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## Role of dissolved nitrate and phosphate in isolates of *Mesodinium rubrum* and toxin-producing *Dinophysis acuminata*

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### Abstract

*Dinophysis acuminata*, a producer of toxins associated with diarrhetic shellfish poisoning (DSP) and/or pectenotoxins (PTXs), is a mixotrophic species that requires both ciliate prey and light for growth. Linkages have been described in the literature between natural abundances of the predator *Dinophysis* and its prey, *Mesodinium rubrum*, and culture experiments have demonstrated that prey, in addition to light, is required for toxin production by *Dinophysis acuminata*; together these suggest *Mesodinium* is a critical component for *Dinophysis* growth and toxicity. However, little is known about the role of dissolved inorganic nutrients on *Mesodinium* growth or that of toxin-producing *Dinophysis*. Accordingly, a series of experiments were conducted to investigate the possible uptake of dissolved nitrate and phosphate by 1) *Dinophysis* starved of prey, 2) *Dinophysis* feeding on *Mesodinium rubrum*, and 3) *M. rubrum* grown in nutritionally-modified media. All single-clone or mixed cultures were monitored for dissolved and particulate nutrient levels over the growth cycle, as well as growth rate, biomass, and toxin production when appropriate. *D. acuminata* did not utilize dissolved nitrate or phosphate in the medium under any nutrient regime tested, i.e., nutrient-enriched and nutrient-reduced, in the absence or presence of prey, or during any growth phase monitored, i.e., exponential and plateau phases. Changes in particulate phosphorus and nitrogen in *D. acuminata*, were instead, strongly influenced by the consumption of *M. rubrum* prey, and these levels quickly stabilized once prey were no longer available. *M. rubrum*, on the other hand, rapidly assimilated dissolved nitrate and phosphate into its particulate nutrient fraction, with maximum uptake rates of 1.38 pmol N/cell/day and 1.63 pmol P/cell/day. While *D. acuminata* did not benefit directly from the dissolved nitrate and phosphate, its growth ( $0.37 \pm 0.01 \text{ day}^{-1}$ ) and toxin production rates for okadaic acid (OA), dinophysistoxin-1 (DTX1) or pectenotoxin-2 (PTX2), 0.1, 0.9 and 2.6 pg /cell/day, respectively, were directly coupled to prey availability. These results suggest that while dissolved nitrate and phosphate do not have a direct effect on toxin production or retention by *D. acuminata*, these nutrient pools contribute to prey growth and biomass, thereby indirectly influencing *D. acuminata* blooms and overall toxin in the system.

## Keywords

*Dinophysis acuminata*, *Mesodinium rubrum*; Diarrhetic Shellfish Poisoning (DSP); okadaic acid (OA); dinophysistoxin (DTX); pectenotoxins (PTXs); dissolved inorganic nutrients; nitrate; phosphate

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## Introduction

*Dinophysis acuminata* Claparède & Lachmann, an obligate mixotrophic dinoflagellate, obtains energy for maintenance and growth through a combination of autotrophy and heterotrophy (Park *et al.*, 2006; Kim *et al.*, 2008; Riisgaard and Hansen, 2009). Mixotrophy is a common phenomenon in the euphotic zone of estuarine and oceanic waters, with mixotrophic species differing in feeding behavior, light requirements and the uptake of dissolved inorganic nutrients (Stoecker, 1999). As *D. acuminata* is a producer of okadaic acid (OA) and dinophysistoxins (DTXs) responsible for diarrhetic shellfish poisoning (DSP), in addition to pectenotoxins (PTXs), there is a history of attempted culturing experiments aimed at investigating the physiology and toxicity of the dinoflagellate; in these studies, experimental growth media were prepared with artificial or enriched natural seawater with additions of vitamins, glycolic acid (Sampayo, 1993), trace inorganic elements, dissolved organic materials, or food organisms (bacteria, pico- and nanoplankton, and yeast, Maestrini *et al.*, 1995). Despite these additions, researchers were unable to establish viable cultures of *Dinophysis*, suggesting that *Dinophysis* spp. were unable to utilize these chemical forms in a manner sufficient to support cell division. Soon thereafter, it was discovered that *Dinophysis acuminata* could be maintained in the laboratory using a specific three-stage food chain: the cryptophyte *Teleaulax amphioxeia* is fed to the ciliate *Mesodinium rubrum*, which in turn, is fed to *Dinophysis* under sufficient light (Park *et al.*, 2006). Subsequent laboratory studies determined that the mixotrophic *D. acuminata* required nutrients and chloroplasts from its prey to sustain photosynthesis (Park *et al.*, 2006; Kim *et al.*, 2008; Riisgaard and Hansen, 2009).

Several *Dinophysis* species, along with a few *Prorocentrum* spp., are responsible for the diarrhetic shellfish poisoning (DSP) syndrome (Lee *et al.*, 1989; Dickey *et al.*, 1990). Now that multiple isolates of *D. acuminata* have been successfully maintained in culture, recent studies have found that both prey and light are required for cell growth and DSP toxin production and that toxin production is further dependent upon growth stage; highest production rates occurred during exponential growth and the highest toxin quotas were coincident with early-mid stationary phase (Tong *et al.*, 2011; Smith *et al.*, 2012). Conversely, production of pectenotoxin-2 does not appear to be directly linked to irradiance or prey consumption in *D. acuminata* (Nielsen *et al.*, 2012). Kamiyama *et al.* (2010) found that the growth rate of *D. acuminata* from Japan increased with increasing temperature from 10–22°C but that pectenotoxin-2 (PTX2) toxin cell quotas showed an inverse trend at these temperatures. No relationship was detected between temperature and okadaic acid (OA) or dinophysistoxin-1 (DTX1) cell quotas. Additionally, it has been determined that toxin profile and content can vary between isolates of *D. acuminata* (Blanco *et al.*, 2007; Kamiyama and Suzuki, 2009; Kamiyama *et al.*, 2010; Fux *et al.*, 2011; Nielsen *et al.*, 2012).

Numerous field studies have demonstrated that *Dinophysis* spp. are adapted to a wide range of chemical, biological and physical conditions, making them geographically wide-spread, with toxic populations reported from western Europe, East and Southeast Asia, South America, North America, Central America, Southern Africa, New Zealand and Australia (Van Dolah, 2000; Reguera *et al.*, 2012). *Dinophysis* spp. are tolerant of a large range of temperatures (5 – 22°C) and salinities (5 – 34.5‰) (Reguera *et al.*, 1993; Nishihama *et al.*, 2000; Setälä *et al.*, 2005; Lindahl *et al.*, 2007). The vertical distribution of *Dinophysis* extends to as deep as 110 m (Fux *et al.*, 2010) ranging from the surface to below the euphotic zone (Gisselson *et al.*, 2002), suggesting that the genera are tolerant of high and low light conditions. *Dinophysis* growth is often associated with a stable water column (Maestrini, 1998; Seeyave *et al.*, 2009; Reguera *et al.*, 2012) and cells have often been shown to accumulate in thin layers or patches due to horizontal and vertical migration, transport driven by wind or currents (Mackenzie, 1991, 1992; Xie *et al.*, 2007), or biological behavior to locate prey or avoid predators (Maestrini, 1998; Campbell *et al.*, 2010; Gonzalez-Gil *et al.*, 2010; Sjoqvist and Lindholm, 2011, Hattenrath-Lehmann *et al.*, 2013). Populations have also been documented within systems containing a range of ambient dissolved nutrient levels: total dissolved nitrogen, 2 – 21 µM; silicate, 0 – 10 µM, and phosphate, 0 – 0.24 µM (Delmas *et al.*, 1992; Maestrini, 1998). Seeyave *et al.*, (2009) further determined that *D. acuminata* was most abundant under nitrogen-deplete conditions, 0.1 – 0.5 µM NO<sub>3</sub><sup>-</sup>, along the western coast of South Africa, and displayed a greater affinity for NH<sub>4</sub><sup>+</sup> and urea, relative to NO<sub>3</sub><sup>-</sup>, based on incubation uptake experiments with field material. Johansson *et al.* (1996) reported that limitation by dissolved nitrate may promote toxin production by *D. acuminata* and *D. acuta* in field incubations of natural populations in the absence of prey, and Nagai *et al.*, (2011) found that the addition of dissolved organic substances (ciliate prey exudate) to culture medium enhanced toxin production. Despite this collection of investigations into *Dinophysis* ecology, no work has yet investigated the relationships between ambient nutrient concentrations, prey availability, *D. acuminata* abundance, and toxicity in one comprehensive study complicating efforts to understand this species' distribution and potential for impact.

Similar to *D. acuminata*, *M. rubrum* is also a mixotroph that feeds on cryptophytes (Gustafson *et al.*, 2000; Yih *et al.*, 2004) and bacteria (Moeller *et al.*, 2011) to acquire organelles or growth factors (Gustafson *et al.*, 2000; Hansen and Fenchel, 2006; Johnson *et al.*, 2007; Park *et al.*, 2007) but relies primarily on phototrophy for growth. Moreover, *M. rubrum* was reported to assimilate nitrate, ammonium and dissolved organic nitrogen in the field (Wilkerson and Grunseich, 1990; Kifle and Purdie, 1993). However, no investigations have been conducted in the laboratory quantifying the uptake rate of dissolved nutrients by *M. rubrum*.

Thus, to better understand the nutritional ecology of *D. acuminata*, it is essential to understand the relationship between ambient dissolved inorganic nutrients, prey growth and dependence upon inorganic nutrients, and *Dinophysis* growth and toxicity. To address these questions, we quantified changes in particulate and dissolved nutrients over growth in batch cultures of *D. acuminata*, in the absence and presence of *M. rubrum*, and in batch cultures of *M. rubrum* alone under different nutrient regimes.

