Paralytic shellfish poisoning in *Haliotis tuberculata* from the Galician coast: geographical distribution, toxicity by lengths and parts of the mollusc

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Abstract

In order to clarify the origin of Paralytic Shellfish Toxins occurring in the ormers, *Haliotis tuberculata*, various studies were conducted to describe toxicity distribution by area, length and parts of the mollusc. Ormer toxicity from different locations of the Galician coast had a confidence interval of 252±25 μg STX eq/100 g of meat (μg of equivalents of saxitoxin/100 g) by mouse bioassay analyses and 454±86 μg STX eq/100 g of meat by High Performance Liquid Chromatography (HPLC). No value of below 140 μg STX eq/100 g of meat was detected. No significant differences were observed among locations for the values obtained by HPLC, while some differences were found by mouse bioassay. Toxin composition showed decarbamoylsaxitoxin (dcSTX) as the most abundant component (83–100%) with saxitoxin (STX) in a much smaller proportion. Significant differences were observed in the toxin content for the different length groups, toxin content increasing very significantly in the largest ormers, of over 85 mm. Toxin analyses in the different parts of the mollusc by HPLC showed significantly high values for the epithelium of the foot in comparison with the gut and the rest of the mollusc. They reached $105x10^2 \pm 15x10^2$ μg of toxin/100 g of epithelium, against 28±5 μg of toxin/100 g of gut and 27±6 μg of toxin/100 g of muscle. Considering the weight and the quantity of toxin in each part of the ormer, the epithelium carried 2.6 times more toxin than the muscle.

Keywords: Haliotis tuberculata; PSP toxicity; Gastropod toxicity; Galician coast

1. Introduction

The ormer, *H. tuberculata* Linnaeus 1758 is a gastropod mollusc with a geographic range in the Eastern Atlantic extending from the English Channel down to the west coast of Africa (Nicklés, 1950; Gaillard, 1958). Ormers are patchily distributed in infralitoral areas, occupying specific rocky habitats. In some areas of the world members of genus *Haliotis* are of commercial importance and aspects such as feeding, growth and reproduction have received considerable attention (Mgaya and Mercer, 1994). In Spain, ormers were not considered commercially important until the 80s, when they began to be caught in Galicia for export. In April 1991, Paralytic Shellfish Poisoning (PSP) was detected in the ormers from this region and in October 1993, the market for this mollusc was closed due to its toxicity being higher than the legally permitted level (Martinez et al., 1993; Bravo et al., 1996).

The toxicity data of ormers from Galicia show that the main toxin in these molluscs is decarbamoylsaxitoxin (dcSTX) and that a higher concentration is found in the muscle of the foot than in the gut (Nagashima et al., 1995; Bravo et al., 1996). These last authors also mention that all the specimens analysed were toxic and exceeded 80 μg STXeq/100 g of meat (μg of equivalents of saxitoxin/100 g), and additionally that no detoxification effect was observed in ormers kept under controlled conditions for 3 months. The data presented here go into greater depth on the distribution of toxicity of *Haliotis tuberculata* in the Galician coast, both geographically and by length, or in different parts of the mollusc.

2. Material and methods

In December 1995, three or four specimens of *H. tuberculata* were collected at three stations 20–50 m apart at different locations situated in the areas shown in Fig. 1. Ormers of 60–90 mm were selected where possible. The value of toxicity for each station, by bioassay and high performance liquid chromatography (HPLC), corresponds to the extracts taken from the previously gutted specimens.

For the study of toxicity by length, performed at the same time as the above, 22 specimens were sampled (four specimens for all length classes except for 55–65 mm, of which only two were found) ranging from 55 to 100 mm at a station situated in location 5 (Fig. 1). The analyses of toxicity by HPLC and bioassay were carried out separately

for each specimen. As in the study of toxicity by area, extracts were taken from the meat of molluscs after gutting.

For the study of toxicity in different parts of the ormers, 24 specimens were used, whose lengths ranged from 65 to 80 mm. Toxin analyses were carried out by HPLC for three body parts of each specimen, specified as follows: gut, epithelium of the foot and muscle of the foot. The contribution of each part to the total mollusc toxicity was estimated based on the toxin content of each part and on the following relationship: weight of each part/total mollusc weight.

