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## **Effect of *Azadinium spinosum* on the feeding behaviour and azaspiracid accumulation of *Mytilus edulis***

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### **Abstract :**

*Azadinium spinosum*, a small toxic dinoflagellate, was recently isolated and identified as a primary producer of azaspiracid toxins (AZAs). Previous experiments related to AZA accumulation in blue mussels upon direct feeding with *A. spinosum* revealed increased mussel mortality and had negative effects on the thickness of the digestive gland tubules. Therefore we conducted follow up experiments in order to study effects of *A. spinosum* on mussel feeding behaviour. Individual assessment of mussel feeding time activity (FTA), clearance rate (CR), filtration rate (TFR), absorption rate (AR), faeces and pseudofaeces production were carried out on mussel fed either toxic (*A. spinosum*) or non-toxic (*Isochrysis* aff. *galbana* (T-Iso)) diets. Furthermore, AZA accumulation and biotransformation in mussels were followed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). *Azadinium spinosum* had a significant effect on mussel feeding behaviour compared to T-Iso: CR was lower by a factor of 6, FTA by a factor of 5, TFR by a factor of 3 and AR even decreased to negative values for the last day of exposure. Even so, a rapid AZA accumulation was observed during the first hours of the trial; less than 6 h of feeding were required to reach AZA concentration in mussel above regulatory level. In consistence with physiological observations, AZA concentration of about 200 µg kg<sup>-1</sup> did not increase further until the end of the study. AZA bioconversion was also found to be a fast process: after 3 h of exposure AZA17, -19 and AZA7-10 were already found, with a proportion of AZA17 equal to AZA2. These results show a negative effect of *A. spinosum* on blue mussel feeding activity and indicate a possible regulation of AZA uptake by decreasing filtration and increasing pseudofaeces production.

**Keywords :** Bivalve molluscs ; Mussel ; Ecophysiology ; AZA biotransformation ; AZA accumulation ; trophic transfer ; dinoflagellate ; azaspiracid

## 1. Introduction

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*Azadinium spinosum* is a small toxic dinoflagellate, which was recently isolated in the North Sea and characterised as AZA1 and -2 producer (Tillmann et al., 2009). Since this first identification, species of the genus *Azadinium* were encountered in Europe (Salas et al., 2011; Tillmann et al., 2011; Tillmann et al., 2010; Tillmann et al., 2009), Asia (Potvin et al., 2012), and also reported from Central and South America (Akselman and Negri, 2012; Hernandez-Becerril et al., 2010). However, among the currently described species, only *A. spinosum* has been identified as AZA1 and -2 primary producer (Salas et al., 2011; Tillmann et al., 2009), whereas *A. obesum* and *A. poporum* have not been shown to produce any known AZAs (Potvin et al., 2012; Tillmann et al., 2011; Tillmann et al., 2010), even though *A. poporum* was recently reported to produce a new type of AZA characterised by a m/z 342 fragment (Krock et al., 2012). In direct feeding trials, *A. spinosum* was recently found to directly contaminate mussels, with a fast AZA accumulation and biotransformation after one day of exposure (Salas et al., 2011). Thus, *A. spinosum* is now considered as an organism responsible for azaspiracid shellfish poisoning (AZP), a recognised widespread phenomenon (Twiner et al., 2008).

AZA1 was isolated from Irish contaminated mussels (Satake et al., 1998) after human intoxications in The Netherlands in 1995 (McMahon and Silke, 1996). Since then, this toxin was considered as relevant for human health and a long list of analogues were subsequently identified. AZA2-5 were purified and subsequently structurally characterised using nuclear magnetic resonance (NMR) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Ofuji et al., 2001; Ofuji et al., 1999), AZA6-12 (Diaz Sierra et al., 2003; James et al., 2003), AZA13-32 (McCarron et al., 2009; Rehmann et al., 2008) and AZA1 and -2 methyl esters (Jauffrais et al., 2012a) were identified in mussels, in *A. spinosum*, or during stability studies on AZA standards or extracts using LC-MS/MS.

Suspension-feeding bivalve molluscs are known to be a major vector for the transfer of amnesic, paralytic and diarrhetic shellfish toxins (respectively ASTs, PSTs, DSTs) to human (Bricelj and Shumway, 1998). Symptomatically, azaspiracid shellfish toxins (AZTs) belong to the last group, as it presents similar symptoms: nausea, stomach cramps, vomiting and diarrhoea (Satake et al., 1998). As the molecular mechanism of action of AZTs is different to that of the toxins belonging to the okadaic acid (OA) group, poisoning with AZTs has also been separately referred to as AZP (Twiner et al., 2008). In Ireland, concentrations of AZAs in bivalve molluscs showed records above the regulatory limit (160 µg kg<sup>-1</sup> AZA1 equiv.) causing large closure periods of shellfish production area and important economic losses to shellfish growers; with maximum concentrations found for blue mussels and pacific oysters of 8970 and 310 µg kg<sup>-1</sup> AZA1 equiv. (Salas et al., 2011).

Phycotoxins may also directly affect bivalve molluscs but mussels are considered to be least sensitive and consequently can accumulate larger amount of toxins than most other bivalve molluscs (Bricelj and Shumway, 1998; Mafra et al., 2010). As other suspension feeding bivalve molluscs, *M. edulis* feeds on seston of various quality and quantity and is able to adapt their filtration and rejection rate (Bayne et al., 1993). Furthermore, it has been shown that the American oyster (*Crassostrea virginica*), when exposed to a mono-specific diet of *Pseudo-nitzschia*, can reduce domoic acid uptake by lowering clearance rate and by rejecting toxic cells in the pseudofaeces (Mafra et al., 2009).

In a previous study (Jauffrais et al., 2012c) using *A. spinosum* to feed mussels, a fast AZA accumulation was observed during the first day of contamination. Within few hours following exposure, AZA concentration in mussels increased above the regulatory limit. This fast accumulation was followed by a slower increase or stabilisation of contamination for the next couple of days. AZA accumulation was also observed when mussels were simultaneously

fed *A. spinosum* and *Isochrysis* aff. *galbana*. Thus, addition of a non-toxic source of food to *A. spinosum* did not allow particle selection by mussels, and AZA accumulation was neither enhanced nor decreased. This observation showed that mussels could not initially regulate AZA accumulation by particle selection. Nevertheless, the short period of fast AZA accumulation was soon displaced by a period of a limited or stopped accumulation, which led us to assume that after a short exposure to *A. spinosum* mussels adjust their feeding activity which then limit AZA accumulation. However, physiological or ecological data on *A. spinosum* and its possible role and effect on trophic interaction are still lacking.

