfeldii</i>	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
<i>Alexandrium tamarense</i> 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
<i>Alexandrium tamarense</i> 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
<i>Alexandrium tamarense</i> (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.

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

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

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

## Figure Legends

Figure 1. Growth rates ( $d^{-1}$ ) of a) *A. tamarensis* Group I b) *A. tamarensis* Group III c) *A. ostenfeldii* d) *A. minutum*, under varying conditions of Salinity, Light ( $\mu E$ ), Nutrients (+N, Control (C), +P) and Temperature ( $^{\circ}C$ ).

Figure 2. Mean RNA yield for *Alexandrium* species under various culture conditions ( $n = 9$  for *A. tamarensis* Group I,  $n = 9$  for *A. tamarensis* Group III,  $n=6$  for *A. 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. tamarensis* NA, **b** *A. tamarensis* WE, **c** *A. ostenfeldii* and **d** *A. minutum*

Figure 4 **a** Calibration curves for *Alexandrium tamarensis* Group I from Taylor et al (2013), showing the *Alexandrium* genus probe (AlexGD01\_25) *Alexandrium tamarensis* 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 tamarensis* NA Group III *Alexandrium* genus probe (AlexGD01\_25), *Alexandrium tamarensis* species complex probe (AtamaS01\_25) and Group I ribotype specific probes (ATNA\_D01\_25, ATNA\_D02\_25) Hybridization was done on the 2<sup>nd</sup> generation MIDTAL chip. (Taylor et al 2013),

Figure 5. Two point calibration curves for *Alexandrium tamarensis* from Taylor et al (2013), using the 3<sup>rd</sup> generation MIDTAL chip and the addition of 10 ng of *Dunaliella tertiolecta*. Group I showing an *Alexandrium* genus probe (AlexGD01\_25\_dT) , *Alexandrium tamarensis* 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 tamarensis* NA



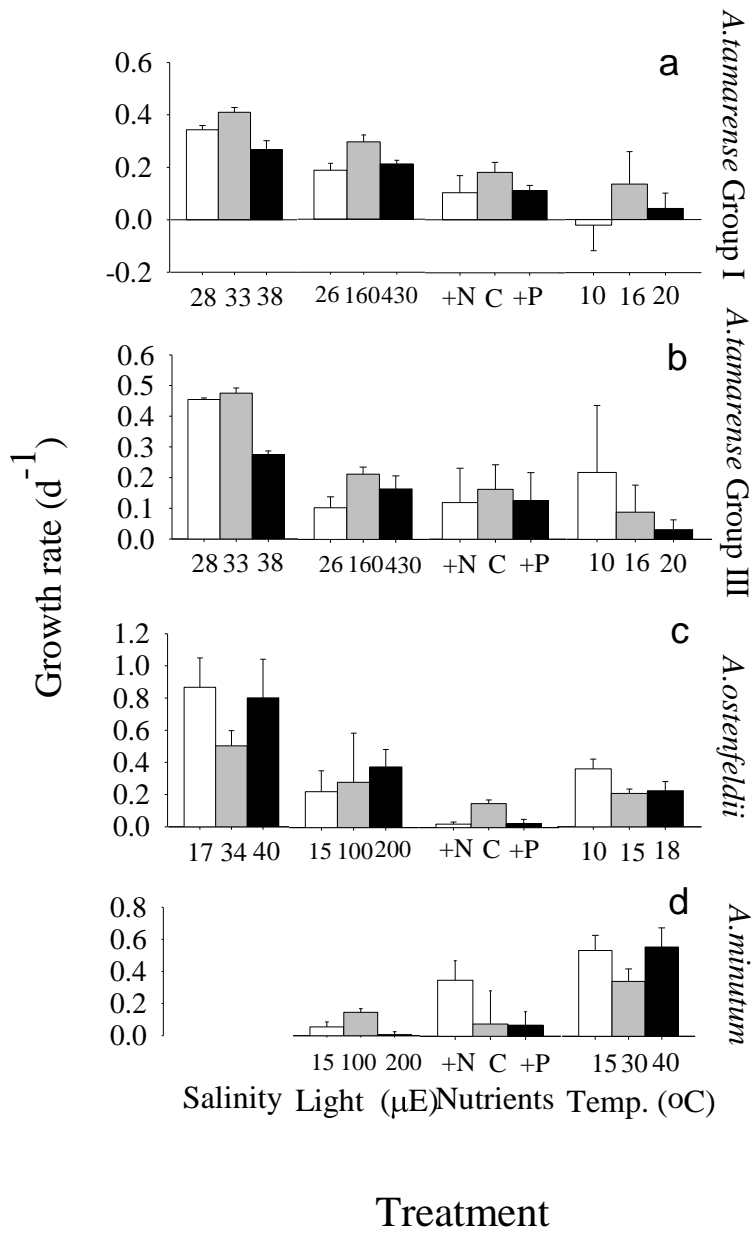
Group III *Alexandrium* genus probe (AlexGD01\_25\_dT), *Alexandrium tamarense* complex probe (AtamaS01\_25) and Group I ribotype specific probes (ATNA\_D01\_25\_dT, ATNA\_D02\_25\_dT) showing normalisation to C) POSITIVE\_25\_dT and D) the *Dunaliella* specific probe DunGS02\_dT. Hybridisation was done 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 3<sup>rd</sup> generation MIDTAL chip.

