



## Accumulation, transformation and breakdown of DSP toxins from the toxic dinoflagellate *Dinophysis acuta* in blue mussels, *Mytilus edulis*

Nielsen, Lasse Tor; Hansen, Per Juel; Krock, Bernd; Vismann, Bent

*Published in:*  
Toxicon

*Link to article, DOI:*  
[10.1016/j.toxicon.2016.03.021](https://doi.org/10.1016/j.toxicon.2016.03.021)

*Publication date:*  
2016

*Document Version*  
Peer reviewed version

[Link back to DTU Orbit](#)

### *Citation (APA):*

Nielsen, L. T., Hansen, P. J., Krock, B., & Vismann, B. (2016). Accumulation, transformation and breakdown of DSP toxins from the toxic dinoflagellate *Dinophysis acuta* in blue mussels, *Mytilus edulis*. *Toxicon*, 117, 84-93. <https://doi.org/10.1016/j.toxicon.2016.03.021>

---

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 **Accumulation, transformation and breakdown of**  
2 **DSP toxins from the toxic dinoflagellate *Dinophysis***  
3 ***acuta* in blue mussels, *Mytilus edulis***

4  
5 Lasse Tor Nielsen<sup>1\*</sup>, Per Juel Hansen<sup>2</sup>, Bernd Krock<sup>3</sup>, Bent Vismann<sup>2</sup>

6  
7 <sup>1</sup> Centre for Ocean Life, National Institute of Aquatic Resources, Technical University of Denmark,  
8 DK-2920 Charlottenlund, Denmark

9 <sup>2</sup> Marine Biological Section, University of Copenhagen, Strandpromenaden 5, DK-3000 Helsingør,  
10 Denmark

11 <sup>3</sup> Alfred-Wegener-Institute for Polar and Marine Research, Am Handelshafen 12, D-27570  
12 Bremerhaven, Germany

13  
14  
15  
16 Running title: DSP toxins in *Mytilus edulis*

17  
18  
19  
20 \*Corresponding author:

21  
22 E-mail: [ltor@aqua.dtu.dk](mailto:ltor@aqua.dtu.dk)

## Abstract

24 Okadaic acid (OA), dinophysistoxins (DTX) and pectenotoxins (PTX) produced by the  
dinoflagellates *Dinophysis* spp. can accumulate in shellfish and cause diarrhetic shellfish poisoning  
26 upon human consumption. Shellfish toxicity is a result of algal abundance and toxicity as well as  
accumulation and depuration kinetics in mussels. We mass-cultured *D. acuta* containing OA, DTX-  
28 1b and PTX-2 and fed it to the blue mussel, *Mytilus edulis* under controlled laboratory conditions  
for a week to study toxin accumulation and transformation. Contents of OA and DTX-1b in mussels  
30 increased linearly with incubation time, and the net toxin accumulation was 66% and 71% for OA  
and DTX-1b, respectively. Large proportions ( $\approx 50\%$ ) of both these toxins were transformed to fatty  
32 acid esters. Most PTX-2 was transformed to PTX-2 seco-acid and net accumulation was initially  
high, but decreased progressively throughout the experiment, likely due to esterification and loss of  
34 detectability. We also quantified depuration during the subsequent four days and found half-life  
times of 5-6 days for OA and DTX-1b. Measurements of dissolved toxins revealed that depuration  
36 was achieved through excreting rather than metabolizing toxins. This is the first study to construct a  
full mass balance of DSP toxins during both accumulation and depuration, and we demonstrate  
38 rapid toxin accumulation in mussels at realistic *in situ* levels of *Dinophysis*. Applying the observed  
accumulation and depuration kinetics, we model mussel toxicity, and demonstrate that a  
40 concentration of only 75 *Dinophysis* cells  $l^{-1}$  is enough to make 60 mm long mussels exceed the  
regulatory threshold for OA equivalents.

42

44 **Keywords:** Diarrhetic shellfish poisoning, Okadaic acid, Dinophysistoxin, Pectenotoxin,  
*Dinophysis*, *Mytilus edulis*

46

## 1. Introduction

48 Contamination of shellfish with various biotoxins can lead to several different shellfish poisoning  
syndromes following human consumption (Landsberg 2002). In all cases, the causative toxins are  
50 *de novo* produced by certain photo- or mixotrophic microalgae – not by the shellfish (Landsberg  
2002, Lewitus et al. 2012). Filter-feeding transfers the toxins to the shellfish, where they may  
52 accumulate to high concentrations. Symptoms range from nausea over paralysis and amnesia to  
death depending on the involved toxins. Consequently, commercial shellfish harvesting (fisheries or  
54 aquaculture) are subject to extensive monitoring of *in situ* concentrations of causative algae and/or  
toxicity of harvested shellfish.

56 Diarrhetic shellfish poisoning (DSP) syndrome is one such syndrome, and the causative  
organisms are a frequent cause for concern in shellfish industries, as they may cause prolonged  
58 closures of mussel harvesting, sometimes lasting several months, with severe economic  
repercussions (Hinder et al. 2011). Predicting DSP toxicity in mussels would be a powerful  
60 mitigation tool, but has so far proved difficult (Reguera et al. 2014). Identification of the causative  
toxins and variability in algal toxicity has received the bulk of the attention, but shellfish toxicity is  
62 also a result of toxin accumulation and depuration kinetics. Nevertheless, comparably little is  
known about accumulation and depuration of DSP toxins in any shellfish species.

64 DSP toxins are produced by species of the two marine dinoflagellate genera *Dinophysis* and  
*Prorocentrum* (Hoppenrath and Elbrächter 2011; Reguera et al. 2014). The toxin producing  
66 *Prorocentrum* species are benthic, and thus unavailable for suspension-feeding mussels in most  
cases. Hence, *Dinophysis* is considered the main source of DSP toxins in marine shellfish. Acute  
68 effects of DSP toxins include diarrhea, nausea, vomiting and cramps, but chronic effects have also  
been reported, including carcinogenic effects and effects on the immune- and nervous systems and  
70 alterations in DNA and cellular components (Valdiglesias et al. 2013).

The DSP toxin group includes okadaic acid (OA) and its analogues the dinophysistoxins (DTX) as well as pectenotoxins (PTX). From the OA-group, OA, DTX-1, DTX-1b, and DTX-2 and their diol-ester precursors have been found in the toxin-producer *Dinophysis* spp. (Miles et al. 2006a, Hackett et al. 2009, Fux et al. 2011, Nielsen et al. 2013, Reguera et al. 2014). From the PTX-group, PTX-2 and PTX-11 - PTX-14 have been found in plankton samples or *Dinophysis* spp. cultures (Draisici et al. 1996, Miles et al. 2004b, Miles et al. 2006b, Suzuki et al. 2006). In shellfish, on the other hand, OA/DTX toxins are often transformed to fatty acid esters (collectively known as DTX-3), and these frequently comprise more than half the total OA/DTX in mussels (Vale and de M. Sampayo 2002a, Vale 2006). Likewise, mussels are known to transform PTX-2 to PTX-2 seco acid (PTX-2sa), but data on PTX in shellfish are scarce (Vale and de M. Sampayo 2002b). PTX-2sa may also be transformed to numerous different fatty acid esters, further adding to the complexity (Wilkins et al. 2006, Torgersen et al. 2008).

Our knowledge on accumulation of DSP toxins in mussels comes almost exclusively from field populations; controlled laboratory experiments are very scarce (Bauder et al. 1996, Rossignoli et al. 2011b). This owes primarily to the fact that culturing of *Dinophysis* spp. was only recently made possible (Park et al. 2006). Depuration (or detoxification) has been studied more intensely using mussels contaminated *in situ*. Effects of various parameters, including food availability (Blanco et al. 1999, Svensson 2003, Svensson and Förlin 2004, Marcaillou et al. 2010), temperature (Shumway and Cembella 1993, Blanco et al. 1999) and mussel size and lipid content (Svensson and Förlin 2004, Duinker et al. 2007) have been evaluated using different mussel species. Reported toxin half-life times vary substantially, from less than a day to 25 days, but despite the efforts, depuration kinetics of DSP toxins is far from well understood. Accumulation kinetics remain virtually unstudied.

94 With the recent discovery of suitable culturing techniques, it is now possible to study  
accumulation kinetics of DSP toxins from the prime *in situ* source, *Dinophysis* spp. Thus, for the  
96 first time, we studied the intoxication of mussels with OA, DTX-1b and PTX-2 supplied via mass  
cultured *Dinophysis acuta*. We chose the blue mussels, *Mytilus edulis*, as the target organism due to  
98 its commercial importance and well-studied physiology. The aim was to establish a mass balance  
under controlled laboratory conditions, quantifying accumulation, transformation, depuration and  
100 excretion of DSP-toxins from *D. acuta*. Based on these results, we model the toxin content of  
various-sized *M. edulis* under different *Dinophysis* spp. concentrations.

