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Growth- and nutrient-dependent gene expression in the toxigenic marine dinoflagellate *Alexandrium minutum*

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ABSTRACT

The toxigenic marine dinoflagellate *Alexandrium minutum* forms toxic blooms causing paralytic shellfish poisoning (PSP), primarily in coastal waters, throughout the world. We examined effects on physiology and gene expression patterns associated with growth and nutrient starvation in a toxic strain of *A. minutum*. Bloom-relevant factors, including growth rate, intracellular toxin content, allelochemical activity and nutrient status were investigated in *A. minutum* cultures grown under different environmental regimes. Allelochemical activity of *A. minutum* cultures, quantified with a cryptomonad *Rhodomonas* bioassay, increased with age but was independent of nutrient status.

The phenotypic data were integrated and compared with gene expression in cell samples taken at selected points along the growth curve. We observed 489 genes consistently differentially expressed between exponentially growing and growth-limited cultures. The expression pattern of stationary-phase cultures was characterized by conspicuous down-regulation of translation-associated genes, up-regulation of sequences involved in intracellular signalling and some indications of increased activity of selfish genetic elements such as transposons. Treatment-specific patterns included five genes regulated in parallel in all nutrient-limited cultures. The conspicuous decrease in photosynthetic performance identified in N-starved cultures was paralleled by down-regulation of chloroplast-associated genes.

The particular gene expression patterns we identified as specifically linked with exponential growth, cessation of growth or nutrient limitation may be suitable biomarkers for indicating the beginning of growth limitation in field- or mesocosm studies.

1. Introduction

Dinoflagellates are ubiquitous protists and key components of marine and freshwater food webs worldwide. In many marine systems, chloroplast-containing dinoflagellates are among the most important biomass producers (Anderson et al., 2008; Thompson et al., 2008; Yallop, 2001). Many dinoflagellate species can form dense blooms, which often pose serious health and ecosystem threats through the production of noxious, toxic or other ecosystem-disruptive substances.

Alexandrium minutum is a widely distributed toxic dinoflagellate that tends to form toxic blooms associated with paralytic shellfish poisoning (PSP) in temperate and subtropical coastal regions worldwide, from the Atlantic and North Sea (McCauley et al., 2009; Touzet et al., 2007a) and the Mediterranean (Bravo et al., 2008) to subtropical Asia (Hwang and Lu, 2000), and New Zealand (Chang et al., 1997). *A. minutum* can grow under a relatively wide range of temperatures and salinities, and under low-turbulence conditions growth and consequent bloom development seems to be largely dependent on nutrient availability (Bravo et al., 2008; Vila et al., 2005). Therefore, data on the physiological and gene expression differences between exponentially growing and nutrient-limited cultures are of high ecological significance. As the different growth stages in laboratory batch cultures correspond to profound physiological differences that develop over a time-scale of several cell cycles (John and Flynn, 2000), this species is also a convenient model to examine transcriptional regulation associated with acclimation of a dinoflagellate over physiologically relevant time-scales.

Dinoflagellates are often considered to exhibit poor nutrient uptake efficiency and relatively slow growth rates when compared with other phytoplankton, such as diatoms (Smayda, 1997), but this may be compensated by the typical dinoflagellate traits of circadian nutrient-retrieval migrations, high prevalence of mixotrophy, and production of allelochemicals and toxins targeted against interspecific competitors and predators (Cembella, 2003; Smayda, 1997). Some of these substances are active against other protists (Tillmann and John, 2002), while others have harmful or toxic effects on other organisms, including humans.

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In order to better understand mechanisms of population dynamics and bloom formation in dinoflagellates, more knowledge of the intrinsic regulation of growth, nutrient uptake and starvation responses, as well as the biosynthesis and regulation of toxins and allelochemical substances, is required. A combination of chemical characterization, physiological experimentation and gene expression comparisons under a variety of environmental regimes seems to be most promising (Cembella and John, 2006). The chemistry of dinoflagellate toxins is well known, and apart from a few newly discovered species (e.g. Tillmann et al., 2009), physiological responses related to growth and toxin production in toxin-producing dinoflagellates have often been well studied (Chang and McClean, 1997; Flynn et al., 1994; Hwang and Lu, 2000; Leong et al., 2004; Touzet et al., 2007b; Yamamoto and Tarutani, 1999).

Genomic studies on dinoflagellates, however, are complicated by profound doubts as to what extent methods and concepts developed in model organisms, including other protists, are applicable to dinoflagellates (Bachvaroff and Place, 2008; Monroe and Van Dolah, 2008; Moreno Díaz de la Espina et al., 2005). Dinoflagellates arguably contain the most unusual eukaryotic genetic machinery known. Their huge genomes (LaJeunesse et al., 2005) comprise both major proportions of apparently random, non-repetitive DNA with very little recognizable gene content (Jaeckisch et al., submitted for publication; McEwan et al., 2008) and unusually high numbers of transcribed genes (Moustafa et al., 2010). For example, *Alexandrium tamarense*, with about three times the nuclear DNA content of *A. minutum*, was shown to contain about 40,000 transcribed genes occurring in complex families (LaJeunesse et al., 2005). Dinoflagellate chromosomes are permanently condensed into a liquid crystal state (Livolant and Bouligand, 1978; Moreno Díaz de la Espina et al., 2005), and transcription as well as most of the coding sequences seem to be restricted to DNA filaments protruding into the nucleoplasm (Anderson et al., 1992). Partly owing to these genomic peculiarities, fundamental aspects about the regulation of dinoflagellate gene expression are currently under debate.

Contradictory evidence exists regarding the extent of gene regulation on the transcriptomic level. Both regulation of mRNA abundances (Hosoi-Tanabe et al., 2005; Okamoto and Hastings, 2003; Taroncher-Oldenburg and Anderson, 2000; Toulza et al., 2010) and a high prevalence of translational regulation (Lapointe and Morse, 2008; Lidie, 2007; Rossini et al., 2003) have been reported in dinoflagellates. The discovery of spliced-leader trans-splicing (Lidie and Van Dolah, 2007; Slamovits and Keeling, 2008; Zhang et al., 2007) and of single-domain transcripts apparently derived from multi-domain genes led to the suggestion of trypanosome-like mechanisms of spliced-leader-associated constitutive translational gene regulation in dinoflagellates (Monroe and Van Dolah, 2008). In analogy to trypanosomes, highly expressed dinoflagellate genes were proposed to be constitutively transcribed and regulated during mRNA processing by a mechanism involving trans-splicing (Bachvaroff and Place, 2008). This model predicts that transcriptional regulation would be restricted to low-copy genes mostly lacking spliced leader sequences. However, this has been challenged by the discovery of spliced leader sequences in the 5′-regions of the genes postulated to lack them (Zhang and Lin, 2009).

We investigated growth-related processes in batch cultures of *A. minutum* in exponential versus stationary growth phase and under nutrient starvation, to gain a deeper understanding of the physiological and transcriptomic processes associated with bloom formation and development. In addition to determining the phenotypic effect on toxin content and allelochemical activity, we compared the transcriptional response of exponentially growing and growth-limited batch cultures under different growth regimes along the growth curve. At three characteristic points of the culture cycle, we determined allelochemical activity, intracellular toxin content and intracellular and extracellular nutrient status. By means of DNA microarrays, we compared gene expression differences among cultures in exponential growth, at the transition to stationary phase, and several days after onset of stationary phase. Cross-comparison of the resulting patterns of differential gene expression enabled us to propose characteristic expression patterns associated with specific physiological phenomena.

2. Methods

2.1. Strain and culture conditions

A. minutum strain AL3T (origin: Gulf of Trieste, Italy) was grown at 20 °C at a photon flux density of 200 μmol m^{-2} s^{-1} on a 16:8 h light:dark cycle. Stock cultures were kept in modified K-medium consisting of aged seawater (salinity ca. 32 practical salinity units) enriched with 440 μmol L^{-1} NO_3^-, 36 μmol L^{-1} NH_4^+, 25 μmol L^{-1} PO_4^{3-}, 10 nmol L^{-1} SeO_3^{2-}, 1000 μmol L^{-1} Trizma-Base (pH 8.3), K trace metal solution and f/2 vitamin solution (Keller et al., 1987). Preparatory cultures were filtered over 10 μm gauze, washed with sterile-filtered seawater to reduce bacterial load and grown under antibiotic treatment (50 μg mL^{-1} ampicillin, 33 μg mL^{-1} gentamicin, 10 μg mL^{-1} ciprofloxacin, 1.13 μg mL^{-1} chloramphenicol and 0.025 μg mL^{-1} streptomycin sulfate) for 13 days, during which they were kept in exponential growth phase by repeated sub-culturing. Only starter cultures in which no bacteria could be detected by Acridine orange staining (Hobbie et al., 1977) followed by fluorescence microscopy were used to inoculate the experimental treatments.

Experimental cultures were grown in 5 L Duran bottles (Schott AG, Mainz, Germany) under constant gentle aeration and sampled with a sterile tube-vacuum system as described in Eschbach et al. (2005). Control cultures were grown in complete K-medium as defined above; for P- or N-limited cultures, the phosphate source or the nitrate and ammonium sources were omitted, respectively, from the medium.

