



# Predator-induced defence in a dinoflagellate generates benefits without direct costs

Fredrik Ryderheim<sup>1</sup> · Erik Selander<sup>2</sup> · Thomas Kjørboe<sup>1</sup>

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## Abstract

Inducible defences in phytoplankton are often assumed to come at a cost to the organism, but trade-offs have proven hard to establish experimentally. A reason for this may be that some trade-off costs only become evident under resource-limiting conditions. To explore the effect of nutrient limitation on trade-offs in toxin-producing dinoflagellates, we induced toxin production in *Alexandrium minutum* by chemical cues from copepods under different levels of nitrogen limitation. The effects were both nitrogen- and grazer-concentration dependent. Induced cells had higher cellular toxin content and a larger fraction of the cells was rejected by a copepod, demonstrating the clear benefits of toxin production. Induced cells also had a higher carbon and nitrogen content, despite up to 25% reduction in cell size. Unexpectedly, induced cells seemed to grow faster than controls, likely owing to a higher specific nutrient affinity due to reduced size. We thus found no clear trade-offs, rather the opposite. However, indirect ecological costs that do not manifest under laboratory conditions may be important. Inducing appropriate defence traits in response to threat-specific warning signals may also prevent larger cumulative costs from expressing several defensive traits simultaneously.

## Introduction

Dinoflagellates of the genus *Alexandrium* produce neurotoxic alkaloids collectively known as paralytic shellfish toxins (PST). The toxins are efficient sodium-channel blockers and among the most potent toxins known [1]. The intracellular toxin content is upregulated in response to the level of threat from zooplankton grazers [2] and, while debated, toxicity as a defence mechanism against grazers is the favoured explanation for the evolution of algal toxins [3–6].

Studies dedicated to defence mechanisms in phytoplankton often focus on the benefits of the defence, but

rarely establish potential costs [7]. So far, experimental assessments have suggested toxin production trade-offs to be insignificant. The growth rate of toxic and non-toxic strains of the same species, or grazer-induced versus non-induced cells with very different toxin contents appears to be identical [2, 8, 9]. Blossom et al. [10] compared several species and strains of *Alexandrium* spp. and did not find any correlation between growth rate and toxin production under light-replete conditions, and even a positive correlation under limiting light. Brown & Kubanek [11] recently demonstrated a negative relation between toxin content and growth rate in *Alexandrium minutum* exposed to lysed cells of various other species of dinoflagellates, thus suggesting a trade-off. However, the correlation may also result from allelochemical substances in the lysed cells reducing growth [12]. Many dinoflagellates produce such dissolved allelochemicals that reduce the growth rate of other cells [13] and when growth is reduced cells commonly become more toxic [14]. Significant costs of predator-induced toxin production have so far only been convincingly demonstrated in diatoms that produce domoic acid, but here the benefits of the toxins are still debated [15]. However, ecological theory predicts associated costs; otherwise, non-defended species or strains would be outcompeted and only defended species would persist. Also, toxin production is inducible, i.e. it is

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✉ Fredrik Ryderheim  
fry@aqu.dtu.dk

<sup>1</sup> Centre for Ocean Life, DTU Aqua, Technical University of Denmark, Lyngby, Denmark

<sup>2</sup> Department of Marine Sciences, University of Gothenburg, Göteborg, Sweden

upregulated in the presence of grazer cues, as seen in *Alexandrium* spp. dinoflagellates [2, 16, 17] and some toxic strains of the diatom *Pseudo-nitzschia* [15, 18]. According to optimal defence theory, inducible defences are favoured when predation risks vary in time and defence costs are significant [19, 20]. While these costs have likely been reduced through evolution, the wide variety of inducible defences found in both marine and terrestrial organisms suggests the presence of influential trade-offs to any beneficial defensive trait [21–24].

The failure of experiments to demonstrate costs may be due to the fact that experimental assessments have often been done under resource-replete conditions, while costs may be more significant when resources are limited [7, 20, 25–27]. The PST molecules are high in nitrogen with N:C ratio 4.6 times higher than average phytoplankton materials [28]. Numerous studies have shown cell toxin content to be low in nitrogen-depleted cells [29, 30] even when exposed to a grazer threat [9]. Trade-off costs may be trivial when nutrients and light are plentiful, but when available nitrogen is limiting and grazer biomass high, a fitness-optimization resource-allocation model predicts a significant growth penalty to toxin production [31]. Nitrogen limitation in temperate shelf regions generally occurs during the summer months [32, 33], and coincidentally it is during these months that defence is most needed due to peaking copepod biomass [33, 34].

Here, we quantify the benefits and costs of toxin production in *Alexandrium minutum* under different degrees of nitrogen limitation using both a chemostat approach and classical batch experiments. The compounds from zooplankton that trigger toxin production are known [35], and we use them for precise manipulation of toxin production without confounding effects of grazing on fitness estimates. The efficiency of the defence is estimated by video recording the response of copepods to induced and non-induced cells. Following the predictions of the model of Chakraborty et al. [31], we hypothesize that the costs of increased toxicity of induced cells will be the highest at intermediate nitrogen limitation, and that cells grown in excess of nitrogen will reap full benefit while paying negligible costs.

## Materials and methods

### Phytoplankton

*Alexandrium minutum* strain GUMACC 83 was grown in B1 medium [36] at salinity 26, 18 °C, and an irradiance of *ca.* 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  on a 12:12 light:dark cycle. To reduce the carry-over of nitrogen from the stock culture, we diluted cells in B1 with reduced  $\text{NO}_3^-$  (80  $\mu\text{M}$ ) for two

weeks prior to inoculation and used cells that were close to the end of the exponential phase.