## Material and Methods

### Culture maintenance

*Dinophysis acuminata* (DAMV01) was isolated from coastal waters near Martha's Vineyard island (41.0° N, 70.5° W), Massachusetts, USA in August of 2008. The ciliate *Mesodinium rubrum* and cryptophyte *Teleaulax amphioxeia* were isolated from Inokushi Bay (131°89' E, 32°79' N) in Oita Prefecture, Japan, in February of 2007 as described in Nishitani *et al.* (2008). All cultures were maintained in modified f/6 medium, which was prepared with 1/3 nitrate, 1/3 phosphate, 1/3 metals, and 1/5 vitamins of modified f/2-Si medium whereby H<sub>2</sub>SeO<sub>3</sub> was added and CuSO<sub>4</sub> was reduced to concentration of 10<sup>-8</sup> M each (Anderson *et al.*, 1994). Cultures were maintained at 15°C with an irradiance of 65 μmol photons m<sup>-2</sup> sec<sup>-1</sup> on a 14h light: 10h dark photocycle.

To maintain cultures of the ciliate, *M. rubrum*, the culture was transferred every two weeks, by mixing 70 mL of two-week old culture (~10,000 cells/mL) and 2 mL of *T. amphioxeia* (containing 1.2–1.6 × 10<sup>6</sup> cells), with 150 mL of modified f/6 medium. *T. amphioxeia* culture was maintained by inoculating 1 mL of the culture (6.0–8.0 × 10<sup>5</sup> cells/mL) into 25 mL of modified f/6 medium. The *Dinophysis* cells were fed a “clean” (cryptophyte free) *M. rubrum* cell suspension every week and transferred every four weeks by adding 2 mL of *M. rubrum* (~10,000 cells mL<sup>-1</sup>) and 2 mL *D. acuminata* (~1,800 cells mL<sup>-1</sup>) to 20 mL of modified f/6 medium.

### Experimental conditions

Here we investigated uptake of dissolved nitrate and phosphate by *M. rubrum* (without prey or predator) and *Dinophysis* (both in presence and absence of prey) and the effect on growth, toxin production, and particulate nutrient quotas. This was accomplished through a series of experiments growing monocultures of *M. rubrum* in nutrient-enriched medium, monocultures of *D. acuminata* in nutrient-enriched and nutrient-reduced medium, and mixed cultures of the ciliate and dinoflagellate in nutrient-enriched medium.

***M. rubrum* monoculture experiment**—To examine nutrient uptake by *M. rubrum* in the absence of cryptophyte prey or dinoflagellate predators, a volume of the ciliate maintenance culture, 220 mL, was transferred into fresh f/6-Si medium, following complete consumption of the cryptophyte cells. The nutrient concentration of the experimental medium (fresh f/6-Si medium in sterile-filtered seawater + nutrient carry over from the *M. rubrum* inoculum) contained 200.80±0.59 μM nitrate and 13.32±0.02 μM phosphate (mean ± STD, n=3). The ciliate monoculture was monitored for growth rate, biomass, dissolved nitrate/nitrite and phosphate, and particulate nitrogen and phosphorus quotas over culture growth. The inclusion of ammonium, 2.08±0.31 μM, in the experimental culture medium was an artifact of the filtered seawater used to make the medium; however, we quantified this nutrient in the initial culture medium and monitored it over the experiment to observe any changes in this additional pool of available nitrogen; this holds true for all experiments presented herein. Concurrently, a second volume of *M. rubrum* culture, 220 mL, was inoculated with *D. acuminata* in fresh f/6-Si medium to begin the mixed culture experiment, i.e., feeding experiment, as described below.

**Mixed culture experiment**—To quantify the utilization of dissolved nitrate and phosphate by *Dinophysis* during periods of rapid division (i.e., in the presence of prey), we conducted a time-course experiment using late plateau phase *M. rubrum* as prey, containing  $96.6 \pm 2.2$  pmol/cell of carbon,  $13.1 \pm 1.1$  pmol/cell of nitrogen and  $0.7 \pm 0.01$  pmol/cell of phosphorus (mean  $\pm$  STD, n=3), in fresh nutritionally-modified f/6 medium. The final nutrient concentrations of the experimental medium for the mixed culture (fresh f/6-Si medium + nutrient carry over from the inoculum *M. rubrum* and *D. acuminata* cultures) were  $222.06 \pm 3.05$   $\mu$ M nitrate,  $0.75 \pm 0.22$   $\mu$ M ammonium and  $11.85 \pm 0.06$   $\mu$ M phosphate (mean  $\pm$  STD, n=3). The mixed experimental cultures of *D. acuminata* and *M. rubrum* were maintained for 40 days, to include early exponential to late-plateau growth phases. Triplicate Fernbach flasks were inoculated with ca. 2,000 and 100 cells mL<sup>-1</sup> of *M. rubrum* and *D. acuminata* (inoculated from early plateau phase), respectively, to achieve a total volume of 1,400 mL. The mixed culture was monitored for growth rate of both organisms, biomass, dissolved nitrate/nitrite, ammonium, and phosphate, particulate nitrogen and phosphorus quotas over culture growth, and toxin production. In preliminary trials, we also conducted the feeding experiment under nutrient deplete conditions (filtered seawater); however, the culturing of *M. rubrum* and *T. amphioxeia* in seawater resulted in no growth and accelerated death (data not shown). Therefore, nutrient uptake of *D. acuminata* with sufficient prey was only possible in nutrient enriched medium.

***D. acuminata* monoculture experiment**—To quantify the utilization of dissolved nitrate and phosphate by *Dinophysis* during periods when ciliate prey were not available, we monitored growth, the concentration of dissolved nitrate/nitrite, ammonium, and phosphate concentrations in the medium, particulate nitrogen and phosphorus quotas, and toxin production in the monoculture over time. The monoculture experiment was conducted in two types of nutritionally-modified medium: nutrient enriched (~f/8-Si medium) and nutrient reduced (~f/15-Si medium), to determine whether dissolved nitrate and phosphate influence the growth and toxin content of *Dinophysis*. Nutrient-enriched medium (including fresh f/6-Si medium in sterile-filtered seawater + nutrient carry over from the *M. rubrum* inoculum) consisted of  $1.70 \pm 0.35$   $\mu$ M ammonium,  $198.73 \pm 2.22$   $\mu$ M nitrate and  $10.01 \pm 0.45$   $\mu$ M phosphate (mean  $\pm$  STD, n=3). The nutrient-reduced medium (including sterile-filtered seawater + nutrient carry over from the *M. rubrum* inoculum) had initial concentrations of  $1.65 \pm 0.24$   $\mu$ M ammonium,  $108.49 \pm 2.02$   $\mu$ M nitrate, and  $5.09 \pm 0.50$   $\mu$ M phosphate (mean  $\pm$  STD, n=3). More specifically, triplicate Fernbach flasks were inoculated with 800 mL of initial culture and 600 mL of either fresh f/2-Si medium or sterile-filtered seawater, depending on the treatment, to reach cell concentrations of 1,500 cells/mL of the dinoflagellate and the desired concentrations of dissolved nitrate and phosphate to begin the *D. acuminata* monoculture experiments.

### Cell enumeration

Triplicate 1.5 mL subsamples were taken for *M. rubrum* and *D. acuminata* enumeration; subsampling occurred daily at the beginning of the experiments, every other day through the middle of the experiment, and once a week near the end of the incubation. Samples were fixed with a 0.2% v/v Acid Lugol's (Tong *et al.*, 2010) and enumerated in a Sedgewick-Rafter chamber using a microscope at 100X total magnification.



### Nutrient sample collection and preparation

Culture was harvested for particulate and dissolved nutrient analyses from each replicate flask and processed separately during the experimental period. Initial particulate nutrient samples were also collected from the inoculum cultures of both *M. rubrum* and *Dinophysis*. For *M. rubrum*, samples for nutrient analysis were collected around 2PM daily the first 4 days and every three days thereafter.

To ensure that the particulate nutrient analyses only reflected those nutrients accumulated by *D. acuminata* in the feeding experiment, the second time point, early plateau phase, was collected following the complete consumption of prey by *D. acuminata* on day 7. Starved *Dinophysis* samples were harvested every two days during the first week and then once a week for the remaining four weeks.

For nutrient analyses, 25 – 50 mL of culture, dependent upon the amount of biomass in the culture, was collected through pre-combusted GF/F filters (450°C for 4 hours, 0.8µm, 25mm) for total particulate organic carbon/nitrogen analysis (CHN). Another 25 – 50 mL of culture was collected through membrane filters (PALL Supor R-800, 0.8µm, 25mm) for the determination of total particulate phosphorus. Filtrate from the CHN samples was collected for dissolved inorganic nutrient analysis ( $\text{NO}_3^-/\text{NO}_2^-$ ,  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$ ). After collection, all filters were placed in a 60°C drying oven for 24 hours and then were stored at –20°C. The particulate phosphorus filters were hydrolyzed by adding 5 mL of 5% potassium persulfate and 10 mL of Milli-Q water and autoclaved (121°C) for 20 min. After hydrolyzation, all particulate phosphorous was converted to, and was measured as, dissolved orthophosphate ( $\text{PO}_4^{3-}$ ). Dissolved inorganic nutrient samples, which were stored frozen at –20°C until analysis, were analyzed on a Lachat QuickChem 8000 at Woods Hole Oceanographic Institution (Woods Hole, MA) using standard US EPA approved methods. For solid phase carbon and nitrogen determination, the particulate CHN samples were analyzed on a Flash EA1112 Carbon/Nitrogen Analyzer using a Dynamic Flash Combustion technique.

### Toxin sample collection and preparation

Both *Dinophysis* cells and media samples in the mixed and monoculture treatments were analyzed for toxin at eight time points as described above for nutrient analysis. Cells (ca. 180,000) were separated from medium using a 15-µm Nitex sieve. The cells and sieved filtrate were thereafter processed separately. The cells were rinsed with fresh seawater, kept wet on the sieve (to minimize cell breakage) and rinsed into a pre-weighed 15-mL centrifuge tube. Triplicate, 200-µL aliquots were pipetted from the mixed sample into separate micro-centrifuge tubes containing 1.3 mL of filtered seawater and 3 µL Acid Lugol's solution (0.2% v/v, Tong *et al.*, 2010) to later determine the cell concentrations in the harvested cell concentrate. The 15-mL tube was reweighed to determine the volume of harvested *Dinophysis* cells (sample weight divided by the density of seawater, 1.03 g/mL) and frozen at –20 °C.

