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Production and excretion of okadaic acid, pectenotoxin-2 and a novel dinophysistoxin from the DSP-causing marine dinoflagellate *Dinophysis acuta* – Effects of light, food availability and growth phase

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ABSTRACT

Diarrhetic shellfish poisoning (DSP) toxins constitute a severe economic threat to shellfish industries and a major food safety issue for shellfish consumers. The prime producers of the DSP toxins that end up in filter feeding shellfish are species of the marine mixotrophic dinoflagellate genus *Dinophysis*. Intraspecific toxin contents of *Dinophysis* spp. vary a lot, but the regulating factors of toxin content are still poorly understood. *Dinophysis* spp. have been shown to sequester and use chloroplasts from their ciliate prey, and with this rare mode of nutrition, irradiance and food availability could play a key role in the regulation of toxins contents and production. We investigated toxin contents, production and excretion of a *Dinophysis acuta* culture under different irradiances, food availabilities and growth phases. The newly isolated strain of *D. acuta* contained okadaic acid (OA), pectenotoxins-2 (PTX-2) and a novel dinophysistoxin (DTX) that we tentatively describe as DTX-1b isomer. We found that all three toxins were excreted to the surrounding seawater, and for OA and DTX-1b as much as 90% could be found in extracellular toxin pools. For PTX-2 somewhat less was excreted, but often >50% was found extracellularly. This was the case both in steady-state exponential growth and in food limited, stationary growth, and we emphasize the need to include extracellular toxins in future studies of DSP toxins. Cellular toxin contents were largely unaffected by irradiance, but toxins accumulated both intra- and extracellularly when starvation reduced growth rates of *D. acuta*. Toxin production rates were highest during exponential growth, but continued at decreased rates when cell division ceased, indicating that toxin production is not directly associated with ingestion of prey. Finally, we explore the potential of these new discoveries to shed light on the ecological role of DSP toxins.

1. Introduction

Diarrhetic shellfish poisoning (DSP) toxins pose a serious threat to both human health and shellfish industries in many areas of the world (Reguera et al., 2012). Although first described from two marine sponges, the primary producers of DSP toxins are species from the two marine dinoflagellate genera *Dinophysis* and *Prorocentrum* (Yasumoto et al., 1980; Tachibana et al., 1981; Murakami et al., 1982). In terms of shellfish poisoning and human health issues, *Dinophysis* is considered the key player, since DSP producing *Prorocentrum* species are benthic, and usually not readily available for suspension feeding mussels.

The genus *Dinophysis* contains more than 100 mixotrophic species and representatives can be found in most oceans and marine environments of the world (Gómez, 2005). *Dinophysis* spp. have long been considered obligate mixotrophs (Jacobson and Andersen, 1994), but Park et al. (2006) were the first to successfully grow a *Dinophysis* species in laboratory culture, by feeding it the marine ciliate *Mesodinium rubrum* (= *Myrionecta rubra*). Prior to that, all studies on *Dinophysis* spp. were limited to in situ populations and single cells picked from natural populations (e.g. Draisci et al., 1996; Miles et al., 2004; Setala et al., 2005).

The mixotrophic nature of *Dinophysis* spp. extends beyond regular mixotrophy, since the genus has recently been shown to sequester and utilize the chloroplasts of its ciliate prey, *M. rubrum* (Park et al., 2007; Wisecaver and Hackett, 2010; Kim et al., 2012). Therefore, high photosynthetic activity of *Dinophysis* spp. relies on continuous food uptake. Starved cells of mixotrophic *Dinophysis* species will remain photosynthetically active, although at reduced rates. This allows mixotrophic *Dinophysis* spp. to survive without food for several months, as long as light is available (Kim et al., 2008; Riisgaard and Hansen, 2009; Nielsen et al., 2012).