### Batch-culture experiment

Six replicate batch cultures of *A. minutum* were initiated at *ca.* 200 cells  $\text{mL}^{-1}$  in 2 L blue-cap glass flasks exposed to *ca.* 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  on a 12:12 light:dark cycle and constant temperature of 18 °C. We used modified B1 medium with reduced nitrogen concentration (60  $\mu\text{M NO}_3^-$ ) to make sure that the cells eventually would be limited by nitrogen rather than other resources. The cultures were gently bubbled to avoid high pH-limiting growth. pH was monitored using a PHM220 Lab pH meter (Radiometer Analytical, Lyon, France). Three bottles were treated with copepodamides to induce increased toxin production [35] and three were used as controls. Copepodamides were extracted from freeze-dried *Calanus finmarchius* (Calanus AS, Tromsø, Norway) as described in Selander et al. [35] and added to the cultures by coating the inside of the bottle with a copepodamide mixture dissolved in methanol to a final concentration of 2 nM. Due to slow release and rapid degradation, the average effective concentration is around 1–2% of the added concentration [18]. The methanol was evaporated using  $\text{N}_2$  gas and the cultures transferred to the bottles after gentle mixing. Cultures were gently transferred to a freshly coated flasks every 24 h during the treatment period to assure a continuous exposure to the cues [18]. The controls received the same treatment but with methanol without copepodamides. Samples were taken every or every second day for cell abundance and nitrogen concentration, while samples for toxin analysis and cellular carbon and nitrogen were taken at inoculation and in conjunction with the six video experiments (see below) during the course of the experiment. Initial samples of cellular toxin-, carbon-, and nitrogen content were taken from the stock culture.

### Chemostat experiments

While nutrient concentration declines over time in batch cultures, they are near constant in a continuous culture, thus allowing us to examine costs and benefits of grazer induction at constant concentrations of nutrients. Dinoflagellates do not tolerate vigorous mixing [37], so a classical chemostat cannot be used. Instead, we used exponentially fed batch cultures [38]. The exponentially fed batch culture (hereafter referred to as ‘chemostat’) is similar to a chemostat, except that there is no continuous outflow. The volume is instead reduced to the initial volume at each sampling occasion after gently mixing the culture. Growth medium is added continuously in a constant proportion of the increasing volume of the culture by exponentially increasing the inflow using a computer-controllable

multichannel peristaltic pump (IPC 16, Ismatec, Wertheim, Germany).

Six replicate chemostat cultures of *A. minutum* were set up as in 1-L blue-cap glass bottles as described above. Depending on the dilution rate (DR), the initial culture volume varied between 250 and 500 mL. Four different DR were used to vary cell growth rate: 0.05, 0.10, 0.20, and 0.40 d<sup>-1</sup>. The inflow medium was B1 with reduced (80 µM) NO<sub>3</sub><sup>-</sup> in all the experiments, except the first one (0.10 d<sup>-1</sup> DR) where the NO<sub>3</sub><sup>-</sup> concentration was 30 µM. We increased the nitrogen concentration in the subsequent experiments as this allowed more cells for analyses and because pH limitation turned out not to be a problem. The resulting ambient nitrogen concentration is independent on nitrogen content of the inflow medium (Appendix 1). The cultures were gently bubbled and pH was measured at each sampling occasion. At each DR, the cultures were allowed 7–10 days to achieve steady-state before starting the experimental treatment. In some cases, perfect steady-state was not achieved.

The cultures were exposed to copepodamides daily as described above, using a nominal concentration of 0.63 nM. For the 0.2 d<sup>-1</sup> DR, a second experiment was run with higher copepodamide concentration of 6 nM to analyse the effect of increased exposure to grazers. Samples for analysis of cell abundance and size, cell toxin content, cellular carbon and nitrogen, dissolved inorganic nitrogen, and copepod rejection rate were taken daily or every 2–3 days during the 6–10-day treatment period. Using the chemostat equations (Appendix 1) and assuming a maximum growth rate of 0.5 d<sup>-1</sup> [39] and a half-saturation constant for nitrate of 0.5 µM [40], the resulting nitrate concentration in the cultures should range from severe limitation to saturation. Thus, while nutrient concentration decreases over time in batch culture, it is more constant and controlled by the DR in chemostats.

In the chemostats, the growth rate is controlled by the DR and any growth rate response will manifest as a change in the steady-state concentrations of cells and nutrients. Thus, if induced toxin production causes lower growth or nutrient affinity, the steady-state concentration of nutrients will increase and cell density decrease. The magnitude of the response can be computed from the chemostat equations (Appendix 1).

### Cell counts and cell size

Cell concentrations were determined in acid Lugol (1%) fixed samples. All cells in 1 mL or at least 400 cells were counted per replicate in a Sedgewick–Rafter chamber using an inverted microscope (Olympus, Tokyo, Japan). Twenty random cells from each sample were measured at 400×

magnification (width–length) and cell volume was estimated by assuming a prolate-spheroid shape [41]. Cell growth was calculated from temporal differences in cell concentration assuming exponential growth. The DR was accounted for when calculating growth in the chemostat experiments.