The toxin extraction process was described in Smith *et al.* (2012). In brief, the cell samples were thawed at room temperature, sonicated in a water bath (Fisher ultrasonic cleaner,

Model FS30H) for 15 min and well mixed by Vortex-Genie 2 mixer before being passed through a solid phase extraction filter (SPE). The filters (Oasis HLB 60 mg; Waters, Milford, MA) were conditioned with methanol (3 mL) and Milli-Q water (3 mL) and then loaded with the cell samples at a flow rate of 1 mL/min, washed with Milli-Q water (6 mL) and eluted with methanol (1 mL) into 1.5-mL high recovery LC vials. The extracts were stored at  $-20^{\circ}\text{C}$  until analysis. The filtrate was immediately loaded onto an Oasis HLB cartridge (60 mg) after sieving, and eluted and stored the same way as the cell extracts. Eluates from the cell and filtrate samples were heated at  $40^{\circ}\text{C}$  in a heating block, dried under a stream of  $\text{N}_2$ , and re-suspended in 1 mL of methanol for toxin analysis.

### Toxin analysis

Toxin analyses were performed on a Quattro Ultima triple quadrupole mass spectrometer (TQ) (Waters Micromass) coupled to an Agilent 1100 HPLC. Separation was achieved on a C8 Hypersil column ( $50 \times 2.1$  mm;  $3.5 \mu\text{m}$  particle size) maintained at  $20^{\circ}\text{C}$ . The flow rate was set at  $0.25 \text{ mL min}^{-1}$  and a volume of  $10 \mu\text{L}$  was injected. Binary mobile phase was used, with phase A (100% aqueous) and phase B (95% aqueous LC-MS grade acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. A gradient elution was employed, starting with 30% B, rising to 100% B over 9 min, held for 3 min, then decreased to 30% B in 0.1 min and held for 3 min to equilibrate at initial conditions before the next run started. The TQ was operated in multiple reaction monitoring (MRM) mode and the following transitions were monitored: OA,  $m/z$   $803.5 > 255.5$  and  $803.5 > 803.5$ ; DTX1,  $m/z$   $817.5 > 255.5$  and  $817.5 > 817.5$  in negative ionization mode and PTX2,  $876.5 > 213.0$  in positive ionization mode. OA and DTX1, or PTX2 were quantified against 8 level calibration curves obtained with OA or PTX2 reference solutions (NRC- Canada), respectively.

### Calculations

**Growth and ingestion rate**—The average growth rates of *D. acuminata* and the ciliate prey, *M. rubrum*, were calculated using the model by Guillard (1973):

$$\mu = \frac{\ln(C_2/C_1)}{t_2 - t_1} \quad (1)$$

In this equation,  $C_1$  and  $C_2$  are the concentrations of cells at time 1 and time 2 (cells/mL), respectively.  $t$  is the experimental time (day) and  $\mu$  ( $\text{day}^{-1}$ ) is the growth rate. The growth rate was calculated over the culture's exponential phase of growth.

The ingestion rate of *D. acuminata*,  $U$  ( $\text{cells predator}^{-1} \text{ d}^{-1}$ ), was calculated using the formula developed by Jakobsen and Hansen, (1997):

$$\frac{dx}{dt} = \mu_x \cdot x - U \cdot y \quad (2)$$



$$\frac{dy}{dt} = \mu_y \cdot y \quad (3)$$

This assumes that the predator concentration  $y$  (*D. acuminata*) and prey  $x$  (*M. rubra*) have an exponential increase, with the growth rate constants  $\mu_y$  and  $\mu_x$ , respectively.

**Nutrient concentration and uptake rate**—The total particulate phosphorous and nitrogen content of *D. acuminata* and *M. rubrum* were presented in units of amount of nutrient (mol) per cell. Dissolved inorganic nutrients were reported as  $\mu\text{M}$  ( $\mu\text{mol/L}$ ). Nutrient uptake rate  $\theta$  (amount of nutrient mol/cell/day) was calculated using the formula:

$$\theta = \frac{(N_1 - N_2)}{(\bar{C})(t_2 - t_1)} \quad (4)$$

where  $N_1$  and  $N_2$  are the dissolved inorganic contents of nutrient, i.e. nitrogen ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{NH}_4^+$ ) and phosphate ( $\text{PO}_4^{3-}$ ), at time 1 and time 2 ( $\mu\text{M}$ ), respectively.  $\bar{C}$  is the natural logarithm (ln) average of the *D. acuminata* cell concentration (Anderson *et al.*, 1990):

$$\bar{C} = \frac{C_2 - C_1}{\ln(C_2/C_1)} \quad (5)$$

**Toxin content, concentration, and production rate**—Intracellular (particulate) content or quotas of OA, DTX1, and PTX2 are presented as toxin per cell of *Dinophysis*, calculated by dividing the toxin concentration by the cell density at each time point of the incubation. Extracellular (dissolved) toxin concentrations and total toxin concentrations were presented as toxin per mL; the latter was calculated by adding the particulate and dissolved toxin concentrations together. The net toxin production rate  $R_{\text{tox}}$  (amount toxin/cell/d) was determined using intracellular quotas with the equation (Anderson *et al.*, 1990; Tong *et al.*, 2011):

$$R_{\text{tox}} = \frac{(T_2 - T_1)}{(\bar{C})(t_2 - t_1)} \quad (6)$$

**Statistical analysis**—After the determination of normality, the effect of dissolved nitrate or phosphate concentrations on the growth rate, biomass, particulate nutrient quotas, toxin content, and total toxin concentration of *Dinophysis* was examined over time using two-way Repeated Measures ANOVA (Sigma Plot, version 12.5). Dissolved nitrate data for *D. acuminata* in the mixed culture were log-transformed prior to analysis. Dissolved ammonium was analyzed for changes over time using a one-way Repeated Measures ANOVA. All measurements were collected in triplicate and alpha was set at 0.05 for all analyses.

## Results

### Nutrient uptake and growth of *M. rubrum* in monoculture

After complete consumption of its cryptophyte prey, *M. rubrum* was transferred into fresh medium where it grew continuously as a monoculture, but slowly, over the seven-day sampling period (Fig. 1a) with mean values ( $n=3$ ) of  $0.062 \text{ day}^{-1}$  in the nutrient-enriched medium. When *M. rubrum* was grown in the absence of prey or predator, the ciliate took up dissolved nitrate and phosphate from the medium and assimilated them into particulate N and P (Fig. 1 b-g). The particulate nitrogen content (PN) and particulate phosphorous (PP) of *M. rubrum* was  $13.1 \text{ pmol/cell}$  (Fig. 1b) and  $0.7 \text{ pmol/cell}$  (Fig. 1c) respectively in the initial monoculture. Within one day of being inoculated into fresh medium, in the absence of food, there was a dramatic increase in both particulate N ( $28.8 \text{ pmol/cell}$ ) and P ( $3.7 \text{ pmol/cell}$ ) within *M. rubrum* with a subsequent decrease in dissolved nitrate (Fig. 1d) and dissolved phosphate concentrations (Fig. 1e). The removal of nitrate from the medium (Fig. 1d, 1f) was pronounced, with nitrate uptake rates of  $1.38 \text{ pmol N/cell/day}$  on Day 1. *Mesodinium rubrum* maintained a cell quota of about  $27.8 \text{ pmol N/cell}$  over the rest of the experimental period. There was also significant uptake of dissolved phosphate on Day 1,  $1.63 \text{ pmol P/cell/day}$  by *M. rubrum* during the monoculture experiment. Cell P quotas of *M. rubrum*, however, rapidly declined with the exhaustion of the dissolved phosphate in the medium and subsequent cell division (Fig. 1c).

### Utilization of nutrients by *D. acuminata* in the mixed culture

In mixed cultures, the mean exponential growth rate of *D. acuminata* was  $0.37 \pm 0.01 \text{ day}^{-1}$  over the first 9 days after inoculation of predator and prey. This exponential growth continued for two days after ciliate prey were completely grazed from the cultures, Day 7 (Fig. 2a). Cultures continued to grow, but at a slower rate,  $0.11 \pm 0.002 \text{ day}^{-1}$ , for the following 7 days, reaching a maximum cell concentration of  $3,986 \text{ cells/mL}$ . The average cell density of *M. rubrum* in the mixed culture upon inoculation was  $2,296 \text{ cells/mL}$  (Fig. 2a).

*D. acuminata* directly acquired nitrogen and phosphorus through the consumption of ciliate prey (Fig. 2b and 2d). As such, nitrogen accumulation by *D. acuminata* significantly increased during the period of prey consumption, rising during early exponential growth from  $12.4 \pm 2.7$  in the initial inoculum culture, to  $47.1 \pm 7.3 \text{ pmol/cell}$  (Mean  $\pm$  STD,  $n=3$ ) on Day 7 when the ciliate prey was completely consumed in the mixed culture (Fig. 2a and 2b). Thereafter, cellular particulate nitrogen (PN) in *Dinophysis* decreased as the result of continued cell division and lack of food; *Dinophysis* particulate N fell to near-initial levels of  $15.4 \text{ pmol/cell}$  in the later stages of the experiment.

The particulate phosphorous (PP) content of *D. acuminata* did not change as dramatically as particulate N through the course of the experiment, with P quotas ranging from  $1.6 - 2.4 \text{ pmol/cell}$ . Unlike the pattern observed with particulate N (Fig. 2b), the particulate phosphorus content of *D. acuminata* cells remained constant during exponential growth. After the consumption of prey, P quotas in *D. acuminata* then followed that of nitrogen and decreased as a result of continued cell division and lack of food (Fig. 2c).

Particulate carbon (PC) followed much the same pattern as particulate phosphorous in the first 26 days of the experiment with no significant change in cell quotas (average of 320.9 pmol/cell, n= 18). At the end of the mixed culture experiment, however, PC in *Dinophysis* increased to 410.7 pmol/cell (mean value, n=3) on Day 33, and finally reached 536.4 pmol/cell (mean value, n=3) on Day 40 (Fig. 2d).