Epithelium, guts, and muscle of foot were extracted, by homogenization with a homogenizer (Ultra-Turrax TP 18-10) in 0.1 N HCl (1:2; weight/volume). Bioassays were performed using the AOAC method (Hollinworth and Wekel, 1990). Previously, the response quantification was carried out with an STX standard provided by the Food and Drug Administration of USA. Extracts were filtered by 0.45 mm membrane filter before HPLC analysis. The separation of toxins was carried out in a 5 mm Lichrospher 100 RP-18 (125x4 mm i.d.) column with an isocratic of 94% 1 mM octane sulfonate in 10 mM ammonium phosphate (pH 7.2): 6% acetonitrile at 1ml:min of flow. Detection and quantification were in a postcolumn system performed in a Teflon coil (10 mx0.5 mm i.d.) at 65°C by delivering reagents with two Eldex A- 30SW-2 pumps. The first reagent, the oxidant, was a solution of 7 mM periodic acid in 50 mM sodium phosphate at pH 9; the second reagent, the acidifiant, was 0.5 M acetic acid. Both were delivered at 0.4 ml/min. The detector was a Waters 474 spectrofluorimeter, set at 330 nm ex., 390 nm em. Millenium software was used for recording and integrating peaks. The standard toxin used in identification and quantification was purchased from the National Research Council of Canada; Gonyatoxins and the neoSTX, dcSTX and STX were supplied by the Bureau Communautaire de Référence of European Union.

Confirmation of the presence of dcSTX and STX was obtained by mass spectrometry with an ion-trap mass spectrometer model LCQ (Finnigan, ThermoQuest). Off-line ‘nanospray’ ionization was carried out using disposable goldcoated capillary probes, as described previously by Marina et al. (1998). Position of the probe, electrospray voltages and typical flows were similar to those described by Wilm and Mann (1996).

The specific detection of toxins at high sensitivities was performed by seeking the appearance of characteristic daughter ions produced by fragmentation of the corresponding molecular ion of the toxins.

In the present paper, the values of toxicity have been expressed in two ways: in μg of toxin/100 g or in μg STX eq/100 g. The first is the sum of the concentrations of STX and dc- STX detected by HPLC. These values were converted to STX eq to enable the comparison of the resulting values of toxicity of analyses carried out by bioassay and HPLC. The specific conversion factors were 1.14 and 1.9 for dcSTX and STX, respectively, which were inferred from the values of specific toxicity given by Oshima (1995).

3. Results

3.1. *Toxicity by location*

No significant differences were observed for ormer toxicity detected in the different studied locations of the Galician coast from values obtained by HPLC, while some differences (significant only at 5%) were found by mouse bioassay. The mean and the confidence interval of toxicity in each location are shown in Fig. 2A. The values of toxicity by bioassay showed a confidence interval of 252±25 μg STX eq/100 g of meat, while no value of below 140 μg STX eq/100 g of meat was detected. By HPLC, the toxin content was 454±86, higher than that observed by bioassay; the coefficient of correlation between HPLC and bioassay values was 0.46 ($P \leq 0.035$). Fig. 2B shows the composition of toxins in the individuals. In all of them, dcSTX was observed as the most abundant toxin (83–100%) with STX in a much smaller proportion.

3.2. *Toxicity by length*

Fig. 3 shows the toxicity of different length groups of *H. tuberculata*, detected by HPLC and mouse bioassay. It is seen that the differences between HPLC and bioassay are greater in the lengths at the extremes (<65 and >95 mm), mainly in the smallest lengths.

The coefficient of correlation of toxin content by HPLC and bioassay was 0.53 ($P \leq 0.022$), but this figure increases up to 0.85 ($P \leq 0.032$) for lengths over 65 mm. In Fig. 4A,B the variations in content of dcSTX and STX detected by HPLC can be seen in relation to ormer length. In Fig. 4C the relative change of both toxins with length is best observed. The quotient of dcSTX/STX diminishes significantly in the larger sizes. Individuals of >95 mm have a mean of approximately 10 times more STX with respect to dcSTX content than the smaller sized individuals. As STX is more toxic than dcSTX, this result may contribute to an increase in toxicity by bioassay in greater lengths which would not correspond to an increase in toxin content by HPLC. Significant differences were observed in the toxin content by HPLC ($P<0.001$) and by bioassay ($P<0.05$) for the different length groups. The differences are significantly high for dcSTX ($P<0.001$) and significant only at 1% for STX.

3.3. *Toxicity in different parts of the mollusc*

The toxin analyses in the different parts of the mollusc by HPLC showed significantly high values for the epithelium in comparison with the gut and the rest of the mollusc. They reached $10^5 x 10^2 \pm 15 x 10^2$ μg of toxin/100 g of epithelium, against 28±5 μg of toxin/100 g of gut and 27±6 μg of toxin/100 g of muscle. Furthermore, Fig. 5 shows the differences in toxin composition of the different parts of the molluscs. Among these differences, the proportion of STX against that of dcSTX is outstanding as observed in gut in comparison with that detected in muscle. The two-way analysis of variance given in Table 1 indicates that the body parts had a significant effect ($P<0.001$) on the total toxin content, while no such effect was found for the length.