### Nitrate analysis

Subsamples for nitrate analysis were filtered through a 0.2-µm syringe filter, and stored frozen at –20 °C until analysis. Nitrate was analysed by reduction to NO<sub>x</sub> with VCl<sub>3</sub> as the reducing reagent [42] on a Smartchem 200 (AMS Alliance, Rome, Italy). Concentrations below 0.5 µM were measured using an extended cuvette (100 mm, FireflySci, New York, New York, USA) by UV–VIS spectrophotometry.

### Toxin analysis

Samples (10–120 mL) for cellular toxin contents were filtered onto 25-mm Whatman GF/F glass fibre filters and frozen at –20 °C until extraction. A total of 750 µL of 0.05 M acetic acid was added and samples were subjected to three freeze–thaw cycles to lyse cells. The extracts were filtered (GF/F) and stored at –20 °C until analysis. The samples from the batch experiment and the 0.2 d<sup>-1</sup> DR experiments (both low and high dose) were analysed with isocratic ion-pair chromatography followed by post-column derivatization and fluorescent detection [43]. We used a reversed phase C18 column (150 × 4 mm C18, 5 µm, Dr. Maisch GmbH, Ammerbuch, Germany). Samples from the 0.05, 0.10, and 0.40 d<sup>-1</sup> DR experiments were separated on an Agilent 1200 HPLC (ZIC-HILIC, 2.1 × 150 mm, 5 µm, Merck KGaA, Darmstadt, Germany) and analysed by tandem mass spectrometry on a triple-quadrupole instrument (Agilent 6470) following the methods described in Ref. [44].

This particular strain of *A. minutum* is known to only produce gonyautoxins (GTX) 1–4 [2, 45]. GTX standards 1–4 were obtained from the Certified Reference Materials Program at National Research Council Canada (Halifax, Canada).

### Cellular carbon and nitrogen

Samples for cellular C and N were filtered onto pre-combusted (550 °C, 2 hours) 13-mm GF/C filters, packed in tin capsules and dried for 24 hours at 60 °C. The samples were kept dry at room temperature in a desiccator until analysis with a Thermo Scientific Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

## Copepod feeding response

We directly observed individual copepod-cell interactions and recorded the fraction of captured cells that were rejected. We used the feeding-current feeding copepod *Temora longicornis* from a continuous culture that was maintained on a phytoplankton diet consisting of *Rhodomonas salina*, *Thalassioria weissflogii*, *Heterocapsa triquetra*, and *Oxyrrhis marina*.

Adult female copepods were tethered to a human hair by their dorsal surface using cyanoacrylate-based super glue [46]. The tethered copepods were starved overnight in darkness at the same temperature (18 °C) and salinity (26) as the cultures before being used for experiments. The tethered copepods are seemingly unaffected by the tethering and can live for many days while feeding, defecating, and producing eggs.

The feeding experiments took place in darkness. The untethered end of the hair was glued to a needle attached to a micromanipulator. The copepod was submerged in a  $10 \times 10 \times 10 \text{ cm}^3$  aquarium and *Alexandrium* cells were added to a final concentration of 100–200 cells  $\text{mL}^{-1}$ . The experiment started when cells were added. The water was gently mixed by a magnetic stirrer to keep the cells suspended. Three 10-min sequences (0–10 min, 25–35 min, and 50–60 min) of 24-fps footage were recorded during a 1-h period using a high-speed camera (Phantom V210; Vision Research, Wayne, New Jersey, USA) connected to a computer. The camera was equipped with lenses to get a field of view of  $1.3 \times 1 \text{ mm}^2$ . The video sequences were analysed to quantify prey capture, ingestion, and the fraction of cells that were rejected by the copepod [46]. A new copepod was used for each replicate culture.

## Statistical analysis

The effect of the copepodamide treatment in the batch-culture experiment was analysed using a generalized additive mixed model (GAMM) in the ‘*gam4*’ R package [47]. ‘Treatment’ and ‘Time’ were used as fixed effects and ‘Replicate’ as the random effect.

To analyse the effect of the copepodamide treatment in the chemostat experiments, we used a linear mixed-effects model with ‘Time’, ‘Treatment’, and ‘Dilution rate’ as fixed effects, and ‘Replicate’ as the random effect, in the ‘*lmerTest*’ R package [48]. The analysis of the repeated ‘High’ copepodamide-dose experiment was done separately. The Akaike information criterion was used to select the model that best fits the data. In case of a significant interaction between ‘Dilution rate’ and ‘Treatment’ a post-hoc test was made via pairwise comparisons by estimated marginal means using the Satterthwaite degrees-of-freedom method. The random-effect-variance component was close to zero for some variables, but retained in the model to

