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Karlodinium veneficum feeding responses and effects on larvae of the eastern oyster *Crassostrea virginica* under variable nitrogen:phosphorus stoichiometry

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ABSTRACT: Mixotrophic feeding can be promoted by nutrient-enriched prey, a nutritional strategy which can provide benefits to some toxic microalgae under nutrient-imbalanced conditions. However, it is unclear how the nutritional condition of the predator or the prey affects the mixotrophy and toxicity of toxin-producing mixotrophs. Laboratory experiments were conducted to measure growth and feeding rates of *Karlodinium veneficum* with addition of *Rhodomonas salina* as prey under varied nitrogen (N):phosphorus (P) stoichiometry (molar N:P of 4, 16 and 32) of both predator and prey and with *K. veneficum* initially in different growth phases (exponential and stationary). Growth rates of initially exponential- and stationary-phase *K. veneficum* were enhanced in the presence of prey with reciprocal nutrient conditions. Feeding rates (measured as prey death rates) were highest for low-NP *K. veneficum* initially growing exponentially and mixed with N-rich prey. Maximum feeding rates of low-NP *K. veneficum* on N-rich prey during exponential growth were ~4-fold higher than the rates of high-NP *K. veneficum* on N-rich prey. The nutritionally different *K. veneficum* were tested with larvae of the eastern oyster *Crassostrea virginica* to compare putative toxicity. Larval mortality was significantly increased in 2 d exposures to high-NP *K. veneficum* monocultures in both growth phases. When mixed with N-rich prey, the presence of *K. veneficum* resulted in significantly enhanced larval mortality, but this was not the case for low-NP *K. veneficum* in exponential phase. Enhanced growth of *K. veneficum* and increased negative effects of *K. veneficum* on larval survival appeared to be highest when fed prey with higher N:P content.

KEY WORDS: Mixotrophy · Harmful algae · *Karlodinium veneficum* · N:P stoichiometry · Larval oyster mortality

INTRODUCTION

Mixotrophy, the process by which algae gain nutrition from both phototrophy and phagotrophy, is a ubiquitous phenomenon in freshwater as well as marine systems from oligotrophic to eutrophic waters (Jeong et al. 2005a,b, Burkholder et al. 2008, Zubkov & Tarran 2008, Stoecker et al. 2009, Flynn et al. 2013). Phagotrophic algae appear to be common among chrysophytes, dinoflagellates and haptophytes; most of these organisms are capable of deriving a substantial proportion of their carbon (C) by ingestion of prey compared to C acquisition from photosynthetic reactions (Schnepf & Elbrächter 1992, Granéli & Carlsson 1998, Legrand 2001, Adolf et al. 2006, Raven et al. 2009, Jeong et al. 2010, Hansen

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2011, Granéli et al. 2012). The understanding that many algae can benefit from prey ingestion and that this metabolic pathway is ecologically important is rapidly advancing (Stoecker et al. 2009, Flynn et al. 2013, Mitra et al. 2014, 2016), but questions of how mixotrophic metabolism can be advantageous to organisms living under varying environmental conditions remain. In particular, linkages between conditions when organisms experience inorganic nutrient and light limitation and/or changes in cellular nutrient content and mixotrophic nutrition are not well understood (Stoecker et al. 1997, Li et al. 2000a, Smalley et al. 2003, Carvalho & Granéli 2010, Lundgren et al. 2016). Although the importance of mixotrophic metabolism is regulated by prey quantity and quality (Hansen et al. 2000), detailed examinations of how these factors affect physiological states of mixotrophic organisms are scarce (Carvalho & Granéli 2010, Lundgren et al. 2016).

Mixotrophic nutrition has been shown to be significant for growth of the dinoflagellate *Karlodinium veneficum* (Li et al. 1996, Adolf et al. 2006, Calbet et al. 2011). This species can produce hemolytic, cytotoxic and ichthyotoxic compounds, named karlotoxins (Kempton et al. 2002), and growth-limited conditions (e.g. stationary growth phase) have been associated with higher cellular quotas of karlotoxin (Adolf et al. 2009). This species is also capable of forming high-biomass blooms of up to 10^5 cells ml^{-1} (e.g. Adolf et al. 2008, Place et al. 2012), leading to fish and shellfish mortality, illness of aquatic organisms and human health concerns (Deeds et al. 2002). Blooms of *K. veneficum* are distributed worldwide in estuaries and coasts from South Africa (Braarud 1957) to Europe (Bjornland & Tangen 1979), China (Dai et al. 2014), Australia (Ajani et al. 2001, Adolf et al. 2015) and the United States (Li et al. 2000b, Adolf et al. 2008, Hall et al. 2008). The importance of mixotrophy to this species under highly variable environments in terms of nutrient availability and its association with toxigenic abilities may contribute to its global success (Adolf et al. 2009, Place et al. 2012).

In the United States, *K. veneficum* is known to be an important member of the Chesapeake Bay phytoplankton community (reviewed in Marshall et al. 2005), and this dinoflagellate is frequently present at levels of $>4 \times 10^3$ cells ml^{-1} (Li et al. 2015). It may be distributed throughout the bay through annual subsurface transport from the southern bay to nutrient-poor surface waters in the middle and upper bay (Li et al. 2000b). This species co-occurs with cryptophytes and develops high concentrations, particularly in the salinity range of 7 to 18 (Li et al. 2000b). It also has been shown to occur during the period in which a median value of ambient dissolved inorganic nitrogen (N):phosphorus (P) ratios bracket the Redfield ratio (~16) in summer, but blooms may also be found during periods well in excess of Redfield proportions (Li et al. 2015). The ability to use organic nutrients, including particulate nutrients via mixotrophy, appears to be essential to the growth and maintenance of high abundances of this species under nutrient limitation. For example, grazing of phycoerythrin-containing cryptophytes by *K. veneficum* based on food vacuole contents is commonly found (Li et al. 1996), and daily removal of up to 4% of the cryptophyte population in Chesapeake Bay has been observed (Li et al. 2001). Growth rates of *K. veneficum* in its mixotrophic mode (~0.52–0.57 d^{-1}) have been reported to be ~2-fold larger than those in its autotrophic mode (~0.22–0.27 d^{-1}) (Li et al. 1999, Adolf et al. 2006, Calbet et al. 2011). The nutritional supply from feeding to the growth of mixotrophic *K. veneficum* in Chesapeake Bay can be significant, as they can gain 10% of their C, 11% of their N and 17% of their P requirements through consumption of cryptophyte biomass (Li 1998). These data support the hypothesis that feeding contributes important nutrient sources to the formation and persistence of *K. veneficum* blooms when inorganic nutrients are limited in supply (Adolf et al. 2008), especially under P-limited environments (Li et al. 2000a).

