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The effect of some habitual cooking processes on the domoic acid concentration in the cockle (*Cerastoderma edule*) and Manila clam (*Ruditapes philippinarum*)

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Abstract

The effect of cooking on the concentration and burden of domoic acid in two bivalve molluscs was studied. The Manila clam (*Ruditapes philippinarum*) and cockle (*Cerastoderma edule*) were subjected to steaming and boiling, respectively. In both cases, factorial plans were used to evaluate the effects of common cooking methods and the variations likely to take place during the cooking procedure (cooking time and salt concentration in both species, in addition to ethanol percentage in Manila clam). The domoic acid concentration and toxin content were affected by cooking in very different ways in the two species studied. The cockle lost a significant part of its domoic acid content, while the clam did not. Since the weight of the soft tissues in cooked bivalves was lower than in the raw samples in both species, the toxin concentration decreased less than the toxin burden in the cockle, while it increased in the clam, where the toxin burden did not change significantly. Among the cooking variables tested, only cooking time had a noticeable effect on the domoic acid content in the clam and cockle, with the bivalves that were cooked for a longer time having smaller amounts of toxin. It is clear that cooking affects the toxin concentration in bivalves in a way that is species-specific. This characteristic must be taken into account when evaluating epidemiological information, establishing allowable toxin levels and in cases where pre-processing treatments such as cooking or similar methods are used in monitoring systems.

Introduction

Filter feeding shellfish such as mussels, clams, oysters and cockles can accumulate domoic acid which is the main toxin responsible for amnesic shellfish poisoning (ASP) in high concentrations. These organisms ingest phytoplankton during algal blooms which are, in some cases, composed of species that produce toxins. Some *Pseudo-nitzschia* species produce domoic acid and consequently, when they bloom in the plankton, these species become the primary sources of domoic acid in bivalve shellfish food. In such cases the toxin is accumulated by the bivalves, which when consumed by humans, may produce a type of poisoning known as amnesic shellfish poisoning (ASP), since one of the symptoms of this syndrome is recent memory loss. In 1987, the first episode of this kind of toxicity was recorded in Canada, causing three