2.2. Sampling and daily measurements

Cultures were monitored daily by pH measurements, microscopic cell counts and measurements of potential quantum efficiency (F_v/F_m) of Photosystem II. The F_v/F_m values were determined by Pulse-Amplitude-Modulated (PAM) fluorometry using a Xenon-PAM-Fluorometer (WALZ GmbH, Effeltrich, Germany) after 15 ± 5 min of dark incubation, following the method detailed by Mock and Hoch (2005). Specific growth rates were calculated as: $\mu = (\ln(N_{t_2}) - \ln(N_{t_1}))(t_2 - t_1)^{-1}$, with N = cells mL^{-1} and t = sampling day. Stationary phase was defined as the growth phase where $\mu < 0.1$ d^{-1}. Samples for nutrient measurements, allelochemical assays, PSP toxin measurements, and RNA extraction were taken on Days 4 and 5 for all cultures and two to three days after each treatment triplicate had entered stationary growth phase. On the last treatment-specific sampling date, aliquots of each culture were transferred into 50 mL Erlenmeyer flasks to serve as follow-up cultures for further monitoring of cell growth. In order to confirm nutrient limitation, two aliquots per nutrient-limited culture were taken, one of which was supplemented with the missing nutrient.

2.3. Nutrient analysis

Filtered medium samples for dissolved nutrient analysis were preserved by adding 3 μL 3.5% (w/w) $HgCl_2$ per mL sample and stored at 4 °C until analysis. Dissolved nutrients were analyzed by

continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany). For total dissolved phosphorus and nitrogen, the analysis was preceded by digestion with peroxodisulfate in an autoclave.

Samples for particulate nutrient analysis were filtered on pre-combusted glass fiber GF/F filters (Whatmann, Omnilab, Bremen, Germany) and stored at −20 °C. Filters for C/N-measurements were dried at 60 °C and encapsulated into chloroform-washed tin containers. Samples were analyzed on an NA 1500C/N Analyzer (Carlo Erba Instrumentazione, Milan, Italy). Particulate phosphate was measured photometrically by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany) after digestion with peroxide and sulfuric acid (Kattner and Brockmann, 1980). Mean C/N values were calculated from the C/N measurements for individual filters; C/P and N/P values were determined from the average of all possible pairs of measurements for each culture at a given sampling point.

2.4. Toxin analysis

PSP toxins were extracted and prepared for analysis following the method of Krock et al. (2007). Briefly, cells were harvested by centrifugation (3000 × *g*, 4 °C). Pellets were suspended in 0.03 N acetic acid and homogenized in FastPrep tubes containing 0.9 g of lysing matrix D with a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France) at maximum speed (6.5) for 45 s. Cell debris was removed by centrifugation at 16,100 × *g* at 4 °C for 15 min. The supernatant was filtered through a 0.45 μm pore-size Ultrafree spin-filter (Millipore, Eschborn, Germany) by centrifugation for 30 s at 800 × *g*. PSP toxins were separated by ion-pair liquid chromatography and detected fluorometrically after post-column derivatization (LC-FD) as described in Krock et al. (2007).

2.5. Determination of allelochemical activity

Allelochemical activity was determined by co-incubation of *A. minutum* cells with intact cultured cells of the cryptophyte *Rhodomonas salina* (Tillmann et al., 2008). Nine to ten different concentrations of *A. minutum* cells (in biological triplicates) were incubated with *R. salina* cells for 24 h in 20 mL glass vials in darkness. Incubations were stopped by addition of Lugol's iodine solution and numbers of intact *R. salina* cells were counted with an inverted microscope (Zeiss, Jena, Germany) at 200–400× magnification. The *Alexandrium* cell concentrations yielding a 50% decline in intact *R. salina* cells (EC_{50}) were estimated by fitting the following equation to the cell count data using the non-linear fit procedure of Statistica (Statsoft, Germany):

$$N_{\text{final}} = \frac{N_{\text{control}}}{1 + (x/\log\ EC_{50})^h}$$

with N_{final} = *R. salina* cell concentration after incubation with *A. minutum*, N_{control} = *R. salina* cell concentration after incubation without *A. minutum*, x = log-transformed cell concentration of *A. minutum*, and the fit parameters $\log EC_{50}$ and h. Results are expressed as EC_{50} including 95% confidence intervals. To increase the number of data points to fit the equation, the data from all three replicate cultures were combined to calculate one EC_{50} value per treatment and time-point.

2.6. RNA extraction and microarray experiments

RNA extraction and microarray hybridization were carried out as described in Yang et al. (2010b). Cells were harvested by filtration upon an 8 μm pore-sized filter (TETP04700, Millipore Schwalbach, Germany) and rinsed with filter-sterilized seawater. Filters were quick-frozen in liquid nitrogen and later thawed by rinsing with heated (60 °C) TriReagent (Sigma–Aldrich, Steinheim, Germany). RNA was extracted according to the TriReagent protocol, following cell lysis by 10 min incubation at 60 °C in TriReagent, aided by repeated vortex mixing with glass beads included in the sample tube. Briefly, after addition of 200 mL chloroform per mL TriReagent, samples were centrifuged for 15 min at 12,000 × *g* at 4 °C. The aqueous phase was mixed with an equal volume of isopropanol and incubated at −20 °C for at least 10 min. An RNA pellet was obtained by centrifugation at 12,000 × *g* for 10 min at 4 °C. The pellet was washed by addition of 75% ethanol, followed by another centrifugation step. After removal of the ethanol, the pellet was dried until hyaline and then dissolved in 100 mL RNAse-free water (Qiagen, Hilden, Germany). RNA cleanup and DNA digestion followed the protocol supplied with the Qiagen RNeasy kit: RNA samples were mixed with 350 mL binding buffer RLT containing 1% β-mercaptoethanol. After mixing with 250 mL ethanol, samples were applied to an RNeasy column (Qiagen) containing a silica membrane. Columns were washed by 1 min incubation with 700 mL RW1 followed by centrifugation before 10 mL DNase I mixed with 70 mL buffer RDD (both Qiagen) were applied for 15 min. To interrupt DNase digestion, columns were washed with 700 mL RW1. Samples were incubated for 1 min in buffer RPE (Qiagen), centrifuged, and washed again with the same buffer. After 2 min centrifugation and another 1 min high-speed centrifugation in a new collection tube, RNA was eluted with 40 mL RNase-free water. To increase final RNA concentration, the flow-through was applied to the membrane a second time. When necessary, an additional cleanup and concentration step using Qiagen MinElute or Microcon Ultracel YM-30 columns was applied. RNA purity and quantity were determined with a NanoDrop ND-1000 Spectrophotometer V3.1.0 (PeqLab, Erlangen, Germany), and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Total RNA (500 ng sample^{-1}) was amplified and labeled using a low-input linear amplification kit (Agilent, Waldbronn, Germany), following the Agilent protocol for synthesis of Cy3- and Cy5-labeled cRNA and microarray hybridization. Agilent custom-made microarrays were based on the oligonucleotide probe set previously developed (Yang et al., 2010b). Day 5 and stationary-phase samples from the nutrient-limitation treatments were hybridized against the corresponding control-treatment samples. Day 5 and stationary-phase control treatment samples were hybridized against Day 4 control-treatment samples. Microarrays were scanned on an Agilent G2565AA scanner, and raw data was extracted with the Agilent Feature Extraction Software version 9.1.3.1 (FE). Array quality was monitored using the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07.

Pre-processed data were analyzed by SAM (Significance Analysis of Microarrays, Tusher et al., 2001) as implemented in MeV 4.0 (Saeed et al., 2006), and SAM-based q-values (Storey, 2003) were calculated. The SAM one-class option served to compare each treatment to the control treatment hybridized on the same arrays. Probes with a q-value of <1% were considered to indicate differential expression of the corresponding genes if the mean fold-change of the sample triplicate was at least 1.5. As the nutrient-limited treatments for each time point had been hybridized against the same control samples, two-class SAM was applied to directly compare between these treatments on Day 5 respectively in stationary phase. As the Day 5 and stationary-phase control samples had also been hybridized against Day 4 control samples, two-class SAM was also applied to directly compare between nutrient-limited and control Day 4 samples. After identification of probes recognized as differentially expressed in several comparisons, the corresponding contig

sequences in the *A. minutum* EST library were manually annotated.

2.7. Statistical analysis

Except for allelochemical activity (see above), physiological values are reported as the mean of biological triplicates with the associated standard deviation. Where not otherwise stated, significance of physiological data was tested according to Student's *t*-test at $p < 0.05$ (*t*-test). Normality was assessed by the Shapiro–Wilk-test as implemented in *R* and variances were compared by Fisher's *F*-test. Significance of physiological data for which Fisher's *F*-test indicated unequal variances was tested according to Welch's *t*-test for unequal variances at $p < 0.05$ (Welch test). Where indicated, significance of differences was tested by ANOVA analysis using *R* ($p < 0.05$). Microarray-based expression values are given as the geometric mean of three microarray measurements based on biological triplicates.

3. Results

3.1. Growth and physiological parameters

The nutrient-limited and control cultures displayed similar growth patterns during the early growth stages (Fig. 1A). None of the cultures exhibited a pronounced lag phase, and mean exponential-phase growth rates were not significantly different between samples and among treatments (two-tailed *t*-test, $p = 0.1$; Fig. 1B). Cell concentrations increased exponentially until Day 4, after which growth rate began to decrease under all treatments. Stationary phase was reached at Day 6 in the N-limited cultures, Day 7 in the P-limited cultures and Day 8 in the control treatment (see Table 1 for stationary-phase cell counts, pH and nutrient ratios). Follow-up cultures after the stationary-phase harvesting point confirmed the specific nutrient limitation: aliquots of both the P- and the N-restricted treatments resumed growth after addition of the limiting nutrient, but those without added nutrients remained in stationary phase (Fig. 1C). Follow-up cultures of the control treatment, presumably containing sufficient extracellular and/or intracellular residual N- and P-nutrients, resumed growth as well. The pH values increased with increasing cell concentration; the highest values were reached in the control treatment just before stationary phase (Figs. 1B and 2).