Cellular toxicity also has implications for *K. veneficum* bloom formation and/or maintenance under varying environments. The toxins (allelochemicals) are found to aid the mixotrophic feeding of *K. veneficum* (Adolf et al. 2008) by inhibiting movement of prey, and negative effects of toxin on other planktonic organisms, including grazers, can be a mechanism for bloom promotion (Mitra & Flynn 2006). Additional nutrient sources via feeding are promoted by these toxic substrates that immobilize prey and enhance the efficiency of prey capture (Place et al. 2012). As *K. veneficum* has the ability to produce toxins that are involved in allelopathic interactions, its temporal and spatial overlap with oyster spawning in Chesapeake Bay has drawn attention to potential impacts of these toxins on oyster larvae (Glibert et al. 2007). Although laboratory studies with a low toxin level strain have examined the adverse effects on early development of oyster larvae as a function of the abundance of *K. veneficum* (Glibert et al. 2007, Stoecker et al. 2008), questions of whether or not allelochemicals produced by this species change with the physiological state of its growth or that of its prey due to varying nutrient supply as well as how

such changes may affect their interactions with oyster larvae remain largely unanswered.

Studies of other mixotrophic algae have demonstrated that high N:P stoichiometry is often associated with increases in cellular toxicity (Granéli & Johansson 2003, Granéli & Flynn 2006, Hardison et al. 2012, Lundgren et al. 2016). Many toxic compounds are N and/or C rich, so production of toxins under high enrichment conditions might be considered a dissipatory mechanism such that cells release the nutrients (N or C) that are not needed (Glibert & Burkholder 2011) or produce secondary metabolites through metabolic processes that may not go to completion via the normal pathway. Toxin production by *K. veneficum* is coupled with cellular C acquisition (Staunton & Weissman 2001) through photosynthesis, and it has been shown that these cells produce toxin and only eat during the light period (Adolf et al. 2008), but the link between this observation and that of N and P nutrition is unknown. A link between feeding and toxicity might be explained by situations in which nutrient limitation triggers nutritional switches, which in turn contribute to production of toxins (Stoecker et al. 2006). From this reasoning, *K. veneficum* would likely be more phagotrophic and more toxic under conditions of nutrient imbalance.

Our objective was to examine if growth rates of *K. veneficum*, or death rates of its cryptophyte prey, change when it and its prey are under different stoichiometric conditions (as defined by N:P ratio). Bioassay experiments with oyster larvae were used to test the hypothesis that under nutrient-imbalanced conditions, *K. veneficum* will increase its mixotrophic metabolism and toxin production, enhancing the detrimental effects on oyster larvae. In all, our data contribute to the understanding of how different growth phases and nutritional conditions of *K. veneficum* affect oyster larval growth under varying environmental conditions.

MATERIALS AND METHODS

Algal cultures

Non-axenic strains of *Karlodinium veneficum* (CCMP 1975, isolated from Chesapeake Bay, Maryland, USA) and *Rhodomonas salina* were provided by the National Center for Marine Algae and Microbiota and the Oyster Hatchery of Horn Point Laboratory (HPL), respectively. Strains were first grown in *f*/2 media (Guillard 1975) at 22°C with a light intensity of 430 µmol photons m^{-2} s^{-1} on a 14 h light:10 h dark cycle. Culture media were prepared with autoclaved artificial water (salinity of 10). Once the cultures (both experimental species) reached high cell densities but were still growing exponentially, the cells were inoculated into new growth media adjusted to give variable N:P ratios. The nitrate (NO_3^-) concentration was held constant at *f*/2 proportions, but different phosphorus (PO_4^{3-}) concentrations were added to achieve 3 nutrient conditions for both species: low NP (molar N:P = 4, [N] = 88 µM, [P] = 22 µM); Redfield ratio (molar N:P = 16, [N] = 88 µM, [P] = 5.5 µM); and high NP (molar N:P = 32, [N] = 88 µM, [P] = 2.75 µM). Trace metals, iron and vitamins (B12, biotin and thiamine) were added to the cultures at levels corresponding to *f*/2 media. Our objective was to establish cultures with variable stoichiometry, not true N or P limitation.

Experimental design

Experimental treatments were designed as 3 by 3 crossmatches of different nutrient conditions of *K. veneficum* and *R. salina* for each growth state of *K. veneficum.* Specifically, experiments were performed using *K. veneficum* grown under 3 N:P ratios (low NP, Redfield ratio and high NP, see 'Algal cultures' above) and in 2 growth phases (exponential and stationary) and with *R. salina* grown under the same N:P conditions but always under exponential growth. Culture flasks containing 250 ml of new growth media (i.e. N:P ratio of 4, 16 and 32) were inoculated with *K. veneficum* and *R. salina* cells to a final concentration of 1500 and 5000 cells ml^{-1}, respectively. The *R. salina* cells were centrifuged at 6000 rpm (1000 × *g*) for 10 min to remove the culture medium and inorganic nutrients and added into culture flasks with *K. veneficum* cultures. Control treatments, of individual species only, were also conducted for the 3 nutrient conditions of each *K. veneficum* and *R. salina*. Thus, this study consisted of 15 treatments, in duplicate, totalling 30 culture flasks for each growth state of *K. veneficum*.

The mixed-culture experiments with *K. veneficum* inoculated during both exponential and stationary phase lasted for 72 and 96 h, respectively. During this time, aliquots (5 ml) were collected for cell enumeration at 0, 6, 12, 24, 36, 48 and 72 h after homogenization from each flask and were preserved with diluted acid Lugol's solution. Additional water samples (40 ml) were collected at the beginning (t_0) and end (t_f) of the time courses and filtered for the analysis of NO_3^- and PO_4^{3-}. Samples of water and cells were also

collected at the end of the experiments to perform the toxicity bioassays with oyster larvae (see 'Bioassay determination of putative toxicity' below).

Cell counts and nutrient analyses

Samples of *K. veneficum* and *R. salina* cells were enumerated using light microscopy at 100× magnification using a Sedgewick-Rafter chamber (Guillard 1978). Replicate counts per sample were performed on 20 random fields for representative cell concentrations (expressed as cells ml^{-1}). Analyses of NO_3^- and PO_4^{3-} were performed on samples that were filtered through precombusted GF/F filters (pore size 0.47 µm). Colorimetric analyses were conducted using 96-well micro-assay plates, based on the methods of Doane & Horwáth (2003) for NO_3^- and Ringuet et al. (2011) for PO_4^{3-}.

Growth, death rates and nutrient consumption

Cell-specific growth rates (d^{-1}) of *K. veneficum* were calculated from the slopes of the regressions of natural log-transformed data during periods of maximum changes in cell densities.

