

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

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ABSTRACT: *Mesodinium rubrum* (as prey) is a critical component, in addition to light, for growth and toxin production by the mixotrophic dinoflagellate *Dinophysis acuminata*. Little is known, however, about the role that dissolved inorganic nutrients play in this predator–prey relationship and system toxicity. A series of experiments were conducted to investigate the possible uptake of dissolved nitrate and phosphate by (1) *D. acuminata* starved of prey, (2) *D. acuminata* feeding on *M. rubrum*, and (3) *M. rubrum* grown in nutritionally modified medium. All single-clone or mixed cultures were monitored for dissolved and particulate nutrient levels over the growth cycle, as well as for growth rate, biomass, and toxin production when appropriate. *Dinophysis acuminata* did not utilize dissolved nitrate or phosphate in the medium under nutrient-enriched or nutrient-reduced regimes, in the absence or presence of prey, or during any growth phase monitored. 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. *Mesodinium rubrum*, in contrast, rapidly assimilated dissolved nitrate and phosphate, with maximum uptake rates of 1.38 pmol N cell^{−1} d^{−1} and 1.63 pmol P cell^{−1} d^{−1}, respectively. While *D. acuminata* did not benefit directly from the dissolved nitrate and phosphate, its growth (0.37 ± 0.01 d^{−1}) and toxin production rates for okadaic acid, dinophysistoxin-1 and pectenotoxin-2 (0.1, 0.9 and 2.6 pg cell^{−1} d^{−1}, 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 toxins in the system.

KEY WORDS: *Dinophysis acuminata* · *Mesodinium rubrum* · Diarrhetic shellfish poisoning · Okadaic acid · Dinophysistoxin · Pectenotoxins · 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 & 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 this genus. In these studies, experimental growth media were pre-

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pared 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. A decade later, it was discovered that *D. acuminata* could be maintained in the laboratory using a specific 3-stage food chain: the cryptophyte *Teleaulax amphioxieia* is fed to the ciliate *Mesodinium rubrum*, which, in turn, is fed to the dinoflagellate 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 & Hansen 2009).

Several *Dinophysis* species, along with a few *Prorocentrum* spp., are responsible for the 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: the highest production rates occurred during exponential growth and the highest toxin quotas were coincident with the early-mid stationary phase (Tong et al. 2011, Smith et al. 2012). Conversely, production of pectenotoxin-2 (PTX2) 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 to 22°C, but that PTX2 toxin cell quotas showed an inverse trend at these temperatures. No relationship was detected between temperature and 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 & 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 widespread, 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 genus is tolerant of both 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, Sjöqvist & 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 NO₃[−], along the western coast of South Africa, and displayed a greater affinity for NH₄⁺ and urea, relative to NO₃[−], 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 on the local ecosystem or public health.

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 & 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 & Grunseich 1990, Kifle & 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 dinoflagellate growth and toxicity. To address these questions, we quantified changes in particulate and dissolved nutrients over multiple growth phases 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.

MATERIALS 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 2008. The ciliate *Mesodinium rubrum* and the cryptophyte *Teleaulax amphioxeia* were isolated from Inokushi Bay (131° 89' E, 32° 79' N) in Oita Prefecture, Japan, in February 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_2SeO_3 was added and CuSO_4 was reduced to a concentration of 10^{-8} M each (Anderson et al. 1994). Cultures were maintained at 15°C with an irradiance of 65 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ on a 14 h light:10 h dark photoperiod.

To maintain cultures of the ciliate, *M. rubrum*, the culture was transferred every 2 weeks, by mixing 70 ml of 2-wk-old culture ($\sim 10\,000$ cells ml^{-1}) and 2 ml of *T. amphioxeia* (containing $1.2\text{--}1.6 \times 10^6$ cells), with 150 ml of modified f/6 medium. *Teleaulax amphioxeia* culture was maintained by inoculating 1 ml of the culture ($6.0\text{--}8.0 \times 10^5$ cells ml^{-1}) 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 4 wk by adding 2 ml of *M. rubrum* ($\sim 10\,000$ cells ml^{-1}) and 2 ml of *D. acuminata* (~ 1800 cells ml^{-1}) 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 predators) and *D. acuminata* (both in the presence and absence of prey) and the effect on growth, toxin pro-

duction, 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.

Mesodinium rubrum monoculture experiment

To examine nutrient uptake by *M. rubrum* in the absence of cryptophyte prey or dinoflagellate predators, 220 ml of the ciliate maintenance culture 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 \pm SD, $n = 3$). The ciliate monoculture was monitored for growth rate, biomass, dissolved nitrate/nitrite and phosphate, and particulate nitrogen and phosphorus quotas over exponential and stationary 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 to 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 ± 2.2 pmol cell^{-1} carbon, 13.1 ± 1.1 pmol cell^{-1} nitrogen and 0.7 ± 0.01 pmol cell^{-1} phosphorus (mean \pm SD, $n = 3$) 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 carryover from the inoculum *M. rubrum* and *D. acuminata* cultures) were 222.06 ± 3.05 μM nitrate, 0.75 ± 0.22 μM ammonium and 11.85 ± 0.06 μM phosphate

(mean \pm SD, $n = 3$). The mixed experimental cultures of *D. acuminata* and *M. rubrum* were maintained for 40 d to include early exponential to late-plateau growth phases. Triplicate Fernbach flasks were inoculated with ca. 2000 and 100 cells ml^{-1} of *M. rubrum* and *D. acuminata* (inoculated from early plateau phase), respectively, to achieve a total volume of 1400 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.