We hypothesize that *A. spinosum* could be considered as a micro-alga either toxic to or of poor nutritional value for mussels, and that, consequently, AZA uptake might be limited by a decrease in clearance rate and an increase in *A. spinosum* rejection *via* pseudofaeces. Hence the present study was designed to compare the feeding behaviour of mussels fed toxic and non-toxic diets based on *A. spinosum* and *I. aff. galbana*, respectively. To do this, we assessed physiological factors such as feeding time activity (FTA), clearance rate (CR) and absorption rate (AR), faeces and pseudofaeces production to confirm the negative effect of *A. spinosum* on mussels.

## 2. Materials and methods

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### 2.1. Collection and maintenance of bivalves

Blue mussels (*Mytilus edulis*) were collected from a mussel farm at La Plaine, French Atlantic coast in October 2011. Through routine official control, this area was known at that time to be free of known marine biotoxins. Mussels (5 kg) were transported alive to aquarium facilities, cleaned of epibionts and maintained before the experiment for five days in circulating filtered seawater (2 aquaria of 20 L capacity; salinity of 35 ppt) to ensure acclimatisation to laboratory conditions. During this time, temperature of the water was gradually decreased from  $16 \pm 1^\circ\text{C}$  to  $12.5 \pm 1^\circ\text{C}$  to be in same conditions than in a previous experiment (Jauffrais et al., 2012c) and to avoid possible bacterial contamination. Mussels were fed *ad libitum* with *Isochrysis* aff. *galbana* (T-Iso) and seawater was renewed daily. Note, one aquarium with environmental and feeding conditions identical to the acclimatisation period was kept during the experiment with 40 mussels, thus they could be taken from the aquarium to replace mussels sampled for toxin analysis during the second period of the experiment (see below).

### 2.2. Micro-algal culture and cell count

*A. spinosum* strain 3D9 used in this study had been isolated in the North Sea near the coast of Scotland by Tillmann et al. (2009). The algae were grown in pH-controlled stirred photobioreactors (100 L) operated in chemostat mode at a dilution rate of  $0.15 \text{ day}^{-1}$  (Jauffrais et al., 2012b). Culture medium was a K modified medium ((Keller et al., 1987), i.e. without  $\text{NH}_4\text{Cl}$  and Tris buffer but with  $\text{Na}_2\text{SeO}_3$  ( $10^{-8} \text{ M}$ )). The following conditions were controlled in the bioreactor: pH was maintained at 7.9 using  $\text{CO}_2$  addition, temperature at  $18^\circ\text{C}$ , photon flux density at  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , photoperiod was 16 h of light and 8 h of dark. The algae were then collected into an aerated harvesting tank (300 L), maintained at  $18^\circ\text{C}$  until the trial.

*Isochrysis* aff. *galbana* (CCAP 927/14, T-Iso) was used as control as it has a size similar to *A. spinosum* and is widely used as algal diets for farmed bivalve molluscs (Marchetti et al., 2012). T-Iso was grown in aerated batch culture, using 10 L flat bottomed flasks with the same environmental conditions as for *A. spinosum*, except using F/2 instead of K culture medium (Guillard, 1975; Guillard and Ryther, 1962).

Cell concentrations (cells mL<sup>-1</sup>) and cellular volume (μm<sup>3</sup> mL<sup>-1</sup>) were determined using a particle counter (Multisizer 3 Coulter counter, Beckman). Subsequently, cell concentration values were used to calibrate and adjust fluorometers during the experiment.

### 2.3. Experimental design

After acclimatisation to laboratory conditions, 22 mussels were randomly chosen for the experiment and placed individually in experimental boxes. At the same time, 30 additionally mussels were also randomly selected to measure condition index (wet tissue weight 2.65 ± 0.86 g per mussel; shell length 45.5 ± 3.8 mm; 27% of dry matter) and 5 others mussels were chosen for initial total flesh toxin analysis.

The experimental design used to follow individual physiological responses was a closed recirculating water system similar to the one already described in Bougrier et al. (2003) and in Lassus et al. (2007). Briefly, the system is build-up of 22 experimental boxes (0.5 L each), 20 containing one live mussel each and 2 containing one mussel shell each for control. Temperature and salinity were the same as during the acclimatisation period. Seawater was flown through the 22 experimental boxes at 80 mL min<sup>-1</sup> before returning to the feeding tank (40 L). Cell concentration level was continuously monitored in the feeding tank using a Turner 10AU 072 fluorometer coupled to a computer software (LabView). To maintain cell concentration constant, an electrovalve connected to the algal culture tank (25 L) was opened or closed according to cell fluorescence level. The fluorometer was calibrated using a particle counter (Multisizer 3 Coulter counter, Beckman). Cell concentration in the system was routinely checked during the experiment using the particle counter. Mussel clearance and filtration were assessed by comparing the algal concentration at the outlet of the control boxes to algal concentration at the outlet of the experimental boxes. The algal concentration in each box was assessed once every 11 min for 1 min using two other Turner fluorometers coupled to the computer software (Fig. 1).

In this experimental system, mussels were first exposed to a non-toxic diet of *T-iso* for 4 days (20000 cells mL<sup>-1</sup>, total particular matter (TPM) = 0.88 ± 0.07 mg L<sup>-1</sup>, particular organic matter (POM) = 0.61 ± 0.07 mg L<sup>-1</sup>, organic matter = 69%) for 4 days. After this period, the non-toxic diet was changed to a toxic diet of *A. spinosum* (5000 cells mL<sup>-1</sup>, TPM = 1.77 ± 0.14 mg L<sup>-1</sup>, POM = 1.61 ± 0.14 mg L<sup>-1</sup>, organic matter = 90%) for another 4 days. To assess TPM and POM, 1 L of algal diet were filtered (twice daily) on pre-weighted burned GF/C Whatman filter and rinsed. They were then dried (24 h, 60°C), weighed and burned (1 h, 450°C) and weighed again to determine by comparison their organic and inorganic matter content.

In order to generate a full time course of AZA accumulation for the four-day period and in sufficient replication and given the limited number of 20 experimental boxes, mussels were sampled after 3, 6, 12, 24, 48, and 72 h and replaced by new mussels originating from the aquaria used for acclimatisation to laboratory conditions. We thus obtained three replicates at 6 h and six replicates for the other time points (3, 12, 24, 48, 72 h) and 5 replicates at 96 h (see electronic supplementary material).

Small amounts of faeces and pseudofaeces were collected with a Pasteur pipette (note, that faeces were rinsed in filtered sea water) and placed on a glass micro-slide for brightfield microscopic observation. These observations allowed to determine the presence of cells in the faeces or pseudofaeces and consequently to assess whether cells were able to pass through the digestive systems of mussels during the experiment or were rejected beforehand.