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3 fatalities. The symptoms included vomiting and diarrhea followed by confusion,
4 memory loss and, in critical cases, coma (Wright et al. 1989).
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9 In September 1994, during a phytoplankton bloom, consisting mainly of *Pseudo-*
10 *nitzschia australis*, ASP toxins were detected in cultured mussels from Galician Rías
11 (Míguez et al. 1996). Since then, the Galician monitoring system has been recording
12 outbreaks of this type of toxicity in spring and autumn practically every year (Correa et
13 al. 2007). The frequent closure of the main shellfish production areas due to these
14 recurrent episodes is having an important economical impact on Galicia and many other
15 areas in Europe.
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23 In order to minimize economic losses while maintaining consumer safety, the maximum
24 allowable level of these toxins needs to be fine-tuned. This is a difficult task, mainly
25 because intoxications of this kind are difficult to detect, but also because, when
26 detection does occur, there is little information on the effect of cooking on the toxin
27 concentration which is generally required to transform the toxicity found in ingested
28 food to raw shellfish toxicity (the latter being the one needed for harvesting regulation).
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35 The effect of cooking (or canning) on the concentration of toxins, other than ASP
36 toxins, in shellfish has been evaluated in several studies. Lawrence et al. (1994) reported
37 reductions of Paralytic Shellfish Poisoning toxins (PST) in cooked (steamed or boiled)
38 lobsters, and Berenguer et al. (1993) and Vieites et al. (1999) examined the
39 concentration of the same group of toxins during canning, in *Acantocardia*
40 *tuberculatum* and *Mytilus galloprovincialis*, respectively. More recently, Hess et al.
41 (2005) studied the effects of cooking on azaspiracids (AZP toxins), okadaic and
42 dinophysistoxin-2- all of which are lipophilic toxins- in the mussel *Mytilus edulis*. In
43 relation to ASP toxins, only two recent studies focused on the effect of some cooking
44 processes on the concentration of domoic acid (the main ASP toxin) in shellfish.
45 McCarron et al. (2006) found that the toxin concentration in the mussel *Mytilus edulis*
46 was unaffected or actually underwent a slight increase (20%) by steaming, depending
47 on the location in which the mussels were harvested. In the other shellfish species
48 studied, the Dungeness crab (*Cancer magister*), Hatfield et al. (1995) found a 67-71%
49 reduction of domoic acid concentration in the viscera after the cooking process (boiling
50 in fresh water or 3% salt water). To our knowledge, no studies have been done on other
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3 species, including the cockle *Cerastoderma edule* and the Manila clam *Ruditapes*
4 *philippinarum*, which are important species in the shellfish market (Anonymous 2004,
5 Anonymous 2008).
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10 Cooking processes have some degree of variability. The amount of water, salt
11 concentration, temperature, time and other factors may sometimes vary, depending on
12 the preferences of the cook. No information has been reported on the effect of these
13 variations on the final toxicity of the cooked food.
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19 Additionally, some monitoring programs have included pre-treatments (i.e. mild
20 steaming) of the samples prior to analysis in order to facilitate shell removal and
21 stabilize the matrix (Hess et al. 2005). This is the case, for example, of the monitoring
22 systems in Denmark and Germany which cook the mussels by light steaming before
23 analysis (McCarron et al. 2008). The current European regulation stipulates that the
24 total content of amnesic toxin in the edible part of molluscs should not exceed 20 mg of
25 domoic acid kg⁻¹ of meat (Community Decision of March 15, 2002), but it does not
26 address the question of pre-treatments. If treatments are found to have a significant
27 effect on the toxicity of the samples and their use is not strictly regulated, they might
28 cause major changes in the current levels of closure used by the different monitoring
29 systems, thus compromising the efforts made to achieve the harmonization of the
30 regulations worldwide.
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42 In this paper we have studied, the effect of: a) the usual cooking procedures, and b)
43 some deviations from the standard procedure likely to take place during the cooking
44 process on domoic acid concentrations in two commercially important species (cockle
45 *Cerastoderma edule* and Manila clam *Ruditapes philippinarum*).
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51 **Material and methods**

52 ***Biological material***

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Cockles (*Cerastoderma edule*) and Manila clams (*Ruditapes philippinarum*) naturally
contaminated with ASP toxins were obtained from the Ría de Camariñas (NW Spain).
A 5-kg sample of cockle and a 6-kg sample of Manila clam were taken and transported

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3 to the laboratory within a few hours of their collection. There, the individuals were
4 carefully mixed and a number of 15-individual subsamples (depending on the
5 experiment) were obtained completely at random. All individuals were weighed prior to
6 being subjected to the experimental treatments.
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10 11 12 13 *Experimental design*

14 The experimental designs used consisted of two parts that focused directly on the two
15 objectives of this work. In order to quantify the effect of variations during the cooking
16 process, 2-level factorial plans with central points were used. The effect of the standard
17 way of cooking was quantified by means of a simple comparison between raw samples
18 and the central points of the factorial designs were planned.
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24 Two different factorial plans were used for each species to account for differences in
25 common cooking methods. Cockles are usually steamed for a short time in a small
26 amount of water (without being submerged), in some cases containing salt. Clams
27 (clams marinière) are usually boiled, with the water containing some salt. Wine is
28 frequently added, but in this experiment it was substituted by 12% (v:v) ethanol.
29 Consequently the factorial design corresponding to cockles included two factors
30 (steaming time and salt content, each having two levels (plus the central points). As
31 regards opening time, the lowest level was the time it took the cockles to open and the
32 highest level was that time plus 5 min. The salt levels were 0 and 35g L⁻¹ (Figure 1A).
33 The design for clams included three factors: boiling time, salt content and percentage of
34 ethanol. Boiling and salt levels were the same as in the cockle, and the ethanol
35 percentages were 0% and 6% (Figure 1B). Cockle treatments were performed in
36 duplicate, while clam treatments were carried out without replication, with the
37 exception of 4 central points that were included in both experiments.
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51 All computations were made with Minitab 15 Statistical package.
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55 56 57 *Chemicals, toxin extraction and analysis*