Figure 7. Cell number against microarray signal for probes specific for *A. minutum*. Hybridization was done on the 3<sup>rd</sup> 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



# Figure 2

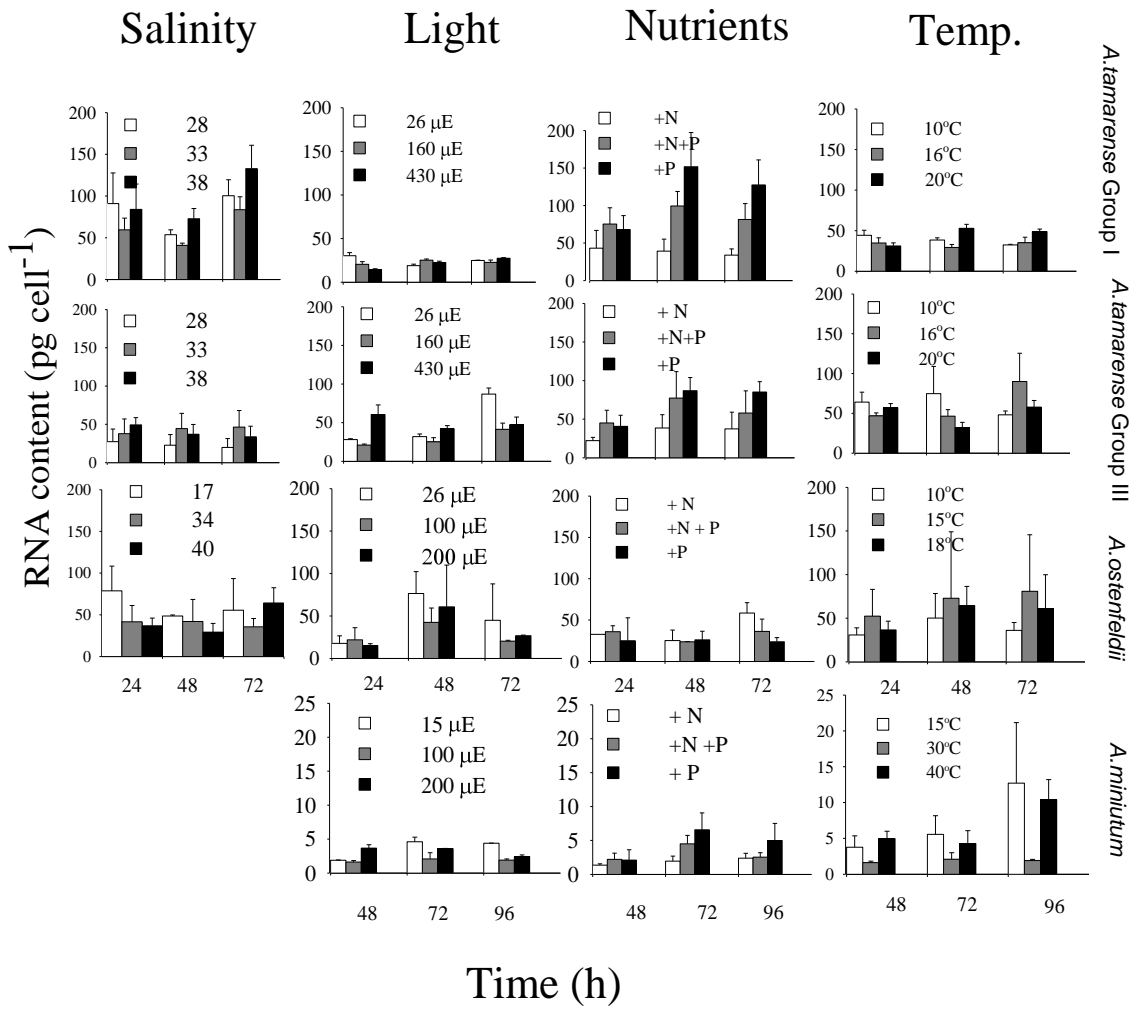
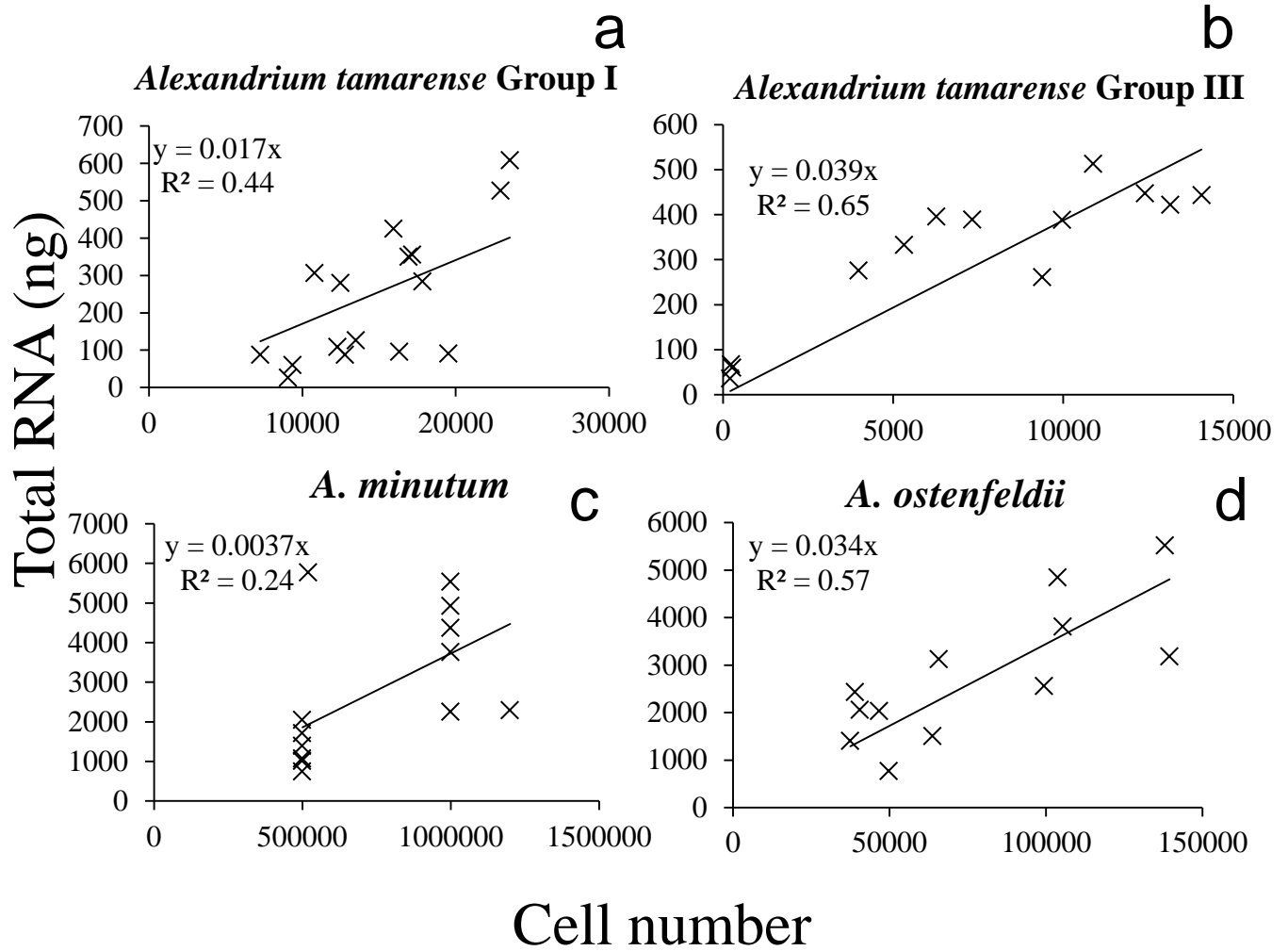
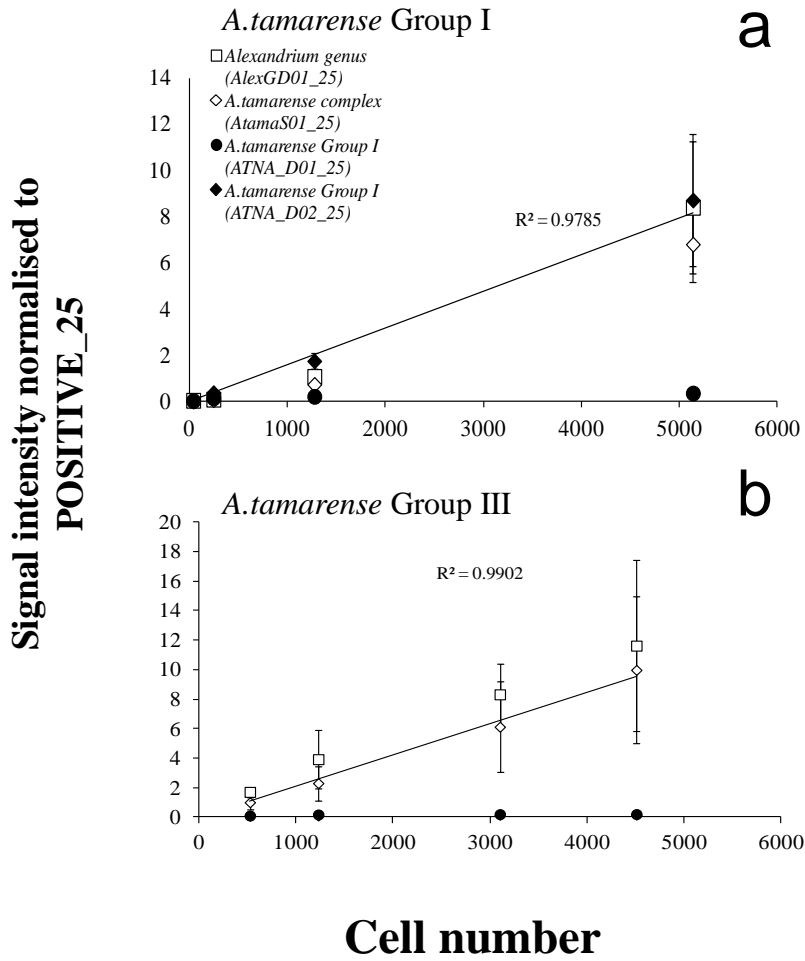