Throughout the experiment, the nutrient-limited culture media contained greatly reduced amounts of the limiting nutrient (Fig. 3A), which was reflected in the intracellular nutrient levels and ratios (Fig. 3B and C). The stationary-phase control cultures had depleted the dissolved phosphate to levels similar to the exponentially growing P-limited cultures by the time of the last harvesting, but intracellular P levels and C/P ratios were not significant different from those of N-limited cultures (Fig. 3C and Table 1).

The potential quantum efficiency of Photosystem II (PSII), measured as F_v/F_m, increased with increasing cell concentrations in exponentially growing control and P-limited cultures (Fig. 4) and slowly decreased during stationary phase. In N-limited cultures, F_v/F_m did not change significantly during exponential phase (one-way ANOVA F_v/F_m versus time) but decreased rapidly during stationary phase.

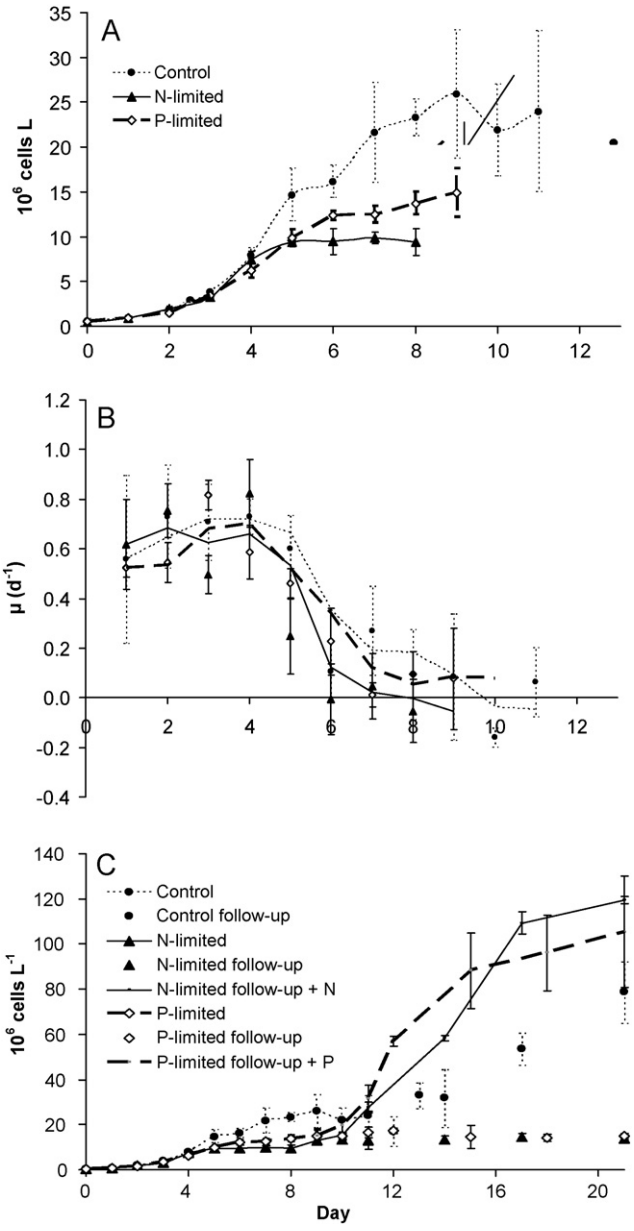

Fig. 1. Growth kinetics of *Alexandrium minutum* AL3T in batch culture experiments: (A) cell concentrations versus day after inoculation; (B) growth rate (μ) versus day after inoculation; lines: two-day moving average; (C) cell concentrations versus day after inoculation including nutrient-spiked (lines only) and non-spiked (symbols only) follow-up cultures. All values are mean ± standard deviation of biological triplicates.

Table 1
Values for physiological variables in stationary phase.

Treatment	Cells L^{-1}	pH	C:N	N:P	C:P
Control	23.7 ± 5.6[a]	9.10 ± 0.13	5.5 ± 0.4[b]	8.5 ± 2.2	45.9 ± 10.5
P-limited	13.7 ± 1.8[a]	9.01 ± 0.21	9.9 ± 0.8[b]	44.6 ± 10.8[b]	432.4 ± 86.6[b]
N-limited	9.6 ± 1.1[a]	8.82 ± 0.24	19.9 ± 2.7[b]	2.6 ± 0.4	50.4 ± 6.8

Stationary phase defined as when $\mu < 0.1\ d^{-1}$. All values are mean ± standard deviation of biological triplicates, with pH measurements and cell counts averaged over the stationary phase, and cellular nutrient ratios determined at the last harvesting point at the end of the experiment.

[a] Significantly different ($p \leq 0.05$) from all other values in this column as determined by Student's *t*-test on ln-transformed data ($p \leq 0.05$).

[b] Significantly different from all other values in this column according to Welch's *t*-test (unequal variances *t*-test, $p \leq 0.05$).

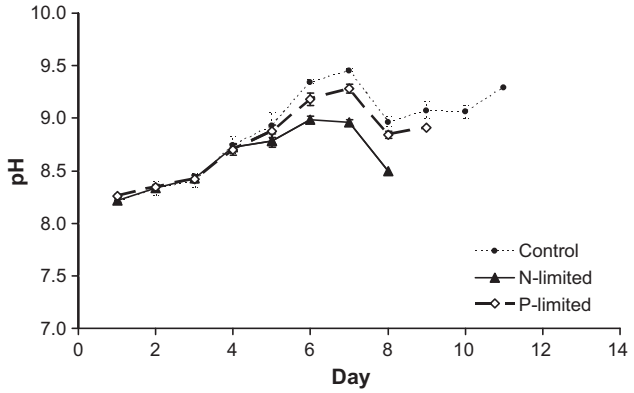

Fig. 2. Variation in pH of the culture medium over time for various treatments (mean ± standard deviation of biological triplicates). The last data point corresponds to the treatment-specific stationary-phase harvesting date.

The changes in intracellular PSP toxin content along the growth curve were strongly treatment-dependent (Fig. 5A; significance tested according to Student's *t*-test at $p < 0.05$). At the first two sampling points, toxin content per cell was not significantly different between nutrient-replete control and P-limitation conditions. Stationary-phase cellular toxin concentrations significantly declined in control cultures. In P-limited cultures, toxin per cell was significantly elevated both with respect to values from control or N-limited cultures and to values at the second sampling point from all treatments. Intracellular toxin content in the N-limited cultures was significantly lower than in the other treatments at all sampling time points, but did not change significantly over the culture cycle (Student's *t*-test, respectively; Welch test at $p < 0.05$).

Allelochemical activity against *R. salina* followed the same trend in all cultures. Whereas the P-limited cultures were much less allelochemically active at the first two sampling points, as indicated by much higher EC_{50} values, allelochemical activity increased with culture age in all three treatments (Fig. 5B).

3.2. Gene expression

Out of 4298 *A. minutum* sequences represented in the database, 1781 (41%) were identified as differentially expressed between exponential-phase control samples and at least one of the treatments at the second or third sampling time-point (Tables 2a and 2b). Among these genes, 1025 were differentially expressed

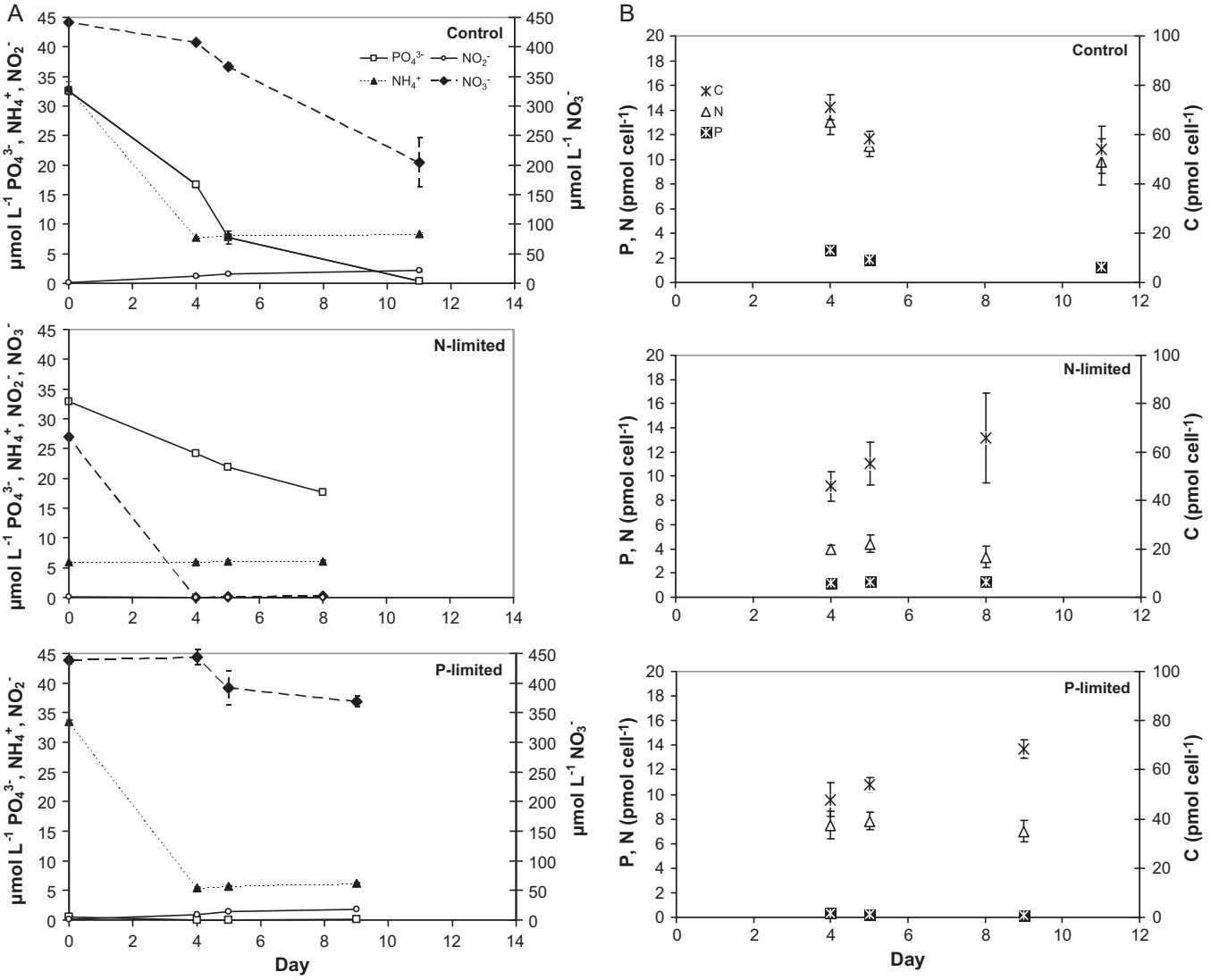

Fig. 3. Nutrient status of cultures: (A) dissolved nutrient concentrations in the culture medium; (B) cellular nutrient concentrations; (C) nutrient element ratios. Values are mean ± standard deviation of biological triplicates. Invisible error bars do not exceed range covered by symbol.