102

## 2. Materials and Methods

### 104 2.1. Culture conditions and specimen collection

106 A laboratory culture of the marine DSP producing dinoflagellate *Dinophysis acuta* (Strain DANA-  
106 2010) was established from water samples collected in the North Atlantic during a research cruise  
in June 2010 (60°24' N; 6°58' W) (Nielsen et al. 2013). Cells were isolated under a dissection  
108 scope by micro manipulation. The culture was non-clonal, as  $\approx 10$  cells were picked and grown  
together in 1 ml 0.2  $\mu\text{m}$  filtered seawater. To facilitate the mixotrophic growth of *D. acuta*, the  
110 ciliate *Mesodinium rubrum* was added as prey twice a week at a prey:predator ratio of 10:1. The *M.*  
*rubrum* culture was fed the cryptophyte *Teleaulax amphioxeia* at similar intervals and ratios. All  
112 protist cultures were grown in a temperature controlled room at 15°C in f/2 seawater-based growth  
medium (Guillard and Ryther 1962) with a pH of  $8.0 \pm 0.05$ , a salinity (psu) of  $30.0 \pm 1.0$  and a  
114 dissolved inorganic carbon content of  $2.3 \pm 0.1 \text{ mmol l}^{-1}$ . A Sentron ArgusX pH-meter equipped  
with a Hot-line cupFET probe was used to determine pH (NBS scale), and salinity was measured  
116 with a handheld visual refractometer. Dissolved inorganic carbon was measured with an infrared  
gas analyzer as described in detail elsewhere (Nielsen et al. 2007). An irradiance of 130  $\mu\text{mol}$   
118  $\text{photons m}^{-2} \text{ s}^{-1}$  was provided by Osram 58W/640 cool-white fluorescent tubes at a 16:8 h light:dark  
cycle. Previous studies of the *Dinophysis acuta* culture had shown that it produced OA and PTX-2  
120 as well as a novel isomer of DTX-1 tentatively termed DTX-1b (Nielsen et al. 2013).

The mussel *Mytilus edulis* was collected in Oresund, Denmark with a dredge deployed from boat  
122 during autumn 2011. The mussels were brought back to the laboratory, where they were kept for at  
least a month in a continuous flow of aerated sea water ( $30 \pm 1.0$  psu, 10°C) before use in  
124 experiments. The collection site typically has low abundances ( $<1 \text{ cell ml}^{-1}$ ) of *Dinophysis* spp.  
almost year round, but only rarely experiences blooms. The mussels were thus not naïve. On the

126 other hand, one month in the laboratory may have removed any adaptations towards DSP toxins  
before the onset of the experiment.

## 128 **2.2. Accumulation, transformation and breakdown of DSP toxins**

The experiment was designed to study accumulation and depuration rates of different DSP toxins in  
130 *Mytilus edulis*. Several initial attempts to intoxicate *M. edulis* with DSP toxins from *Dinophysis*  
*acuta* resulted in production of pseudo-faeces and correspondingly low (<1%) net toxin retention.  
132 These observations were most likely due to high concentrations of *D. acuta* (>20,000 l<sup>-1</sup>), and the  
present experiment was thus designed to work with low *D. acuta* concentrations.

134 Four replicate 12 l aquaria were setup in a 15°C temperature controlled room. Five litre GF/C  
filtered seawater (salinity = 30 ± 1) and 12 *M. edulis* (length 23.4 ± 1.0 mm) were added to each.  
136 Mussels were allowed 6 h to attach to the bottom, after which centrifugal aquarium pumps were  
deployed to ensure adequate mixing. The aquaria were all aerated with atmospheric air (≈40 ml  
138 min<sup>-1</sup>). To limit the growth of *D. acuta* in both supply cultures and the four aquaria, the experiments  
were conducted at relatively low irradiance levels (≈10 μmol photons m<sup>-2</sup> s<sup>-1</sup>).

## 140 **2.3. *Dinophysis acuta* inflow**

Once the experiment started, *D. acuta* was pumped into each aquarium by a Gilson Minipuls 3  
142 peristaltic pump (Biolab A/S, Denmark), equipped with a 4 channel pump head. A well-mixed *D.*  
*acuta* supply culture was ensured by continuous stirring with a magnetic stirrer. Glass funnels,  
144 custom-made from Pasteur pipettes, were positioned in the narrow intake of the tubes to avoid  
clogging of *D. acuta* cells. In order to design the experiment with low cell concentrations of *D.*  
146 *acuta*, a simple model was developed to predict the steady-state concentration of *D. acuta* as a  
function of *D. acuta* inflow rate:

$$148 \quad A_t = \frac{A_{t-1} \times V_{t-1} + F - (M \times C \times A_{t-1})}{V_t} \quad (1)$$



Where  $A$  is *D. acuta* concentration (cells  $l^{-1}$ ) at times  $t$  and  $t-1$ ,  $V$  is total water volume (l),  $F$  is  
150 inflow of *D. acuta* in (cells  $h^{-1}$ ),  $M$  is number of mussels and  $C$  is clearance rate ( $l\ ind^{-1}\ h^{-1}$ ). For  
designing the experiment,  $C$  was, according to Kiørboe and Møhlenberg (1981), assumed to be  $1.0\ l$   
152  $ind^{-1}\ h^{-1}$  for the size of *M. edulis* used.

The experiment was designed to produce an asymptotic rise to a maximum steady-state *D. acuta*  
154 cell concentration as a result of the continuous *D. acuta* inflow and the constant clearance by  
mussel. The inflow was set to be  $1000\ D. acuta\ cells\ mussel^{-1}\ h^{-1}$ , which, according to eq. 1, would  
156 result in a steady-state *D. acuta* concentration of  $1000\ cells\ l^{-1}$ . The continuous food supply was  
maintained for a week, only interrupted by the quick daily samplings. After the first week, the food  
158 supply was stopped, and the mussels remaining in the aquaria were left without additional food.  
During the first week, where *D. acuta* was supplied, the removal of mussels at each sampling was  
160 accompanied by a reduction of the inflow rate of *D. acuta* according to eq. 1, and a steady inflow  
per mussel was thus maintained.

## 162 **2.4. Sampling**

One mussel was harvested from each aquarium on day 0, 1, 2, 3, 4, 7, 7½, 8, 9, 10 and 11. Shell  
164 length and wet weight of soft parts were recorded. The soft parts were transferred to cryotubes and  
immediately frozen at  $-18^{\circ}C$  for later toxin extraction and analysis. Mussel filtering activity was  
166 inspected visually twice on each day of sampling by scoring each mussel as either open or closed.

Cell concentrations of *D. acuta* in supply cultures were determined before and after each  
168 sampling, by fixing 3 ml subsamples with Lugol's (final conc. 1%) and enumerating *D. acuta* by  
normal light microscopy in 1 ml Sedgwick-Rafter sedimentation chambers. Combined with  
170 volumes of *D. acuta* supply cultures before and after sampling, this allowed total inflow of *D. acuta*  
to be calculated.

172 The toxin contents of *D. acuta* were determined on day 0 and 7 by taking triplicate 0.5 ml  
subsamples from the supply culture. The subsamples were added to Eppendorf tubes with 0.45 µm  
174 spin filter inserts (VWR, Denmark). These were centrifuged at 800 × g for 1 minute, after which the  
filtrate was discarded, and the tubes with filter inserts were stored at -18°C until later toxin  
176 extraction and analysis. Water temperature and oxygen saturation were monitored daily in all four  
aquaria with a WTW OXI 96 oximeter equipped with an EO 96 sensor.

178 At each sampling, the *D. acuta* inflow was stopped, and all the water in each aquarium was  
replaced with 5 l of pre-filtered seawater at similar salinity, temperature and pH. For determination  
180 of cell concentrations of *D. acuta*, 100 ml subsamples of the discarded water were fixed with  
glutaraldehyde (final concentration 2%), and filtered onto 25 mm black polycarbonate filters (pore  
182 size 2 µm). These were mounted in immersion oil on microscopy slides, and inspected under  
epifluorescence microscopy (Olympus BX50) for enumeration of *D. acuta* at 100x magnification.  
184 Due to the complete exchange of water at samplings, the *D. acuta* concentrations in the aquaria  
changed over time; immediately after samplings, concentrations would have been close to zero, and  
186 as *D. acuta* were added, the concentrations would have increased asymptotically towards a steady-  
state maximum, where inflow and clearance cancelled each other out.

188 Dissolved toxins were sampled during the depuration period (Day 7-11) by mixing the remaining  
≈5 l of discarded water with 2 g of Diaion® HP-20 resin (Sigma-Aldrich, MO, USA) in 10L glass  
190 flasks and bubbling heavily for 12 h. The resin was mixed freely with the water and collected with a  
20 µm mesh following the 12 h period.

192 After termination of the experiment, average clearance rates could be calculated from the  
observed steady-state concentrations of *D. acuta* by simple reorganization of equation 1:

194 
$$C = \frac{A_{t-1} \times V_{t-1} + F - (A \times V)}{M \times A_{t-1}} \quad (2)$$

## 2.5. Control experiment without mussels

196 On day 7, after feeding was stopped, a control experiment was setup, to determine the reduction (or  
198 growth) in *D. acuta* without mussels. A flow of *D. acuta* similar to the highest one used in the  
200 experiment was directed into four additional aquaria with 5 l seawater, identical aquarium pumps  
and aeration. The control experiment lasted for 24 h, corresponding to the sampling interval in the  
experiment. After 24 h, cell concentrations of *D. acuta* were determined as described above, and  
these were compared to the total inflow of *D. acuta* during the same period.