Dinophysis acuminata monoculture experiment

To quantify the utilization of dissolved nitrate and phosphate by *D. acuminata* during periods when ciliate prey were not available, we monitored (1) growth, (2) the concentration of dissolved nitrate/nitrite, ammonium, and phosphate in the medium, (3) particulate nitrogen and phosphorus quotas, and (4) toxin production in the monoculture over time. The monoculture experiment was conducted in 2 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 carryover from the *M. rubrum* inoculum) consisted of 1.70 ± 0.35 μM ammonium, 198.73 ± 2.22 μM nitrate, and 10.01 ± 0.45 μM phosphate (mean \pm SD, $n = 3$). The nutrient-reduced medium (including sterile-filtered seawater + nutrient carryover from the *M. rubrum* inoculum) had initial concentrations of 1.65 ± 0.24 μM ammonium, 108.49 ± 2.02 μM nitrate, and 5.09 ± 0.50 μM phosphate (mean \pm SD, $n=3$). 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 1500 cells ml^{-1} 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 solution (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 *D. acuminata*. For *M. rubrum*, samples for nutrient analysis were collected around 14:00 h daily for the first 4 d and every 3 d 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 2 d during the first week and then once a week for the remaining 4 wk.

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 h, 0.8 μm , 25 mm) for total particulate organic carbon/nitrogen analysis (CHN). Another 25–50 ml of culture was collected through membrane filters (PALL Supor R-800, 0.8 μm , 25 mm) for the determination of total particulate phosphorus. Filtrate from the CHN samples was collected for dissolved inorganic nutrient analysis ($\text{NO}_3^-/\text{NO}_2^-$, NH_4^+ and PO_4^{3-}). After collection, all filters were placed in a 60°C drying oven for 24 h and were then 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 hydrolization, all particulate phosphorous was converted to, and measured as, dissolved orthophosphate (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, USA) 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 *D. acuminata* cells and media samples in the mixed and monoculture treatments were analyzed for toxin at 8 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⁻¹) 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 a Vortex-Genie 2 mixer before being passed through a solid-phase extraction (SPE) filter. The filters (Oasis HLB 60 mg; Waters) 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⁻¹, washed with Milli-Q water (6 ml), and eluted with methanol (1 ml) into 1.5-ml high recovery vials. The extracts were stored at -20°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°C in a heating block, dried under a stream of N₂, 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

(TQMS) (Waters Micromass) coupled to an Agilent 1100 HPLC. Separation was achieved on a C8 Hypersil column (50 × 2.1 mm; 3.5 μm particle size) maintained at 20°C. The flow rate was set at 0.25 ml min⁻¹ and a volume of 10 μl was injected. A binary mobile phase was used, with Phase A (100% aqueous) and Phase B (95% aqueous liquid chromatography–mass spectrometry 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, holding for 3 min, then decreasing to 30% B in 0.1 min and holding for 3 min to equilibrate at initial conditions before the next run started. The TQMS was operated in multiple reaction monitoring 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, m/z 876.5>213.0 in positive ionization mode. Both 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 *Dinophysis acuminata* and the ciliate prey *Mesodinium 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⁻¹), respectively, t is the experimental time (d), and μ (d⁻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 (cells predator⁻¹ d⁻¹), was calculated using the formulas developed by Jakobsen & 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 concentration x (*M. rubra*) have an exponential increase, with the growth rate constants μ_y and μ_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 μM ($\mu\text{mol l}^{-1}$). Nutrient uptake rate θ (amount of nutrient $\text{mol cell}^{-1} \text{d}^{-1}$) 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 (NO_3^- , NO_2^- and NH_4^+) and phosphate (PO_4^{3-}), at Time 1 and Time 2 (μM), respectively, and \bar{C} is the natural logarithm (ln) average of the *D. acuminata* cell concentration, which can be calculated using (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 milliliter; the latter was calculated by adding the particulate and dissolved toxin concentrations together. The net toxin production rate R_{tox} (toxin $\text{cell}^{-1} \text{d}^{-1}$) was determined using intracellular quotas with the equation (Anderson et al. 1990, Tong et al. 2011):

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

where T_1 and T_2 are the total toxin concentrations (intracellular + extracellular, toxin ml^{-1}) at Time 1 and Time 2, respectively.

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 *D. acuminata* was examined over time using 2-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 *Mesodinium 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 7-d sampling period (Fig. 1a) with mean values ($n = 3$) of 0.062 d^{-1} in the nutrient-enriched medium. When *M. rubrum* was grown in the absence of prey or predators, the ciliate took up dissolved nitrate and phosphate from the medium and assimilated them into particulate N and P (Fig. 1b–g). The particulate nitrogen (PN) and particulate phosphorous (PP) contents of *M. rubrum* were $13.1 \text{ pmol cell}^{-1}$ (Fig. 1b) and $0.7 \text{ pmol cell}^{-1}$ (Fig. 1c), respectively, in the initial monoculture. Within 1 d of being inoculated into fresh medium, in the absence of food, there was a dramatic increase in both PN ($28.8 \text{ pmol cell}^{-1}$) and PP ($3.7 \text{ pmol cell}^{-1}$) 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,f) was pronounced, with nitrate uptake rates of $1.38 \text{ pmol N cell}^{-1} \text{d}^{-1}$ on Day 1. *Mesodinium rubrum* maintained a cell quota of about $27.8 \text{ pmol N cell}^{-1}$ over the rest of the experimental period. There was also significant uptake of dissolved phosphate on Day 1, $1.63 \text{ pmol P cell}^{-1} \text{d}^{-1}$, 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 *Dinophysis acuminata* in the mixed culture

In mixed cultures, the mean exponential growth rate of *D. acuminata* was $0.37 \pm 0.01 \text{ d}^{-1}$ over the first 9 d after inoculation of predator and prey. This exponential growth continued for 2 d after ciliate prey were completely grazed from the cultures, i.e. on Day 7 (Fig. 2a). Cultures continued to grow, but at a slower rate, $0.11 \pm 0.002 \text{ d}^{-1}$, for the following 7 d, reaching a maximum cell concentration of 3986 cells