Dissolved inorganic nitrogen ( $\text{NO}_3^-/\text{NO}_2^-$ ,  $\text{NH}_4^+$ , Fig. 2e) concentrations in the mixed culture medium were constant during the exponential growth phase of *Dinophysis*. The concentration of dissolved phosphate, on the other hand, decreased in the medium while *M. rubrum* prey were present in the mixed culture and actively utilizing this dissolved nutrient, from day 0 ( $11.87 \pm 0.06 \mu\text{M}$ ) to Day 7 ( $6.41 \pm 0.20 \mu\text{M}$ , Fig. 2f). Upon the removal of ciliate prey from the mixed culture on Day 7, concentrations of dissolved phosphate stabilized. Both dissolved nitrate and phosphate then remained constant until late plateau phase, when concentrations increased in the medium of the mixed culture.

By applying the measured nutrient uptake rates in *M. rubrum* monocultures to the mixed culture experiment, we were able to show that *M. rubrum* was solely responsible for the uptake of dissolved phosphate during co-incubation with *D. acuminata* (Table 1); dissolved nitrate concentrations in the medium did not change during co-incubation, however, we calculate that *M. rubrum* could have depleted the source by  $6.9 \mu\text{M}$  in 7 days. Similarly, *M. rubrum* had the potential to decrease the dissolved phosphate pools in the mixed culture by  $6.5 \mu\text{M}$  in 7 days. As there was only an absolute decrease in dissolved phosphate of  $5.5 \pm 0.3 \mu\text{M}$  from the medium during this period, we conclude that *M. rubrum* was solely responsible for the removal of dissolved inorganic phosphorus from the medium during the initial stage of the mixed culture treatment. A 7-day timeframe was chosen as this represents the period of co-incubation before *M. rubrum* was completely consumed by *Dinophysis*, and therefore, were able to impact the dissolved nutrient concentrations through uptake. This result supports the conclusion that the mixotrophic dinoflagellate, *D. acuminata* acquires both nitrogen and phosphorus from prey consumption, and not from the uptake of dissolved nitrate and phosphate in the culture media (Fig. 2f).

#### Utilization of dissolved nutrients by *D. acuminata* in monoculture

In the absence of prey, *Dinophysis* had minimal growth,  $0.002$  and  $0.003 \text{ day}^{-1}$ , under both nutrient regimes, nutrient enriched and nutrient reduced, respectively (Fig. 3a).

Similar to the mixed culture experiment, monocultures of *D. acuminata* did not utilize dissolved nitrate or phosphate in the culture media: dissolved inorganic nitrogen ( $\text{NO}_3^-/\text{NO}_2^-$ ,  $\text{NH}_4^+$ ) and phosphate concentrations held constant through the duration of the experimental period (Fig. 3d and 3e). At the start of the monoculture experiment, i.e., the inoculation of well-fed *D. acuminata* into fresh nutrient-enriched and nutrient-reduced media, initial particulate nitrogen concentrations in *Dinophysis* were  $24.2 \pm 3.1$  and  $23.4 \pm 1.2$  pmol/cell (Mean  $\pm$  STD, n=12), respectively, and remained constant for at least 6 days. By day 12, particulate nitrogen significantly decreased to  $8.1 \pm 1.9$  and  $14.9 \pm 5.6$  pmol/cell (Mean  $\pm$  STD, n=12) in the two treatments, respectively, and then again remained relatively constant for the remainder of plateau phase. An exception occurred on day 19, when particulate nitrogen decreased further to  $6.8 \pm 4.6$  and  $7.4 \pm 2.1$  pmol/cell (Mean  $\pm$  STD, n=6)

(Fig. 3b), respectively. Particulate phosphorus, however, remained steady during the entire experimental period (Fig. 3c), with the average cellular levels of  $2.08 \pm 0.29$  and  $1.98 \pm 0.19$  pmol/cell (Mean  $\pm$  STD,  $n=24$ ) in the nutrient-enriched and nutrient-reduced treatments, respectively.

### Toxin production

When incubated with prey, *Dinophysis* displayed similar patterns of OA, DTX1 and PTX2 production, whereby cell quotas (pg/cell) remained low during exponential growth, and rose during early (PTX2) to mid-plateau phase (DSP toxins) (Fig. 4). Interestingly, OA and DTX1 toxin quotas continued an increasing trend over the remainder of plateau phase, while PTX2 cell quotas decreased as the culture aged. Maximum OA, DTX1, and PTX2 quotas were  $0.59 \pm 0.03$ ,  $8.82 \pm 0.23$  pg/cell, and  $15.30 \pm 1.76$  pg/cell, respectively (Fig. 4a, 4b, 4c). Dissolved OA and DTX1 accumulated in the medium over exponential and early plateau phase (Fig. 4d, 4e), and then either continued to increase or plateaued as the culture aged. Concentrations of dissolved PTX2, instead, peaked at early to mid-plateau phase (Days 20–30) and rapidly declined into late plateau phase (Days 30–40, Fig. 4f). For each toxin, production rates were greatest during exponential growth of *D. acuminata* (Fig. 4g, 4h, and 4i). Maximum production rates were 0.065 pg OA/cell/day; 0.86 pg DTX1/cell/day; and 2.61 pg PTX2/cell/day in the mixed culture experiment.

Patterns of toxin accumulation in the cells and medium varied between the mixed and monoculture experiments with *Dinophysis*; however, we observed no effect of nutrient regime on toxin production or exudation in the *Dinophysis* monoculture trials, suggesting prey availability was critical to toxin production, not the uptake of dissolved nutrients. Unlike in the mixed cultures, where toxin quotas and concentrations changed with growth phase, experiments consisting of monocultures of *Dinophysis*, with reduced growth rates and biomass, displayed only minimal changes in OA, DTX1 or PTX2 toxin quotas, concentrations, and total toxin over the experimental period. Cellular OA, DTX1, and PTX2 levels were relatively constant over the starvation period, or plateau phase, with slight decreases in toxin contents on the last day of sampling (Fig. 5a, 5b, 5c). Concentrations of dissolved OA, DTX1, and PTX2 were similarly constant over time in the starvation treatments (Fig. 5d, 5e, 5f), and this lack of variation was reflected in similarly stable total toxin concentrations for each toxin quantified (Fig. 5g, 5h, 5i). The total OA, DTX1 and PTX2 concentrations at the end of incubation reached 140.9, 2,357 and 2,799 ng/mL, respectively. We were unable to detect a significant difference in any of the parameters tested (intracellular, extracellular and total OA, DTX1 or PTX2) between the nutrient-enriched and nutrient-reduced treatments in the monocultures of *Dinophysis*, i.e., in the absence of prey.

No DSP toxins or PTXs were detected in *M. rubrum* cultures, confirming that toxins were indeed produced by *D. acuminata* and not prey.

### Discussion

These experiments investigated the role of dissolved nitrate and phosphate in two mixotrophic organisms, *Dinophysis acuminata* and *Mesodinium rubrum*, in regards to

growth, particulate nutrient content, and toxin production under conditions of varying nutrient and prey availability. This was accomplished through a series of experiments growing monocultures of *M. rubrum* in nutrient-enriched medium, monocultures of *D. acuminata* in nutrient-enriched and nutrient-reduced medium, and mixed cultures of the ciliate and dinoflagellate in nutrient-enriched medium. The extensive and rapid decline in *M. rubrum* biomass that occurred within days upon inoculation of the ciliate into sterile-filtered seawater, prohibited us from including nutrient-reduced treatments of mixed culture or *M. rubrum* monoculture. Nonetheless, the collection of treatments and experiments conducted conclusively determined that *M. rubrum* utilized dissolved nitrate and phosphate in the medium immediately upon inoculation, whereas *D. acuminata*, alternatively, incorporated nitrogen and phosphorus through the ingestion of prey. We did not detect a direct effect of dissolved nitrate and phosphate on toxin production by *D. acuminata*.

### Nutrient uptake by *M. rubrum*

*M. rubrum*, a planktonic ciliate, incorporates plastids (Johnson and Stoecker, 2005; Hansen and Fenchel, 2006; Johnson *et al.*, 2007) and acquires additional growth factors by ingesting cryptophyte algae (Gustafson *et al.*, 2000). This organism can also survive and grow for long periods at low irradiance without feeding (Johnson and Stoecker, 2005; Smith and Hansen, 2007) although bacteria may potentially provide an important source of organic material under light-limited conditions (Moeller *et al.*, 2011). Dissolved organic nitrogen (Wilkerson and Grunseich, 1990) as well as dissolved nitrate and phosphate (this study) can be utilized by *M. rubrum* for enhanced growth when plastids and other promotional factors were previously obtained from their cryptophyte prey, *Teleaulax/Geminigera* spp. Using isotope uptake experiments,  $^{15}\text{N}$ , with field material from the upper euphotic zone off the coast of Peru, Wilkerson and Grunseich (1990) measured average nitrate uptake rates by *M. rubrum* to be  $2.08 \pm 1.42 \mu\text{g/L/h}$  under various irradiance conditions. In our laboratory study, not utilizing  $^{15}\text{N}$ , the nitrate uptake rate,  $1.38 \text{ pmol N/cell/day}$ , equates to  $1.61 \mu\text{g/L/h}$  (when *M. rubrum* concentration was 2,000 cells/mL in the monoculture), falling within the range of the previous field-based study. Dissolved phosphate was removed from culture medium at a maximum rate of  $1.63 \text{ pmol P/cell/day}$  in the monoculture.

Under nutrient-enriched conditions, monocultures of *M. rubrum* achieved stabilized nitrogen and phosphorous quotas, 25–30 pmol N/cell and 3–4 pmol P/cell, respectively, within two days of inoculation into fresh medium (Fig. 1b and 1c). These values equate to an N:P ratio of 8–10 (Fig. 6a), suggesting that either a higher cell quota of phosphorous, or lower quota of nitrogen, is desired by this ciliate relative to other “Redfield ratio (16:1) species”. Another mixotrophic, dinoflagellate *Gyrodinium galatheanum* (Li *et al.*, 2000) (*Gymnodinium galatheanum*, Nielsen 1996) reportedly had similarly low N:P ratios, indicating that this species had a large storage capability for phosphorus. Our data suggest that *M. rubrum* may also be able to luxuriously utilize phosphorus (Fig. 1e, 2f), skewing its ratio below Redfield values (Fig. 6a), requiring its predator to then assimilate nitrogen at a greater rate to balance nutrient availability and support division.