Cell-specific death rates of *R. salina* (Rs *K. veneficum*$^{-1}$ d^{-1}) were determined as the difference between growth rates of prey in the control and experimental flasks with the corresponding nutrient conditions, based on the equations of Frost (1972) and Heinbokel (1978) to account for grazer growth. Death rates of *R. salina* were reported rather than ingestion rates because of the difficulty in differentiating between cells that were actually grazed and those cells that may have burst due to putative toxic effects.

Rates of consumption of NO_3^- and PO_4^{3-} were calculated based on the change in concentration from different time point samples, t_1 to t_2, to determine the extent to which the NO_3^-:PO_4^{3-} drawdown ratio varied between the different growth phases of the predator and between the different nutritional status of both predator and prey cells in the mixed cultures.

Bioassay determination of putative toxicity

Oyster larvae (provided by the Oyster Hatchery at HPL) were used as bioassay organisms to assess alleged *K. veneficum* toxicity. Spawning oysters were collected in filtered natural seawater with a salinity of 10 and a temperature of 28°C. The larvae were tested within 4 h of fertilization, in triplicate, in 3 ml 12-well culture plates with flat bottoms.

Cells were obtained at t_0 and t_f of the mixed-culture experiments to initiate the bioassay tests. The oyster larvae (60 larvae cells ml^{-1}) were exposed to a fixed density of *K. veneficum* (9×10^2 cells ml^{-1}) obtained from the 3 monoculture treatments and the 9 mixed-culture treatments from each growth phase. After 48 h of exposure of the larvae to the algal cultures, each well was fixed with dilute acid Lugol's solution, and the samples were analyzed using an Utermöhl chamber (Edler & Elbrächter 2010) through an inverted light microscope (Nikon Eclipse TE2000-U) at 100×. Both live and dead larvae were counted to estimate larval mortality.

Statistical analyses

All statistical analyses were performed with R. The Shapiro-Wilks test was used to check normality of the data, while the Levene's test was used to assess homogeneity of variance. Maximal growth rates of *K. veneficum* were compared for statistical differences in the slopes of the regressions of natural log-transformed data among the variables measured in the replicates of the treatments during the same periods of time (ANCOVA test). Comparisons between the 2 growth-phase conditions considering a single variable were verified using the Student's *t*-test, while correlations between 2 variables were estimated by significance of the Pearson's product moment coefficients. Differences among larval mortality in the triplicate monocultures and mixed cultures were analyzed using an ANOVA test followed by Tukey's HSD test for pairwise comparison.

RESULTS

Cell densities, growth and prey death rates

Mixed-culture experiments were performed using stock cultures of *Karlodinium veneficum* grown under 3 N:P ratios (low-NP, Redfield ratio and high-NP conditions), and cells were inoculated during the exponential and stationary phases (Fig. 1). Since the variable N:P growth conditions did not initially yield N or P limitation, similar patterns of growth of *K. veneficum* from exponential phase to early stationary phase were observed (Fig. 1). The specific growth rates of monocultures of *K. veneficum* under the 2

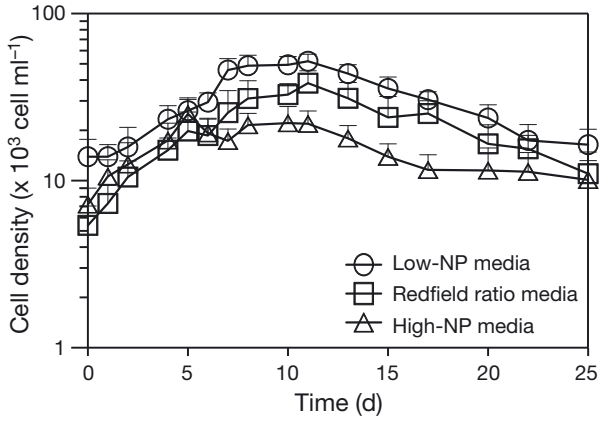

Fig. 1. Growth curves of the stock cultures of *Karlodinium veneficum* grown on low-NP, Redfield ratio and high-NP culture media. Inocula of *K. veneficum* cells were transferred to the mixed-culture experiments on Day 5 (exponential growth) and Day 11 (stationary phase growth). N: nitrogen; P: phosphorus

growth phases and 3 nutrient conditions ranged from 0.25 to 0.50 d^{-1} (Table 1). While monocultures of exponential-phase *K. veneficum* had similar growth rates when transferred to low-NP, Redfield ratio and high-NP growth media, the cultures of stationary-phase *K. veneficum* showed significantly different cell densities and growth rates when transferred into different media (ANCOVA test, $p < 0.01$; Fig. 2). Maximum growth rates increased when cells were transferred into the high-NP media compared to the other 2 conditions when *K. veneficum* was initially in stationary phase (Fig. 2).

With additions of prey, cell densities and growth rates of *K. veneficum* changed with time for both conditions of *K. veneficum* cells, exponentially growing and stationary phase (Fig. 3). After transfer to the plus-prey condition, maximal growth rates of initially exponential-phase *K. veneficum* cultures under low-NP condition (0.70 ± 0.10 d^{-1}) and initially stationary-phase *K. veneficum* cultures under high-NP condition (0.66 ± 0.16 d^{-1}) were similar (Table 1). Specific growth rates between the monoculture and mixed-culture treatments were compared, and it was found that there was a significant increase in growth when exponentially growing, low-NP *K. veneficum* cells were mixed with prey of high-NP (ANCOVA test, $p < 0.05$) and when high-NP *K. veneficum* cells

Table 1. Specific growth rates (μ, d^{-1}) calculated from the slopes of the regression of cell density vs. time for initially exponential- and stationary-phase *Karlodinium veneficum*. ANCOVA were used to compare statistical differences in slopes for the low-NP, Redfield ratio and high-NP *Rhodomonas salina* additions of each predator growth condition. N: nitrogen; P: phosphorus