## 202 2.6. Clearance rate with *Rhodomonas salina* as food source

A second control experiment was set up with the cryptophyte *Rhodomonas salina* as food source, to  
204 compare with the clearance rates of *M. edulis* obtained with *D. acuta* as a food source. This  
experiment also served to prove the principal of the clearance rate calculations. Biovolume  
206 equivalents comparable to the steady-state levels observed in the *D. acuta* experiment were set as  
the target ( $\approx 2000$  *R. salina* cells  $\text{ml}^{-1}$ ). Triplicate 10 l, aerated aquaria were added 5 l filtered  
208 seawater and 10 *M. edulis* individuals each. Mussels were allowed a few hours to attach by byssus  
before submersible aquarium pumps were started in order to ensure mixing. A continuous flow of  
210  $35 \text{ ml h}^{-1}$  of a  $455.000 \text{ cells ml}^{-1}$  *R. salina* culture was provided, and the concentrations of *R. salina*  
in the aquaria were monitored every hour. The experiment was terminated when a steady-state  
212 concentration of *R. salina* had been attained (4 h). *Rhodomonas salina* was enumerated using a  
Multisizer 3 Coulter Counter (Beckman Coulter) equipped with a  $100 \mu\text{m}$  aperture and with  
214 background samples subtracted. Shell length and wet weight of soft parts of each mussel was  
determined after the experiment was completed.

## 216 **2.7. Toxin extraction and analysis.**

Upon toxin extraction, 0.5 ml of circonia beads and 1.3 ml methanol were added to each cryotube-  
218 sample containing the soft parts of a mussel. These were homogenized with a FastPrep instrument  
(Thermo-Savant, Illkirch, france) for 45 sec at max speed ( $6.5 \text{ m s}^{-1}$ ). Samples were left to extract  
220 for 5 min after which they were centrifuged and the supernatant transferred to a 20 ml glass  
scintillation vial. An additional 1.3 ml methanol was added, and the sample was re-homogenized, -  
222 extracted and -centrifuged. The supernatant was transferred and combined with the first. A third  
extraction was performed in the same way, resulting in a total of ca. 3.8 ml methanol. Additional  
224 methanol was added to a total of 4.0 ml (by weight,  $\rho = 0.79 \text{ g cm}^{-3}$ ). Half this volume was removed  
for hydrolysis of toxin esters; the other half was processed without hydrolysis.

226 Hydrolysis of toxin esters was achieved by adding 250  $\mu\text{l}$  NaOH (2.5 M) to the 2 ml methanol  
toxin extract and heating the samples to  $76^\circ\text{C}$  for 40 min. After cooling, samples were neutralized  
228 with 250  $\mu\text{l}$  HCl (2.5 M).

### *2.7.1. Cleanup with solid phase extraction (SPE)*

230 Milli-Q water was added to all samples before clean-up with 3 ml LC-18 SPE cartridges (Sigma-  
Aldrich, Germany). Four ml were added to non-hydrolyzed aliquots and 3.5 ml to hydrolyzed ones,  
232 thus bringing the total methanol content down to  $<33\%$ . Organic solvent content should not exceed  
50% in order for SPE cartridges to bind DSP toxins. SPE cartridges were pre-treated with 1 ml  
234 methanol for 10 min, and washed twice with Milli-Q water, before the sample extract was added, all  
the time taking care not to dry the cartridges out. Slowly ( $\approx 1 \text{ ml min}^{-1}$ ) the extract was passed  
236 through the SPE cartridge, and the toxin thus adsorbed onto the SPE column. The cartridges were  
continuously refilled with extract until all 6 ml had been added, and were then washed thrice with 1  
238 ml Milli-Q water. Finally, the toxins were eluted with 1 ml 80% methanol directly into 2 ml glass  
HPLC vials.

240 2.7.2. *Algal samples*

The spin filters containing *D. acuta* were extracted with 100  $\mu$ l 100% methanol for one hour after  
242 which the Eppendorf tubes and filters were centrifuged at  $800 \times g$  for two minutes. The filtrate was  
then transferred to a 250  $\mu$ l glass insert in a 2 ml HPLC vial that was closed with a lid.

244 2.7.3. *Dissolved toxins*

Aliquots of one to two g of wet HP20 resin were weighed into 15 ml centrifugation tubes and 10 ml  
246 methanol were added to the resin and left over night. The next day the resin methanol slurry was  
transferred into a chromatography glass column (24 cm length, 1 cm ID) with frit topped with a 1  
248 cm quartz sand layer and the centrifugation tube was rinsed with an additional 5 ml methanol. The  
column filling was topped with another 1 cm quartz sand layer and the supernatant methanol was  
250 eluted to a glass beaker. Additional 15 ml methanol was added to the column and subsequently  
slowly eluted (ca. 10 drops  $\text{min}^{-1}$ ). Combined eluates were homogenized by vortexing and a 1 ml  
252 aliquot was used for LC-MS/MS analysis.

2.7.4. *Hydrolysis of HP20 extracts and HP20 resin*

254 10 ml of HP20 extracts from the samples taken at day 9 were mixed with 1.25 ml of 2.5 N sodium  
hydroxide solution and heated to 76  $^{\circ}\text{C}$  for one hour and subsequently left to cool down to room  
256 temperature overnight. The reaction mixture was neutralized with 1.25 ml of 2.5 N acetic acid and  
solvents removed in a rotary evaporator. The residue was adjusted with methanol to 1 ml and  
258 passed through a spin filter (0.45  $\mu\text{m}$ ) prior to LC-MS/MS analysis. As quantitative control an  
HP20 extract was treated identically, but without alkaline hydrolysis.

260 Approximately 5g of resin of the four same samples from day 9 were accurately weighed and  
suspended in 1 ml of methanol. The mixtures were treated with sodium hydroxide and subsequently  
262 neutralized as described above. After neutralization, additional 4 ml of methanol were added to the  
resin suspension. The supernatant was removed from the resin after centrifugation and the

264 remaining resin was re-extracted with another 4 ml methanol. The extracts were combined and  
prepared for measurement as described above.

266 LC-MS/MS analysis followed the protocol of Nielsen et al. (2013), with the added detection of  
PTX-2sa at the mass transition  $m/z$  894>213.

### 268 3. Results

Salinity, temperature and oxygen conditions of the four aquaria were stable and non-detrimental to  
270 the mussels (*Mytilus edulis*) throughout the experiment (table 1). Two of the initial 48 mussels died  
during the experiment, and this caused the experiment to be shortened from 12 to 11 days (Fig. 1A).

272 Visual inspections confirmed that mussels were generally open (and thus active) during the first  
week where *Dinophysis acuta* were supplied ( $89 \pm 5$  % [mean  $\pm$  S.D.] mussels scored as open). The

274 24 h control experiment without mussels, recorded a 21% loss of *D. acuta* cells during a 24 h period  
(data not shown), and this was accounted for in subsequent calculations of mussel ingestion and

276 clearance. The *D. acuta* culture contained OA, DTX-1b and PTX-2 at cell quotas of  $3.0 \pm 0.5$ ,  $7.6 \pm$   
 $1.3$  and  $39.0 \pm 4.6$  pg cell<sup>-1</sup>, respectively, with no statistically significant differences during the

278 experimental period.

280 **Table 1. Experimental conditions in the four replicate aquaria and lengths and weight of the 44 *Mytilus edulis* sampled.**

Values are means  $\pm$  SD.

<b>Parameter</b>	<b>Value</b>
Salinity (psu)	$30 \pm 1.0$
Temperature (°C)	$16.0 \pm 1.8$
O <sub>2</sub> (% sat.)	$100 \pm 3.5$
Mussel length (mm)	$23.4 \pm 1.0$
Mussel wet weight (g)	$0.23 \pm 0.05$

286

### 3.1. Clearance and ingestion

288 The average steady-state *D. acuta* concentration reached was  $6,762 \pm 2,317$  cells  $l^{-1}$  (Fig. 1B).  
 Based on equation 2, this translates to a clearance rate of  $0.15 \pm 0.04$   $l\ ind^{-1}\ h^{-1}$  (table 2). Combined,  
 290 these values demonstrate a maximum ingestion rate of  $1,028 \pm 240$  *D. acuta* cells  $ind^{-1}\ h^{-1}$  once  
 steady-state concentrations of *D. acuta* were reached.