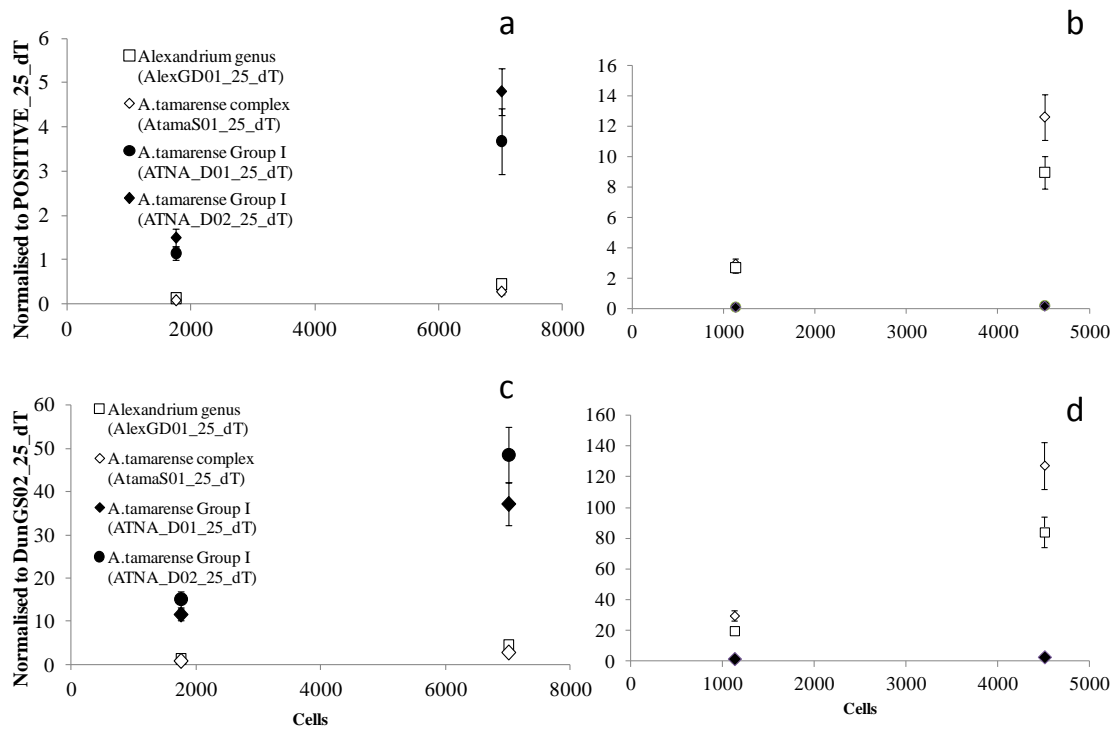
Figure 3



# Figure 4



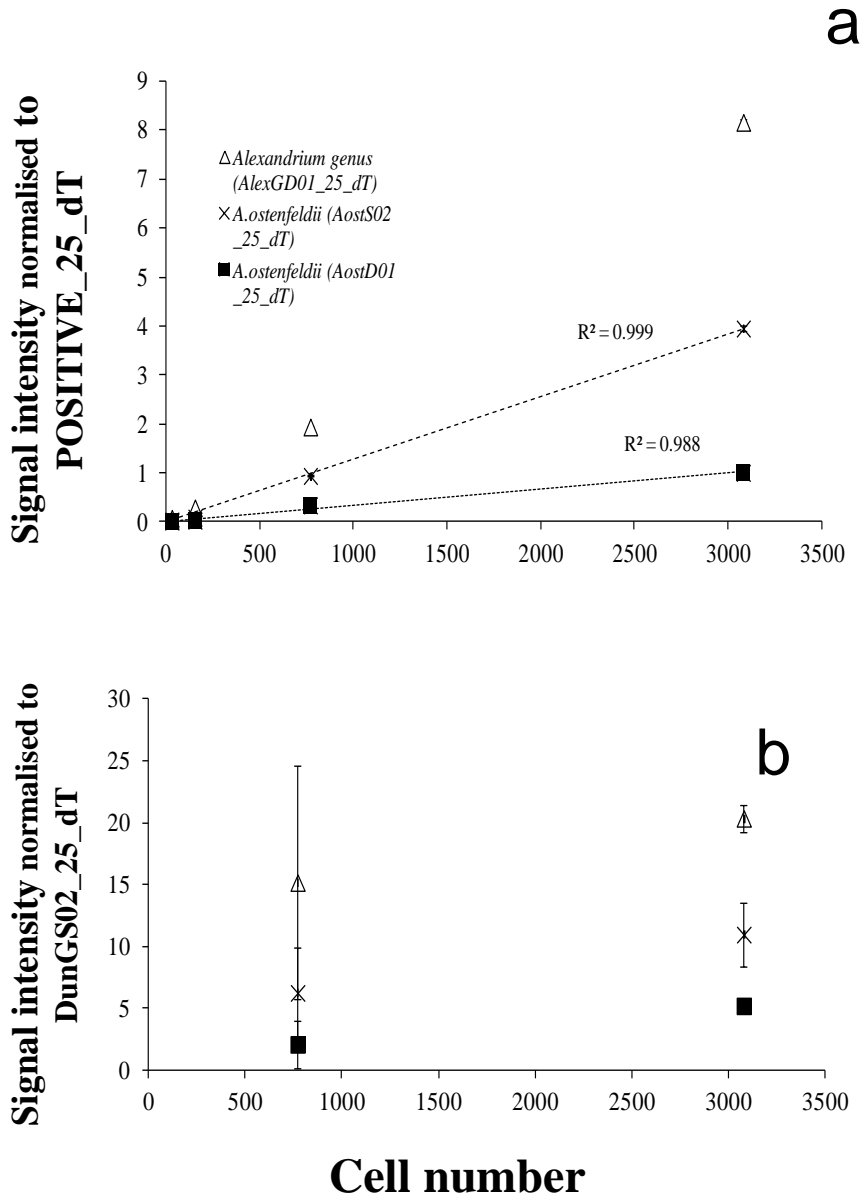