In the monoculture, particulate carbon quotas in *M. rubrum* were constant as cell concentrations increased, suggesting that the ciliate was able to maintain internal carbon

levels during cell division in the absence of prey, i.e., *M. rubrum* can assimilate carbon by other methods such as by photosynthesis or the uptake of dissolved or particulate organic matter (Johnson and Stoecker, 2005; Smith and Hansen, 2007; Moeller *et al.*, 2011). Previous studies have found that the ingestion of cryptophytes represents less than 5% of the required carbon requirements for *M. rubrum* growth and maintenance (Yih *et al.*, 2004; Johnson and Stoecker, 2005), indicating that plastid transfer is the primary benefit of grazing by *M. rubrum*. As a result of this acquired photosynthetic capability, it requires prey only when ambient nutrients are not sufficient for autotrophic growth. This is characteristic of the organisms classified by the mixotrophy model IIIA (Stoecker, 1998).

### Nutrient uptake by *D. acuminata*

Unlike *M. rubrum* which can utilize dissolved nitrate and phosphate, our results demonstrate that *D. acuminata* cannot directly assimilate these dissolved nutrients, and instead is a mixotrophic species that must continually acquire its nutrition and plastids from prey to grow photosynthetically (Park *et al.*, 2006; Kim *et al.*, 2008; Tong *et al.*, 2010). Our calculations reveal that *M. rubrum* was solely responsible for the uptake of dissolved phosphate in the mixed experimental cultures with *Dinophysis*, as determined by comparing nutrient utilization by *M. rubrum* in the monoculture experiment (Table 1). Interestingly, there was no discernable decrease in dissolved nitrate or ammonium in the mixed culture when *M. rubrum* prey was present (Fig. 2e) even though *M. rubrum* rapidly removed dissolved nitrate from culture medium when grown as a monoculture (Fig. 1d). We do not have an explanation for why the ciliate would utilize dissolved nitrate in monoculture, but not in the presence of a predator, but suspect that the observed contradiction may be correlated to the recycling between pools of bioavailable nitrogen in the system. Isotope-enrichment experiments could provide additional information regarding uptake rates and nutrient recycling in the medium, and should be considered as a future research direction.

Nitrogen content in *Dinophysis* rapidly increased with the consumption of prey, with no apparent increase in cellular phosphorus (Fig. 2b and 2c), suggesting a preferential assimilation of nitrogen by the dinoflagellate despite its phosphorus-rich ciliate prey (see above). After the removal of prey from the mixed culture, cellular nitrogen in *D. acuminata* rapidly declined as dinoflagellate cells continued to divide, albeit at a slower rate. This finding may help explain why *Dinophysis* in the mixed culture appeared to demonstrate “luxury” uptake of nitrogen, relative to phosphorus, bringing its N:P ratio temporarily above 16:1 (Fig. 6b). In contrast, *Dinophysis* appeared to only assimilate enough phosphorus to hold internal quotas constant in dividing cells, as demonstrated in the strain’s inefficient uptake of particulate nutrients from prey (Table 2). Based on our calculations, *D. acuminata* only assimilated 65 and 25 % of the prey’s particulate N and P, respectively, into their own biomass. Gisselson *et al.* (2001) investigated intracellular nutrient variation in field isolates of *Dinophysis norvegica*, showing that N and P quotas were 11.7 – 24.3 pmol/cell and 1.1 pmol/cell, respectively, with N:P ratios ranging from 6.26 – 36.3. In our study, *D. acuminata* possessed comparable quotas of cellular N and P (11.5 – 47.1 pmol N/cell, 1.6 – 2.4 pmol P/cell) and a comparable range of N:P ratios (5.7 – 21.5). And in agreement with our findings, cellular P quotas in *D. norvegica* were stable over time (1.1 pmol/cell, see Table 1 in



Gisselson *et al.*, 2001), suggesting that the phosphorus content of *Dinophysis* is far less variable than cellular N quotas.

At the end of the mixed culture experiment, i.e., during late plateau phase, cellular quotas of N, P, and C increased. This increase in nutrient concentrations is likely not solely a reflection of *Dinophysis* cell quotas, as these cells were transitioning from late plateau to decline, but instead, includes increased heterotrophic bacterial growth promoted by cell exudates and detrital matter in the aged, mixed batch culture that could contribute to our bulk measurements of particulate CNP, i.e., during filtration of culture (Nielsen *et al.* 2012; 2013). The presence of heterotrophic bacteria in all of our non-axenic batch cultures likely contributed to the particulate and soluble N and P concentrations during late-plateau phase, resulting from the breakdown and remineralization of cell exudates and detrital matter. For example, the concentrations of dissolved nitrate and phosphate remained relatively constant in the mixed culture until late plateau phase, when they both increased (Fig. 2e and 2f) perhaps due to the biotransformation of organic exudates from *Dinophysis* and/or *M. rubrum* into  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , and  $\text{PO}_4^{3-}$  (Tezuka 1989; John and Flynn 1999; Collos *et al.*, 2004). In contrast to the mixed culture experiment, dissolved nitrate and phosphate did not increase in the aged *Dinophysis* monoculture, likely reflecting a lack of organic debris available for remineralization. *Dinophysis* biomass was significantly reduced in the monoculture relative to the mixed culture, and ciliate organic matter and cellular exudates were removed and/or significantly diluted upon inoculation into fresh nutrient-enriched and nutrient-reduced, leaving relatively little material for bacterial remineralization (Fig. 3d, 3e).

Seeyave *et al.* (2009) determined that *D. acuminata* had a higher affinity for ammonium and urea, relative to nitrate in a field incubation experiment using natural populations. The cultures used in our study were only exposed to very low background concentrations of ammonium, and as such, it is difficult to determine if they could indeed utilize this form of nitrogen. However, our data provide convincing evidence that dissolved nitrate and phosphate are not assimilated by *D. acuminata* when incubated in the presence or absence of its prey. Given this uncertainty, additional studies into *Dinophysis* nutritional ecology, with a focus on ammonium, urea and other forms of organic nitrogen, should be considered, especially in light of the findings of Nagai *et al.* (2011), who report on the utilization of filtered ciliate exudate by *Dinophysis*.

### Toxin production by *D. acuminata*

Prey and light, and not dissolved nitrate and phosphate, are the direct drivers of growth and toxin production in this northwestern Atlantic strain of *D. acuminata*. An increase in the number of *D. acuminata* cells, in the presence of prey, led to elevated total toxin concentration (intra + extracellular toxins, ng/mL of culture, Fig. 5) of OA, DTX1 and PTX2. Simply put, more *Dinophysis* cells in the system resulted in more total toxin. In the absence of prey, *Dinophysis* growth rates slowed or ceased, and not surprisingly, no changes in intracellular or extracellular toxin were observed. Together, these results suggest that prey availability influenced the total amount of OA, DTX1, and PTX2. This finding is in agreement with previous reports on other species of *Dinophysis* in the field and in culture (Kim *et al.*, 2008; Riisgaard and Hansen, 2009; Campbell *et al.*, 2010; Gonzalez-Gil *et al.*,

2010; Minnhagen *et al.*, 2011; Sjoqvist and Lindholm, 2011; Hattenrath-Lehmann *et al.*, 2013), that reported maximum abundances of *Dinophysis* spp. occurring shortly after the peak and subsequent depletion of prey. With the use of an automated imaging sampler, Campbell *et al.* (2010) further linked prey and *Dinophysis* abundance to toxicity, and with the help of shellfish toxicity data (Deeds *et al.*, 2010), documented the first *Dinophysis*-related DSP closure in North America, Gulf of Mexico, TX, USA.

Dissolved nitrate and phosphate concentrations in the medium did not affect toxin profiles or quotas in *D. acuminata* or the exudation of toxins into the medium (Fig. 5). In agreement with previous findings (Fux *et al.*, 2011), the toxin profile of the Martha's Vineyard *D. acuminata* isolate, DAMV01, contained OA, DTX1, and PTX2 in all treatments (Figs. 4 and 5). Toxin quotas of OA and DTX1 showed similar patterns over the growth of *D. acuminata* in the mixed culture, with the lowest toxin quotas occurring during early exponential phase and increasing by early to mid-plateau phase (Fig. 4a and 4b). Intracellular levels of PTX2 peaked earlier and reached a maximum during late exponential phase to early plateau phase (Fig. 4c). This general pattern and dependency of toxin content upon growth stage has been documented previously for another northwestern Atlantic isolate of *D. acuminata* (Tong *et al.*, 2011) and multiple studies have shown that during plateau phase, or bloom maintenance, the most toxic cells are found (Pizarro *et al.*, 2009; Nagai *et al.*, 2011). Toxin production rates of OA, DTX1 and PTX2 in the mixed culture were also in agreement with previous findings, with maximum rates occurring during exponential growth and quickly declining upon transition into plateau phase (Fig. 4g, 4h, and 4i). In the absence of prey, no difference was observed in cellular, dissolved or total OA, DTX1 or PTX2 values over time (Fig. 5). A new finding, however, was that significantly more OA and DTX1 was retained in the cell, possibly as a carbon resource, when cells were lacking food (80% of toxins were intracellular) versus when prey were available (27.2% – 49.9% OA; 31.5% – 64.2% DTX1). In agreement with Nielsen *et al.* (2012), intracellular PTX2 quotas were similar between the starved and food-sufficient *Dinophysis* cultures.

Dissolved DSP toxins consistently, but slowly, accumulated as the *Dinophysis* cultures aged (Fig. 4d, 4e, 5d, 5e); however, the cell concentration data do not support extensive cell death during plateau phase (Fig. 2a, Fig. 3a). As we have calculated previously (Smith *et al.*, 2012), concentrations of dissolved toxins can be overestimated during the late plateau phase due to artificial cell lysis during the harvesting of cells for toxin analyses, i.e., sieving, and/or during periods of rapid growth when smaller cells were not retained by the sieve. This error was minimal, i.e., did not lead to any significant changes over-time.

