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RE	Restricted to a group specified by the consortium (including the Commission	
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Summary

The objective of the study was to pinpoint a possible effect of the food on the decontamination speed up of mussels containing DTX toxins.

Studying the possible impact of food on the detoxification rate consists in i) following the toxin content over time for both feeding conditions and ii) checking the physiological state of the animals.

For this purpose, an experimental protocol has been designed to describe the kinetics and to compare the detoxification rate in fed and non fed toxic mussels. The experimental disposal was available in an experimental oyster farm where the water and food supply qualities were controlled. The food supply consisted in a monospecific culture of the diatom *Skeletonema costatum* which is widely used in the shellfish aquaculture. The diatom mother culture was distributed at two levels of dilution.

Due to a lack of available *Dinophysis spp* culture, contaminated shellfish was collected on site for the purpose of the study. The probability for *Dinophysis spp* blooms in French coastal waters is relatively high : Atlantic and Mediterranean areas are concerned by this and mussel farms are severely affected every year.

The bivalve physiological state was evaluated from two parameters: the condition index and biochemical parameters, lipid and glycogen, constituting a major form of energy storage in the mussel. The chemical analysis was performed mainly on digestive glands but it was also planned to verify whether the meat was toxin free.

A possible toxin release in the medium was investigated qualitatively via toxin detection in bio-deposits by collecting samples at various times during the trial. In order to detect any dissolved toxins. SPATT resin sachets (Solid Phase Adsorption Toxin Tracking) were suspended between the tray stacks and retrieved several times during the experiment. Three experiments were conducted during the duration of the contract.

The experimental facility made it possible to maintain a significant mass of live mussels in proper conditions. In act, the mortality rates and the relatively stability – and even the trend towards an increase- of the biochemical parameters attested that the experimental conditions did not affect the animals. The lipid concentrations in the digestive glands show fluctuations that could be the result of an adaptation of the new medium (mainly the water and food quality) but they stabilized at the initial value in the tank without food whereas they tended to increase in the tank with food.

On the whole, the study describes a decrease in the toxins concentration of mussels of approximately 90% when they are fed and of approximately 65% when they are kept in clear water after 3 weeks in the experimental medium.

Since there has been no increase in the biomass whatever the trial as shown either by the condition indices or the individual mean weights of dry flesh, decontamination is not the result of a dilution effect. It is the results of a true loss of toxin content.

The analyses of the results obtained on mussels from Britanny, suggest a distinction of two phases in the evolution of the mussel toxicity. The first days, an over-contamination was observed in some cases, interpreted as a release of toxins from the phytoplanktonic cells in the gut of the bivalves. After the third day, the attempts to describe the decontamination kinetics led to two possible models i.e, a linear relationship and a decreasing exponential, meaning . that the model cannot be definitively established.

The bio-deposit analysis showed an toxin elimination mainly as an ester form. Concerning the passive adsorption of OA on the resin (SPATT bags), the results from the last two experiments were diverging : in June 2006 no toxin or traces were detected while a variable amount of OA was measured in June 2007. A toxin release is therefore detectable in the decontamination processes.

Considering the field situation for the two consecutive years, the shellfish market was re-opened on the fifth week after the beginning of the experiments in accordance with the monitoring survey.

Consequently, in the conditions of this study, the time to decontaminate the mussels with artificial means is not very different from the time needed in the field to collect safe mussels. Nevertheless, the work has demonstrated the positive role of the food on the detoxification rate and shows future ways of investigation to improve the decontamination processes.

1 Introduction

The shellfish industry occupies the number one place out of all the marine aquaculture p roduction in Europe. It relies mainly on bivalve mollusc production amounting to 756 095 tonnes (live weight) in 2004. 78% of which are mussels, 17% oysters and 5% of which are other bivalves. This sector underscores all socio-economic considerations concerning coastal areas as demand for either fresh or processed products continues to increase. In 2006, the estimated turnover approaches 1,100 millions Euros and directly employs 37,000 workers. Moreover, this production complies with the criteria of a sustainable activity, given that it operates and optimises natural processes.

Nevertheless, the production of shellfish farming is being drastically hampered by the development of toxic micro-algae. Among the phytoplankton flora, certain toxic species proliferate sporadically in unknown environmental conditions. As filter-feeders, the bivalves accumulate toxins and may become unsafe for human consumption. Three kinds of shellfish intoxication are commonly encountered in Europe : Paralytic Shellfish Poisoning (PSP), Diarrhoeic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP). Whereas PSP presents the most serious risk in term of public health, DSP is the most serious issue pertaining to shellfish farming.

The toxins involved in the DSP syndrome comprise those toxins mainly belonging to the okadaïc acid family and the azaspiracid family. The producer species for the first family belong to the *Dinophysis* genus but there are currently no controls for this culture in Europe and the origin of the azaspiracids is still being investigated. The data from the monitoring surveys shows that the phenomenon affects almost all European countries with unforeseen frequency, duration and spread.

The resulting economical losses are difficult to quantify, given that the stock is not suddenly destroyed. In most cases, the bans on shellfish harvesting or marketing last several weeks or months depending on the areas. This delay unsteadies the market and customers are compelled to source other suppliers or chose other products. In addition, the shellfish replacement is prevented by the obligation of having to keep the stock in water as is the case for the mussels reared on Bouchots and must be replaced by new spawn between May-July.

As a result, several attempts have been made in order to curb the economic losses.

Firstly, different methods based on contamination avoidance have been prospected.

- The submersion of the bivalves into deeper layers where the concentrations of the toxic algae is smaller.
- The transfer of the bivalves to a natural area free of toxic algae.

Secondly, because of the lobbying of the shellfish farmers, research has been launched in order to attempt to improve the decontamination process. That implies the transfer of the bivalves to inshore facilities permitting more or less the control of certain parameters (water quality, varied food supply).

The controlling of temperature was firstly considered as a favourable factor but the effect has not been demonstrated. On the contrary, the studies performed in Northern Europe have related mussel decontamination during the Spring diatom bloom when the water temperature was approximately zero degrees (Dahl and Yndestadt, 1985 ; Lindahl and Hageltorn, 1986; Svensson and Förlin, 2004).

On the other hand, food supply seems to be significant in the decontamination processes but it was mainly described for PSP toxins. As a matter of fact, despite their great interest , the decontamination of lipophilic toxins is far less documented and in particular, there exists no available information for AZA depuration in bivalves. Concerning the dinophysitoxins, certain studies have been carried out to estimate the depuration rate of naturally contaminated mussels by transplantation from contaminated areas to a free toxic algae environment or to shellfish farming units using re-circulating seawater with or without food. On the whole, the observations converge towards a positive effect of the food upon toxin elimination but over a more or less long period of time. A review of these studies (Blanco et *al*., 2005), relates the high variability of the registered detoxification rates. The commonly accepted model is that of biphasic depuration with a rapid first phase followed by a slow phase which can last several weeks and even several months. Nevertheless, the authors draw attention to the relevance of such a comparison by taking into account the heterogeneity in the study conditions (field or laboratory) and in the parameters measured (esters or not, bivalve condition, food quantity if any,etc.).

In fact, recent studies performed in the field, do not lend credence to the fact that the food enhances decontamination speed-up. In Sweden, Susanne Svensson (2003) does not observe a difference in the depuration rates between fed and non fed mussels over a 32 day period. However, she has only measured the okadaic acid. When comparing the speeds of decontamination, other authors, (Duinker *et al*., 2007) do not describe any difference in the two age classes of mussels whereas their feeding behaviour is normally different. They conclude that the food is not a preponderant factor in the elimination of dinophysistoxins.

Referring to the review of Blanco et al (2005).and to our previous work (Marcaillou *et al*., 1993), it seemed opportune to propose, within the framework of sixth PCRD, a study on the food effect within a controlled environment by taking into account the acyled toxins and the bivalve condition. Mussel was chosen as a biological model because this bivalve is the most affected by the lipophilic toxins in comparison with other edible bivalves.

As the culture of a *Dinophysis* strain is not available, we were dependent on natural events for the supply of the toxic material. What is more, the difficulty in the advance planning of the experiments was not a negligible constraint in the achievement of the study.

2 Materials and Methods

2.1 Context of monitoring survey at the moment of the toxic mussel sampling

An examination of all the data recorded by the phycotoxin network (REPHY) all along the French coasts, shows that toxic events due to *Dinophysis* proliferation are recorded almost every year with a variable frequency and duration. Two sites were particularly targeted for the purpose of the project because they dispayed a relatively high probability for harvesting toxic mussels. They are the shellfish production areas located in South Britanny (Atlantic coast) and the Leucate coastal lagoon along the western Mediterranean coast (Figure 1). In fact, we succeeded in setting up three experiments with toxic mussels from these two areas.