# Figure 5



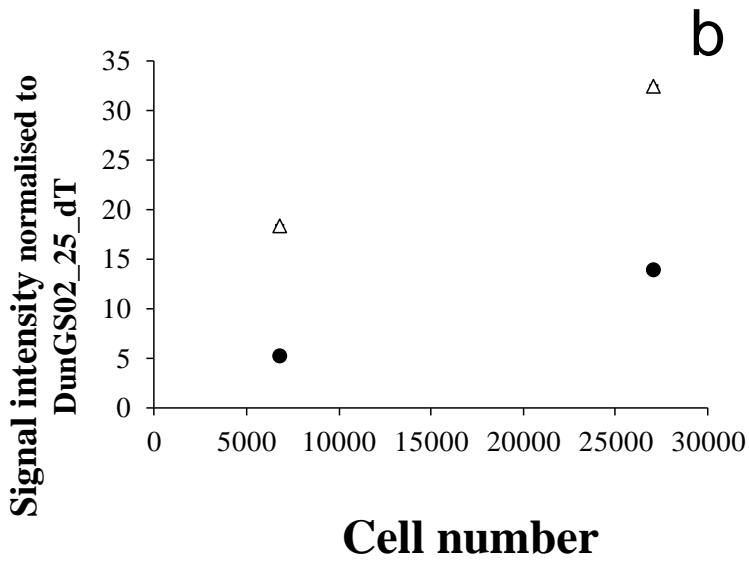
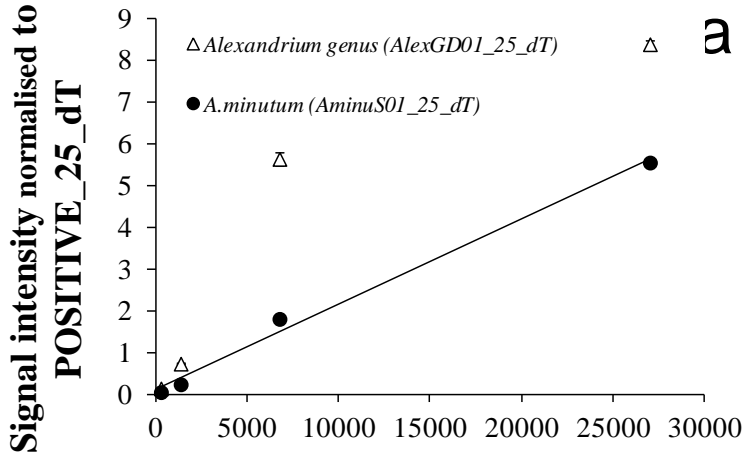
*A. tamarensis* Group I

