

Harmful Algae

July 2013, Volume 27, Pages 138–148

<http://dx.doi.org/10.1016/j.hal.2013.05.009>

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Archimer
<http://archimer.ifremer.fr>**Effect of environmental and nutritional factors on growth and azaspiracid production of the dinoflagellate *Azadinium spinosum***Thierry Jauffrais^{a, b, *}, Véronique Séchet^a, Christine Herrenknecht^b, Philippe Truquet^a,
Savar Véronique^a, Urban Tillmann^c, Philipp Hess^{a, *}^a IFREMER, Laboratoire EMP/PHYC, Rue de l'Île d'Yeu, 44311 Nantes, France^b LUNAM, Université de Nantes, MMS EA2160, 2 rue de la Houssinière, 44035 Nantes, France^c Alfred Wegener Institute, Am Handelshafen 12, D-27570 Bremerhaven, Germany*: Corresponding authors : Thierry Jauffrais, email addresses : Thierry.Jauffrais@univ-nantes.fr ; thierry.jauffrais@ifremer.fr ; Philipp Hess, email address : Philipp.Hess@ifremer.fr ; tel.: +33 2 40 37 42 57 ; fax: +33 2 40 37 40 26**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 1 °C to 6 °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 1 °C and 3 °C, and with aeration. Concerning cell quota, the most significant effect was observed at low temperature: the cell quota was more than 20% higher at 1 °C (220 fg cell⁻¹) than at temperatures between 1 °C and 6 °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.

Keywords: AZA ; Chemostat ; Dinophyceae ; Nutrients ; N/P ratio ; Photobioreactor**Highlights**

► *Azadinium spinosum* was exposed to different environmental and nutritional factors ► Growth, biomass and AZA cell quota were observed in continuous and or batch culture ► *A. spinosum* grew in a wide range of conditions ► Temperature had the main effect on AZA cell quota.

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 *A. obesum* and *A. polongum*, which are not known to produce AZAs (Tillmann et al., 2010; Tillmann et al., 2012b), and *A. 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, 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 Harbour (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 diarrhetic shellfish poisoning (DSP). However, concentration of okadaic acid and analogues 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, *A. 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 to 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, 2006).

This work on *A. 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. Material 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 to 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 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 day^{-1} in 2.5 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 *A. 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

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

To evaluate the effect of aeration on growth, *A. spinosum* was cultured in triplicate 10 L flat bottomed glass flasks with and without aeration ($\sim 1 \text{ L min}^{-1}$ for a 10 L culture volume, air was 0.2 μm prefiltered and administered using a glass tube, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 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 $1 \cdot 10^{-8}\text{M}$) (Guillard and Hargraves, 1993) and the last one an L1 medium with addition of 3 mL of soil extract (following the protocol SE1 of the Culture Collection of Algae and Protozoa (CCAP)). Before measuring growth, cell yield and toxin cell quota, cultures were pre-adapted to the experimental media for several weeks.

Next, potential effects of using K modified medium of different strength were assessed in photobioreactors (100 L, dilution rate: 0.2 day^{-1}) using the following medium concentration: 0.5Kmod, Kmod and 2Kmod.

2.3.2. Nitrogen and phosphate sources

An experiment was carried out in duplicate to assess the effect of the addition of ammonium and/or urea to a culture medium already containing nitrate (initial concentration of nitrate: 50 μM , initial concentration of ammonium: 0 or 100 μM and initial concentration of urea: 0 or 100 μM) on *A. spinosum* growth, cell concentration and toxin cell quota in 50 mL batch culture. Concerning the other nutrients, trace metals and vitamins were at the same concentration as used in the K medium (Keller et al., 1987)

Subsequently, nitrate (882 μM) or urea (100 μM) as sole nitrogen source on *A. spinosum* production and toxin cell quota were compared using two photobioreactors (2.5 L, dilution rate: 0.3 day^{-1}).

In one experiment, addition of organic (Na₂ β-glycerophosphate) or inorganic (NaH₂PO₄ · H₂O) phosphate was compared, to evaluate their respective effect on *A. spinosum* grown in batch cultures (50 mL).

Three N/P ratios (NO₃⁻/PO₄³⁻ in μM: 40/5, 160/10, 882/10) were tested using the 2.5 L chemostat at a dilution rate of 0.3 day⁻¹.

2.4. Cell growth

Cell concentrations (cell mL⁻¹), mean estimated spherical diameter (ESD, μm) and total biovolume (μm³ mL⁻¹, spherical equivalent) were assessed daily using a particle counter (Multisizer 3 Coulter counter, Beckman).

In batch culture: growth rates (μ in day⁻¹) were calculated following the equation (Guillard, 1973):

$$\mu = (\ln X_t - \ln X_0) / t$$

Where X₀ is the initial cell density and X_t is the cell density after t days (the day chosen for t are represented by a star (*) in the different graphics).

One experiment (i.e. section 2.2. aeration) yielded a sufficient number of points to apply a Gompertz model to assess maximum growth rate (μ_{max} in d⁻¹), maximum cell concentration (α = ln(X/X₀) with X in cell mL⁻¹) and latency time (λ in day, if present) with a MatLab software:

$$f(t) = \alpha \times \exp(-\exp(\mu_{\max} \times \exp(1)/\alpha \times (\lambda - t) + 1))$$

In continuous culture, the bioreactors were considered to have reached steady state after a minimum of 3 days at the same microalgal concentration (± 10%).

2.5. AZA analysis

2.5.1. Reagents

Acetone and methanol (MeOH) were obtained as HPLC grade solvents from JT Baker. Water for analysis was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality) and ammonium formate (Purity for MS) were from Sigma Aldrich. AZA1-2 calibrants for LC-MS/MS analysis were dilutions of certified AZA1 solutions obtained from the National Research Council Canada.

2.5.2. *A. spinosum*

The analytical procedure had previously been optimized (Jauffrais et al., 2012a). Briefly, *A. spinosum* cells were collected by centrifugation (10 ml, 2500 g, 20 min, 4° C). The supernatant was collected (see below) and the pellet re-suspended using 0.5 mL of acetone, transferred to an Eppendorf tube (1.5 mL) and bath sonicated (10 min). After sonication, the aliquot was centrifuged (15000 g, 10 min, 4°C). The supernatant was transferred into a 5 mL glass tube and the pellet was re-suspended in 0.5 mL of acetone, homogenized and centrifuged again. The supernatants were transferred again into the same 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35°C. This process was repeated three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol, filtered with NANOSEP MF centrifugal filter 0.2 μm (PALL) (6000 g, 3 min, 4°C), and transferred into a HPLC vial for analysis.