4. Discussion

The toxin composition of Haliotis tuberculata described in the present work is very similar to that detected by Bravo et al. (1996) (83–100% and 79–100% of dcSTX). Our values of toxicity for the ormers collected in December 1995 in the same area as sampled by the previous authors show remarkable similarity to their results, mainly to those from sampling in December 1994. In neither study were specimens detected with values of toxicity <80 μg STX eq/100 g. Nagashima et al. (1995) also mention dcSTX

as the most abundant toxin detected in ormers from the Galician coast. Apart from dcSTX and STX, these authors also found a small proportion of neoSTX.

Bravo et al. (1996) give a notably higher correlation for the data from HPLC and bioassay than that given by the data presented here. Nevertheless, in the study of toxicity by length the coefficient of correlation increases considerably on eliminating the data corresponding to lengths below 65 mm. The correlation also increases remarkably if it is made between the concentration of STX and bioassay. These two facts would support the hypothesis of the previous authors, who suggest that the low toxicity of dcSTX for the mouse compared with STX may be a cause of low correlation between HPLC and bioassay in the case of the toxicity of *Haliotis tuberculata*. As our results indicate, dcSTX is the most abundant toxin by far in relation to STX, which is accentuated in lengths below 65 mm. This, together with the fact that the concentration of dcSTX is much greater for these small lengths than for the remaining lengths, would bring about a greater discrepancy between HPLC and bioassay for the smaller ormers.

If the ranges of values of toxin concentration (dcSTX+STX) are compared from the studies by area and by length, it may appear that great differences exist. Nevertheless, taking into account that in the study of toxicity by area, the specimens were within the length range of 65–85 mm, the values are very similar. For this length range, mean toxicities were 384±162 and 397±168 μg of toxin/100 g of meat for the data by area and length, respectively. All data over 1000 μg of toxin/100 g corresponded to specimens of less than 65 mm or greater than 85 mm.

On the basis of our earlier comments, there seems to be a length effect influencing the proportion dcSTX/STX and thus the correlation data of HPLC/bioassay and the quantity of total toxins. Although the unknown origin of toxins in these gastropods makes any interpretation difficult, the different proportion between the two toxins according to the length of the specimens may suggest a metabolic transformation of dcSTX to STX as the molluscs age. There now follows a discussion on whether the differences found in toxin content in the different parts of the ormers can explain this observed length effect.

Our results reveal that the toxin content per gram in the epithelium of *Haliotis tuberculata* is more than 300 times greater than in the gut or muscle. However, taking

into account the weight relationship of each of these parts and the total weight of the mollusc, the proportion of the quantity of total toxin in each part per specimen changes significantly. Considering the weights and the quantity of toxin in each part of the ormer, the contribution of each of these to the total toxicity of the mollusc was calculated (Fig. 6). The fact that the epithelium carried 2.6 times more toxin than the muscle may be very important in interpreting the data previously presented. The relationship epithelium weight/muscle weight in the different lengths may help to interpret toxicity distribution by length. Owing to the fact that our study of toxicity in the different parts was carried out with specimens within a length range of 65–85 mm, it was not sufficient to determine whether toxin content in these parts of *H. tuberculata* are affected by length, as is seen for whole molluscs (Fig. 3).

The distribution of points in Fig. 7 shows that the epithelium weight/muscle weight relationship of *Haliotis* is inversely proportional to length (r^2=0.56), above all for the smallest sizes. That is to say, there is more epithelium per unit weight of muscle in lengths below 65 mm than in the rest. This may be the reason for the high quantity of dcSTX detected for the ormers of the length group <65 mm compared with those of lengths up to 85 mm (Figs. 3 and 4B). Nevertheless, for *Haliotis* greater than 85 cm, this relationship epithelium: muscle would not explain the increase in toxicity which is also observed in these graphs. On the other hand, the possibility cannot be dismissed that the concentration of toxins in the epithelium and in the muscle increases with age, as the results shown in the previously mentioned graphs would appear to suggest.

Data presented above may open up new possibilities in the search for a solution to the commercial moratorium to which this species is subject in Galicia because of its toxicity. Attempts at natural detoxification, keeping ormers under controlled laboratory conditions, did not work (Bravo et al., 1996); nor have any specimens been found which do not contain toxins. Tests are being performed to try to detoxify these molluscs through the elimination of the epithelium by biochemical means (Murado, unpublished data). According to the data presented here, specimens of between 65 and 85 mm would be the most suitable to be treated with some kind of methodology to diminish toxin content due to their lower initial level of toxicity. The elimination of the epithelium and the gut would bring about a fall in toxicity of around 75%, which would bring the

previously mentioned length range below the legal maximum of 80 µg STX eq/100 g of meat.