58 Domoic acid calibration solutions were prepared from certified reference materials
59 (NRC Canada CRM-DA-d). Methanol extra pure was obtained from Scharlab; ethanol
60 96 % (v/v) from Panreac, and acetic acid (glacial), acetonitrile of isocratic grade for

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3 liquid chromatography and sodium chloride for analysis were purchased from Merck.
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5 Ultrapure water from a Milli-Q system (Millipore) was used in all preparations and
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7 manipulations requiring aqueous solutions.
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10 Domoic acid was extracted from shellfish meat following the method of Quilliam et al.
11 (1995). The tissues in each sample were homogenized and 4 g of this homogenate were
12 disrupted with Ultraturrax (IKA), for 3 min, with 16 mL of methanol-water (50:50,
13 v:v). The resulting slurry was then centrifuged at 4000 rpm for 10 min and the
14 supernatant filtered through a Millipore filter (Millex-HN, Nylon 0.45 μ m). The extracts
15 were directly injected in the HPLC. Omitting the SPE-SAX purification step in the
16 method because no interferent was detected by the DAD purity test, and because its use
17 increases the variation coefficient of the determinations.
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26 HPLC analyses were carried out by the method of Quilliam et al. (1995) with some
27 minor modifications in order to adapt the method to existing equipments: 1) a Waters
28 HPLC system including a photo diode array detector (PDA) was used for analysis; 2) a
29 Lichrospher RP18 125 x 4mm, 5 μ m, reverse phase C18 column was used; 3) the
30 isocratic elution was carried out with a mobile phase of water, acetonitrile and acetic
31 acid, at a ratio of 89:10:1 v:v:v; and 4) the flow rate was 0.6 mL \cdot min⁻¹.
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39 Domoic acid was identified by comparing the retention time with that of a reference
40 solution obtained from NRC, Halifax, Canada, and by examining the UV spectrum
41 (maximum at 242nm). It was quantified by means of a comparison of the response of
42 the samples at 242 nm, with the above-mentioned reference solution, and the purity of
43 the peaks was checked by means of the wavelength (242.0 \pm 2.4nm) and by visual
44 examination of the spectrum.
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51 **Results**

52 ***Experiment A: Steamed Cockles***

53 *Effect of standard cooking.*

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56 Meat weight decreased (P=0.001) by 38% with the standard cooking process. This
57 decrease was mainly due to dehydration, as no significant amount of particulate matter
58 was found in the cooking water after the process (Figure 2A). The domoic acid burden
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3 of the cockle was also substantially reduced ($P= 0.001$). This reduction was estimated to
4 be 52 % of the initial toxin amount (Figure 2B). The domoic acid concentration also
5 decreased but to a lesser extent than the toxin burden (23%, $P=0.032$), as would be
6 expected given the fact that toxin burden and meat weight have opposite effects on
7 concentration, as it is the ratio between the two variables).
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12 13 14 15 *Effect of variations in cooking conditions*

16 Steaming time was the only cooking variable with a relevant effect on tissue weight and
17 toxin burden, producing a significant decrease in both variables ($P = 0.002$, in both
18 cases). The other factor tested (salt concentration) and the interaction between the two
19 factors (salt and time) (Figure 3A, B) did not have any statistically significant effects
20 (Table 1).
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26 Although the domoic acid concentrations observed after cooking for the longest time
27 period and with the highest salt level were *ca.* 10% smaller than those corresponding to
28 the lowest levels of these factors, (Figure 3C), the differences were not statistically
29 significant. Moreover these two variables do not seem to interact with one another,
30 since no statistical significance for the interaction term was found.
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36 ***Experiment B: Clams marinière***

