TITLE: Detoxification of paralytic shellfish poisoning toxins in naturally contaminated mussels, clams and scallops by an industrial procedure

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Abstract

Paralytic shellfish poisoning (PSP) episodes cause important economic impacts due to closure of shellfish production areas in order to protect human health. These closures, if are frequent and persistent, can seriously affect shellfish producers and the seafood industry, among others. In this study, we have developed an alternative processing method for bivalves with PSP content above the legal limit, which allows reducing toxicity to acceptable levels. A modification of the PSP detoxifying procedure stablished by Decision 96/77/EC of the European Union in *Acanthocardia tuberculatum*, was developed and implemented for PSP elimination in other species of bivalves. The procedure was applied to 6 batches of mussels, 2 batches of clams and 2 batches of scallops, achieving detoxification rates of around 85%. A viable industrial protocol which allows the transformation of a product at risk into a safe product was developed. Although a significant reduction was obtained, in a sample circa 9000 µg STX diHCl equiv/kg, the final toxin level in these highly toxic mussels did not fall below the European limit. The processing protocol described may be applied efficiently to mussels, clams and scallops and it may be a major solution to counteract the closure of shellfish harvesting areas, especially if persistent.

Highlights

- An industrial protocol aimed at reducing PSP toxin levels was developed and optimized in mussels, clams and scallops.
- The procedure was applied to some batches of PSP-contaminated molluscs obtaining ± 85 % detoxification and a safe product.
- However, one sample with an exceptionally high toxicity, 9000 µg STX diHCl equiv/kg, did not fall below the European limit.
- An economically feasible bivalve canning processing was implemented, guaranteeing the manufacture of a safe product.

Keywords: *Paralytic shellfish poisoning,* detoxification, industrial protocol**,** *mollusks, LC-FLD. (maximum 6 descriptive keywords)*

Graphical abstract (optional)

Abbreviations:

C1-4, N-sulfo-carbamoyl gonyautoxins

CRM, certified reference material

dcGTX decarbamoyl gonyautoxin

dcNEO, decarbamoyl neosaxitoxin

dcSTX, decarbamoyl saxitoxin

equiv, equivalent

GTX1-6, gonyautoxins

HABs, harmful algal blooms

i.p., intraperitoneal

LC-FLD, liquid chromatography-fluorescence detection

NEO, neosaxitoxin

PSP, paralytic shellfish poisoning

STX, saxitoxin

TEF, toxicity equivalency factor

1 Introduction

Paralytic shellfish poisoning (PSP) is caused by consumption of shellfish containing PSP toxins of the family of saxitoxins (STX) (EFSA, 2009). These toxins are produced by microalgae, mainly toxic marine dinoflagellates such as species of the genera *Alexandrium* and *Gymnodinium*, and also by certain freshwater cyanobacteria (Gracia Villalobos et al., 2019) (Pitois et al., 2018) (Fabre et al., 2017). These toxins are accumulated and sometimes metabolized into toxin derivatives in many species of filter-feeding bivalves, as mussels, clams and scallops, making them potentially toxic to humans. Harmful algal blooms (HABs) can also induce other ecological damage and adverse effects to living marine resources. In fact, some bivalves can be impaired during intense toxic episodes. For instance, a population of the surf clam *Mesodesma donacium* with high PSP toxic levels, died due to the desiccation caused by the incapability of the clams to burrow (Álvarez et al., 2019).

To protect public health and ensure the quality of seafood, monitoring programs are implemented worldwide in order to detect and quantify these toxins, and eventually forbidding shellfish harvesting when levels of toxins exceed the legal limit laid down in current regulations. In Europe for example, harvesting and commercialization of bivalves is prohibited above the threshold of 800 µg STX diHCl equiv/kg of shellfish tissues (EC, 2004). Closure of shellfish production areas has an important economic impact for producers and other associated industries. No solutions have been found to prevent these important episodes which are seldom predictable, and despite the influence of PSP events on human health and fisheries, studies on shellfish detoxification to mitigate this problem are still very scarce.

Natural detoxification occurs very slowly and it is conditioned by the presence of toxin producing microalgae in the water column. Lipophilic toxins are retained longer than the hydrophilic toxins, such as PSP toxins, although the detoxification rate depends on the species, concentration of toxins and environmental conditions (Lee et al., 2008). Several studies described that the concentration of some PSP toxin analogues in bivalves, but not all of them, can be reduced by exposing contaminated shellfish to a non-toxic diet (Reis Costa et al., 2018). Nevertheless, mitigating or modulating the presence of microalgae in the field is currently not possible, so this eventual solution should be applied by maintaining large stocks of shellfish in a closed space for several days, and the feasibility of this would be dubious.

Once harvested, toxin reduction or elimination from shellfish is mainly affected by the chemical properties of the toxins. In the particular case of PSP toxins, a regulation was published after performing scientific studies which proved that a suitable heat treatment decreased the levels of PSP toxins and guaranteed the safety of the cockle *Acanthocardia tuberculata* (Berenguer et al., 1993; EC, 1996).

A detoxification procedure would result in an economically feasible solution for a shellfish canning industry in locations where PSP toxic episodes occur very often or are persistent, and large amounts of shellfish are affected. Besides, in view of the changing environmental conditions related to climate change, a rise in the incidence of these episodes could take place in the near future (Barbosa et al., 2019). Changes in the profiling and behavior of PSP toxic episodes, leading to lower toxicity values but longer toxic episodes have been proposed (Braga et al., 2018). It is important to mention that it would not be necessary to

perform important modifications in factory installations to accomplish the PSP detoxification protocol. The required equipment is the same usually employed by the canning industry and factories applying this protocol do actually exist in the case of giant cockle. If a regulation for this detoxification protocol was finally approved, the importance of such modifications will depend on each individual factory and the decision to implement it or not would be due more to economic than technical reasons. Only the duration of the whole thermal process would be slightly increased.

In this paper, naturally PSP contaminated mussels, clams and scallops were specifically harvested in order to implement the thermal procedure described in the EU decision. Slight modifications were applied, in order to obtain a better efficiency of detoxification and yield of mussels, clams and scallops.

2 Material and methods

2.1 Sampling of contaminated mussels and scallops

Samples were obtained from different sampling points along the Spanish and Portuguese coasts from July 2018 to March 2019. Mussels (*Mytilus galloprovincialis*) were acquired from several mussel raft cultures in: a) Galicia, (samples coming from two different floating rafts in the Ría of Vigo, Pontevedra); b) Andalucía, (one batch of mussels from Benalmádena, Málaga), and c) Portugal, (one batch of mussels from Portinho da Costa, near Lisbon). In addition, other mussel batches were obtained in Catalonia, one sample, after exposure to a toxic bloom of the dinoflagellate *Alexandrium minutum*, inside a harbour, as explained in this article. The phytoplanktonic species involved in the naturally contaminated batches of shellfish are depicted in Table 1. Special

permissions from the local authorities were obtained in order to harvest the toxic molluscs from the closed areas. Two batches of Japanese littleneck clams (*Ruditapes philippinarum*) were obtained from Pontevedra, Galicia (Spain) and both batches of scallops (*Pecten maximus*) were obtained from Málaga, Andalucia (Spain). Sampling zones where toxic mussels, clams and scallops were harvested are depicted in Figure 1.

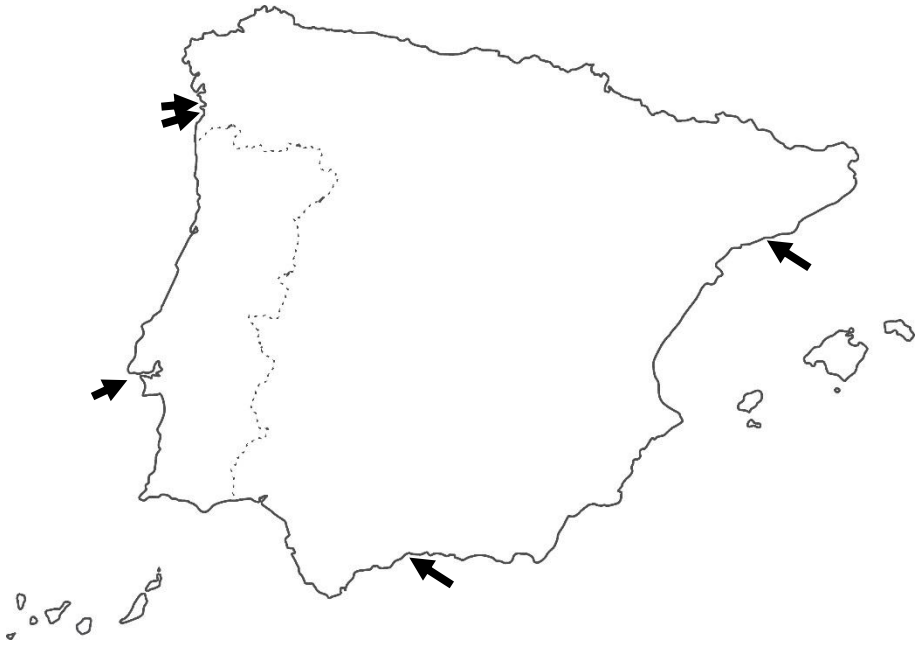


Fig 1: sampling points (marked by arrows) where PSP contaminated mussels and scallops were obtained during the study.

Samples were refrigerated in thermally isolated boxes with cold accumulators after collection and shipped to the laboratory. Upon arrival, samples were processed as indicated in “Detoxification study” and analyzed as described below. Some subsamples of the different batches of mollusks were frozen at -20 °C and processing and analysis was performed after days or weeks until a maximum of 10 weeks.

2.2 Mussel exposure to a toxic bloom of the dinoflagellate Alexandrium minutum

A controlled field study was carried out in the Catalonian coast exposing 50 kg of edible mussel for 5 days to a toxic bloom of *Alexandrium minutum*, a known producer of PSP toxins. The objective was to allow high levels of PSP toxins to bioaccumulate in the mussels. Levels of *A. minutum* were always above 200000 cells/L and, as a result, the concentration of PSP toxins in mussels was higher than 4000 µg STX diHCl equiv/kg.

2.3 Procedure for PSP mussels, clams and scallops detoxification

The regulated procedure (EC, 1996), was applied to all different batches of PSP naturally contaminated mussels, clams and scallops with some modifications:

- Preliminary cleaning in running fresh water for two minutes.
- Pre-cooking in fresh water for three minutes at a temperature of 95 ± 5 °C.
- Separation of flesh and shells.
- Second cleaning in fresh water for 30 seconds.
- Cooking in fresh water for nine minutes at a temperature of 98 ± 5 °C.
- Cooling in running fresh water for approximately 90 seconds.
- Conditioning in containers closed hermetically in a non-acidified liquid medium.
- Sterilization in autoclave at 116 °C for 51 min (referred as “Canning”) or Pasteurization at 90 °C for 10 min.

Separation of the edible parts (foot) from the non-edible parts (gills, viscera and mantle), in mussels and clams, was omitted in order to increase the yielding of

the process. In the case of scallops, edible parts correspond to the sum of adductor muscle and roe. During the different cleaning steps, mollusk flesh was submerged in fresh water, including a last rinse step. To facilitate toxin analysis, drinking water was employed as covering sauce, since the habitual covering medium used in processed *A. tuberculata* products (brine) can interfere with HPLC columns. Samples subjected to the detoxification method are identified along the text as “EC”. Aliquots of the same batches of mussels, clams or scallops were sterilized or pasteurized without applying the detoxification procedure and are identified along the text as “normal”.

2.4 Toxin extraction

Two laboratories, ANFACO and IRTA, were involved in the extraction and the analysis of PSP toxins in the samples, either processed or not. Both laboratories performed the same extraction and analysis protocol described below, only with variations related to the chromatographic columns and LC equipment used.

2.4.1 Chemicals

Milli-Q ultrapure water, acetonitrile LC-MS grade (Scharlau), methanol LC-MS grade (Fisher), ammonium formate HiPerSolv Chromanorm® for LC-MS (VWR), glacial acetic acid reagent grade (Scharlau), periodic acid analytical reagent AnalaR NORMAPUR (VWR), Na_2HPO_4 analysis grade (MERCK), sodium hydroxide reagent grade (Scharlau), hydrogen peroxide solution 30% (v/v) reagent grade (Scharlau), ammonium acetate reagent grade (Scharlau), sodium chloride for analysis (MERCK).

SPE cartridges: SPE C18 sep-pack (3 mL, 500 mg) (Waters), SPE COOH (3 mL, 500 mg) (Bakerbond).

Certified PSP standards used at ANFACO: CRM-00-STX, gonyautoxins 1-5 (CRM-00-GTX1&4, CRM-00-GTX2&3, CRM-00-GTX5), neosaxitoxin (CRM-00-NEO), decarbamoylneosaxitoxin (CRM-00-dcNEO), decarbamoylsaxitoxin (CRM-00-dcSTX), N-sulfocarbamoyl gonyautoxin-2&3 (CRM-00-C1&2), and decarbamoylgonyautoxin-2&3 (CRM-00-dcGTX2&3) and gonyautoxins 6 (CRM-00-GTX6) were purchased from Cifga (Lugo, Spain).

Certified standards PSP used at IRTA: CRM-00-dcGTX2&3, CRM-00-C1&2, CRM-00-dcSTX, CRM-00-GTX2&3, CRM-00-GTX5, CRM-00-STX, CRM-00-GTX1&4, CRM-00-NEO and CRM-00-dcNEO were purchased from the National Research Council (NRC, Halifax, NS, Canada) and CRM-00-GTX6 was purchased from Cifga (Lugo, Spain).