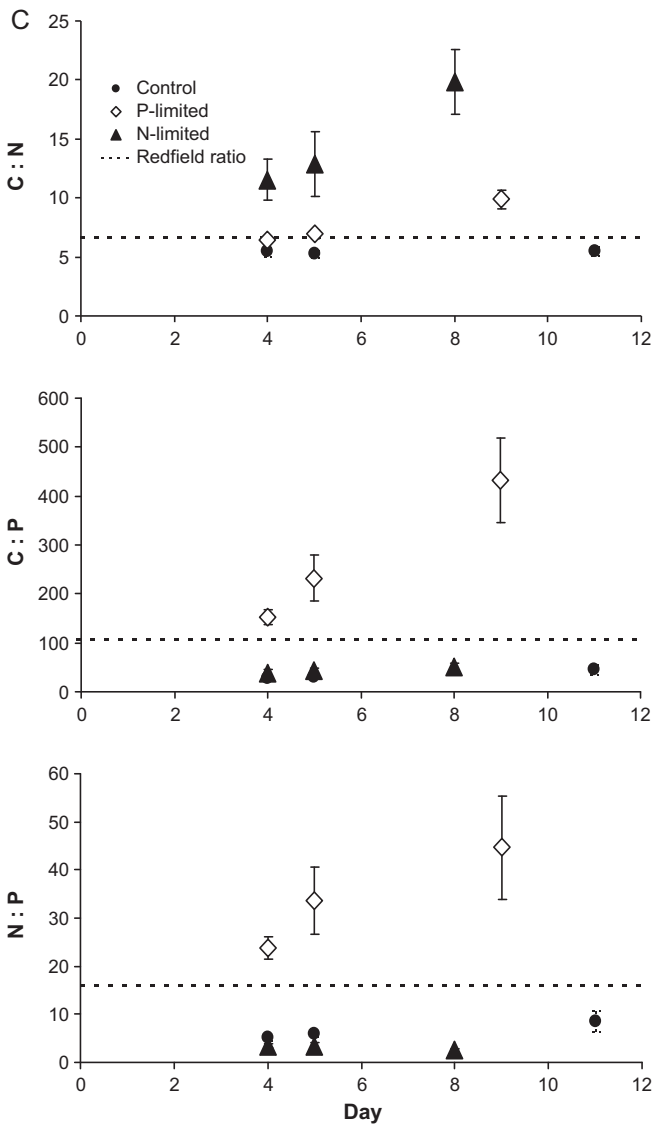

Fig. 3. (*Continued*).

between one or both of the nutrient-limited treatments and the exponentially growing control.

The set of 1565 genes up-regulated in the stationary phase compared to exponentially growing control cultures contained

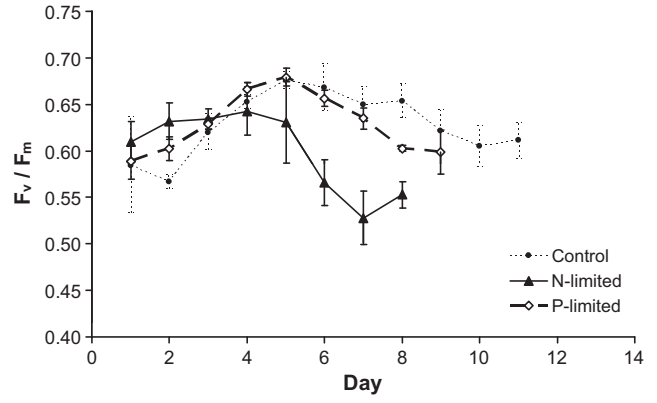

Fig. 4. Potential quantum efficiency of PSII expressed as dark-adapted F_v/F_m. Mean of triplicates ± standard deviation.

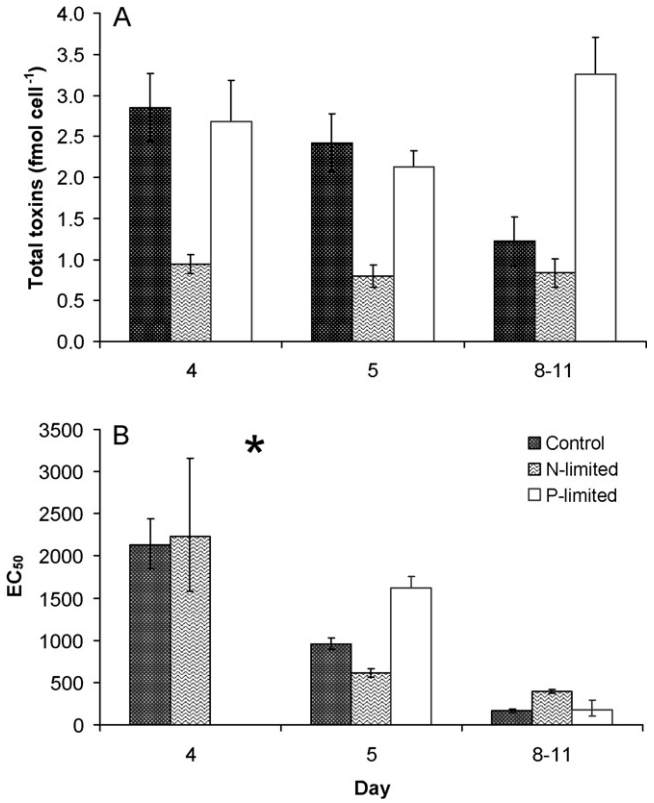

Fig. 5. PSP toxin content and allelochemical activity. (A) Cellular toxins (fmol cell^{-1}) as measured by liquid chromatography with fluorescence detection (LC-FD). Mean of biological triplicates ± standard deviation; (B) allelochemical activity against *Rhodomonas salina*, calculated as half-effective concentration (EC_{50}) of *A. minutum* cells, with 95% confidence intervals. *At limit of measured range, between 3500 and 7200 – no fit to model was possible.

two carbonic anhydrase (CA) sequences. A dinoflagellate-type extracellular delta-CA (Amin_85n03r) was expressed 1.65-fold higher in the stationary phase control. This sequence was also 2.09-fold up-regulated in the stationary-phase N-limited cultures when compared to exponentially growing controls. The second sequence Amin_77b06f is an intracellular CA similar to a sequence known from the pennate diatom *Phaeodactylum tricornutum*. In *A. minutum* this gene was 2.12-fold up-regulated in the stationary-phase control relative to the exponentially growing control. For both Amin_85n03r and Amin_77b06f, expression differences between all other tested pairs of growth conditions remained non-significant.

In searching for consistent patterns of up- and down-regulation among datasets, we identified 554 sequences repeatedly associated with one of the tested physiological regimes (Table 3). The expression of 489 genes was linked to the difference between exponential growth and all tested growth-limiting conditions for *A. minutum* cultures. Table 4 depicts a selection of these genes for which a function was assignable.

In stationary-phase control cultures, 8 genes were down-regulated relative to exponential-phase controls and relative to both nutrient-limited treatments (Table 5); these were identified as characteristic for the stationary phase in control cultures.

In both the comparisons with exponential- and stationary-phase cultures (Tables 3 and 6), 87 genes were associated with N- or P-limitation. Among these genes, 5 were regulated in parallel under all nutrient-limited regimes (Table 3, Supplementary Table 1), but none of them could be annotated to function.

Analysis of the frequency of complete spliced leader (SL) sequences in the set of differentially expressed genes in comparison with the whole underlying EST library revealed no apparent

Table 2a
Numbers of genes differentially expressed between treatments at transition to stationary phase and when compared to exponentially growing control cultures.

		Transition to stationary phase		
		Control culture	N-limited	P-limited
Transition to stationary phase	N-limited	430	–	13
	P-limited	6	13	–
Exponential phase	Control culture	6	126	21

Table 2b
Numbers of genes differentially expressed between treatments in stationary phase and when compared to exponentially growing control cultures.

		Stationary phase		
		Control culture	N-limited	P-limited
Stationary phase	N-limited	143	–	19
	P-limited	77	19	–
Exponential phase	Control culture	1565	778	764

pattern. Examination of the associated EST contigs showed that 4.4% of the genes differentially expressed between one of the treatments and the exponential-phase control samples contained a complete SL sequence. The same was true for 5.8% of the sequences that could be linked to physiological conditions. Both percentage values are similar to the 4.7% SL-containing sequences in the whole library.