Toxin quotas and profiles produced by cultured *Dinophysis* spp. can vary greatly among isolates. In the present study, our isolate of *D. acuminata* from the northwestern Atlantic was characterized as having low levels of OA (0.18 – 0.58 pg/cell) and DTX1 (2.2 – 8.8 pg/cell), but moderate amounts of PTX2 (7.8 – 15.3 pg/cell). In contrast, *D. acuminata* isolated from Japan had high levels of OA (2.1 – 12.2 pg/cell) and PTX2 (14.7 – 107.1 pg/cell), and low DTX1 content (0.2 – 4.8 pg/cell) (Kamiyama and Suzuki, 2009; Kamiyama *et al.*, 2010; Nagai *et al.*, 2011). An isolate from Denmark had a unique toxin profile, only producing PTX2, with toxin quotas ranging from 12.7 to 35.6 pg PTX2/cell (Nielsen *et al.* 2012). These isolates were grown under similar experimental conditions, e.g., temperature (14 –

18°C) and light intensity (65 – 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), in the presence of *Mesodinium* spp. and sampled at similar growth stages, suggesting that toxin production and retention is controlled by intrinsic factors specific to a *Dinophysis acuminata* strain or a driver that has not yet been investigated (e.g., prey isolate or prey nutritional quality).

In summary, while *D. acuminata* did not utilize dissolved nitrate and phosphate in our study, these pools of inorganic nutrients supported *M. rubrum* growth and elevated biomass. Additionally, active toxin production by *Dinophysis* was only observed in the presence of ciliate prey. Together these data suggest that while dissolved nitrate and phosphate do not have a direct effect on toxin production or retention by *D. acuminata*, these nutrient pools may contribute to prey growth and biomass, thereby indirectly promoting *D. acuminata* blooms and overall toxin in the system. In light of recent work by Nagai *et al.* (2011) demonstrating a direct relationship between the uptake of organic substances by *D. acuminata* and increased toxin concentration, we conclude that prey abundance and dissolved inorganic and organic nutrients should be considered in monitoring or modeling *D. acuminata* bloom dynamics and toxicity.

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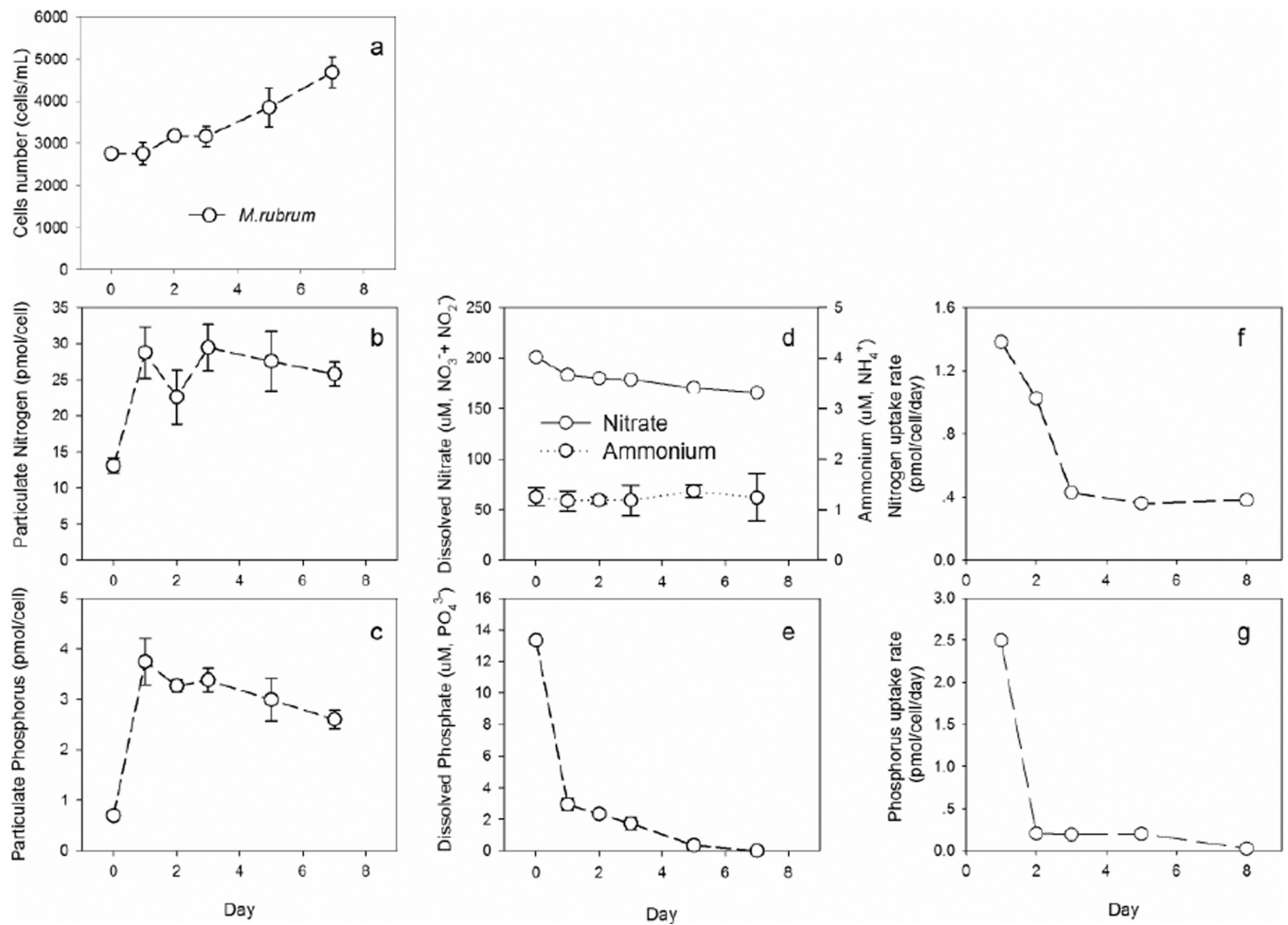
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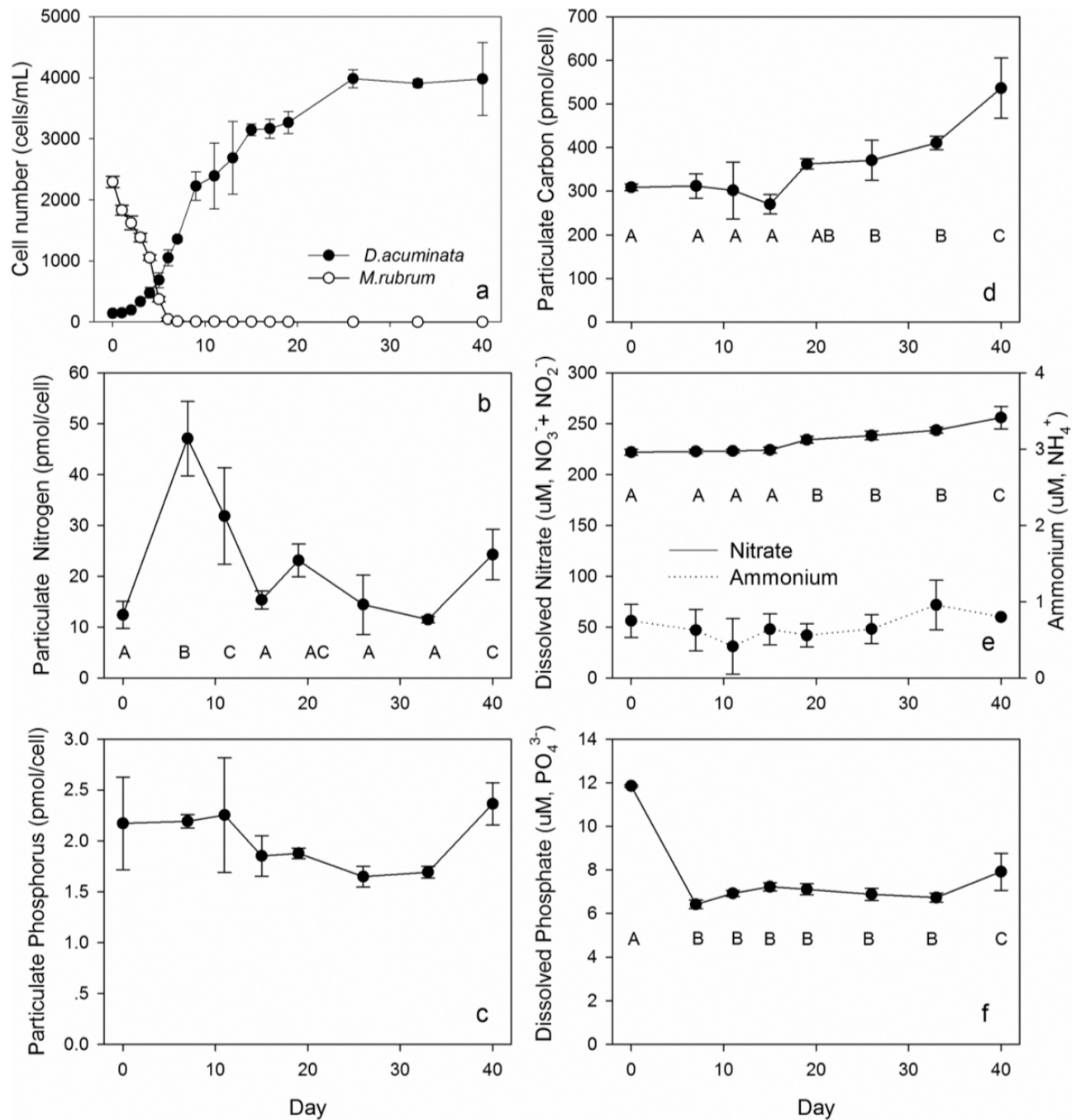
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**Fig 1.**

The monoculture growth response of *Mesodinium rubrum* in nutrient-enriched medium, expressed as (a) cell concentration, and the particulate and dissolved nutrient levels and uptake rates of *M. rubrum*: (b) particulate nitrogen and (c) particulate phosphorus; (d) dissolved concentrations of nitrate/nitrite and ammonium, and (e) phosphate in the medium (Mean  $\pm$  STD, n=3); (f) uptake rates of nitrogen and (g) phosphorus by *M. rubrum* during the experimental growth period.