It is suggested here that *H. tuberculata* has a remarkable capacity for retaining PSP toxins in the epithelium. To date, there are no other reports of such retention by gastropods. A bivalve, the butter clam *Saxidomus giganteus*, has been reported to accumulate STX in the siphon tips, where the toxin can remain active for years (Beitler and Liston, 1990). Immunohistochemical studies of toxic butter clam siphon showed the location of STX to be in the columnar epithelium of the outer surface of the siphon (Smolowitz and Doucette, 1995). Kvitek (1993) suggests that this capacity is a defence mechanism to reduce predation on clams. Further studies on the epithelium of *H. tuberculata* and on its tendency to retain toxins are being performed, both to explain these ecological mechanisms and to manage this mollusc as a food resource.

The source or origin of toxins of H. tuberculata remain unknown. Bravo et al. (1996) mention the low probability of PSP-producing dinoflagellates known in the area being the source of toxicity in the ormers, as neither the geographical distribution nor the toxic profile coincide. PSP toxin analyses of other mollusc and crustacean species by mouse bioassay in different seasons of the year at a station where ormers remain toxic during this time gave negative results (Cacho, pers. comm.). Furthermore, the study of cyanobacteria present in the substrata of the same area did not provide any data to explain the origin of toxins. Up to the present time, no organism has been found which may bring about the intoxication of the ormers through the food chain. We hope that the studies currently being carried out on the epithelium of H. tuberculata help to determine the location of toxins and open up new areas of research into their origin.

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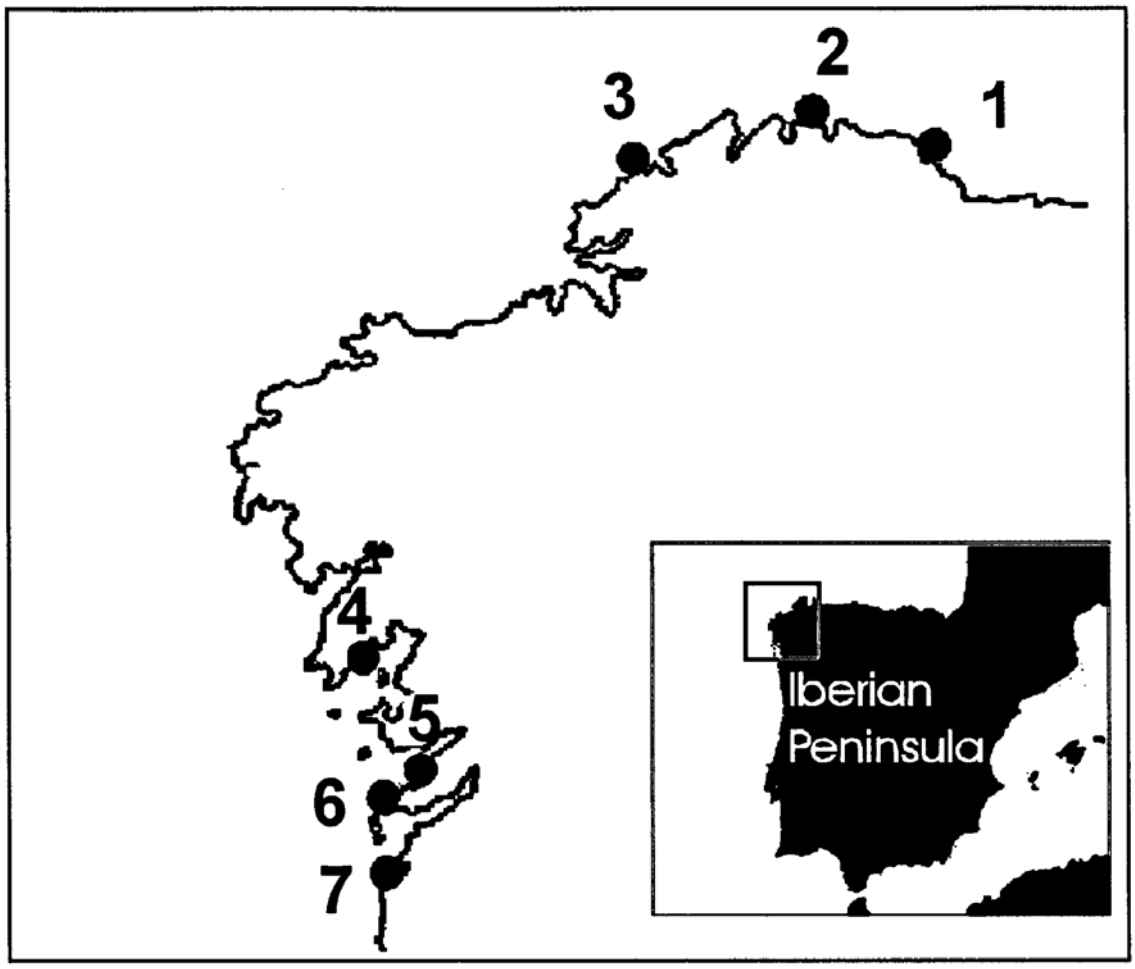

Fig. 1. Location of *Haliotis tuberculata* sampling.

