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Effect of ciliate strain, size, and nutritional content on the growth and toxicity of mixotrophic *Dinophysis acuminata*

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ABSTRACT

Previous studies indicate differences in bloom magnitude and toxicity between regional populations, and more recently, between geographical isolates of *Dinophysis acuminata*; however, the factors driving differences in toxicity/toxigenicity between regions/strains have not yet been fully elucidated. Here, the roles of prey strains (i.e., geographical isolates) and their associated attributes (i.e., biovolume and nutritional content) were investigated in the context of growth and production of toxins as a possible explanation for regional variation in toxicity of *D. acuminata*. The mixotrophic dinoflagellate, *D. acuminata,* isolated from NE North America (MA, U.S.) was offered a matrix of prey lines in a full factorial design, 1 × 2 × 3; one dinoflagellate strain was fed one of two ciliates, *Mesodinium rubrum*, isolated from coastal regions of Japan or Spain, which were grown on one of three cryptophytes (*Teleaulax/Geminigera* clade) isolated from Japan, Spain, or the northeastern USA. Additionally, predator: prey ratios were manipulated to explore effects of the prey's total biovolume on *Dinophysis* growth or toxin production. These studies revealed that the biovolume and nutritional status of the two ciliates, and less so the cryptophytes, impacted the growth, ingestion rate, and maximum biomass of *D. acuminata*. The predator's consumption of the larger, more nutritious prey resulted in an elevated growth rate, greater biomass, and increased toxin quotas and total toxin per mL of culture. Grazing on the smaller, less nutritious prey, led to fewer cells in the culture but relatively more toxin exuded from the cells on per cell basis. Once the predator: prey ratios were altered so that an equal biovolume of each ciliate was delivered, the effect of ciliate size was lost, suggesting the predator can compensate for reduced nutrition in the smaller prey item by increasing grazing. While significant ciliate-induced effects were observed on growth and toxin metrics, no major shifts in toxin profile or intracellular toxin quotas were observed that could explain the large regional variations observed between geographical populations of this species.

1. Introduction

Diarrhetic shellfish poisoning (DSP) toxins, i.e., okadaic acid (OA) and dinophysistoxins (DTXs), and/or the less-potent pectenotoxins (PTXs) have been detected in ten of the 75+ species of *Dinophysis* identified worldwide (Reguera et al., 2012; Gómez, 2012). While other DSP toxin-producing species of this genus appear to have a more limited geographical range (e.g., *D. ovum*, Raho et al., 2008; Campbell et al., 2010), *Dinophysis acuminata* poses a threat to seafood safety along most major coastlines, including European, Atlantic coasts, Adriatic Sea, NE Japan, Australia, New Zealand, South Africa, California, Tasmania, NE and Mid-Atlantic North America (Reguera et al., 2014 and references therein). Previous field and culture studies indicate significant differences in DSP toxin content associated with *D. acuminata*, i.e., over an order of magnitude difference in amount of DSP toxin per *D. acuminata* cell, among geographical populations and/or isolates (Lee et al., 1989; Cembella, 1989; Masselin et al., 1992; Tango et al., 2004; Park et al., 2006; Lindahl et al., 2007; Kim et al., 2008; Kamiyama and Suzuki, 2009; Riisgaard and Hansen 2009; Hackett et al., 2009; Suzuki et al., 2009; Hattenrath-Lehmann et al., 2013; Trainer et al., 2013; Tong et al., 2015b). Even within a region, significant variation exists; for example, *D. acuminata* populations from NE North America (i.e., coasts of ME, MA and NY, U.S.) contain DSP toxins and PTX2, however, the relative contributions of the toxin congeners varied between isolates: e.g., one isolate did not produce OA (Tong et al., 2015b), and the intracellular level of OA was similar, greater, or less than DTX1, depending on the isolate (Tong et al., 2015b; Hattenrath-Lehmann and Gobler, 2015; Hattenrath-Lehmann et al., 2015). Further emphasizing

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intraspecific variability, seven isolates of *D. acuminata* from Denmark and isolated cells from Chile contained only PTX2, with no DSP toxins present (Blanco et al., 2007; Fux et al., 2011; Nielsen et al., 2012). These inconsistencies in toxin profile and significant differences in toxin content between regions are reflected in the observed, cross-regional variations in incidence of shellfish harvesting closures due to DSP toxins (Reguera et al., 2014). The factors driving these differences in geographical toxicity of *D. acuminata*, however, have not yet been completely explained.