Nutritional status of predator	Prey addition	Slope ± SE	r^2	n	p-value
Exponential-phase culture					
Low-NP *K. veneficum*	+ no prey	0.25 ± 0.07	0.70	8	
	+ low-NP *R. salina*	0.28 ± 0.09	0.53	8	
	+ Redfield ratio *R. salina*	0.50 ± 0.08	0.85	8	<0.01
	+ high-NP *R. salina*	0.70 ± 0.10	0.83	8	
Redfield ratio *K. veneficum*	+ no prey	0.26 ± 0.05	0.48	14	
	+ low-NP *R. salina*	0.35 ± 0.05	0.82	14	
	+ Redfield ratio *R. salina*	0.45 ± 0.04	0.90	14	<0.01
	+ high-NP *R. salina*	0.23 ± 0.01	0.82	14	
High-NP *K. veneficum*	+ no prey	0.36 ± 0.07	0.73	14	
	+ low-NP *R. salina*	0.32 ± 0.04	0.64	14	
	+ Redfield ratio *R. salina*	0.44 ± 0.05	0.87	14	0.12
	+ high-NP *R. salina*	0.30 ± 0.06	0.50	14	
Stationary-phase culture					
Low-NP *K. veneficum*	+ no prey	0.28 ± 0.06	0.63	12	
	+ low-NP *R. salina*	0.47 ± 0.10	0.62	12	
	+ Redfield ratio *R. salina*	0.17 ± 0.07	0.64	12	0.25
	+ high-NP *R. salina*	0.33 ± 0.08	0.59	12	
Redfield ratio *K. veneficum*	+ no prey	0.37 ± 0.04	0.89	12	
	+ low-NP *R. salina*	0.51 ± 0.21	0.36	12	
	+ Redfield ratio *R. salina*	0.42 ± 0.09	0.69	12	0.17
	+ high-NP *R. salina*	0.33 ± 0.07	0.71	12	
High-NP *K. veneficum*	+ no prey	0.50 ± 0.07	0.80	12	
	+ low-NP *R. salina*	0.66 ± 0.16	0.62	12	
	+ Redfield ratio *R. salina*	0.25 ± 0.05	0.37	12	0.09
	+ high-NP *R. salina*	0.43 ± 0.12	0.56	12	

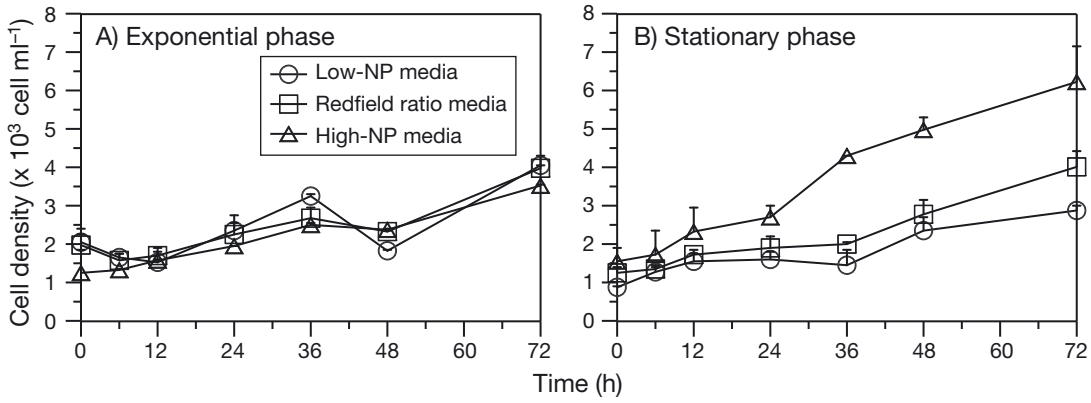

Fig. 2. Growth curves of initially (A) exponential-phase and (B) stationary-phase *Karlodinium veneficum* when transferred into low-NP, Redfield ratio and high-NP culture media during monoculture experiments. N: nitrogen; P: phosphorus

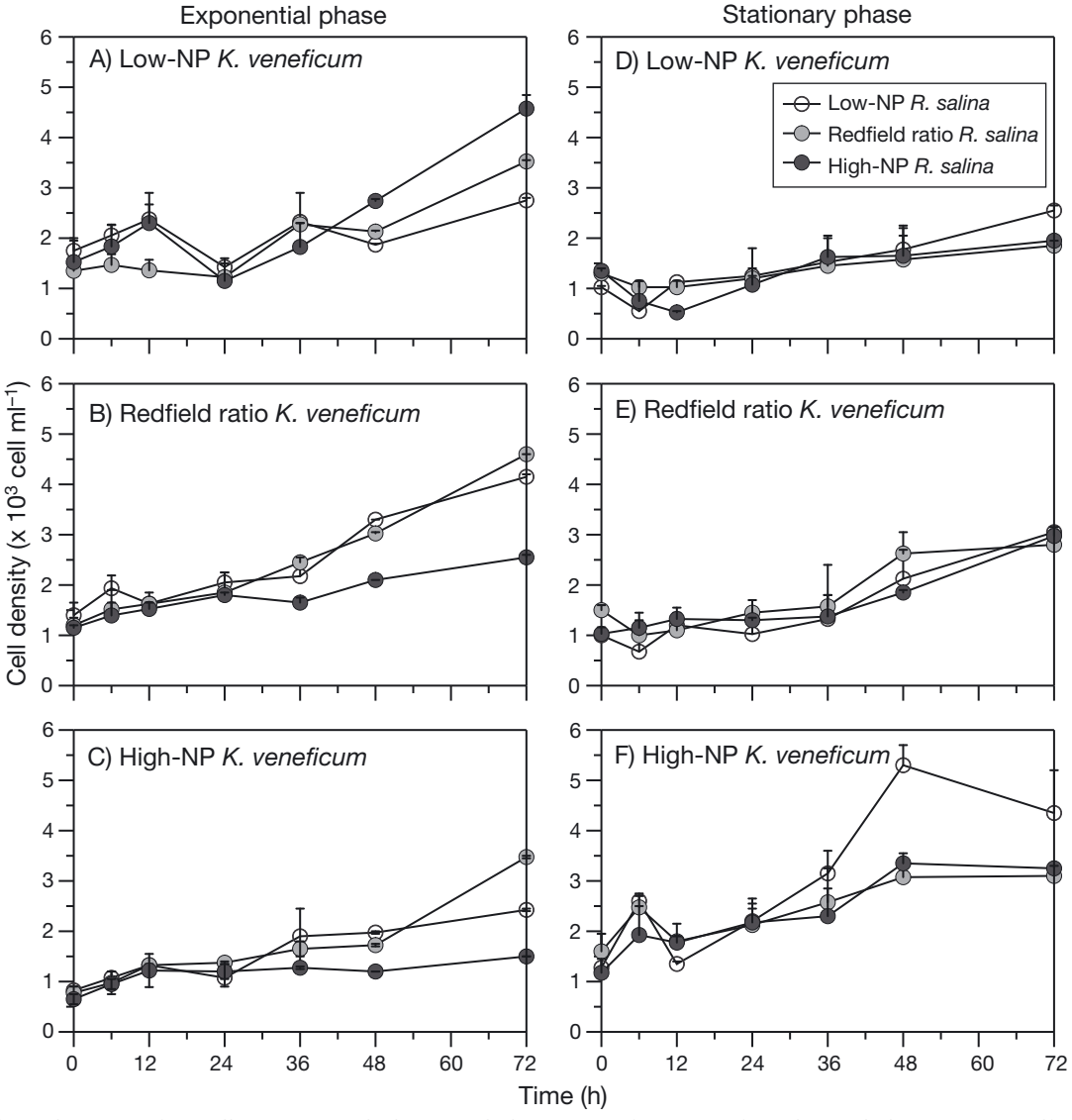

Fig. 3. Growth curves of initially exponential-phase *Karlodinium veneficum* transferred into (A) low-NP, (B) Redfield ratio and (C) high-NP conditions and of initially stationary-phase *K. veneficum* transferred into (D) low-NP, (E) Redfield ratio and (F) high-NP conditions and provided with low-NP, Redfield ratio and high-NP prey *Rhodomonas salina* during mixed-culture experiments. N: nitrogen; P: phosphorus