2.5.3. Supernatant

Solid Phase extraction (SPE) was carried out on Oasis HLB cartridges (Waters) to estimate AZA concentration in the 10 mL supernatant. Oasis HLB cartridges (6 cc, 200 mg) were activated with methanol (10 mL) and washed with a solution of water-methanol (9:1 v/v, 10 mL). The sample was loaded dropwise (gravity), once loaded the cartridge was washed with the solution of water-methanol (9:1 v/v, 10 mL) and eluted with 3 mL of methanol in a glass tube. After evaporation of the methanol, the residue was reconstituted in 0.5 mL methanol, filtered with NANOSEP MF centrifugal filter 0.2 μm (PALL) (6000 g, 3 min, 4°C), and transferred into a HPLC vial for analysis.

2.5.4. LC-MS/MS analysis

The samples were analyzed by LC-MS/MS using an Agilent 1100 model coupled to a triple quadrupole mass spectrometer (API 2000, SCIEX-Applied Biosystems) for quantification of AZAs. Chromatography was performed using a Hypersil BDS C8 column (50 x 2 mm, 3 μm , Thermo scientific) and an isocratic elution at 250 $\mu\text{L}\cdot\text{min}^{-1}$ at 20°C for 10 min. Mobile phase A was 100% water (25%) and mobile phase B (75%) consisted of acetonitrile–water (95:5) both containing 2 mM ammonium formate and 50 mM formic acid. The injection volume was 5 μL and the column and sample temperatures were 20 and 5°C, respectively.

The declustering potential was 140 V, the entrance potential 10 V, the cell exit potential 35 V, and the collision cell were 50 and 70 V for fragmentation 1 and 2 respectively. The electrospray ionisation interface (ESI) was operated using the following parameters: curtain gas: 19 psi; temperature: 350°C; gas 1: 30 psi; gas 2: 50 psi; CAD gas: 5 psi; ion spray voltage: 5800 V.

Azaspiracids were quantified against NRC certified AZA1 standards. The two most intense product ions were selected with the following transitions: AZA1 m/z 842.5→824.5 and 842.5→672.4, and AZA2 m/z 856.5→838.5 and 856.5→672.4.

2.6. Nutrient analysis in culture medium

For the analysis of dissolved nitrate and inorganic phosphate, the culture medium was filtered through a 0.22 μm syringe filtered (FP30/0.2 Whatman) in 50 mL pretreated plastic tubes (Sarstedt, pre-washed with 1 M HCl and then rinsed three times with Milli-Q water) and stored at -20°C until analysis (Aminot and Kerouel, 2007).

2.7. Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Depending on the data, statistical analyses consisted either in one-way analysis of variance (ANOVA), or in a Kruskal–Wallis test, followed, when necessary, by a Fisher's least significant difference procedure or a box-and-whisker plot. Differences were considered significant at $p < 0.05$. Before each ANOVA analysis or Kruskal–Wallis test, normality and equality of variance were tested to decide which test was going to be used.

Statistical analyses were carried out using Statgraphics Centurion XV.I (StatPoint Technologies, Inc.).

3. Results

3.1. Environmental factors

3.1.1. Temperature

The largest differences of *A. spinosum* growth of this study were observed when using different temperatures (Fig. 1a and Table 1). *A. spinosum* was able to grow in the whole range of temperatures tested (10 to 26°C) and always reached cell concentration above $50 \times 10^3 \text{ cell mL}^{-1}$, but growth clearly was temperature dependent. The best growth was found at 22° C (0.46 d^{-1}), whereas growth was much slower (0.12 d^{-1}) at 10°C.

AZA cell quota were found to be antagonist to growth, as at slow growth a high AZA cell quota was found (10°C, 220 fg cell^{-1}) whereas fast growing algae at higher temperatures had a lower AZA cell quotas (e.g. 22°C, 8 fg cell^{-1}).

3.1.2. Salinity

A. spinosum inoculated at a salinity of 10 were not measurable with the particle counter on the following day, and cells inoculated at a salinity of 20 were only measurable until the second day of the experiment but no further; however, growth of *A. spinosum* was observed between 30 and 40 of salinity (Fig. 1b and Table 1), with growth rates assessed between 0.22 and 0.34 d^{-1} . Fastest growth and highest final cell yield was obtained at a salinity of 35 whereas the highest toxin cell quota was obtained at the two extreme salinities, 30 and 40.

3.1.3. Photon flux density

When grown at different PFDs ranging from 50 to $250 \mu\text{mol m}^{-2} \text{ s}^{-1}$, growth of *A. spinosum* was comparable for irradiance down to $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and slightly but significantly reduced at $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Concerning AZA cell quota and the maximum cell concentration, no differences were found between the different PFD tested (Fig. 1c and Table 1).

In addition, *A. spinosum* was grown in continuous culture at different light conditions (Fig. 3). In stirred photobioreactors, a slightly higher steady state cell concentration was observed at 200 and $400 \mu\text{mol m}^{-2} \text{ s}^{-1}$ compared to growth at $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$. However, the main effect found was on the AZA cell quota, where an increase by a factor of 3 was observed between the lowest PFD and the highest one (Table 2). At the same time an increase in ESD was observed as PFD increased.

3.1.4. Aeration

In 10 L batch culture flasks with aeration, *A. spinosum* had a growth rate of 0.41 d^{-1} , reached maximum cell concentration of $315 \times 10^3 \text{ cell mL}^{-1}$ and aeration prolonged the length of the stationary phase (Table 1 and Fig 1d). Without aeration, growth was 0.66 d^{-1} and the final cell yield was $90 \times 10^3 \text{ cell mL}^{-1}$ (Table 1 and and Fig 1d). Maximum AZA cell quota was substantially higher with aeration than without aeration, i.e. 122 ± 2 and $15 \pm 2 \text{ fg cell}^{-1}$) but cell quota were similar when compared for the same sampling day (Fig1d, Fig 2d, Table 1).

The data set of the batch culture in 10 L aerated flasks was also used to apply a Gompertz-model to assess *A. spinosum* maximum growth rate, maximum cell concentration, and

latency time, and to follow the variation of the mean ESD, the AZA cell quota and the nitrate and phosphate consumption during a batch culture (Fig. 2).