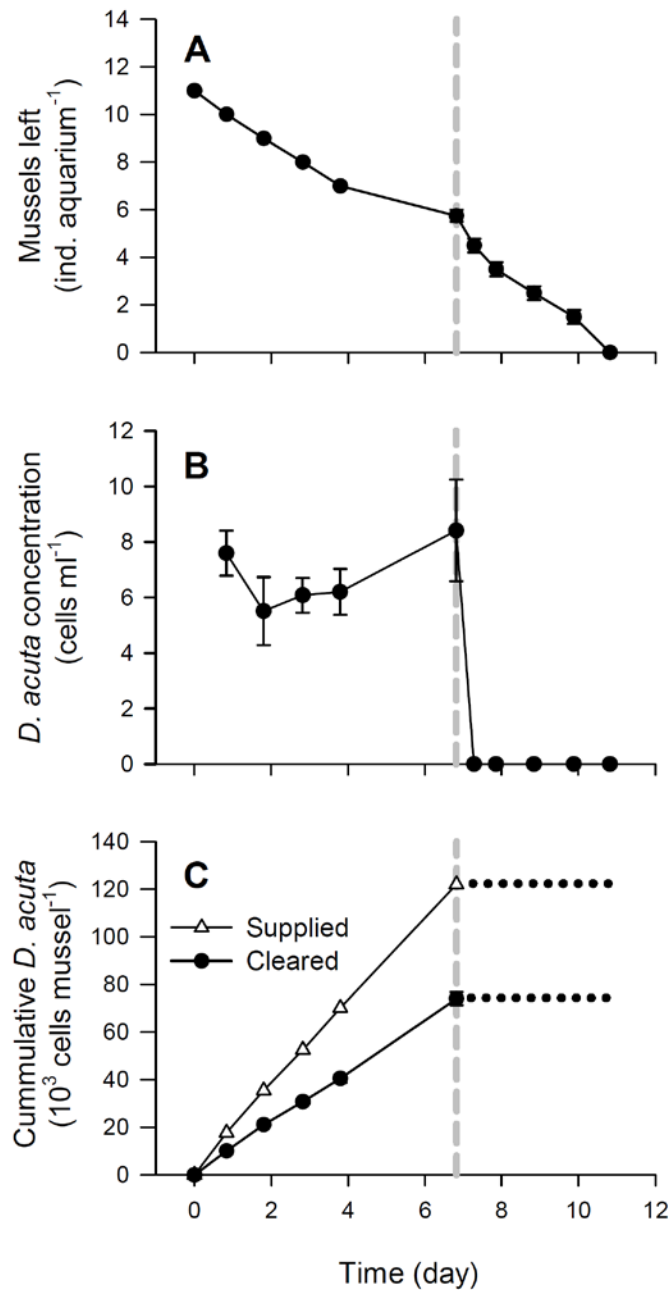
292 The inflow of *D. acuta* was  $17,711 \pm 267$  cells mussel $^{-1}$  day $^{-1}$ , but only  $10,719 \pm 149$  of these  
 were cleared by the mussels (Fig. 1C). Correspondingly, on day 7, a total of 122,000 *D. acuta* cells  
 294 had been provided per mussel, but only  $74,000 \pm 5,580$  of these had been cleared. Thus, the average  
 ingestion rate across the whole experiment was  $452 \pm 34$  *D. acuta* cells  $ind^{-1}\ h^{-1}$  (compared with the  
 296 maximum ingestion rates of  $1,028 \pm 240$  *D. acuta* cells  $ind^{-1}\ h^{-1}$  at steady state concentrations).

298

Table 2. Feeding and filtration characteristics of *Mytilus edulis* in the intoxication experiment with *Dinophysis acuta* and in  
 300 the control experiment using *Rhodomonas salina*. The steady-state concentration of *D. acuta* was also expressed as *R. salina*  
 equivalent using a biovolume conversion factor of 278 *R. salina* for each *D. acuta*. Note the distinction between the steady-  
 302 state ingestion rate (achieved once *D. acuta* concentrations had stabilized) and average ingestion rate over the whole  
 experiment. Values are means  $\pm$  SD.

	Steady state Cell concentration (cells $ml^{-1}$ )	<i>R. salina</i> equivalents (cells $ml^{-1}$ )	Clearance rate ( $l\ ind^{-1}\ h^{-1}$ )	Steady-state ingestion (cells $ind^{-1}\ h^{-1}$ )	Average ingestion (cells $ind^{-1}\ h^{-1}$ )
<i>D. acuta</i>	$6.8 \pm 2.3$	$1894 \pm 649$	$0.15 \pm 0.04$	$1028 \pm 240$	$452 \pm 34$
<i>R. salina</i>	$1898 \pm 495$	$1898 \pm 495$	$0.86 \pm 0.19$	-	-

304



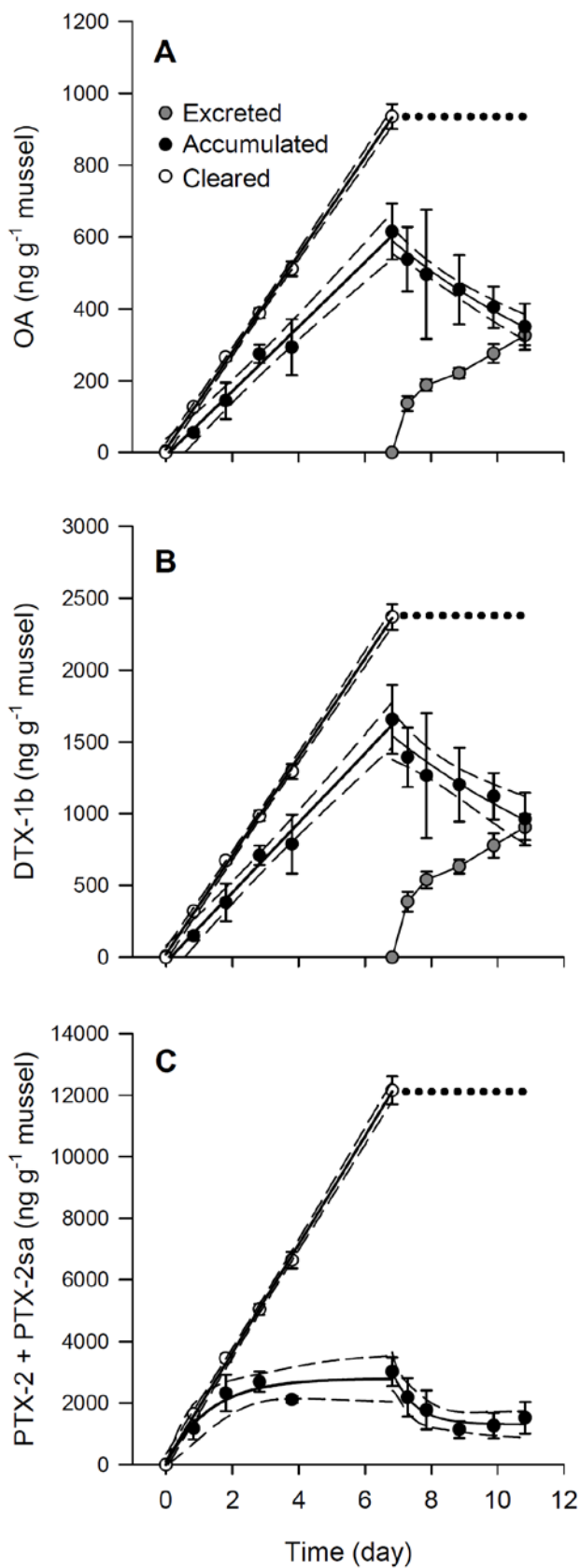
**Fig. 1.** Intoxication experiment with *Mytilus edulis* fed *Dinophysis acuta*. Mussel sampling scheme (A), steady-state *D. acuta* concentrations at each sampling (B) and cumulative *D. acuta* supplied and cleared (C) in the intoxication and depuration experiment with *M. edulis*. The vertical dashed line indicates when the *D. acuta* supply was stopped. Note that only the steady-state concentrations are depicted in B, not the increase between samplings. Symbols and bars are means  $\pm$  SD (n=4) except for the supplied amount of *D. acuta* (C), which are values from the *D. acuta* common supply culture (n=1).



306 **3.2. Intoxication**

All three toxins clearly accumulated in *M. edulis* during the seven day intoxication period (Fig. 2).  
308 Toxin clearance and accumulation in *M. edulis* were fitted with linear regression. Based on these  
fits, mussels cleared  $135.5 \pm 1.9$ ,  $343.2 \pm 4.8$  and  $1,760 \pm 24.5$  ng toxin  $\text{g}^{-1}$  mussel  $\text{d}^{-1}$  of OA, DTX-  
310 1b and PTX-2, respectively (Fig. 2 & table 3). Contents of both OA and DTX-1b (total, i.e.  
free+esters) increased linearly throughout the seven days of feeding, at rates of  $89.79 \pm 4.9$  and  
312  $242.3 \pm 12.0$  ng toxin  $\text{g}^{-1}$  mussel  $\text{d}^{-1}$ , respectively. This corresponds to a net retention of 66% for  
OA and 71% for DTX-1b. Thus, approximately two thirds of the cleared OA and DTX-1b was  
314 accumulated in the mussels. Most of the PTX-2 was immediately converted to PTX-2sa in the  
mussels (Fig. 3 C&D), and in contrast to OA and DTX-1b, the increase in [PTX-2 + PTX2sa]  
316 content was not linear but rather resembled an asymptotic rise towards a maximum value (Fig. 2 C).  
Thus, the net retention of PTX-2 observed during the first day was almost 100%, but this value  
318 decreased as the experiment progressed. On the final day of feeding, day 7, it had dropped to 25%  
(table 2).

320 The majority of the accumulated OA found in *Mytilus edulis* was found as esters; only  $27.1 \pm 4.2$   
% was found as free OA (Fig. 3A). Free DTX-1b accounted for  $45.9 \pm 5.3$  % of the total DTX-1b  
322 pool (Fig. 3B). Thus, esters of DTX-1b were less frequent compared to OA, but still comprised the  
majority of the total DTX-1b pool. PTX-2 and PTX-2sa were apparently both lower in hydrolyzed  
324 samples compared to non-hydrolyzed ones. This demonstrates that PTX-2 and PTX-2sa are  
unstable under the strong alkaline conditions present during the hydrolysis step. There may thus  
326 have been a significant pool of unquantified fatty acid esters of PTX-2 and PTX-2sa.