2.4.2 Standard solutions

Standard mixtures were prepared from the commercial standards*,* at ANFACO: MIX I (STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 and C1,2), MIX II (NEO and GTX1,4), MIX III (dcNEO), MIX IV (dcSTX) and MIX V (GTX6). For each MIX, 6 standard calibration levels were prepared by dilution with Milli-Q water in the range 0.006 – 1 µM (MIX I) and 0.015 – 1 µM (the remaining MIX). These standard solutions were preserved at -20°C. Individual LQ were 45 µg equiv. STX diHCl/kg for dcGTX2,3; 5 µg equiv. STX diHCl/kg for C1,2; 40 µg equiv. STX diHCl/kg for dcSTX; 25 µg equiv. STX diHCl/kg for GTX2,3; 5 µg equiv. STX diHCl/kg for GTX5; 40 µg equiv. STX diHCl/kg for STX; 150 µg equiv. STX diHCl/kg for GTX1,4; 140 µg equiv. STX diHCl/kg for NEO; 80 µg equiv. STX

diHCl/kg for dcNEO; 13 µg equiv. STX diHCl/kg for GTX6 and 20 µg equiv. STXdiHCl/kg for C3,4. Sum of individual LQ of PSP toxins at ANFACO-CECOPESCA was 563 µg equiv. STX diHCl/kg. At IRTA, standard mixtures were prepared from the commercial standards*:* MIX I (STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 and C1,2), MIX II (NEO and GTX1,4), MIX III (dcNEO), MIX IV (dcSTX) and MIX V (GTX6). For each MIX, 6 standard calibration levels were prepared by dilution with Milli-Q water in the range LQ-800 µg STX diHCl equiv/kg. Individual LQ were 46 µg equiv. STX diHCl/kg for dcGTX2,3; 6 µg equiv. STX diHCl/kg for C1,2; 40 µg equiv. STX diHCl/kg for dcSTX; 26 µg equiv. STX diHCl/kg for GTX2,3; 5 µg equiv. STX diHCl/kg for GTX5; 40 µg equiv. STX diHCl/kg for STX; 150 µg equiv. STX diHCl/kg for GTX1,4; 140 µg equiv. STX diHCl/kg for NEO; 80 µg equiv. STX diHCl/kg for dcNEO and 13 µg equiv. STX diHCl/kg for GTX6. Sum of individual LQ of PSP toxins was 546 µg equiv. STX diHCl/kg. These standard solutions were preserved at -20°C.

2.4.3 Extraction, clean-up, hydrolysis and oxidation

The method was based on the HPLC-FLD Official Method (AOAC, 2005; Lawrence et al., 2005), and refined as described by Turner et al. and Ben-Gigirey et al. (Ben-Gigirey et al., 2012; Turner et al., 2009). The method involves an acetic acid extraction through clean-up with SPE C18 cartridge extraction followed by periodate oxidation and analysis by HPLC-FLD. If the presence of any toxin is observed, peroxide oxidation and/or fractionation (F1, F2, F3) are then carried out by using COOH ion exchange SPE cartridges with periodate oxidation, injecting the obtained extracts in the HPLC-FLD.

2.4.4 PSP toxin quantitation

The toxins dcGTX2,3, C1,2, dcSTX, GTX2,3, GTX5, STX were quantified in the C18 extract after peroxide oxidation; GTX1,4 and GTX6 in F2 fraction; NEO and dcNEO in F3 fraction and C3,4 in F1 hydrolyzed fraction after periodate oxidation. Total PSP toxicity, expressed as STX diHCl equivalents/kg, is calculated by summing individual toxin concentrations and applying toxicity equivalents factors (TEF) that are established for each toxin according to EFSA Scientific Opinion (EFSA, 2009).

In the samples where no PSP toxins has been detected (below LODs), the histograms were left blank.

2.4.5 HPLC-FLD equipment and chromatographic conditions

PSP toxins analyses, at ANFACO, were carried out using an HPLC Alliance 2695 model and fluorescence detector 2474 model (Waters Corporation). A XSelect CSH C18 3.5 µm, 4.6 mm x 150 mm column and a XSelect CSH C18 3.5 µm, 3.9 mm x 5 mm precolumn from Waters were used. Chromatography conditions are described in the AOAC Method (Lawrence et al. 2005).

At IRTA, PSP toxins analyses were carried out using an UPLC Acquity H-Class model and FLR Acquity fluorescence detector (Waters Corporation). A Kinetex C18 4.5 µm, 4.6 x 150 mm column and a XSelect CSH C18 4.5 µm guard column from Phenomenex were used. Chromatography conditions used are those described in the rapid method by Hatfield et al. (Hatfield et al., 2016).

2.4.6 Method performance

The method acceptability criteria were selected to ensure the performance of the method, according to the International Organization for Standardization (ISO) 17025:2005 standards and the screening and semi-quantitation of PSP toxins EURLB-SOP quality requirements (EURLMB, 2019). The minimum performance criteria were checked out throughout the study such as retention time deviation ± 0.2 min, peak area deviation (RSD ≤3.0%), linearity (R^2 ≥0.98), sensitivity (individual toxin LOD should be equal or lower than 1:20th of regulatory level), precision intra-batch ≤20% and inter-batch ≤25%.

2.5 Statistics

For all naturally contaminated samples, differences in total PSP toxicity obtained in both laboratories were analyzed by two-tailed t-test with the significance level set at 5%. Prior to t-test analysis, it was checked normality and variance homogeneity (SigmaPlot v12.0, Systat software, Inc., CA, USA). For each naturally contaminated samples, differences in the total PSP toxicity obtained between raw and normal canning (NC), raw and EC canning, raw and normal pasteurization (NP), and raw and EC pasteurized were evaluated by one-tailed t-test with the significance level set at 5%.

3 Results

The different batches of cultivated mussels (*M. galloprovincialis*), clams (*R. philippinarum*) and scallops (*P. maximus*), origin and sampling place, toxic phytoplankton involved, date of harvesting and analytical results initially obtained

in the raw mollusks, are summarized in Table 1. In this table, mean values ± standard error of the mean (SEM) obtained for each sample analyzed by both laboratories are included.

The different batches were split and samples were processed by the different thermal treatments as described above. A standard canning, a standard pasteurization, as usually performed in an industrial situation, and the detoxification procedure followed by canning or pasteurization were carried out. These treatments are referred, respectively, as Normal canning, Normal pasteurization, EC canning and EC Pasteurization. Normal pasteurization was not performed in all the batches, so in those cases, it is not depicted in the corresponding figure (Figs 3, 4, 6, and 9). The different keys used in figure's legend are summarized in Table 2. All samples were analyzed by HPLC by the two laboratories (ANFACO and IRTA). The HPLC results obtained by both laboratories showed good agreement, and good correlation was obtained, as shown in Figure 2. No significant differences were observed between results from both laboratories (t-Student, $p > 0.05$).

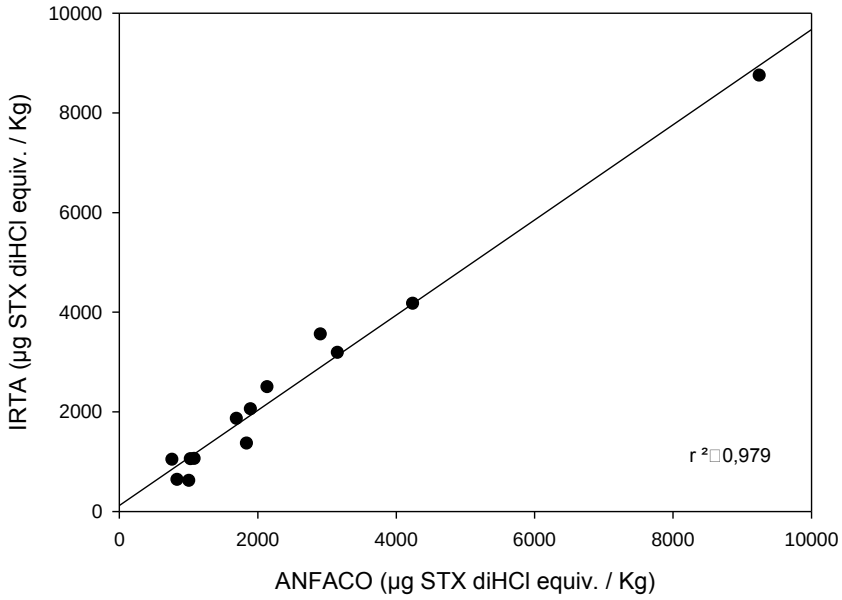


Fig 2: Sigma-plot correlation chart for the total PSP toxicity present in raw bivalves set analyzed by HPLC-FLD (µg STX diHCl equiv/kg) (n=13) showing a good correlation between results obtained at ANFACO and IRTA laboratories.

Table 1. Origin and date of harvesting of live PSP contaminated mussels and scallops. Results of PSP toxins (mean values ± standard deviation, n=2) in raw bivalves analyzed by HPLC-FLD, at both laboratories.

Species	**Location**	**Harvesting date**	**Present phytoplankton**	**Average result (µg STX diHCl equiv/kg) (n=2)**
Mussel (*Mytilus galloprovincialis*)	Ría of Vigo (Vigo A)	09/07/2018	*Alexandrium spp*	1072 ± 11

Mussel (*M. galloprovincialis*)	Ría of Vigo (Redondela C)	23/07/2018	*Alexandrium spp*	1604 ± 330
Mussel (*M. galloprovincialis*)	Ría of Vigo (Redondela C)	23/07/2018	*Alexandrium spp*	737 ± 134
Mussel (*M. galloprovincialis*)	Andalucía (Benalmádena)	02/08/2018	*Gimnodinium catenatum*	812 ± 270
Mussel (*M. galloprovincialis*)	Portinho da Costa (Lisbon)	22/10/2018	*Gymnodinium catenatum*	9001 ± 345
Mussel (*M. galloprovincialis*)	Catalonia	05/03/2019	*Alexandrium minutum*	4205 ± 43
Mussel (*M. galloprovincialis*), frozen	Catalonia	05/03/2019	*Alexandrium minutum*	2317 ± 261
Clam (*R. philippinarum*)	Ría of Pontevedra	27/07/2018	*Alexandrium spp*	1041 ± 23
Clam (*R. philippinarum*), frozen	Ría of Pontevedra	27/07/2018	*Alexandrium spp*	903 ± 204
Scallop (*Pecten maximus*)	Andalucía	22/08/2019	*Gimnodinium catenatum*	3232 ± 466
Scallop (*P. maximus*) eviscerated	Andalucía	22/08/2019	*Gimnodinium catenatum*	1976 ± 117
Scallop (*P. maximus*)	Andalucía	22/08/2019	*Gimnodinium catenatum*	3171 ± 30
Scallop (*P. maximus*), eviscerated	Andalucía	22/08/2019	*Gimnodinium catenatum*	1779 ± 126

Table 2 keys used in figure legends along the text

Key word	Use of PSP detoxification procedure	Thermal processing
Raw	No	None.
Normal Canning	No	116 °C, 51 min
Normal Pasteurization	No	90 °C, 10 min
EC Canning	Yes	116 °C, 51 min
EC Pasteurization	Yes	90 °C, 10 min

Fig 3A, shows the levels of PSP toxins, expressed as µg STX diHCl equiv/kg (total PSP toxicity), in raw and thermally processed mussels harvested during a *Alexandrium* bloom from Redondela, Galicia. Results show that the normal canning procedure, as well as the application of the detoxification process (EC) followed by sterilization or pasteurization, were able to decrease PSP levels below the limit of detection when applied to raw mussels containing around 1604 µg STX diHCl equiv/kg.

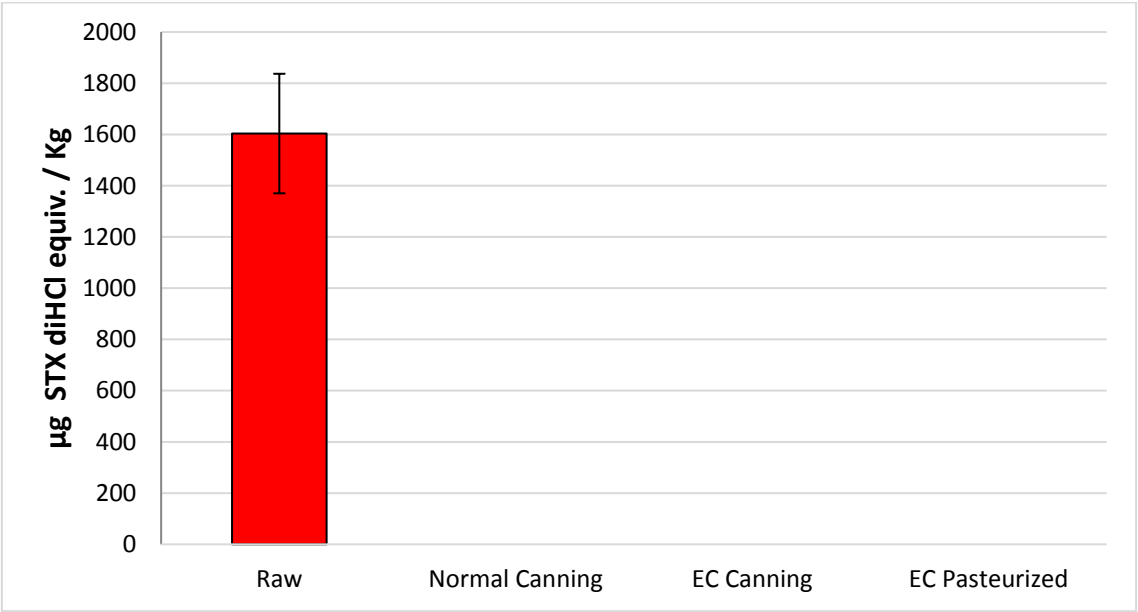


Fig 3A. PSP toxins (total PSP toxicity) in naturally contaminated mussels from Redondela, NW Spain analyzed by HPLC. Mean values (n=2) are represented, and vertical bars indicate the SEM.

Fig 3B shows the quantification of the different PSP analogues of samples from fig 3A, expressed as STX diHCl equiv/kg. It is worth to mention that in the sample, GTX1,4 was the dominant toxin. Very low concentration of other analogues, such as dcSTX, GTX2,3 and C3,4 toxins were detected as well. Similar results were obtained in another batch of mussels from the same location and bloom with a PSP concentration near the legal limit, 800 µg STX diHCl equiv/kg due to GTX1,4; C3,4 and GTX2,3 as show Fig 3C. In this batch, no PSP toxins were detected after processing. A similar behavior, was found with a new lot of mussels harvested during a *Alexandrium* bloom from the same ría but from a different location, Vigo, also in Galicia, as illustrates Fig 4.