*A. tamarensis* Group III

# Figure 6

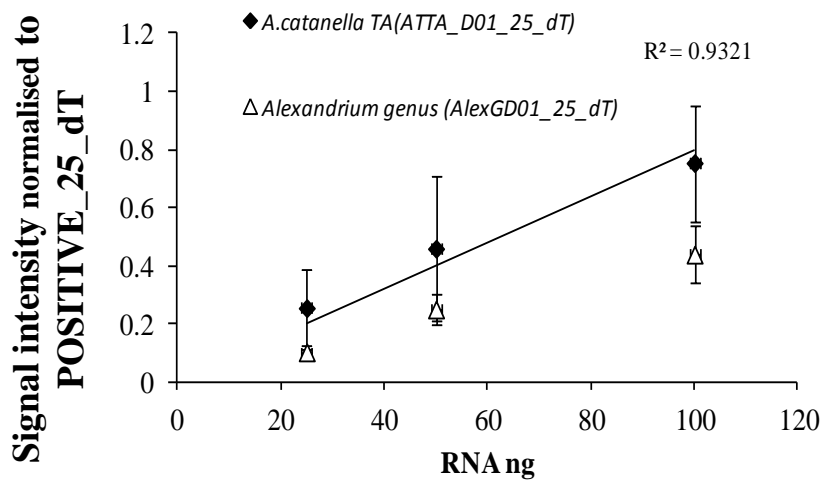


# Figure 7





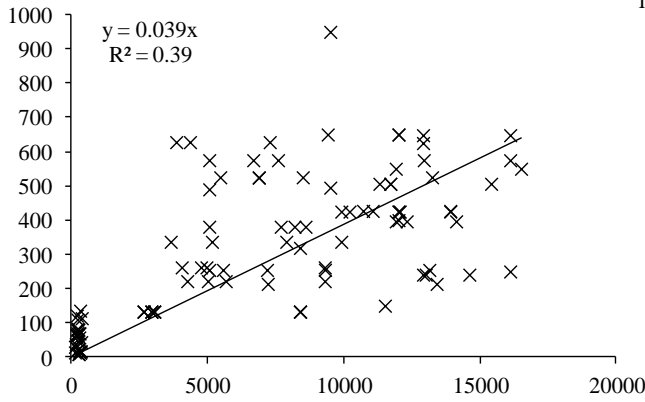
# Figure 8



# Supplementary Figure 1

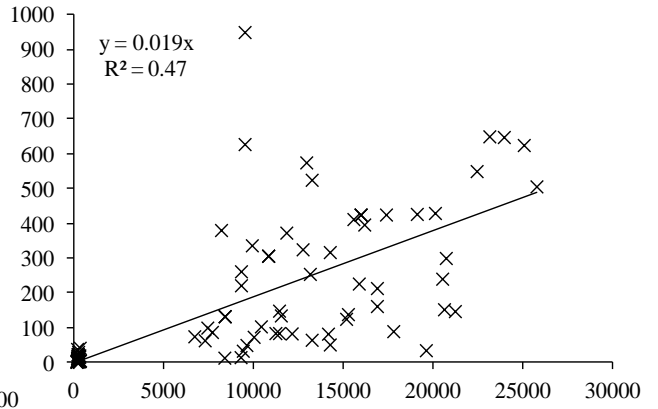
a

*Alexandrium tamarense* Group I



b

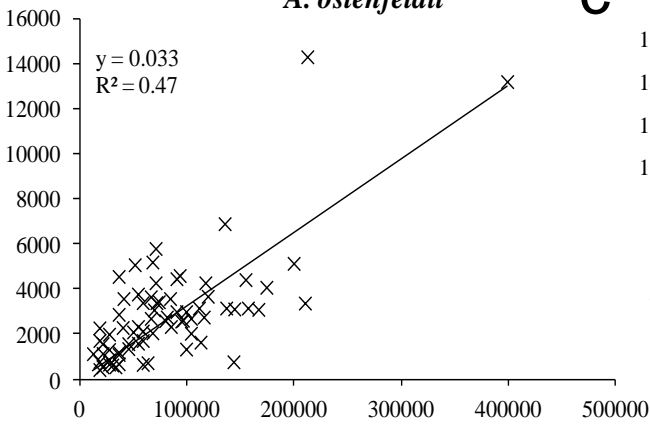
*Alexandrium tamarense* Group III



Total RNA (ng)

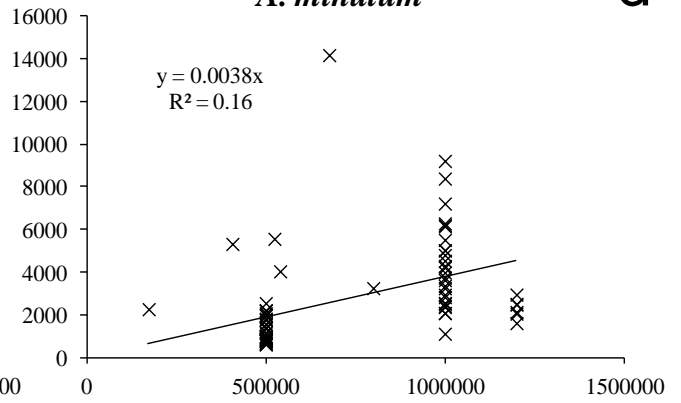
*A. ostensfeldii*

c



*A. minutum*

d



Cell number