The ESD decreased during the first days of the culture until growth rate begun to decrease (day 4) and then increased until the end of the stationary phase and finally decreased as senescence phase begun (Fig. 2a). The maximum growth rate found by the model under this condition was equal to 0.57 day^{-1} , with a maximum cell concentration of $302 \times 10^3 \text{ cell mL}^{-1}$, and a negligible latency time (Fig. 2b). Using the K modified medium, phosphate with concentration close to $0 \mu\text{M}$ was found to be depleted after 14 days of culture (Fig. 2c). Interestingly, AZA cell quota increased from the beginning of the culture to the end of the stationary phase where it drastically decreased. This final drop of intracellular toxin was linked to an increase of extracellular toxin dissolved in the culture medium. Effectively, until day 14 the percentage of dissolved AZAs was below 1% of total AZAs, whereas for the last day of the experiment this percentage increased up to 35% (data not shown). However, we also noticed that the total amount of AZAs (intra + extracellular) decreased from day 14 to 17 (-31%), indicating possible transformation/degradation of AZA1 and -2 in seawater. The intracellular AZA2/AZA1 ratio was almost identical during the whole growth curve (0.30 ± 0.02).

3.2. Nutritional factors

3.2.1. Medium

A. spinosum grew with all four different growth media tested with growth rates ranging between 0.39 and 0.45 day^{-1} (Fig. 4, Table 2). Even though growth rates were similar to each other, statistical differences ($P = 0.02$) were found, and the K modified medium (Kmod) yielded the highest growth rate. No statistically significant differences were found concerning the *A. spinosum* maximum concentration. Some small but statistically significant differences were noticed in AZA cell quota ($K_{\text{mod}2} > K_{\text{mod}} = L1 = L1 + \text{SE}$).

Kmod was tested at different concentrations in continuous culture. Cell concentration at steady state was positively related to the medium concentration (Table 2), and AZA cell quota were negatively correlated to it. Differences were also found in ESD as at a lower medium concentration the ESD was larger than at high medium concentration.

3.2.2. Nitrogen and phosphate sources

When simultaneously providing different nitrogen sources (nitrate or nitrate with ammonium and/or urea), *A. spinosum* showed a reduction of growth with the addition of ammonium (Fig. 5), whereas addition of urea had no effect on growth. For all combinations, comparable maximum cell concentrations ($100 \times 10^3 \text{ cells mL}^{-1}$) and AZA cell quota ($10\text{-}20 \text{ fg cell}^{-1}$) were observed (Table 4).

The production of *A. spinosum* on culture media containing nitrate or urea was also studied in photobioreactors (Table 2), and results found between the two conditions were close; still, a slight difference was found in favor of nitrate in cell concentration as well as in mean ESD and cellular volume, but no differences were found in AZA cell quota (41 vs 42 fg cell^{-1}).

Two sources of phosphate were also tested in batch culture and results were found to be similar in terms of growth, maximum cell concentration and AZA cell quota using either NaH_2PO_4 or β -glycerophosphate (Table 3).

A. spinosum grown in photobioreactors at different NO_3/PO_4 ratios showed that steady state cell concentration increased with the absolute amount of nitrate. Likewise, AZA cell quota was maximized at the highest N/P ratio tested (Table 2).

4. Discussion

There is a general need for purified AZA toxins for either LC-MS/MS calibration in the frame of monitoring programs and for extended and detailed toxicological studies, and the availability of *A. spinosum* opened the possibility to use cultures as a sustained source of toxins. Understanding of environmental and nutritional factors affecting *A. spinosum* growth and toxin production/accumulation constitutes a pre-requisite to allow and improve large-scale high biomass culturing and AZA production and thus was the main motivation for this study. It is important to note that the experiments were therefore started at relatively high cell densities for dinoflagellate culture. Batch cultures thus only initially (if ever) showed exponential growth with a density independent maximum rate but were generally characterized by a more linear increase in density typical for the transition from the initial maximum exponential increase to growth cessation at the stationary phase. Nevertheless, growth of different treatments, albeit not representing the maximum growth rate of that particular environmental condition, allowed a direct comparison and yielded some first insights into the ecological characteristics of *A. spinosum*. We found AZA-toxins with an unchanged toxin profile in significant amounts for all environmental conditions tested, indicating that toxin production of *A. spinosum* is constitutive. Nevertheless, quantitative differences in the toxin cell quota were found to vary more than 20 fold. Generally, when comparing growth and AZA cell quota at different environmental conditions, lower growth were consistently coupled with higher toxin cells quotas underlining the notion that toxin production is not strictly coupled to growth. The extended data set of aerated 10 L batch cultures allow for a detailed analysis of the dynamics of toxin cell quota during growth. As it has been determined for PSP producers (Boyer et al., 1987; Guillard, 1975; Kim et al., 1993), AZA toxin quota of *A. spinosum* significantly increased with decreasing growth rate during the transition from the initial maximum exponential increase to growth cessation and peaked in the late stationary phase, which is comparable to other dinoflagellate producers of polyether toxins (Bomber and Tindall, 1988; Guerrini et al., 2010; Pan et al., 1999). Such a higher toxin cell quota in stationary phase has often been related to phosphate depletion (McLachlan et al., 1994; Morlaix and Lassus, 1992; Vanucci et al., 2010). Given the high surplus of nitrate in K-medium and the very low phosphate concentrations at stationary phase (see Fig. 2c), P-limitation also was likely the cause of growth cessation in the aerated high biomass batch cultures. Likewise, phosphorous is assumed to be the growth limiting factors in chemostat-mode bioreactors (see below). In any case, the high dynamic variability of toxin cell quota over the growth phases shown above, hinder a direct and straightforward comparison of toxin cell quota of different treatments when cultures were just once sampled at slightly different growth phases. Nevertheless, as cultures for cell quota determination were always sampled at early stationary phase, an interpretation of the major differences in cell quota as a response to the respective environmental factor is certainly possible.