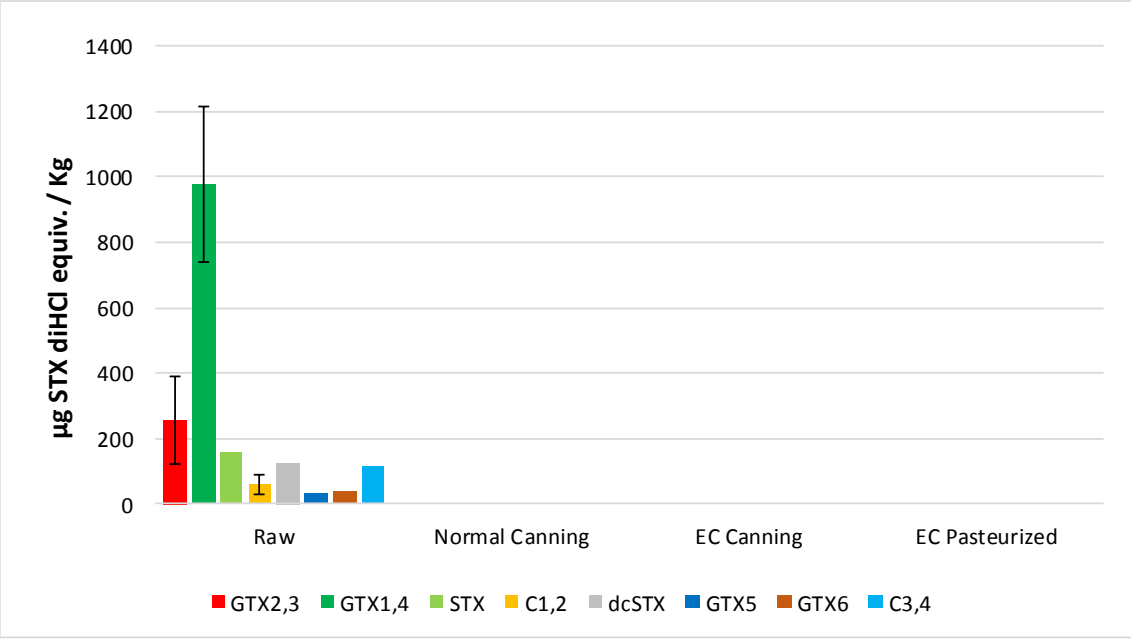


Fig 3B. PSP toxins in naturally contaminated mussels from Redondela, NW Spain. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM

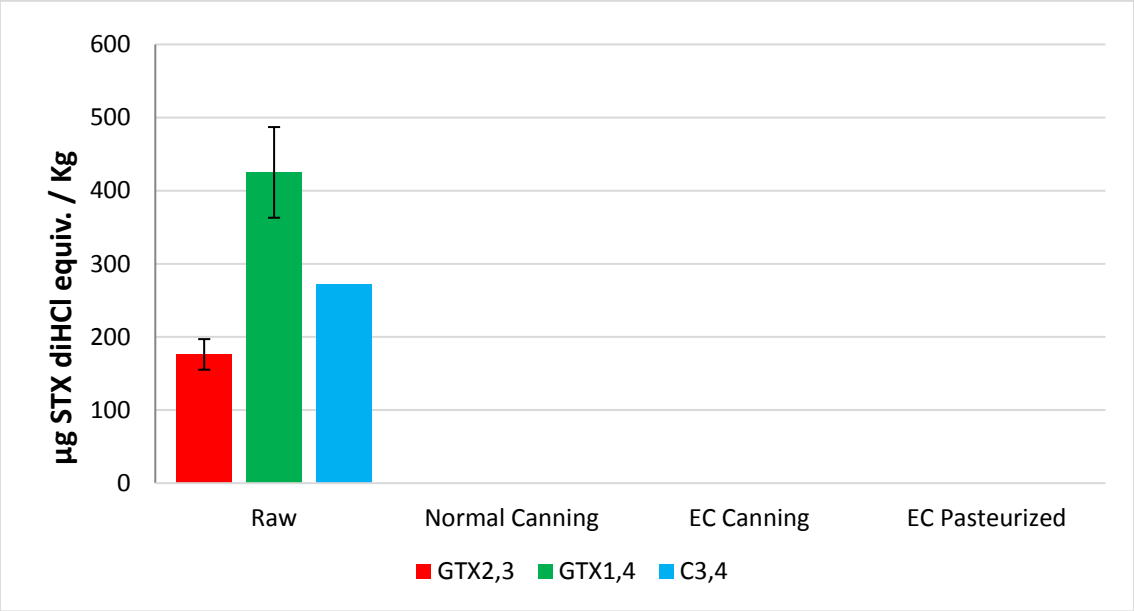


Fig 3C. Naturally contaminated mussels from Redondela, NW Spain analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM.

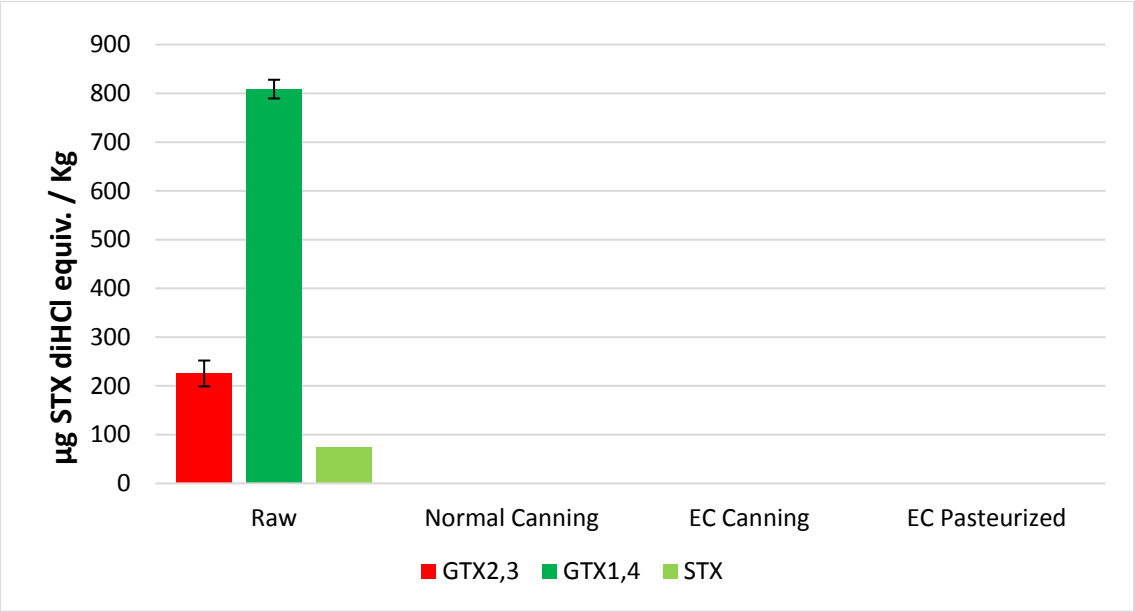


Fig 4. Naturally contaminated mussels from Vigo, NW Spain analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM.

A batch of mussels harvested during a *Gimnodinium catenatum* bloom from the South of Spain, Málaga, in Andalucía, was also processed, obtaining an important reduction of PSP toxins concentration, as expected. All the applied protocols allowed to decrease initial levels of PSP toxins below the legal limit, as shows Fig 5A. Significant differences were observed between raw and the other four thermal treatments applied in this study (t-Student, $p < 0.05$).

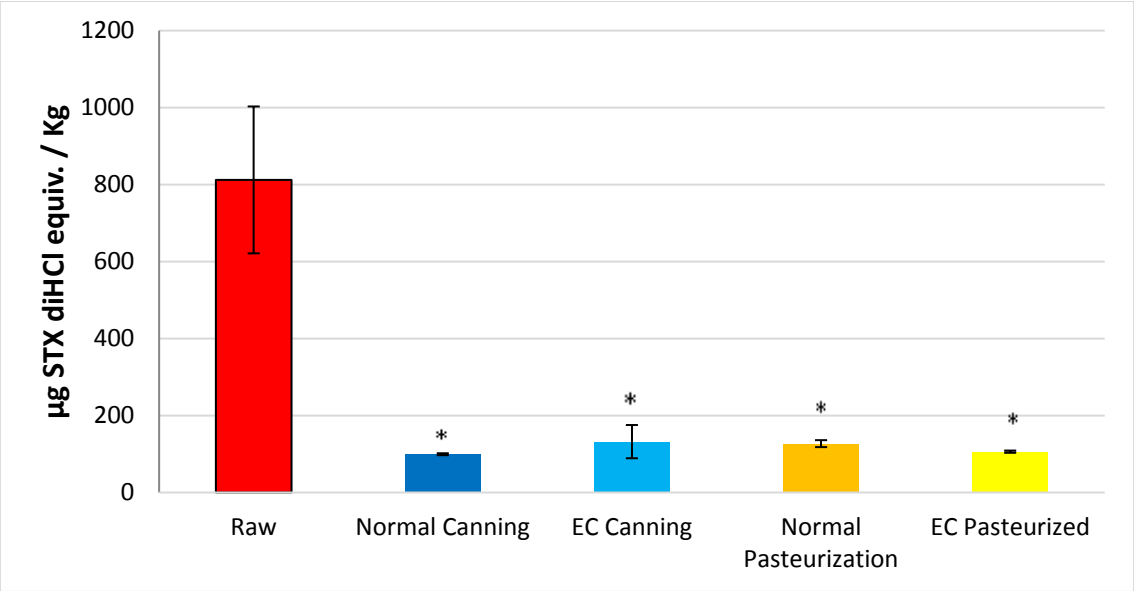


Fig 5A. Naturally contaminated mussels from Andalucía, S Spain analyzed by HPLC (total PSP l toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the four thermal treatments (t-Student, $p < 0.05$).

Fig 5B illustrates all PSP analogues identified in the samples represented in Fig 5A. Raw sample contained mostly GTX1,4 and in lower concentration GTX2,3, dcSTX, C1,2 and GTX5. All analogues except for dcSTX after the EC canning protocol showed a significant decrease ($p < 0.05$). It is remarkably that after processing dcSTX is the dominant analogue.

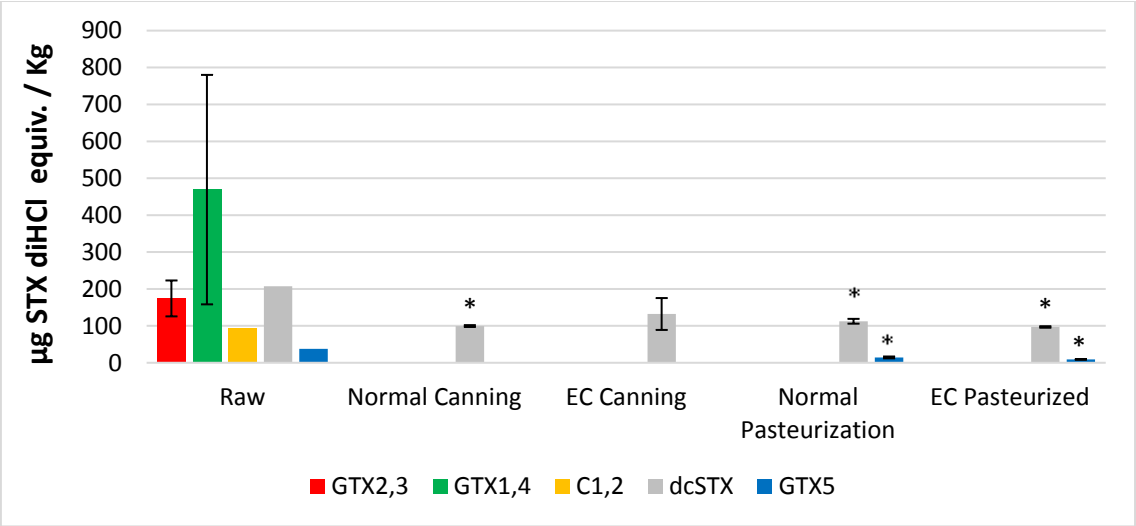


Fig 5B Naturally contaminated mussels from Andalucía, Spain. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the four thermal treatments (t-Student, $p < 0.05$).

A new batch of mussels with an extremely high concentration of PSP toxins was harvested from Portugal during a *Gymnodinium catenatum* bloom, as shows Fig 6A. In this case, mussels exposed to the toxic episode for a long time, presented a huge toxin concentration (9000 µg STX diHCl equiv/kg), exceeding more than 10 times the legal limit. Although after application of the detoxification procedure, PSP toxins concentration decreased in a significant way (t-Student, $p < 0.05$), reaching 90 % of detoxification, no safe products were attained in this case. PSP concentration in mussels elaborated with the detoxification protocol and then canned was 1054±33 µg STX equiv/kg, higher than the legal limit, whereas those samples pasteurized after the detoxification procedure showed lower PSP concentrations (783±183 µg STX diHCl equiv/kg), which was a surprising finding.