## 2.4. Physiological measurements

During the two feeding periods of this study (T-Iso and *A. spinosum*), physiological responses were assessed daily and rates calculated according to the formulas of (Hawkins et al., 1998); Hawkins et al. (1996).

Clearance rate ( $L h^{-1} gDW^{-1}$ ) was defined as the volume of water cleared of particles per unit of time per dry weight of mussel (CR, *equation 1*). CR was assessed using comparison of the fluorescence of each algal species at the output of the experimental ( $C_1$ ) and control boxes ( $C_0$ ); flow rate (D) and mussel dry weight (DW) were subsequently incorporated.

$$CR (L h^{-1} gDW^{-1}) = ((C_0 - C_1) / C_0) \times D / DW \quad \text{equation 1}$$

TPM and POM ( $mg L^{-1}$ ) were assessed daily in the water according to Hawkins et al. (1996). These values were subsequently used to estimate the total (TFR) and organic (OFR) filtration rate ( $mg h^{-1} gDW^{-1}$ , *equation 2* and *3*).

$$TFR (mg h^{-1} gDW^{-1}) = CR \times TPM \quad \text{equation 2}$$

$$OFR (mg h^{-1} gDW^{-1}) = CR \times POM \quad \text{equation 3}$$

Pseudofaeces were observed during feeding periods and they were collected (see below) to estimate total (TPs) and organic (OPs) pseudofaeces production (subsequently expressed in  $mg h^{-1} gDW^{-1}$ ). TPs and OPs were then used to calculate total (TIR, *equation 4*) and organic (OIR, *equation 5*) ingestion rates, as well as the accumulation of OPs.

$$TIR (mg h^{-1} gDW^{-1}) = TFR - TPs \quad \text{equation 4}$$

$$OIR (mg h^{-1} gDW^{-1}) = OFR - OPs \quad \text{equation 5}$$

The last parameter assessed for both conditions was the absorption rate (AR) using the organic matter weighed in the faeces (OFs, subsequently expressed in  $mg h^{-1} gDW^{-1}$ ) as presented in *equation 6*. Faeces and pseudofaeces were carefully sampled twice daily using a pipette, filtered and rinsed on pre-weighted burned GF/C Whatman filter. They were then dried, weighed and burned and weighed again to determine their organic and inorganic matter content.

$$AR (mg h^{-1} gDW^{-1}) = OIR - OFs \quad \text{equation 6}$$

Feeding time activity (FTA) was also assessed to determine the time during which the mussels were active daily as well as during their time of exposure to *A. spinosum* (3, 6, 12, 24, 48, 72 and 96 h). Mussels were considered active when the difference between the outlet of the control box and the experimental box was above 5% according to Bougrier et al. (2003).

*Notice:* Boxes 1-6 were not used for to assess CR, TIR, AR, faeces and pseudofaeces measurement during exposure to *A. spinosum* due to the large number of sample and replacement to measure AZA accumulation (see electronic supplementary material).

## 2.5. Chemical 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. AZA1-3 standards for LC–MS/MS analysis were dilutions of certified AZA1-3 solutions obtained from the National Research Council Canada.

#### 2.5.2. *A. spinosum*

Triplicate samples of *A. spinosum* were taken after each addition of algae in the 25 L algal tanks at day 4 and 6. The extraction procedure previously optimised for AZA analysis in cultures of *A. spinosum* (Jauffrais et al., 2012a) was used in the present study. Briefly, *A. spinosum* culture (10 mL) was collected and centrifuged (2500 g, 20 min, 4°C). The supernatant was discarded and the pellet transferred to an Eppendorf tube (1.5 mL), bath sonicated, and extracted three successive times using acetone (0.5 mL). After centrifugation, the organic phases were gently evaporated, reconstituted in 1 mL methanol, filtered (0.2 µm) and transferred into a HPLC vial for analysis.

#### 2.5.3. Mussel

Blue mussels were collected at the different time points of the contamination period (3, 6, 12, 24, 48, 72 and 96 h). Total flesh was carefully removed using a dissection scalpel, drained for 5 min and weighed to record wet weight. Samples were then placed into labelled 50 mL centrifuge tubes and stored at -80°C until extraction. Prior to extraction, the flesh was defrosted, methanol (9 mL) was added to each vial and samples were extracted using a high-speed homogeniser (Polytron PT1300D) at 15000 rpm for 5 min. Samples were then centrifuged at 4500 g for 5 min at 4°C, and the supernatant was transferred into 20 mL volumetric flasks. Another 9 mL of methanol was added to the remaining pellet and homogenised again. Centrifugation was repeated at above parameters, and supernatants were transferred into the same 20 mL volumetric flasks. Volumetric flasks were then made up to the mark using methanol, homogenised and aliquots were filtered and analysed by LC-MS/MS. (This extraction procedure was adapted according to Villar-Gonzalez et al. (2011) as explained in Jauffrais et al. (2012c)).

#### 2.5.4. LC-MS/MS analysis

The samples were analysed by LC-MS/MS to quantify AZAs using a HPLC (model UFLCxr, Shimadzu) coupled to a triple quadrupole mass spectrometer (API 4000Qtrap, Applied Biosystems). Separation and LC-MS/MS procedures were the same as used in the previous experiment (Jauffrais et al., 2012c) and were based on a previous study (Rehmann et al., 2008). Quantifications were carried out using external calibration against AZA1 to 3, with Analyst 1.5 software (Applied Biosystems), assuming that all analogues had a similar response factor as AZA1, exception made for AZA2 and -3 for which the respective standards were used. Toxin equivalent factors (TEFs) were also applied to estimate the true toxic potential of mussel samples during the experiment (TEQ = toxin equivalent). AZA1 TEFs applied were equal to 1.8 and 1.4 for AZA2 and -3, respectively (Ofuji et al., 1999) and Concerning the relative in vitro potency of AZA6, it appears to be not unlike that of AZA1 (Dr. M. Twiner, personal communication). Thus, a provisional TEF of 1 was used for crude estimation of the toxicity represented by AZA6. Consequently, a TEF of 1.4 and 1 were applied to AZA17 and -19, respectively, as AZA17 and -19 transform into -3 and -6 after cooking (McCarron et al., 2009).

### 2.6. Statistical analysis and model

Data were expressed as mean ± standard deviation (SD) and, when specified, standardised to 1 g dry weight (DW). Statistical analyses were multi-factor ANOVA and differences were considered significant at  $P < 0.05$ . Statistical analyses were carried out using Statgraphics Centurion XV.1 (Statpoint Technologies, INC). Before each ANOVA analysis, normality and equality of variance were tested. Only data on AZA concentration in mussel tissues did not

respect equality of the variance, consequently a non-parametric analysis was carried out (Kruskal Wallis) followed by a box and whisker plot to discriminate the differences between values.