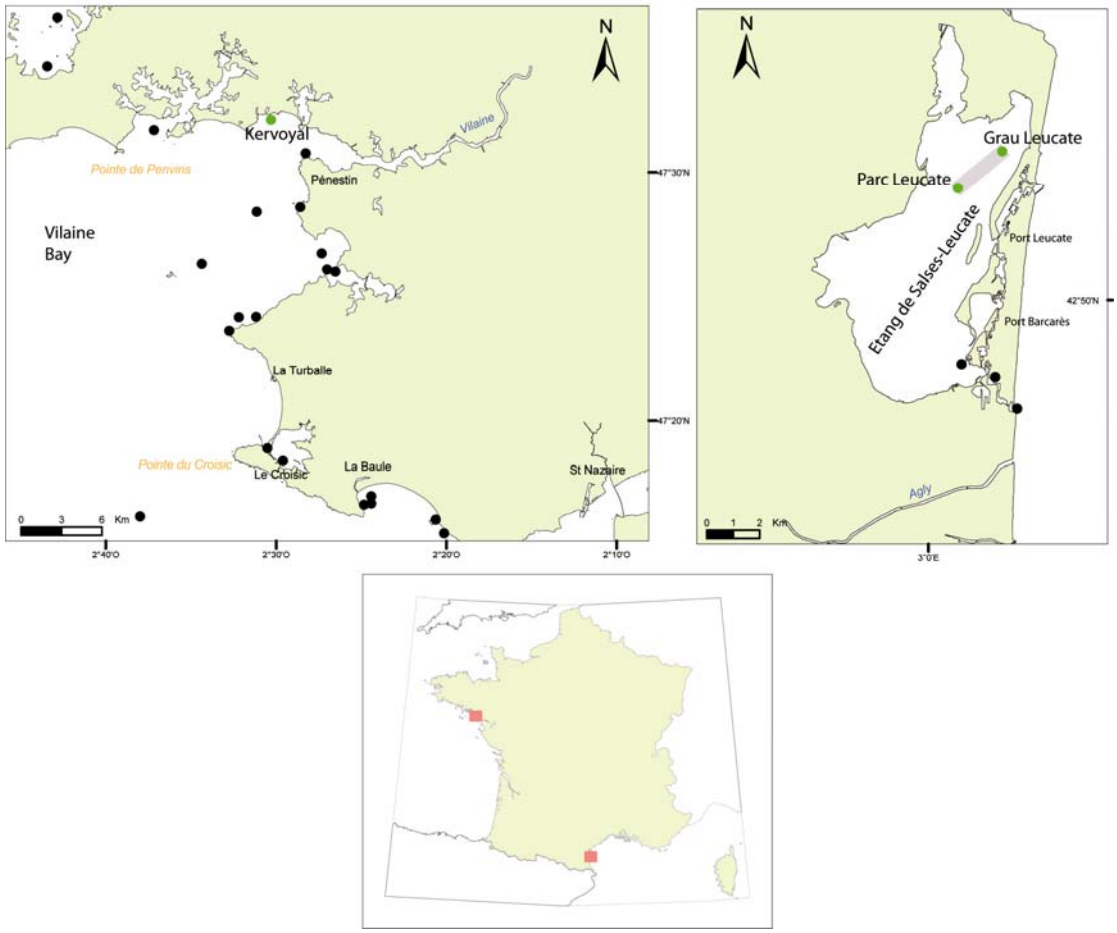


Figure 1 : Localisation of the sites where the toxic mussels were harvested for the experiments

The year 2005, the first year of the research contract, was unusual from a climatic standpoint. We had no opportunity of conducting a decontamination trial during the period when toxic blooms are most likely to occur. A *Dinophysis acuta* bloom

nevertheless occurred late that year in the Leucate lagoon at concentrations exceeding a thousand cells per litre. In order not to miss an opportunity it was decided to launch

the first experiment in early January 2006 in spite of an estimation of the mussel toxicity close to the sanitary threshold. As planned, the following two experiments were conducted in June/July, on mussels harvested on the South Britanny coast.

The data recorded by the monitoring survey during these three toxic events is described in Table 1, 2 and 3 :

Sampling dates 2006	***Dinophysis* concentration cell.l^{-1}**		**Mouse test (+++ : positive test)**	**Chemical analysis results µg eq.AO. kg^{-1} fresh flesh**
	Salses	Parc Leucate		
19-23 December	1200	300	+++	176
26-30 December	700	200	+++	211
2-6 January	500	400	+++	No analysis
9-13 January	**200**	**0**	**+++**	**183**
16-20 January	500	100	+++	-
23-27 January	0	0	-	-

Table 1 : Trend in *Dinophysis spp* concentration and mussel toxicity recorded by the monitoring network (REPHY) before and after sampling at Leucate in 2006 (shown in grey)

Sampling dates 2006	***Dinophysis* concentration cell.l^{-1} Kervoyal**	**Mouse test (survival time)**	**Chemical analysis results µg eq. OA.kg^{-1}**
2 May	0	——	
9 May	400	——	
15 May	2300	——	
22 May	No sampling	+++ (10h)	?
29 May	4900	+++ (4h)	272
5 June	100	+++ (2h)	508
12 June	800	+++ (1h15)	1012
19 June	600	+++ (10mn)	?
26 June	100	+++ (14h)	
3 July	0	——	

Table 2 : Trends in *Dinophysis spp* concentration and mussel toxicity recorded by the monitoring network (REPHY) before and after mussel sampling at Kervoyalin 2006 (shown in grey)

Sampling dates 2007	*Dinophysis* concentration Cell.l^{-1}	Mouse test (survival time)	Chemical analysis µg eq.OA.kg^{-1}
4 May	No sampling	Negative	
11 May	No sampling	+++ (23h40)	59
18 May		+++ (4 minutes)	61
25 May	200	Negative	
1st June	0	Negative	
8 June	400	Negative	
15 June	200	+++ (1h25)	
22 June	**1800**	**+++ (1h20)**	
29 June	11100	+++ (3h40)	
6 July	No sampling	+++ (4h10)	
13 July	400	+++ (5h35)	
20 July	100	Negative	

Table 3 : Trends in *Dinophysis* concentration and mussel toxicity recorded by the monitoring network (REPHY) before and after the mussel sampling at Kervoyal in 2007 (shown in grey)

The characteristics of the mussels that were the object of the experiment are described in Table 4. The Mediterranean mussels distinguish themselves from the Atlantic mussels by their species and their toxin profile. Their size range was roughly estimated on 90% of the total sample.

Site of origine	Phytoplankton species	Mussel species	Size Min-max *(cm)*	Initial toxicity (wet flesh)
Leucate lagoon January 2006 **Leu 06**	*Dinophysis acuta*	*Mytilus galloprovincialis*	3 classes 3-5 5-7 8-9	92 µg.eq.OA .kg^{-1} + 91.µg PTX .kg^{-1}
Kervoyal June 2006 **Kerv 06**	*Dinophysis acuminata*	*Mytilus edulis*	4-7	916 µg eq.OA .kg^{-1}
Kervoyal June 2007 **Kerv 07**	*Dinophysis acuminata*	*Mytilus edulis*	4-7	340µg OA.kg^{-1}

Table 4 : Characteristics of the toxic samples used for the experiments

In order to clarify the writing, the experiments performed with the mussels from Leucate in January 2006 and those performed with the mussels from Kervoyal in June 2006 and June 2007 will be entitled respectively: Leu 06, Ker 06, Ker 07.

2.2 Experimental protocol

2.2.1 Mussel sampling

Approximately 250 kg of mussel were collected by a shellfish farmer on the day chosen as the most suitable for the decontamination. They were transported without prior sorting to avoid any stress, to the Bouin experimental station located at approximately one hundred kilometers from IFREMER in Nantes.

On arrival, the mussels were washed and roughly sorted to discard any coarse waste and dead animals. About 15 kg were set aside for the preparation of samples for parameter measurements. The remaining mussels were dispatched in 13-15 kg trays. No significant mortality was observed on that day.

2.2.2 Experimental conditions

The experiment was conducted in two 0.8m^3 tanks in which two stacks of trays were immersed. Each tank contained 100 kg of bivalves (Figure 2). Both tanks were supplied with a continuous flow (2.5 m^3.h^{-1}) of subterranean water comprising the following characteristics : salinity : 33-34%0, temperature : 14-15°C, Ph : 7.

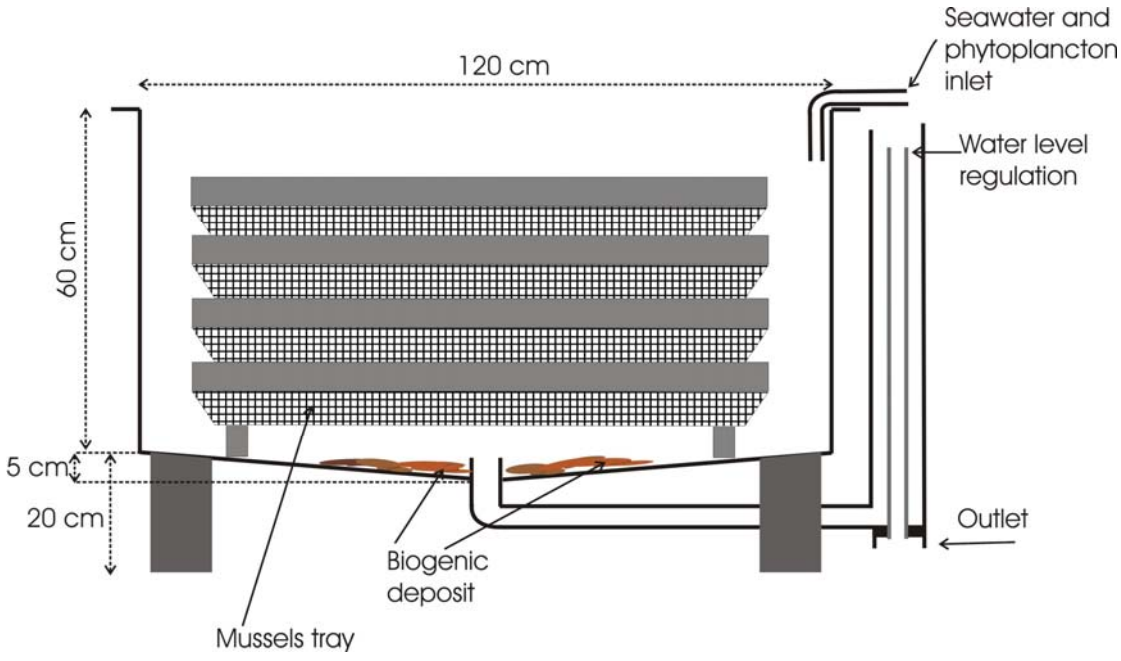


Figure 2 : Diagram of a tank

This water is free from any micro-organisms, it is highly anoxic and contains nitrogen in ammonia form and is iron-rich. Because of these highly stable properties, it is

unsuitable for use inits raw state during a depuration trial. It was therefore filtered on a biological filter before use (NH4 is converted into NO3 which is less toxic for shellfish) and oxygenated in order to precipitate iron (98% decrease).

2.2.3 Food supply

The experimental station at Bouin contains the appropriate facilities to continuously produce a large biomass of a monospecific culture of the diatom *Squeletonema costatum,* extensively used in aquaculture. The raw subterranean water is perfectly suited to the diatom culture which is kept in $80m^3$ tanks, located outside and available at an initial concentration of approximately 1 million cells per litre.

The fodder algae was delivered into the experimental tank, called feeding tank via a floodgate regulating the concentration. Two levels of food have been tested in the Leu 06 and Ker 06 experiments and a more concentrated third level, was added in the last Ker 07 experiment. The diatom concentrations corresponding to the three levels were : zero, about $15\text{-}20.10^3$ and $30\text{-}40.10^3$ millions of cells per millilitre. Dilutions of 1/80 and 1/40 were established to obtain the diatom densities respectively in Tank A and Tank B of the third trial. The food supply was indirectly controlled via chlorophyll measurement at the inlet and outlet of each tank.