**Fig. 2.** Total toxin concentrations (hydrolyzed samples) of *Mytilus edulis* in the intoxication and depuration experiment compared to the amount cleared from *D. acuta*. OA (A), DTX-1b (B) and PTX-2 (C). Toxin accumulation until day 7 was fitted to a linear increase, and the subsequent toxin depuration was fitted either to a simple exponential decay function (OA and DTX-1b) or a two-compartment exponential decay model (PTX-2). Blue lines denote 95% confidence interval around the means. Also depicted are values of cumulative excreted OA (A) and DTX-1b (B) during the depuration period from day 7 onwards. This was not included for PTX-2 due to the inability to measure total PTX-2 (see text and Fig. 3). Symbols and bars are means  $\pm$  SE (n=4).

330 **Table 3. Regression parameters of DSP toxin clearance, accumulation and loss in *Mytilus edulis*. Slopes and coefficients are**  
 332 **reported as means  $\pm$  SD. TAE = toxin accumulation efficiency.  $t_{1/2}$  = half-life of each toxin calculated as exponential decay**  
**functions with the exponential coefficients, bshown in the table. OA and DTX-1b includes fatty acid esters, and PTX-2**  
**includes PTX-2sa, the primary form of PTX-2 in the mussels. \* = TAE of PTX-2 decreased over time and the value reported**  
**here is that observed for the entire 7-day feeding period.**

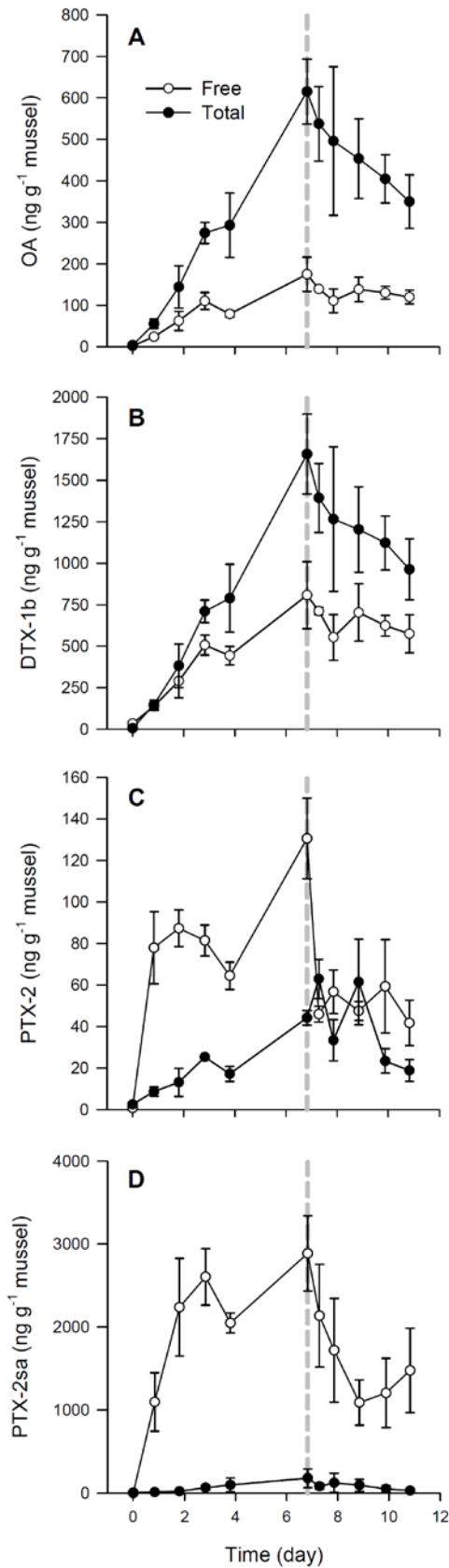
	Cleared		Accumulated			Exponential loss		
	$r^2$	Slope ( $\text{ng g}^{-1} \text{d}^{-1}$ )	$r^2$	Slope ( $\text{ng g}^{-1} \text{d}^{-1}$ )	TAE %	$r^2$	$b \pm \text{S.D.}$	$t_{1/2}$ (days)
OA	0.99	$136 \pm 2$	0.99	$90 \pm 5$	66%	0.97	$0.13 \pm 0.01$	5.3
DTX-1b	0.99	$343 \pm 5$	0.99	$242 \pm 12$	71%	0.90	$0.12 \pm 0.02$	5.8
PTX-2	0.99	$1760 \pm 25$	-	-	25%*	0.69	$0.24 \pm 0.01$	2.9

### 334 **3.3. Depuration**

Total OA and DTX-1b contents in *M. edulis* began to decrease immediately after feeding was  
 336 terminated (Fig. 2B & C). The decreases were fitted to simple exponential decay functions ( $y = a \times e^{-bx}$ ) and these gave good  $r^2$  values (table 3). The fitted exponential decay function yielded  
 338 exponential coefficients of  $0.131 \pm 0.012$  for OA and  $0.120 \pm 0.021$  for DTX-1b. This corresponds to half-life times of 5.3 and 5.8 days, respectively (table 3). Depuration of [PTX-2 + PTX-2sa] was  
 340 poorly described ( $r^2 = 0.69$ ) by the simple one-compartment exponential decay function used for  
 OA and DTX-1b. This likely owes to the difficulties measuring PTX-2sa fatty acid esters discussed  
 342 later.

### **3.4. Dissolved toxins**

344 Hydrolysis of the HP20 resin and HP20 resin extracts showed that toxins dissolved in the water  
 consisted exclusively of the free-forms. No toxin esters were found in the water during the  
 346 depuration period. Dissolved toxins were found during the entire depuration period (day 7-11). The  
 cumulative amounts of dissolved (excreted) toxins increased to  $326 \pm 53 \text{ ng OA g}^{-1}$  mussel and  $905$   
 348  $\pm 180 \text{ ng DTX-1b g}^{-1}$  mussel. This slightly overestimated the observed decrease in mussel toxicity,  
 accounting in total for 123 % and 130 % of the loss in mussel toxicity of OA and DTX-1b,  
 350 respectively (Fig. 2A & B). PTX-2 and PTX-2sa were not measured in the dissolved fraction.

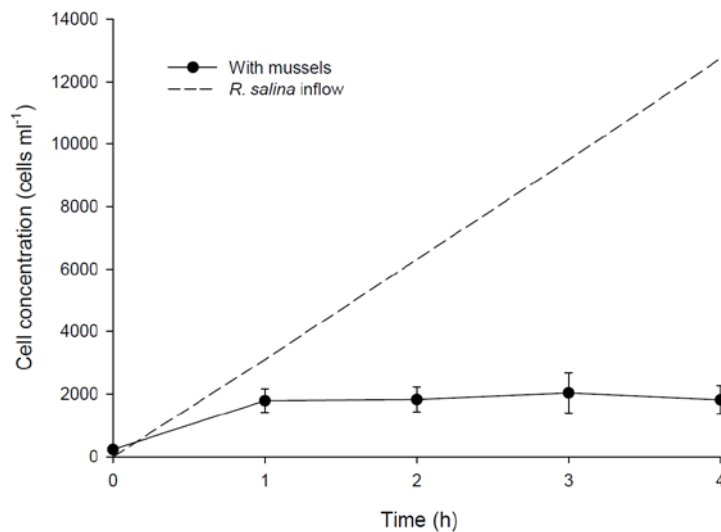


**Fig. 3.** Concentration of total and free toxins in *Mytilus edulis* during the intoxication and depuration experiment. OA (A), DTX-1b (B), PTX-2 (C) and PTX-2sa (D). The difference between total and free toxin represent toxin esters. Pectenotoxins are destroyed during the hydrolysis process, thus giving lower values in the ‘total’ samples compared to the ‘free’ samples. Symbols and bars are means  $\pm$  SE (n=4).

352 **3.5. Clearance rate with non-toxic prey**

The *Rhodomonas salina* concentrations stabilized within the first hour of the control experiment, 354 indicating that the mussels were active (Fig. 4). The steady-state concentrations of *R. salina* in the last three hours of the control experiment was  $1898 \pm 452$  cells  $\text{ml}^{-1}$  (mean  $\pm$  SD). From equation 2, 356 this corresponds to an average clearance rate of  $0.86 \pm 0.19$  l  $\text{ind}^{-1}$   $\text{h}^{-1}$ . This was statistically significantly higher than the clearance rate of  $0.15 \pm 0.04$  l  $\text{ind}^{-1}$   $\text{h}^{-1}$  observed when eating *D. acuta* 358 (t-test,  $p < 0.001$ ,  $n = 4$ ). The average shell length of the mussels used in the control experiment was  $22.83 \pm 1.58$  mm and the average wet weight was  $0.175 \pm 0.038$  g. Thus, the mussels used in the 360 control experiment were slightly smaller than those used in the DST intoxication experiment (compare with table 1).

362



**Fig. 4.** Clearance of the non-toxic cryptophyte *Rhodomonas salina* by *Mytilus edulis*. The dashed line indicates cumulative *R. salina* inflow in the absence of *M. edulis*. Values are means  $\pm$  SD ( $n = 4$ ).

364

## 4. Discussion

### 366 4.1. Accumulation

Okadaic acid and DTX are powerful inhibitors of serine/threonine protein phosphatases and may in addition cause various chronic effects (Valdiglesias et al. 2013). Pectenotoxins disrupts actin in the cytoskeleton, and may cause cell cycle arrest and cell death (Spector et al. 1999; Ares et al. 2005; Anonymous 2009). Thus, accumulation of both toxin groups is undesirable at best. Nevertheless, blue mussels, *Mytilus edulis*, used in this experiment accumulated large amounts of both OA and DTX-1b when feeding on *Dinophysis acuta*.