Dinophysis spp. produce two groups of DSP toxins: (1) okadaic acid (OA) and the structurally similar dinophysistoxins (DTXs) and

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(2) pectenotoxins (PTXs) (Yasumoto et al., 1985). OA and DTXs are free polyether acids that inhibit serine/threonine phosphatase, and affect the secretion and gene transcription of nerve growth factor (Pshenichkin and Wise, 1995; Garcia et al., 2003). PTXs are polyether lactones, but the actual toxicity, and their status as DSP toxins, is currently under debate. For now, however, the toxin group remains included in the 160 $\mu g\ kg^{-1}$ regulatory threshold set for all commercial shellfish (EC, 2004; Miles et al., 2004; Reguera et al., 2012).

Currently, two DTXs (DTX-1 and DTX-2) have been described with full molecular structures from species of *Dinophysis*. In addition, other DTXs with yet undisclosed exact molecular structures have been reported from Irish waters, and the list of known DTXs could grow within the next years (James et al., 1997; Draisci et al., 1998). PTX-2 is the primary PTX produced by *Dinophysis* spp. but PTX can be found in a variety of forms, with at least 15 different derivatives presently identified (Miles, 2007; Anonymous, 2009). Most of these are believed to occur only as metabolites in shellfish, however (Suzuki et al., 1999). *Dinophysis acuta* normally contains OA and PTX-2 as well as either DTX-1 or DTX-2, but the cellular content of each toxin can vary a lot (Lee et al., 1989; James et al., 1999; Lindahl et al., 2007; Pizarro et al., 2008, 2009; Fux et al., 2010).

The ecological role and significance of DSP toxins are currently largely unknown (Reguera et al., 2012). Several functions seem plausible, including food capture, grazer defense, allelopathy and anti-bacterial deterrent, and some of these have already been visited (Nagai et al., 1990; Carlsson et al., 1995; Gross, 2003). The theory of DSP toxins as a defense against grazing is supported by the finding that some copepods seem to discriminate against *D. acuminata* as a food source, whereas another non-discriminating copepod species experienced reduced survival rates (Carlsson et al., 1995). The theory of allelopathic effects has also received backing (Windust et al., 1996), but both ideas remain unproven, and the ecological role of DSP toxins is still undisclosed.

Many harmful microalgae are mixotrophic (i.e. use particulate food for growth; e.g. *Prymnesium parvum*, *Alexandrium* spp., *Karenia* spp., and *Karlodinium* spp.). In fact, only the non-motile algal groups like diatoms (e.g. *Pseudo-nitzschia* spp.) and cyanobacteria (e.g. *Nodularia spumigena*) can be considered autotrophs or auxotrophs (Flynn et al., 2013). *Dinophysis* spp. are among the very few toxic microalgae that rely on chloroplasts sequestered from their prey. *Pfisteria* spp. may be another group with similar abilities, but data on rates of photosynthesis are still lacking. The available data on *Dinophysis* spp. suggest they are obligate mixotrophs. Hence they 1) cannot live in the long run without food and 2) cannot live in complete darkness even when supplied excess amounts of food (Park et al., 2008; Nagai et al., 2008; Nishitani et al., 2008; Kim et al., 2008; Riisgaard and Hansen, 2009). This raises questions about the role of food uptake and light for toxin production. It also raises questions about the possible excretion of DSP toxins and ultimately about the ecological role of DSP toxins.

Here, we investigate the dependence of toxin contents and production of *D. acuta* upon irradiance, food availability and growth phase. We measure both intra- and extracellular levels of DSP toxins in order to quantify toxin excretion under various conditions. The aim is to understand *Dinophysis* spp. toxicity better and ultimately to unravel the ecological function of DSP toxins.

2. Materials and methods

2.1. Cultures and culturing conditions

Cultures of the cryptophyte *Teleaulax amphioxeia* (K-0434 (SCCAP)) and the ciliate *M. rubrum* (MBL-DK2009) were established from water samples from Helsingør Harbor in 2009. Cultures of *M. rubrum* were fed *T. amphioxeia* at a predator:prey ratio of ≈1:10 twice a week. During a scientific cruise in the North Atlantic ca. 100 km south of the Faroe Islands (60°24′N; 6°58′W), a non-clonal culture (DANA-2010) of the DSP producing dinoflagellate *D. acuta* was established in June 2010, by picking and washing several cells. *M. rubrum* was added as prey organism twice per week at a predator:prey ratio of ≈1:10 to allow mixotrophic growth.