4.1. Environmental factors

A. spinosum was found to grow in a wide range of temperatures. The highest growth rate was observed at 22°C, and a drop of the temperature corresponded to a decrease in growth that concurred with a very pronounced increase in toxin content. This observation was also mentioned in the literature with other toxic dinoflagellates (Anderson et al., 1990; Guerrini et al., 2007; Navarro et al., 2006; Ogata et al., 1987). A temperature above the optimum growth temperature (e.g. 26°C in the present case) can also lead to an increase in toxin quota in

some species (*Protoceratium reticulatum* (Guerrini et al., 2007), *Prorocentrum hoffmannianum* (Morton et al., 1994) and *A. spinosum* in the present study). With a more than 20 fold difference, low temperature caused the by far largest AZA cell quota of *A. spinosum*. As for any increase in cell quota, this observation can be due to either an increase in toxin production rate as a response to an (environmental) stress and/or due to a general decrease in growth rate causing toxin accumulation even with unchanged toxin production. To follow this up calculations were made to relate AZA cell quota to the time necessary for cell division and thus to the amount of light received and to cell volume (table 5). The lower growth and thus the time the cells were illuminated before cell division as well as the larger cell volume of low temperature grown cells may partly explain the differences in AZA cell quota; however, a factor of five is still unexplained when normalizing to both the amount of light received and the cell volume obtained. Thus, lower temperature appears to also increase the absolute rate of biosynthesis of azaspiracids. This large effect of low temperature elevating cell quota to more than 200 fg cell⁻¹ in the stationary phase when grown at 10° C may help to explain the rather unexpected but repeated AZA problems in Irish mussels during winter month. In terms of mass culturing for toxin harvesting, application of a low growth temperature seems promising to maximize total toxin yield, even when considering the long time needed to reach high biomass and the lower final cell yield found in batch culture. On the other hand, however, it will also impose a technical challenge to cool large scale photobioreactors so the practical implementation for routine large scale culturing is quite unlikely.

A. spinosum cells acclimatized to a salinity of 35 were able to grow well in a range of proximate salinities (30 to 40), and were able to survive for a few days at a salinity of 20. Yet better survival could possibly be obtained with a progressive acclimation of the organism to different salinities. Even though this range does not classify *A. spinosum* as a euryhaline species (at a salinity of 10 no survival was observed), the tolerance for short and sudden periods to decreased salinity may at least partially explain why *A. spinosum* fares relatively well in Irish bays or Norwegian fjords, where rainfall may temporarily contribute to large freshwater inputs (and hence drop in salinity). While cells grown at a salinity of 30 and 40 were found to contain slightly more toxins (Table 1), this difference may be confounded by the fact that toxin analysis was carried out on the same day for all cultures, yet some cultures were at that stage in senescence phase whereas others were still in stationary phase (see also figure 2 for decreased toxin content at the beginning of the senescence phase). Furthermore, the significant difference in AZA cell quota found at a salinity of 40 can be partially explained by a slightly larger mean ESD and thus a larger cellular volume found at a salinity of 40 than at 35 (Table 1).

Growth response of *A. spinosum* at different light levels gave no signs of photoinhibition of growth at higher light levels (tested up to 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Growth was almost saturated down to the lowest light level tested (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$), where only a slight decrease in growth was noticed. However, the characteristics of a growth-vs-light-curve at low light (e.g. to determine the light compensation point or the light leading to half μ_{max}) has yet to be determined so a general classification of *A. spinosum* as a “low light” or “high light” adapted species is currently not possible. It is often assumed that photosynthesis in dinoflagellates plays an important role in the synthesis of toxins (Ogata et al., 1987). However, for *A. spinosum* different PFD in batch culture was apparently not affecting AZA cell quota, suggesting that when growth is light saturated, excess energy at higher light is not used to increase AZA production. In contrast, when grown in chemostat at potentially nutrient limited conditions (low dilution rate), different PDFs clearly affected AZA cell quota. An increase by a factor of 3 was observed between the lowest photon flux density and the highest one, indicating that under nutrient limiting growth, surplus light energy lead to an increased AZA accumulation, a fact that can be used to further improve AZA mass production (Jauffrais et al., 2012b).

Addition of turbulences by either aeration, agitation or shaking of a dinoflagellate culture is known to either increase cell concentration and toxin production (Morton and Bomber, 1994) or to inhibit growth (Berdalet, 1992; Pollinger and Zemel, 1981). In the case of *Alexandrium minutum*, turbulence caused a decrease in growth rate, biomass, and lower concentration of paralytic shellfish toxins and production of ecdysed cysts (Bolli et al., 2007). However, all authors do not agree with these effects of turbulence and some believe that some of these experiments on turbulence were conducted above a growth inhibition threshold (Burkholder et al., 2006). Indeed, turbulence seemed to be dependent upon the species and the applied intensity. Using turbulence intensities comparable to natural conditions, a wide range of bloom forming dinoflagellate species, their growth rate and cell concentration were either affected (positively or negatively) or unaffected (Sullivan and Swift, 2003). *A. spinosum* growth was only slightly affected by a significant, albeit unquantified, level of turbulence as introduced here by aeration, which was somewhat unexpected given the cursory notion of *A. spinosum* as a small and fragile thin thecate dinoflagellate. However, turbulence resistance is an important prerequisite for mass culturing in photobioreactors, where stirring and aeration (CO₂ addition) are used to regulate pH and to keep cells in suspension. In batch culture, aeration drastically increased final cell yield (biomass) and prolonged the length of the stationary phase. We could not detect a direct short-term effect of turbulence on AZA cell quota as values measured on day 4 were similar (16 fg cell unaerated vs 20 fg cell aerated). The enormous increase of late stationary phase AZA cell quota and final biomass in aerated cultures compared to non-aerated batches might be due to lowering or preventing carbon limitation (enhanced gas exchange) or by avoiding direct negative effects of high pH potentially prevailing in non-aerated cultures (Hansen et al., 2007; Rost et al., 2006) or to nutrient depletion due to the prolonged growth and stationary phase.

4.2. Nutritional factors

Although the nutritional requirements of *A. spinosum* in terms of trace elements/vitamins etc. have not yet been defined precisely, culture work performed so far showed that the species is easy to grow on a number of different standard culture media. These include K-medium of various strength (but with omission of ammonium, (Tillmann et al., 2009) and this study), F/2 medium (Salas et al., 2011), or L1 medium with or without addition of soil extract (this study). The omission of tris buffer (Kmod), as tested here for technical reasons (in photobioreactors the pH is already controlled by addition of CO₂) was no problem and even slightly enhanced growth for unknown reasons, however, pH was not continuously analysed in these cultures and direct effects of pH on *A. spinosum* still need to be addressed. In conclusion, the use of different media, including the addition of soil extract, did not largely affect growth and toxin cell quota and did not indicate any unusual nutrient requirement. In terms of major nutrient, *A. spinosum* was able to grow and to produce toxins with different sources of nitrogen (nitrate, urea and ammonium) and phosphate (NaH₂PO₄ and Na-βglycerophosphate). The potential to use urea is important as during the last four decades the amount of urea in fertilizers has increased drastically in many agricultural regions of the world and has been linked to increasing paralytic shellfish poisoning events (Glibert et al., 2006). Addition of ammonium, however, a nutrient known to be rapidly toxic to some dinoflagellates (Chang and McClean, 1997; Leong and Taguchi, 2004), reduced growth and thus conformed the empirical notion that omitting ammonium from the standard K-recipe improved growth for *A. spinosum* (Tillmann et al., 2009).