Laboratory studies into the physiology of *Dinophysis* spp. were logistically impossible until a critical discovery by Park et al. (2006) led to the successful isolation and culturing of this genus in the laboratory; mixotrophic *Dinophysis* require a unique multi-stage feeding regime whereby a cryptophyte (photosynthetic nanoflagellate of the *Teleaulax/Geminigera* clade) is fed to *Mesodinium rubrum* (a photosynthetic, mixotrophic ciliate) before the ciliate is fed to *Dinophysis*. Over the subsequent decade, there has been a surge in laboratory studies investigating the relative importance of prey, light, and dissolved nutrients in cell growth and/or toxin production by *Dinophysis acuminata*. As a mixotrophic species, *D. acuminata* requires both prey, i.e., for particulate nutrients and pigment function, and light to sustain photosynthesis, growth, and toxin production when incubated in nitrate- and phosphate-rich medium (Park et al., 2006; Kim et al., 2008; Riisgaard and Hansen, 2009; Tong et al., 2011; Nielsen et al., 2012). Cells, however, could survive on reserves (with no toxin production) for an additional two months after prey were removed as long as sufficient light was provided, or only one month without light (Smith et al., 2012). More research is required to investigate the importance of dissolved inorganic and organic nutrients in toxin production, but in regards to growth, recent studies indicate that ammonium likely plays a direct role in *D. acuminata* growth and bloom development (Hattenrath-Lehmann et al., 2013, 2015; Hattenrath-Lehmann and Gobler, 2015). Elevated levels of phosphate and nitrate, however, may indirectly impact *D. acuminata* by promoting blooms of prey, *M. rubrum*, capable of rapid assimilation (Tong et al., 2015a; Hattenrath-Lehmann et al., 2015). The dinoflagellate may also be impacted by elevated levels of dissolved organic nutrients, as growth increased when provided filtered, lysed ciliates (Nagai et al., 2011), urea, an amino acid, or waste water organic matter (Hattenrath-Lehmann and Gobler, 2015; Hattenrath-Lehmann et al., 2015). While Kim et al., (2008) and Riisgaard and Hansen (2009) clearly demonstrated a direct relationship between prey abundance and *D. acuminata* growth rate, the effects of prey abundance, prey nutrition, or prey strain, on DSP toxin production remain uncharacterized. The latter, i.e., prey strain, is of particular interest as 1) a possible impediment to invasion if *D. acuminata* is a highly selective grazer on *M. rubrum* strains or is unable to sustain growth equally amongst strains, or 2) a driver of regional toxicity due to variability in nutrition, e.g., a more or less nutritious prey strain leads to more or less toxic *D. acuminata* in that region. Recent molecular evidence also supports this line of investigation as it points toward a more diverse array of cryptophyte-ciliate prey than originally proposed (Kim et al., 2012a,b).

The effect of prey strain, prey nutritional content, and prey biovolume on the growth, toxin production, and toxin exudation by an isolate of *D. acuminata* were investigated. The overall goal of this work was to assess if intrinsic differences between geographically-isolated prey strains (e.g., differences in maximum cell abundances, cell size, and nitrogen, carbon and phosphorus content) could potentially account for the observed variability in toxin profiles and bloom toxicity levels observed across regions. From these data, new hypotheses can be formed regarding whether local prey species could serve as barriers to *D. acuminata* immigration.

2. Methods

An isolate of *Dinophysis acuminata* from the northeastern USA was offered a matrix of prey lines in a full factorial design, 1 × 2 × 3; where one dinoflagellate isolate was fed one of two ciliates as prey, at a ratio of 1:15 predator:prey. The two ciliates, *Mesodinium rubrum*, were isolated from coastal regions of Japan or Spain, which were grown on three cryptophytes, *Teleaulax/Geminigera* clade, isolated from Japan, Spain, or the northeastern USA. As the biovolume of *M. rubrum* from Japan was 3.3x greater than the isolate from Spain, a second set of experiments was conducted where predator to prey ratios were changed from 1:15, to represent equal prey biovolume (1:33 in the Spanish treatment and 1:10 in the Japanese treatment). Intracellular and extracellular toxin levels, cell abundances, grazing rates, and the nutritional content of the ciliate prey were monitored over time, with a focus on exponential and plateau growth phases of *D. acuminata*.

2.1. Culture maintenance

The mixotrophic dinoflagellate *D. acuminata* (DA) used in these experiments was isolated from Eel Pond, MA U.S. in 2006 (strain DAEP01, Hackett et al., 2009). Two isolates of the ciliate *Mesodinium rubrum* (MR), and three isolates of cryptophyte, identified as either *Teleaulax amphioxeia* (TA) or *Geminigera cryophila* (GC) were also cultured for the experiments (Table 1). Two prey lines were utilized, consisting of *T. amphioxeia* and *M. rubrum* from Japan (JA, Nishitani et al., 2008), and *T. amphioxeia* and *M. rubrum* from Spain (SP, Rodríguez et al., 2012). An isolate of *G. cryophila* isolated from the U.S. (strain USGC, originally isolated as GCEP02 from Eel Pond, MA in 2008) was also included in the experimental design. A local ciliate was not utilized as attempts to isolate from this location have been unsuccessful.

Two additional isolates, from Antarctica, were utilized in maintenance culturing only (Table 1); *D. acuminata* cultures were maintained at 6 °C with the addition of Antarctic *Mesodinium rubrum*

Table 1
Maintenance culturing conditions for isolates, including two lines of *Teleaulax amphioxeia* (TA), two lines of *Geminigera cryophila* (GC), three lines of *Mesodinium rubrum* (MR) and one strain of *Dinophysis acuminata* (DA).

Origin	Culture ID	Taxonomic group	Species	Medium[a]	Light[b] (uE)	Temperature (°C)
Antarctic	CCMP2564[c]	cryptophyte	*Geminigera cryophila*	f/2-Si	50	4
	CCMP2563[c]	ciliate	*Mesodinium rubrum*	f/2-Si	50	4
Japan	JATA	cryptophyte	*Teleaulax amphioxeia*	f/2-Si	50	15
	JAMR	ciliate	*Mesodinium rubrum*	f/12-Si	50	15
Spain	SPTA	cryptophyte	*Teleaulax amphioxeia*	f/2-Si	50	19
	SPMR	ciliate	*Mesodinium rubrum*	f/12-Si	50	19
USA	USGC	cryptophyte	*Geminigera cryophila*	f/2-Si	50	15
	DAEP01	dinoflagellate	*Dinophysis acuminata*	filtered seawater	65	6

JA = Japan, SP = Spain, US = United States.
[a] Culture medium, f/2-Si and f/12-Si, modified as described in Anderson et al., 1994.
[b] All cultures grown on a 14 h light:10 h dark photocycle.
[c] Identifies isolates not used in any experiments, only in maintenance culturing.