Mussel toxicity relative to the total amount of ingested toxins demonstrated net retention of 66 and 71 % for OA and DTX-1b, respectively. This is similar to the net retention of 63 % previously found for an unspecified mussel species, when using OA encapsulated in gelatin-acacia microcapsules (Rossignoli et al. 2011a). It is, however, much higher than the 1-10% found in the only previous study to use live dinoflagellates as prey (Bauder et al. 2001), but this study did not include toxin esters and may consequently have missed a substantial part of the toxin pool. Available evidence thus suggests that *M. edulis* accumulate the majority of both OA and DTX ingested.

PTX-2 also accumulated rapidly in the mussels (mostly as PTX-2sa), although the net retention appeared to decrease during the seven-day intoxication period, from nearly 100% after the first day to  $\approx 25\%$  at the end of the seven-day intoxication period. This is the first study to examine accumulation kinetics of PTX in a shellfish species. All three toxins can be transformed to various analogue compounds following ingestion by mussels, and a transformation of PTX-2 and PTX-2sa to compounds not included in the LC/MS-MS analysis could provide a compelling explanation of the apparent decrease in net retention of PTX-2 during the seven days of *D. acuta* exposure.

388 **4.2. Transformation**

OA and DTX may be transformed to various fatty acid esters analogues upon ingestion by mussels.  
390 In our experiment, approximately half the OA and DTX-1b in *M. edulis* were found as esters, and  
previous studies have shown high variability in the proportion of esters in various shellfish species  
392 (Vale and de M. Sampayo 2002a, Morono et al. 2003, Vale 2006, Torgersen et al. 2008). Rossignoli  
et al. (2011) argued that esterification is a step in the depuration process based on the observation  
394 that almost all the OA found in faeces of *Mytilus galloprovincialis* were esters. In our experiment,  
however, all excreted OA and DTX-1b were on the free form – not esters. The apparent discrepancy  
396 may illustrate species specific differences, but it may also reflect differences between excretion as  
faeces and in the dissolved form (e.g. urine).

398 PTX-2sa constituted the majority of the total PTX-2 pool in mussels at all times, indicating that  
PTX-2 was rapidly transformed into PTX-2sa. Large fractions of PTX-2sa have been found in  
400 mussels before, and shellfish (including mussels) have previously been demonstrated to facilitate  
the conversion of PTX-2 into PTX-2sa (Suzuki et al. 2001a, b, Vale and de M. Sampayo 2002a,  
402 Vale 2004). Our results support this, and further demonstrate that the process is rapid, and that large  
amounts of PTX-2 can be transformed via this process daily, even in the small mussels examined  
404 here. Transformation to seco acid may be a protective measure as PTX-2sa has a low toxicity  
compared to PTX-2 (Miles et al. 2004a, 2006c)

406 The net accumulation of [PTX-2 + PTX-2sa] apparently decreased as the experiment progressed  
and by the end of the feeding period, only 25% of the ingested PTX-2 could be accounted for in the  
408 mussels. This is most likely explained by a further transformation of PTX-2sa into fatty acid esters  
as recently observed by others (Wilkins et al. 2006, Torgersen et al. 2008). The build-up of PTX-  
410 2sa suggests that the conversion from PTX-2sa to the different PTX-2sa esters is a slower process  
than the conversion from PTX-2 to PTX-2sa.

412 We also found that PTX-2 and PTX-2sa are unstable under the strong basic conditions present  
during the hydrolysis step. This is in accordance with earlier attempts to hydrolyse PTX-2 (Vale and  
414 de M. Sampayo 2002; Miles 2007; Anonymous 2009). To quantify PTX-2 esters, one can either (1)  
hydrolyse samples enzymatically as demonstrated by Doucet et al. (2007) or (2) include all known  
416 PTX esters in the LC-MS analysis as done by Torgersen et al. (2008). Only esters with known mass  
transitions can be included, however, and standards of PTX esters are not yet commercially  
418 available. Thus, with the LC/MS-MS method applied here, we most likely overlooked the majority  
of the total PTX-2 pool, and this should be considered when monitoring shellfish toxicity using  
420 LC/MS-MS.

### 4.3. Depuration

422 Mussel toxin contents started decreasing immediately after feeding with *D. acuta* was terminated.  
The calculated half-life times of 5.3 and 5.8 days for OA and DTX-1b respectively, are somewhat  
424 fast compared to literature values: Marcaillou et al. (2010) found a half-life time of 20 days for 4-7  
cm long, unfed *Mytilus edulis*. However, this discrepancy could potentially be related to the size  
426 difference of the mussels used. Mussel size has previously been speculated (but not proven) to be  
negatively correlated with toxin depuration rate (Duinker et al. 2007). The depuration rates in the  
428 present study cover only the first few days of depuration, and if there is both a labile and a more  
slowly depurated toxin pool as suggested by Blanco et al. (1999) and Moroño et al. (2003), then our  
430 one-compartment model could overestimate the depuration rates.

PTX depuration is difficult to evaluate due to the fact that we did not measure PTX esters. The  
432 half-life times acquired from the applied two-compartment model are likely flawed.

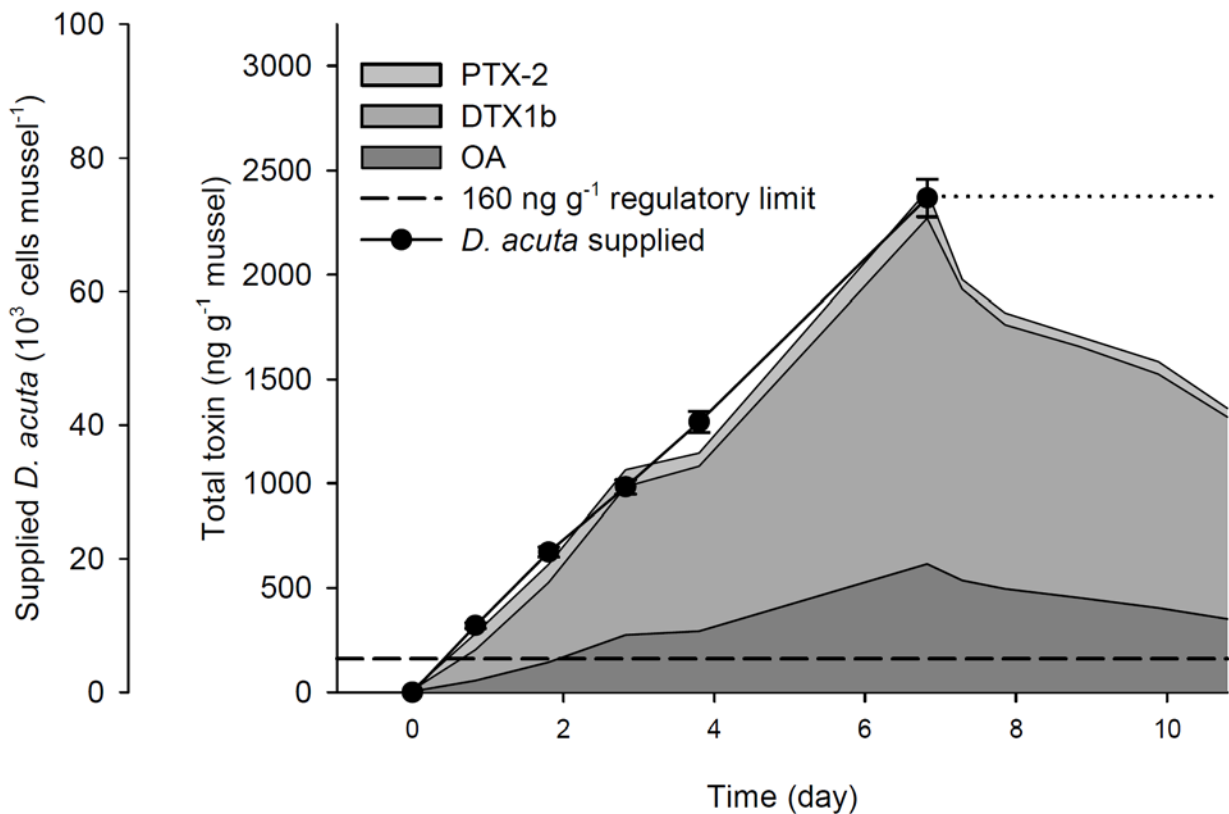
Gross rates of toxin accumulation can be estimated by combining net rates of toxin accumulation  
434 with depuration rates. By the end of the feeding period, the gross accumulation accounted for 95 %  
and 99 % of the ingested OA and DTX-1b, respectively. Thus, effectively all ingested OA and



436 DTX-1b seems to be taken up in the digestive tract of *M. edulis*. No significant amount was  
excreted directly with remains of the food source without first being absorbed by the intestine. On  
438 the same note, the amount of excreted toxins found in the water during the depuration period  
accounted for all of the observed loss in mussel toxicity. Thus, we have demonstrated that *M. edulis*  
440 depurated solely through excretion of toxins rather than metabolizing. This is the first study to  
establish a mass balance of DSP toxins in a mussel species during accumulation and subsequent  
442 depuration. It should be mentioned, that the situation described here is a controlled laboratory  
experiment, where several factors diverge from *in situ* conditions. Most importantly these include,  
444 but are not limited to, initial starvation and the single-species diet offered.