Without doubt, growth and toxin production of dinoflagellates are influenced by the supply with nitrogen and phosphate (Anderson et al., 1990; Flynn et al., 1994; John and Flynn, 2000). Increasing the amount of nutrient in the inflowing medium in chemostat bioreactors increased the steady state cell concentration (e.g. a 1.8 fold increase when using double strength K-medium compared to normal K) but also caused an almost two-fold decreased

AZA cell quota (see Table 2). Thus, beside nutrient limitation, another factor related to the higher steady state biomass must have simultaneously affected the AZA cell quota. Although negative effects of autoinhibitory compounds cannot be ruled out, self shading is probably the most important aspect here as decreasing light intensity at nutrient limited chemostat growth clearly decreased toxin cell quota (Table 2). Such a synergistic combinations of effects (phosphate limited growth at high light intensities) may further improve the large-scale culturing approach developed by Jauffrais et al. (2012b), where an increase in PFD applied during the “maturation” period might further enhance AZA production once high cell concentrations are reached.

Anthropogenic addition of nitrogen or phosphate into the coastal water unbalanced the Redfield N/P ratio (16/1), commonly found in the ocean, and might modify growth and toxin production of toxic dinoflagellates (Glibert et al., 2006; Heisler et al., 2008). In polyether toxin producers, P limiting conditions seem to enhance toxin quota as already mentioned and N limiting conditions may also maximize toxin quota in some organisms (Vannucci et al., 2010), but in both cases, N or P limitation reduced growth and even toxin quota for *Ostreopsis cf. ovata* (Vanucci et al., 2012). Our preliminary experiments using three different N/P ratios in chemostat continuous cultures with higher AZA cell quota at the highest N/P ratio (Table 3) indicate that phosphorous limitation might play a role in promoting high toxin cell quotas, but it still need to be determined if this is caused by accumulation (reduced growth) or/and by an increase in toxin production rate. In the future, low biomass continuous culture experiment using different dilution rates (different degrees of nutrient limitations) are needed to more thoroughly clarify the effects of nutrient limitation on *A. spinosum* toxin production/accumulation.

5. Conclusions

A. spinosum was found to grow with all media tested and in a wide range of conditions, from 10 to 26°C, 30 to 40 psu, 50 to 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and with or without aeration; with best growth and/or highest final cell yield found at 35 psu, from 18 to 22°C and with aeration. AZA-toxins with an unchanged toxin profile were found in significant amounts for all environmental conditions tested and thus indicate that toxin production of *A. spinosum* is constitutive. Nevertheless, quantitative differences in the toxin cell quota may be large. AZA cell quota were most significantly affected by low temperature: the AZA cell quota was more than 20 times higher at 10°C (220 fg cell⁻¹) than at temperatures comprised between 18 and 26°C. Likewise, but to a lesser degree, AZA cell quota were enhanced in aerated stationary phase cultures and at high irradiance when cells were grown in chemostats. Decreasing the nutrient concentration in the ingoing medium (0.5Kmod) in chemostats improved AZA cell quota while higher concentration (2Kmod) improved maximal cell concentration.

These experiments on the effects of environmental and nutritional factors on *A. spinosum* improved our knowledge on its culture and allowed its growth in pilot scale photobioreactors for AZA1 and -2 production (Jauffrais et al., 2012b). Furthermore, the now well defined ability of this species to support conditions applied in continuous cultures will enable more detailed future studies on the effect of nutrient limitations on growth, AZA toxin production and *A. spinosum* biochemical composition. Finally, the large differences in toxin cell quota observed under different environmental and nutritional conditions open possible routes of comparative gene expression studies for the elucidation of the genes involved in toxinogenesis.

Acknowledgement

This project, ASTOX2 (Grant-Aid Agreement No. PBA/AF/08/001), is carried out under the Sea Change strategy with the support of the Marine Institute and the Marine Research Sub-programme of the National Development Plan 2007–2013, co-financed under the European Regional Development Fund. Further funding was obtained through Ifremer from the French Ministry of Education, Research and Technology through Programme 187 of the National Finance Law.

The authors would also like to thank all the members of the laboratory EMP/PHYC at the Atlantic Centre of Ifremer for their help and technical advice during this study as well as Yoann Le Merrer and Isabelle Truquet from the LER MPL at the Atlantic Centre of Ifremer for the nutrient analysis in culture media.

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Tables

Table 1. **Batch culture:** *A. spinosum* specific growth rate, μ (d^{-1}), maximum cell concentration, C_{max} (10^3 cell mL^{-1}), AZA cell quota (fg cell $^{-1}$) and ESD (mean estimated spherical diameter (μm)) in batch culture at different salinities, photon flux densities (PFD, $\mu mol\ m^{-2}\ s^{-1}$), temperatures ($^{\circ}C$) and with the presence or absence of aeration (values with different letters are statistically different at $P < 0.05$). Standard batch culture conditions were applied for all conditions unless specified in a given treatment ($n=3$).

Environmental factors		μ^* (d^{-1})	C_{max} (10^3 cell mL^{-1})	AZAs (fg cell $^{-1}$)	ESD § (μm)
Temperature ($^{\circ}C$)	10	0.12 ± 0.01^e	53 ± 5^d	220 ± 38^a	10.8 ± 0.5^a
	14	0.25 ± 0.01^d	80 ± 2^c	80 ± 10^b	10.2 ± 0.4^b
	18	0.33 ± 0.03^c	86 ± 7^{bc}	14 ± 2^c	9.4 ± 0.2^c
	22	0.46 ± 0.03^a	90 ± 5^a	8 ± 1^d	9.1 ± 0.3^c
	26	0.40 ± 0.02^b	58 ± 7^d	14 ± 1^c	9.2 ± 0.2^c
Salinity	30	0.26 ± 0.01^b	48 ± 1^c	31 ± 2^b	8.9 ± 0.2^b
	32	0.33 ± 0.00^a	65 ± 2^b	29 ± 3^b	9.2 ± 0.3^{ab}
	35	0.34 ± 0.00^a	77 ± 3^a	23 ± 1^c	9.3 ± 0.4^{ab}
	40	0.22 ± 0.01^c	32 ± 2^d	37 ± 3^a	9.8 ± 0.6^a
PFD ($\mu mol\ m^{-2}\ s^{-1}$)	50	0.31 ± 0.01^c	95 ± 7	16 ± 2	9.5 ± 0.4
	100	0.42 ± 0.01^b	94 ± 2	22 ± 1	9.9 ± 0.3
	150	0.41 ± 0.01^b	95 ± 2	16 ± 4	9.7 ± 0.4
	200	0.41 ± 0.01^b	94 ± 3	16 ± 1	9.8 ± 0.4
	250	0.41 ± 0.02^b	98 ± 1	21 ± 1	9.7 ± 0.3
Aeration	Yes	0.41 ± 0.02^b	316 ± 15^a	122 ± 2^a	9.5 ± 0.2
	No	0.66 ± 0.02^a	90 ± 2^b	15 ± 2^b	9.3 ± 0.4