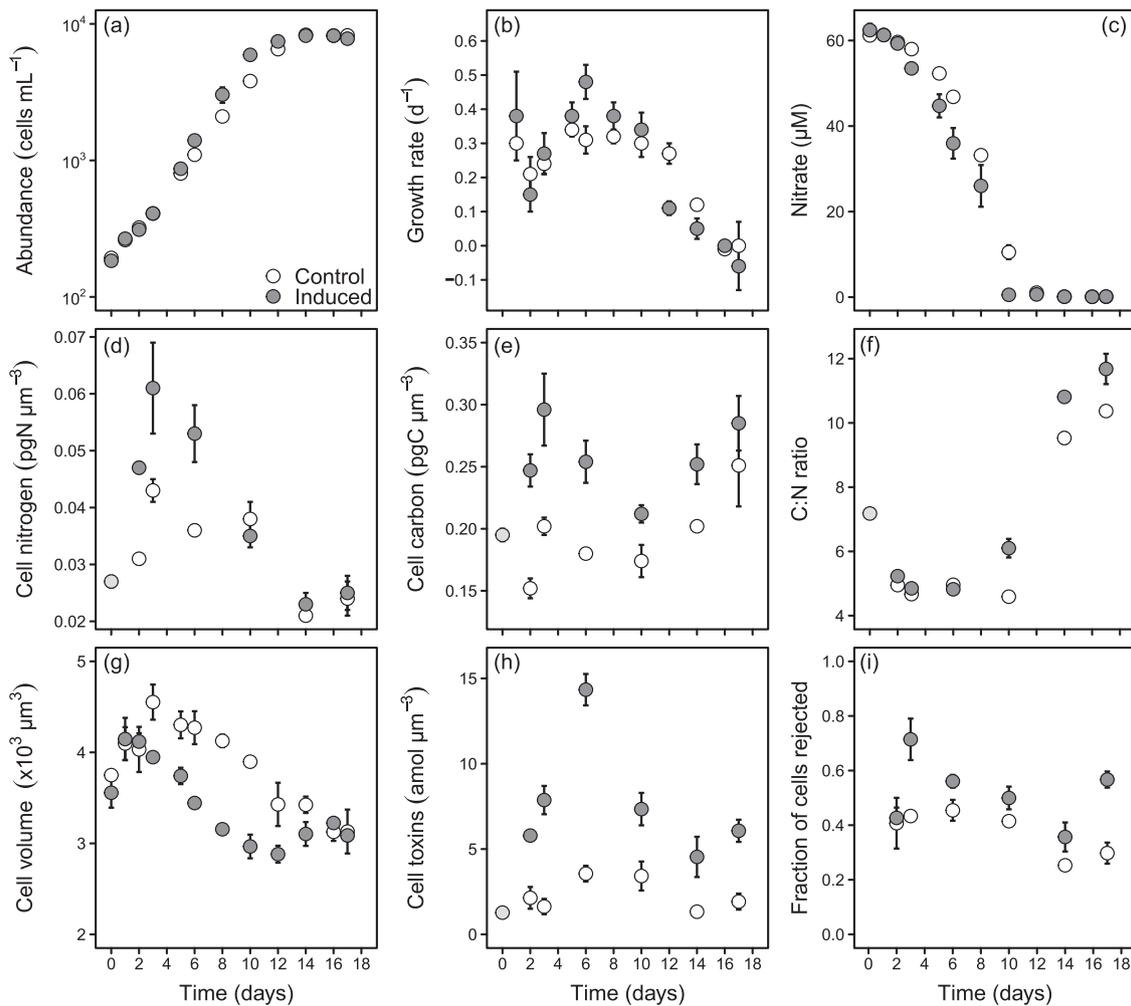
incorporate the dependency of the response variable on the replications. Some variables were log-transformed to homogenize variances. Statistical tests were considered significant at the 0.05 level and are summarized in Appendix Tables S1, S2, S3 and S4.

## Results

### Batch-culture experiment

Cell abundance in the grazer-induced cultures increased faster than controls during the exponential phase (days 3–12; GAMM,  $F = 14.0$ ,  $p < 0.001$ ) and reached the stationary phase after around 14 days as the inorganic nitrogen in the cultures became depleted (Fig. 1a, c). The available nitrate in the culture medium was used up at a significantly higher rate in the induced treatment (Fig. 1c; GAMM,  $F = 9.7$ ,  $p = 0.003$ ), because cell accumulation rate in terms of cellular nitrogen was also faster in induced than in non-induced cultures (Appendix 2 Fig. S1; GAMM,  $F = 11.0$ ,  $p = 0.003$ ). Cellular nitrogen content and cell sizes initially increased and then decreased as nutrients were exhausted and growth ceased, but induced cells had a significantly higher nitrogen content (Fig. 1d) and were significantly smaller (Fig. 1g, Appendix 2 Table S1) than non-induced cells during the exponential growth phase but converged with control cells after 10 and 16 days, respectively. Cellular carbon content was significantly higher in induced than in non-induced cells (Fig. 1e, Appendix 2 Table S1), resulting also in a faster accumulation of biomass during the exponential phase (Appendix 2 Fig. S1; GAMM,  $F = 61.8$ ,  $p < 0.001$ ). The differences in cellular C and N contents between induced and non-induced cultures on a per-cell level showed the same pattern, but were less pronounced (Appendix 2 Fig. S1). The carbon-to-nitrogen ratio also increased in response to nitrate depletion but more markedly so in induced treatments (Fig. 1f). Overall, cellular nitrogen content increased and cellular carbon content decreased with increasing growth rate and the contents of both nitrogen and carbon were higher in induced cells (Fig. 2, Appendix Table S2).

Cell toxicity peaked after 6 days of exposure in the induced treatments with 400% higher GTX content than controls, after which it decreased throughout the rest of the exponential phase (Fig. 1h). Toxin production essentially reached zero after 14 days but cell toxicity remained stable at around  $5\text{--}7 \text{ amol } \mu\text{m}^{-3}$  due to the low cell division rates. In the control treatment, cell toxicity followed the same temporal patterns but was lower throughout than in induced cells (Fig. 1h). Finally, a significantly higher fraction of induced than non-induced cells was rejected by copepods, demonstrating a clear benefit (Fig. 1i).