4. Discussion

4.1. Physiology of the experimental cultures

4.1.1. Growth-limiting factors

For both nutrient-limited and control cultures, cellular nutrient quotas and ratios were in the range of values previously published for *A. minutum* in laboratory experiments (Flynn et al., 1994; Maguer et al., 2007), and also agreed with those reported for other *Alexandrium* species (John and Flynn, 2000; Juhl, 2005). Molar nutrient ratios (C:N, C:P, N:P) in the control cultures were at the lower range of the reported values for nutrient-replete cultures of various marine microalgal species (Geider and La Roche, 2002). Control culture values were also lower (Fig. 3C) than the canonical Redfield ratios (Redfield, 1958) of mol C:N:P = 106:16:1 considered to represent balanced growth conditions in natural populations. Similar to other *Alexandrium* species, *A. minutum* is known to be a specialist for intracellular storage of P (Labry et al., 2008), and to a lesser extent of N (Flynn et al., 1996; Maguer et al., 2007), during nutrient pulses. The nutrients stored during the periods of "luxury consumption" can later be mobilized for growth when extracellular nutrients are depleted. Laboratory cultures are typically grown on "excess" inorganic N and P, often at high external N:P ratios. Our *A. minutum* control cultures were inoculated into K-medium at 476 μmol L^{-1} total inorganic N and 25 μmol L^{-1} PO_4^{3-}, apparently triggering significant intracellular nutrient storage and correspondingly reduced nutrient ratios.

As expected, in the nutrient-limited cultures, the intracellular amount of the limiting nutrient decreased both in absolute and in relative terms (Fig. 3B and C), indicating that acclimation to these conditions involves major changes in intracellular biochemistry. The N:P ratio of 44.6 ± 11.8 reached in the P-limited cultures were somewhat lower than those typically attained by a variety of marine microalgae under P-limitation (Geider and La Roche, 2002). However, given the high DNA content of about 29.9 pg DNA per cell in *A. minutum* (Figueroa et al., 2010) and the approximate elemental composition of DNA (Geider and La Roche, 2002), the genomic DNA alone should account for about 56% of the intracellular P in stationary phase P-limited cultures (0.16 ± 0.03 pmol $cell^{-1}$).

Even with abundant aeration, high pH in batch cultures and natural populations of microalgae is both a cause and indicator of insufficient biologically available dissolved C to sustain further growth (Berman-Frank et al., 1994). High pH in itself has been shown to be a possible limiting factor for dinoflagellate growth (Hansen et al., 2007; Søderberg and Hansen, 2007). In the stationary-phase control cultures, where N- and P-nutrients were replete, growth might have been limited by pH stress or low availability of dissolved CO_2, as suggested by the high pH in the culture vessels (Fig. 2) and the resumption of growth after transfer to the small-volume follow-up culture. Nevertheless, pH in the follow-up cultures was not measured, and thus limitation of the control cultures by other factors cannot be ruled out.

4.1.2. Photosynthetic performance

The potential quantum efficiency of Photosystem (PS) II, measured as F_v/F_m, is a sensitive indicator of photosynthetic performance and as such is often used as a general stress indicator for photosynthetic cells (Hsu, 2007; Kim et al., 2006; Krell et al., 2007; Nedbal et al., 2000). The F_v/F_m increase in exponentially growing control- and P-limited cultures was associated with increasing cell concentrations (Fig. 3), and apparently results from acclimation to decreasing light availability in increasingly dense cultures. Similar effects are known from the chlorophyte macroalga *Cladophora* sp., where increased F_v/F_m values are reported for light-limited environmental samples (Hiriart-Baer et al., 2008), and from cultured isolates (Clades A, B and F) of the symbiotic dinoflagellate *Symbiodinium*, for which acclimatization to high light was associated with reduced F_v/F_m values (Robison and Warner, 2006).

Table 3
Numbers of differentially expressed genes (SAM-based q-value < 1% and fold-change ≥ 1.5) showing the same trend in several comparisons.

Stationary phase		N-limitation		P-limitation		Nutrient limitation		Stationary phase-control		Transition to stationary phase	
Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up
197	292	26	35	3	19	2	3	8	0	5	0

Stationary phase: significantly up- or down-regulated in all stationary-phase samples in relation to the control culture in exponential phase; N-/P-limitation: differentially expressed and showing the same trend between stationary-phase N- or P-limited cultures and both exponentially growing and stationary-phase control samples; nutrient limitation: identified for N- and P-limitation and same trend in both; stationary phase-control: differentially expressed between stationary and exponentially growing control and stationary phase control and both stationary-phase N- and P-limited cultures; transition to stationary phase: differentially expressed and showing the same trend in all comparisons between Day 5 samples and the control cultures in exponential phase.

Table 4
Selection of genes identified as differentially expressed in all stationary-phase regimes when compared to control in exponential growth.

Contig name	Stationary versus exponential control			Gene product	Function
	Control	P-limited	N-limited		
Amin_09g12r	−2.91	−2.33	−3.65	ABC (ATP-binding-cassette) transporter protein	Transport
Amin_15e01r	−2.98	−2.62	−2.78	Calcium-activated potassium channel	Transport
Amin_51h24f	−3.74	−2.66	−3.31	Putative sugar transporter family protein	Transport
Amin_07f04r	−2.38	−2.76	−2.50	Calcium/calmodulin-dependent protein kinase	Intracellular signalling
Amin_08d07f	−9.27	−5.28	−6.98	Putative intracellular signalling protein	Intracellular signalling
Amin_42g08r	−3.74	−4.11	−3.93	Putative mitochondrial protein, PRR repeat-containing	Organellar RNA-binding protein (putative)
Amin_12a02r	−4.38	−2.79	−2.12	60S ribosomal protein L10a	Organellar translation
Amin_34c10r	−1.99	−2.42	−6.07	Photosystem I P700 chlorophyll a apoprotein A1	Chloroplast
Amin_26m06r	−1.82	−2.05	−6.43	Photosystem II D2 protein	Chloroplast
Amin_06c10r	−3.82	−4.22	−4.59	Malate dehydrogenase	Citric acid cycle
Amin_57n13r	−5.78	−8.05	−23.75	Cytochrome b	Mitochondrial
Amin_38k24f	−1.75	−2.11	−8.58	Cytochrome c oxidase polypeptide I	Mitochondrial
Amin_52g07r	−1.69	−2.42	−3.87	DNA-3-methyladenine glycosylase	DNA repair
Amin_26g08f2	−2.17	−2.04	−2.29	Pre-mRNA-processing-splicing factor 8, C-terminal domain	Splicing
Amin_34h03r	−1.88	−2.15	−2.33	Putative small nuclear ribonucleoprotein polypeptide E	Splicing
Amin_33f12f	−6.92	−9.07	−9.81	18S rRNA	Translation
Amin_44f03f	−3.72	−2.41	−2.01	40S ribosomal protein S11	Translation
Amin_06b02r	−3.02	−2.94	−2.71	40S ribosomal protein S15	Translation
Amin_83a11r	−3.81	−2.35	−2.18	40S ribosomal protein S16	Translation
Amin_03c10r	−3.34	−2.50	−2.15	40S ribosomal protein S4	Translation
Amin_57f03r	−3.99	−3.10	−2.56	40S ribosomal protein S7	Translation
Amin_90e06f	−10.52	−7.02	−6.06	40S ribosomal protein S7.	Translation
Amin_48e09r	−1.94	−2.70	−3.48	50S ribosomal protein L14, mitochondrial or chloroplast	Translation
Amin_14a02r	−5.53	−7.67	−6.25	60S ribosomal protein L12	Translation
Amin_24d11f2	−4.33	−3.65	−2.89	60S ribosomal protein L6	Translation
Amin_13g03r	−4.49	−3.24	−2.21	60S ribosomal protein L6	Translation
Amin_78c12f	−4.21	−2.76	−2.80	60S ribosomal protein L7a	Translation
Amin_26m14r	−8.93	−15.02	−16.98	Ribosomal operon external transcribed spacer	Translation
Amin_75d07r	−4.67	−3.08	−2.64	Ribosomal protein S13	Translation
Amin_36k19f	−4.41	−3.29	−3.43	Translation elongation factor-like protein	Translation
Amin_07g02r	−3.07	−2.21	−2.52	tRNA (guanine-N1-)-methyltransferase	Translation
Amin_95c08r	−4.34	−2.26	−3.27	Sialyltransferase involved in protein glycosylation	Protein glycosylation
Amin_95c05r	−1.59	−2.15	−2.34	Probable E3 ubiquitin-protein ligase, HECT domain-containing	Protein degradation
Amin_11c08r	−2.76	−3.36	−3.70	Aspartyl proteinase family protein	Protein degradation
Amin_24h08f2	1.53	1.75	1.77	ABC-transporter protein	Transport
Amin_13d06r	3.76	3.59	3.27	ABC-transporter family protein	Transport
Amin_32d04f	2.18	2.18	2.27	Ion channel similar to voltage-gated cation channels	Transport
Amin_93m06f	1.61	2.40	1.91	TPT transporter family protein	Transport
Amin_68f12f	2.97	2.66	2.66	Inorganic H^+ pyrophosphatase, vacuolar-type	Intracellular pH regulation
Amin_08d12r	2.19	3.48	4.19	Vacuolar ATP synthase subunit B	Intracellular pH regulation
Amin_03h02f	2.18	2.33	3.96	14-3-3 protein	Intracellular signalling
Amin_84i17r	3.13	2.40	5.91	3′5′-Cyclic nucleotide phosphodiesterase family member	Intracellular signalling
Amin_56m09r	2.12	2.00	2.19	Calmodulin-like protein	Intracellular signalling
Amin_49d23r	2.25	2.00	1.86	cGMP-dependent protein kinase	Intracellular signalling
Amin_52f11f	1.60	2.05	2.04	Dual specificity phosphatase	Intracellular signalling
Amin_98e10f	2.06	2.53	2.47	Predicted Traf-like protein	Intracellular signalling
Amin_01h11r	3.94	3.05	6.62	Protein kinase similar to shaggy-related protein kinases	Intracellular signalling
Amin_88g07r2	3.54	2.85	3.53	Protein kinase, putatively calcium-dependent	Intracellular signalling
Amin_07c10r	6.15	5.15	6.04	Ras small GTPase, Rab type, probably involved in vesicle trafficking	Intracellular signalling
Amin_06f11f	1.59	2.42	2.65	Serine/threonine-protein kinase	Intracellular signalling
Amin_09a03r	3.11	2.70	2.76	Serine/threonine-protein phosphatase	Intracellular signalling
Amin_68g05r	3.86	5.22	6.06	Pentatricopeptide (PPR) repeat-containing protein	Organellar RNA-binding protein (putative)
Amin_84m07f	4.21	2.42	4.20	Pentatricopeptide (PPR) repeat-containing protein	Organellar RNA-binding protein (putative)
Amin_34g06f	2.61	4.45	4.10	Putative PPR repeat protein	Organellar RNA-binding protein (putative)
Amin_64f10f	2.36	3.33	6.73	Caroteno-chlorophyll a–c-binding protein	Chloroplast
Amin_78d01f	6.17	4.41	4.83	Light-harvesting chlorophyll a–c binding protein	Chloroplast
Amin_41p17r	2.87	2.87	5.50	Light-harvesting chlorophyll a–c binding protein	Chloroplast
Amin_06b05r	1.80	2.00	2.63	Phosphofructokinase family protein	Chloroplast
Amin_61f12r	2.23	3.02	2.55	Fumarate hydratase, putative	Mitochondrial
Amin_07a03r	2.28	1.92	1.98	Arp2/3 complex, subunit 2 (p34-Arc)	Cytoskeleton
Amin_21f05r	2.05	2.21	3.03	Actin	Cytoskeleton
Amin_09e07f	2.69	2.95	2.66	C-terminal motor kinesin	Cytoskeleton
Amin_74g08f	3.59	5.16	4.85	Dynein heavy chain family protein	Cytoskeleton
Amin_07f07r	1.76	1.96	4.48	LisH domain-containing protein	Cytoskeleton
Amin_55d08r	2.05	2.16	2.51	Putative myosin, N-terminal WD 40-repeats	Cytoskeleton
Amin_06h09r	2.54	2.36	2.19	Ribonuclease HII	Putatively reverse transcription
Amin_58c02r	2.81	3.45	3.74	Putative reverse transcriptase	Reverse transcriptase
Amin_09f06f	1.71	1.96	2.08	Amine oxidase	Amine metabolism
Amin_33f09r	2.59	2.97	3.45	Putative D-3-phosphoglycerate dehydrogenase	Amino acid biosynthesis
Amin_77b01r	3.53	2.93	3.16	Putative sterol 3-beta-glucosyltransferase, partial sequence	Sterol modification
Amin_60e01f	2.02	4.96	3.30	Glycoside hydrolase family 28 family member	Glycoside hydrolase
Amin_47c09f	3.42	2.87	2.67	Adenylosuccinate lyase	Nucleotide metabolism
Amin_83d02r2	2.87	2.25	2.15	Guanine deaminase	Nucleotide metabolism