* calculated between the initial cell density the day represented by a star (*) in the different graphics

§ mean ESD over the experiment

Table 2. **Continuous culture:** *A. spinosum* concentration (10^3 cell mL⁻¹), mean estimated spherical diameter (ESD, μm), cellular volume (μm^3 mL⁻¹) and AZA cell quota (AZA1+2 fg cell⁻¹) in continuous culture at steady state using different photon flux densities (PFD, $\mu\text{mol m}^{-2} \text{s}^{-1}$), K medium concentration, nitrogen sources (nitrate and urea) and N/P ratios (values with different letters are statistically different at $P < 0.05$). Standard continuous culture conditions were applied for all conditions unless specified in a given treatment (nine sampling occasions for each of the two bioreactors).

Factors		Concentration (10^3 cell mL ⁻¹)	ESD (μm)	Biovolume ($10^6 \mu\text{m}^3$ mL ⁻¹)	AZAs (fg cell ⁻¹)
PFD ($\mu\text{mol m}^{-2} \text{s}^{-1}$) $\mu = 0.2 \text{ day}^{-1}$	100	185 ± 7 ^b	9.5 ± 0.1 ^c	85 ± 3 ^c	21 ± 2 ^b
	200	194 ± 5 ^a	9.9 ± 0.1 ^b	101 ± 3 ^b	58 ± 9 ^a
	400	195 ± 7 ^a	10.1 ± 0.1 ^a	108 ± 4 ^a	69 ± 11 ^a
Kmod $\mu = 0.2 \text{ day}^{-1}$	0.5	122 ± 7 ^c	10.1 ± 0.1 ^a	66 ± 3 ^c	58 ± 5 ^a
	1	196 ± 7 ^b	9.9 ± 0.1 ^a	104 ± 3 ^b	47 ± 6 ^b
	2	346 ± 29 ^a	9.7 ± 0.2 ^b	170 ± 17 ^a	25 ± 7 ^c
Nitrogen source $\mu = 0.3 \text{ day}^{-1}$	Nitrate (882 μM)	207 ± 13 ^a	10.2 ± 0.2 ^a	119 ± 8 ^a	41 ± 8
	Urea (100 μM)	182 ± 10 ^b	9.9 ± 0.1 ^b	95 ± 7 ^b	42 ± 6
NO ₃ ⁻ /PO ₄ ³⁻ ratio (μM) $\mu = 0.3 \text{ day}^{-1}$	40/5	59 ± 6 ^c	9.6 ± 0.3 ^b	29 ± 4 ^c	18 ± 3 ^b
	160/10	187 ± 11 ^b	9.7 ± 0.1 ^{ab}	92 ± 4 ^b	14 ± 1 ^b
	882/10	216 ± 10 ^a	10.0 ± 0.3 ^a	117 ± 6 ^a	36 ± 2 ^a

Table 3. *A. spinosum* specific growth rate, μ (d⁻¹), maximum cell concentration, Cmax (10^3 cell mL⁻¹), AZA cell quota (AZA1+2fg cell⁻¹) and ESD (mean estimated spherical diameter (μm)) in batch culture using different culture medium and phosphate sources (values with different letters are statistically different at $P < 0.05$). Standard batch culture conditions were applied for all conditions unless specified in a given treatment (n=3).

Nutritional factors		μ * (d ⁻¹)	Cmax (10^3 cell mL ⁻¹)	AZAs (fg cell ⁻¹)	ESD [§] (μm)
Culture medium	Kmod	0.45 ± 0.004 ^a	98 ± 3	20 ± 2 ^b	9.5 ± 0.1 ^a
	Kmod2	0.39 ± 0.006 ^c	88 ± 1	27 ± 1 ^a	9.3 ± 0.1 ^a
	L1	0.40 ± 0.004 ^c	101 ± 1	22 ± 1 ^b	8.9 ± 0.2 ^b
	L1+SE	0.42 ± 0.001 ^b	100 ± 3	14 ± 4 ^b	8.9 ± 0.2 ^b
Phosphate	NaH ₂ PO ₄	0.40 ± 0.01	78 ± 1	17 ± 1 ^b	10.1 ± 0.5
	β glycerophosphate	0.42 ± 0.02	81 ± 1	24 ± 1 ^a	9.9 ± 0.2

* calculated between the initial cell density the day represented by a star (*) in the graphic

[§] mean ESD over the experiment

Table 4. *A. spinosum* specific growth rate, μ (d^{-1}), maximum cell concentration, C_{max} (10^3 cell mL^{-1}), AZA cell quota (AZA1+2 fg cell $^{-1}$) in batch culture using different source of nitrogen (Nitrate, ammonium and urea at the indicated concentration in the table). Standard batch culture conditions were applied for all conditions unless specified in a given treatment (n=2).

NO ₃ ⁻ (μ M)	NH ₄ ²⁺ (μ M)	Urea (μ M)	μ * (d^{-1})	C_{max} (10^3 cell mL^{-1})	AZAs (fg cell $^{-1}$)	ESD [§] (μ m)
50	0	0	0.39 ± 0.01	95 ± 1	17.3 ± 0.1	9.6 ± 0.1
50	100	0	0.28 ± 0.01	101 ± 2	10.1 ± 0.1	10.2 ± 0.1
50	0	100	0.40 ± 0.01	95 ± 1	12.3 ± 0.8	9.6 ± 0.1
50	100	100	0.27 ± 0.01	109 ± 9	11.3 ± 0.8	10.3 ± 0.1

* calculated between the initial cell density the day represented by a star (*) in the graphic

§ mean ESD over the experiment

Table 5. *A. spinosum* AZA1+2 cell quota (fg cell $^{-1}$), estimated cell volume (μ m³), number of cell divisions, time for one cell division (h), time of light for one cell division (h) and AZA1+2 expressed in fg cell $^{-1}$ h $^{-1}$ of light and in fg μ m⁻³ h $^{-1}$ of light observed and estimated at 10, 14, 18, 22 and 26°C, and the calculated ratio between the values found at 10°C and 22°C.