**Fig. 1** Changes over time in the batch-culture experiment. (a) cell abundance (cells mL<sup>-1</sup>), (b) growth rate (d<sup>-1</sup>), (c) culture nitrate concentration (µM), (d) cell nitrogen content (pg N µm<sup>-3</sup>), (e) cell carbon content (pg C µm<sup>-3</sup>), (f) C:N ratio, (g) cell volume (µm<sup>-3</sup>), (h)

cell toxin content (amol µm<sup>-3</sup>), and (i) the fraction of cells rejected by copepods. The light-grey points in (d), (e), (f), and (h) are initial values from the stock culture. Values are means and error bars are standard error (*n* = 3).

### Chemostat experiments

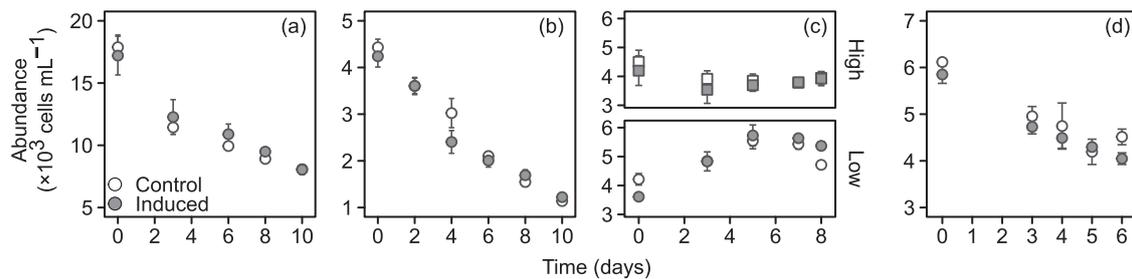
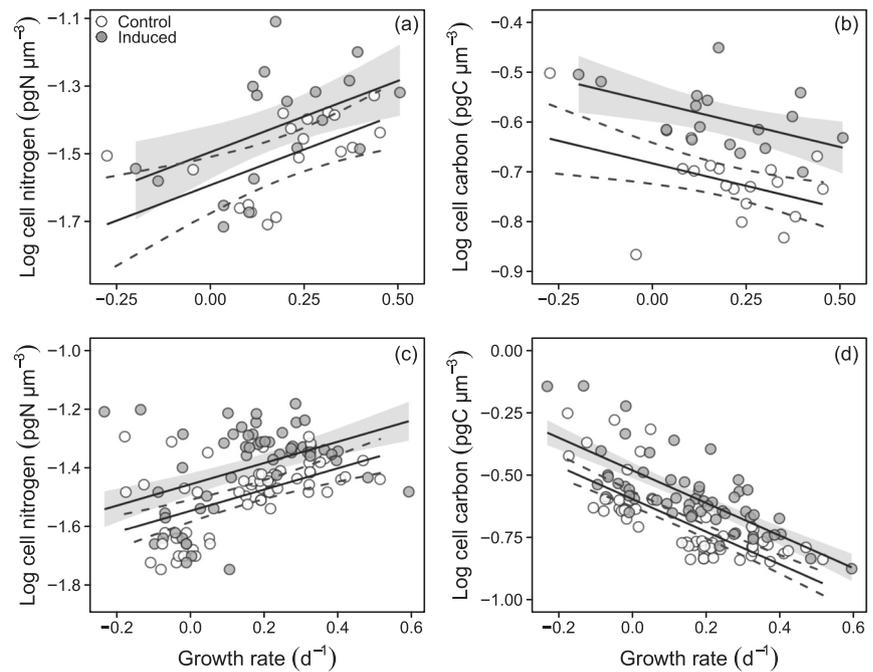
Growth rate in the 0.05, 0.10, and 0.40 d<sup>-1</sup> DR experiments was lower than the DR and cell concentrations thus decreased over time (Fig. 3). It was only in the two 0.20 d<sup>-1</sup> DR experiments that the cells were able to keep up with the DR (Fig. 3c, Fig. 4a). However, growth rates calculated from cell-bound nitrogen values per culture volume (µg N mL<sup>-1</sup>) were nearly constant over time at the three lowest DR, and the growth rates were similar to the DR, except at the lowest and highest DR (Fig. 4b, Appendix 2 Fig. S2). The small differences in cell concentrations and the low sensitivity of estimates of affinity and maximum growth rate parameters to changes in cell concentration at low DR makes the estimation of these parameters meaningless (Appendix 1).

Consistent with the results of the batch-experiment results, induced cells were significantly smaller than non-induced cells at intermediate DR but similar at the lowest

and highest rates. (Fig. 4c). Cellular carbon increased and nitrogen contents decreased with growth rate and both were significantly higher in induced than non-induced cells, particularly at intermediate DR (Fig. 4d, e, Fig. 2). Cellular C:N ratio varied inversely with DR and was slightly higher in induced than non-induced cells, all again consistent with the patterns in the batch experiment (Fig. 4f, Fig. 2). As in the batch experiment, effects were similar but less pronounced when expressed on a per-cell basis (Appendix 2 Table S5, S6). The effect of varying the copepodamide dose from low (0.63 nM) to high (6 nM) in the 0.2 d<sup>-1</sup> DR experiment had a significant effect on (reduced) cell volume and also further increased toxin content relative to the controls (Fig. 4c, g; Appendix 2 Table S4), consistent with the result of a more comprehensive dose–response experiment (Appendix 3; Appendix 3. Fig. S1).