37 38 39 *Effect of standard cooking*

40 The response of the meat weight of Manila clams to the cooking process was
41 similar to that of cockles, but the responses of toxin burden and concentration were
42 substantially different. Cooking produced a noticeable reduction (29 %) in the weight of
43 the soft tissues ($P = 0.006$). As in cockles, this transformation was not due to a loss of
44 particulate matter, since its presence in the cooking liquids was found to be negligible.
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51 The domoic acid burden of the soft tissues remained unchanged after cooking,
52 indicating that it was not degraded or released into the cooking liquid (Figure 4B). As
53 the content of domoic was constant, the toxin concentration was only affected by the
54 decrease in meat weight which, having diminished with cooking, produced a significant
55 increase in concentration ($P = 0.045$) which was 26.5% higher in cooked molluscs than
56 in raw ones (Figure 4C).
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Effect of variations in cooking conditions

Cooking time was found to have a negative effect on the toxin burden and toxin concentration but not on meat weight. In the latter variable a slight decrease (*ca.* 5% of the average) was observed but it was not statistically significant (Figure 5, Table 2). The above-mentioned effects of time were only found to be significant when the factorial experiment was simplified by removing salt concentration and ethanol concentration as factors.

Discussion

Cooking can affect the domoic acid concentration and burden of different species in different ways. Toxin concentration decreased in the cockle while it increased in the Manila clam. This difference is due to two facts: 1) during cooking, the cockle loses a significant proportion of its toxin, but the Manila clam does not; and 2) in both species the weight of the soft tissues decreased, thus causing the toxin to concentrate in less biomass. In both cases weight undergoes a decrease. However, while in the cockle it partially counteracts the effect of the decrease of toxin burden in the concentration, in the case of Manila clams, where no reduction in toxin burden was detected, it produces an increase in concentration. The two species examined in this study, would seem to be in terms of response to cooking, like the mussel *Mytilus edulis* (McCarron et al. 2006), since the toxin concentration is maintained or increases only slightly, depending on the balance between the increase produced by weight reduction and the decrease derived from toxin loss.

Although the cooking processes studied here were not exactly the same, they share the same basic characteristics, i.e., they are thermal treatments in an aqueous environment, which would suggest that the differences found are more likely related to the species rather than to the differences in treatment. Only small differences in domoic acid concentration were able to be attributed to the likely deviations from the standard cooking procedure. In fact, only the cooking time seems to have had a significant effect, i.e., a reduction in toxin burden and concentration. Salt concentration during cooking had no effect either, as was also reported by (Hatfield et al. 1995) in the Dungeness crab *Cancer magister*, where the effect of using salt water (3%NaCl) versus fresh water on the resulting domoic acid content was investigated.