	10°C	14°C	18°C	22°C	26°C	ratio 10°C/22°C
AZA1+2 (fg cell $^{-1}$)	220 ± 38	80 ± 10	14 ± 2	8 ± 1	14 ± 1	27.5
Estimated cell volume (μ m ³)*	650	561	439	392	410	1.7
Number of cell division [§]	3.1	3.1	3.6	3.9	3.5	0.8
Time for one cell division ^{§§} (h)	348	166	163	108	119	3.2
Time of light for 1 cell division (h)	232	111	108	72	80	3.2
AZA1+2 (fg cell $^{-1}$ h $^{-1}$ of light)	0.9	0.7	0.1	0.1	0.2	8.5
AZA1+2 (fg μ m ⁻³ h $^{-1}$ of light)	0.0015	0.0013	0.0003	0.0003	0.0004	5.1

* Estimated cell volume = $4/3 \times \pi \times (ESD/2)^3$

§ Number of cell division from the beginning of the experiment until AZA analysis

§§ Average time for one cell division from the beginning of the experiment until AZA analysis

Figures

Figure 1. Growth kinetics for the different condition of (A) temperature (10 to 26°C), (B) salinity (30 to 40), (C) photon flux density (50 to 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and (D) aeration (with and without), arrows indicate at which day samples were taken for AZA analysis and * the day used to calculate growth rates.

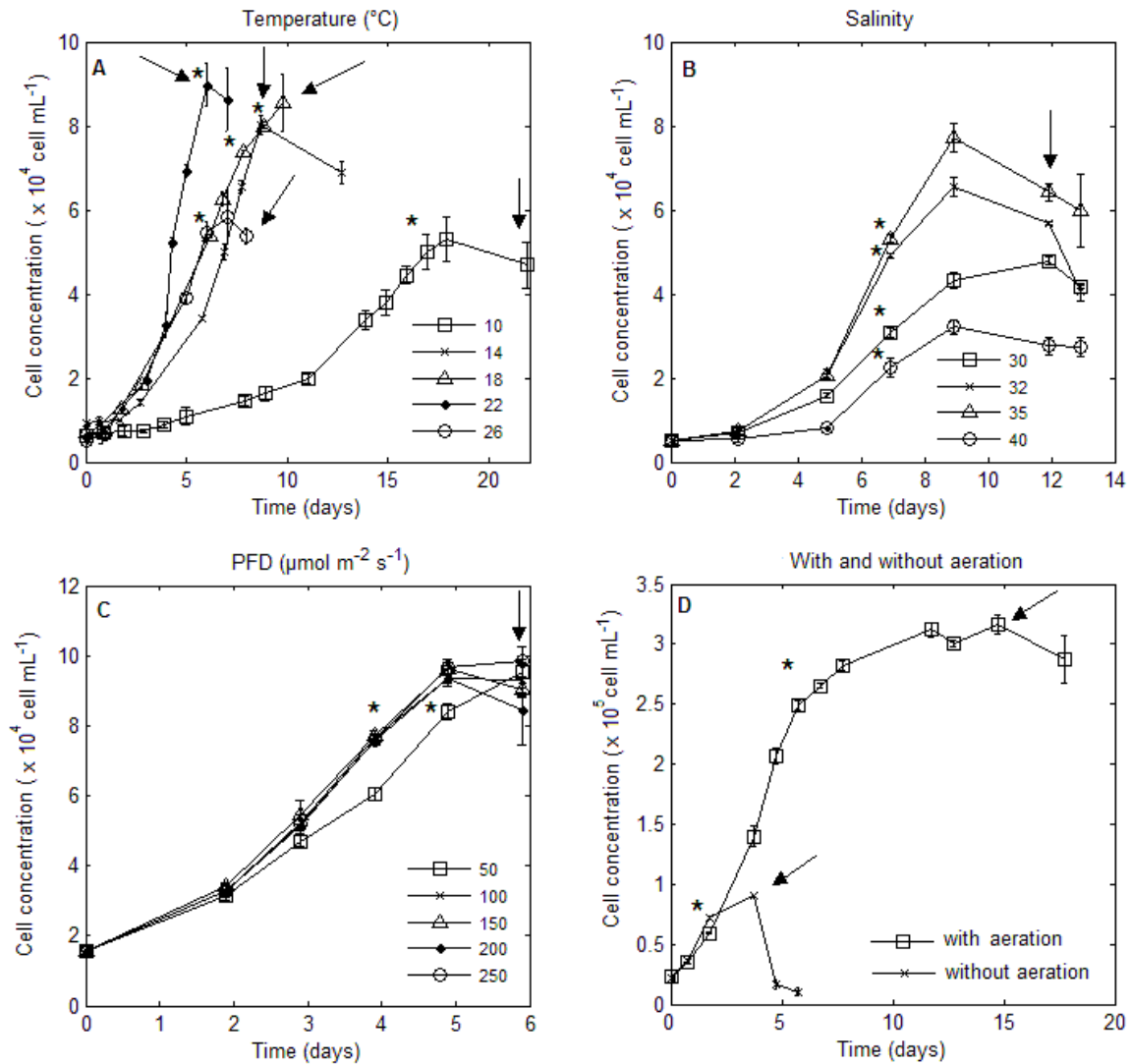


Figure 2. (A) *A. spinosum* cell concentration and mean spherical diameter (ESD) as a function of time in aerated 10 L culture flasks, (B) Gompertz model fitted to the cell concentration with its 95% confident bounds for the maximum cell concentration (C_{max}), growth rate (μ_{max}), latency time and its adjusted R^2 , (C) NO_3^- and PO_4^{3-} consumption by *A. spinosum* as a function of time and (D) AZA1+2 cell quota as a function of time (error bars = SD, $n=3$).