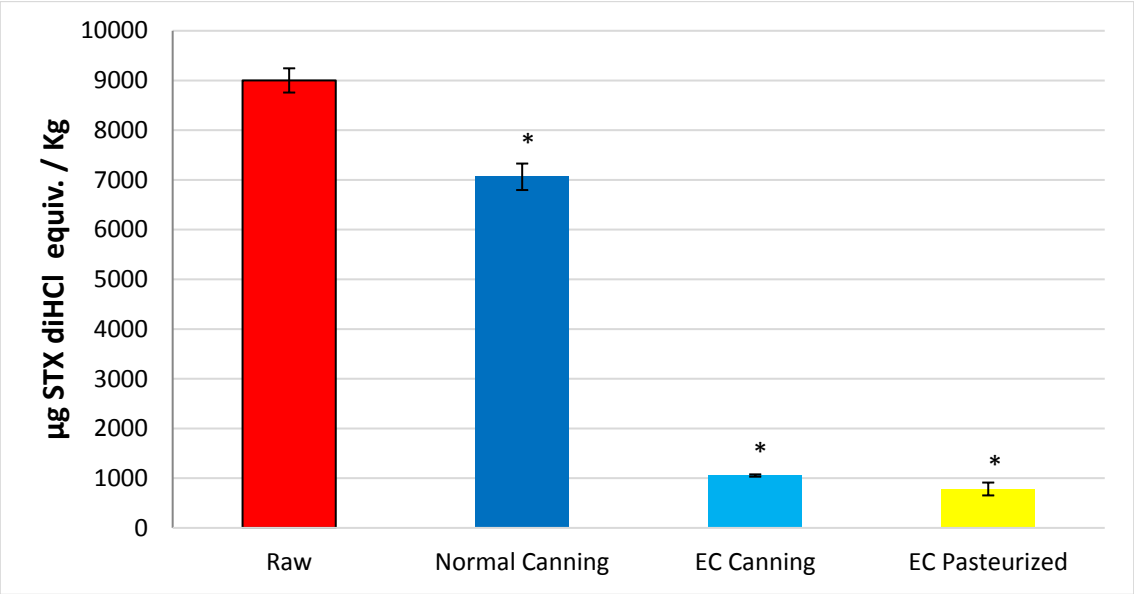


Fig 6A. Naturally contaminated mussels from Lisbon, Portugal, analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the three thermal treatments (t-Student, $p < 0.05$).

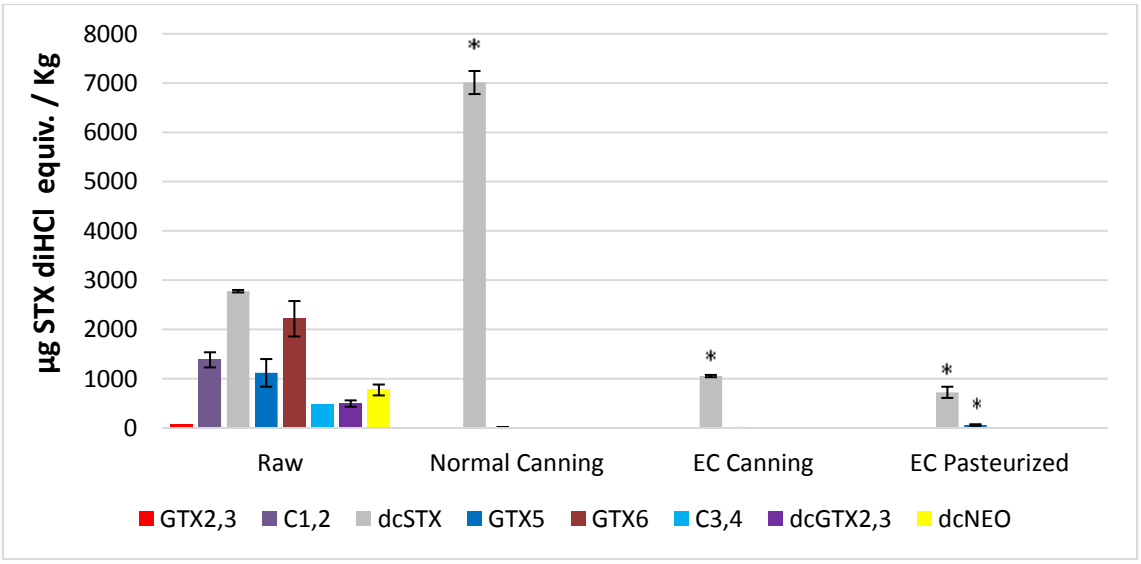


Fig 6B. Naturally contaminated mussels from Lisbon, Portugal, analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the three thermal treatments (t-Student, $p < 0.05$).

Fig 6B shows all PSP analogues identified in the samples represented in Fig 6A. The raw sample contained several toxins of the group, mainly dcSTX; GTX6; GTX5, C1,2 and dcGTX2,3. Again, dcSTX was the dominant analogue in the processed samples, even at higher levels than in the raw sample after “Normal Canning”. This fact suggests that a transformation of other toxins to dcSTX takes place due to the thermal process.

Similar results were obtained in the batch of mussels from Catalonia containing a final concentration of 4206 µg STX diHCl equiv/kg (Fig 7A). It is worth mentioning that, the same sample, after frozen storage at -20ºC for 3 weeks, showed a decrease in toxicity to 2318 µg STX diHCl equiv/kg (Fig 8A). Both treatments, the normal sterilization and the detoxification protocol, followed by sterilization or pasteurization, produced a significant decrease in PSP levels, below the legal limit (t-Student, $p < 0.05$).

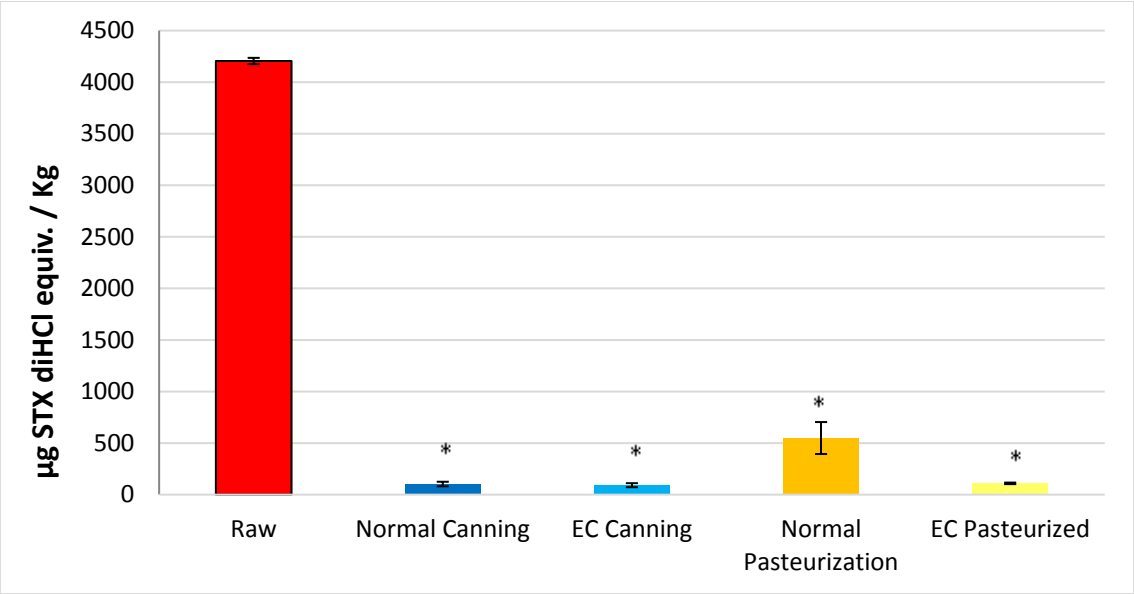


Fig 7A. Naturally contaminated mussels after controlled immersion into an area with *A minutum* in Catalonia, Spain analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the four thermal treatments (t-Student, $p < 0.05$).

The raw sample of immersed mussels coming from Catalonia contained several toxins of the group, mainly GTX1,4 and GTX2,3. STX was the dominant analogue in all four processed samples, and was not present in the raw sample. This fact suggests that a transformation to STX takes place due to the thermal process (Figure 7B).

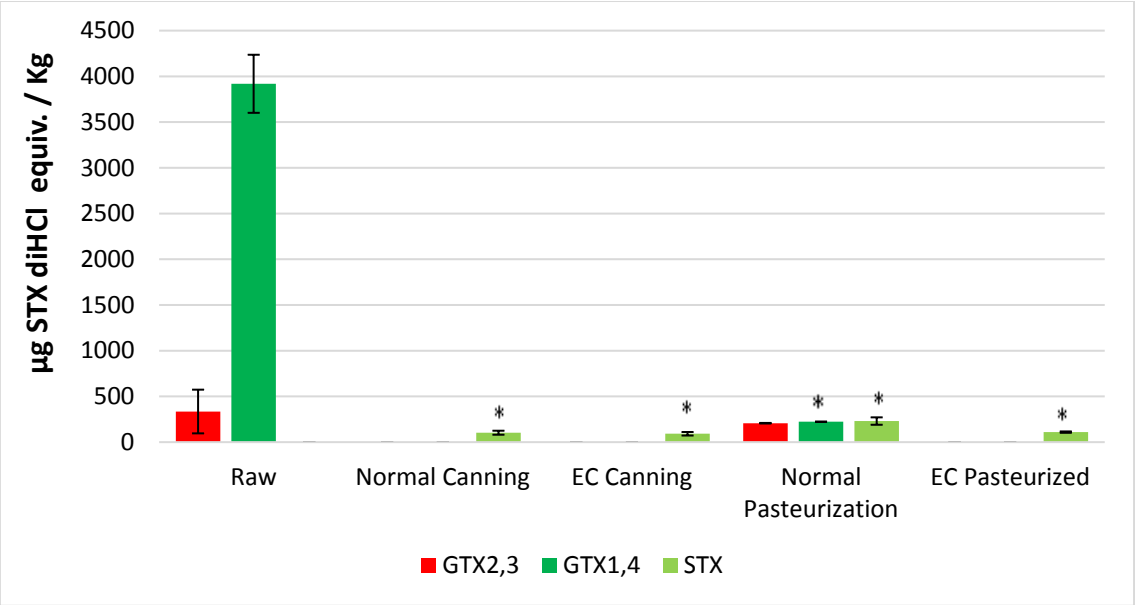


Fig 7B. PSP toxins in naturally contaminated mussels after controlled immersion into an area with *A minutum* from Catalonia, Spain, fresh sample. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the four thermal treatments (t-Student, $p < 0.05$).

Analysis of the same sample after preservation at -20ºC, showed an important decrease in total PSP toxicity in comparison with the fresh, unfrozen sample (t-Student, $p < 0.05$). From a qualitative point of view, both samples, fresh and frozen and the processed samples obtained, showed a similar behavior regarding decrease of toxins after processing (Figure 8A) and the type of toxins present, mainly GTX1,4; GTX2,3 and STX (Figure 8B). STX was again the dominant analogue in the processed samples, even at higher levels than in the raw sample after all thermal processing. This fact suggests that a transformation to STX takes place due to the canning and pasteurization process (Figure 7B and 8B).

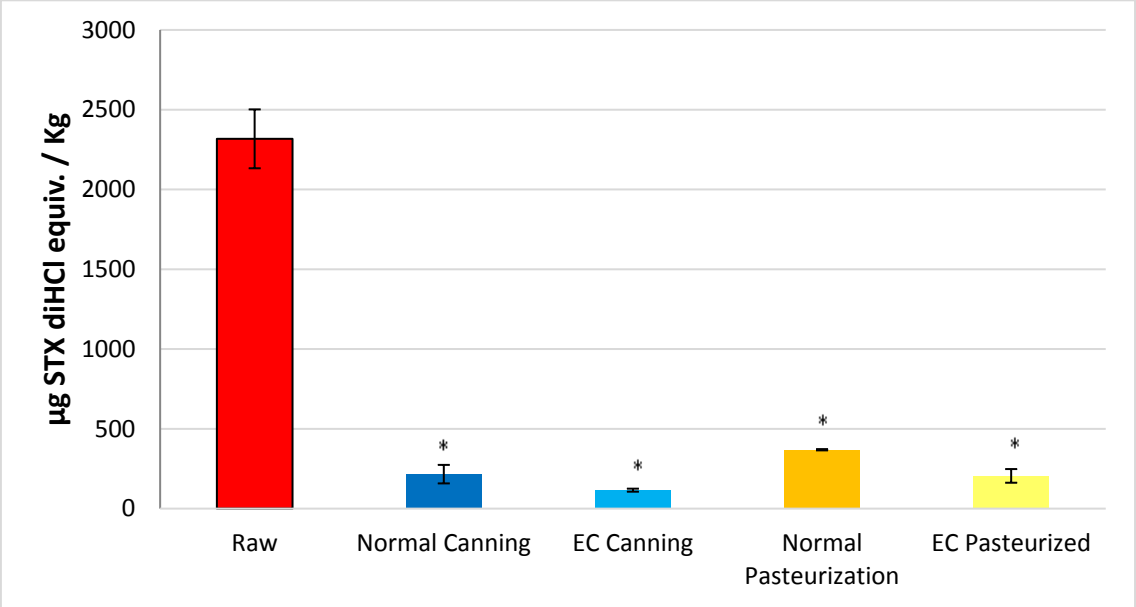


Fig 8A. Naturally contaminated mussels from Catalonia after storage at -20ºC analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the four thermal treatments (t-Student, $p < 0.05$).

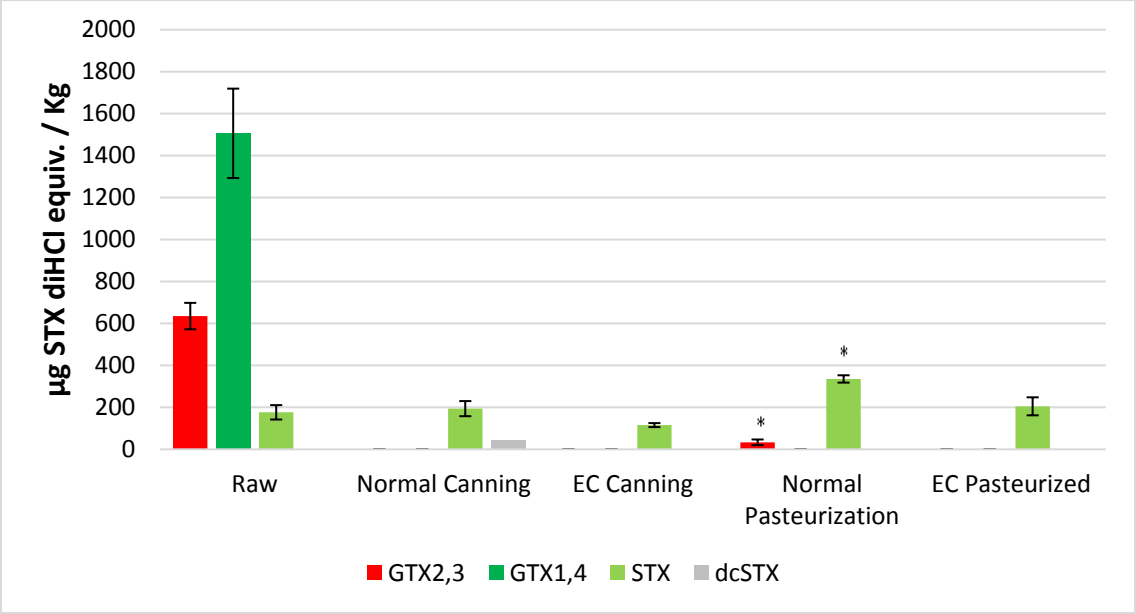


Fig 8B. PSP toxins in naturally contaminated mussels from Catalonia after storage at -20ºC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the four thermal treatments (t-Student, $p < 0.05$).