#### 4.4. Total toxicity

446 Okadaic acid, dinophysistoxins and pectenotoxins are currently still considered together for  
regulatory purposes (European Commission 2011). Using a toxicity equivalent factor (TEF) for  
448 each compound, the total toxicity is expressed in OA equivalents. The TEF of PTX-2sa is set to  
zero rendering this compound unregulated and excluded from a total toxicity measure. OA and  
450 PTX-2 are both regulated, whereas DTX-1b is not because it was only recently discovered and a  
TEF has yet to be assigned (Nielsen et al. 2013). If we assume a TEF similar to DTX-1 (TEF=1),  
452 it would be necessary to regulate DTX-1b in order to protect consumer health. Fig. 5 shows the  
cumulative amount of OA, DTX-1b and PTX-2 in *M. edulis* during the experiment. We included  
454 DTX-1b since it will likely add to total toxicity, but refrain from calculating a total toxicity in OA  
equivalents since DTX-1b does not yet have a TEF. Even under the low *Dinophysis* spp.  
456 concentrations used in the present study, mussels exceeded the regulatory limit of  $160 \mu\text{g kg}^{-1}$   
within the first day of exposure to *D. acuta* (Fig. 5). This was unexpected, and largely owes to the  
458 finding that practically all ingested toxin was absorbed in the intestine, leaving nothing to  
immediate excretion with faeces.



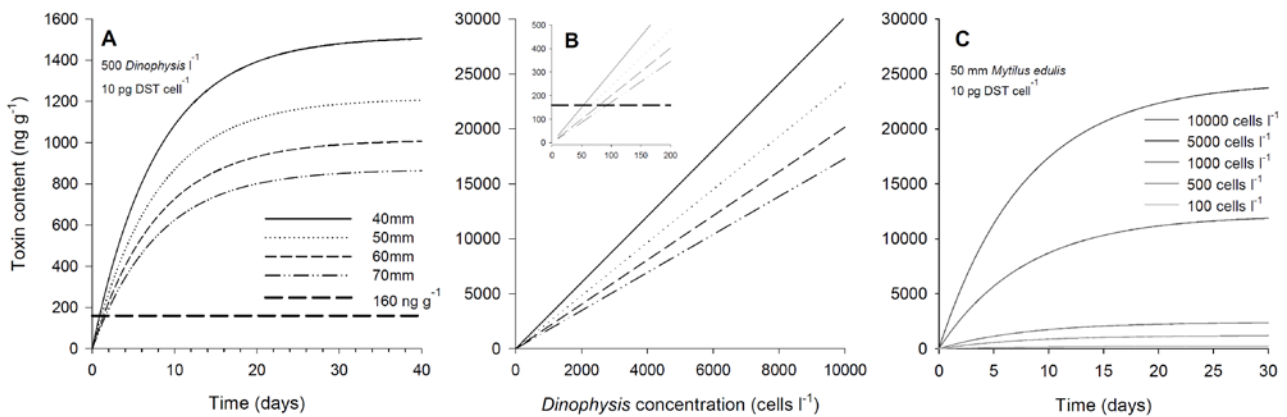
**Fig. 5.** Total toxin (OA + DTX-1b + PTX-2) content of *Mytilus edulis* and the cumulative amount of ingested *Dinophysis acuta* during the intoxication and depuration experiment. Mussels exceeded the combined OA+DTX+PTX regulatory limit of 160 ng g<sup>-1</sup> wet weight within the first day.

#### 460 **4.5. Modelled toxin content**

Accumulation and depuration kinetics are useful in understanding DST intoxication events of *M.*  
 462 *edulis*. It is currently unknown, to what extent the observed toxin accumulation efficiencies and  
 depuration rates can be extrapolated to different sized mussels in natural populations and to  
 464 situations where *Dinophysis* spp. are ingested at different concentrations and in combination with  
 other food particles. Adopting the observed accumulation and depuration kinetics (of OA and DTX-  
 466 1b), and using literature values of clearance (Kiørboe and Møhlenberg 1981) and wet weight (Jones  
 et al. 1992) of different sized mussels, one can model the toxin content of various sized mussels

468 under different conditions (Fig. 6) Such calculations reveal, that a moderately toxic (similar to the  
 OA+DTX-1b content of *D. acuta* in this study = 10 pg DST cell<sup>-1</sup>) population at sub-bloom  
 470 densities (500 *D. acuta* cells l<sup>-1</sup>) would make *M. edulis* of any size exceed the regulatory threshold  
 of 160 ng g<sup>-1</sup> within only one or two days of exposure (Fig. 6A). Also, a concentration of only ≈75  
 472 *D. acuta* cells l<sup>-1</sup> of moderate toxicity (10 pg DST cell<sup>-1</sup>) is enough to make 60 mm long *M. edulis*  
 exceed the regulatory threshold within ≈10 days (Fig. 6B). Due to the increased weight-specific  
 474 clearance rate, smaller mussels would require even fewer *D. acuta* per litre in order to potentially  
 exceed the threshold. Finally, as an example, a 50 mm long mussel would exceed the regulatory  
 476 threshold within a day at concentrations from 500 *D. acuta* cells l<sup>-1</sup>, but it may take up to 20 days to  
 approach steady-state toxin concentrations in the mussels (Fig. 6C).

478



**Fig. 6.** Modelled toxin contents of *Mytilus edulis* based on the observed toxin accumulation efficiencies of OA and DTX-1b and literature values of clearance rates and wet weight of different sized mussels. (A) Toxin content as a function of time when subjected to a stable 500 cells l<sup>-1</sup> *Dinophysis acuta* concentration of moderate toxicity (10 pg DST cell<sup>-1</sup>), corresponding to the total OA and DTX-1b found for *D. acuta* in this study. (B) Maximum achievable toxin content as a function of *D. acuta* cell concentration of the same toxicity. Legend as in A. (C) Toxin content of a 50 mm long *M. edulis* at five different *D. acuta* concentrations from 100-10,000 cells l<sup>-1</sup> as a function of time. Note the different y-axis scales.

#### 480 **4.6. Feeding physiology and effects of DSTs on mussels**

Clearance rates of *M. edulis* were much lower when feeding on *D. acuta* compared to the non-toxic  
482 cryptophyte *Rhodomonas salina* (Fig. 4, table 3). Thus, intake of *D. acuta* seems to affect the  
filtration of *M. edulis*. Similar effects are caused by other toxic algae, including saxitoxin  
484 containing *Alexandrium* spp. (e.g. May et al. 2010), azaspiracid containing *Azadinium spinosum*  
(Jauffrais et al. 2012) and karlotoxin containing *Karlodinium veneficum* (Brownlee et al. 2008).  
486 Further studies are needed, however, in order to determine the extent and severity of the effect of *D.*  
*acuta* on mussel filtration, and to determine whether the effect owes to one or more of the DSP  
488 toxins.

#### 490 **Conflict of interest**

The authors declare no conflict of interest.

492

#### **Acknowledgements**

494 We thank Wolfgang Drebing for technical assistance with toxin measurements. The work was  
funded by project no. 483 2101-07-0084 and project no. 10-078561 from The Danish Counsel for  
496 Strategic Research for PJH and BV.

## Literature cited

- 498 Ares IR, Louzao MC, Vieytes MR, Yasumoto T, Botana LM (2005) Actin cytoskeleton of rabbit intestinal cells is a target for potent marine phycotoxins. *J Exp Biol* 208:4345–4354
- 500 Bauder AG, Cembella AD, Bricej VM, Quilliam MA (2001) Uptake and fate of diarrhetic shellfish poisoning toxins from the dinoflagellate *Prorocentrum lima* in the bay scallop *Argopecten*  
502 *irradians*. *Mar Ecol-Prog Ser* 213:39-52
- Blanco J, Fernandez ML, Miguez A, Morono A (1999) Okadaic acid depuration in the mussel  
504 *Mytilus galloprovincialis*: one- and two-compartment models and the effect of environmental conditions. *Mar Ecol-Prog Ser* 176:153–163
- 506 Brownlee EF, Sellner SG, Sellner KG, Nonogaki H, Adolf JE, Bachvaroff TR, Place AR (2008)  
508 Responses of *Crassostrea virginica* (Gmelin) and *C. ariakensis* (Fujita) To Bloom-Forming Phytoplankton Including Ichthyotoxic *Karlodinium veneficum* (Ballantine). *J Shellfish Res* 27:581–591
- 510 Doucet E, Ross NN, Quilliam MA (2007) Enzymatic hydrolysis of esterified diarrhetic shellfish poisoning toxins and pectenotoxins. *Anal Bioanal Chem* 389:335–342
- 512 Draisci R, Lucentini L, Giannetti L, Boria P, Poletti R (1996) First report of pectenotoxin-2 (PTX-2) in algae (*Dinophysis fortii*) related to seafood poisoning in Europe. *Toxicon* 34:923–935
- 514 Duinker A, Bergslien M, Strand Ø, Olseng CD, Svardal A (2007) The effect of size and age on depuration rates of diarrhetic shellfish toxins (DST) in mussels (*Mytilus edulis* L.). *Harmful*  
516 *Algae* 6:288–300
- European Commission (2011) Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs Text with EEA relevance. <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32011R0015>  
518  
520
- 522 Fux E, Smith JL, Tong M, Guzmán L, Anderson DM (2011) Toxin profiles of five geographical isolates of *Dinophysis* spp. from North and South America. *Toxicon* 57:275–287
- 524 Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Grun. *Can J Microbiol* 8:229–239
- 526 Hackett JD, Tong M, Kulis DM, Fux E, Hess P, Bire R, Anderson DM (2009) DSP toxin production *de novo* in cultures of *Dinophysis acuminata* (Dinophyceae) from North America. *Harmful Algae* 8:873–879
- 528 Hinder SL, Hays GC, Brooks CJ, Davies AP, Edwards M, Walne AW, Gravenor MB (2011) Toxic marine microalgae and shellfish poisoning in the British isles: history, review of epidemiology,  
530 and future implications. *Environ Health* 10:54