**Fig 2.** The mixed culture growth response of *D. acuminata* and *M. rubrum* in nutrient-enriched medium, expressed as (a) cell concentrations, and the associated particulate and dissolved nutrient levels: (b) particulate nitrogen, (c) particulate phosphorus and (d) particulate carbon in *D. acuminata*; (e) dissolved concentrations of nitrate/nitrite and ammonium, and (f) phosphate in the medium (Mean  $\pm$  STD, n=3). One way repeated measurement ANOVA was run for the statistical analysis of the particulate and dissolved inorganic nutrient levels (2b–f)

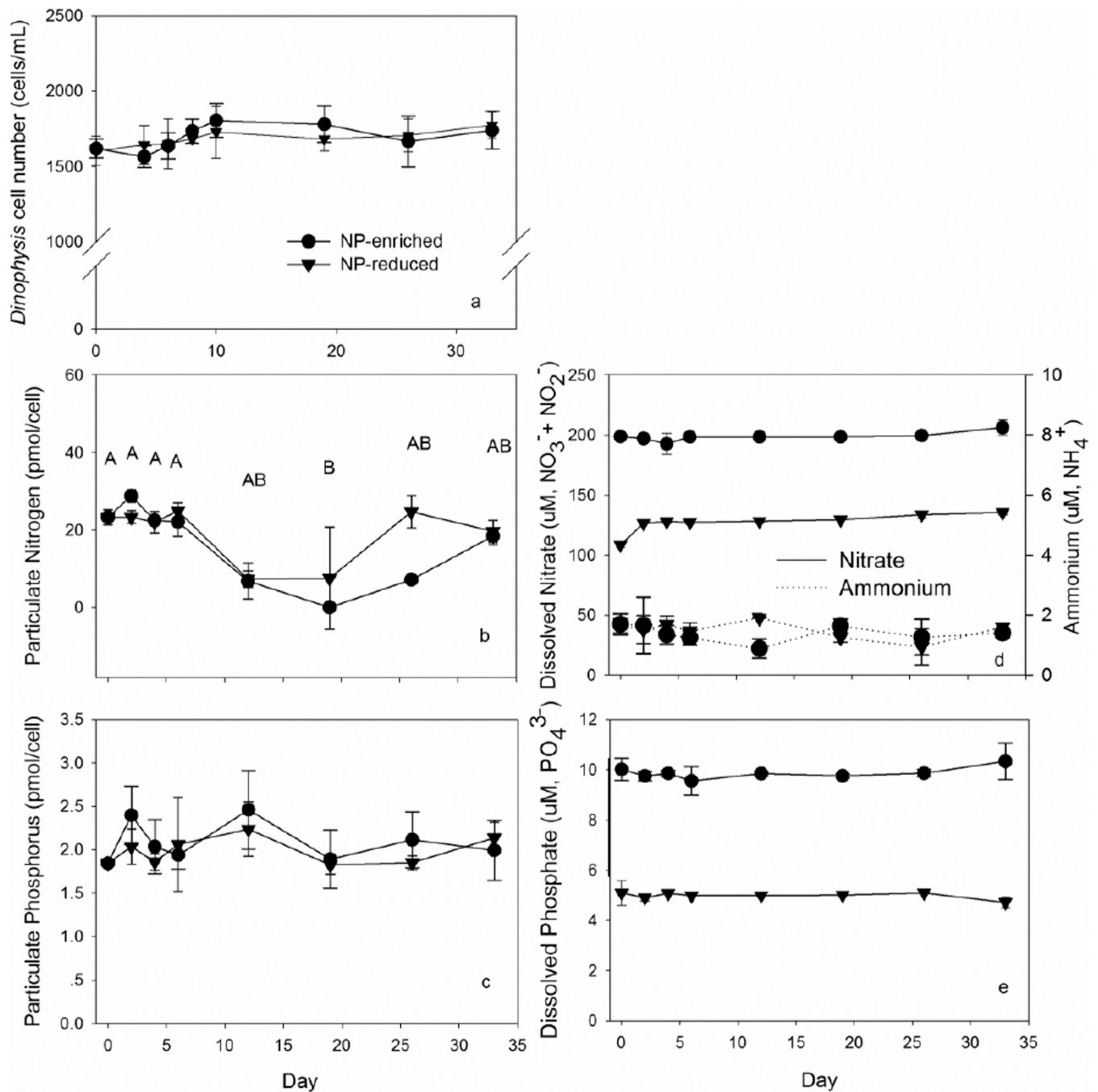
and significance is indicated with uncommon letters. No significance was detected in panel 2c.

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**Fig 3.** The monoculture growth response of *D. acuminata* under two nutrient regimes (nutrient-enriched medium and nutrient-reduced medium), expressed as (a) cell concentration, and the associated particulate and dissolved nutrient levels: (b) particulate nitrogen and (c) particulate phosphorus of *D. acuminata*; (d) dissolved concentrations of nitrate/nitrite and ammonium, and (e) phosphate in the medium (Mean  $\pm$  STD, n=3). One way repeated measurement ANOVA was run for the statistical analysis of the particulate and dissolved

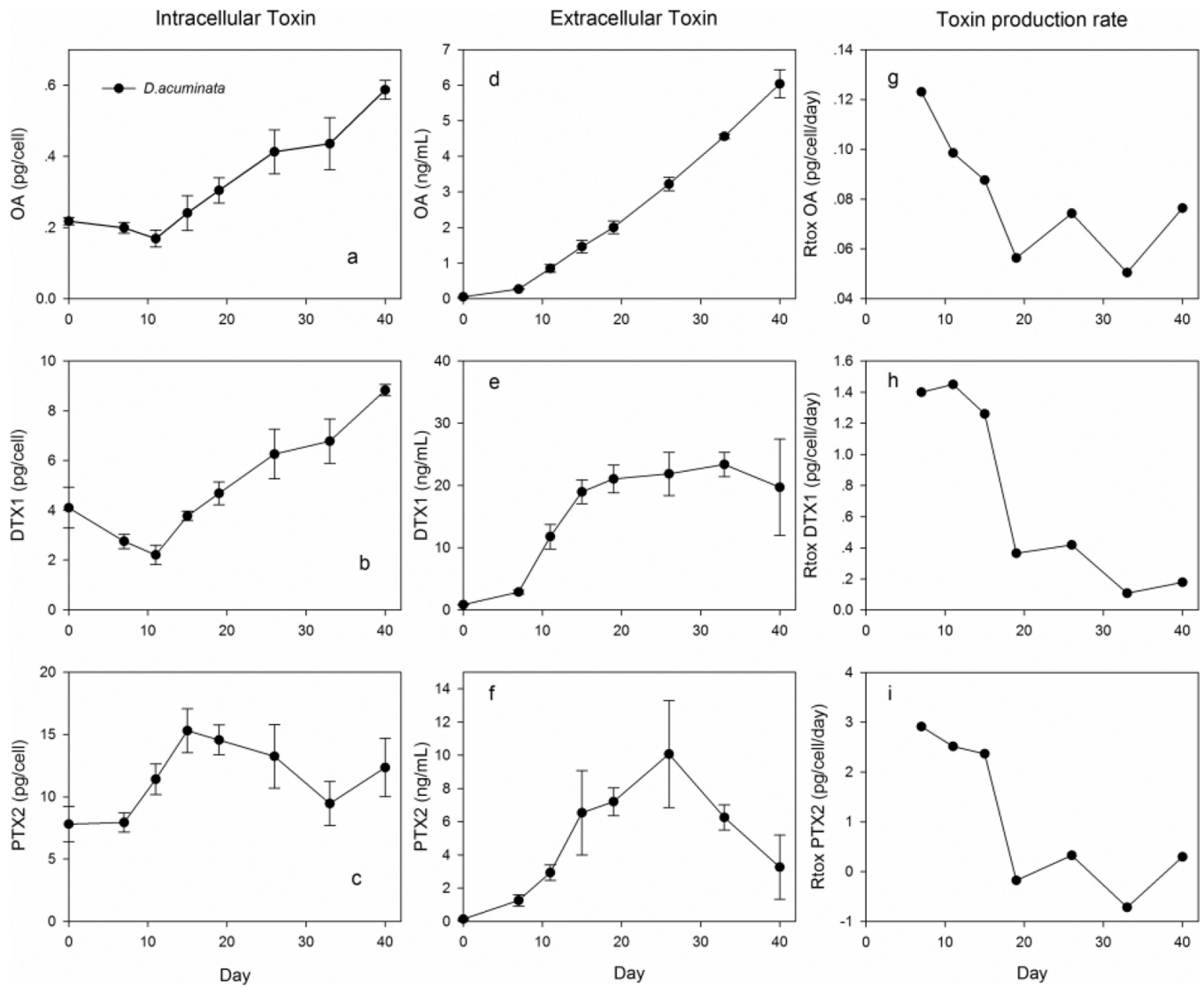
inorganic nutrient levels (3b–e) and significance is indicated with uncommon letters. No significance was detected in panels 3c, 3d and 3e.