2.2.4 Experimental model and parameters

Studying the possible impact of food on the detoxification rate consists in i) following the toxin content over time for both feeding conditions, ii) and in checking the physiological state of the animals. The chemical analysis was performed mainly on digestive glands but it was also planned to verify whether the meat was toxin free. The physiological state was evaluated by the measurement of the condition index and biochemical parameters constituting a major form of energy storage in the mussel (Gosling, 1992). In addition, toxin concentration was measured in dry tissue.

A possible toxin release in the medium was qualitatively investigated qualitatively via toxin detection in bio-deposits and in the water. The bio-deposits consist of a mixture of feces, pseudo-feces, inorganic matter and culture waste. Bio-deposit samples were collected at various times during the trial for dry weight estimates and toxin analysis. In order to detect any dissolved toxins SPATT resin sachets (Solid Phase Adsorption Toxin Tracking) were suspended between the tray stacks and retrieved several times during the experiment. This technique, inspired from a New Zealand study (Mac Kenzie *et al*., 2004), was tested in a preliminary study conducted within the framework of this project and previously submitted for publication (the 12^{th} Conference in Copenhagen, 2006).

Three experiments were carried out in the course of the study using a very similar protocol. The first mussel sample of the experiment (Leu 06) displayed a fairly low initial toxin concentration and it was not worth extending the trial beyond a fortnight. For the first two trials (Leu 06 and Ker 06) only one food level was compared to the absence of food. For the third experiment (Ker 07), two levels of food were tested in comparison with a control without food. Over the duration of this last trial, the number of the samplings was reduced to fit with the possible work load and there was no sampling in the first two days.

Taking into account these considerations, a type of experimental schedule is set out in Table 5.

Sampling day	Use of samples collected on day Tn						
	Condition index	Ratio DG/flesh	Toxins in mussels	Toxins in bio-deposits	Toxins adsorbed	Lipid and/or Glygo	Chlor.a
T0	X	X	X		Suspension of sachets	X	
T1		X	X	X		X	X
T2		X	X	X			X
T3		X	X	X	X		X
T6 ou T7		X	X	X	X		X
T10 ou T11	X	X	X	X	X	X	X
T14		X	X	X	X		X
T20 ou T21	X	X	X	X	X	X	X

Table 5 : Example of an experimental model designed for the decontamination of mussels.
Tn indicates the sampling day in the tanks: T0 being the starting day. X indicates sample collection.

2.3 Methods

2.3.1 Sample collection and processing

On sampling days, the trays were removed from the tanks and the water drained very slowly in order to allow for the sedimentation of bio-deposits and the collection of one aliquot. Approximately 100 individuals of different sizes were removed from each tray to form the sample (2-3 kg per tank) submitted to the day's measurements. Dead animals were discarded and counted. For the index condition estimate, this amount was increased by around 2 kg of mussels for each tank.

The mussels were then dissected, separating the digestive glands (DG) from the remaining tissues regarded as meat. After draining, the tissues were transferred into tubes for immersion into liquid nitrogen: digestive glands were stored at -80°C and meat tissues at –30°C in the laboratory. The tissues were ground and dispatched in aliquots for measurements of the various parameters. Replicates were formed from this pool of ground DG or meat.

2.3.2 Dry matter content

Dry weight toxicity was calculated separately for DG, meat and bio-deposits after freeze-drying of various amounts of these ground matrices. Analyses on three replicates have shown that variability was negligible for this measurement.

2.3.3 Chlorophyll a monitoring

Water samples were collected at the inlet and outlet of each tank. Chlorophyll a concentration was measured by the Lorenzen method (1980) .

2.3.4 Biochemical parameters

Total lipid content. Two methods were applied to measure the total lipids Three aliquots of around 1g of fresh tissue were used for the total lipid analysis performed on a nuclear magnetic resonance spectrometer (Minispec, Bruker), with an external calibration curve on oil dilutions. Digestive glands and the remaining meat were analyzed separately with this method.

On the other hand, nine aliquots of whole individuals (used for the condition indices) were analysed in accordance with the method of Bligh and Dyer (1959).

Glycogen content. Nine aliquots were analysed according to the method described by Dubois et al.(1956).

2.3.5 Condition index

The condition index (CI) was used to characterize the apparent health of farmed bivalves at a given time. Various indices exist but we opted for the index most widely used in aquaculture which involves calculating the ratio of dry flesh weight vs. dry shell weight (*100) for each individual. It can be easily standardized and is thus much more universal (Lucas and Beninger, 1985). The CIs were estimated from 90 individuals at the beginning, the middle and the end of the experiment. The comparison o the values were treated with the non parametric Kolmogorov-Smirnov test (Stagraphic).

2.3.6 Toxin extraction and detection

The tissues were extracted in accordance with a lab-designed protocol (Mondeguer, 2004) validated against certified contaminated standards (AOCS-1 and MUS-2 IBM, CNRC, Canada). A 4g aliquot from the homogenate was extracted with 10.8 and 6ml of pure acetone. The combined acetone layers were topped up to 25ml and then split into two equal fractions and evaporated to dryness. One fraction was intended for alkaline hydrolysis for okadaic acid derivatives (DTX-3) detection. The dry residue was re-suspended in 1ml of pure acetone and 125µl of NaOH water solution (2.5N) were added. After one night at room temperature, the reaction was neutralized with 125 µl of HCl (2.5N) and the solvent was evaporated.

The dry hydrolyzed and non-hydrolyzed extracts were dissolved into 10ml of hexane/chloroform (50/50vol.).The solid-phase extraction process was automated on a ASPEC Xli robotized unit (GILSON) fitted with silica cartridge (Lichrolut 500mg, 40-60 µm), conditioned with 15ml ml of chloroform and then 10ml of hexane. 1.5ml of the extract was eluted with a mixture of methanol/chloroform (50/50 vol.)

Detection and quantification were carried out by HPLC coupled with ion trap spectrometry (Finnigan LCQ ion trap), under the following conditions: column and pre-column: Kromasil: C18 (250mm x 2.0mm, ID 0.5µm); temperature: 40°C; isocratic mobile phase: acetonitrile/water+0.1% TFA, (75/25 v/v); flow: 0.2ml.mn-1. Data acquisition was conducted in the positive mode, with alternating full MS and full MS2. The analysis quantifies the daughter ions by comparing the response obtained

with that of a methanolic dilutions of a reference solution of okadaic acid (OA) provided by the IMB of the National Research Council (Halifax, Canada). The quantification limit is 0.2ng OA injected. The mean value recorded on the certified reference material (NRC/CMR-DSP-MUS-b) measured on 8 extracts is : 9.47 ± 2.28 $OA.g^{-1}$ for a certified value of 10.1 ± 0.8 $\mu g.g^{-1}$.

2.3.7 Bio-deposit

Bio-deposits were removed at each sampling day. As a release of water could not be avoided, the collection of a more or less aqueous sludge was not measured accurately. Nevertheless, after decanting , some water was removed and the remaining mud was homogenized and an aliquot was kept frozen.

After thawing, the deposit volumes were reduced by centrifugation and an aliquot from the bottom was freeze-dried for water content analysis.

The toxin analysis was carried out on 1 to 5g of wet deposit, depending on the amount available. The extraction was then conducted in the same way as for the tissues. One aliquot was subjected to alkaline hydrolysis for acyl detection with the same procedure used for the digestive glands .

2.3.8 Solid Phase Adsorption Toxins Tracking (SPATT).

The procedure and the efficiency of the resin sachets were previously established (Marcaillou et al, 2008; Fux et al., 2008). After thawing, the sachets were rinsed and vigorously shaken to remove salts and water. The resin was eluted with25ml of methanol from which 400µl were inserted in LC-MS vials and 5µL were injected in the LC-MS system. Then, the remaining volum (24,6ml) was evaporated to dryness and the residue was re-suspended in 1ml of methanol from which 5µl were used for LC-MS analysis.

3 Results

3.1 Mortality

The mussel mortality rates, were evaluated from the cumul of dead individuals recorded in the tray used for this measurement at the end of the experiment. These are described in Table 6 for the three trials.

	Tank without food	**Tank with food**	
		Tank A (1/80)	Tank B (1/40)
Leucate 06	2	0	———
Kervoyal 06	12	6	———
Kervoyal 07	2	4	4

Table 6 : Pourcentage of dead individuals recorded at the end of the three experiments in the different tanks.

Taking into account the transfer (sampling, washing, immersion in water of different quality), the mortality rates can be considered as lowin addition, tThe experimental design was suitable for the maintenance of live mussels.

3.2 Mussel condition indices

The results of comparisons of mean indices at the beginning and at the end of experiment for the three trials are described in Table 7.