Table 2. Death rates of prey, *Rhodomonas salina* (Rs *Karlodinium veneficum*$^{-1}$ d^{-1}), based on equations of Frost (1972) and Heinbokel (1978) to account for *K. veneficum* growth in mixed cultures. Significant differences in prey death rates between 3 nutritional states of prey are marked as different letters (ANOVA test, $p < 0.01$). N: nitrogen; P: phosphorus

Nutritional status of predator	Prey addition	Exponential phase	Stationary phase
Low-NP *K. veneficum*	+ low-NP *R. salina*	0.35 ± 0.02^a	0.70 ± 0.04^a
	+ Redfield ratio *R. salina*	0.28 ± 0.01^a	1.24 ± 0.22^a
	+ high-NP *R. salina*	0.68 ± 0.07^b	1.21 ± 0.66^a
Redfield ratio *K. veneficum*	+ low-NP *R. salina*	0.35 ± 0.04^a	0.70 ± 0.26^a
	+ Redfield ratio *R. salina*	0.35 ± 0.05^a	1.06 ± 0.09^a
	+ high-NP *R. salina*	0.20 ± 0.07^a	0.47 ± 0.08^a
High-NP *K. veneficum*	+ low-NP *R. salina*	0.25 ± 0.13^a	0.51 ± 0.03^a
	+ Redfield ratio *R. salina*	0.59 ± 0.15^a	1.00 ± 0.75^a
	+ high NP *R. salina*	0.18 ± 0.12^a	0.63 ± 0.92^a

were mixed with Redfield ratio prey (ANCOVA test, $p < 0.01$). In contrast, significant increased growth rates of initially stationary-phase *K. veneficum*, when transferred into all nutrient conditions, were found only when cells were mixed with prey that were low NP compared to monocultures (ANCOVA test, $p < 0.05$ for all tests).

The effects of the nutrient condition of the prey on growth rates of *K. veneficum* were determined as the slope of the rate of changes over the exposure time course, and it was found that the rate of change was higher in initially exponential-phase *K. veneficum* than in initially stationary-phase *K. veneficum* (Fig. 3). In particular, exponential-phase *K. veneficum* grown under both Redfield ratio and low-NP conditions had significantly different growth rates with nutritionally distinct prey (ANCOVA test, $p < 0.01$; Table 1), while only the growth rates of stationary-phase *K. veneficum* under high NP responded differently to nutritional distinct prey *Rhodomonas salina* (ANCOVA test, $p < 0.1$; Table 1).

Regardless of the nutritional status of *R. salina* or *K. veneficum*, death rates of prey were significantly higher when *K. veneficum* was initially in stationary phase compared to exponential phase (*t*-test for all comparisons, $p < 0.01$; Table 2) and were the same regardless of the nutritional status of the prey (ANOVA test, $p > 0.01$; Table 2). In addition, the maximal growth rates of initially exponential-phase *K. veneficum* were positively correlated with prey death rates ($r = 0.77$, $n = 18$, $p < 0.01$; Fig. 4), while those of the mixed cultures for initially stationary-phase *K. veneficum* showed a negative relationship with prey death rates ($r = -0.65$, $n = 18$, $p < 0.05$; Fig. 4). When looking only at the initially exponential-phase *K. veneficum*, highest prey death rates were observed when low-NP *K. veneficum* were combined with high-NP *R. salina* (ANOVA test, $p < 0.01$; Table 2).

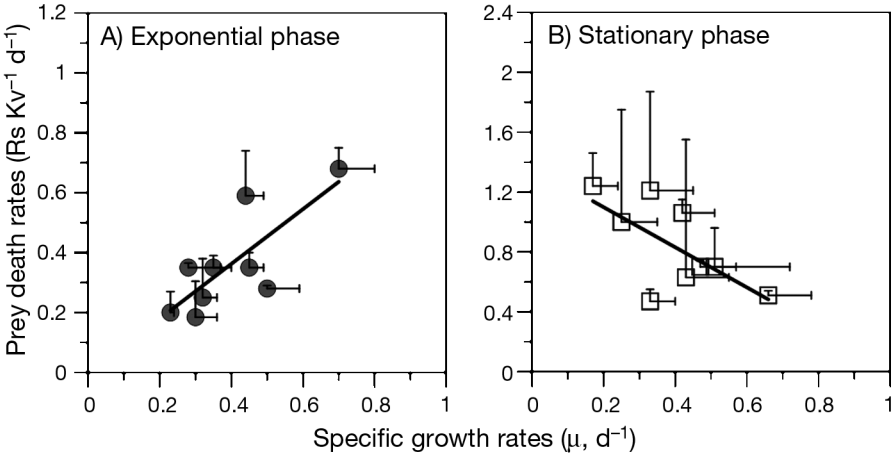

Fig. 4. Relationship between death rates of *Rhodomonas salina* (Rs) prey relative to *Karlodinium veneficum* (Kv) specific growth for *K. veneficum* that were initially grown to (A) exponential phase and (B) stationary phase

Nutrient depletion

The ratios of consumption of NO_3^- and PO_4^{3-} (drawdown ratios) in monoculture and mixed cultures varied between preconditioned exponential- and stationary-grown *K. veneficum*, and there were also slight differences in the nutrient consumption ratios of the monocultures of *K. veneficum* compared to nutritionally distinct prey *R. salina* (Fig. 5). In mixed cultures, the NO_3^-:PO_4^{3-} drawdown ratios of initially stationary-phase *K. veneficum* were significantly higher than those of