### 3. Results

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#### 3.1. Feeding behaviour responses

##### 3.1.1. Clearance rate

The mean clearance rate of mussel fed T-Iso was higher ( $0.84 \pm 0.50 \text{ L h}^{-1} \text{ gDW}^{-1}$ ) than the CR of mussels fed *A. spinosum* ( $0.14 \pm 0.17 \text{ L h}^{-1} \text{ gDW}^{-1}$ ). Concerning mussels fed T-Iso, a decrease was observed during the first day of exposure even though mussels were adapted to T-Iso before this feeding trial. However, during most of this period, the CR was ranging between 0.5 and  $1.5 \text{ L h}^{-1} \text{ gDW}^{-1}$  (Fig. 2; note that the daily drop in CR is due to the daily seawater renewal). Concerning mussels fed *A. spinosum*, a sharp decrease from 1 to  $0.05 \text{ L h}^{-1} \text{ gDW}^{-1}$  was observed during the first 12 hours of exposure to *A. spinosum*. Subsequently, the CR stabilised until the next day where seawater was renewed. After water was renewed, CR increased to  $0.5 \text{ L h}^{-1} \text{ gDW}^{-1}$  for 3 to 5 hours, then decreased again to  $0.05 - 0.1 \text{ L h}^{-1} \text{ gDW}^{-1}$ .

However this daily increase of CR following seawater renewal was only observed during the first 2 days of the exposure to *A. spinosum* and was almost negligible at the end of the experiment.

##### 3.1.2. Feeding time activity, organic filtration, ingestion and absorption rates

The daily mussel feeding time activity (FTA) (Tab. 1) was clearly affected by diet. Mussel fed T-Iso had a FTA of more than 50%, which significantly decreased when mussels were fed *A. spinosum*. During the exposure to *A. spinosum*, the mussel FTA further decreased from 16 to 2%.

Mussel TFR and TIR presented in table 1 were closely related and varied as a function of pseudofaeces production. A production of pseudofaeces was observed from the first day to the end of the experiment under the toxic diet, whereas, only small amount of pseudofaeces were observed and weighed when mussels were fed T-Iso on day 4 of the experiment, explaining that both TFR and TIR are very similar for the non-toxic diet. The multifactor ANOVA showed that both factors, the type of food and the time (where time refer to the days within a particular diet), had a significant effect: *A. spinosum* significantly decreased mussel TFR and TIR compared to T-Iso. The effect of time was also significant. Furthermore, TIR was negative during the last day of feeding *A. spinosum*.

The mussel absorption rate (AR) also underlined the negative effect of the *A. spinosum* diet. Mussels significantly decreased their AR when the diet was switched from T-Iso to *A. spinosum*. This lower AR continuously decreased until the end of the experiment to even negative values (Tab. 1). The production of faeces and pseudofaeces were assessed daily. Production of faeces was the only parameter influenced neither by diet nor by time. However, the production of pseudofaeces was significantly affected by the diet supplied. Mussels exposed to *A. spinosum* produced significantly higher amounts of pseudofaeces. Furthermore, mussel pseudofaeces production increased as a function of time ( $P = 0.07$ ).

Microscopic analysis of mussel faeces revealed that they contained a few ecdysed *A. spinosum* cells while pseudofaeces contained many *A. spinosum* cells, ecdysed cells and empty thecae (Fig. 3a and b).

## 3.2. AZA analysis

### 3.2.1. *A. spinosum*

AZA cell quota of *A. spinosum* during the contamination period was  $93.9 \pm 10.4$  fg cell<sup>-1</sup>, with a proportion of 81% of AZA1 ( $76.6 \pm 8.3$  fg cell<sup>-1</sup>) and 19% of AZA2 ( $17.3 \pm 2.2$  fg cell<sup>-1</sup>), the SD represents the variation observed during the four days of the contamination period.

### 3.2.2. AZA accumulation and profile in mussel

A rapid accumulation of AZAs in mussels was observed during the first hours of exposure to *A. spinosum*. Less than six hours were necessary to reach AZA concentration above the regulatory level ( $160 \mu\text{g kg}^{-1}$ ). However, after this fast AZA accumulation, the concentration stabilised during the experiment as no significant differences were found between values from 6 hours to the end of the study (Fig. 4). Mean feeding time activity (FTA) of mussels fed *A. spinosum* and used for AZA analysis was initially as high 60% but rapidly declined to about 10% after two days of exposure. Consequently, it is possible to correlate the stagnation in AZA accumulation to the decrease of the FTA (Fig. 4).

Toxin profiles in mussels presented in table 2 showed a fast AZA biotransformation, as some AZA1 and -2 metabolites were already found after 3 hours. AZA17 was found to have a proportion almost equal to AZA2, whereas AZA19 and a group of metabolites (AZA7-10) were also detected, but in minor proportion. During the first 6 hours, the number of metabolites as well as their relative proportions did not change. However, as AZA accumulation began to stabilize, the proportion and number of metabolites started to increase. AZA17 reached a maximum proportion of 35% and AZA19 of 6% after two days of contamination, and these two metabolites were the major ones found during the experiment. Other AZAs found were AZA3 to -10 and AZA21, with proportions below 3% for each of them during the study.

## 4. Discussion

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In a previous study on AZA accumulation in blue mussels fed *A. spinosum*, a fast initial toxin accumulation was followed by a slow further increase or a stabilisation of accumulated toxins. Furthermore, we observed a negative effect of *A. spinosum* on mussel digestive tissues (Jauffrais et al., 2012c). Consequently, we hypothesised that *A. spinosum* is a micro-alga either toxic to mussels or of poor nutritional quality for mussels and thus AZA uptake was limited by a decrease in clearance rate and an increase in the *A. spinosum* rejection via the pseudofaeces. This hypothesis was established following mechanisms contributing to low toxin uptake by oyster (Mafra et al., 2009) and the ability of *M. edulis* to reject bad seston quality due to a high content in inorganic particles (Bayne et al., 1993). Subsequently, this study was designed to compare feeding responses of mussels when fed non-toxic and toxic diets based on *I. aff. galbana* and *A. spinosum*, respectively.