- 532 Hoppenrath M, Elbrächter M (2011) Prorocentrales. In: IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae, <http://www.marinespecies.org/HAB>
- 534 Jauffrais T, Contreras A, Herrenknecht C, Truquet P, Séchet V, Tillmann U, Hess P (2012) Effect of *Azadinium spinosum* on the feeding behaviour and azaspiracid accumulation of *Mytilus edulis*. *Aquat Toxicol* 124-125:179–187
- 536 Jones HD, Richards OG, Southern TA (1992) Gill dimensions, water pumping rate and body size in the mussel *Mytilus edulis* L. *J Exp Mar Bio Ecol* 155:213–237
- 538 Kiørboe T, Møhlenberg F (1981) Particle Selection in Suspension-Feeding Bivalves. *Mar Ecol-Prog Ser* 5:291–296
- 540 Landsberg JH (2002) The Effects of Harmful Algal Blooms on Aquatic Organisms. *Rev Fish Sci* 10:113–390
- 542 Lewitus AJ, Horner RA, Caron DA, Garcia-Mendoza E, Hickey BM, Hunter M, Huppert DD, Kudela RM, Langlois GW, Largier JL, Lessard EJ, RaLonde R, Jack Rensel JE, Strutton PG, Trainer VL, Tweddle JF (2012) Harmful algal blooms along the North American west coast region: History, trends, causes, and impacts. *Harmful Algae* 19:133–159
- 546 Marcaillou C, Haure J, Mondeguer F, Courcoux A, Dupuy B, Pénisson C (2010) Effect of food supply on the detoxification in the blue mussel, *Mytilus edulis*, contaminated by diarrhetic shellfish toxins. *Aquat Living Resour* 23:255–266
- 550 May SP, Burkholder JM, Shumway SE, Hégaret H, Wikfors GH, Frank D (2010) Effects of the toxic dinoflagellate *Alexandrium monilatum* on survival, grazing and behavioral response of three ecologically important bivalve molluscs. *Harmful Algae* 9:281–293
- 552 Miles CO, Wilkins AL, Munday R, Dines MH, Hawkes AD, Briggs LR, Sandvik M, Jensen DJ, Cooney JM, Holland PT, Quilliam MA, MacKenzie AL, Beuzenberg V, Towers NR (2004a) Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon* 43:1–9
- 556 Miles CO, Wilkins AL, Samdal IA, Sandvik M, Petersen D, Quilliam MA, Naustvoll LJ, Rundberget T, Torgersen T, Hovgaard P, Jensen DJ, Cooney JM (2004b) A novel pectenotoxin, PTX-12, in *Dinophysis* spp. and shellfish from Norway. *Chem Res Toxicol* 17:1423–1433
- 560 Miles CO, Wilkins AL, Hawkes AD, Jensen DJ, Cooney JM, Larsen K, Petersen D, Rise F, Beuzenberg V, Lincoln MacKenzie A (2006a) Isolation and identification of a cis-C8-diol-ester of okadaic acid from *Dinophysis acuta* in New Zealand. *Toxicon* 48:195–203
- 562 Miles CO, Wilkins AL, Hawkes AD, Jensen DJ, Selwood AI, Beuzenberg V, Lincoln MacKenzie A, Cooney JM, Holland PT (2006b) Isolation and identification of pectenotoxins-13 and -14 from *Dinophysis acuta* in New Zealand. *Toxicon* 48:152–159

- 566 Miles CO, Wilkins AL, Munday JS, Munday R, Hawkes AD, Jensen DJ, Cooney JM, Beuzenberg  
568 V (2006c) Production of 7-epi-pectenotoxin-2 seco acid and assessment of its acute toxicity to  
mice. *J Agric Food Chem* 54:1530–1534
- Moroño A, Arévalo F, Fernández ML, Maneiro J, Pazos Y, Salgado C, Blanco J (2003)  
570 Accumulation and transformation of DSP toxins in mussels *Mytilus galloprovincialis* during a  
toxic episode caused by *Dinophysis acuminata*. *Aquat Toxicol* 62:269–280
- 572 Nielsen LT, Lundholm N, Hansen PJ (2007) Does irradiance influence the tolerance of marine  
phytoplankton to high pH? *Mar Biol Res* 3:446–453
- 574 Nielsen LT, Krock B, Hansen PJ (2013) Production and excretion of okadaic acid, pectenotoxin-2  
576 and a novel dinophysistoxin from the DSP-causing marine dinoflagellate *Dinophysis acuta* –  
Effects of light, food availability and growth phase. *Harmful Algae* 23:34–45
- Park M, Kim S, Kim H, Myung G, Kang Y, Yih W (2006) First successful culture of the marine  
578 dinoflagellate *Dinophysis acuminata*. *Aquat Microb Ecol* 45:101–106
- Reguera B, Riobó P, Rodríguez F, Díaz PA, Pizarro G, Paz B, Franco JM, Blanco J (2014)  
580 *Dinophysis* toxins: causative organisms, distribution and fate in shellfish. *Mar Drugs* 12:394–  
461
- 582 Rossignoli AE, Fernández D, Acosta CP, Blanco J (2011a) Microencapsulation of okadaic acid as a  
tool for studying the accumulation of DSP toxins in mussels. *Mar Environ Res* 71:91–93
- 584 Rossignoli AE, Fernández D, Regueiro J, Mariño C, Blanco J (2011b) Esterification of okadaic acid  
in the mussel *Mytilus galloprovincialis*. *Toxicon* 57:712–720
- 586 Shumway SE, Cembella AD (1993) The impact of toxic algae on scallop culture and fisheries. *Rev*  
*Fish Sci* 1:121–150
- 588 Spector I, Braet F, Shochet NR, Bubb MR (1999) New anti-actin drugs in the study of the  
organization and function of the actin cytoskeleton. *Microsc Res Tech* 47:18–37
- 590 Suzuki T, Mackenzie L, Stirling D, Adamson J (2001a) Pectenotoxin-2 seco acid: a toxin converted  
592 from pectenotoxin-2 by the New Zealand Greenshell mussel, *Perna canaliculus*. *Toxicon*  
39:507–514
- Suzuki T, Mackenzie L, Stirling D, Adamson J (2001b) Conversion of pectenotoxin-2 to  
594 pectenotoxin-2 seco acid in the New Zealand scallop, *Pecten novaezelandiae*. *Fish Sci* 67:506–  
510
- 596 Suzuki T, Walter JA, LeBlanc P, MacKinnon S, Miles CO, Wilkins AL, Munday R, Beuzenberg V,  
598 MacKenzie AL, Jensen DJ, Cooney JM, Quilliam MA (2006) Identification of pectenotoxin-11  
as 34S-hydroxypectenotoxin-2, a new pectenotoxin analogue in the toxic dinoflagellate  
*Dinophysis acuta* from New Zealand. *Chem Res Toxicol* 19:310–318

- 600 Svensson S (2003) Depuration of okadaic acid (Diarrhetic Shellfish Toxin) in mussels, *Mytilus edulis* (Linnaeus), feeding on different quantities of nontoxic algae. *Aquaculture* 218:277–291
- 602 Svensson S, Förlin L (2004) Analysis of the importance of lipid breakdown for elimination of  
604 okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis*: Results from a field study  
and a laboratory experiment. *Aquat Toxicol* 66:405–418
- 606 Torgersen T, Sandvik M, Lundve B, Lindegarth S (2008) Profiles and levels of fatty acid esters of  
okadaic acid group toxins and pectenotoxins during toxin depuration. Part II: Blue mussels  
(*Mytilus edulis*) and flat oyster (*Ostrea edulis*). *Toxicon* 52:418–427
- 608 Valdiglesias V, Prego-Faraldo MV, Pásaro E, Méndez J, Laffon B (2013) Okadaic acid: more than  
a diarrhetic toxin. *Mar Drugs* 11:4328–49
- 610 Vale P (2004) Differential dynamics of dinophysistoxins and pectenotoxins between blue mussel  
612 and common cockle: A phenomenon originating from the complex toxin profile of *Dinophysis*  
*acuta*. *Toxicon* 44:123–134
- 614 Vale P (2006) Detailed profiles of 7-O-acyl esters in plankton and shellfish from the Portuguese  
coast. *J Chromatogr A* 1128:181–188
- 616 Vale P, de M. Sampayo MA (2002a) Esterification of DSP toxins by Portuguese bivalves from the  
Northwest coast determined by LC-MS - A widespread phenomenon. *Toxicon* 40:33–42
- 618 Vale P, de M. Sampayo MA (2002b) Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and  
pectenotoxin-2 in shellfish and plankton from Portugal. *Toxicon* 40:979–987
- 620 Wilkins AL, Rehmann N, Torgersen T, Rundberget T, Keogh M, Petersen D, Hess P, Rise F, Miles  
CO (2006) Identification of fatty acid esters of pectenotoxin-2 seco acid in blue mussels  
(*Mytilus edulis*) from Ireland. *J Agric Food Chem* 54:5672–5678