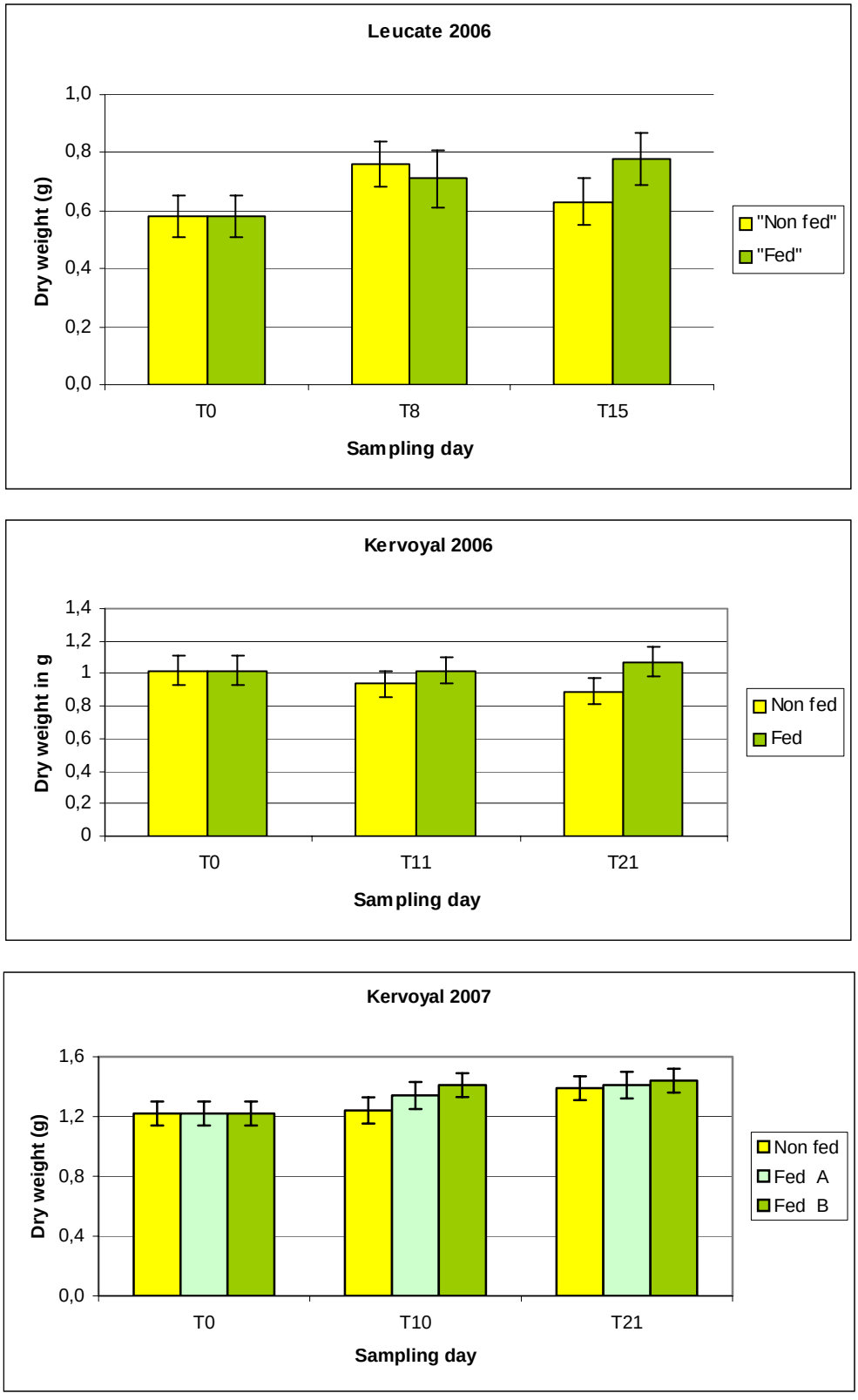
Date/code experiment	Sampling day	Food level	Condition index n=90			Significance Kolmogorov-Smirnov test
			Mean	Standard deviation	-Tn-T0	
January2006 Leu 06	T0		9.62	2.59		
	T16	Non fed	7.16	1.88	-2.46	Significative Probability : $1.5.10^{-8}$
		Fed	9.30	2.99	- 0.30	Non significative
June 2006 Kerv 06	T0		25.18	5.87		
	T21	Non fed	20.24	4.65	- 4.94	Significative Probability $2.4.10^{-6}$
		Fed	23.24	4.75	-1.94	Significative Probability 0.015
June 2007 Kerv 07	T0		30.27	7.31		
	T21	Non fed	27.15	5.97	-3.12	Significative Probability 0.0056
		Fed level A	27.56	6.65	-2.71	Significative Probability 0.00192
		Fed level B	29.74	6.44	-0.53	Non significative

Table 7: Summary of the results of the condition indices comparison for the three trials.

The condition index decreases in the non fed mussels during the three experiments. In addition, this decreases in the tanks receiving food in the Ker 06 and Ker 07 trials (dilution of 1/80 of the mother culture). It remains stable in the tanks where the food supply is at the dilutions 1/80 for Leu 06 and at 1/40 for Ker 07.

Unsurprisely, the lack of food causes a loss of biomass while the food supply does not produce an increase in the condition index. In fact, this lack induces a stability at best and a slight decrease in the biomass at worth. It is particularly surprising to observe no difference by doubling the food flow in the third experiment.

Disregarding the ponderation of the shell , it is of interest to note that there is no difference in the values of dry matter between the start and the end of the trials whatever the food level including the absence of food (Figure 3).



3 : Evolution of mean weight of mussel dry flesh for the three experiments. Vertical bars indicate confident intervals of the averages calculated from 90 individuals.

Nevertheless, whereas the individual variations do not prompt any conclusion, there is a trend towards a slight increase in the tanks with food.

3.3 Food supply

The recording of the chlorophyll a several times throughout the trials is described in t Table 8.

Sampling day	Leucate 2006			Kervoyal 2006			Kervoyal 2007					
	Inlet	Outlet	retention %	Inlet	Outlet	retention %	Inlet		Outlet		retention %	
							A	B	A	B	A	B
T1	20.48	1.64	92	16.71	3.35	80						
T2							6.99	9.94	1.08	3.23	85	68
T3	14.6	0.95	93	12.71	1.28	90						
T4							9.09	14.18	1.19	1.74	87	88
T7	18.23	1.48	92	17.24	1.97	89						
T8							7.03	11.9	1.2	1.2	83	90
T9				15.35	2.24	85						
T10	14.57	4.76	67				7.68	12.48	0.87	2.22	88	82
T13	14.89	2.96	80	11.47	1.79	84						
T15	15.08	1.27	92				7.29	14.5	1.07	1.43	85	90
T16				8.46	1.3	85						
T18							5.26	8.42	0.93	2.14	82	75

Table 8 : Chlorophyll a values at the inlet and outlet of each tank in the course of thee experiments

As the mother diatom culture is produced outside, it is influenced by the natural light. That explains the fluctuations in the values. Nevertheless, the differences between the inlet and outlet flows show that the retention rate of the fodder algae is of approximately 75-90% . It can be assumed that this filtration activity is linked to an ingestion of the diatoms.

3.4 Biochemical parameters

3.4.1 Total lipids

The lipid content is a parameter of interest if we consider the lipophilic nature of the DTXs and a possible dilution effect in the tissues.

The total lipid concentrations in the whole flesh have only been measured for Ker 06 and Ker 07. The data is not shown because no evolution was observed in the 3 weeks. On the contrary, the lipids content in the digestive glands is shown in Figure 4.

In the tank without food for the three trials : the lipids percentage varies but the value recorded after 2-3 weeks does not differ from that recorded at the beginning.

In the tank with food : in the course of time and after fluctuations similar to those observed in absence of food, the lipid rates show an increase at the end of the experiment in all cases ranging between 19 and 35 %. The highest value corresponds to the highest level of food (Ker 07, level B).

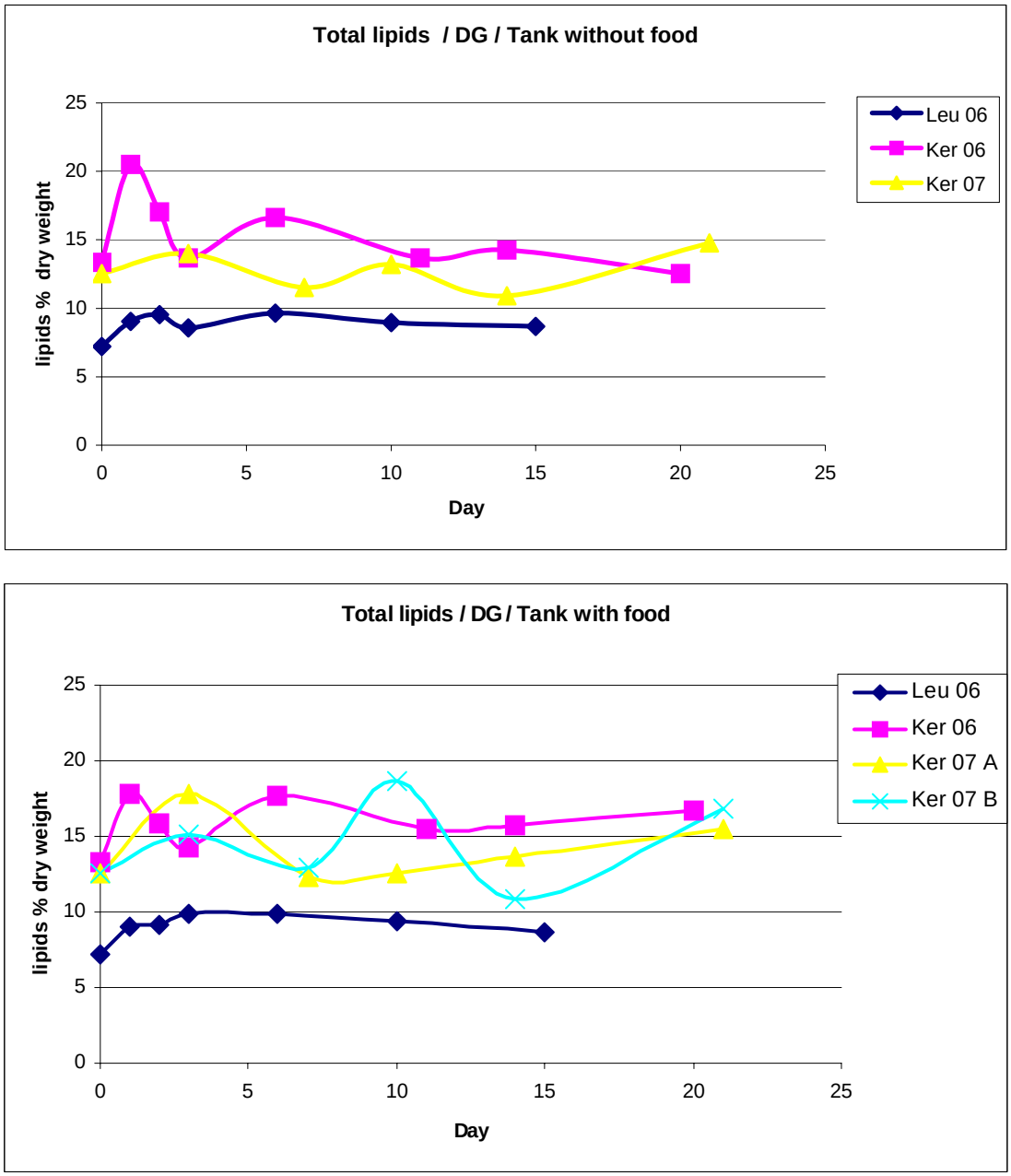


Figure 4 : Percentage of total lipid in the digestive glands (DG) in non fed mussels (above) and in fed mussels (below) in the course of the three experiments.

3.4.2 Glycogen

The glycogen is the main available source of energy outside the reproductive period. The *Mytilus* genus normally exhibits a reproductively quiescent period in temperate water, when the reserve is replenished during the month of June. It was thus useful to verify whether the experimental conditions would affect this parameter (Table 9).

In spite of the variability of the measurements, the values do not pinpoint a decrease in the contrary they tend to increase in fed mussels.