Non-toxic diet based on T-Iso had different cell densities (on purpose due to different cell size) and slightly different TPM and cellular volume compared to the toxic diet based on *A. spinosum*. However, both were in the same order of magnitude and at adequate concentration for mussels (Bayne et al., 1987; Bayne et al., 1993; Gosling, 2003), and thus cell size or TPM did not impact our results and conclusion. *Azadinium spinosum* concentration applied in this experiment was similar to concentrations used in a previous one (Jauffrais et al., 2012c); Toxin profile of *A. spinosum* with almost 80% AZA1 was also similar to previous experiments (Salas et al. 2011). AZA cell quota has been shown to be quite variable in the few studies presenting results on *A. spinosum*; from 5 to 50 fg cell<sup>-1</sup> for cells



grown in batch culture (Salas et al., 2011; Tillmann et al., 2009) and up to 100 fg cell<sup>-1</sup> in stirred photobioreactors at limited or stagnant growth (Jauffrais et al., 2012b). In the present experiment AZA cell quota was approximately 50% higher (94 fg cell<sup>-1</sup>) compared to our first experiment (59 fg cell<sup>-1</sup> (Jauffrais et al., 2012c)). In the present experiment, *A. spinosum* was grown under the condition developed by Jauffrais et al. (2012b) to improve AZA production, so this elevated AZA cell quota is easy to explain. In any case, this variation could at least partly explain the difference of AZA accumulation between the two studies (400 to 600 µg kg<sup>-1</sup> in Jauffrais et al. (2012c) against a maximum of 250 µg kg<sup>-1</sup> in the present one) and could enhance a possible negative effect of AZA cell quota on AZA uptake by mussels. Effectively, significant inhibitions of the clearance rate are mentioned in *M. edulis*, *C. gigas* and *Mya arenaria* above a threshold of cell toxicity with *Alexandrium* isolates (Bricelj and Shumway, 1998). Generally, comparison to the situation in the field is still hampered by the lack of data on *A. spinosum* concentration and AZA cell quota of natural populations. Nevertheless, the cell concentration corresponds to bloom densities of a species of *Azadinium* in coastal Argentinian waters, where concentration from 0.5 to 9 × 10<sup>6</sup> cells L<sup>-1</sup> were observed (Akselman and Negri, 2012).

Concerning the effect of toxin cell quota, it has been shown for mussels fed *Alexandrium* species (PST producers) with high and low toxicity that toxin accumulation rate was positively correlated to cell toxicity (Bricelj and Shumway, 1998). Furthermore, the effect of toxic dinoflagellates on feeding activity and PSP accumulation is species-specific (Bricelj and Shumway, 1998): insensitive species like mussels can accumulate large amount of toxins (Bricelj et al., 1990), whereas more sensitive species as oyster showed feeding responses that reduce their toxin accumulation (Bardouil et al., 1996; Lassus et al., 1999; May et al., 2010). Based on maximal concentration of AZA found in shellfish species cultured in Ireland (between 2003 and 2010 analysed by the Irish monitoring program using LC–MS/MS), AZA accumulation seemed also to be species-specific and *M. edulis* was found to accumulate the largest concentration of AZA among the different bivalve molluscs monitored (Hess et al., 2003; Salas et al., 2011).

Furthermore, bivalve mollusc responses to toxic dinoflagellates are known to differ in their CR. Several species showed reduction of filtration rate (e.g. oyster and clams) whereas others even increased their filtration rate (*M. edulis* and *O. edulis*) (Shumway and Cucci, 1987), explaining the higher accumulation of toxins in some species compared to others.

Based on literature, mussels were not supposed to be significantly affected by toxic dinoflagellates. However, our present results confirmed previous experimental observations (Jauffrais et al., 2012c) regards a negative effect of *A. spinosum* on mussels. Consequently, AZA accumulation was limited after a short time of exposure to *A. spinosum* by a sharp decrease in CR and FTA and an increase in pseudofaeces production.

Interestingly, negative ingestion and absorption rate were measured on the last day of the experiment. Negative TIR and AR are closely related to absorption efficiency, a parameter found to be negative when mussels were fed on seston of low quality, e.g.: *M. edulis*, *Aulacomya maoriana*, *M. galloprovincialis* and *Perna canaliculus* (Bayne et al., 1987; Gardner, 2002; Helson and Gardner, 2007). This observation was linked to a high inorganic content of seston that led to metabolic faecal loss; however, the seston used in our study (toxic or not) had a high organic content and thus should not negatively affect the absorption efficiency.

These results confirmed our hypothesis and show the negative effect of *A. spinosum* on feeding responses and a possible regulation of AZA uptake by decreasing filtration and increasing pseudofaeces production. It is also important to note than no significant differences were observed in faeces production, even though IR and AR were lower with the

second and toxic diet. An explanation might be that the experiment was not long enough to observe differences on faeces production.

Thus, bioactive compounds, either AZAs or other allelochemical substances produced by *A. spinosum* could explain the differences in absorption rate measured between the two diets.

Contrarily to mussels fed PST producers, *i.e.* *Alexandrium* species (Contreras et al., 2012; Marsden and Shumway, 1992; Navarro and Contreras, 2010; Navarro et al., 2008), *A. spinosum* had a short term effect as only 6-12 hours were necessary to drastically reduce CR and also a longer term effect as over the experiment (4 days) no recovery was observed.

Thus, from the present study we can conclude that mussels might reduce AZA accumulation by lowering filtration rate and enhancing pseudofaeces production. However, it cannot be explained by the present experiment if these responses are due to intracellular or dissolved AZA or other allelochemical substances.

Another experimental observation was made during the experiment; after daily water change we found an increase in CR for three to five hours when mussels were fed *A. spinosum* (see fig. 2, day 5 and 6). This time might be necessary to reach a threshold of dissolved AZA detectable by mussels. Such dissolved AZAs or bioactive compounds could derive and accumulate from degrading cells (theca, ecdysed cells and other detritus were microscopically observed in pseudofaeces). Effectively, qualitative analysis of mussel faeces and pseudofaeces during the exposure to *A. spinosum* indicated that *A. spinosum* cells are egested under an ecdysed form by *M. edulis* (Fig. 3a and 3b). Pseudofaeces presented the largest concentration of intact cells, ecdysed cells and theca; however, most of them were embedded in mucus. This was already shown for other toxic dinoflagellates (Hegaret et al., 2008) and the present study provides another indication that relocation of bivalve molluscs during a bloom of *A. spinosum* could be a vector for the transport of *A. spinosum* to shellfish grounds free of this toxic species.

These observations, the negative effect of *A. spinosum* on mussels feeding behaviour and the pseudofaeces production that can be potentially linked to a release of dissolved AZAs in the water raised question on the bioavailability and effect of AZAs (dissolved or not) on other aquatic organisms (e.g. bivalve larvae, plankton, fish embryo). The phenomenon might be of particular importance to the first stage of larval development of aquatic organisms, as AZAs were found to have a teratogenic effect after microinjection on Japanese medaka (*Oryzias latipes*) embryos (Colman et al., 2005). Further studies on bivalve larvae should be undertaken to investigate the effect on this class.

