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Effect of environmental and nutritional factors on growth and azaspiracid production of the dinoflagellate *Azadinium spinosum*

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ABSTRACT

Azadinium spinosum, a small dinoflagellate isolated from the North Sea, is a producer of azaspiracids (AZAs), a group of biotoxins associated with human illness following ingestion of contaminated shellfish. Using batch and continuous cultures of *A. spinosum*, the present study investigated the effects of different environmental and nutritional factors (salinity, temperature, photon flux density, aeration, culture media, nitrogen sources, phosphate source, and N/P ratios) on growth, maximum cell concentration, and AZA cell quota.

Azadinium spinosum grew in a wide range of conditions; from 10°C to 26°C and salinities from 30 to 40, under irradiances ranging from 50 μmol m^{-2} s^{-1} to 250 μmol m^{-2} s^{-1}, with or without aeration. Growth and maximum cell concentration were highest at a salinity of 35, at temperatures between 18°C and 22°C, and with aeration. Concerning AZA cell quota, the most significant effect was observed at low temperature; the AZA cell quota was more than 20 times higher at 10°C (220 fg $cell^{-1}$) than at temperatures between 18°C and 26°C. *A. spinosum* grew on all media tested with only slight differences in growth rate and AZA cell quota. In continuous culture, lowering the concentration of nutrients (0.5 strength of a modified K-medium) in the inflow improved AZA cell quota whereas higher concentration (doubling the normal strength of K-medium) improved maximal cell concentration. *A. spinosum* grew on different sources of nitrogen tested (nitrate, urea, ammonium) with almost no effect on toxin cell quota and growth, except that adding ammonium caused a decrease in growth.

These first experiments on *Azadinium spinosum* increased our knowledge on factors affecting its growth and toxin production; furthermore, these results allowed and improved particularly *A. spinosum* production in pilot scale photobioreactors for AZA isolation.

1. Introduction

Azadinium spinosum is a small dinoflagellate (12–16 μm length and 7–11 μm width) producing azaspiracid-1 and -2 (AZAs) (Tillmann et al., 2009). This dinoflagellate was first isolated from the North Sea off Scotland during a research cruise, with on-board LC–MS/MS to screen different size fractions of phytoplankton for the characterization and isolation of AZA producing organisms (Krock et al., 2008). Following its discovery, a new genus *Azadinium* was erected (Tillmann et al., 2009), which rapidly was expanded by the identification of other new species. These are *Azadinium obesum* and *Azadinium polongum*, which are not known to produce AZAs (Tillmann et al., 2010, 2012b), and *Azadinium poporum* (Tillmann et al., 2011) including a strain designated as *A.* cf. *poporum* (Potvin et al., 2012) isolated from Korea and additional strains of *A. poporum* isolated from China (Gu et al., 2013). *A. poporum* has recently been found to produce AZAs, partly of a new type, which also was detected in *Amphidoma languida* (Krock et al., 2012) a species closely related to *Azadinium* (Tillmann et al., 2012a). Generally, *A. poporum* was shown to be quite variable in terms of AZA profile, with a number of the Chinese strains producing AZA-2 (Gu et al., 2013). Other strains of *A. spinosum* subsequently isolated in Denmark (Tillmann et al., 2011), Ireland (Salas et al., 2011), and from the Shetland Islands (Tillmann et al., 2012b) were all found to produce AZA1 and -2 and another AZA of a different mass, termed AZA-716 (Tillmann et al., 2012b). A species of *Azadinium*, described as *A.* cf. *spinosum* morphotype was reported from Argentina (Akselman and Negri,

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2012). The authors reported a bloom (up to 9.03×10^6 cell L^{-1}) in 1990; however, intoxications following shellfish consumption were not reported at that time.

Concerning AZAs, the toxin was found 12 years before the primary producing organism. In 1995, contaminated mussels (*Mytilus edulis*) from Killary Harbor (Ireland) were consumed in the Netherlands and caused human illness (McMahon and Silke, 1996). The symptoms were diarrhea, nausea, vomiting and stomach cramp, typical for diarrheic shellfish poisoning (DSP). However, concentration of okadaic acid and analogs were below the regulatory limit. Subsequently, AZA1 was discovered (Satake et al., 1998) and later structurally revised (Nicolaou et al., 2006). The base structure of this toxin group is composed of an cyclic amine group (aza group), a tri-spiro ring assembly and a carboxylic acid group (Satake et al., 1998). Since its identification, the toxin was found to be responsible for additional human intoxications in Europe (Furey et al., 2010) and in the US (Klontz et al., 2009). AZAs were identified throughout Northern and Western Europe (Amzil et al., 2008; James et al., 2002; Magdalena et al., 2003; Vale et al., 2008) and in Morocco (Taleb et al., 2006), Chile (Lopez-Rivera et al., 2009), Japan (Ueoka et al., 2009), and China (Yao et al., 2010), indicating a worldwide distribution of the toxin and consequently of the producing organism.

In feeding experiments, *Azadinium spinosum* was found to directly contaminate mussels, with a fast AZA accumulation and biotransformation after a day or more of exposure to *A. spinosum* (Jauffrais et al., 2012c; Salas et al., 2011). In spite of the importance of *A. spinosum* as an AZA producer, little is known on factors affecting *A. spinosum* physiological responses in terms of growth, cell yield and AZA cell quota, which arguably are most relevant in directly affecting AZA accumulation rate by suspension feeding bivalves. Preliminary information on AZA cell quota just pointed out a general variability of roughly 5–40 fg $cell^{-1}$ in batch culture (Salas et al., 2011; Tillmann et al., 2009), but cell quota has not yet been systematically related to environmental growth conditions.