All three species were grown in f/2 medium based on autoclaved seawater, and with a salinity of 32 ± 1, a dissolved inorganic carbon (DIC) concentration of 2.3 ± 0.1 mmol L^{-1} and a pH of 8.0 ± 0.05 (Guillard and Ryther, 1962). They were grown at 15.0 ± 1.0 °C in a temperature controlled room, at an irradiance of 130 μmol photons $m^{-2}\ s^{-1}$ (PAR), controlled by a timer to a light:dark cycle of 16:8 h. All cultures were non-axenic.

DSP toxins of a *D. acuta* stock culture were sampled by transferring 0.5 ml subsamples to spin filters (pore size = 0.45 μm, VWR, Denmark), and centrifuging these at $400 \times g$ for 2 min. Filtrates were removed, and spin filters were stored at −18 °C until extraction and analysis.

2.2. Experiment 1 – effects of irradiance on growth, photosynthesis and toxin production

D. acuta was kept well-fed for a minimum of 14 days at four different irradiances to evaluate the effects of light on photosynthesis, growth rate and toxin content. The four irradiances were 7, 15, 30 and 130 μmol photons $m^{-2}\ s^{-1}$ (PAR), henceforth termed I_7, I_{15}, I_{30} and I_{130} respectively. All treatments were setup in the same room, in front of the same light source consisting of Osram cool white 58 W/640 fluorescent tubes, but with different combinations of neutral density filters in front of I_7, I_{15} and I_{30} in order to achieve the designated irradiances. All four treatments were run in triplicate 65 ml polystyrene bottles filled to capacity. The ciliate, *M. rubrum*, was cultured at I_{130}, but was light acclimated for a week at the appropriate irradiances before being used as prey for *D. acuta*. The same was done with the cryptophyte *T. amphioxeia*. Initial cell concentrations at the experimental setup were 200 and 2500 cells ml^{-1} of *D. acuta* and *M. rubrum*, respectively.

Every 2–4 days, 3 ml subsamples were removed from each flask for enumerations of *D. acuta*, *M. rubrum* and *T. amphioxeia*. 1 ml Sedgewick–Rafter sedimentation chambers were used, and cells were counted on an Olympus CK2 inverted microscope at 40–400×. A minimum of 200 cells were counted, unless cell concentrations were below 200 ml^{-1} at which point a maximum 1 ml was inspected. After each sampling, cell concentrations of *D. acuta* and *M. rubrum* were adjusted to 200 and 2000 cells ml^{-1} respectively, by adding f/2 medium and light acclimated, well fed *M. rubrum*.

On the final day, 1 ml subsamples were removed from each triplicate bottle for determination of photosynthetic activity. 80 *D. acuta* cells were picked from each subsample under a stereo microscope, and photosynthetic activities were determined exactly like presented earlier, including ^{14}C addition (as HCO_3^-) to both light and dark samples, 3 h incubations and checks of specific activity (Nielsen et al., 2012).

Toxin samples were also taken from each flask on the final day of the first experiment. Subsamples of 0.5 ml were transferred to spin filters, and these were centrifuged at $400 \times g$ for 2 min. Filtrates were removed, and spin filters stored at −18 °C until extraction and analysis. This toxin extraction method has previously been shown not to affect intracellular toxins quotas of *Dinophysis acuminata* at centrifugal forces up to $12{,}800 \times g$ (Nielsen et al., 2012).

For growth rates and toxin contents, averages of the last three values were defined as the well-fed, light acclimated values (hereafter termed 'steady-state'), and these were used for comparisons between irradiances.