AZA accumulation of mussels fed *A. spinosum* was found to be a fast process since the regulatory limit was reached in less than 6 h, thus confirming our previous results (Jauffrais et al., 2012c; Salas et al., 2011). In the present study the accumulation increased until 6 h and tended to stabilize to  $\sim 200 \mu\text{g kg}^{-1}$ . These toxin contents in blue mussels were within the range of what is frequently found in the Irish monitoring program (Salas et al., 2011). However, mussels did not build up very high levels over the 4 days of exposure to *A. spinosum*. AZA concentration was found to be low compared to the maximal concentration found in Irish mussels (up to  $8970 \mu\text{g Kg}^{-1}$  AZA1-equiv).

To enhance AZA accumulation by mussels, a first experiment had already been carried out with a 50/50 *A. spinosum*/T-Iso mixture (Jauffrais et al., 2012c) to reflect natural conditions. Effectively, for some species (e.g.: *Mya arenaria*, *Mercenaria mercenaria*, *C. gigas*) toxin accumulation is influenced by the presence of non-toxic microalgae in a mixed assemblage which enhance or not feeding on toxic dinoflagellates (e.g.: *Alexandrium sp.*) (Bardouil et al., 1996; Bricelj and Shumway, 1998; Bricelj et al., 1991). Similar to the mono-specific diet made of *A. spinosum*, AZA accumulation was also observed when mussels were

simultaneously fed *A. spinosum* and *Isochrysis* aff. *galbana* (Jauffrais et al., 2012c). However, AZA accumulation was neither enhanced nor decreased by the toxic/non-toxic microalgal assemblage. Nonetheless, a lower ratio of toxic/non-toxic microalgal assemblage (i.e. <50/50) might enhance CR and possibly AZA accumulation.

Consequently, to explain differences between high natural AZA concentrations observed in Irish mussels compared to the “low” concentration found in the present experiment other possibilities have to be verified. It is suggested that mussels which are periodically exposed to *A. spinosum* blooms (which is the case of Irish mussels compared to French one) may have developed mechanisms allowing them to digest the micro-algae without adverse effects, thus enhancing their ability to accumulate the toxin. In some ways they could have developed a mechanism of adaptation to the toxins, a similar hypothesis was raised and confirmed for mussels from different locations exposed to *Alexandrium tamarense* (Shumway and Cucci, 1987). Another possibility could be a more complex trophic interaction. *A. spinosum* is now known to be a potential prey for other heterotrophic plankton organisms such as *Protooperidinium crassipes* or *Favella ehrenbergii* (Krock et al., 2009; Tillmann et al., 2009). It is then possible to imagine an AZA biomagnification through the food web allowing for a faster accumulation by mussel than observed in this study.

Nevertheless, the limited amount of time necessary for mussels to reduce its AZA accumulation, by modifying its feeding activity, was sufficient to reach EU regulatory level. This rapid accumulation of AZAs underlines difficulties in monitoring toxins for sanitary purposes and the necessity to predict and to track *A. spinosum* blooms.

Biotransformation of AZA1 and -2 in mussels was also found to be a fast process. These results confirmed the two first feeding experiments carried out during 24 h and over a week of exposure to *A. spinosum* (Jauffrais et al., 2012c; Salas et al., 2011). In both studies rapid bioconversion of AZA1 and -2 into AZA17, -19 was found, with AZA17 as the major bioconversion product after exposure to *A. spinosum* (Jauffrais et al., 2012c; Salas et al., 2011). In the present study, AZA17 and -19 as well as the group AZA7-10 were already found after 3 h of exposure, with a proportion of AZA17 equal to that of AZA2. Bioconversion of AZA in mussels has to be considered as a fast process, and consequently AZA17 and -19 are two major metabolites that should be taken into account by official monitoring programs in the EU.

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## Tables

Table 1. Mussel daily feeding time activity (FTA), total filtration rate (TFR), total ingestion rate (TIR) absorption rate (AR), organic faeces (OFs) and organic pseudofaeces (OPs) when fed *A. spinosum* (toxic diet) or T-Iso (non-toxic diet) and *P* value of the main effects and interaction.

| Time               | FTA (%)          | TFR (mg.h <sup>-1</sup> .gDW <sup>-1</sup> ) | TIR (mg.h <sup>-1</sup> .gDW <sup>-1</sup> ) | AR (mg.h <sup>-1</sup> .gDW <sup>-1</sup> ) | OFs (mg.d <sup>-1</sup> .gDW <sup>-1</sup> ) | OPs (mg.d <sup>-1</sup> .gDW <sup>-1</sup> ) |
|--------------------|------------------|--|--|---|--|--|
| <i>T-Iso</i>       |                  |  |  |   |  |  |
| 1                  | 63.6 ± 19.8      | 0.91 ± 0.32                                  | 0.91 ± 0.32                                  | 0.42 ± 0.18                                 | 2.34 ± 0.59                                  | -  |
| 2                  | 65.3 ± 27.5      | 0.92 ± 0.52                                  | 0.92 ± 0.52                                  | 0.47 ± 0.28                                 | 1.94 ± 1.11                                  | -  |
| 3                  | 56.0 ± 26.6      | 0.68 ± 0.39                                  | 0.68 ± 0.39                                  | 0.37 ± 0.24                                 | 1.40 ± 0.62                                  | -  |
| 4                  | 54.5 ± 25.0      | 0.43 ± 0.24                                  | 0.41 ± 0.25                                  | 0.19 ± 0.14                                 | 1.29 ± 0.40                                  | 0.09 ± 0.24                                  |
| <i>A. spinosum</i> |                  |  |  |   |  |  |
| 5                  | 16.7 ± 13.3      | 0.31 ± 0.19                                  | 0.20 ± 0.18                                  | 0.10 ± 0.16                                 | 1.36 ± 0.73                                  | 1.04 ± 0.78                                  |
| 6                  | 13.8 ± 10.0      | 0.27 ± 0.13                                  | 0.16 ± 0.14                                  | 0.07 ± 0.17                                 | 1.61 ± 1.01                                  | 0.88 ± 0.45                                  |
| 7                  | 10.3 ± 6.4       | 0.24 ± 0.06                                  | 0.12 ± 0.07                                  | 0.00 ± 0.10                                 | 1.89 ± 0.92                                  | 1.06 ± 0.58                                  |
| 8                  | 2.1 ± 1.20       | 0.08 ± 0.03                                  | <b>-0.10 ± 0.06</b>                          | <b>-0.14 ± 0.08</b>                         | 1.42 ± 0.73                                  | 1.47 ± 0.87                                  |
| ANOVA              |                  |  |  |   |  |  |
| Diet               | <i>P</i> < 0.001 | <i>P</i> < 0.001                             | <i>P</i> < 0.001                             | <i>P</i> < 0.001                            | <i>P</i> = 0.284                             | <i>P</i> < 0.001                             |
| Time               | <i>P</i> = 0.140 | <i>P</i> < 0.001                             | <i>P</i> < 0.001                             | <i>P</i> < 0.001                            | <i>P</i> = 0.184                             | <i>P</i> = 0.07                              |
| Diet × Time        | <i>P</i> = 0.913 | <i>P</i> = 0.291                             | <i>P</i> = 0.422                             | <i>P</i> = 0.927                            | <i>P</i> < 0.05                              | <i>P</i> = 0.312                             |