Table 4 (*Continued*)

Contig name	Stationary versus exponential control			Gene product	Function
	Control	P-limited	N-limited		
Amin_46e08r	4.05	4.23	2.56	Putative uridine-binding protein	Nucleotide metabolism
Amin_08h02r	1.79	2.11	2.16	DNA-directed RNA polymerases I, II, and III subunit RPABC3	Transcription
Amin_03c05r	2.17	2.47	3.81	Ribosomal operon external transcribed spacer	Translation
Amin_44h09f	2.46	2.97	3.13	Glycyl-tRNA synthetase	Translation-tRNA-related
Amin_69g10f	1.74	2.73	2.00	Phenylalanine-tRNA synthetase	Translation-tRNA-related
Amin_74a06r	1.82	2.09	2.19	tRNA-dihydrouridine synthase 3-like	Translation-tRNA-related
Amin_08c10f	2.74	2.72	3.19	Peptidylprolyl isomerase	Protein folding
Amin_46g06r	2.42	2.22	2.35	Diphthine synthase	Protein modification
Amin_07g04f	2.07	1.94	2.47	Aspartic protease	Protein degradation
Amin_07g03r	2.21	2.36	2.44	Proteasome subunit alpha	Protein degradation
Amin_42o14f	1.51	2.35	1.90	Ubiquitin-specific protease, putative	Protein degradation

The decrease in F_v/F_m measured in all stationary cultures may correspond to a higher proportion of damaged PSII reaction centers due to impaired repair mechanisms (Lippemeier et al., 2001; Takahashi and Murata, 2008) or to down-regulation of photosynthesis-associated processes in non-growing cells. The lower demand for carbon compounds, ATP and redox equivalents (see Geider et al., 1993) in combination with the reduced availability of dissolved CO_2 at higher pH values in older cultures, can lead to a diversion of photosynthetic electrons to oxygen, resulting in the production of reactive oxygen species (ROS) (Vardi et al., 1999). Limitation of photosynthetic CO_2 fixation can decreases the consumption of the reducing agent NADPH, potentially leading to depletion of its reduced form $NADP^+$, the major acceptor of electrons for PSI. This increases the transfer of electrons from PSI to molecular oxygen. The generated ROS impede the repair of photodamaged PSII by inhibiting the synthesis of new D1 protein at the elongation step of translation, leading to photoinhibition (reviewed by Takahashi and Murata, 2008).

4.1.3. Factors influencing toxin content and allelochemical activity

Similar to other studies on various PSP toxin-producing *Alexandrium* species (John and Flynn, 2000; Leong et al., 2004; Lippemeier et al., 2003), intracellular PSP toxin content in our cultures was closely linked to nutritional status. This sensitivity to nutrient limitation has been attributed to variation in intracellular concentrations of arginine (John and Flynn, 2000), which is a biosynthetic precursor of PSP toxin biosynthesis in cyanobacteria and likely also in dinoflagellates (Kellmann and Neilan, 2007; Shimizu, 1982). Anderson et al. (1990) found that under most growth conditions, in laboratory cultures of *Alexandrium fundyense*, cellular concentrations of free arginine were low when toxin content peaked but increased rapidly as toxin content declined. This relationship also held for P-limited cultures. While intracellular toxin quota cannot be interpreted as a direct measure for the rate of toxin production (Cembella, 1998), P-limitation in *Alexandrium* tends to lead to an increase in toxin not only on a cellular but also on a culture volume basis (Lippemeier et al., 2003). Anderson et al. (1990) explained this observation by the existence of a saxitoxin biosynthetic pathway that continues to operate even after the cessation of cell division, and which then depletes cellular arginine pools with greatly reduced competition from other pathways. Lippemeier et al. (2003) attributed the elevated toxin production during P-limitation to a potential arrest of the cell cycle in G1, the cell cycle stage when toxin synthesis occurs (Taroncher-Oldenburg et al., 1997). This arrest in G1 would imply a continuous expression of G1-specific genes, which should include at least some of the genes coding for PSP toxin biosynthetic enzymes.

Allelochemical activity against *R. salina*, unlike PSP toxin content, did not respond in a notably treatment-specific pattern (Fig. 4B). In contrast to the situation in the haptophyte *Prymnesium parvum*, in which lytic activity is considerably induced by P- and often also by N-starvation (Beszteri et al., submitted for publication; Granéli and Johansson, 2003), the pattern observed in *A. minutum* is more reminiscent of an accumulation with culture age that might be secondarily influenced by nutrient availability (Fig. 5B). Simple calculations assuming a constant production rate and the decreasing growth rates from Fig. 1B predict a roughly 4-fold increase in per cell extracellular toxicity from initial to stationary phase cultures. A roughly 10-fold increase (Fig. 5) thus indicates that an increase in production by growth limitation acting non-discriminately under P- and N-limitation and in the stationary-phase control cultures cannot be ruled out. This would be in contrast to *A. tamarense*, where a maximal two-fold increase in lytic activity per cell in stationary phase was measured (Ma et al., 2010). In order to further illuminate the factors influencing synthesis and accumulation of the lytic compounds of *Alexandrium* spp., highly targeted datasets combining a high number of measurements of allelochemical activity with finely graded variations of the physiological parameters seem most promising. The elucidation of the chemical composition and structure of those compounds is currently under way (Ma et al., 2009, 2011).

Table 5
Genes in stationary phase control cultures which were down-regulated relative to both exponential-phase and nutrient-limited cultures.

Contig name	Control stationary phase versus control exponential	Stationary versus stationary		Gene product
		N-limited/control	P-limited/control	
Amin_17h12f	−6.50	16.60	7.04	Hypothetical protein similar to subtilase family peptidases
Amin_09f12f	−5.93	5.47	2.58	Putative aminopeptidase
Amin_07e10f	−5.13	5.97	8.56	Hypothetical protein similar to alcohol dehydrogenase
Amin_12a02r	−4.38	2.07	1.57	60S ribosomal protein L10a
Amin_64e04r	−4.04	2.44	1.89	60S ribosomal protein L22
Amin_61a05r	−2.95	1.77	1.99	60S ribosomal protein L21
Amin_07h11f	−4.52	3.10	2.48	Hypothetical protein
Amin_54e08r	−2.67	2.38	1.85	Hypothetical protein

Table 6
Selection of genes identified as differentially expressed in stationary-phase nutrient-limited cultures when compared to control cultures in both exponential and stationary growth phase.