The analysis of contaminated clams and the products obtained after processing offered similar results to those obtained in mussels. Fresh raw clams obtained from Pontevedra (NW of Spain) during an *Alexandrium* spp bloom showed a PSP concentration of 1041 ± 22 µg STX diHCl equiv/kg (Fig 9A). The same sample after frozen storage at -20ºC for 7 weeks, contained 903 ± 204 µg STX diHCl equiv/kg (data not shown). Application of both treatments: the normal sterilization procedure and the detoxification protocol followed by sterilization or pasteurization, produced a decrease in PSP levels to not detectable levels (Fig.9B).

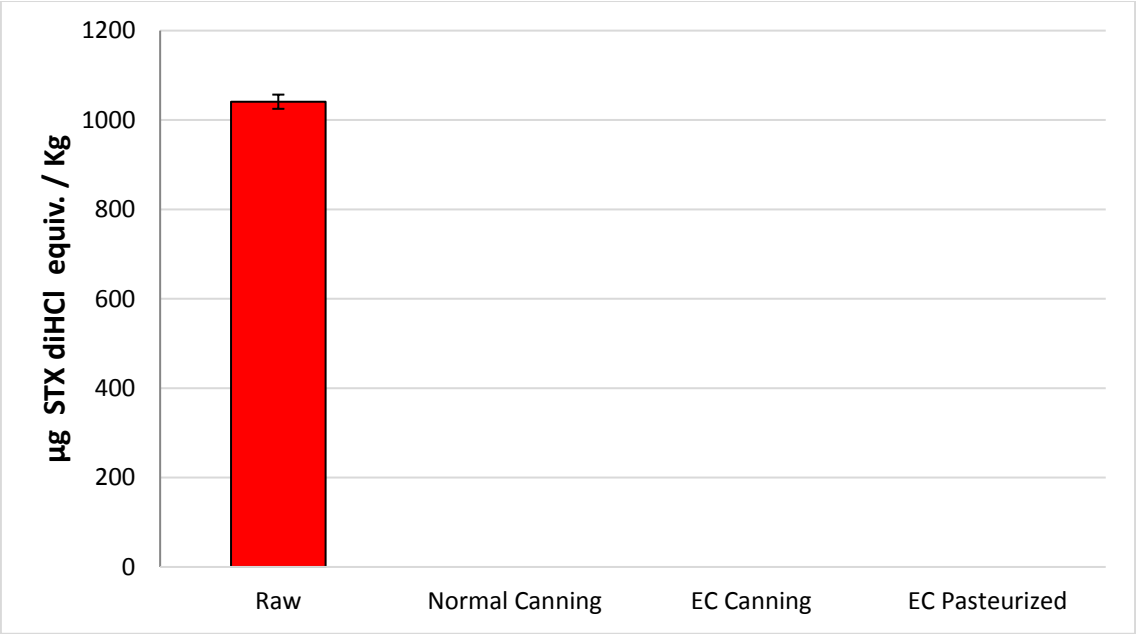
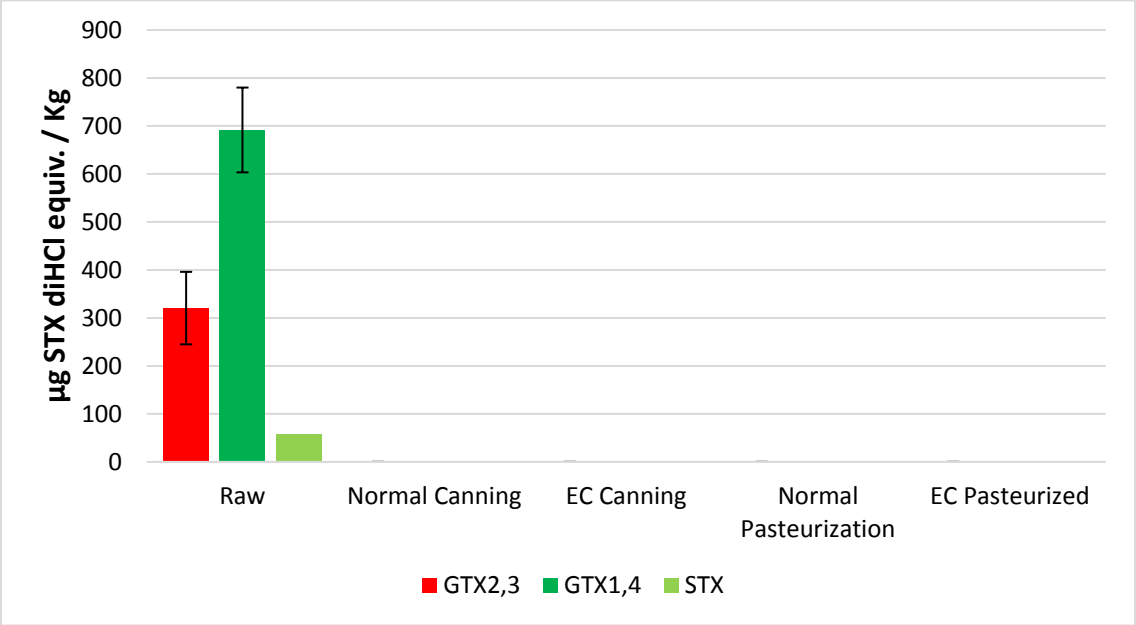


Fig 9A. PSP toxins in naturally contaminated clams from Pontevedra, NW Spain analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM.



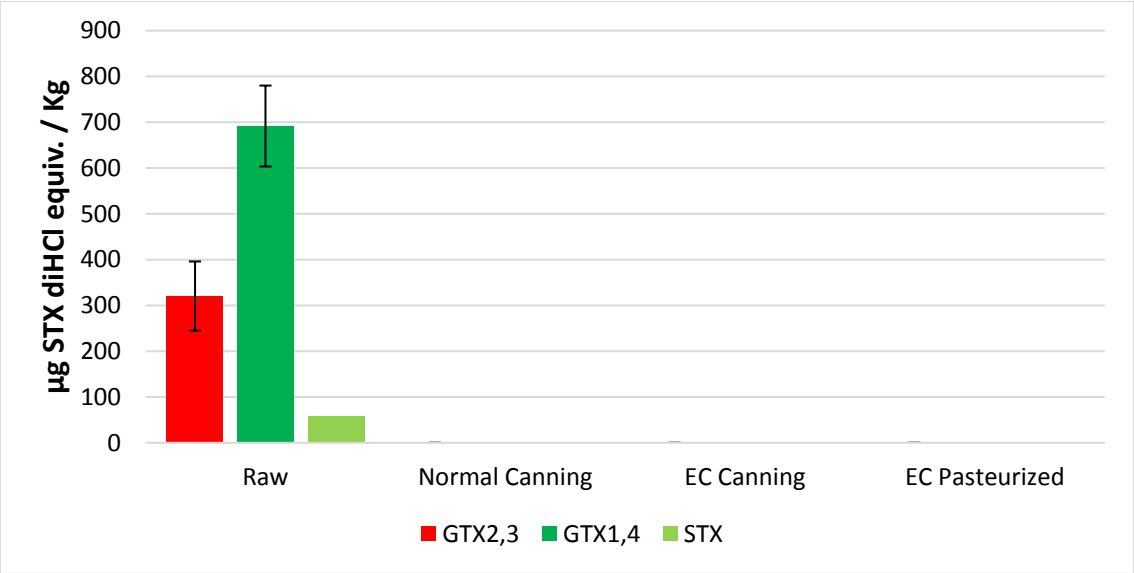


Fig 9B. Naturally contaminated clams from Pontevedra, Galicia, Spain, analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw, eviscerated or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM.

In addition to mussels and clams, scallops contaminated with PSP were harvested during a *Gimnodinium catenatum* bloom. In this species, evisceration of raw scallops reduced significantly PSP concentration, as expected (t-Student, $p < 0.05$) (Fig 10A). Evisceration was performed previously to all the thermal processes applied and reduced drastically all the congeners to non-detectable levels, in canned samples (with or without detoxification protocol). Also, it reduced to levels well below the legal limit in pasteurized samples. The different analogues detected in the raw mollusk, whole body or eviscerated, were GTX2,3; STX; C1,2; dcSTX, GTX5, and dcGTX2,3). Mostly dcSTX (327±69 µg STX diHCl equiv/kg) and, in a much lower level, GTX5 (28±9 µg STX diHCl equiv/kg) were detectable when pasteurization was carried out after a conventional pre-cooking step. In addition, only dcSTX was detectable to a lower level (144±19 µg STX

diHCl equiv/kg) when pasteurization was carried out after the detoxification protocol (Fig 10B).

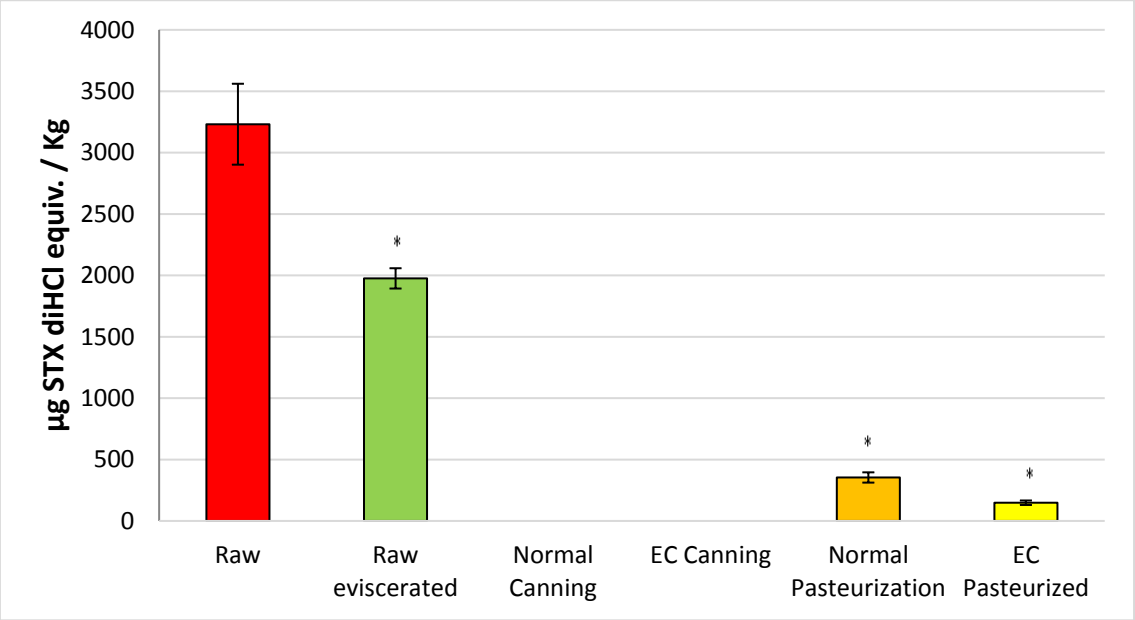


Fig 10A. Naturally contaminated scallops (*Pecten maximus*) from Marbella, Andalucía , Spain (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw, raw eviscerated and the four thermal treatments (t-Student, $p < 0.05$).

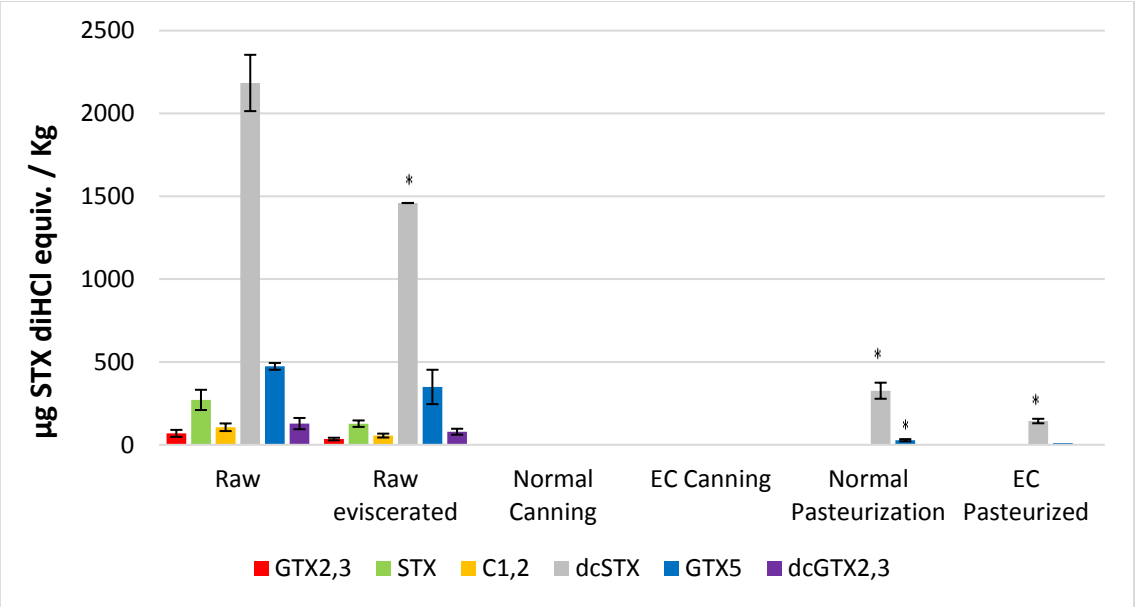


Fig 10B. Naturally contaminated scallops from Marbella, Andalucía, Spain, analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw, eviscerated or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw, raw eviscerated and the four thermal treatments (t-Student, $p < 0.05$).