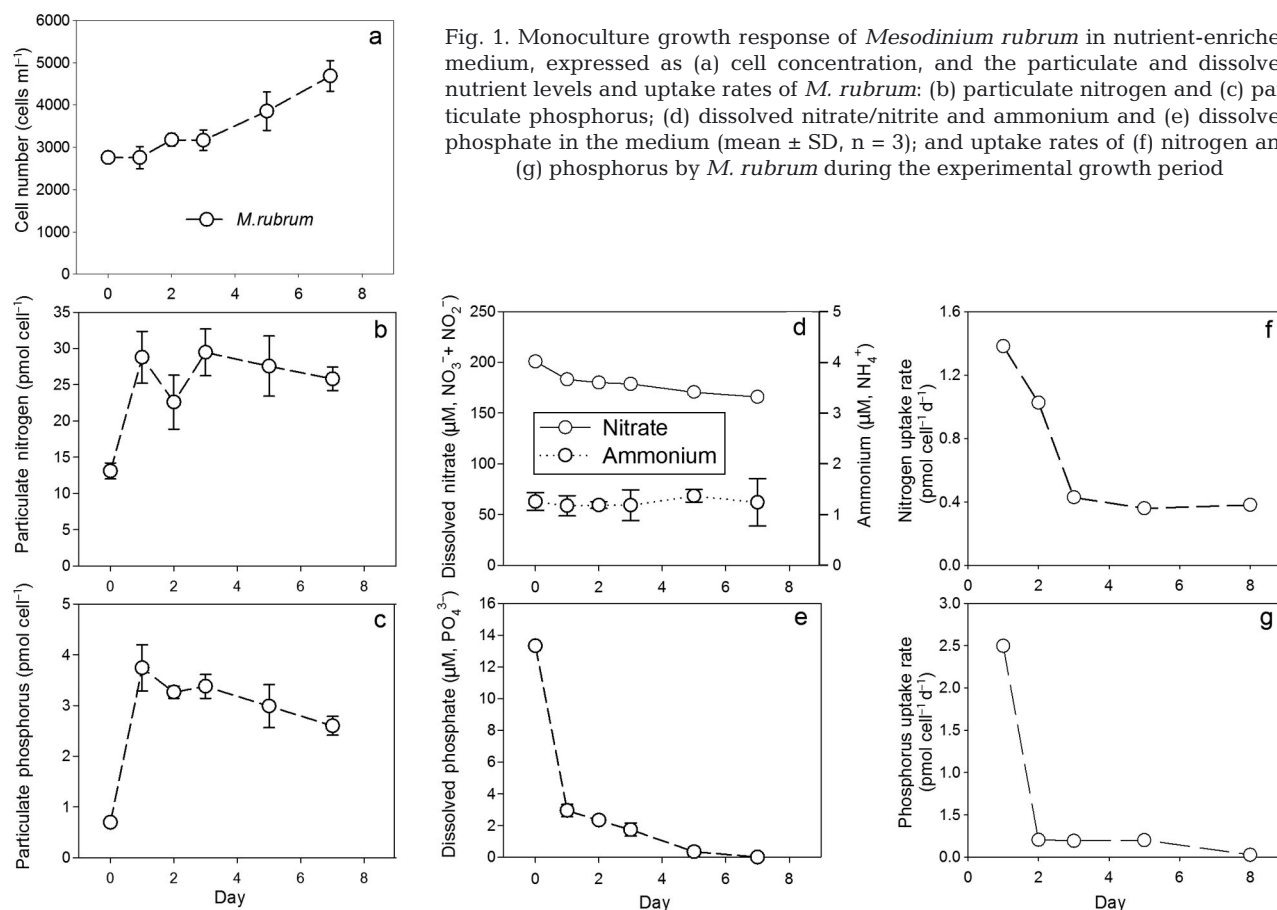


Fig. 1. Monoculture growth response of *Mesodinium rubrum* in nutrient-enriched medium, expressed as (a) cell concentration, and the particulate and dissolved nutrient levels and uptake rates of *M. rubrum*: (b) particulate nitrogen and (c) particulate phosphorus; (d) dissolved nitrate/nitrite and ammonium and (e) dissolved phosphate in the medium (mean \pm SD, $n = 3$); and uptake rates of (f) nitrogen and (g) phosphorus by *M. rubrum* during the experimental growth period

ml⁻¹. The average cell density of *M. rubrum* in the mixed culture upon inoculation was 2296 cells ml⁻¹ (Fig. 2a).

Dinophysis acuminata directly acquired nitrogen and phosphorus through the consumption of ciliate prey (Fig. 2b,d). As such, nitrogen accumulation by *D. acuminata* significantly increased during the period of prey consumption, rising during early exponential growth from 12.4 ± 2.7 pmol cell⁻¹ in the initial inoculum culture to 47.1 ± 7.3 pmol cell⁻¹ (mean \pm SD, $n = 3$) on Day 7, when the ciliate prey was completely consumed in the mixed culture (Fig. 2a,b). Thereafter, cellular PN in *Dinophysis* decreased as the result of continued cell division and lack of food; *Dinophysis* PN fell to near-initial levels of 15.4 pmol cell⁻¹ in the later stages of the experiment.

The PP content of *D. acuminata* did not change as dramatically as PN throughout the course of the experiment, with P quotas ranging from 1.6 to 2.4 pmol cell⁻¹. Unlike the pattern observed with PN (Fig. 2b), the PP content of *D. acuminata* cells remained constant during exponential growth. After the consumption of prey, P quotas in *D. acuminata* then fol-

lowed 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 PP in the first 26 d of the experiment, with no significant change in cell quotas (average of 320.9 pmol cell⁻¹, $n = 18$). At the end of the mixed culture experiment, however, PC in *Dinophysis* increased to 410.7 pmol cell⁻¹ (mean, $n = 3$) on Day 33, and finally reached 536.4 pmol cell⁻¹ (mean, $n = 3$) on Day 40 (Fig. 2d).