Contig name	N-limitation	P-limitation	Stationary versus exponential control			Stationary versus stationary			Gene product
			Control	P-limited	N-limited	N-limited/ control	P-limited/ control	P-limited/ N-limited	
Amin_57f07r		Down		−3.31			−3.21		Sodium-glucose cotransporter
Amin_60e01f		Up	2.02	4.96	3.30		2.51		Glycoside hydrolase family 28 family member
Amin_69g10f		Up	1.74	2.73	2.00		1.57		Phenylalanine-tRNA synthetase
Amin_37h11f		Up		2.08	2.67		1.54		Putative NALP-related protein similar to NOD3
Amin_53g06f	Down				−1.85	−1.81			Aldo-keto reductase family protein
Amin_57n13r	Down		−5.78	−8.05	−23.75	−4.22		2.96	Cytochrome b
Amin_14a08f	Down				−3.14	−2.39		3.02	Cytochrome b6
Amin_04f11f	Down		−2.35		−10.25	−4.76		5.85	Cytochrome b6–f complex subunit 4
Amin_38k24f	Down		−1.75	−2.11	−8.58	−4.34			Cytochrome c oxidase polypeptide I
Amin_34c10r	Down		−1.99	−2.42	−6.07	−3.06			Photosystem I P700 chlorophyll a apoprotein A1
Amin_89e05r	Down			−2.26	−6.28	−3.48		2.97	Photosystem I P700 chlorophyll a apoprotein A1
Amin_26m06r	Down		−1.82	−2.05	−6.43	−3.50		3.05	Photosystem II D2 protein
Amin_46g07r	Down			−2.43	−9.71	−5.69			Photosystem Q(B) protein
Amin_83b01r	Down			−2.23	−4.26	−3.47			Putative esterase
Amin_64f10f	Up		2.36	3.33	6.73	2.62			Caroteno-chlorophyll a–c-binding protein
Amin_41p17r	Up		2.87	2.87	5.50	1.74			Light-harvesting chlorophyll a–c binding protein
Amin_98b11f	Up		1.90		3.92	1.93		−2.94	Ribulose-bisphosphate carboxylase form II
Amin_11a02f	Up		2.35	3.62	6.69	2.94			Hypothetical protein similar to metal-dependent hydrolase ElsH
Amin_18f03f	Up				2.50	2.39			Hypothetical protein similar to sodium channel proteins
Amin_100c02r	Up			2.03	2.70	2.18			Major intrinsic protein, putative aquaporin
Amin_71d02r	Up		1.72		2.88	1.68	−2.77	−4.57	Pre-mRNA-processing factor
Amin_36g07r	Up		1.51		2.61	1.70			skp1 family protein
Amin_84i17r	Up		3.13	2.40	5.91	1.84		−2.48	3′5′-Cyclic nucleotide phosphodiesterase family member
Amin_95f08r	Up				1.96	1.58			ADP-ribosylation factor-like protein 3
Amin_71f07r	Up				1.92	1.98			vWF domain-containing protein

4.2. Differential gene expression

Dinoflagellates are evolutionarily distant from most genomic model organisms, even among alveolates. Functional annotation of their genes is therefore often difficult (John et al., 2004). With the *A. minutum* EST library herein analyzed, BLAST-based automated annotation as a starting point for manual annotation was available for only 28% of the sequences (Yang et al., 2010b). In any case, physiological responses of the toxigenic dinoflagellate *A. minutum*, including growth kinetics and nutrient-dependent limitation, PSP toxin biosynthesis and cell content, and allelochemical activity, are reflected in comparisons of gene expression among cultures grown under different environmental regimes. While this restricted availability of genomic information precluded pathway reconstruction, we nevertheless observed patterns characteristic for the processes involved in acclimation to the different treatments.

41% of all tested sequences were differentially expressed between any of the growth-limited treatments and the exponential-phase control samples (Tables 2a and 2b).

This is a somewhat higher change rate than reported from the related species *A. tamarense* in a recent massively parallel signature sequencing (MPSS) study (Moustafa et al., 2010): in *A. tamarense*, 73% of transcripts remained uniformly abundant irrespective of N-or P-limitation or the presence or absence of bacteria. Whether this difference is due to true differences in the prevalence of transcriptome-level gene regulation, or due to the different experimental setups, remains to be tested.

4.2.1. Growth stage dependent gene expression

The high number of genes consistently up- or down-regulated in all stationary-phase cultures when compared to the exponentially growing control (Table 3) mirrors the fundamental physiological differences between the different culture growth stages. Gene expression differed considerably more among growth stages than between different treatments at the same growth stage (Tables 2a, 2b and 3), demonstrating an essential dissimilarity between actively growing and non-growing cells. An equivalent effect is documented in yeast, where the stages of fermentation, which correspond to the different growth stages in batch cultures, can affect the overall gene expression to a considerably greater degree than the fermentation medium or even the strain examined (Rossouw and Bauer, 2009).

Not unexpectedly, the largest functional group among the genes higher expressed in exponential phase comprised genes involved in translational processes (Table 4). This is in agreement with the high number of sequences involved in protein synthesis and translational regulation identified in a recent study in *Alexandrium catenella*, which involved a gene expression library of exponential-phase *A. catenella* subtracted with a stationary-phase sample (Toulza et al., 2010). These authors considered the expression of the ribosomal component 5.8S rRNA as reflecting the whole-cell metabolic activity. Following this suggestion, the reduced expression of 14 ribosome-connected sequences is an indication of reduced translational and metabolic activity associated with stationary phase. While a high incidence of post-

transcriptional regulation in dinoflagellates has frequently been reported (Lapointe and Morse, 2008; Lidie, 2007; Rossini et al., 2003), we found only one translation elongation factor-like protein and two splicing-related proteins to be down-regulated across all treatments.

In all stationary-phase treatments, gene expression patterns revealed evidence of down-regulation of photosystem components. Reduction of the number of photosystem reaction centers, otherwise known as a photoacclimation strategy (e.g. Ragni et al., 2010), is apparently linked to the reduced F_v/F_m values in stationary-phase cultures. This is likely to be either a response to photoinhibition due to ROS production linked to reduced photosynthetic CO_2 fixation, or an acclimation response to avoid such damage. The main causes for the stationary-phase reduction in carbon fixation differ among treatments. In control and N-limited cultures, this effect may be directly caused by limiting CO_2 concentrations (see above) and reduced amounts of the CO_2-fixing enzyme (Huang et al., 2004), respectively. The reason for the reduced carbon fixation in P-limited cultures is less well understood, but appears to be related to a decrease in photosynthetic electron transport capacity, which decreases the ATP content and hence CO_2 fixation, while increasing the likelihood of damage from excess absorbed excitation energy (Lin et al., 2009; Moseley et al., 2006). Both photosynthetic performance and the related gene expression are most affected in the N-limitation treatment.

The most prevalent functional group among the genes higher expressed in all of the stationary-phase cultures were sequences involved in intracellular signalling, which might be due to an increase in stress-related effects. Like the differential expression of translation genes and photosynthesis-related gene expression, the importance of intracellular signalling-related sequences has also been noted in *A. catenella* (Toulza et al., 2010).

As the group of growth-state indicative genes identified here is correlated with the difference between exponentially growing cells and cells limited by different environmental factors, it provides an interesting starting point to identify marker genes or marker gene expression profiles to identify the level of growth limitation in mesocosm experiments or in potential *A. minutum* bloom populations.

4.2.2. Gene expression specifically linked to stationary phase in control cultures

While a high number of genes were differentially expressed between exponentially growing controls and the same cultures in stationary phase, significant expression differences between the stationary-phase samples from different treatments were rare (Table 2b). Of the 8 genes identified as specifically down-regulated in the stationary-phase control cultures as compared to both the exponential-phase control and the nutrient-limited cultures, three sequences were identified as ribosomal proteins, indicating a further down-regulation of the translational machinery when compared to the nutrient-limited cultures (Table 5).

Control cultures reached higher pH levels than the other treatments, and their growth might have been limited by availability of dissolved organic carbon (see above). The typical dinoflagellate response to a reduced concentration of dissolved CO_2 is an increase in amount and per-cell-activity of the enzymes involved in the dehydration of HCO_3, the extracellular and intracellular carbonic anhydrases (CAs) (Berman-Frank et al., 1994; Lapointe et al., 2008; Ratti et al., 2007). We identified an extracellular δ-CA similar to that of the bloom-forming marine dinoflagellate *Lingulodinium polyedrum* (Lapointe et al., 2008; see Toulza et al., 2010), as well as an intracellular CA, which were both significantly higher expressed in the stationary-phase control cultures than in the exponentially growing control (Supplementary Table 2).

4.2.3. Nutrient limitation-related gene expression patterns

Metabolic pathways and related gene expression conceivably affected by nutrient supply include mechanisms of active transport, nutrient assimilation and sequestration, as well as biosynthetic pathways leading to core components (proteins, nucleic acids, lipids) and secondary metabolites. Indeed, this is reflected in the gene expression data for *A. minutum*, as 23% of the genes tested were differentially expressed between at least one of the nutrient-limiting treatments and exponentially growing control cultures. This ratio is comparable to the percentage of differentially expressed genes between exponentially growing cultures at different salinities (Yang et al., 2010a).

Most of the changes were associated with the difference between exponentially growing and stationary-phase cultures irrespective of the limiting factor, but 87 genes specifically responding to N- or P-starvation were identified (Tables 3 and 6).