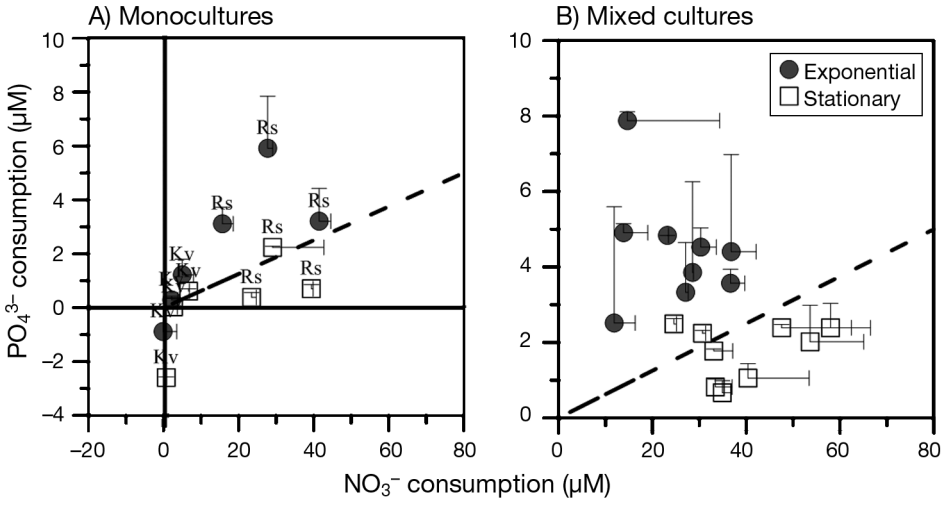

Fig. 5. Relationship between the concentrations of nitrate (NO_3^-) and phosphorus (PO_4^{3-}) for *Karlodinium veneficum* (Kv) and *Rhodomonas salina* (Rs) in (A) monocultures and (B) mixed cultures for both initially exponential and stationary phases of *K. veneficum*. The Redfield proportion of 16:1 is included for reference (dashed line)

exponential-phase *K. veneficum* (Student's *t*-test for all comparisons, $p < 0.01$) and ~2-fold greater than the Redfield ratio of 16 (Fig. 5B).

Putative toxic effects of *Karlodinium veneficum* on larval growth

The monocultures of *K. veneficum* grown under high-NP (i.e. N-rich) media in both exponential and stationary phases induced high larval mortality (on average 76 ± 15%, n = 12) under most growth conditions (Fig. 6A,C). However, stationary-phase *K. veneficum* grown on low-NP media, and exponentially growing *K. veneficum* initially grown on all media combinations, did not induce significant mortality greater than the larvae-only controls (Fig. 6A,C). Overall, larval mortality rates caused by the exponential-phase *K. veneficum* (on average 75 ± 21% for the 3 culture media) were ~2-fold higher than those of stationary-phase *K. veneficum* (43 ± 26%, $p < 0.001$) at t_0. In contrast, by t_f, *K. veneficum* monocultures showed a different pattern of larval mortality depending on their growth phase, with the highest mortality (82 ± 4%) found for cultures grown in high-NP media (Fig. 6C). Overall, larval mortality rates were lower for the exponential-phase *K. veneficum* treatments (54 ± 4%) compared to those of the stationary-phase *K. veneficum* (66 ± 18%) at the end of experiments, but there was not a significant difference (Student's *t*-test, $p = 0.11$).

In the presence of *R. salina*, the bioassay tests with both exponential- and stationary-phase *K. veneficum* indicated increases in larval mortalities, except for the condition of N-deficient *K. veneficum* mixed cultures in exponential phase (Fig. 6B,D). Mixed cultures (irrespective of their nutrient conditions) resulted in high larval mortality in exponential phase (Fig. 6B) but even higher rates of mortality in stationary phase under most nutrient conditions (Fig. 6D). In exponential phase, only low-NP *K. veneficum* mixed cultures resulted in low larval mortality (Fig. 6B). With the presence of *R. salina* with the low-NP condition excepted, stationary-phase *K. veneficum* mixed cultures exhibited significantly higher larval mortality rates (average of 81 ± 7%) compared to those exponential-phase *K. veneficum* mixed cultures (average of 67 ± 12%; Student's *t*-test, $p < 0.001$). Highest larval mortalities typically occurred with the presence of high-NP (i.e. N-rich) *R. salina* when exponential- and stationary-phase *K. veneficum* was grown under Redfield ratio and high-NP conditions, respectively (Fig. 6B,D).

DISCUSSION

Mixotrophy is clearly far more common in dinoflagellates than previously recognized (Jeong et al. 2005a,b, Flynn et al. 2013) and has advantages for the cells with synergistic, not just additive, effects of phototrophic and heterotrophic growth (Mitra & Flynn 2010). Even though mixotrophic nutrition has been emphasized as a major mode for harmful algal species in eutrophic environments and feeding has been linked to toxin production (Adolf et al. 2008, Burkholder et al. 2008), there is still much we do not fully understand about why foods of certain nutritional content are eaten, what the effects of variable nutrition are on growth and putative toxicity and how nutrition or physiological state affect growth of the harmful algal bloom or other organisms (e.g. oyster