Cell toxin content increased in all but the 0.05 and 0.10 d<sup>-1</sup> DR experiments in response to copepodamides

**Fig. 2 Relation between cellular nitrogen (N,  $\text{pg N } \mu\text{m}^{-3}$ ) or cellular carbon (C,  $\text{pg C } \mu\text{m}^{-3}$ ), and growth rate (GR,  $\text{d}^{-1}$ ). (a, b) batch-culture and (c, d) chemostat experiments. Growth rate is calculated from change in biovolume. Multiple linear regression (with 95% confidence intervals) was fit to the data (a) control:  $\log \text{ cell } N = -1.594 + 0.420 \times \text{GR}$ , induced:  $\log \text{ cell } N = -1.497 + 0.420 \times \text{GR}$  ( $R^2 = 0.27$ ,  $p = 0.002$ ); (b) control:  $\log \text{ cell } C = -0.684 - 0.181 \times \text{GR}$ , induced:  $-0.561 - 0.181 \times \text{GR}$  ( $R^2 = 0.539$ ,  $p < 0.001$ ); (c) control:  $\log \text{ cell } N = -1.546 + 0.363 \times \text{GR}$ , induced:  $-1.456 + 0.363 \times \text{GR}$  ( $R^2 = 0.28$ ,  $p < 0.001$ ); and (d) control:  $\log \text{ cell } C = -0.594 - 0.654 \times \text{GR}$ , induced:  $-0.479 - 0.654 \times \text{GR}$  ( $R^2 = 0.61$ ,  $p < 0.001$ ).**



**Fig. 3 Change in cell abundance ( $\text{cells mL}^{-1}$ ) in the chemostats at the different dilution rates. (a)  $0.05 \text{ d}^{-1}$ , (b)  $0.10 \text{ d}^{-1}$ , (c)  $0.20 \text{ d}^{-1}$  with high (6 nM) and low (0.63 nM) dose of copepodamides,**

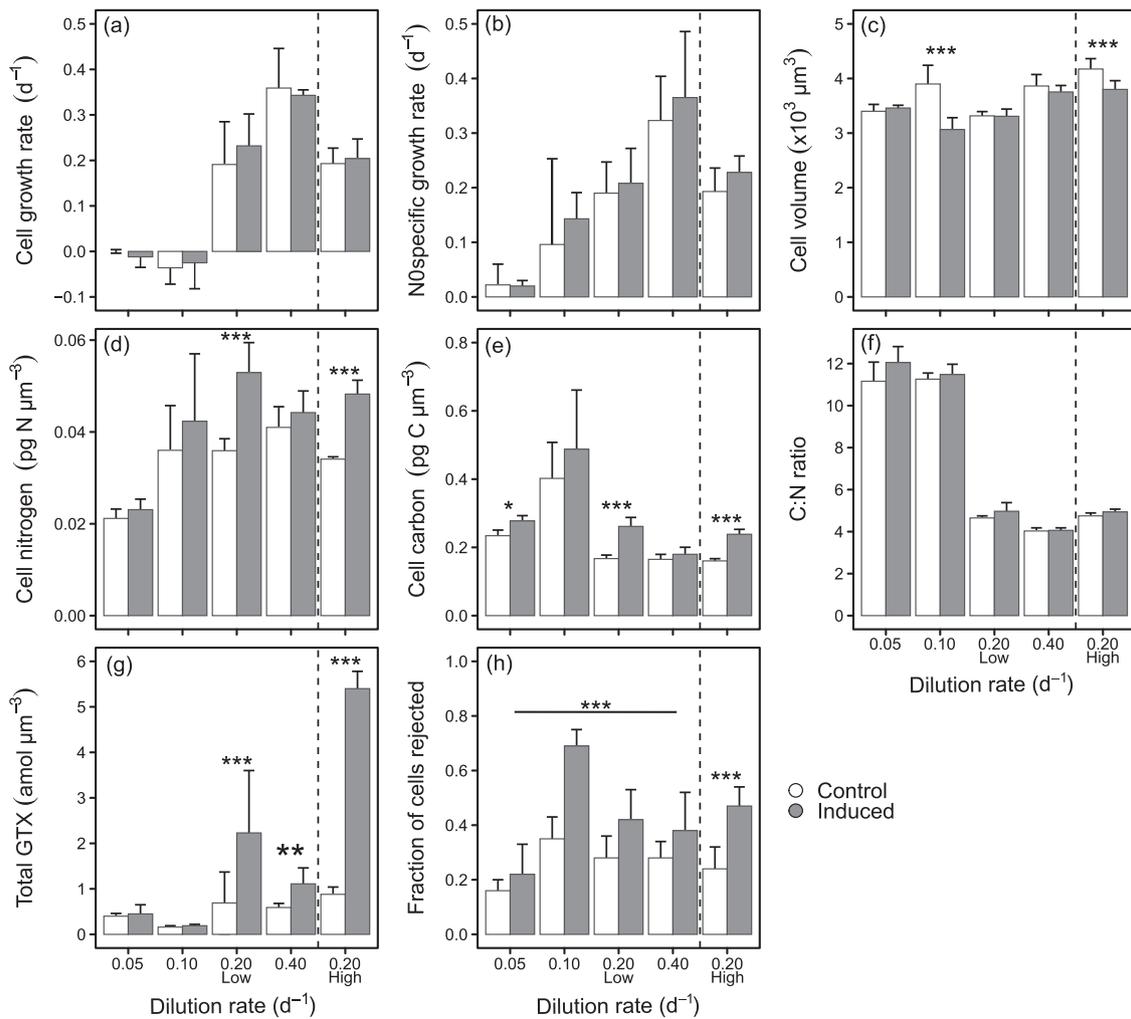
**(d)  $0.40 \text{ d}^{-1}$ .** The values are means and error bars show standard error ( $n = 3$ ). Note the different y-axis scales.