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5 In view of the information presented above, it seems clear that the actual banning levels
6 based on raw bivalves might over- or under-protect the consumers when shellfish are
7 cooked. Moreover, epidemiological data based on toxin or toxicity quantification of
8 raw shellfish, should be carefully evaluated if the intoxications are produced by cooked
9 specimens. The use of cooked samples for monitoring purposes or the inclusion of
10 species-specific factors to transform raw toxicity to cooked toxicity would be useful for
11 the optimization of shellfish aquaculture or fisheries while safeguarding consumer
12 health, provided that similar cooking methods are generally used for all species.
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21 It is clear that monitoring the resources using heating-related pre-treatment of samples
22 might in fact be more or less permissive than using raw samples, depending on the
23 species and the toxin group. In the cockle, for example, control carried out using cooked
24 samples would be more permissive than with raw samples and the opposite would be
25 expected to be the case in Manila clams. Mussels *Mytilus edulis* would probably not be
26 affected at all in view of the results of McCarron et al. (2006). While the approach
27 might be correct, in the sense that it could approach more closely the actual levels that
28 the consumers ingest, the direct introduction of these pre-processing steps without
29 adapting the banning levels, (that are in general defined for raw shellfish) could produce
30 differences in the actual banning levels used in the different monitoring systems or
31 when dealing with different species. This fact should be taken into account in order to
32 harmonize the regulation in the different countries and to prevent food alerts originating
33 from the importation of products subjected to different (not theoretical but practical),
34 banning levels.
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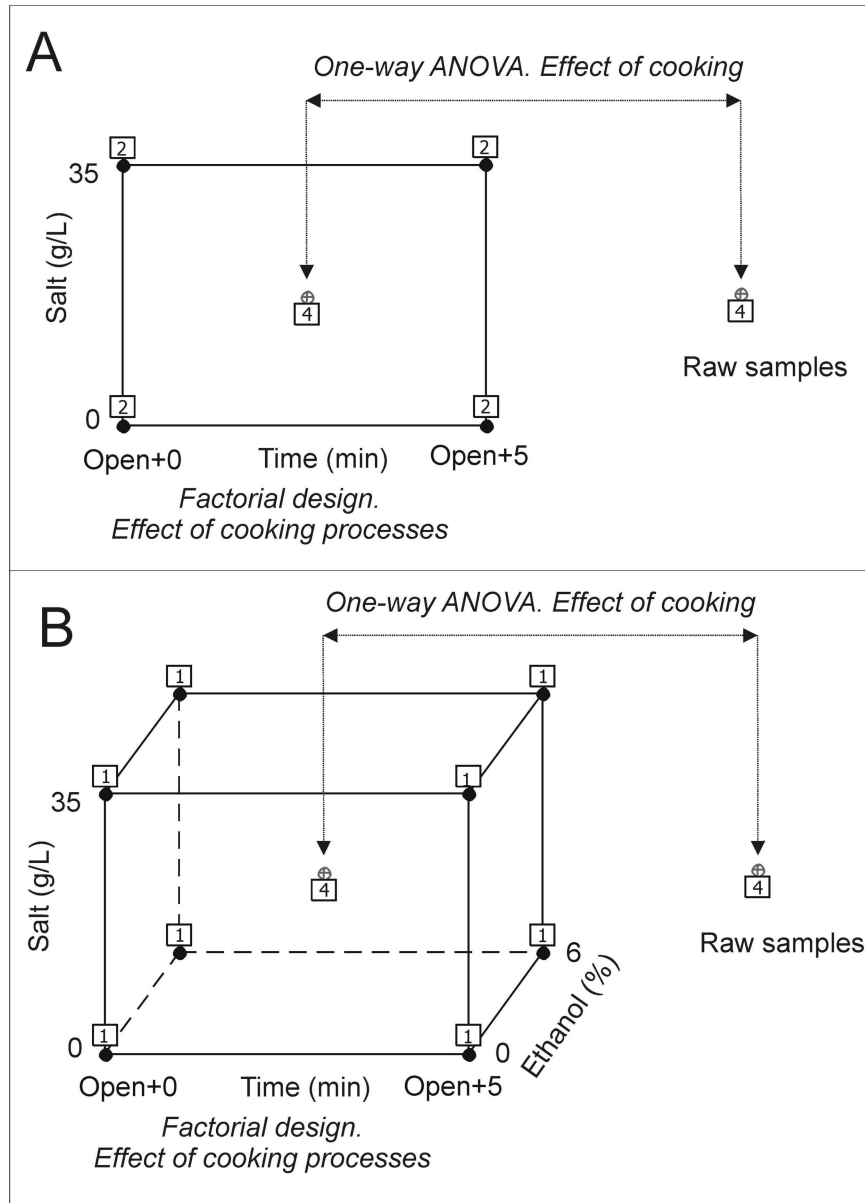


Figure 1. Diagram of the two experiments. (A) Effect of cooking on cockle; and (B) on Manila clam. In both cases raw samples are compared to the central point of the factorial part of the experiment. Numbers inside the small squares indicate the number of replicates of each design point. 104x146mm (600 x 600 DPI)