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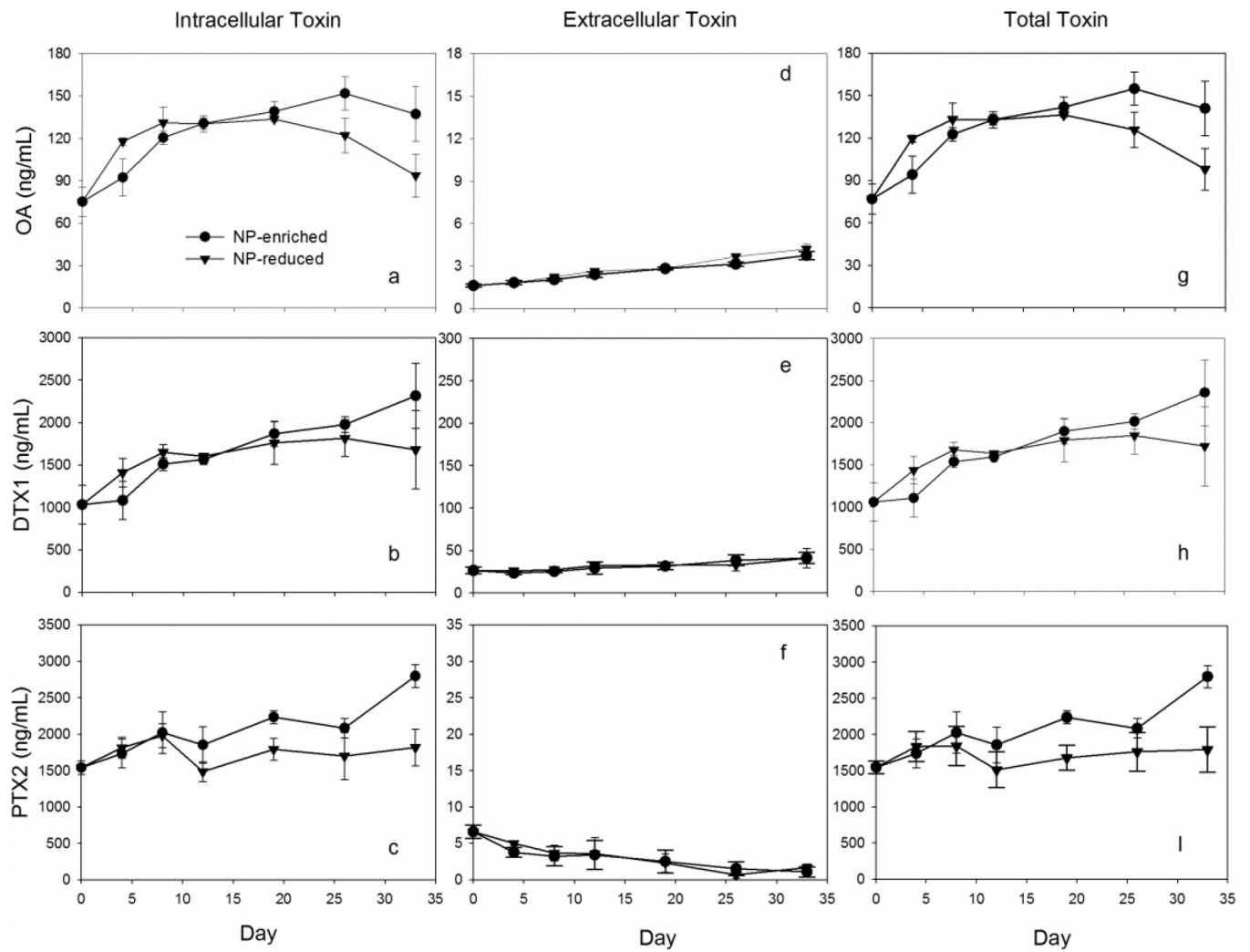
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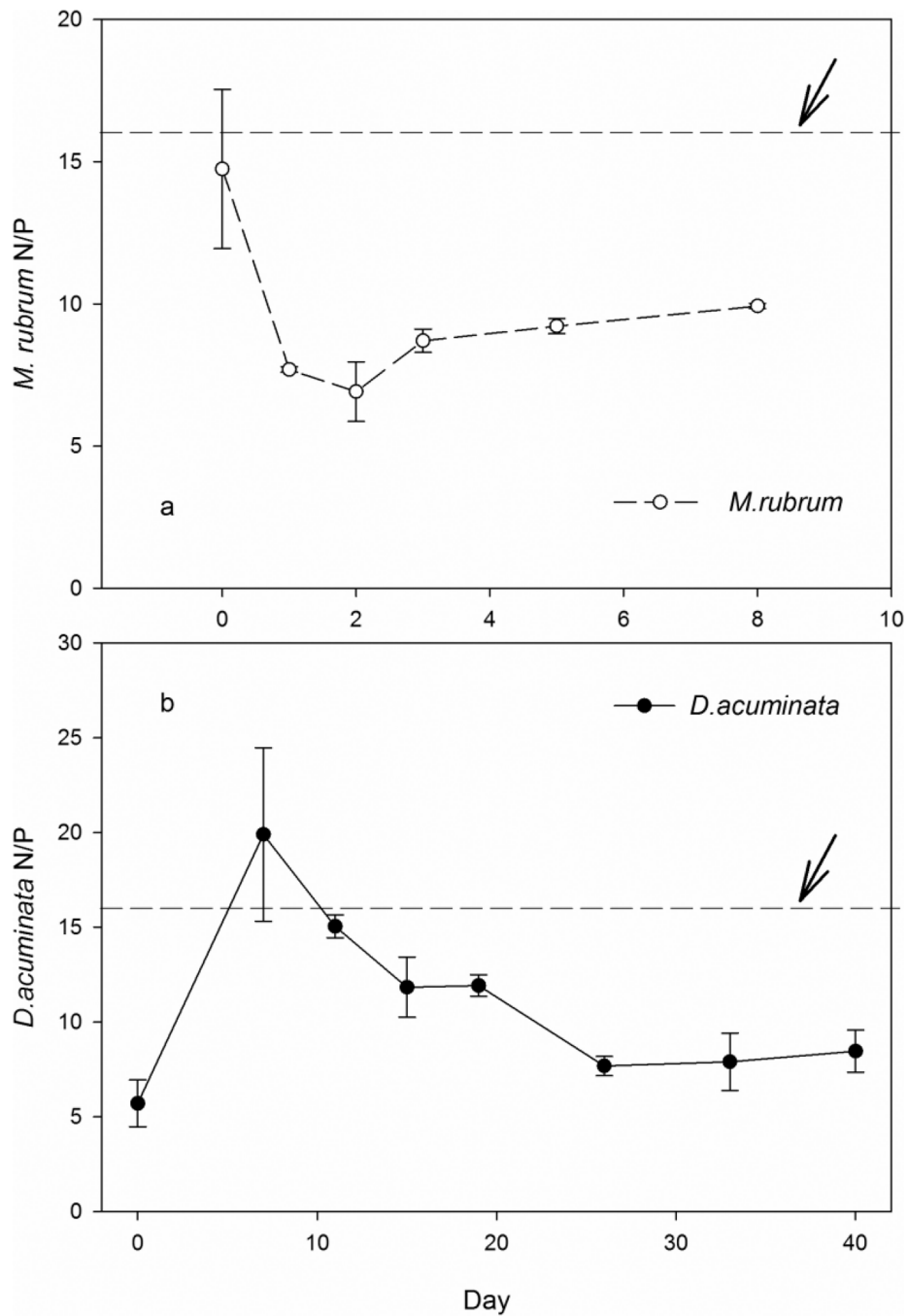


**Fig 4.** Intracellular (a, b, c) toxin quotas, (d, e, f) extracellular toxin concentrations in the medium, and (g, h, i) toxin production by *D. acuminata* in the mixed culture, i.e., in the presence of ciliate prey (Mean  $\pm$  STD, n=3). Toxins quantified include okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2).





**Fig 5.** Intracellular (a, b, c) toxin concentrations, (d, e, f) extracellular toxin concentrations in the medium, and (g, h, i) total toxin concentration (intra + extracellular) in the *D. acuminata* monoculture experiments, i.e., in the absence of ciliate prey. Monocultures were conducted under two nutrient regimes (nutrient-enriched medium and nutrient-reduced medium; Mean  $\pm$  STD, n=3). Toxins quantified include okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2).



**Fig 6.** Nitrogen to phosphorus ratio (N:P) of (a) *M. rubrum* in monoculture (b) and *D. acuminata* in the mixed culture in nutrient-enriched medium (Mean  $\pm$  STD, n=3). Arrows indicate the N:P Redfield ratio of 16:1.

**Table 1**

Predicted amount of dissolved nitrate (\*Nm) and phosphate (\*Pm) that could be utilized by *M. rubrum* during the first seven days of the mixed culture experiment, based upon the *M. rubrum* monoculture experiment.

	Day 1	Day 2	Day 3	Day 5	Day 7	Total
$\Theta N$ (pmol/cell/d)	1.38	1.03	1.06	0.85	0.38	
$\Theta P$ (pmol/cell/d)	1.63	0.80	0.92	0.45	0.48	
$\bar{C}$ (cells/mL)	2055	1725	1501	771	105	
*Nm ( $\mu M$ )	2.84	1.78	1.59	0.66	0.04	6.91
*Pm ( $\mu M$ )	3.35	1.38	1.38	0.35	0.05	6.51

$\Theta N$ : observed nitrogen uptake rate of *M. rubrum* in monoculture

$\Theta P$ : observed phosphorus uptake rate of *M. rubrum* in monoculture

$\bar{C}$ : natural logarithm (ln) average of the observed *M. rubrum* cell concentration in the mixed culture as they are consumed by *D. acuminata*

Table 2

Ingestion rate, calculated and observed nutrient uptake, and feeding efficiency of *Dinophysis* during the 7-day feeding period of the mixed culture experiment.

	Average <i>Dinophysis</i> prey ingestion during the 7-day feeding period ( <i>Mesodinium/Dinophysis</i> )	Average cellular nutrient content of <i>Mesodinium</i> (pmol/ <i>Mesodinium</i> )	Calculated nutrient uptake by <i>Dinophysis</i> assuming 100% feeding efficiency (pmol/ <i>Dinophysis</i> )*	Observed nutrient content of <i>Dinophysis</i> before feeding (pmol/ <i>Dinophysis</i> )	Observed nutrient content of <i>Dinophysis</i> after feeding (pmol/ <i>Dinophysis</i> )	Observed nutrient uptake by <i>Dinophysis</i> during the 7-day feeding period (pmol/ <i>Dinophysis</i> )**	Feeding efficiency***
Nitrogen	6.72	26.24	176.33	12.4	47.1	115.35	65.42%
Phosphorus		2.93	19.69	2.17	2.19	4.94	25.09%

\* Calculated nutrient uptake of *Dinophysis* assuming that nutrient assimilation from prey to predator is 100% = average *Dinophysis* prey cell during the 7-day feeding period multiplied by the average cellular nutrient contents of prey.

\*\* Observed assimilation of particulate nutrients by *Dinophysis* = (Observed nutrient content of *Dinophysis* after feeding multiplied by the number of *Dinophysis* after feeding - Observed nutrient content of *Dinophysis* before feeding multiplied by the number of *Dinophysis* before feeding) divided by the average number of *Dinophysis* during the 7 day feeding period.

\*\*\* Feeding efficiency = Observed nutrient uptake divided by the calculated nutrient uptake assuming that nutrient assimilation from prey to predator was 100%