	Glycogen concentrations (n=9) mg.g^{-1} dry weight of total flesh			
		T0	**T10**	**T21**
Kervoyal 06	Non fed	234 ± 57	191 ± 34	194 ± 34
	Fed	234 ± 57	264 ± 42	228 ± 96
Kervoyal 07	Non fed	209 ± 29	192 ± 15	248 ± 39
	Fed A	209 ± 29	206 ± 14	239 ± 52
	Fed B	209 ± 29	188 ± 28	255 ± 50

Table 9 : Glycogen concentrations in the mussels from Kervoyal at three moments of the experiments (standard deviation of 9 measures)

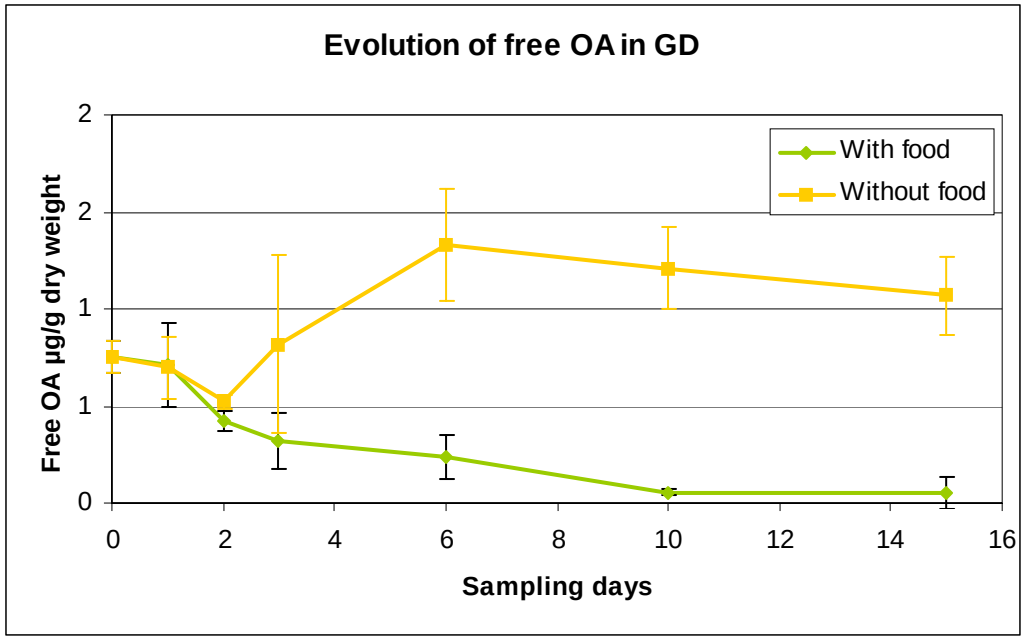
3.5 Toxin evolution in the digestive glands

The analysis of the remaining flesh performed at certain times during the trials (T0, T8, T10 and T21) never detected any toxin presence. We will therefore consider the evolution of the toxicity only in the digestive glands, distinguishing the two forms of toxins: free OA detected in the non-hydrolysed extracts and total OA detected in the hydrolysed extracts.

3.5.1 Mussels from Leucate (Leu 06)

The Leu 06 experiment, , took place in winter with M. g*alloprovincialis* as the mussel species. The starting toxin level was close to the sanitary threshold. The sample also contained pectenotoxins (PTXs) at the level of 0.5 µg.g^{-1} (DG, dry weight). As was the case after the tenth day, the PTX concentration dropped below the quantification threshold. However, these results are not shownd on Figure 9 which separately shows the comparison between fed and non fed bivalves for only free OA and total OA .

As the free OA and total OA concentrations do not differ significantly before re-immersing of the mussels, we assume that they do not contain esterified DTX (DTX-3). The esters do not appear in the course of the trial given that we do not observe a systematic and significant difference between both forms of OA.



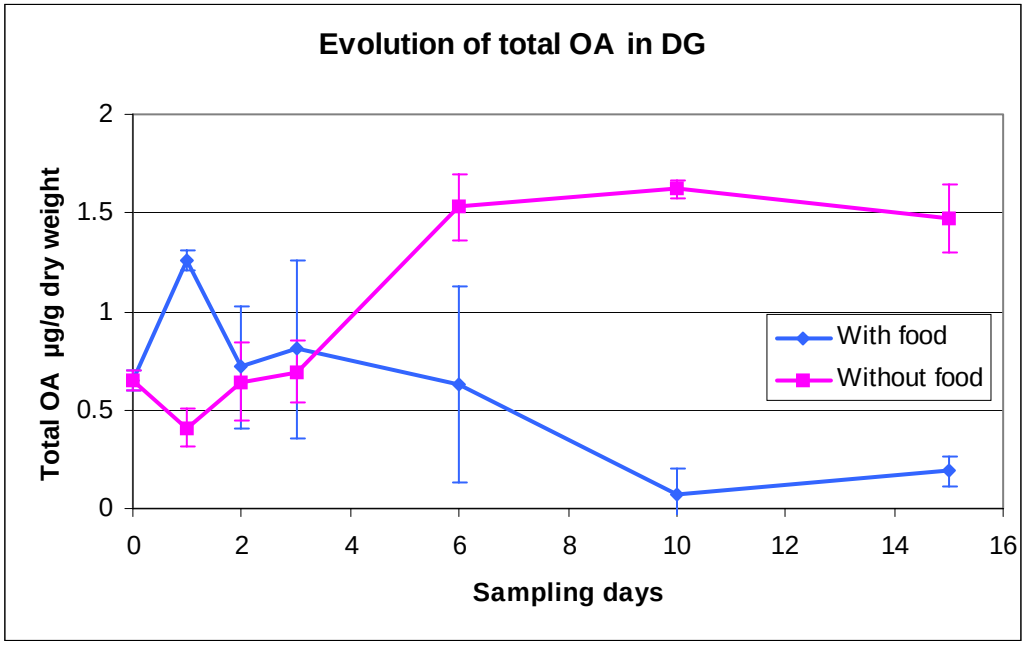


Figure 5 : Evolution of toxin concentrations in DG over time. Error bars represent S.D of 3 independent extracts

The free OA elimination begins in the two tanks as early as the first day but the kinetics reverse from the third day for mussels in the tank without food and in which the concentrations increase. This apparent increase could be the result of a loss of biomass in the starved mussels but this assumption is not borne out by the evolution of the dry weight as previously established (see 3.2).

The abatement for free OA concentration is about 70% in the fed mussels.

For total OA, the kinetics diverge in both tanks from the first day. In the absence of food, the concentrations increase from the third day after a slight decrease as was the case for free OA whereas in the presence of food, the evolution of total OA

concentrations shows a peak and then a slow decrease which attains 58% of the initial value after the tenth day. The peak in toxin suggests a toxin release from the phytoplankton cells, remaining in the gut. In fact, the esters from the algal cells have seemingly escaped from the enzymatic hydrolysis and could not be detected as free OA only until the alkaline hydrolysis.

3.5.2 Mussels from Kervoyal 2006

The results of the second experiment performed in June 2006 on mussels belonging to the species *M. edulis* are described in Figure 10.

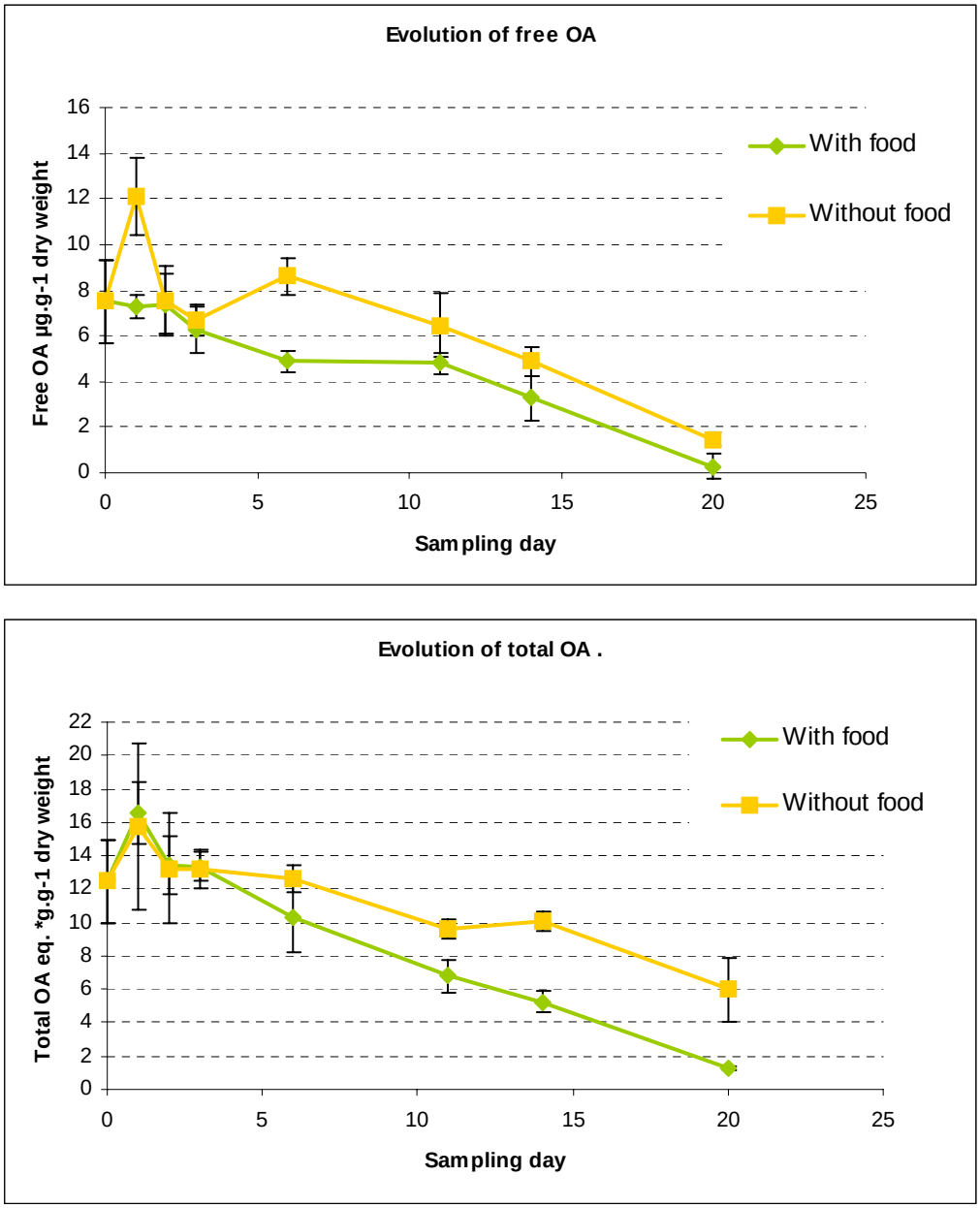


Figure 6 : Evolution of toxin concentrations in DG over time. Error bars represent S.D of 3 independent extracts.