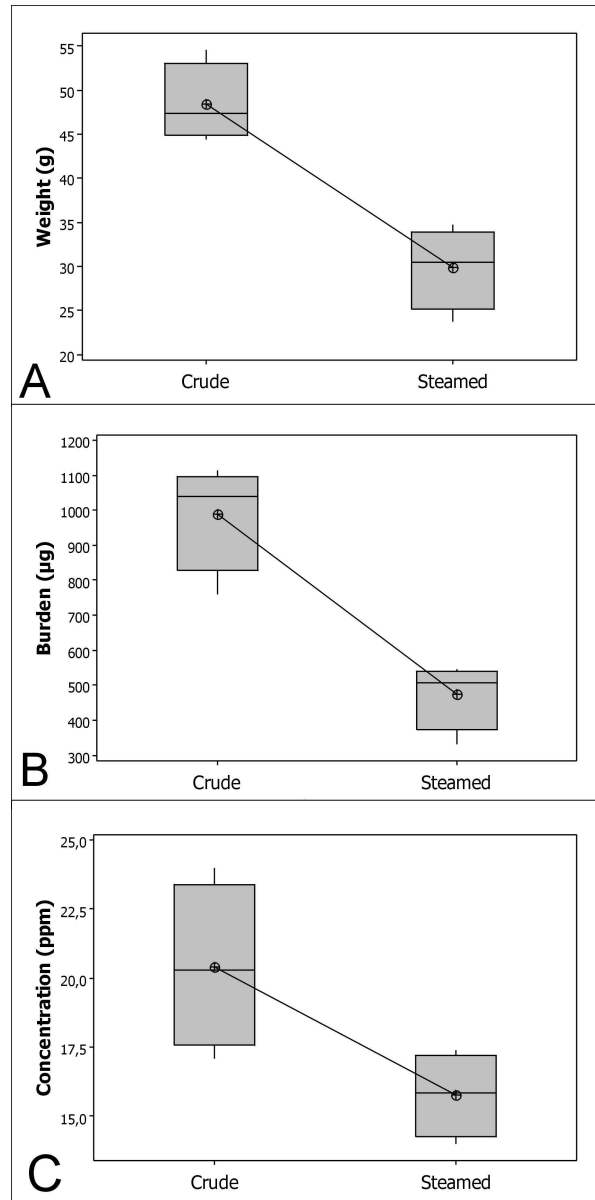


Figure 2. Weight (A), burden (B) and domoic acid concentration (C) of the soft tissues (edible part) of the cockle *Cerastoderma edule* after (steamed) and before (raw) the standard cooking of this species (steaming).