Dissolved inorganic nitrogen ($\text{NO}_3^-/\text{NO}_2^-$, NH_4^+ ; Fig. 2e) concentrations in the mixed culture medium were constant during the exponential growth phase of *Dinophysis*. In contrast, the concentration of dissolved phosphate decreased in the medium while *M. rubrum* prey were present in the mixed culture and actively utilizing this nutrient, from Day 0 (11.87 ± 0.06 μM) to Day 7 (6.41 ± 0.20 μM; 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 mixed culture medium.

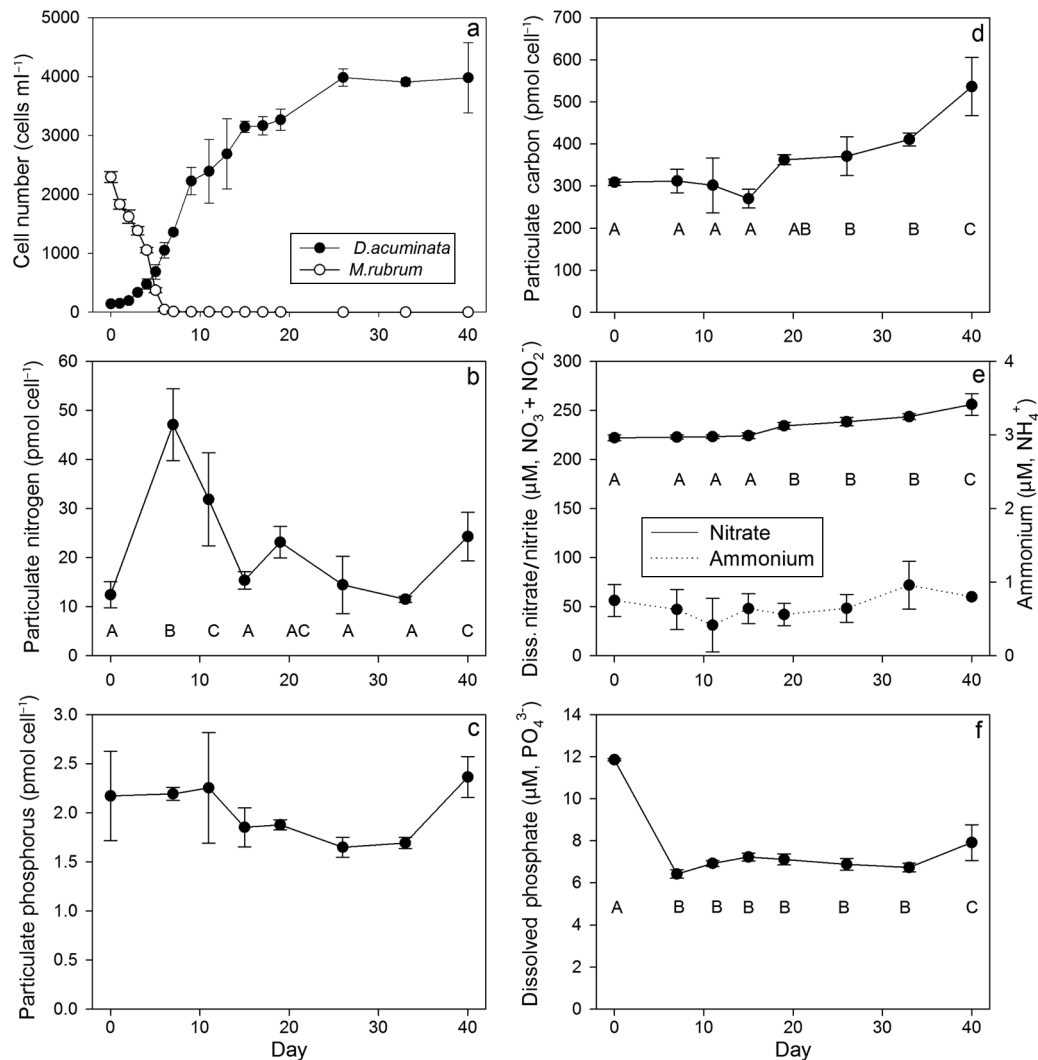


Fig. 2. Mixed culture growth response of *Dinophysis acuminata* and *Mesodinium rubrum* in nutrient-enriched medium, expressed as (a) cell concentration, and the associated particulate and dissolved (diss.) nutrient levels: (b) particulate nitrogen, (c) particulate phosphorus, and (d) particulate carbon in *D. acuminata*; and dissolved concentrations of (e) nitrate/nitrite and ammonium and (f) phosphate in the medium (mean \pm SD, n = 3). One-way repeated-measures ANOVA was run for the statistical analysis of the particulate and dissolved inorganic nutrient levels (b–f) and significance is indicated with different letters. No significance was detected in (c)

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 μ M in 7 d. Similarly, *M. rubrum* had the potential to decrease the dissolved phosphate pools in the mixed culture by 6.5 μ M in 7 d. As there was only an absolute decrease in dissolved phosphate of 5.5 ± 0.3 μ M from the medium dur-

Table 1. Predicted amount of dissolved nitrate (*Nm) and phosphate (*Pm) that could be utilized by *Mesodinium rubrum* during the first 7 d of the mixed culture experiment, based on the *M. rubrum* monoculture experiment. θ N: observed nitrogen uptake rate of *M. rubrum* in monoculture; θ 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 *Dinophysis acuminata*

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

ing 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-d time frame was chosen, as this represents the period of co-incubation before *M. rubrum* were completely consumed by *D. acuminata* 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, *D. acuminata* had minimal growth under both nutrient regimes: 0.002 and