In this second experiment, the ester content was estimated at the level of 5µg.g^{-1} DG (dry weight) by the difference between the concentrations of hydrolysed and non hydrolysed extracts.

The elimination of the free OA is marginally higher in the tank with food in which a decrease of 96% is recorded than in the tank without food in which the decrease is 80% . As was observed in Leu 06, an increase in can be observed on the first day that could correspond to an phytoplanktonic ester hydrolysis but, in this case, thist takes place in the tank without food, contrarily to the first trial.

The elimination of total OA becomes significantly different between both tanks after the fifth day. Before this date, the total OA evolution is similar in fed and in non fed mussels, particularly as the concentration increases the first day for both lots of mussel. After 3 weeks, the decontamination rate is 90% for the fed mussels and only 52% for the non fed mussels.

The shape of the kinetics therefore suggests two phases

a-in the course of the first days: the decontamination is not visible and an increase of the OA concentration can occur, except for the free OA in the fed mussels where concentration remains at the same level as that of the beginning.

b- after the third day, the concentrations seem to follow a linear decrease over time. Disregarding the values of the first two days, the relationship is significant as shown in Figure 7.

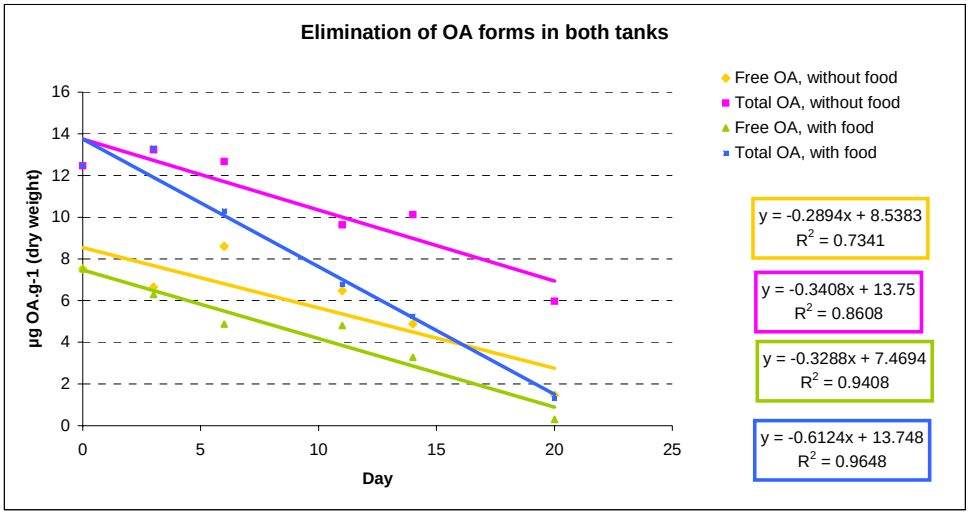


Figure 7 : Presentation of a linear relationship between the OA concentrations and the time in both tanks. The points corresponding to the first and second days have been discarded.

The comparison of the slopes obtained for fed and non fed mussels illustrates two points.

For free OA, the slopes are not different. In the presence or absence of food, the free OA elimination appears to have the same speed-up (slope test , 90%).

For total OA, the difference between the slopes is significant (99%) indicating a more rapid total OA elimination quicker in the fed mussels than in the starved mussels.

The ester estimates in the tank without food reveal values in the same range at the beginning and at the end of the experiment (5.0 and 4.5 µg.g^{-1} DG respectively).

The results of this second trial suggests that the food affects the ester depuration.

Apart from the description of the kinetics, the results recorded at the end of the trial must be compared with the sanitary threshold which is 160 µg. eq.OA.kg^{-1} of total meat. These values for Ker 06, expressed according to the total fresh flesh are the following : 290µg OAeq..kg-1 for the non fed mussel and 74 µg OAeq.;kg^{-1} for the fed mussel. Consequently, only the fed mussel would have been sold on the market.

3.5.3 Mussel from Kervoyal 2007

The third experiment can be considered as a replica because several points are similar to the second one : the same mussel species harvested at the same site and at almost the same date, but with an additional level of food being tested (level B). On the other hand, the follow up of the decontamination only accountedfor 6 sampling days over the 3 weeks of experiment.

Figure 8 displays the decontamination diagrams for free OA and total OA separately.

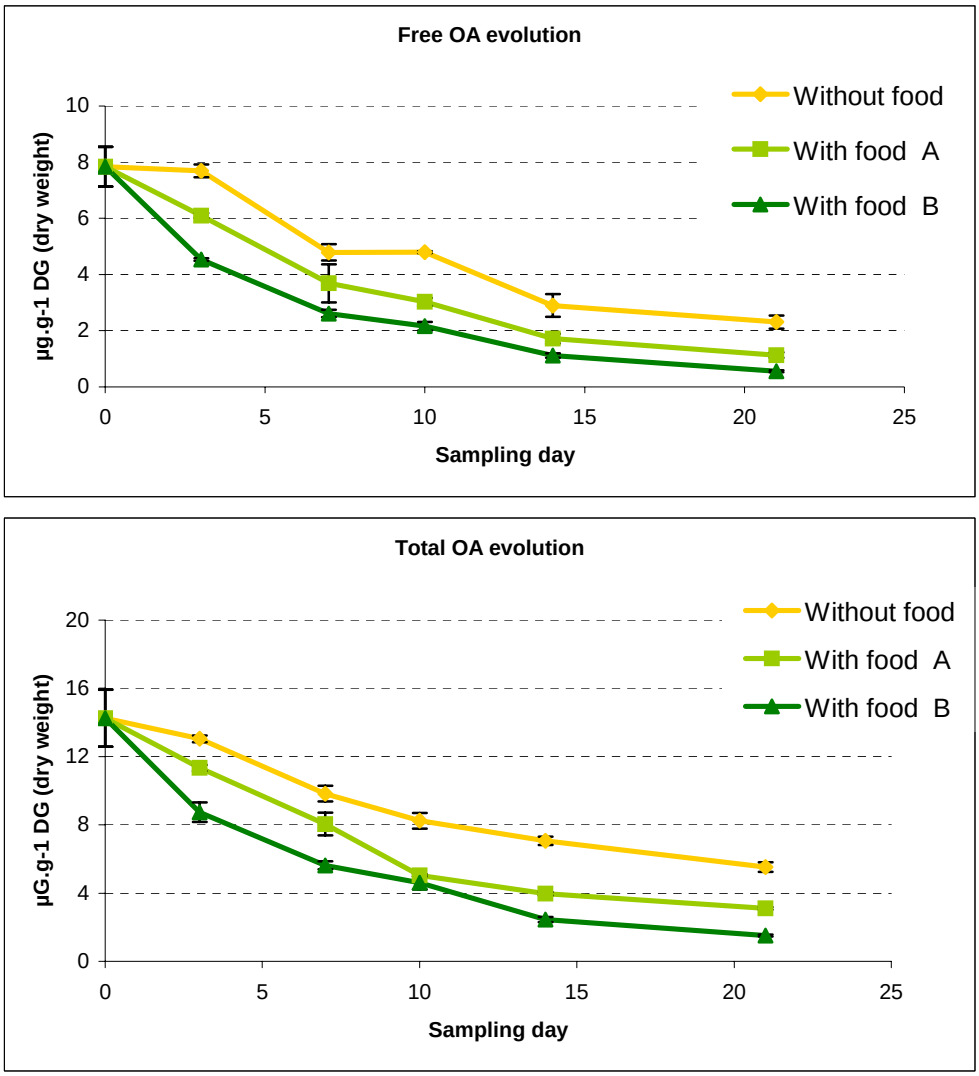


Figure 8 : Presentation of a linear relationship between the OA concentrations and the time in both tanks.

The toxin elimination either for free OA or total OA, is all the more quicker as the diatom supply is more significant.

Contrary to 2006, the whole shape of the relationship between the concentrations and the time fits well with a decreasing exponential curve for both forms of toxin as it is shown in Figure 9.