The most conspicuous group among these nutrient-limitation responsive sequences comprised the chloroplast-associated genes differentially regulated under N-limitation. For example, 8 mRNAs encoding cytochromes and photosystem proteins were on average 9.3 times less expressed under N-limitation than in the exponentially growing control, and those for two chlorophyll a–c-binding proteins and a ribulose-bisphosphate carboxylase (RubBisCO) were up-regulated by an average factor of 5.4 (Table 6). This is consistent with the marked N-dependency of these two core components of the photosynthetic apparatus. The key carbon fixation enzyme RubBisCO catalyzes the rate-limiting step of light-independent photosynthetic reaction and is generally the most abundant protein in photosynthetic organisms. Levels of RubBisCO can be severely compromised under N-limitation (Huang et al., 2004; Sims et al., 1998), which can limit overall photosynthesis (Parry et al., 2008). The reduced CO_2 fixation can lead to substantially increased ROS production (see above) and cause enhanced photoinhibition (Takahashi and Murata, 2008). An up-regulation of RubBisCO on the mRNA level might partially compensate this effect. Synthesis of the chlorophyll tetrapyrrole skeleton is dependent on the availability of amino acid precursors (reviewed in Nogaj et al., 2005), and lower chlorophyll quotas in N-limited cells are common (Sciandra et al., 2000; Verhoeven et al., 1997). This can be associated with reduced amounts of light harvesting complexes, while other components of the light harvesting machinery shift abundance (Peltier and Schmidt, 1991). The observed adjustments of the mRNA levels of chlorophyll-binding proteins in connection with reduced mRNA amounts of other photosystem proteins and different cytochromes are consistent with these phenomena. They are reflected in the decreased potential quantum efficiency of PSII (Fig. 4) in the N-limited cultures. Similar strong effects of N-starvation on photosynthetic performance are known from a variety of phytoplankton species (Flynn et al., 1994; Juhl, 2005; Lippemeier et al., 2001).

4.2.4. Transition to stationary phase

The 5 genes up-regulated at the transition to stationary phase in all treatments (Table 3) are associated with the onset of limitation or with a slowing of growth. As such, they constitute putative candidates as markers for the onset of growth limitation and potentially bloom breakdown.

In *A. tamarense*, nutrient limitation can lead to bloom breakdown by induction of resting cyst formation, which tends to remove considerable fractions of the vegetative population (Anderson and Lindquist, 1985; Ichimi et al., 2001; Yamamoto et al., 2002). While N- and P-limitation can promote cyst production in *A. minutum* under laboratory conditions (Blanco, 1995; Figueroa et al., 2007), bloom decline cannot usually be attributed to encystment (Garcés et al., 2004), as cyst production in

natural bloom populations is apparently a constant process, involving a continual small percentage of cells throughout most of the bloom duration (Garcés et al., 2004; Pitcher et al., 2007).

Nevertheless, in addition to the obvious implications of a marker for restricted growth, physiological state might influence the susceptibility of *A. minutum* to parasites and pathogens (Llaveria et al., 2010). This would indicate an increased likelihood of bloom termination by otherwise tolerated (but see Chambouvet et al., 2008) levels of infection (Figueroa et al., 2008). Furthermore, a decrease in growth rate can lead to significant reductions of the dinoflagellate population by microzooplankton grazing (Calbet et al., 2003), which might otherwise have little effect on an established bloom (Estrada et al., 2010; Van Lenning et al., 2007).

The genes associated with the transition to stationary phase included a serine/threonine-protein phosphatase, potentially a regulator of enzyme activity, and an ABC transporter probably involved in active transport across membranes. The transporter sequence was the only one of the transition phase-associated sequences that was not identified as associated with stationary phase as well. This reinforces the potential utility of these genes as markers for early stages of limitation.

4.2.5. Toxin-related gene expression

Our gene expression results contribute little to the elucidation of the regulation of PSP toxin content. The recently discovered dinoflagellate PSP toxin-associated genes *sxtA1* and *sxtA4* (Stüken et al., 2011) cannot be identified by BLAST searches against our EST database, which is probably due to its limited size. While more than half of the genes differentially expressed between the highly toxin-containing P-limited cultures and the low-toxin N-limited cultures could be annotated, all inferred functions suggested involvement in the regulation of core metabolic responses (Table 7). Nevertheless, the differential expression pattern of two of the non-annotatable sequences was consistent with an involvement in the regulation of PSP toxin levels; Amin_21a03r was up-regulated under both beginning and severe P-limitation and down-regulated under severe N-limitation when compared to any other condition, and Amin_78e07r was higher expressed under P-limited than N-limited conditions, less expressed under N-limited than under stationary phase control conditions, and not significantly differentially expressed in any other comparison (Table 7). Additionally, earlier BLAST comparisons had identified a sequence similar to Amin_78e07r in the related PSP toxin-producing *A. tamarense*, but not in any other dinoflagellate, including several other PSP toxin producing and non-PSP-toxin-producing *Alexandrium* species (Yang et al., 2010b). However, these BLAST comparisons were based on non-exhaustive EST databases, and absence of a sequence in these databases is not conclusive evidence of absence in the respective organism. Nevertheless, as these two sequences are genes of unknown function with an expression pattern suggestive of involvement in PSP toxin regulation, they warrant further examination in more targeted experiments.

Apart from the effect of nutrient status, PSP toxin cell quotas in *Alexandrium* can also be manipulated by the presence of certain species of copepod grazers (Selander et al., 2006). Two recent studies investigated changes in the transcriptome of *A. tamarense* and *A. minutum* in cultures with elevated cell toxin quotas after exposure to such grazers (Wohlrab et al., 2010; Yang et al., 2011). The same microarray probes were used in these investigations as in the present study, either alone or as part of a larger set. Comparing these results to the transcriptomic dataset presented here revealed a number of genes associated both with stationary phase and with response to copepod presence (Supplementary Table 1). Interesting gene expression patterns include those of contig Amin_56a03r, which was induced to higher expression levels by copepod presence in both *Alexandrium* species, were earlier identified to be specifically associated with toxigenic strains of *A. minutum*, and were associated with stationary phase. Contig Amin_14a08f, which was higher expressed in *A. tamarense* exposed to the copepod *Calanus helgolandicus*, was consistently down-regulated in the low-toxin-containing N-limited cultures.

4.2.6. Gene expression and spliced leader-transsplicing

In analogy to the situation in trypanosomes, the phenomenon of mRNA transsplicing in dinoflagellates has been suggested to be associated with post-transcriptional control of gene expression (Bachvaroff and Place, 2008; Monroe and Van Dolah, 2008). Our data on the percentages of differentially expressed *A. minutum* sequences that contain the typical transsplicing-associated spliced leader (SL) sequence suggests that this mechanism in dinoflagellates is not associated with a lack of regulation at the transcriptional level.

Table 7
Complete list of genes differentially expressed between P-limited and N-limited cultures.

Contig name	Stationary versus exponential control			Stationary versus stationary			Gene product
	Control	P-limited	N-limited	N-limited/control	P-limited/control	P-limited/N-limited	
Amin_71d02r	1.72		2.88	1.68	−2.77	−4.57	Pre-mRNA-processing factor
Amin_84i17r	3.13	2.40	5.91	1.84		−2.48	3′5′-Cyclic nucleotide phosphodiesterase family member
Amin_97h03r	1.64		2.73			−2.39	High-affinity nitrate transporter, putative
Amin_95g04f	−2.76		−3.36		2.73	3.39	Ubiquitin conjugation factor E4 B
Amin_98b11f	1.90		3.92	1.93		−2.94	Ribulose-bisphosphate carboxylase form II
Amin_57n13r	−5.78	−8.05	−23.75	−4.22		2.96	Cytochrome b
Amin_89e05r		−2.26	−6.28	−3.48		2.97	Photosystem I P700 chlorophyll a apoprotein A1
Amin_14a08f			−3.14	−2.39		3.02	Cytochrome b6
Amin_26m06r	−1.82	−2.05	−6.43	−3.50		3.05	Photosystem II D2 protein
Amin_04f11f	−2.35		−10.25	−4.76		5.85	Cytochrome b6–f complex subunit 4
Amin_09e10r						−4.46	Hypothetical protein
Amin_60e06r	−1.88	−2.28		2.13		−2.60	Hypothetical protein
Amin_78e07r				−1.72		2.16	Hypothetical protein
Amin_93o20f	−4.47	−6.92	−18.07	−4.17		2.68	Hypothetical protein
Amin_22d03r			−2.19			2.79	Hypothetical protein
Amin_42m14r	2.69	6.78	2.46		2.51	2.82	Hypothetical protein
Amin_66a07f	−2.13	−2.25	−6.69	−3.33		2.99	Hypothetical protein
Amin_25j23r			−5.59	−3.87		3.32	Hypothetical protein
Amin_21a03r		3.37	−2.06	−2.72	2.58	7.08	Hypothetical protein

Whereas 4.7% of all the sequences in our normalized EST library contained an SL sequence, the same was true for 4.4% of the genes differentially expressed between one of the treatments and the exponential-phase control samples, and for 5.8% of those showing the same trend of differential expression in several comparisons. As many of these ESTs are incomplete and 3′end-biased representations of the corresponding mRNAs, SL sequences are probably hugely underrepresented. The mentioned percentages should therefore not be taken as indicative of the prevalence of SLs in *A. minutum* mRNAs. While the equal representation of spliced leader sequences among the differentially expressed genes does not imply any specific function of this sequence, the data presented here are in agreement with those of Zhang and Lin (2009), who found SLs associated with all sequences examined. This indicates that even if SL trans-splicing is involved in the post-transcriptional regulation of gene expression, e.g. in mRNA stabilization or in the recruitment into the actively translated mRNA pool, it is probably not restricted to constitutively transcribed genes.

5. Conclusion

The dataset presented here constitutes a comprehensive series of measurements related to bloom-relevant factors such as toxin content, allelochemical activity, nutrient limitation and growth in *A. minutum*. While the growth stages of batch cultures are a rather crude model for the stages of bloom development, they nevertheless permit a comparison between actively proliferating and non-growing cells. Using extensive cross-comparisons of the differential gene expression responses, we identified narrow gene expression patterns linked with specific physiological factors such as exponential growth, cessation of growth or nutrient limitation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2011.08.012.

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