0.003 d⁻¹ in the nutrient-enriched and nutrient-reduced media, 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 ($\text{NO}_3^-/\text{NO}_2^-$, NH_4^+) and phosphate concentrations were constant through the duration of the experimental period (Fig. 3d,e). 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 PN concentrations in *Dinophysis* were 24.2 ± 3.1 and 23.4 ± 1.2 pmol cell⁻¹ (mean \pm SD, $n = 12$), respectively, and remained constant for at least 6 d. By Day 12, PN significantly decreased to 8.1 ± 1.9 and 14.9 ± 5.6 pmol cell⁻¹ (mean \pm SD, $n = 12$) in the 2 treatments, respectively, and then again remained relatively constant for the remainder of the plateau phase. An exception occurred on Day 19, when PN decreased further to 6.8 ± 4.6 and 7.4 ± 2.1 pmol

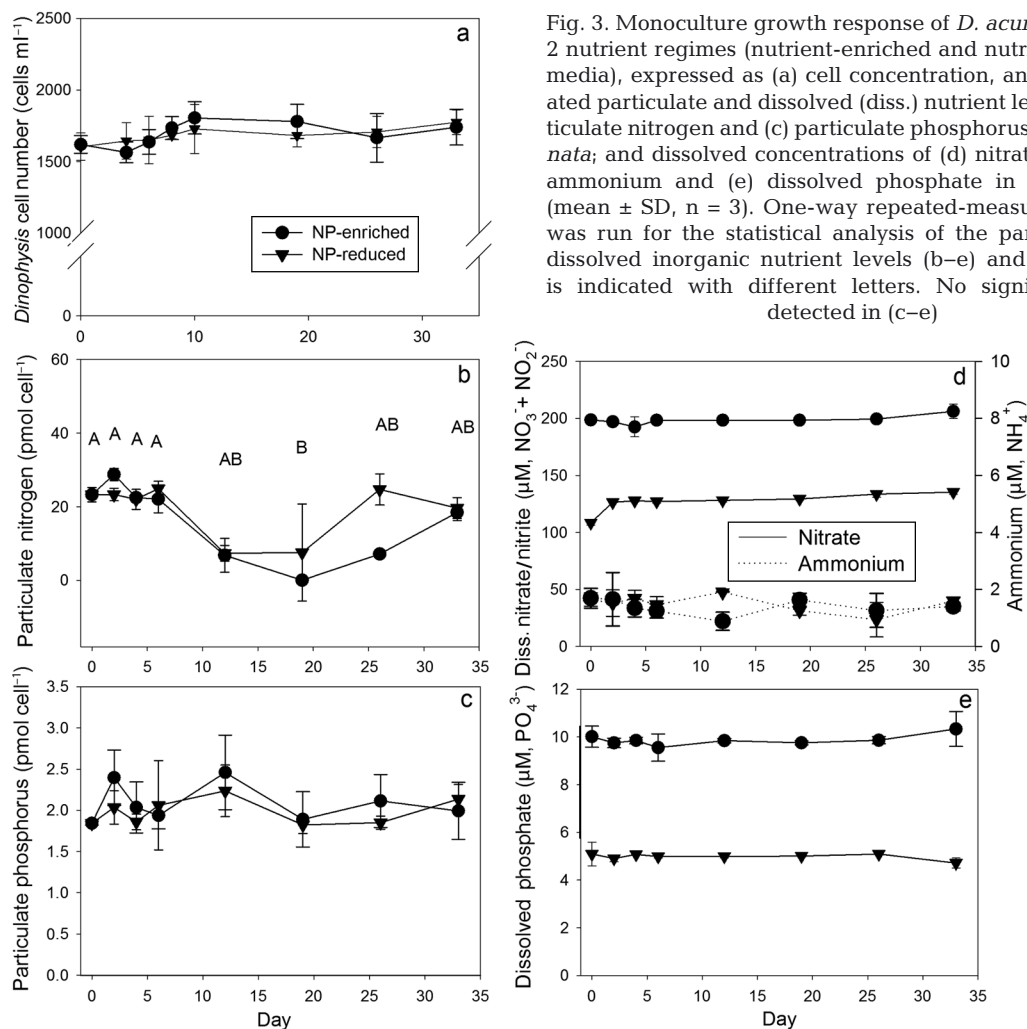


Fig. 3. Monoculture growth response of *D. acuminata* under 2 nutrient regimes (nutrient-enriched and nutrient-reduced media), expressed as (a) cell concentration, and the associated particulate and dissolved (diss.) nutrient levels: (b) particulate nitrogen and (c) particulate phosphorus in *D. acuminata*; and dissolved concentrations of (d) nitrate/nitrite and ammonium and (e) dissolved phosphate in the medium (mean \pm SD, $n = 3$). One-way repeated-measures ANOVA was run for the statistical analysis of the particulate and dissolved inorganic nutrient levels (b–e) and significance is indicated with different letters. No significance was detected in (c–e)

cell⁻¹ (mean \pm SD, $n = 6$; Fig. 3b), respectively. PP, however, remained steady during the entire experimental period (Fig. 3c), with average cellular levels of 2.08 ± 0.29 and 1.98 ± 0.19 pmol cell⁻¹ (mean \pm SD, $n = 24$) in the nutrient-enriched and nutrient-reduced treatments, respectively.

Toxin production

When incubated with prey, *D. acuminata* displayed similar patterns of OA, DTX1 and PTX2 production, whereby cell quotas (pg cell⁻¹) 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 the plateau phase, while PTX2 cell quotas decreased as the culture aged. Maximum OA, DTX1, and PTX2 quotas were 0.59 ± 0.03 , 8.82 ± 0.23 , and 15.30 ± 1.76 pg cell⁻¹, respectively (Fig. 4a–c). Dissolved OA and DTX1 accumulated in the medium over exponential and early plateau phase (Fig. 4d,e), and then either continued to increase or plateaued as the culture aged. In contrast, concentrations of dissolved PTX2 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–i). Maximum production rates were 0.065 pg OA cell⁻¹ d⁻¹, 0.86 pg DTX1 cell⁻¹ d⁻¹, and 2.61 pg PTX2 cell⁻¹ d⁻¹ in the mixed culture experiment.