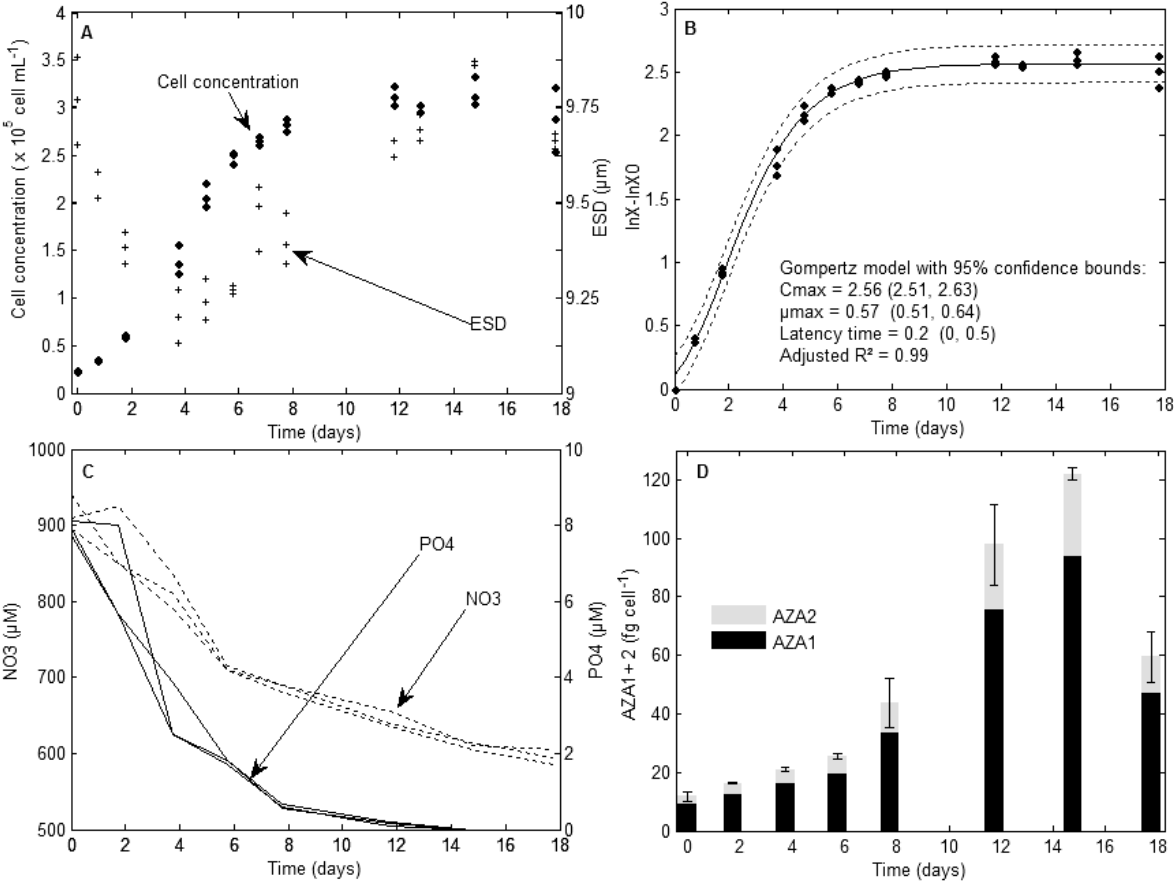


Figure 3. *A. spinosun* concentration (cell mL^{-1}) and cellular volume ($\mu\text{m}^3 \text{mL}^{-1}$) kinetics in 100 L photobioreactors at a dilution rate of 0.2 day^{-1} and at three different irradiance conditions 100, 200 and $400 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The steady states are represented by the red lines and the arrows represent the day where the irradiance was changed.

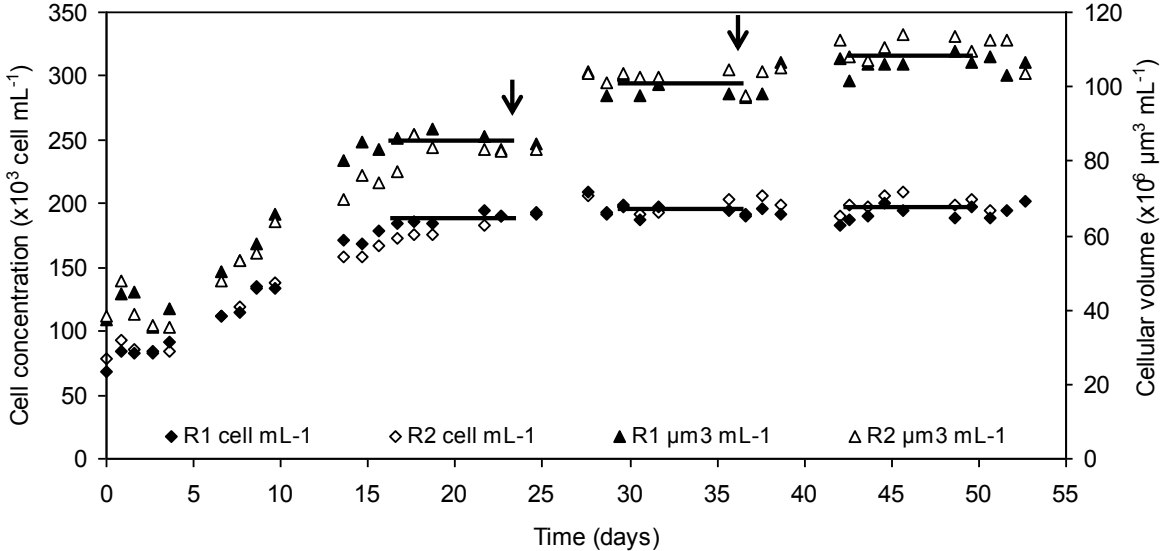


Figure 4. Growth kinetics for the different condition culture medium tested (Kmod1, Kmod2, L1, L1 + SE), arrows indicate at which day samples were taken for AZA analysis and * the day used to calculate growth rates.

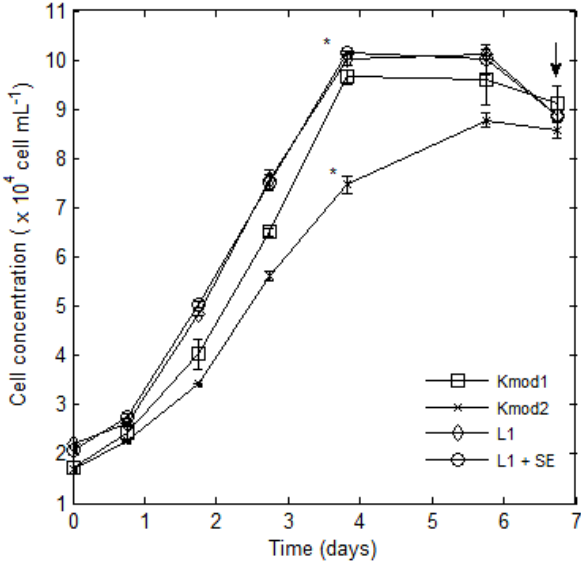


Figure 5. Effect of the addition of ammonium (NH_4^{2-}) and/or urea to a medium with nitrate on growth and cell concentration (nitrate (NO_3^-): $50 \mu\text{M}$, ammonium and urea: $100 \mu\text{M}$), arrows indicate at which day samples were taken for AZA analysis and * the day used to calculate growth rates