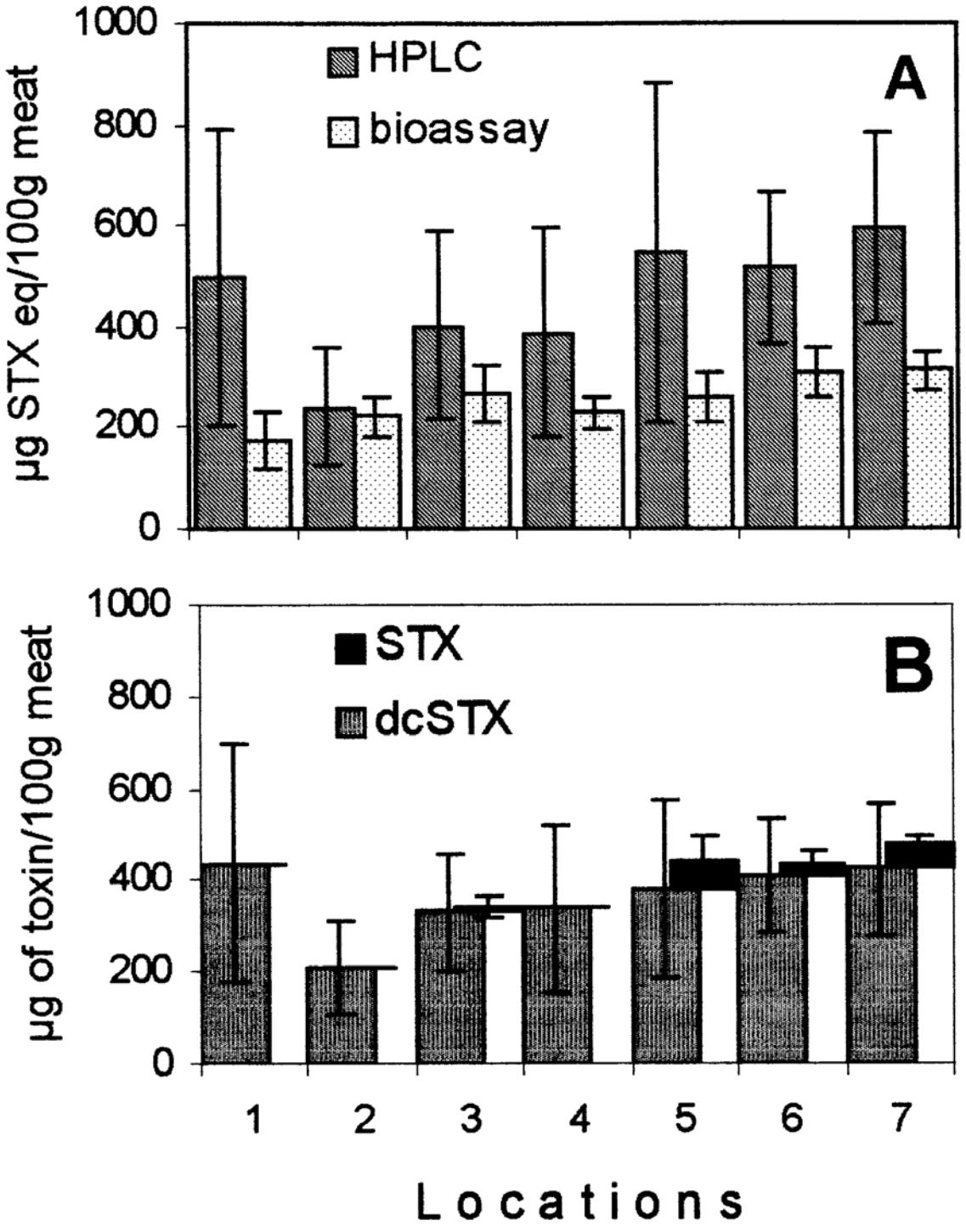

Fig. 2. Toxicity of *Haliotis tuberculata* (mean and confidence interval of three stations, α=0.05) at the different locations shown in Fig. 1. (A) Micrograms STX eq/100 g by HPLC and mouse bioassay; (B) dcSTX and STX contents by HPLC.