4 Discussion

Some studies have been conducted to reduce or eliminate PSP toxins in mollusks and other invertebrates. The influence of thermal processing in naturally contaminated bivalves has already been studied by our group and by other authors, finding PSP detoxification in shellfish after application of high temperatures (Berenguer et al., 1993) (Lawrence et al., 1994; Reboreda et al., 2010; Vieites et al., 1999).

In this study, an approved thermal procedure to decrease PSP toxins in the giant cockle, *Acanthocardia tuberculata*, (EC, 1996), was evaluated on mussels, clams and scallops naturally contaminated with PSP toxins. We applied the heat

treatment (so called “detoxification procedure”) that consists on several cleaning and cooking steps, as establishes the European legislation but with certain modifications (EC, 1996). Mainly, in this legislated procedure, the edible parts are separated from the non-edible parts. In the case of mussels, the digestive viscera constitute 30 % of the total tissue weight (FAO, 2004), and removing it is not realistic regarding the commercial practices by the industry for this species (as well as for clams), while it is accepted for scallops. Hence, we did eviscerate the scallops, since this is a common practice.

A standard canning or pasteurization, as usually performed in an industrial situation, without previous washes and cooking, was carried out to compare the results with those obtained after application of the “detoxification procedure”.

Different batches of mussels, clams and scallops containing PSP levels above the regulated limit (800 µg STX equiv/kg) and coming from several areas of Spain and Portugal, were thermally processed. In our hands, scallops, clams and almost all mussels unless one batch, underwent an important detoxification process, leading to fulfill PSP legislated limit. Only one sample of mussels, with the highest concentration, 9000 µg STX diHCl equiv/kg, more than 10 times the legal concentration, (Fig 6), showed a statistically significant decrease in total PSP toxicity after processing, but in a minor extent for normal canning, with a decrease of 22% in total PSP toxicity. Otherwise, the rest of mollusk batches processed along this study, either after standard canning or pasteurization, even without the application of the “detoxification procedure”, reached levels of detoxification higher than 85 %. This was a much unexpected finding. These results suggest that in some circumstances, if concentrations of toxins in shellfish are not very elevated, it is not necessary to apply the “detoxification

procedure", since a normal canning or pasteurization seems sufficient to reduce PSP levels. Nevertheless, in our opinion, detoxification of mussels depends not only on the initial concentration, but also on the exposure time to toxins and the seawater conditions as well. So, the detoxification procedure should be applied in all cases. In contrast, when shellfish are contaminated with high levels (>5300 µg STX diHCl equiv/kg), even the "detoxification procedure" application is not enough to reduce PSP levels below the legislated limit. Taking this into account, a threshold level should be established in mussels if a detoxification legislative proposal is expected. Based on data found in this work, with detoxification levels of 85%, a maximum level of 5300 µg STX diHCl equiv/kg is proposed to be established for the application of this procedure. Nevertheless, in order to obtain a higher amount of data to support this proposed limit, further work would be necessary. The highest level allowed in the European Legislation authorized for the harvesting of *A. tuberculata* is 3000 µg STX diHCl equiv/kg, if the product is intended to the canning industry, and analytical control of each batch, which "must not contain a PSP level detectable by the bioassay method after the application of this heat treatment" is mandatory (EC, 1996). The analytical control of produced batches should be maintained if the present detoxification procedure was extended to other molluscan species. In addition to this Decision, applied in Spain for the giant cockle, a legislation in Canada allows canning of soft shell clams and mussels with levels between 800 and 1600 µg STX equiv/kg (Fernández et al., 2003). Also, butter clams containing 3000 to 5000 µg STX equiv/kg may be commercialized after removing the entire siphon, whereas butter clams containing 800 to 3000 µg STX equiv/kg may be marketed

after removing the distal half of the siphon (Fernández, 1998), cited in (FAO, 2004).

A preliminary article shows that the standard canning process resulted in a significant and reproducible reduction of PSP toxicity in mussel meat (> 50%), decreasing toxin levels under the limit of detection (Vieites et al., 1999). In this paper, authors observed that the decrease of toxicity was not dependent on toxin levels of raw material, although it is worth to mention that raw samples in this work contained PSP toxin levels below 4000 µg STX diHCl equiv/kg. These results are in good agreement with ours, since only when the concentrations of PSP toxins are very high (9000 µg STX diHCl equiv/kg), we find that the traditional canning did not allow detoxifying mussels below the legal limit. A subsequent work confirmed the detoxification after canning, although no very relevant results were obtained in that study due to the unavailability of highly PSP contaminated shellfish (Reboreda et al., 2010); in this study a mollusk type-dependent decrease in toxicity after freezing was observed, and the conclusion was that PSP toxins leaked with thawing water.

In lobsters, boiling or steaming reduced toxicity by approximately 65% compared to values obtained in the raw samples (Lawrence et al., 1994).

Our results suggest that thermal treatment induces chemical modifications of toxins, changing the toxic potency of processed shellfish, converting toxins into more or less toxic analogues, as occurs by biotransformation or metabolic transformations in shellfish (Reis Costa et al., 2018).

In the present study, GTX1,4 was the dominant detected toxin in raw samples of mussels, except for the most toxic sample from Portugal where a mixture of analogues was identified and dcSTX and GTX6 were the major toxins with

similar levels. Among the different PSP toxins, dcSTX was the major analogue obtained in processed samples, including the one with the highest PSP concentration except for the mussel from Catalonia where the major analogues obtained in the processed samples was STX. GTX1 and GTX4 together are less toxic (TEF=1 and 0.7, respectively) than dcSTX (TEF=1) (EFSA, 2009). This was taken into account for the calculation of total PSP toxicity since HPLC results were expressed as STX diHCl equiv/kg.

More studies will be necessary to assess if toxins in cooked mussels and scallops are removed through chemical decomposition, leached out during the loss of water or transferred to packing medium.

Regardless of these possibilities, our study demonstrates that processing of PSP contaminated shellfish, significantly reduces the PSP toxicity and industrial processes may be a solution in shellfish harvesting areas affected by intense and frequent PSP closures. The proposed method has been already implemented in the transformation of *Acanthorcardia tuberculatum* and should be easily transferred to other bivalve transforming facilities.

5 Conclusions

In conclusion, an efficient and inexpensive “detoxification procedure” can be applied in PSP contaminated mussels, clams and scallops to decrease PSP toxins below the legal limit (800 µg STX diHCl equiv/kg). However, a maximum threshold level in raw material should be previously established to define if the processing will efficiently reduce PSP toxins below the legal limit. Based on our data, 5300 µg STX diHCl equiv/kg would be the highest level. Although it is still necessary that the industry should proceed with quality controls of the final

product to ensure that it responds to the legal requirements and levels of PSP toxins are safe, in the same way as stated in the reference legislation for *Acanthocardia tuberculatum*.

ACKNOWLEDGEMENTS

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement no. 773400 (SEAFOODTOMORROW). This output reflects the views only of the author(s), and the European Union cannot be held responsible for any use which may be made of the information contained therein.

The authors acknowledge the Departament d'Agricultura, Ramaderia, Pesca i Alimentació (DARP) of the Generalitat de Catalunya for financing of the Shellfish Harvesting Areas Monitoring Program. We also appreciate the support of the CERCA program of the Generalitat de Catalunya. The authors acknowledge Margarita Fernández-Tejedor for her assistance in the mussel exposure to a toxic bloom of the dinoflagellate *Alexandrium minutum.*

REFERENCES

Álvarez, G., Díaz, P.A., Godoy, M., Araya, M., Ganuza, I., Pino, R., Álvarez, F., Rengel, J., Hernández, C., Uribe, E., Blanco, J., 2019. Paralytic Shellfish Toxins in Surf Clams *Mesodesma donacium* during a Large Bloom of *Alexandrium catenella* Dinoflagellates Associated to an Intense Shellfish Mass Mortality. Toxins (Basel) 11, 188.

AOAC, 2005. Official Method 2005.06. Paralytic Shellfish Poisoning Toxins in Shellfish. Prechromatographic Oxidation and Liquid Chromatography with fluorescence detection. First Action 2005. J AOAC INT 88, 1714.

Barbosa, V., Santos, M., Anacleto, P., Maulvault, A.L., Pousao-Ferreira, P., Costa, P.R., Marques, A., 2019. Paralytic Shellfish Toxins and Ocean Warming: Bioaccumulation and Ecotoxicological Responses in Juvenile Gilthead Seabream (Sparus aurata). Toxins (Basel) 11.

Ben-Gigirey, B., Rodriguez-Velasco, M.L., Gago-Martinez, A., 2012. Extension of the validation of AOAC Official Method 2005.06 for dc-GTX2,3: interlaboratory study. J AOAC INT 95, 111-121.

Berenguer, J.A., Gonzalez, L., Jimenez, I., Legarda, T.M., Olmedo, J.B., Burdaspal, P.A., 1993. The effect of commercial processing on the paralytic shellfish poison (PSP) content of naturally-contaminated Acanthocardia tuberculatum L. Food Additives & Contaminants 10, 217-230.

Braga, A.C., Camacho, C., Marques, A., Gago-Martinez, A., Pacheco, M., Costa, P.R., 2018. Combined effects of warming and acidification on accumulation and elimination dynamics of

paralytic shellfish toxins in mussels Mytilus galloprovincialis. Environmental research 164, 647-654.
EC, 1996. Commission Decision of 18 January 1996 establishing the conditions for the harvesting and processing of certain bivalve molluscs coming from areas where the paralytic shellfish poison level exceeds the limit laid down by Council Directive 91/492/EEC (96/77/EC). Off. J. Eur. Communities L 15, 46-47.
EC, 2004. REGULATION (EC) No 853/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 laying down specific hygiene rules for food of animal origin. Off. J. Eur. Communities L 226, 55-205.
EFSA, E.F.S.A., 2009. Marine biotoxins in shellfish – Saxitoxin group. EFSA Journal 7, 1019.
EURLMB, 2019. AOAC 2005.06 Standard Operating Procedure. Simplified version for the screening and semi-quantitation of PSP toxins. Avaliable at http://www.aecosan.msssi.gob.es/en/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/SOP_AOAC_2005_06_semi.pdf.
Fabre, A., Lacerot, G., de Paiva, R.R., Soares, M.C.S., de Magalhães, V.F., Bonilla, S., 2017. South American PSP toxin-producing Cylindrospermopsis raciborskii (Cyanobacteria) decreases clearance rates of cladocerans more than copepods. Hydrobiologia 785, 61-69.
FAO, 2004. Marine Biotoxins. In FAO Food and Nutrition Paper 80. p. 295. Available at http://www.fao.org/3/y5486e/y5486e00.htm in: F.a.A. Organization (Ed.), Rome, Italy.
Fernández, M.L., 1998. Phycotoxins: Regulatory Limits and Effects on Trade. , in: M. Miraglia, H.P.v. Egmond, C. Brera, J. Gilbert (Eds.), Mycotoxins and Phycotoxins – Developments in Chemistry, Toxicology and Food Safety. Alaken, Inc., Fort Collins, Colorado, pp. 503-516.
Fernández, M.L., Shumway, S.E., J., B., 2003. Managementof shellfish resources., in: H. M., A.A. D., A.D. M. (Eds.), Manual on Harmful Marine Microalgae. UNESCO Publishing, Paris, pp. 657-692.
Gracia Villalobos, L., Santinelli, N.H., Sastre, A.V., Marino, G., Almandoz, G.O., 2019. Spatiotemporal distribution of paralytic shellfish poisoning (PSP) toxins in shellfish from Argentine Patagonian coast. Heliyon 5, e01979.
Hatfield, R.G., Punn, R., Algoet, M., Turner, A.D., 2016. A Rapid Method for the Analysis of Paralytic Shellfish Toxins Utilizing Standard Pressure HPLC: Refinement of AOAC 2005.06. Journal of AOAC International 99, 475-480.
Lawrence, J.F., Maher, M., Watson-Wright, W., 1994. Effect of cooking on the concentration of toxins associated with paralytic shellfish poison in lobster hepatopancreas. Toxicon 32, 57-64.
Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J AOAC INT 88, 1714-1732.
Lee, R., Lovatelli, A., Ababouch, L., 2008. Bivalve Depuration: Fundamental and Practical Aspects. FAO. Fisheries Technical Paper No 511. Rome, FAO. , 139.
Pitois, F., Fastner, J., Pagotto, C., Dechesne, M., 2018. Multi-Toxin Occurrences in Ten French Water Resource Reservoirs. Toxins (Basel) 10.
Reboreda, A., Lago, J., Chapela, M.-J., Vieites, J.M., Botana, L.M., Alfonso, A., Cabado, A.G., 2010. Decrease of marine toxin content in bivalves by industrial processes. Toxicon 55, 235-243.
Reis Costa, P., Braga, A.C., Turner, A.D., 2018. Accumulation and Elimination Dynamics of the Hydroxybenzoate Saxitoxin Analogues in Mussels Mytilus galloprovincialis Exposed to the Toxic Marine Dinoflagellate *Gymnodinium catenatum*. Toxins (Basel) 10, 428.
Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., Lees, D.N., 2009. Refinement and extension of AOAC Method 2005.06 to include additional toxins in mussels: single-laboratory validation. J AOAC INT 92, 190-207.
Vieites, J.M., Botana, L.M., Vieytes, M.R., Leira, F.J., 1999. Canning Process that Diminishes Paralytic Shellfish Poison in Naturally Contaminated Mussels (*Mytilus galloprovincialis*). Journal of Food Protection 62, 515-519.