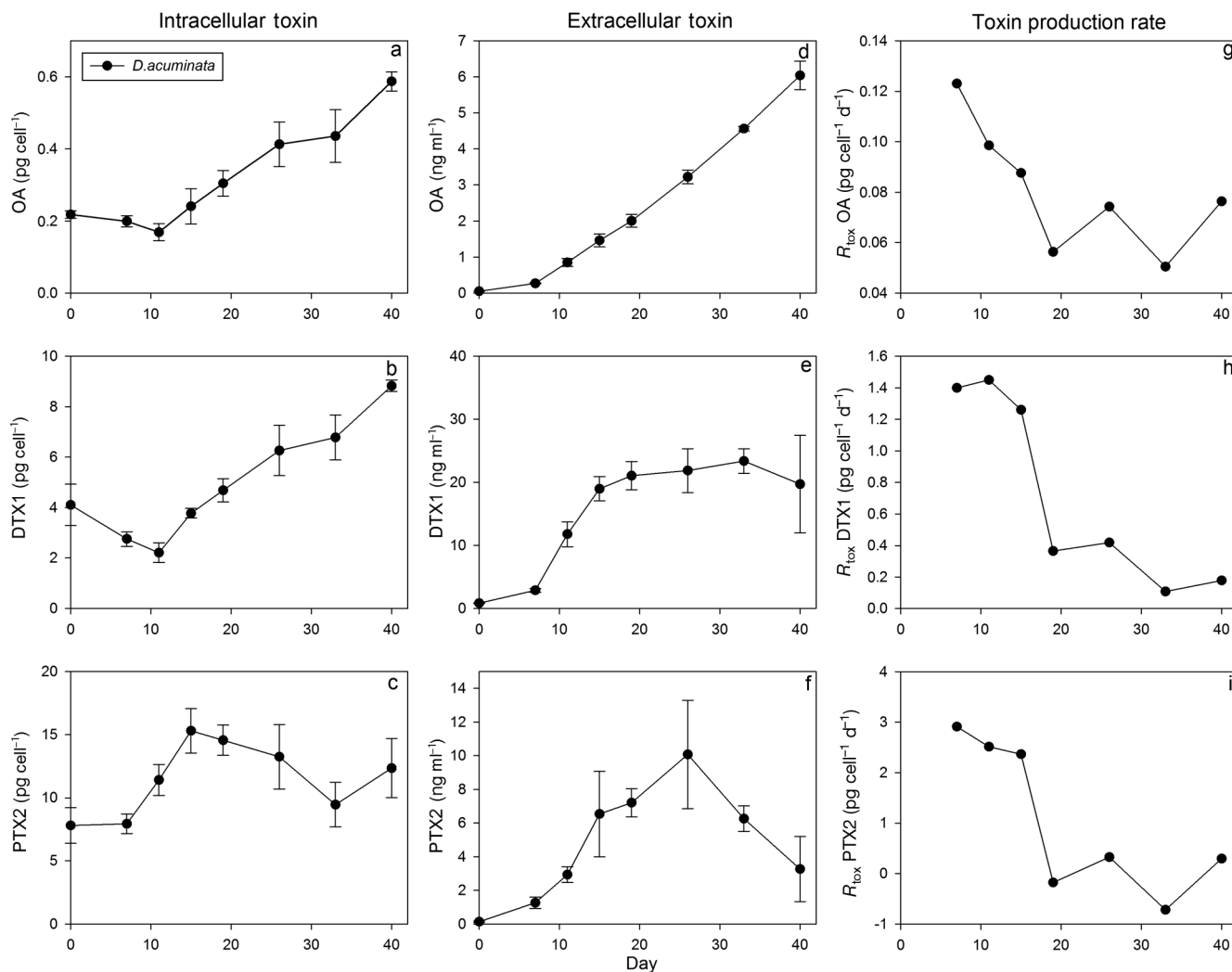


Fig. 4. (a–c) Intracellular toxin quotas, (d–f) extracellular toxin concentrations in the medium, and (g–i) toxin production by *Dinophysis acuminata* in the mixed culture, i.e. in the presence of ciliate prey (mean \pm SD, $n = 3$). Toxins quantified include okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2). R_{tox} : net toxin production rate

Patterns of toxin accumulation in the cells and medium varied between the mixed and monoculture experiments with *D. acuminata*; however, we observed no effect of nutrient regime on toxin production or exudation in the *Dinophysis* monoculture trials, suggesting prey availability, and not the uptake of dissolved nutrients, was critical to toxin production. 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–c). Concentrations of dissolved OA, DTX1, and PTX2 were similarly constant over time in the starvation treatments (Fig. 5d–f), and this lack of variation was reflected in similarly stable total toxin concentrations for each toxin quantified (Fig. 5g–i). The total OA, DTX1 and PTX2 concentrations at the end of incubation reached 140.9, 2357, and 2799 ng ml⁻¹, 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 by prey.