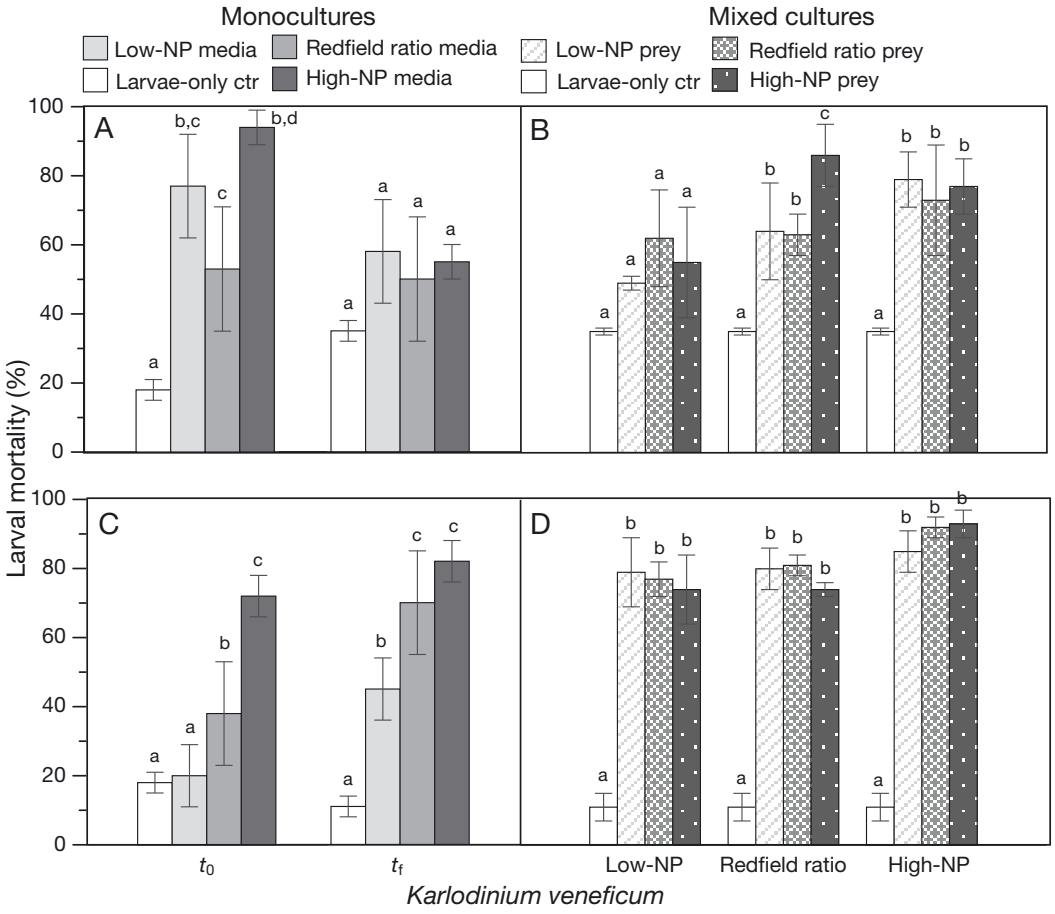

Fig. 6. Mortality of *Crassostrea virginica* larvae after 2 d of exposure to low-NP, Redfield ratio and high-NP *Karlodinium veneficum* at final concentrations (0.9×10^3 cells ml^{-1}) in the absence and presence of low-NP, Redfield ratio and high-NP prey *Rhodomonas salina.* Monocultures of initially (A) exponential-phase and (C) stationary-phase *K. veneficum* with 3 nutrient conditions were collected for larval bioassay tests at the beginning (t_0) and end (t_f) of the time courses. Mixed cultures of initially (B) exponential-phase and (D) stationary-phase *K. veneficum* were collected for larval tests at t_f. Control treatments (ctr) contained *C. virginica* larvae alone. Statistical tests were conducted separately for the monocultures and mixed cultures of 3 nutrient conditions. Different letters show significant differences between treatments (ANOVA test, $p < 0.01$). N: nitrogen; P: phosphorus

larvae) under eutrophic conditions. Our experiments show that the intracellular balance of nutrients and growth phases of the mixotroph as well as its prey have important effects on the growth of *Karlodinium veneficum* blooms and the development of oyster larvae.

Growth and grazing responses of mixotrophic *Karlodinium veneficum*

Mixotrophic nutrition can yield significantly enhanced growth rates compared to those achievable in autotrophic mode (Li et al. 1999, Jeong et al. 2005a,b, Adolf et al. 2006, Glibert et al. 2009). The growth rates of *K. veneficum* cultures that were grown only phototrophically or mixotrophically with additions of *Rhodomonas salina* (Table 1) were similar to those reported for different strains of *K. veneficum* by a wide range of investigators in batch cultures (Li et al. 1999, Adolf et al. 2006, Calbet et al. 2011). The growth benefit of mixotrophy to *K. veneficum* has been shown to be upwards of 2- to 3-fold. For example, Li et al. (1999) measured maximum autotrophic growth rates of *K. veneficum* strain CCMP 1974 (maintained at 20°C at a salinity of 10) of 0.32 ± 0.02 d^{-1} but rates of 0.94 ± 0.06 d^{-1} in mixotrophic cultures with the presence of the cryptophyte *Storeatula major*. In the present study, growth of *K. veneficum* strain CCMP 1975 with additions of *R. salina* had a maximal increase of ~2.8-fold for exponentially growing *K. veneficum* under low-NP condition compared to monoculture growth (Table 1).

Growth rates of *K. veneficum* have been shown to be dependent on their food source and its quality. Previous investigators have also found that nutrient availability is one of the triggering factors for mixotrophy in this dinoflagellate. For example, feeding appears under nutrient-replete conditions but increases when under N and/or P deficiency (Li et al. 2000a). The growth rates of *K. veneficum* for both exponentially growing *K. veneficum* under low-NP condition (0.70 ± 0.10 d^{-1}) and stationary-phase *K. veneficum* under high-NP condition (0.66 ± 0.17 d^{-1}) were significantly enhanced in the presence of prey with reciprocal nutritional status when compared to maximal autotrophic growth in exponential phase (0.25 ± 0.07 d^{-1}) and stationary phase (0.50 ± 0.07 d^{-1}), respectively (Table 1). These results suggest that enhanced growth performance of *K. veneficum* depends on both prey and predator nutritional status (defined here in terms of N:P ratio) and is ultimately determined through a dynamic balance between internal factors of *K. veneficum* cells under different growth phases and external nutrient supplies (e.g. culture media and high-quality food).

It has been shown for other species that mixotrophy can be influenced by both cellular and external nutrient concentrations and ratios as well as prey quality (Smalley et al. 2003, Lundgren et al. 2016). Our results showed significant increases in growth performance for exponentially growing *K. veneficum* in mixed cultures when supplied with high levels of inorganic P- and/or N-rich prey (Table 1); however, the growth rates of exponentially growing *K. veneficum* in monocultures were not profoundly influenced by the N:P ratio of the culture media (Fig. 2A). This indicates that when the N was depleted in the ambient culture media, exponentially growing *K. veneficum* presumably resorted to feeding on N-rich *R. salina* to compensate. In contrast, the growth performance of initially stationary-phase *K. veneficum* was strongly influenced by the N:P ratio of culture media in monocultures, and the highest growth rates were also found when stationary-phase *K. veneficum* grown with P-rich prey were resupplied with a high level of inorganic N (Table 1). In particular, it is recognized that N:P drawdown ratios for monoculture exponentially growing *K. veneficum* were higher than those *K. veneficum* in stationary phase; those conditions were enhanced in the mixed cultures, although in the latter the contributions between prey and predator to total inorganic nutrient consumption were difficult to distinguish from each other (Fig. 5). Those results might suggest that the N and P in proportions incorporated into particulate matter of *K. veneficum* from the ambient culture media are different regarding the different cellular metabolic and structure requirements of the different growth phases. Nielsen (1996) assessed the cellular composition of *Gymnodinium galatheanum*, a harmful algal bloom species regarded as a synonym of *K. veneficum,* and found a comparatively larger capability for P storage compared to that of other dinoflagellates. This stored P consequently is thought to allow them to survive long periods in stationary phases under conditions not otherwise conducive for sustaining growth. With internally stored P, stationary-phase *K. veneficum* can survive a condition of high N:P in the external media. Such a strategy has also been suggested for other dinoflagellates, including *Prorocentrum minimum* and *Ostreopsis* cf. *ovata*, both of which appear to be sustained for long periods of time when P is seemingly depleted in their natural waters (Glibert et al. 2012, Accoroni et al. 2015). Although significantly higher prey death rates were observed with *K. veneficum* in stationary phase than in exponential phase, the short-term benefits of the mixotrophy on growth rates were mainly observed in the latter phase (Fig. 4). The data suggest that feeding by exponential-phase *K. veneficum* could improve growth performance immediately compared to stationary-phase cells in which metabolic processes are generally slower. In this regard, nutritional or metabolic status of *K. veneficum* may play an important role in determining the capability to absorb particulate and/or use dissolved organic material released from prey (Glibert & Legrand 2006) as well as to produce algal toxins with which it can immobilize or kill its prey as part of its nutritional strategy (Sheng et al. 2010, Place et al. 2012). Thus, these differential relationships between exponential-phase and stationary-phase *K. veneficum* could have implications for bloom formations due to different responses and strategies in utilization of nutrient supplies.