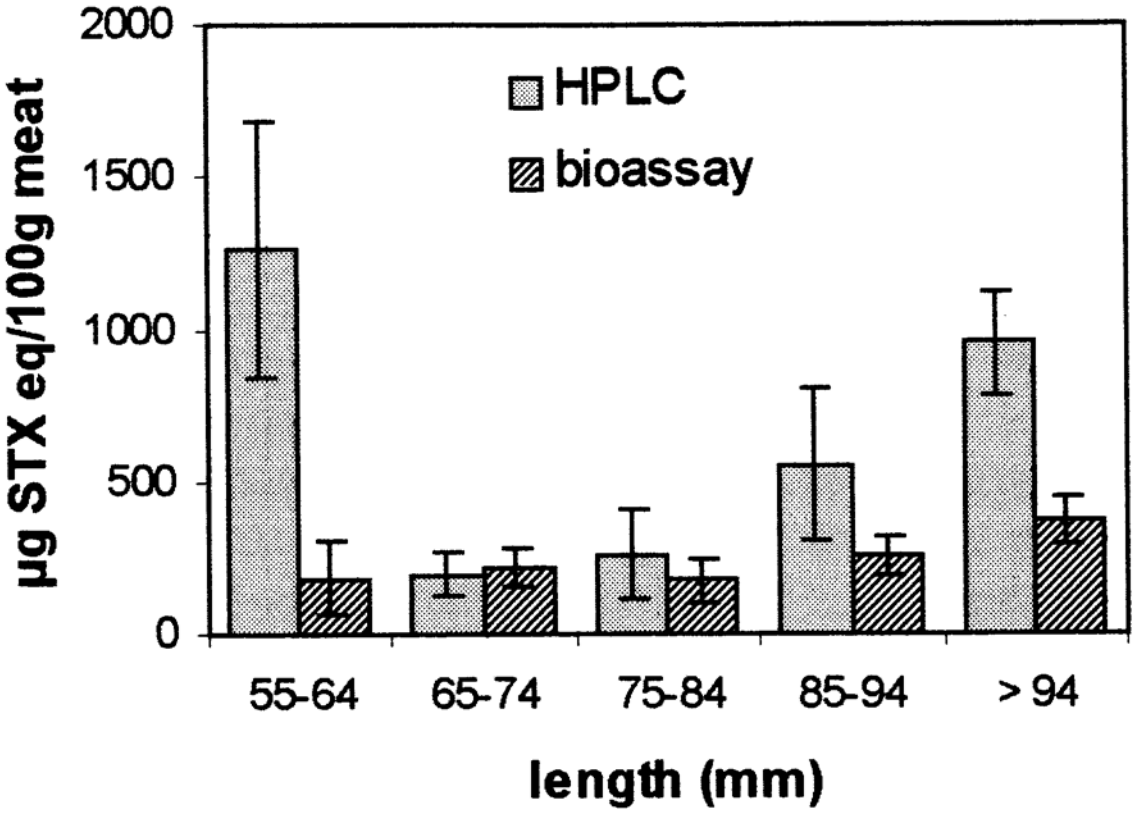

Fig. 3. Toxin content by HPLC and bioassay of different length groups of *Haliotis tuberculata* (mean and confidence interval, α=0.05).