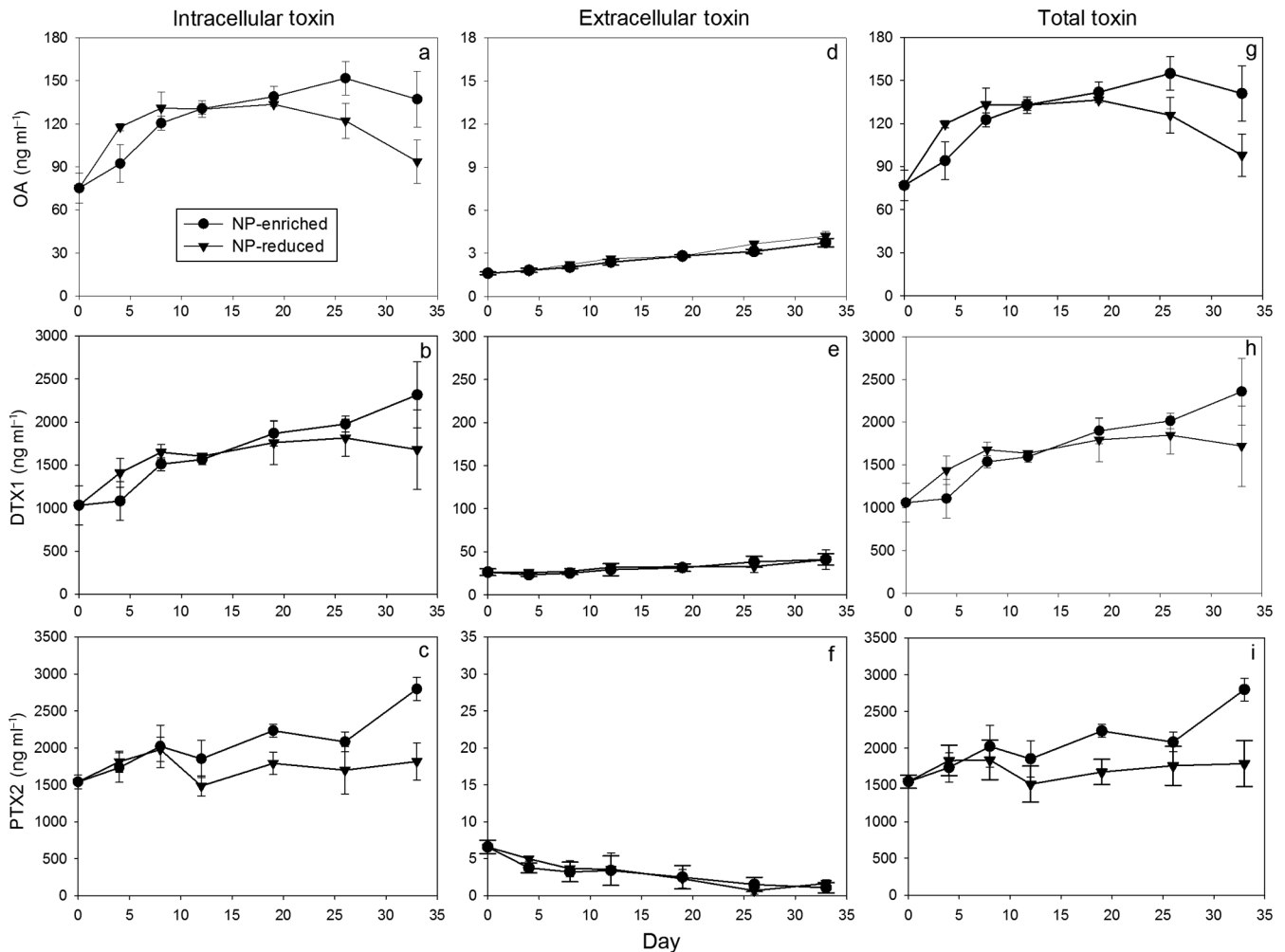


Fig. 5. (a–c) Intracellular toxin concentrations, (d–f) extracellular toxin concentrations in the medium, and (g–i) total toxin concentration (intracellular + extracellular) in the *Dinophysis acuminata* monoculture experiments, i.e. in the absence of ciliate prey. Monocultures were conducted under 2 nutrient regimes (nutrient enriched and nutrient reduced; mean \pm SD, $n = 3$).

Toxins quantified include okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2)

DISCUSSION

These experiments investigated the role of dissolved nitrate and phosphate in 2 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 media, 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 of 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* 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*

Mesodinium rubrum, a planktonic ciliate, incorporates plastids (Johnson & Stoecker 2005, Hansen & 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 & Stoecker 2005, Smith & 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 & Grunseich 1990) as well as dissolved nitrate and phosphate (present study) can be utilized by *M. rubrum* for enhanced growth when plastids and other promotional factors are first obtained from their cryptophyte prey, i.e. *Teleaulax/Geminigera* spp. Using isotope uptake experiments (^{15}N) with field material from the upper euphotic zone off the coast of Peru, Wilkerson & Grunseich (1990) measured average nitrate uptake rates by *M. rubrum* of $2.08 \pm 1.42 \mu\text{g l}^{-1} \text{h}^{-1}$ under various irradiance conditions. In our laboratory study, not utilizing ^{15}N , the nitrate uptake rate of $1.38 \text{ pmol N cell}^{-1} \text{d}^{-1}$ equates to $1.61 \mu\text{g l}^{-1} \text{h}^{-1}$

(when *M. rubrum* concentration was $2000 \text{ cells ml}^{-1}$ 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 $1.63 \text{ pmol P cell}^{-1} \text{d}^{-1}$ in the monoculture.

Under nutrient-enriched conditions, monocultures of *M. rubrum* achieved stabilized nitrogen and phosphorous quotas, $25\text{--}30 \text{ pmol N cell}^{-1}$ and $3\text{--}4 \text{ pmol P cell}^{-1}$, respectively, within 2 d of inoculation into fresh medium (Fig. 1b,c). These values equate to an N:P ratio of 8–10 (Fig. 6a), suggesting that either a higher cell quota of phosphorous or a 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 (Figs. 1e & 2f), skewing its ratio below Redfield val-