The concept that N is preferably obtained through feeding is supported by some studies on haptophytes *Prymensium parvum* (Legrand 2001, Lindehoff et al. 2010, Lundgren et al. 2016). Lundgren et al. (2016) found that mortality rates are higher when prey is N rich, regardless of the nutritional states of *P. parvum*. The present study agreed with the works involving *P. parvum* of varying nutritional states, as evidenced by the highest values of prey death rates occurring when mixed with high-NP *R. salina* for both initially exponential- and stationary-phase *K. veneficum* grown under low-NP conditions (Table 2). However, only low-NP *K. veneficum* had high feeding rates of N-rich prey in exponential phase (0.68 ± 0.07 Rs *K. veneficum*$^{-1}$ d^{-1}), a likely consequence of prey selec-

tion that can serve to rectify nutrient deficiency (Mitra & Flynn 2005).

Moreover, incidences of feeding in mixotrophic dinoflagellates seem to be influenced by ambient nutrient concentrations and ratios (Li et al. 2000a,b, Smalley & Coats 2002). In Chesapeake Bay, feeding in *Ceratium furca* and *K. veneficum* (referred to as *Gyrodinium galatheanum* by the author) are enhanced when the N:P ratio deviates from the Redfield ratio with either N or P deficiency (Li et al. 2000a, Smalley & Coats 2002). The present study agreed with these observations. If only considering the feeding on Redfield ratio prey, increased prey death rates were observed when initially exponential- and stationary-phase *K. veneficum* were grown under high-NP and low-NP conditions, respectively (Table 2). However, the effects of inorganic nutrient on feeding responses of *K. veneficum* were inversed, which suggests that feeding is mediated through the cellular status of the initial growth phase. In fact, *K. veneficum* have shown a 95% increase in fatty acid content, as high-quality lipids, during stationary- compared to exponential-phase growth (Fuentes-Grünewald et al. 2009), so the cellular nutrient stoichiometry and energy metabolism might have changed between the distinct growth phases. This underscores the fact that the intracellular nutrient history and growth conditions of mixotrophic dinoflagellates can influence the ability to utilize different nutrient supplies and supports the study of Smalley et al. (2003) in terms of difficulty of inferring feeding dynamics based on inorganic nutrient data alone.

The difference between prey cells that were actually grazed and involved in burst release due to allelopathic interactions is difficult to resolve for the mixed algae cultures (Carvalho & Granéli 2006, Lundgren et al. 2016). Further studies on the mechanisms of allelopathic interactions among microalgae may be warranted, especially considering the varying nutritional status and different toxin production during different growth phases as well as how they may be involved in prey capture. For example, karlotoxins of the dinoflagellate *K. veneficum* released into the surrounding media have been shown to cause prey immobilization and improve ingestion rates (Adolf et al. 2007, Place et al. 2012).

Implication for natural blooms and oyster restoration

In Chesapeake Bay, *K. veneficum* blooms mostly occur during summer from June to September when the ambient dissolved inorganic phosphorus is high and N:P ratio is lower than the Redfield ratio (N:P = 1:16; Li et al. 2000a, 2015). Our study, in which higher P consumption rates for exponential-phase *K. veneficum* were observed, supports the field observations and suggests that the first phases of the bloom (i.e. when cells are in exponential phase) often occur at N:P ratios lower than 16 but still with measurable N in the water column. In contrast, in the latter phases of *K. veneficum* blooms (i.e. when cells are in stationary phase), cells appear to have adaptive physiological mechanisms involving mixotrophic and/or allelopathic interactions that enable them to be maintained at less than maximal growth rates and at higher N:P ratios (Glibert et al. 2012, Accoroni et al. 2015), as is the case with *Prorocentrum minimum* and *Ostreopsis* cf. *ovata*. Abundances of *K. veneficum* have been related to patterns in subsurface transport from southern Chesapeake Bay to upper and middle regions of the bay (Li et al. 2000b), and well-mixed conditions in the shallow upper bay could bring low-NP oceanic waters into the waters with reciprocal nutrient conditions (e.g. high-NP freshwater). Also, the dinoflagellates may encounter cryptophytes of differing nutritional content if they have originated in one of the tributaries with different patterns of nutrient loading. In this regard, according to our experiments, the possibility exists for improvement in the growth performance of *K. veneficum* inoculated from southern waters if chance encounters with prey with reciprocal nutrient content should occur in the northern reaches of the bay.

Nutrient-limited growth conditions for *K. veneficum* have previously been shown to be associated with toxin production (Adolf et al. 2009) and impacts on larval mortality (Stoecker et al. 2008). The overlap of *K. veneficum* blooms in space and time with larval spawning has emphasized the need for understanding the growth of mixotrophic *K. veneficum* and its negative effects on oyster larvae survival and development of embryos and young larvae (Glibert et al. 2007). Herein, the bioassay experiments indicated that both exponential- and stationary-phase *K. veneficum* caused higher mortality when cells were grown under N-rich (i.e. P-deficient) conditions (Fig. 6A,C). These findings suggest that growth-limiting conditions, especially under P-deficiency conditions, would enhance the adverse effects on larval survival. In addition, rates of larval mortality were generally enhanced with the presence of prey and when *K. veneficum* was inoculated from initially stationary-phase growth compared to rates in the presence of *K. veneficum* from exponential phase. In par-

ticular, P-deficient *K. veneficum* mixed with N-rich prey resulted in the highest oyster larval mortality at stationary phase (Fig. 6B,D). In this regard, the potential for this dinoflagellate to inhibit larval growth seems to be high when shellfish spawning coincides with late-stage blooms (i.e. cells are at stationary growth phase) and with prey of nonreciprocal nutrient status. Although these links have not yet been tested in the field, the relationships between eutrophication and *Crassostrea virginica* need to be highlighted especially in subregions of estuaries such as Chesapeake Bay, which have excessive N inputs and a succession from dense cryptophyte blooms to *K. veneficum* blooms (Adolf et al. 2008).

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