(Fig. 4g). Consequently, the copepods generally rejected a larger fraction of induced cells, except at the lowest DR (Fig. 4h; Appendix 2 Table S3, S4). Overall, the fraction of rejected cells increased with increasing toxin content but the effect saturates at a rather low toxin content of ca.  $10 \text{ amol GTX } \mu\text{m}^{-3}$  (Fig. 5).

## Discussion

We set out to quantify the costs and benefits of toxin production in a dinoflagellate by comparing the performance of cells induced to express their defence with those that were not under different degrees of nitrogen limitation. Our experiments produced decreasing growth rates with increasing nitrogen limitation in both batch and chemostat cultures (Fig. 2a, c), and high C:N ratios in N-limited cells (Fig. 1f, Fig. 4f). We have utilized that toxin production in many dinoflagellates, including *A. minutum*, can be induced

by grazer cues [2, 35], thus allowing us to examine the same strain at different toxin-production rates. This is important because different strains of the same species may differ in many traits, including in their toxin profiles [45, 49]. We also note that we examined the ‘private-good’ [50] grazer-deterrent effect of the defence at the level of the individual. That is, we quantify the benefits that only the individual cell that produces the toxin may benefit from. This is different from any toxic effects on the copepod that reduce its ability to graze on further cells, which is a ‘public good’, as also cells not producing the toxin may benefit [50]. We had predicted that both benefits and costs would be small in nutrient-starved cells, that benefits would be large but costs relatively small in nutrient-replete cells and that both benefits and costs would be high at intermediate nitrogen levels. In line with our hypothesis, toxin induction was highest at intermediate nitrogen limitation, but we found no evidence of direct costs in terms of reduced growth rate.



**Fig. 4 Summary of the chemostat experiments.** (a) cell growth rate ( $d^{-1}$ ), (b) N-specific growth rate ( $d^{-1}$ ), (c) cell volume ( $\times 10^3 \mu m^3$ ), (d) cell nitrogen ( $pg N \mu m^{-3}$ ), (e) cell carbon ( $pg C \mu m^{-3}$ ), (f) C:N ratio, (g) cell toxin content ( $amol \mu m^{-3}$ ), and (h) the fraction of cells rejected by copepods. Values are averaged over time during the treatment period and error bars show standard deviation ( $n = 4$  in 0.05, 0.20, and

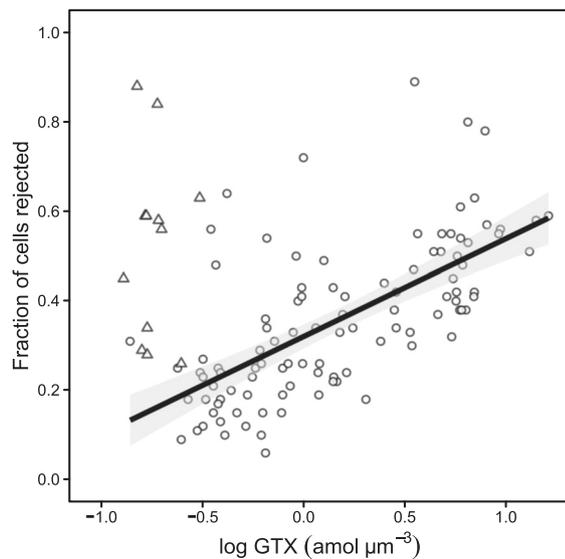
0.40  $d^{-1}$ ;  $n = 5$  in 0.10  $d^{-1}$ ;  $n = 3$  in 0.10  $d^{-1}$  C/N measurements). Asterisks above bars indicate significant differences between treatments within dilution rates ( $***p < 0.001$ ,  $**p < 0.01$ ,  $*p < 0.05$ ). In (h) and all 0.2  $d^{-1}$  high they indicate significant effect of the ‘Treatment’ factor. Further statistical analysis is reported in the appendix.

### Defence trade-offs

The benefits of toxin production were clear and largely followed the pattern predicted. Moreover, the results were consistent between the two types of experiments: induced cells have up to three times higher chance of being rejected by the copepod than non-induced cells, and the chance of rejection was positively correlated to the toxin content of the cells. This confirms previous reports of reduced grazing on induced dinoflagellates [2], but the demonstration at the individual cell level is novel. It is well established that nitrogen-starved cells produce no or very little toxins [9, 51] and, hence, gain little or no defensive benefits. In the batch experiment, cells remained toxic even in the stationary phase due to low cell division rate, but they did not produce new toxins. With low toxin content but high rejection

fraction, the 0.1  $d^{-1}$  DR experiment shows some odd behaviour. Thus, we cannot exclude the possibility that other defences, such as allelochemicals [52, 53], are also at play.

The efficiency of the defence was not as high as that reported for other strains and species of *Alexandrium* spp. Thus, Xu and Kiørboe [5] found that >90% of the cells of some toxic *Alexandrium* species/strains were rejected by a copepod, but also that other strains containing toxins were readily consumed by the copepod. Neither Xu et al. [46] nor Teegarden et al. [54] were consequently able to relate the efficiency of the defence to the composition and concentration of specific toxins in the cells of *Alexandrium* spp. Here, we have established a direct correlation between the cells’ content of the GTX toxin and the efficiency of the defence in the same strain of *Alexandrium minutum*.