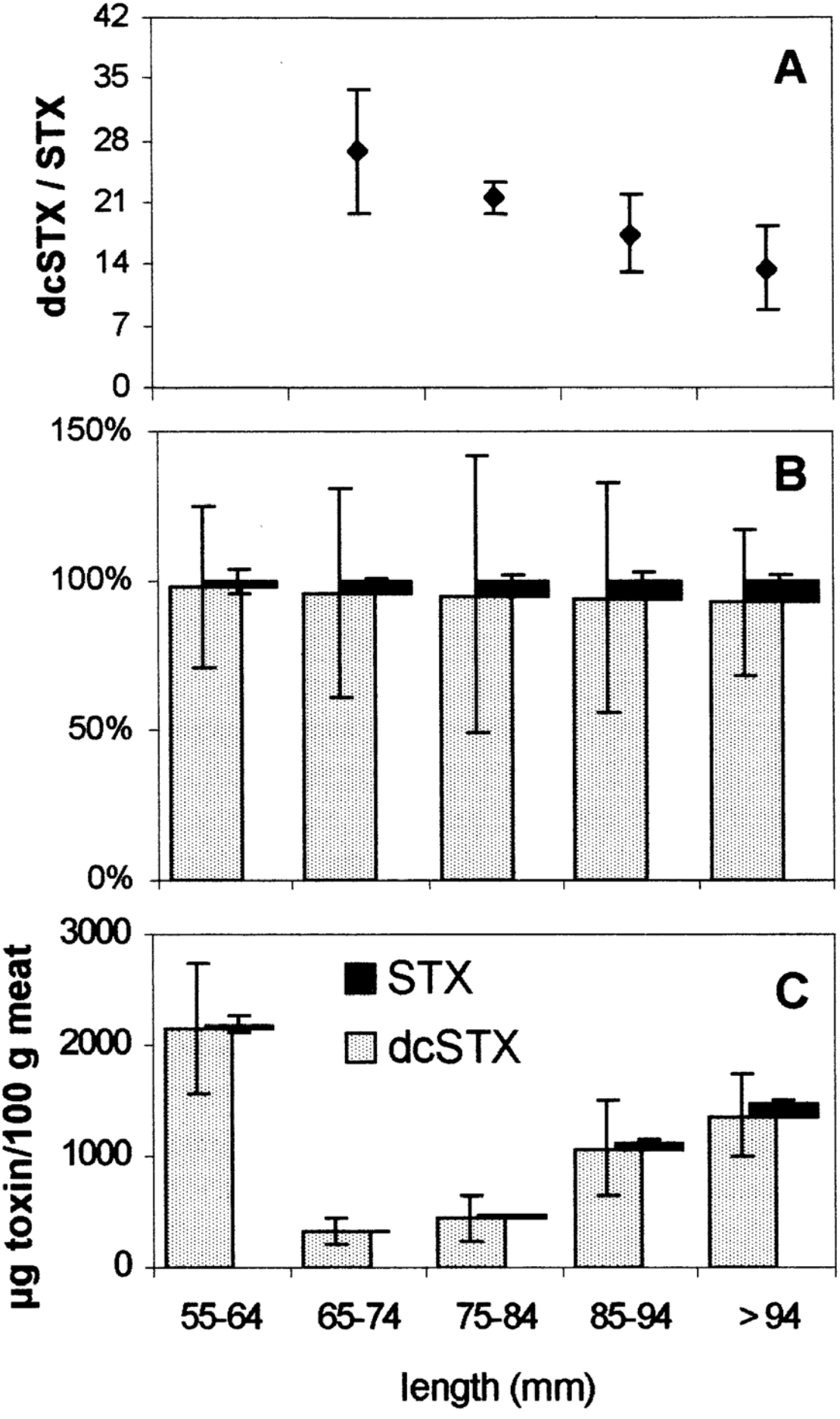
A
dcSTX / STX
42
35
28
21
14
7
0
B
150%
100%
50%
0%
C
STX
dcSTX
µg toxin/100 g meat
3000
2000
1000
0
55-64
65-74
75-84
85-94
> 94
length (mm)

Fig. 4. Toxin composition of different length groups of *Haliotis tuberculata* (mean and confidence interval, α=0.05). (A) dcSTX/STX ratio; (B) toxin content percentage; (C) weight concentrations.

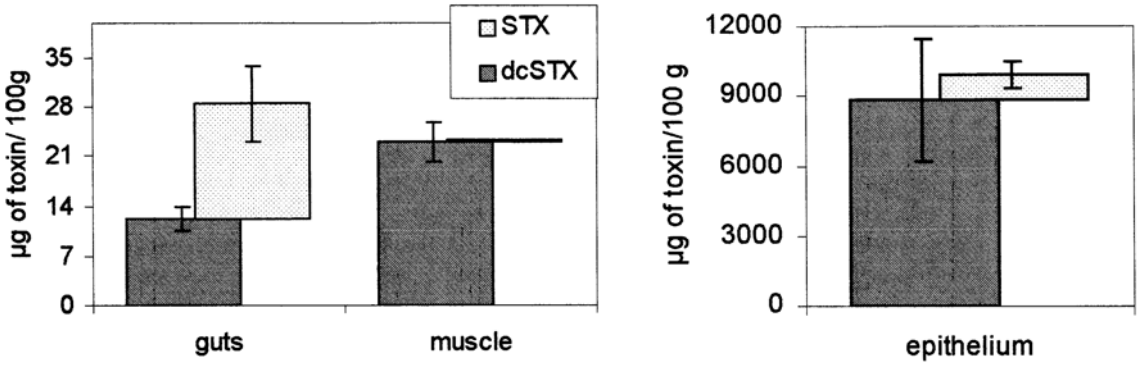

Fig. 5. Toxin concentrations from different parts of *Haliotis tuberculata* (mean and confidence interval, α=0.05).