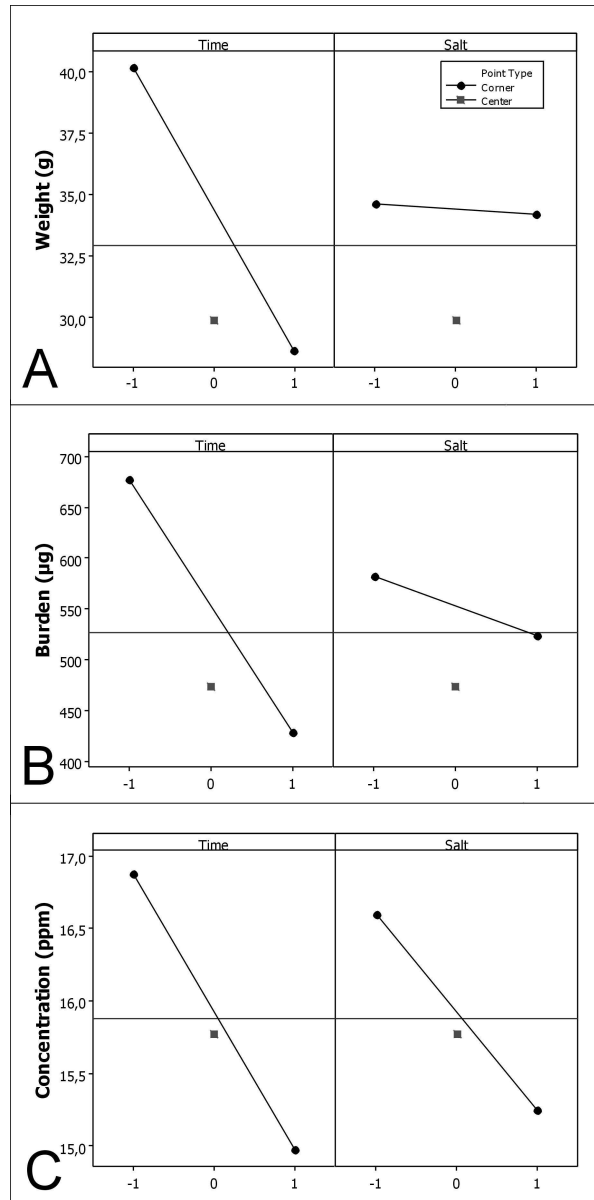


Figure 3. Main effects of steaming time and salt concentration on weight (A), domoic acid concentration (B) and burden (C) of the soft tissues (edible part) of the cockle *Cerastoderma edule*. 553x984mm (96 x 96 DPI)

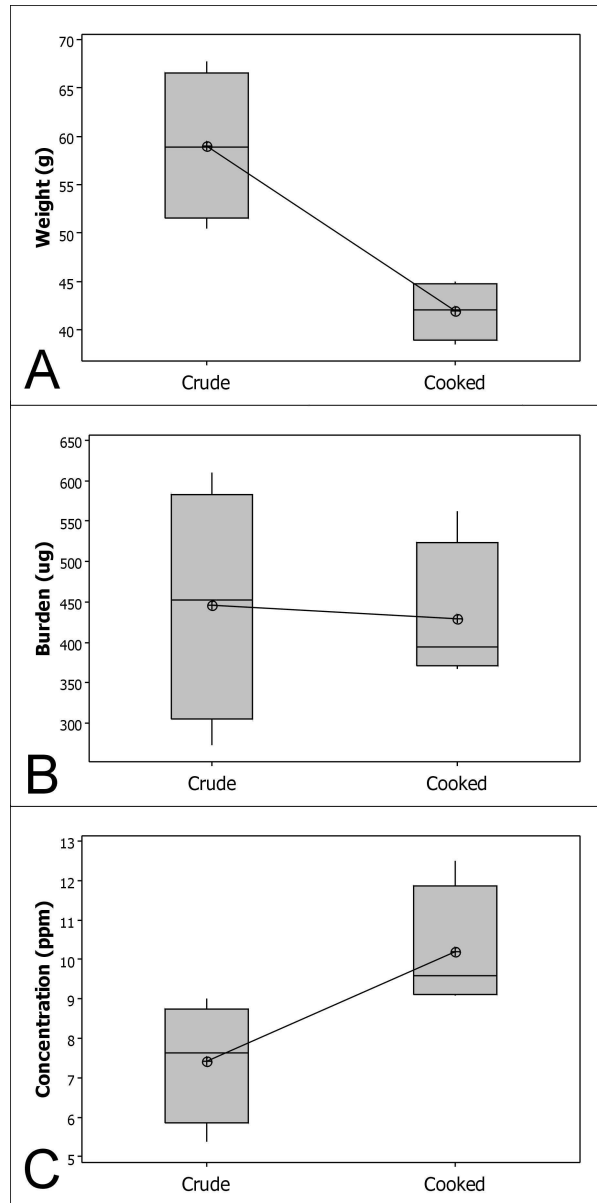


Figure 4. Weight (A), burden (B) and domoic acid concentration (C) of the soft tissues (edible part) of the Manila clam *Ruditapes philippinarum* after (cooked) and before (raw) the standard cooking of this species (cooking).
526x980mm (96 x 96 DPI)