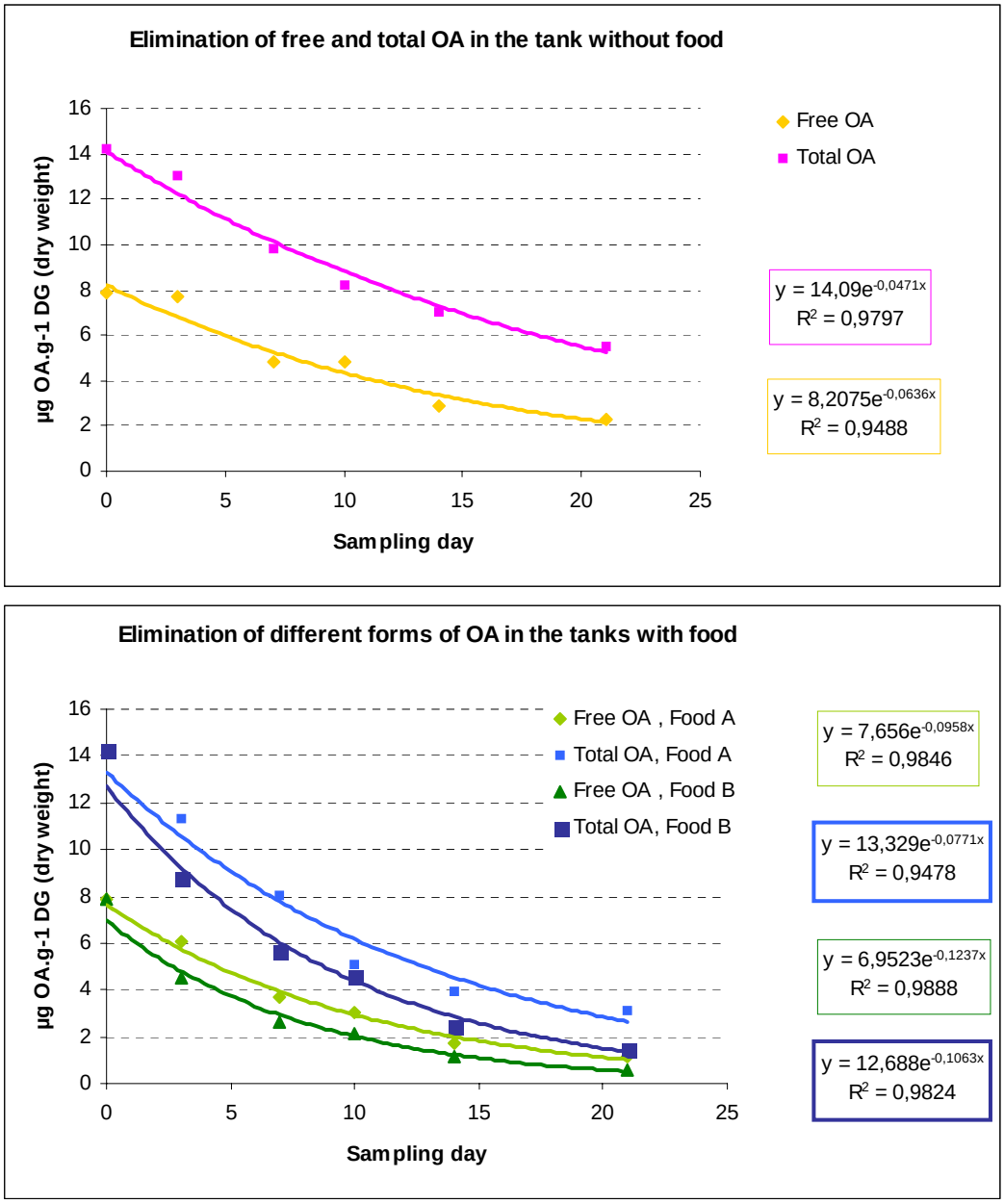


Figure 9 : Comparison of the decontamination kinetics in non fed and fed mussels for free OA and total OA.

The parameters of the equation $y = a.e^{-kt}$ have been defined as follows :

Y : concentration at the time t

a : concentration at T0

k : exponential constant

In order to verify the difference in the decontamination of fed and non fed mussels, the initial concentration was fixed for all equations and the confidence intervals of the exponential coefficients has been calculated . This data ise compiled in the table

Toxin	Food supply	Equation : $y = a.e^{-kt}$	Confidence interval of k	
			Min. limit	Max. limit
Free OA	Without food	Y=7.84exp(-0.0583x)	-0.06928	-0.04734
	With food A	Y=7.84exp(-0.0996x)	-0.1139	-0.0854
	With food B	Y=7.84exp(-0.1487x)	-0.1707	-0.1267
Total OA	Without food	Y=14.25exp(-0.0488x)	-0.05656	-0.04106
	With food A	Y=14.25exp(-0.0872x)	-0.09973	-0.07467
	With food B	Y=14.25exp(-0.1487x)	-0.1464	-0.1082

Table 10 : Exponential equations of the kinetics defined from the data of the three experiments.

As no overlap is recorded in the confidence intervals it can be recognised that the exponential coefficients differ between them. As a result, the food effect is clearly confirmed in this third trial whatever the form of OA but there is no difference in the elimination speed up between free OA and total OA.

Concerning the mussel toxicity at the end of the experiment , the final concentrations were respectively: 227, 139, 88.5 µg OAeq;kg^{-1} (total fresh flesh) for the non fed mussels and the fed mussels with the diatom concentration A and B. Only the fed mussels can be considered as safe for human consumption but in this case, the official mouse test was performed on the same samples as those used for the chemical analysis. The results were all positive. Two of the three mice died after the injection of the extract performed with the mussels fed with the food level B.

3.6 Toxin detection in bio-deposits and in the passive adsorption devices (SPATT bags)

Although the experimental facilities used were not suitable for accurately quantifying the bio-deposits, the OA detection in the bio-deposits has provided some useful information.

The first deposit collection, the third day, is roughly the same amount in the two or three tanks. Following this, the quantity decreases notably in the tank without food as expected, while it remains relatively stable in the tanks receiving the food during the trials. In addition, Tank B contains twice the amount of Tank A.

The results of the analysis are described in Figure 10. They only deal with the total OA concentrations in the tank with food because no toxin or only traces were detected in the non hydrolysed extracts except for one day (14th June 2006).

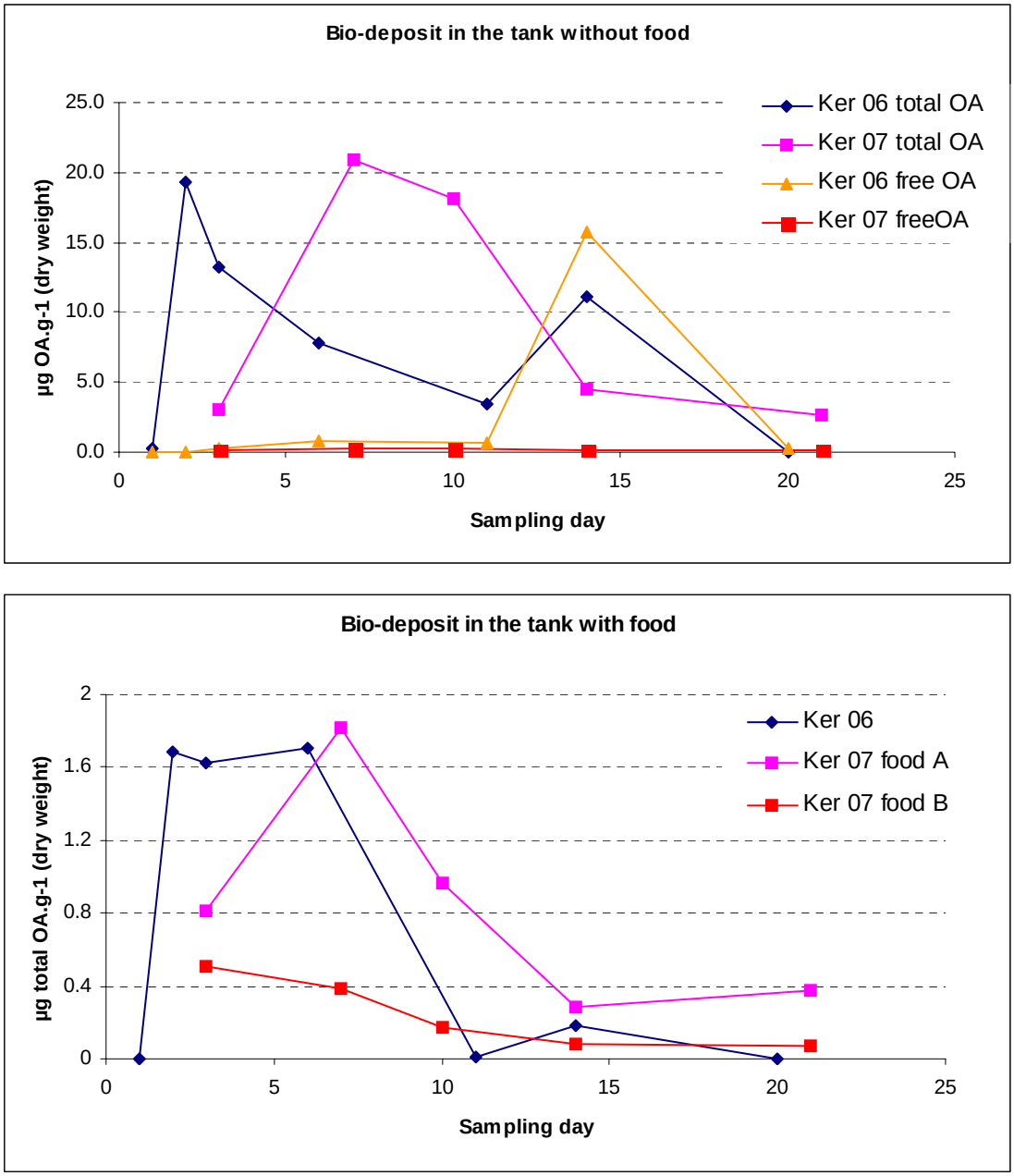


Figure 10 : Evolution of total OA concentration in the bio-deposits collected in the tanks during the experiments Ker 2006 and Ker 2007.

The same trend in the evolution of total OA concentrations is observed in the tanks with or without food.. After an increase occurring in the first days, the values decrease from around the twelfth day for both experiments.

The results are roughly coherent with the decontamination kinetics described above and suggest that ester is the form preferably eliminated at the beginning and during the first ten days of the decontamination.

Concerning the passive adsorption of OA on the resin sachets, the results of Ker 06 and Ker 07 significantly differ.

In June 2006 no toxin or traces were detected in the SPATT bags. In June 2007, however, the variability between the three replicates was very high. For this reason, only the maximum value is recorded in Table 11.

Food supply	Adsorption duration (max in ng OA per sachet)			
	7 days	10 days	14 days	21 days
Without food	59	44	48	59
Food level A	53	95	88	74
Food level B	62	0	77	66

Table 11 : Maximum quantity of OA in ng, adsorbed in one SPATT bag in accordance with the immersion duration.

The release of OA does not increase in the course of the experimental duration. The resin used for the purpose of the study can accumulate more toxin than measured in this kind of resin was proved to concentrate up to several hundred ng of OA in the field. Therefore, it is impossible to ascertain whether the adsorption on the resin was prevented by the water flow or if the toxin release in water only occurred to a significant extent during the first days of the experiment. Nevertheless, a toxin release is detectable in water in the decontamination processes.

4 Discussion Conclusion

The objective of the study was to pinpoint a possible effect of the food on the decontamination speed-up of mussels containing DTXs toxins. An experimental protocol has been designed to compare the detoxification rate in fed and non fed toxic mussels. Three experiments were conducted over the duration of the contract using the facilities of an aquaculture station which were adapted to the relevant protocol.

The experimental set-up made it possible to maintain a sizeable mass of live mussels in good conditions. In fact, the mortality rates and the relatively stability – and even the trend towards an increase- of the biochemical parameters attested that the experimental conditions did not affect the animals. The lipid concentrations in the digestive glands show fluctuations that could be the result of an adaptation of the new medium (mainly the water and food quality) but they stabilized at the initial value in the tank without food while they tended to increase in the tank with food.