**Fig. 5 Relation between copepod rejection rate and cell toxin content (GTX) normalized by volume ( $\text{amol } \mu\text{m}^{-3}$ ).** Data are from both batch culture and chemostat experiments. The regression line (with 95% confidence intervals) is  $\text{Rejection} = 0.320 + 0.219 \times \log \text{GTX}$  ( $R^2 = 0.42$ ,  $p < 0.001$ ). Due to odd behaviour, data from the  $0.10 \text{ d}^{-1}$  chemostat experiment (triangles) are not included in the regression. Including them does not change the significant effect ( $R^2 = 0.12$ ,  $p < 0.001$ ).

It has been notoriously difficult to demonstrate the cost part of defence trade-offs in phytoplankton [7], and this study is no exception, despite a novel approach. Ideally, ‘costs’ should be quantified in terms of reduced cell division rate. We found no reduction in the growth rate nor in nutrient affinity of the cells, even at nutrient-deplete conditions. The proportion of cellular nitrogen invested in toxins increased the more toxic the cells were (Appendix 2 Fig. S3), but this did not affect the growth rate.

However, we document a number of very clear effects of induction in addition to enhanced toxin production, i.e. elevated cellular contents of C and N, a reduction in cell size, and even an increase in cell division rate, with the effects being most pronounced at intermediate nutrient levels. The responses are consistent between the batch and the continuous cultures.

The expectation of reduced cell division rate of grazer-induced, nitrogen-limited cells is based on the nitrogen requirements for PST production in *Alexandrium tamarense* as worked out by Chakraborty et al. [31]. However, *A. tamarense* produces 1–2 orders-of-magnitude more toxins than the *A. minutum* used here. Thus, the biochemical syntheses costs and the N requirements for toxins are correspondingly smaller in our experiment and the higher N-uptake of induced cells cannot be explained by direct requirements for toxin production.

It is well established that cell size decreases when nutrient is limited [55, 56], as also seen most clearly in the

batch experiment (Fig. 1g). However, cell volume shrinks in response to grazer cues by up to 25% relative to non-induced cells. This has two implications. First, a smaller cell volume results in a higher concentration of toxins in the cells. It is reasonable to assume that the copepods respond to the concentration rather than the contents of toxins, and the shrinking of the cells may therefore be adaptive and part of the defence. A similar consistent response in cell size to grazer cues has been found in four species of diatoms [24]. For the diatoms, smaller cell sizes imply higher concentrations of biogenic silica and, therefore, a stronger protective shell that makes the cells less susceptible to copepod grazing [23].

The second potential implication of cell shrinking is a higher specific affinity for dissolved nutrients. To the first order, specific affinity scales inversely with cell radius due to the nature of molecular diffusion [57], and it is well established experimentally that the volume-specific nutrient uptake indeed increases with decreasing cell size [58, 59]. Thus, a 25% decrease in cell volume corresponds to an 8% decrease in radius and a corresponding increase in specific affinity. This, in fact, may account for the elevated nitrogen uptake, nitrogen content, and growth rate of induced cells when cells start being nutrient limited, as most clearly seen in the batch experiment (Fig. 1). If the decrease in cell size is an adaptation to increased toxin concentration, then the elevated nitrogen assimilation and growth rate of induced cells is a secondary and beneficiary effect.

The increased carbon content found in induced cells may be due to thickening of their thecal plates, providing them with another possible defence. It is unclear if this has an effect on the copepods, but it has been shown that diatoms that increase their silica shell thickness experience reduced grazing from both juvenile and adult copepods [23].

### Ecological and indirect costs of defence

While we were unable to demonstrate direct costs of toxin production even in nitrogen-starved cells, defences may come with indirect ecological costs that do not manifest in simplified laboratory settings [21]. This includes, e.g. increased sinking rate or reduced swimming speed that may inflict fitness costs in nature [60, 61]. A possible ecological cost of the reduced cell size recorded here is elevated predation risk. In general, mortality rate of plankton organisms scales inversely with their volume to power 0.25 [62], and a 25% decrease in volume thus implies a 7.5% increase in predation mortality from other predators than copepods. Copepods and other larger herbivores are probably the most important grazers on dinoflagellates, thus >50% decrease in copepod grazing pressure of induced cells more than outweighs the cost in most situations, and toxin production increases the fitness of the cell.

Theory of defences predicts that defences should only be inducible if they are associated with a cost [63]. The number of studies unable to detect costs associated with induced toxin production in phytoplankton suggests that additional factors may be at play. Recent advancements in genome sequencing reveal that a substantial part of the genome may be dedicated to secondary metabolism, up to one-fifth in some cyanobacteria [64]. Keeping a single biosynthetic pathway active may inflict a very limited cost, whereas the cost for constant activation of one-fifth of the genome will be substantial [65]. Thus, the evolution of inducible defence may be driven not by the allocation of resources to a single pathway, but the necessity to avoid allocation to all defence systems simultaneously. This is but a corollary hypothesis to the optimal defence theory, but one that may explain the lack of detectable costs in some induced responses to herbivory.

In conclusion, we found a diverse nutrient-dependent response of a dinoflagellate to copepod cues: increased toxicity with implied lower predation risk, higher cellular contents of carbon and nitrogen, reduced cell size, and higher growth rate. Most of these responses may be beneficial to the cells, while we found no indication of direct costs. Because dinoflagellates are not Darwinian demons, the necessary costs are most likely indirect or ecological that are apparent only in nature.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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