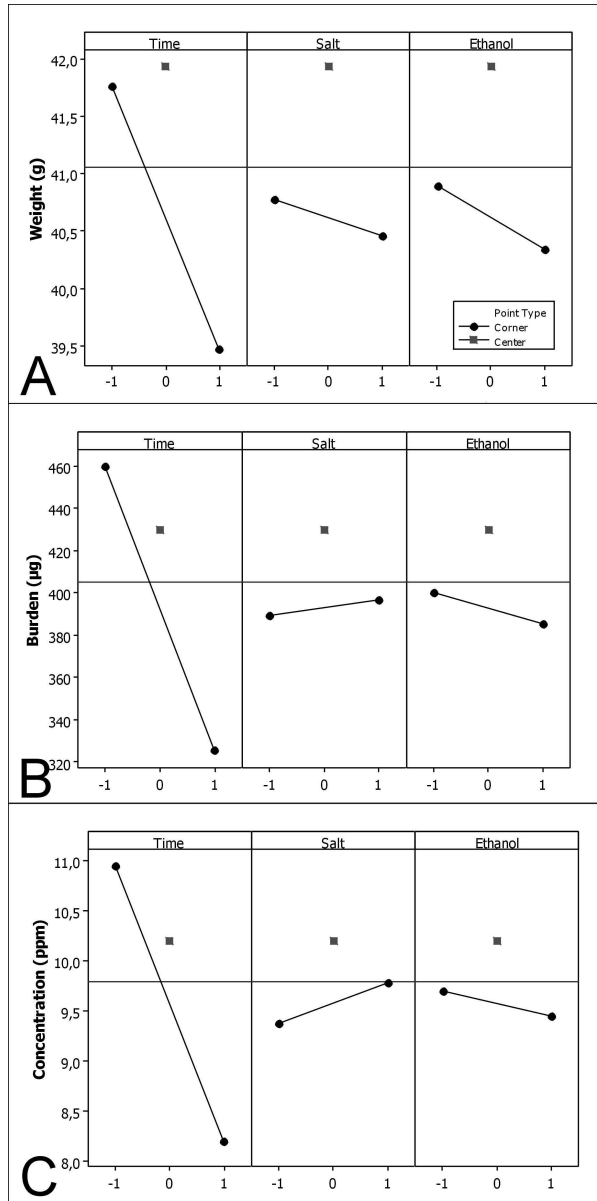


Figure 5. Main effects of weight (A), burden (B) and domoic acid concentration (C) on the soft tissues (edible part) of the Manila clam *Ruditapes philippinarum* after cooking.
600x979mm (96 x 96 DPI)

Table 1. - Results of the analyses of the factorial experiment for cockle, for the two variables studied. The upper part of the table shows the magnitude of the effects, their corresponding coefficients and Ts and their probabilities. The lower part shows the ANOVA of the design indicating the degrees of freedom (DF), the mean square (MS), F and its probability.

Analysis of variance	Weight				Burden				DA Concentration			
	Effect	Coef	T	P	Effect	Coef	T	P	Effect	Coef	T	P
Time	-11.5	-5.7	-5.0	0.002	-249.2	-124.6	20.5	0.002	-1.9	-0.95	-1.4	0.211
Salt	-0.39	-0.2	-0.2	0.868	-58.3	-29.1	-4.6	0.317	-1.3	-0.67	-1.0	0.361
Time*salt	2.29	1.1	1.0	0.352	63.4	31.7	-1.2	0.279	0.5	0.25	0.4	0.728
Source	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P
Main effects	2	132.06	12.51	0.005	2	65501	11.22	0.007	2	5.4325	1.42	0.303
2-Way Interac.	1	10.49	0.99	0.352	1	8043	1.38	0.279	1	0.5000	0.13	0.728
Error	7	10.56			7	5840			7	3.8139		

Table 2. - Results of the analyses of the factorial experiment for clams (simplified to one factor). The upper part of the table shows the magnitude of the effects, their corresponding coefficients and Ts and their probabilities. The lower part shows the ANOVA of the design indicating the degrees of freedom (DF), the mean square (MS), F and its probability.

Analysis of variance	Weight				Burden				DA Concentration			
Term	Effect	Coef	T	P	Effect	Coef	T	P	Effect	Coef	T	P
Time	-2.282	-1.141	-0.92	0.378	-134.04	-67.02	-2.28	0.046	-2.75	-1.37	-2.72	0.022
Source	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P
Main effects	1	10.420	0.85	0.378	1	35934	5.19	0.046	1	15.12	7.37	0.022
Error	9	13.082			9	7297			9	2.163		