Since the toxic episodes are linked to natural events and cannot be planned, neither the date of the beginning of the experiments nor the starting toxic level of the bivalves could be decided in advance. The first experiment suffered because of these constraints and displays a less significant interest compared to the following two experiments due to the low initial toxicity. Nevertheless, it pinpointed a drop of 90% in the total OA concentration in the fed mussels while this concentration slightly increased in the starved mussels. The discussion will therefore focus on the results of the following two experiments performed on the mussels from Britanny.

These experiments clearly confirm a decrease in the DTXs concentrations, significantly higher in the fed mussels than in the starved mussels. The detoxification

takes place essentially in the digestive glands for the flesh that is not contaminated at the outset and remains uncontaminated throughout the study whatever the mussel origin.

The detoxification rates calculated from the difference between the concentrations obtained at the end and beginning of the experiments summarise the results (Fig.11).

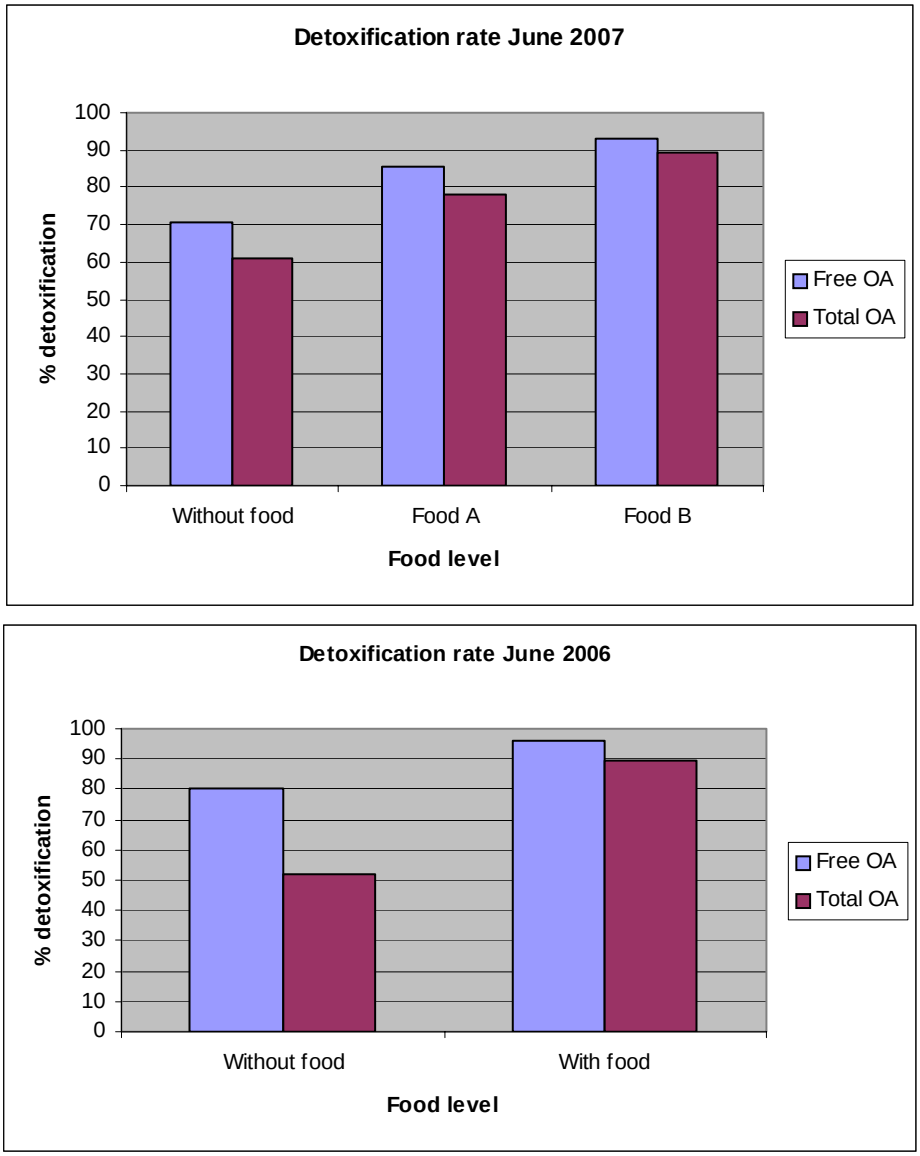


Figure 11 : Detoxification rates calculated for the free OA and total OA from the difference between the concentrations registered the first and the last day of the experiments.

Since there has been no increase in the biomass whatever the trial, as shown either by the condition indices or the individual mean weights of dry flesh, decontamination is not the result of a dilution effect. This is the result of a true loss of toxin content contrary to findings from previous work (Svensson *et al*., 2003).

The analyses of the results obtained on mussels from Kervoyal in June 2006 and 2007, suggest a distinction of two phases in the evolution of the mussel toxicity.

a - The first days.

In certain cases, it was somewhat surprising (Leucate and Kervoyal 2006), to observe a slight increase in the toxicity the first and second days of the experiment. This phenomenom has been already described in previous works (Marcaillou *et al*., 1993; Blanco *et al*., 1999). This toxin increase is interpreted as the release of toxins from the phytoplanktonic cells in the gut of the bivalves which are submitted to digestion. The gut content probably included *Dinophysis* cells, in an unknown proportion, since the mussels had been sampled during a toxic episode. This residual contamination appears to be highly variable according to the individuals and could more or less conceal the decontamination process. This could explain why this observation is not systematically mentioned in the literature. Moroño *et al* (2003) introduce this early over-contamination in their model assuming that it corresponds to the natural enzymatic hydrolyse of the OA esterified forms from the phytoplankton cells. The visible decontamination seemingly occurs just after the third day in our trials.

This residual decontamination was not shown in 2007 because no sampling was carried out during the first few days but the free OA concentration in the starved mussels was very close to the starting value on the third day as if the elimination had balanced the residual contamination.

b - After the third day

The attempts to describe the decontamination kinetics lead to two possible models i.e. : a linear relationship and a decreasing exponential. This latest relationship is generally used in the theoretic modelling in the previous works (Blanco *et al*. 2005).

That means that the model cannot be definitively established.

The comparison between both experiments draws attention to certain diverging points in the decontamination kinetics.

From Ker 06, the food supply does not affect the free OA elimination speed up while it accelerates the detoxification process in Ker 07. In the tank without food, the total OA and free OA evolutions displays a similar kinetic. Contrary to this, it is clearly demonstrated in this trial that total OA depurate more rapidelyr in the tank receiving the food. This result suggests that the food acts on the esters elimination. This hypothesis is lent credence by two others observations recorded in the course of this study :

- the bio-deposit analysis show an toxin elimination mainly as esters form.
- The concentrations estimates in esters in the course of the time, for Ker 06 and Ker07 (Figure) show that the values recorded at the outset and at the end of the experiments are in the same range for the non fed mussels while they are much lower in the fed mussels.

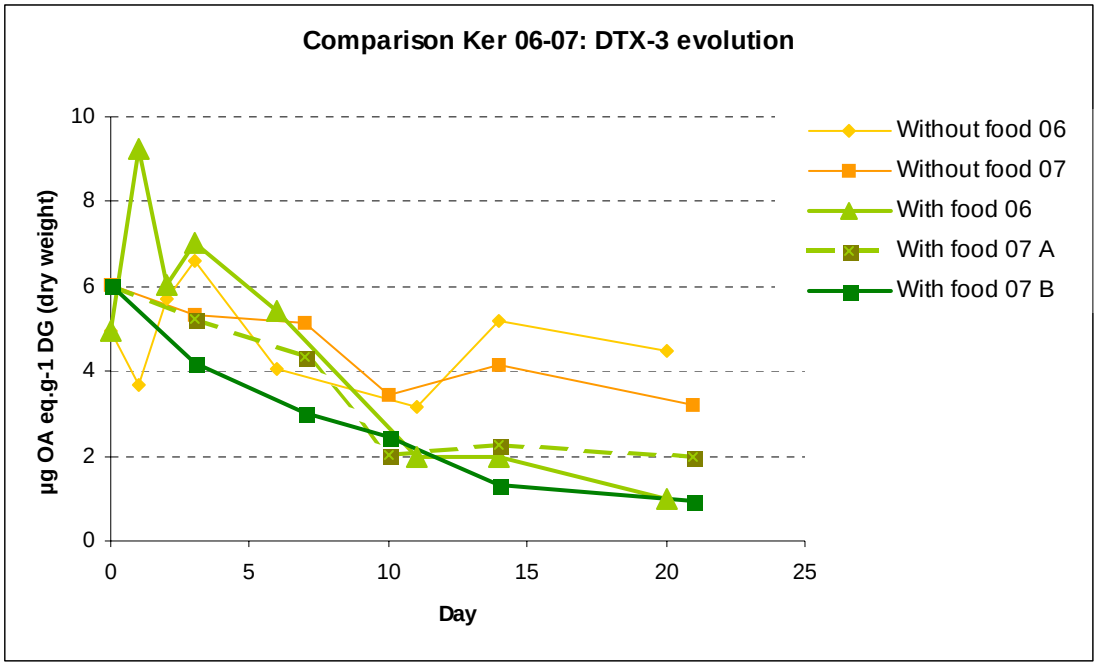


Figure 12 : Esters evolution over the time in the mussels digestive glands for the experiments of June 2006 and 2007.

Nevertheless, the influence of the food supply on esters eliminations is not confirmed in June 2007. The comparison of the free OA and total OA kinetics does not give grounds for drawing any conclusions.

On the other hand, if the food supply enhanced the ester elimination, the detoxification rates for free OA would be lower than those of total OA and then the Figure 8 would show an opposite trend. Nevertheless, the difference narrows when the food supply increases.

In conclusion, the study describes a decrease in the toxins concentration of mussels of approximately 90% when they are fed and of approximately65% when they are maintained in clear water after 3 weeks in the experimental medium.

These results must be considered within the framework of the regulation concerning the acceptable toxin level in the bivalves. In 2006, the mussel toxicity at the end of the experiment was below the sanitary threshold but in 2007 the residual toxicity was high enough after 3 weeks to provide a positive result with the official mouse test. An longer deadline is required to attain the objective of a complete detoxification.

Considering the field situation for the two consecutive years, the shellfish market was re-opened on the fifth week after the beginning of the experiments in accordance with the monitoring survey.

Consequently, in the conditions of this study, the time to decontaminate the mussels with artificial means is not very different from the time needed in the field to collect safe mussels. Nevertheless, the work has demonstrated the positive role of the food on the detoxification rate and paves the way for future investigation to enhance the decontamination processes.

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FIGURES :

TABLES :