TITLE: Detoxification of paralytic shellfish poisoning toxins in naturally contaminated mussels, clams and scallops by an industrial procedure

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Abstract

Paralytic shellfish poisoning (PSP) episodes cause important economic impacts due to closure of shellfish production areas in order to protect human health. These closures, if are frequent and persistent, can seriously affect shellfish producers and the seafood industry, among others. In this study, we have developed an alternative processing method for bivalves with PSP content above the legal limit, which allows reducing toxicity to acceptable levels. A modification of the PSP detoxifying procedure stablished by Decision 96/77/EC of the European Union in *Acanthocardia tuberculatum*, was developed and implemented for PSP elimination in other species of bivalves. The procedure was applied to 6 batches of mussels, 2 batches of clams and 2 batches of scallops, achieving detoxification rates of around 85%. A viable industrial protocol which allows the transformation of a product at risk into a safe product was developed. Although a significant reduction was obtained, in a sample circa 9000 µg STX diHCl equiv/kg, the final toxin level in these highly toxic mussels did not fall below the European limit. The processing protocol described may be applied efficiently to mussels, clams and scallops and it may be a major solution to counteract the closure of shellfish harvesting areas, especially if persistent.

Highlights

- An industrial protocol aimed at reducing PSP toxin levels was developed and optimized in mussels, clams and scallops.
- The procedure was applied to some batches of PSP-contaminated molluscs obtaining ± 85 % detoxification and a safe product.
- However, one sample with an exceptionally high toxicity, 9000 µg STX diHCl equiv/kg, did not fall below the European limit.
- An economically feasible bivalve canning processing was implemented, guaranteeing the manufacture of a safe product.

Keywords: *Paralytic shellfish poisoning,* detoxification, industrial protocol**,** *mollusks, LC-FLD. (maximum 6 descriptive keywords)*

Graphical abstract (optional)

Abbreviations:

C1-4, N-sulfo-carbamoyl gonyautoxins

CRM, certified reference material

dcGTX decarbamoyl gonyautoxin

dcNEO, decarbamoyl neosaxitoxin

dcSTX, decarbamoyl saxitoxin

equiv, equivalent

GTX1-6, gonyautoxins

HABs, harmful algal blooms

i.p., intraperitoneal

LC-FLD, liquid chromatography-fluorescence detection

NEO, neosaxitoxin

PSP, paralytic shellfish poisoning

STX, saxitoxin

TEF, toxicity equivalency factor

1 Introduction

Paralytic shellfish poisoning (PSP) is caused by consumption of shellfish containing PSP toxins of the family of saxitoxins (STX) (EFSA, 2009). These toxins are produced by microalgae, mainly toxic marine dinoflagellates such as species of the genera *Alexandrium* and *Gymnodinium*, and also by certain freshwater cyanobacteria (Gracia Villalobos et al., 2019) (Pitois et al., 2018) (Fabre et al., 2017). These toxins are accumulated and sometimes metabolized into toxin derivatives in many species of filter-feeding bivalves, as mussels, clams and scallops, making them potentially toxic to humans. Harmful algal blooms (HABs) can also induce other ecological damage and adverse effects to living marine resources. In fact, some bivalves can be impaired during intense toxic episodes. For instance, a population of the surf clam *Mesodesma donacium* with high PSP toxic levels, died due to the desiccation caused by the incapability of the clams to burrow (Álvarez et al., 2019).

To protect public health and ensure the quality of seafood, monitoring programs are implemented worldwide in order to detect and quantify these toxins, and eventually forbidding shellfish harvesting when levels of toxins exceed the legal limit laid down in current regulations. In Europe for example, harvesting and commercialization of bivalves is prohibited above the threshold of 800 µg STX diHCl equiv/kg of shellfish tissues (EC, 2004). Closure of shellfish production areas has an important economic impact for producers and other associated industries. No solutions have been found to prevent these important episodes which are seldom predictable, and despite the influence of PSP events on human health and fisheries, studies on shellfish detoxification to mitigate this problem are still very scarce.

Natural detoxification occurs very slowly and it is conditioned by the presence of toxin producing microalgae in the water column. Lipophilic toxins are retained longer than the hydrophilic toxins, such as PSP toxins, although the detoxification rate depends on the species, concentration of toxins and environmental conditions (Lee et al., 2008). Several studies described that the concentration of some PSP toxin analogues in bivalves, but not all of them, can be reduced by exposing contaminated shellfish to a non-toxic diet (Reis Costa et al., 2018). Nevertheless, mitigating or modulating the presence of microalgae in the field is currently not possible, so this eventual solution should be applied by maintaining large stocks of shellfish in a closed space for several days, and the feasibility of this would be dubious.

Once harvested, toxin reduction or elimination from shellfish is mainly affected by the chemical properties of the toxins. In the particular case of PSP toxins, a regulation was published after performing scientific studies which proved that a suitable heat treatment decreased the levels of PSP toxins and guaranteed the safety of the cockle *Acanthocardia tuberculata* (Berenguer et al., 1993; EC, 1996).

A detoxification procedure would result in an economically feasible solution for a shellfish canning industry in locations where PSP toxic episodes occur very often or are persistent, and large amounts of shellfish are affected. Besides, in view of the changing environmental conditions related to climate change, a rise in the incidence of these episodes could take place in the near future (Barbosa et al., 2019). Changes in the profiling and behavior of PSP toxic episodes, leading to lower toxicity values but longer toxic episodes have been proposed (Braga et al., 2018). It is important to mention that it would not be necessary to

perform important modifications in factory installations to accomplish the PSP detoxification protocol. The required equipment is the same usually employed by the canning industry and factories applying this protocol do actually exist in the case of giant cockle. If a regulation for this detoxification protocol was finally approved, the importance of such modifications will depend on each individual factory and the decision to implement it or not would be due more to economic than technical reasons. Only the duration of the whole thermal process would be slightly increased.

In this paper, naturally PSP contaminated mussels, clams and scallops were specifically harvested in order to implement the thermal procedure described in the EU decision. Slight modifications were applied, in order to obtain a better efficiency of detoxification and yield of mussels, clams and scallops.

2 Material and methods

2.1 Sampling of contaminated mussels and scallops

Samples were obtained from different sampling points along the Spanish and Portuguese coasts from July 2018 to March 2019. Mussels (*Mytilus galloprovincialis*) were acquired from several mussel raft cultures in: a) Galicia, (samples coming from two different floating rafts in the Ría of Vigo, Pontevedra); b) Andalucía, (one batch of mussels from Benalmádena, Málaga), and c) Portugal, (one batch of mussels from Portinho da Costa, near Lisbon). In addition, other mussel batches were obtained in Catalonia, one sample, after exposure to a toxic bloom of the dinoflagellate *Alexandrium minutum*, inside a harbour, as explained in this article. The phytoplanktonic species involved in the naturally contaminated batches of shellfish are depicted in Table 1. Special

permissions from the local authorities were obtained in order to harvest the toxic molluscs from the closed areas. Two batches of Japanese littleneck clams (*Ruditapes philippinarum*) were obtained from Pontevedra, Galicia (Spain) and both batches of scallops (*Pecten maximus*) were obtained from Málaga, Andalucia (Spain). Sampling zones where toxic mussels, clams and scallops were harvested are depicted in Figure 1.

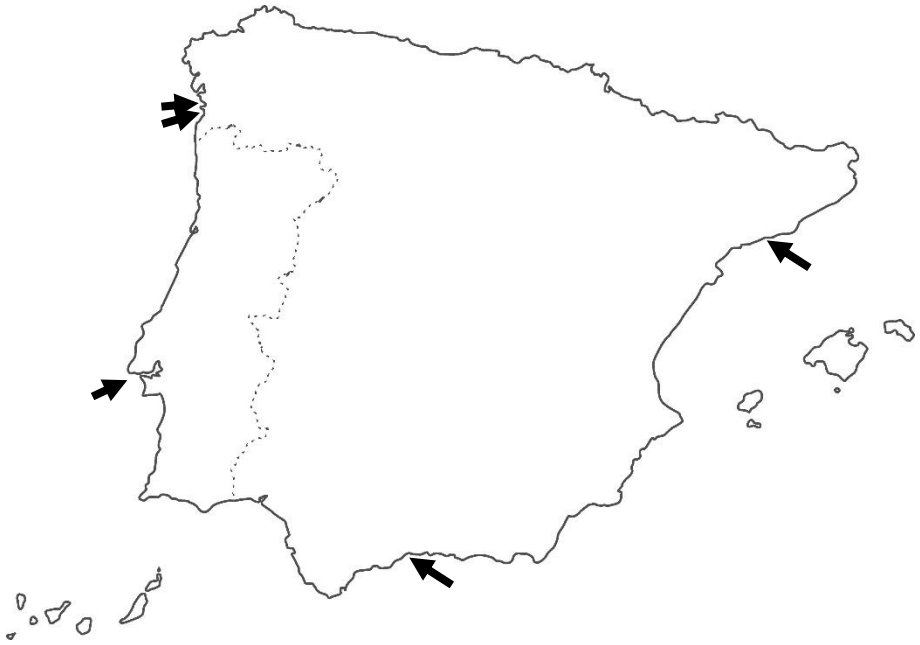


Fig 1: sampling points (marked by arrows) where PSP contaminated mussels and scallops were obtained during the study.

Samples were refrigerated in thermally isolated boxes with cold accumulators after collection and shipped to the laboratory. Upon arrival, samples were processed as indicated in “Detoxification study” and analyzed as described below. Some subsamples of the different batches of mollusks were frozen at -20 °C and processing and analysis was performed after days or weeks until a maximum of 10 weeks.

2.2 Mussel exposure to a toxic bloom of the dinoflagellate Alexandrium minutum

A controlled field study was carried out in the Catalonian coast exposing 50 kg of edible mussel for 5 days to a toxic bloom of *Alexandrium minutum*, a known producer of PSP toxins. The objective was to allow high levels of PSP toxins to bioaccumulate in the mussels. Levels of *A. minutum* were always above 200000 cells/L and, as a result, the concentration of PSP toxins in mussels was higher than 4000 µg STX diHCl equiv/kg.

2.3 Procedure for PSP mussels, clams and scallops detoxification

The regulated procedure (EC, 1996), was applied to all different batches of PSP naturally contaminated mussels, clams and scallops with some modifications:

- Preliminary cleaning in running fresh water for two minutes.
- Pre-cooking in fresh water for three minutes at a temperature of 95 ± 5 °C.
- Separation of flesh and shells.
- Second cleaning in fresh water for 30 seconds.
- Cooking in fresh water for nine minutes at a temperature of 98 ± 5 °C.
- Cooling in running fresh water for approximately 90 seconds.
- Conditioning in containers closed hermetically in a non-acidified liquid medium.
- Sterilization in autoclave at 116 °C for 51 min (referred as “Canning”) or Pasteurization at 90 °C for 10 min.

Separation of the edible parts (foot) from the non-edible parts (gills, viscera and mantle), in mussels and clams, was omitted in order to increase the yielding of

the process. In the case of scallops, edible parts correspond to the sum of adductor muscle and roe. During the different cleaning steps, mollusk flesh was submerged in fresh water, including a last rinse step. To facilitate toxin analysis, drinking water was employed as covering sauce, since the habitual covering medium used in processed *A. tuberculata* products (brine) can interfere with HPLC columns. Samples subjected to the detoxification method are identified along the text as “EC”. Aliquots of the same batches of mussels, clams or scallops were sterilized or pasteurized without applying the detoxification procedure and are identified along the text as “normal”.

2.4 Toxin extraction

Two laboratories, ANFACO and IRTA, were involved in the extraction and the analysis of PSP toxins in the samples, either processed or not. Both laboratories performed the same extraction and analysis protocol described below, only with variations related to the chromatographic columns and LC equipment used.

2.4.1 Chemicals

Milli-Q ultrapure water, acetonitrile LC-MS grade (Scharlau), methanol LC-MS grade (Fisher), ammonium formate HiPerSolv Chromanorm® for LC-MS (VWR), glacial acetic acid reagent grade (Scharlau), periodic acid analytical reagent AnalaR NORMAPUR (VWR), Na_2HPO_4 analysis grade (MERCK), sodium hydroxide reagent grade (Scharlau), hydrogen peroxide solution 30% (v/v) reagent grade (Scharlau), ammonium acetate reagent grade (Scharlau), sodium chloride for analysis (MERCK).

SPE cartridges: SPE C18 sep-pack (3 mL, 500 mg) (Waters), SPE COOH (3 mL, 500 mg) (Bakerbond).

Certified PSP standards used at ANFACO: CRM-00-STX, gonyautoxins 1-5 (CRM-00-GTX1&4, CRM-00-GTX2&3, CRM-00-GTX5), neosaxitoxin (CRM-00-NEO), decarbamoylneosaxitoxin (CRM-00-dcNEO), decarbamoylsaxitoxin (CRM-00-dcSTX), N-sulfocarbamoyl gonyautoxin-2&3 (CRM-00-C1&2), and decarbamoylgonyautoxin-2&3 (CRM-00-dcGTX2&3) and gonyautoxins 6 (CRM-00-GTX6) were purchased from Cifga (Lugo, Spain).

Certified standards PSP used at IRTA: CRM-00-dcGTX2&3, CRM-00-C1&2, CRM-00-dcSTX, CRM-00-GTX2&3, CRM-00-GTX5, CRM-00-STX, CRM-00-GTX1&4, CRM-00-NEO and CRM-00-dcNEO were purchased from the National Research Council (NRC, Halifax, NS, Canada) and CRM-00-GTX6 was purchased from Cifga (Lugo, Spain).