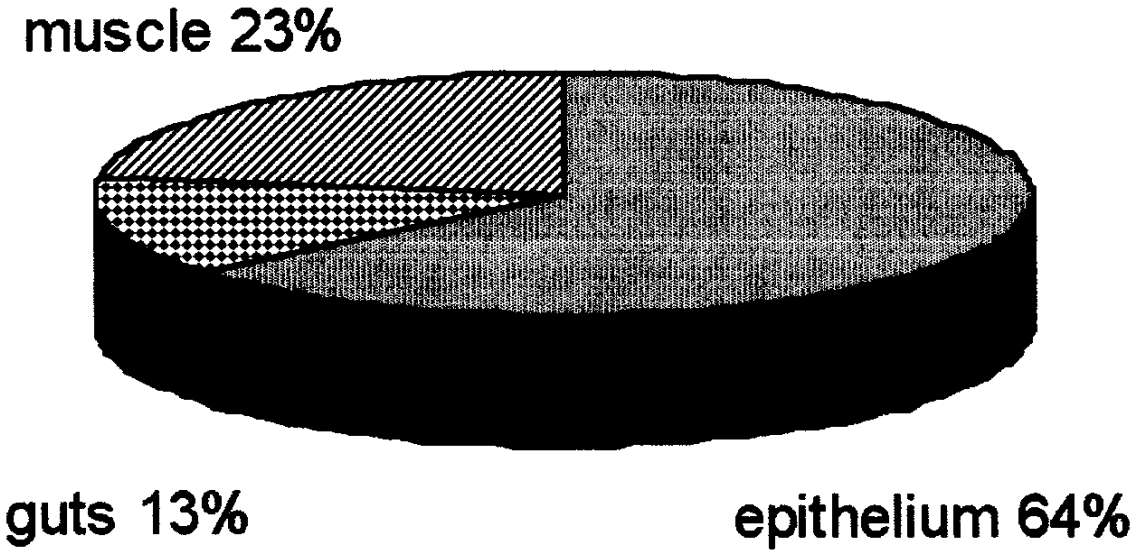

Fig. 6. Contribution of each part of the ormer to total mollusc toxicity.

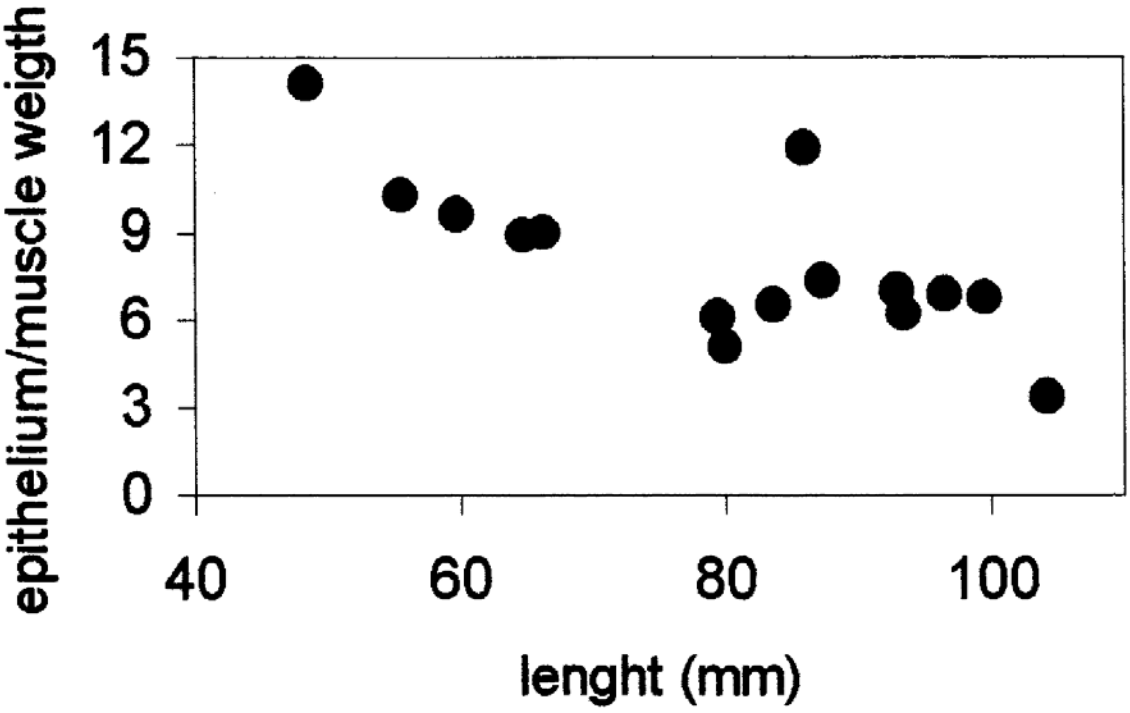

Fig. 7. Distribution of the epithelium/muscle weight ratios by length of *Haliotis tuberculata*

Table 1

Non-orthogonal analysis of variance for the total toxin content

Source of variation	d.f.	SS	MS	*F*-value
Model	4	128775.5	44693.9	24.15*
Length	2	3315.3	1657.5	0.90
Parts	2	175460.5	87730.2	47.41*
Residual	67	123985.6	1850.5	-
Total	71	302761.1	-	-

* $P<0.001$.