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Growth and toxin production of *Azadinium poporum* strains in batch cultures under different nutrient conditions

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ABSTRACT

Azaspiracid-2 (AZA2) is the dominant toxin produced by *Azadinium poporum* strains AZDY06 and AZFC22 isolated from the South China Sea. Biomass and AZA2-production were examined within batch cultures with variation in experimental concentrations of nitrate (0, 88, 882, and 2647 μM) or phosphate (0, 3.6, 36, and 109 μM), different nitrogen sources (nitrate and urea) and media (f/2-Si, L1-Si, and K-Si) in the present study. Growth of both strains positively responded to nitrate or phosphate nutrients, but the growth status was significantly repressed by the highest additional level of phosphate (109 μM). Both AZDY06 and AZFC22 grew well with higher specific growth rates, but with shorter growth periods, within f/2-Si medium spiked with urea than that within media spiked with nitrate. L1-Si medium with relatively high concentrations of trace metals was relatively favorable to both strains of *A. poporum* tested here. No obvious change within the toxin profile occurred in all cultures of both strains under the various nutrient conditions, although trace amounts of some suspicious derivatives of AZA2 occurred in some cultures. AZA2 cell quotas within both strains significantly ($p < 0.05$) increased at the stationary phase under lower additional phosphate (0 and 3.6 μM). Significant differences were not found within AZA2 cell quotas in cultures with additional nitrate ranging from 0 to 2647 μM. The highest AZA2 cell quota and maximum AZA2 quantity per culture volume occurred in batch culture at the stationary phase under phosphate concentrations at 3.6 μM. Neither *A. poporum* strain exhibited significant changes in AZA2 cell quotas within f/2-Si media spiked with urea or nitrate as nitrogen sources. The AZA2 cell quota of strain AZDY06 also did not change remarkably within f/2-Si, L1-Si, and K-Si media, however the AZA2 cell quota of strain AZFC22 within L1-Si medium was significantly ($p < 0.05$) higher than that within f/2-Si medium.
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1. Introduction

At least eight people got sick due to consumption of mussels (*Mytilus edulis*) cultivated at Killary Harbor, Ireland, in 1995. The major causative agent (azaspiracid-1, AZA1) of that incident was elucidated as a new toxin having two spiro ring assemblies, a cyclic amine and a carboxylic acid. Additional analogues of AZA1 were determined from toxic mussels in later studies (James et al., 2003a). Combined more than twenty analogues of azaspiracids (AZAs) have been reported (Krock et al., 2014). Various species of cultured filter-feeding shellfish contaminated by AZAs have been reported from coasts of Ireland, Norway, England, Spain, France, Denmark, Portugal, Morocco, Sweden, Chile, and China (Twiner et al., 2008; López-Rivera et al., 2010; Wu et al., 2014). Azaspiracid shellfish poisoning (AZP) incidents are caused by toxic shellfish seafood consumption (Twiner et al., 2008). A limit for maximum concentration of AZAs in shellfish was set at 160 μg AZA1 eq./kg and is regulated by the European Food Safety Authority in order to protect human health.

Dinoflagellate, *Protoperidinium crassipes*, blooms were regarded as the causative source of AZAs (James et al., 2003b), however, Krock et al. (2009) failed to find a correlation between the occurrence of AZAs in shellfish and blooms of *Protoperidinium* spp. Subsequently, dinoflagellate, *Azadinium spinosum*, was isolated from the waters of Ireland, Scotland, and Denmark, and was described as the azaspiracid toxin producer (Tillmann et al., 2009; Salas et al., 2011). Another species of *Azadinium* was also isolated and identified as *A. poporum* from coastal waters of China, which was regarded as a potential source of AZAs in shellfish cultured in the Asian Pacific (Gu et al., 2013). The dinoflagellate *A. dexteroporum* isolated from the Mediterranean Sea was also reported to be a potential producer of AZAs (Percopo et al., 2013). AZA production was not found in other species of *Azadinium* such as *A.*

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caudatum var. *margalefii* from Scottish waters (Tillmann et al., 2014a), *A. trinitatum* sp. nov., *A. cuneatum* sp. nov., and *A. concinnum* sp. nov. from the Irminger Sea (Tillmann et al., 2014b), *A. dalianense* from the Yellow Sea of China (Luo et al., 2013), *A. polongum* from Shetland Islands (Tillmann et al., 2012), or *A. obesum* from the North Sea (Tillmann et al., 2010). Within *A. poporum* non-toxic strains have been identified (Tillmann et al. 2011; Gu et al., 2013) and the toxin profile of AZAs in the various strains of *A. poporum* was also variable (Krock et al., 2014).

The effect of environmental and nutritional factors on the growth and azaspiracid production of *A. spinosum* has been reported using batch and continuous cultures. *A. spinosum* grows in a wide range of conditions, from 10 °C to 26 °C (8–220 fg cell^{-1}), 30–40 psu (23–37 fg cell^{-1}), 50–250 μmol m^{-2} s^{-1} (16–22 fg cell^{-1}), and the toxin profile did not change under all environmental conditions tested although the AZA cell quota was significantly enhanced by low temperatures (10 °C, 220 fg cell^{-1}) (Jauffrais et al., 2013). Growth response and toxin production has been examined in multiple species of phytoplankton such as *Karlodinium veneficum* (Adolf et al., 2009), *Alexandrium tamarense* (Wang and Hsieh, 2002; Leong et al., 2004; Lee et al., 2012), *Prorocentrum donghaiense* (Lai et al., 2011), *Karenia mikimotoi* (Lei and Lu, 2011), *Amphidinium klebsii* (Long and Long, 2012), and *Karenia brevis* (Hardison et al., 2013). Commonly algal growth was repressed by nitrogen or phosphate limitation and toxin production was stimulated by phosphate deprivation in these studies. However, growth and toxin production of *A. poporum* in batch cultures under different nutrient conditions have not been examined until now. In the present study, the effect of medium nutrients on growth and AZA2-production in two *A. poporum* strains, isolated from the South China Sea, was explored using batch culture under controlled laboratory conditions.