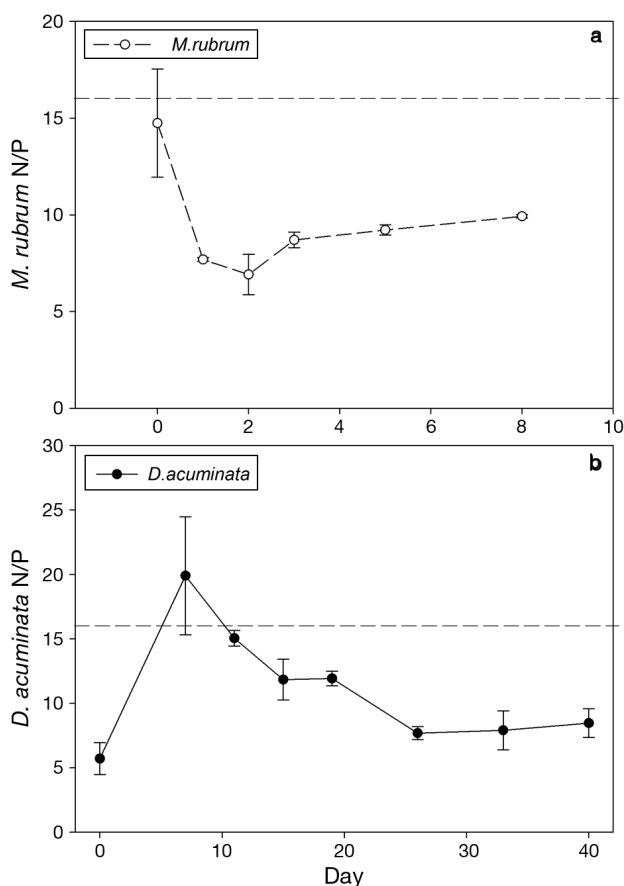


Fig. 6. Nitrogen to phosphorus ratio (N:P) of (a) *Mesodinium rubrum* in monoculture and (b) *Dinophysis acuminata* in the mixed culture in nutrient-enriched medium (mean \pm SD, $n = 3$). Dashed lines indicate the N:P Redfield ratio of 16:1

ues (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 photosynthesis or the uptake of dissolved or particulate organic matter (Johnson & Stoecker 2005, Smith & 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 & Stoecker 2005), indicating that plastid transfer is the primary benefit of grazing by *M. rubrum*. As a result of this acquired photosynthetic capability, *M. rubrum* 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 *D. acuminata* rapidly increased with the consumption of prey, with no apparent increase in cellular phosphorus (Fig. 2b,c), 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 and 1.1 pmol cell⁻¹, respectively, with N:P ratios ranging from 6.26 to 36.3. In our study, *D. acuminata* possessed comparable quotas of cellular N and P (11.5–47.1 pmol N cell⁻¹, 1.6–2.4 pmol P cell⁻¹) 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⁻¹; 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 the late plateau phase, cellular quotas of N, P, and C increased. This increase in nutrient concentrations is likely not solely a reflection of *D. acuminata* 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 PC, PN and PP, 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,f), perhaps due to the biotransfor-

Table 2. Ingestion rate, calculated and observed nutrient uptake, and feeding efficiency of *Dinophysis* during the 7-d feeding period of the mixed culture experiment

	Avg <i>Dinophysis</i> prey ingestion during the 7-d feeding period (Mesodinium/ <i>Dinophysis</i>)	Avg cellular nutrient content of Mesodinium (pmol Mesodinium ⁻¹)	Calculated nutrient uptake (pmol <i>Dinophysis</i> ⁻¹)	Observed nutrient content of <i>Dinophysis</i> (pmol <i>Dinophysis</i> ⁻¹)		Observed nutrient uptake by <i>Dinophysis</i> during the 7-d feeding period (pmol <i>Dinophysis</i> ⁻¹) ^b	Feeding efficiency (%) ^c
				Before feeding	After feeding		
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

^aCalculated nutrient uptake of *Dinophysis* — assuming that nutrient assimilation from prey to predator is 100% — is the average number of prey cells during the 7-d feeding period multiplied by the average cellular nutrient contents of prey

^bObserved nutrient uptake by *Dinophysis* = (observed nutrient content of *Dinophysis* after feeding × number of *Dinophysis* after feeding) – observed nutrient content of *Dinophysis* before feeding × number of *Dinophysis* before feeding/average number of *Dinophysis* during the 7-d feeding period

^cFeeding efficiency is the observed nutrient uptake divided by the calculated nutrient uptake, assuming that nutrient assimilation from prey to predator was 100%

mation of organic exudates from *Dinophysis* and/or *M. rubrum* into NO₃⁻, NO₂⁻, NH₄⁺, and PO₄³⁻ (Tezuka 1989, John & 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 media, leaving relatively little material for bacterial remineralization (Fig. 3d,e).

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 the present study were only exposed to very low background concentrations of ammonium; as such, it is difficult to determine whether 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 concentrations (intracellular + extracellular toxins, ng ml⁻¹ 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 & Hansen 2009, Campbell et al. 2010, Gonzalez-Gil et al. 2010, Minnhagen et al. 2011, Sjöqvist

& Lindholm 2011, Hattenrath-Lehmann et al. 2013), which 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 & 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 the early exponential phase and increasing by early to mid-plateau phase (Fig. 4a,b). 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 the 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 the plateau phase (Fig. 4g–i). 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 in the present study were similar between the starved and food-sufficient *Dinophysis* cultures.

Dissolved DSP toxins consistently, but slowly, accumulated as the *D. acuminata* cultures aged (Figs. 4d,e & 5d,e); however, the cell concentration data do not support extensive cell death during the plateau phase (Figs. 2a & 3a). As we have calculated previously (Smith et al. 2012), concentrations of dis-

solved 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. it 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⁻¹) and DTX1 (2.2–8.8 pg cell⁻¹), but moderate amounts of PTX2 (7.8–15.3 pg cell⁻¹). In contrast, *D. acuminata* isolated from Japan had high levels of OA (2.1–12.2 pg cell⁻¹) and PTX2 (14.7–107.1 pg cell⁻¹), and low DTX1 content (0.2–4.8 pg cell⁻¹) (Kamiyama & 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⁻¹ (Nielsen et al. 2012). These isolates were grown under similar experimental conditions, e.g. temperature (14–18°C) and light intensity (65–100 µmol photons m⁻² 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 *D. acuminata* strain or a driver that has not yet been investigated (e.g. prey isolate or prey nutritional quality).

CONCLUSIONS

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 *D. acuminata* 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 concentration 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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