Table 2. AZA proportion (%) and concentration (µg.kg<sup>-1</sup>, µg.kg<sup>-1</sup> TEQ) in mussel fed *A. spinosum* as a function of time

|                      | Time (days)             | 0.125  | 0.25  | 0.5   | 1     | 2     | 3     | 4     |       |
|----------------------|-------------------------|--------|-------|-------|-------|-------|-------|-------|-------|
| AZA (%)              | AZA1                    | 66.9   | 65.5  | 51.0  | 47.5  | 38.6  | 40.2  | 45.1  |       |
|                      | AZA2                    | 14.3   | 15.1  | 13.4  | 15.0  | 15.6  | 14.8  | 14.1  |       |
|                      | AZA3                    | 0.0    | 0.0   | 0.0   | 0.5   | 0.6   | 0.7   | 0.6   |       |
|                      | AZA4                    | 0.0    | 0.0   | 0.0   | 0.3   | 0.2   | 0.6   | 0.3   |       |
|                      | AZA5                    | 0.0    | 0.0   | 0.0   | 0.3   | 0.9   | 0.6   | 0.8   |       |
|                      | AZA6                    | 0.0    | 0.0   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |       |
|                      | AZA7-10                 | 2.5    | 2.6   | 4.1   | 2.2   | 1.8   | 2.2   | 2.2   |       |
|                      | AZA17                   | 13.7   | 14.0  | 26.7  | 29.2  | 35.0  | 33.1  | 29.4  |       |
|                      | AZA19                   | 2.5    | 2.7   | 4.6   | 4.9   | 5.9   | 5.8   | 5.2   |       |
|                      | AZA21                   | 0.0    | 0.0   | 0.0   | 0.1   | 1.3   | 2.1   | 2.2   |       |
|                      | µg.kg <sup>-1</sup>     | AZAs   | 123.7 | 242.8 | 180.3 | 225.6 | 173.3 | 250.4 | 161.2 |
|                      | µg.kg <sup>-1</sup> TEQ | AZA1-3 | 114.7 | 225.2 | 135.6 | 169.7 | 116.9 | 169.5 | 115.2 |
| AZA1-3, -6, -17, -19 |                         | 137.3  | 270.8 | 181.9 | 231.9 | 165.0 | 235.7 | 156.6 |       |

## Figures

Figure 1. Experimental design used to follow the effect of the non-toxic and toxic diets on the feeding behavior of mussels (for clarity: connections 3 to 10 and 13 to 20 were not shown).

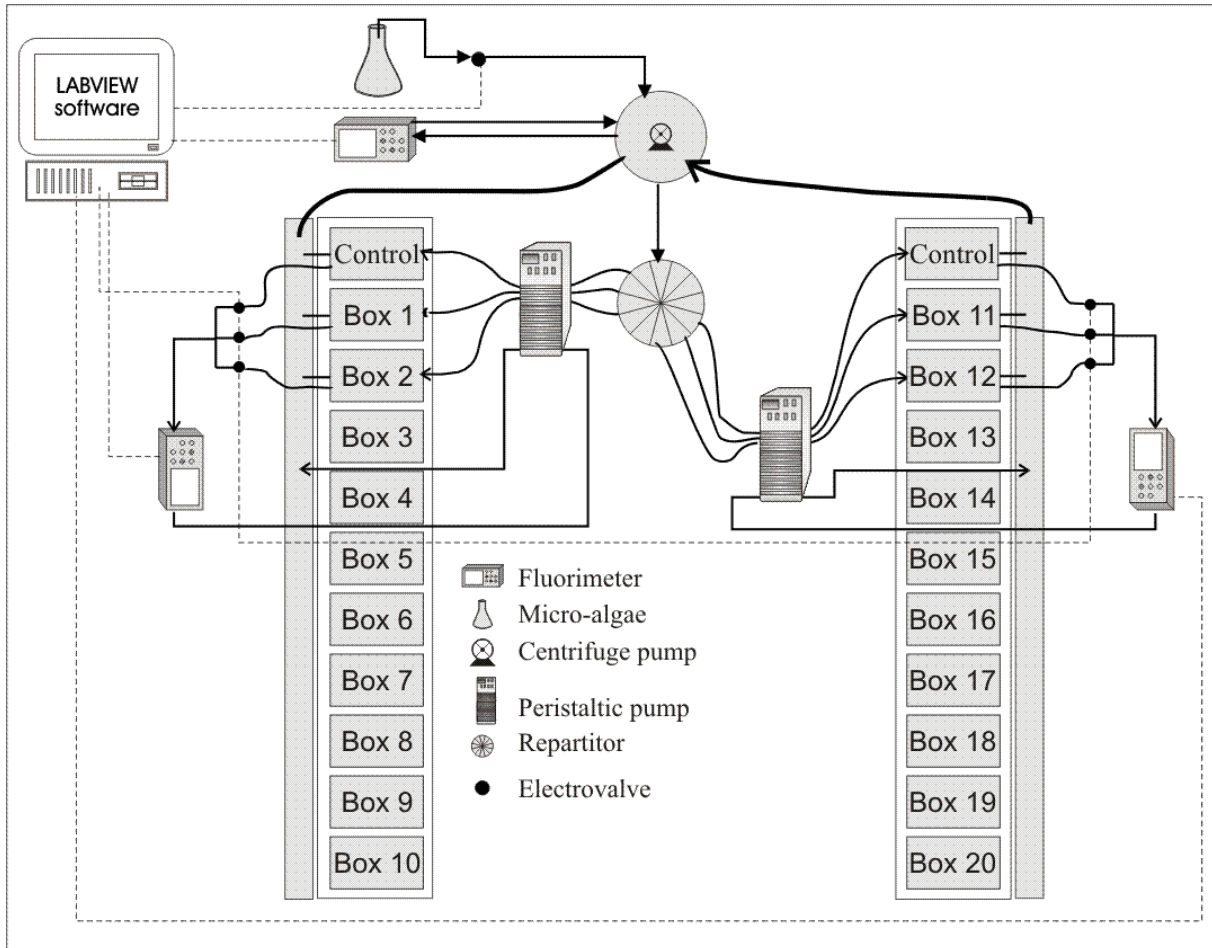


Figure 2. Mussel clearance rate (CR) during the exposure to the non-toxic diet (*T-Iso*) and to the toxic diet (*A. spinosum*). Note that the daily water renewal at every full day coincides with a sudden decrease and/or increase in clearance rate (day 1 to 6).