Dinoflagellate growth and toxin content have repeatedly been shown to be affected by environmental factors such as salinity, temperature, and light (Flynn et al., 1996; Gedaria et al., 2007; Maclean et al., 2003; Parkhill and Cembella, 1999; Xu et al., 2010), and by the use of aeration (Hu et al., 2006b; Morton and Bomber, 1994). Moreover, nutritional factors, and especially nitrogen and phosphate ratio, also affect dinoflagellate growth and toxin cell quota (Flynn et al., 1996; Hu et al., 2006a; Hwang and Lu, 2000; Lartigue et al., 2009). However, chemical structures of algal biotoxins differ between toxic species of microalgae and thus factors influencing toxin cell quota might vary for different group and/or species (Graneli and Turner, 2006).

This work on *Azadinium spinosum* aims at understanding the effect of environmental factors (temperature, salinity, light and aeration) as well as nutritional factors (culture medium, nitrate, urea, ammonium, phosphate, N/P ratios) on growth, cell yield and AZA cell quota in batch culture and/or photobioreactors run in continuous culture mode with the main aim to facilitate and enhance a sustainable production of AZAs from *A. spinosum* culture. These toxins are still necessary for instrument calibration in continuous monitoring programs and for toxicological studies. In addition, this study will improve our understanding on *A. spinosum* and will give initial informations on factors that might influence *A. spinosum* bloom formation and their toxicological effects.

2. Materials and methods

2.1. A. spinosum

2.1.1. Stock culture condition

A. spinosum (strain 3D9) was maintained in 50 mL batch cultures (70 mL sterile polystyrene flask) at 18 °C, at a salinity of 35, a photon flux density (PFD) of 200 μmol m^{-2} s^{-1} and a photoperiod of 16 h of light and 8 h of dark. Cultures were grown in 0.22 μm filtered seawater, enriched with K medium (Keller et al., 1987), which was slightly modified (without addition of NH_4Cl, silicate and tris buffer, and with addition of Na_2SeO_3 (10^{-8} M)). The stock culture was maintained in the growth phase by transferring weekly into fresh medium.

2.1.2. Experimental culture conditions

For experimental purposes, batch and continuous culture were used:

Batch cultures were prepared in two different volumes, 70 mL sterile polystyrene flasks or 10 L flat bottomed glass flasks. Initial cell concentrations were between 5×10^3 cell mL^{-1} and 20×10^3 cell mL^{-1} and the cultures were grown until stationary phase. Growth conditions were as described for the stock culture with the exception of the respective treatment condition.

Continuous cultures were maintained in 2.5 L or 100 L chemostats. The photobioreactors were made of transparent polymethyl methacrylate and operated under the following conditions: the pH was regulated at 7.9 using CO_2 addition, the temperature was kept constant at 18 °C, light was provided on one side of the reactor using neon tubes at identical PFD and photoperiod as the stock cultures, and a Rushton turbine was used to homogenize the culture medium. Prior inoculation, photobioreactors were sterilized using peroxyacetic acid at 5 ppm for 30 min and rinsed twice using filtered sea water (0.22 μm). The photobioreactors were inoculated with *A. spinosum* culture to reach an initial concentration of 70×10^3 cell mL^{-1} and a fixed dilution rate (0.3 and 0.2 d^{-1} in 2.5 L or 100 L chemostats, respectively) was applied and maintained until steady state.

2.2. Environmental factors

To independently assess the effect of temperature, salinity and PFD on *Azadinium spinosum* growth and toxin production in batch culture, the cells were inoculated using an aliquot from the linear growth phase of a stock culture, and grown under the different conditions in triplicate (50 mL) until stationary phase.

Effect of temperature was assessed at 10, 14, 18, 22, 26 °C with a PFD at 50 μmol m^{-2} s^{-1}.

Effect of salinity was assessed at: 10, 20, 30, 32, 35, and 40. The salinity was adjusted by dilution with Milli-Q® water (Millipore) or evaporation, verified with a refractometer (Atago S/Mill) and nutrients were added subsequently.

Effect of the PFD was assessed at 50, 100, 150, 200 and 250 μmol m^{-2} s^{-1} as quantified with a spherical quantum sensor (LI-250 light meter, LI-COR). In addition, the effect of the PFD on *Azadinium spinosum* growth and toxin production in continuous culture was evaluated using two photobioreactors (100 L, dilution rate: 0.2 d^{-1}). PFD was gradually increased: 1st step 100, 2nd step 200, and 3rd step 400 μmol m^{-2} s^{-1}.

To evaluate the effect of aeration on growth, *Azadinium spinosum* was cultured in triplicate 10 L flat bottomed glass flasks with and without aeration (~1 L min^{-1} for a 10 L culture volume, air was 0.2 μm prefiltered and administered using a glass tube, $NaH_2PO_4 \cdot H_2O$ was the source of phosphate in this experiment).

2.3. Nutritional factors

2.3.1. Culture medium

Three different culture media were tested, The first was K-medium (Keller et al., 1987) in its modified version used to maintain the stock culture, the second medium was the same but now contained tris buffer, the third one was an L1 medium (using a K_2CrO_4 concentration in final medium equal to $1 \cdot 10^{-8}$ M) (Guillard