2. Materials and methods

2.1. Chemicals

Acetonitrile, methanol, and monosodium orthophosphate (NaH_2PO_4) were purchased from Merck Ltd. (White-house Station, NJ, USA). Formic acid (FA) and ammonium formate (AF) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium nitrate ($NaNO_3$) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were HPLC grade. AZA1 (CAS Registry No.: 214899-21-5), AZA2 (CAS registry No.: 265996-92-7), and AZA3 (CAS registry No.: 265996-93-8) standard reference materials were purchased from the Certified Reference Materials Program (CRMP) of the National Research Council of Canada (Halifax, NS, Canada). Pure water was filtered by a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA) to 18.2 MΩ cm quality or better.

2.2. Strains of A. poporum

Two strains of *A. poporum* (AZDY06 and AZFC22) were isolated by Dr. Haifeng Gu from sediment samples collected along the coast of South China Sea. Strain AZDY06 was isolated from Daya Bay (latitude 22°36′08″, longitude 114°37′21″) and AZFC22 was isolated from Beibu Bay (latitude 21°29′58″, longitude 108°13′53″). Both strains were cultured within f/2-Si medium (Guillard and Ryther, 1962) at 18 °C, 5000 lux under a 12-h light/12-h dark cycle before inoculation for experimental batch culture. Seawater collected from the Shilaoren bathing beach, Qingdao, was filtered (0.22 μm) and spiked with different concentrations of nutrients in the experimental media. Salinity and pH of natural seawater was approximately 30 ± 1 and 8.0 ± 0.1, respectively.

2.3. Culture of A. poporum with various concentration of nitrate and phosphate

Various concentrations of nitrate or phosphate were separately spiked into medium used for single factor experiments. No effort was made to remove nitrate and phosphate from the natural seawater used here due to their relatively low concentrations (nitrate 1.53–2.02 μM, phosphate 0.52–0.62 μM). Phosphate concentrations were kept constant at 36.2 μM, following the f/2-Si recipe, during experimental manipulation of nitrate concentrations representing zero, one-tenth, one, and three times the standard nitrate concentration (882.4 μM). Similarly, the concentration of nitrate (882.4 μM) was held constant during experiments altering phosphate concentrations. In these experiments, spiked phosphate concentrations varied with additions equaling, zero, one-tenth, one, and three times the normal phosphate concentration (36.2 μM). Each nutrient treatment had seven replicate cultures for each strain. From these replicates, three cultures were used to record growth rates and the other four cultures were used for collection of algal cells at the exponential and stationary phases, respectively. All cultures were maintained under a 12-h light/12-h dark scheme in 300 mL of sterile medium within 500-mL borosilicate flasks. The physical conditions of temperature (18 °C) and illumination intensity (5000 ± 1000 lux) were held constant for all cultures in this study. The initial cell density of microalgae was approximately 1000 cells mL^{-1} for each culture.

2.4. Culture of A. poporum with different nitrogen source and different medium

Another batch culture experiment was also carried out in order to compare the effect of different nitrogen sources on growth and toxin production. In seven treatments nitrate (882.4 μM) was replaced by its atomic equivalent urea (441.2 μM) within the f/2-Si medium. All cultures of *A. poporum* were pre-adapted in the experimental media for several weeks before using them in formal experiments to observe growth rates and measure toxin cell quota. Initial cell density of microalgae was approximately 2000 cells mL^{-1} for each culture.

Three different media including f/2-Si, K-Si, and L1-Si were adopted and compared in batch culture of *A. poporum*. The f/2 medium without silicate was described above. K medium containing a 10-fold higher EDTA chelation than most common marine media (Keller et al., 1987) was used to discover the effect of lower concentrations of trace metals on growth and toxin production. L1 medium had enriched trace metals (exclusively additional components H_2SeO_3, $NiSO_4 \cdot 6H_2O$, Na_3VO_4, and K_2CrO_4 at 1.0×10^{-8} M) in comparison with f/2 medium which is the general purpose marine medium for growing coastal microalgae (Guillard and Hargraves, 1993). Initial cell density of microalgae was approximately 5000 cells mL^{-1} for each culture.

2.5. Record of growth curve of A. poporum

Cell density of *A. poporum* was recorded once every two days using an optical microscope. One mL of algal culture was removed for calculation following a thorough shake. One droplet of Lugol's solution was added to the solution and mixed completely. Triplicate solutions (50 μL) were subsequently removed and cell density was quantified by optical microscope. The total number of cells was counted twice for each triplicate sample. The growth curves were compiled from the final average density. Specific growth rate (SGR or μ) was calculated using the empirical equation. $\mu = (\mathrm{Ln}N_t - \mathrm{Ln}N_0)/(t - t_0)$ where N_0 = the initial culture density, and N_t = the recorded density after t days.

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