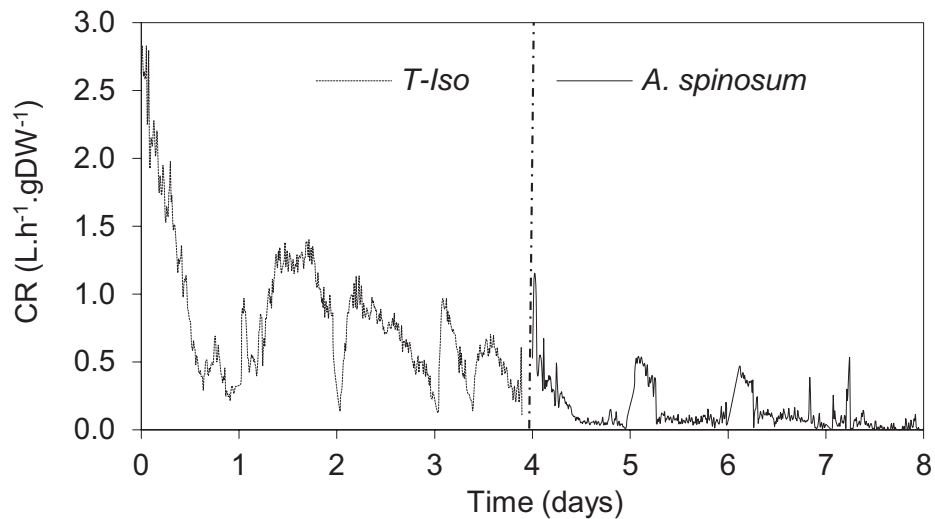


Figure 3. Mussel faeces (a) and pseudofaeces (b) when fed the toxic diet *A. spinosum*. White arrow shows intact *A. spinosum* cell, black arrow ecdysed cell and black dashed arrows empty theca (scale = 20  $\mu\text{m}$ ).

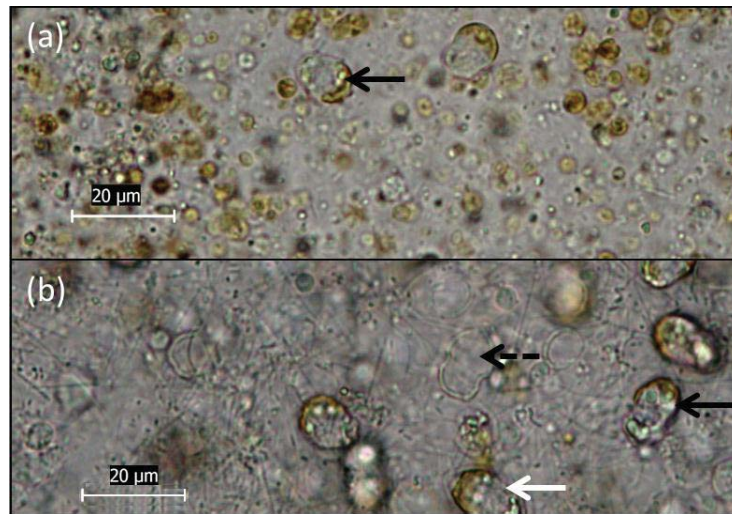
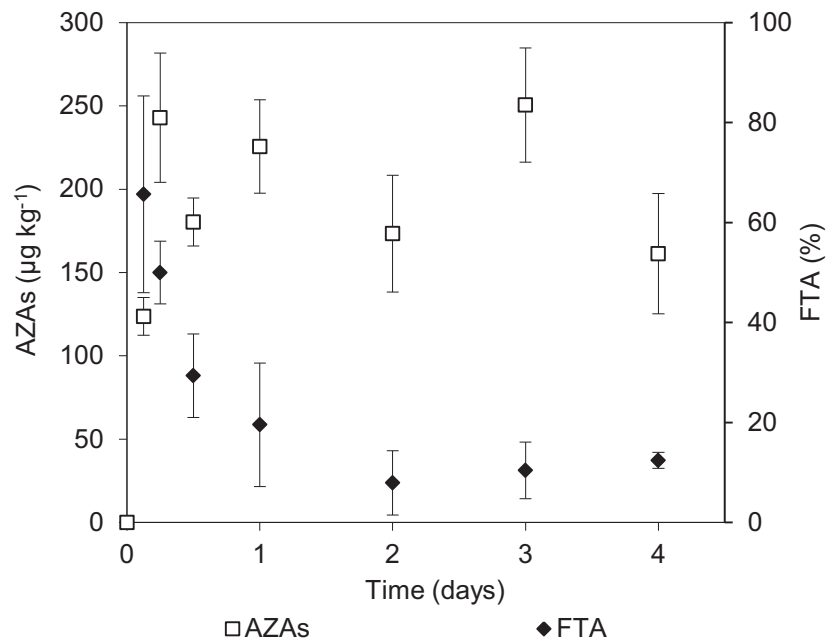


Figure 4. AZA concentrations ( $\mu\text{g kg}^{-1}$ ) in mussel fed *A. spinosum* and mean feeding time activity (FTA, %) of the same mussels during their exposure to *A. spinosum*. Error bars represent 6 replicates (with the exception of the 6h time point for which there are only 3 replicates)



## 1. Appendix A. Supplementary data

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Table 1. Box number and time when mussels were sampled and replaced in each experimental box during the feeding period with *A. spinosum* (notice; boxes 1-6 were not used to assess physiological parameters due to the larger number of samples and replacements taking place).

| Box number | Time (h):<br>sampled + replaced |                        |                        |
|------------|---------------------------------|------------------------|------------------------|
|            | 1 <sup>st</sup> sample          | 2 <sup>nd</sup> sample | 3 <sup>rd</sup> sample |
| 1          | 3                               | 6                      | 18                     |
| 2          | 3                               | 6                      | 18                     |
| 3          | 3                               | 6                      | 18                     |
| 4          | 6                               | 18                     |                        |
| 5          | 6                               | 18                     |                        |
| 6          | 6                               | 18                     |                        |
| 7          | 24                              | 96                     |                        |
| 8          | 24                              | 96                     |                        |
| 9          | 24                              | 96                     |                        |
| 10         | 48                              | 96                     |                        |
| 11         | 48                              | 96                     |                        |
| 12         | 48                              | 96                     |                        |
| 13         | 72                              | 96                     |                        |
| 14         | 72                              | 96                     |                        |
| 15         | 72                              | 96                     |                        |
| 16         | 96                              |                        |                        |
| 17         | 96                              |                        |                        |
| 18         | 96                              |                        |                        |
| 19         | 96                              |                        |                        |
| 20         | 96                              |                        |                        |

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