2.4.2 Standard solutions

Standard mixtures were prepared from the commercial standards*,* at ANFACO: MIX I (STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 and C1,2), MIX II (NEO and GTX1,4), MIX III (dcNEO), MIX IV (dcSTX) and MIX V (GTX6). For each MIX, 6 standard calibration levels were prepared by dilution with Milli-Q water in the range 0.006 – 1 µM (MIX I) and 0.015 – 1 µM (the remaining MIX). These standard solutions were preserved at -20°C. Individual LQ were 45 µg equiv. STX diHCl/kg for dcGTX2,3; 5 µg equiv. STX diHCl/kg for C1,2; 40 µg equiv. STX diHCl/kg for dcSTX; 25 µg equiv. STX diHCl/kg for GTX2,3; 5 µg equiv. STX diHCl/kg for GTX5; 40 µg equiv. STX diHCl/kg for STX; 150 µg equiv. STX diHCl/kg for GTX1,4; 140 µg equiv. STX diHCl/kg for NEO; 80 µg equiv. STX

diHCl/kg for dcNEO; 13 µg equiv. STX diHCl/kg for GTX6 and 20 µg equiv. STXdiHCl/kg for C3,4. Sum of individual LQ of PSP toxins at ANFACO-CECOPESCA was 563 µg equiv. STX diHCl/kg. At IRTA, standard mixtures were prepared from the commercial standards*:* MIX I (STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 and C1,2), MIX II (NEO and GTX1,4), MIX III (dcNEO), MIX IV (dcSTX) and MIX V (GTX6). For each MIX, 6 standard calibration levels were prepared by dilution with Milli-Q water in the range LQ-800 µg STX diHCl equiv/kg. Individual LQ were 46 µg equiv. STX diHCl/kg for dcGTX2,3; 6 µg equiv. STX diHCl/kg for C1,2; 40 µg equiv. STX diHCl/kg for dcSTX; 26 µg equiv. STX diHCl/kg for GTX2,3; 5 µg equiv. STX diHCl/kg for GTX5; 40 µg equiv. STX diHCl/kg for STX; 150 µg equiv. STX diHCl/kg for GTX1,4; 140 µg equiv. STX diHCl/kg for NEO; 80 µg equiv. STX diHCl/kg for dcNEO and 13 µg equiv. STX diHCl/kg for GTX6. Sum of individual LQ of PSP toxins was 546 µg equiv. STX diHCl/kg. These standard solutions were preserved at -20°C.

2.4.3 Extraction, clean-up, hydrolysis and oxidation

The method was based on the HPLC-FLD Official Method (AOAC, 2005; Lawrence et al., 2005), and refined as described by Turner et al. and Ben-Gigirey et al. (Ben-Gigirey et al., 2012; Turner et al., 2009). The method involves an acetic acid extraction through clean-up with SPE C18 cartridge extraction followed by periodate oxidation and analysis by HPLC-FLD. If the presence of any toxin is observed, peroxide oxidation and/or fractionation (F1, F2, F3) are then carried out by using COOH ion exchange SPE cartridges with periodate oxidation, injecting the obtained extracts in the HPLC-FLD.

2.4.4 PSP toxin quantitation

The toxins dcGTX2,3, C1,2, dcSTX, GTX2,3, GTX5, STX were quantified in the C18 extract after peroxide oxidation; GTX1,4 and GTX6 in F2 fraction; NEO and dcNEO in F3 fraction and C3,4 in F1 hydrolyzed fraction after periodate oxidation. Total PSP toxicity, expressed as STX diHCl equivalents/kg, is calculated by summing individual toxin concentrations and applying toxicity equivalents factors (TEF) that are established for each toxin according to EFSA Scientific Opinion (EFSA, 2009).

In the samples where no PSP toxins has been detected (below LODs), the histograms were left blank.

2.4.5 HPLC-FLD equipment and chromatographic conditions

PSP toxins analyses, at ANFACO, were carried out using an HPLC Alliance 2695 model and fluorescence detector 2474 model (Waters Corporation). A XSelect CSH C18 3.5 µm, 4.6 mm x 150 mm column and a XSelect CSH C18 3.5 µm, 3.9 mm x 5 mm precolumn from Waters were used. Chromatography conditions are described in the AOAC Method (Lawrence et al. 2005).

At IRTA, PSP toxins analyses were carried out using an UPLC Acquity H-Class model and FLR Acquity fluorescence detector (Waters Corporation). A Kinetex C18 4.5 µm, 4.6 x 150 mm column and a XSelect CSH C18 4.5 µm guard column from Phenomenex were used. Chromatography conditions used are those described in the rapid method by Hatfield et al. (Hatfield et al., 2016).

2.4.6 Method performance

The method acceptability criteria were selected to ensure the performance of the method, according to the International Organization for Standardization (ISO) 17025:2005 standards and the screening and semi-quantitation of PSP toxins EURLB-SOP quality requirements (EURLMB, 2019). The minimum performance criteria were checked out throughout the study such as retention time deviation ± 0.2 min, peak area deviation (RSD ≤3.0%), linearity (R^2 ≥0.98), sensitivity (individual toxin LOD should be equal or lower than 1:20th of regulatory level), precision intra-batch ≤20% and inter-batch ≤25%.

2.5 Statistics

For all naturally contaminated samples, differences in total PSP toxicity obtained in both laboratories were analyzed by two-tailed t-test with the significance level set at 5%. Prior to t-test analysis, it was checked normality and variance homogeneity (SigmaPlot v12.0, Systat software, Inc., CA, USA). For each naturally contaminated samples, differences in the total PSP toxicity obtained between raw and normal canning (NC), raw and EC canning, raw and normal pasteurization (NP), and raw and EC pasteurized were evaluated by one-tailed t-test with the significance level set at 5%.

3 Results

The different batches of cultivated mussels (*M. galloprovincialis*), clams (*R. philippinarum*) and scallops (*P. maximus*), origin and sampling place, toxic phytoplankton involved, date of harvesting and analytical results initially obtained

in the raw mollusks, are summarized in Table 1. In this table, mean values ± standard error of the mean (SEM) obtained for each sample analyzed by both laboratories are included.

The different batches were split and samples were processed by the different thermal treatments as described above. A standard canning, a standard pasteurization, as usually performed in an industrial situation, and the detoxification procedure followed by canning or pasteurization were carried out. These treatments are referred, respectively, as Normal canning, Normal pasteurization, EC canning and EC Pasteurization. Normal pasteurization was not performed in all the batches, so in those cases, it is not depicted in the corresponding figure (Figs 3, 4, 6, and 9). The different keys used in figure's legend are summarized in Table 2. All samples were analyzed by HPLC by the two laboratories (ANFACO and IRTA). The HPLC results obtained by both laboratories showed good agreement, and good correlation was obtained, as shown in Figure 2. No significant differences were observed between results from both laboratories (t-Student, $p > 0.05$).

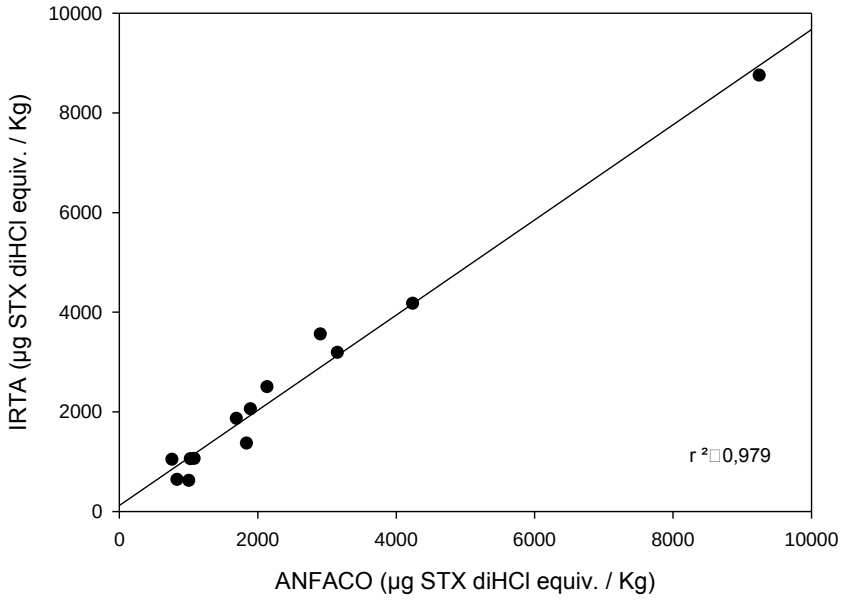


Fig 2: Sigma-plot correlation chart for the total PSP toxicity present in raw bivalves set analyzed by HPLC-FLD (µg STX diHCl equiv/kg) (n=13) showing a good correlation between results obtained at ANFACO and IRTA laboratories.

Table 1. Origin and date of harvesting of live PSP contaminated mussels and scallops. Results of PSP toxins (mean values ± standard deviation, n=2) in raw bivalves analyzed by HPLC-FLD, at both laboratories.

Species	**Location**	**Harvesting date**	**Present phytoplankton**	**Average result (µg STX diHCl equiv/kg) (n=2)**
Mussel (*Mytilus galloprovincialis*)	Ría of Vigo (Vigo A)	09/07/2018	*Alexandrium spp*	1072 ± 11

Mussel (*M. galloprovincialis*)	Ría of Vigo (Redondela C)	23/07/2018	*Alexandrium spp*	1604 ± 330
Mussel (*M. galloprovincialis*)	Ría of Vigo (Redondela C)	23/07/2018	*Alexandrium spp*	737 ± 134
Mussel (*M. galloprovincialis*)	Andalucía (Benalmádena)	02/08/2018	*Gimnodinium catenatum*	812 ± 270
Mussel (*M. galloprovincialis*)	Portinho da Costa (Lisbon)	22/10/2018	*Gymnodinium catenatum*	9001 ± 345
Mussel (*M. galloprovincialis*)	Catalonia	05/03/2019	*Alexandrium minutum*	4205 ± 43
Mussel (*M. galloprovincialis*), frozen	Catalonia	05/03/2019	*Alexandrium minutum*	2317 ± 261
Clam (*R. philippinarum*)	Ría of Pontevedra	27/07/2018	*Alexandrium spp*	1041 ± 23
Clam (*R. philippinarum*), frozen	Ría of Pontevedra	27/07/2018	*Alexandrium spp*	903 ± 204
Scallop (*Pecten maximus*)	Andalucía	22/08/2019	*Gimnodinium catenatum*	3232 ± 466
Scallop (*P. maximus*) eviscerated	Andalucía	22/08/2019	*Gimnodinium catenatum*	1976 ± 117
Scallop (*P. maximus*)	Andalucía	22/08/2019	*Gimnodinium catenatum*	3171 ± 30
Scallop (*P. maximus*), eviscerated	Andalucía	22/08/2019	*Gimnodinium catenatum*	1779 ± 126

Table 2 keys used in figure legends along the text

Key word	Use of PSP detoxification procedure	Thermal processing
Raw	No	None.
Normal Canning	No	116 °C, 51 min
Normal Pasteurization	No	90 °C, 10 min
EC Canning	Yes	116 °C, 51 min
EC Pasteurization	Yes	90 °C, 10 min

Fig 3A, shows the levels of PSP toxins, expressed as µg STX diHCl equiv/kg (total PSP toxicity), in raw and thermally processed mussels harvested during a *Alexandrium* bloom from Redondela, Galicia. Results show that the normal canning procedure, as well as the application of the detoxification process (EC) followed by sterilization or pasteurization, were able to decrease PSP levels below the limit of detection when applied to raw mussels containing around 1604 µg STX diHCl equiv/kg.

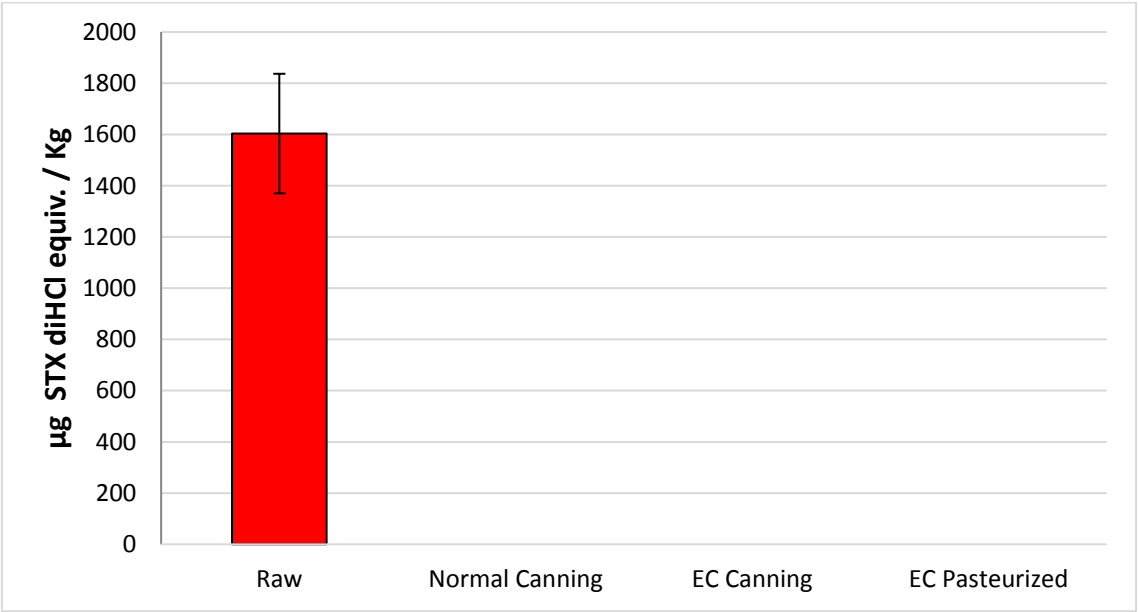


Fig 3A. PSP toxins (total PSP toxicity) in naturally contaminated mussels from Redondela, NW Spain analyzed by HPLC. Mean values (n=2) are represented, and vertical bars indicate the SEM.

Fig 3B shows the quantification of the different PSP analogues of samples from fig 3A, expressed as STX diHCl equiv/kg. It is worth to mention that in the sample, GTX1,4 was the dominant toxin. Very low concentration of other analogues, such as dcSTX, GTX2,3 and C3,4 toxins were detected as well. Similar results were obtained in another batch of mussels from the same location and bloom with a PSP concentration near the legal limit, 800 µg STX diHCl equiv/kg due to GTX1,4; C3,4 and GTX2,3 as show Fig 3C. In this batch, no PSP toxins were detected after processing. A similar behavior, was found with a new lot of mussels harvested during a *Alexandrium* bloom from the same ría but from a different location, Vigo, also in Galicia, as illustrates Fig 4.

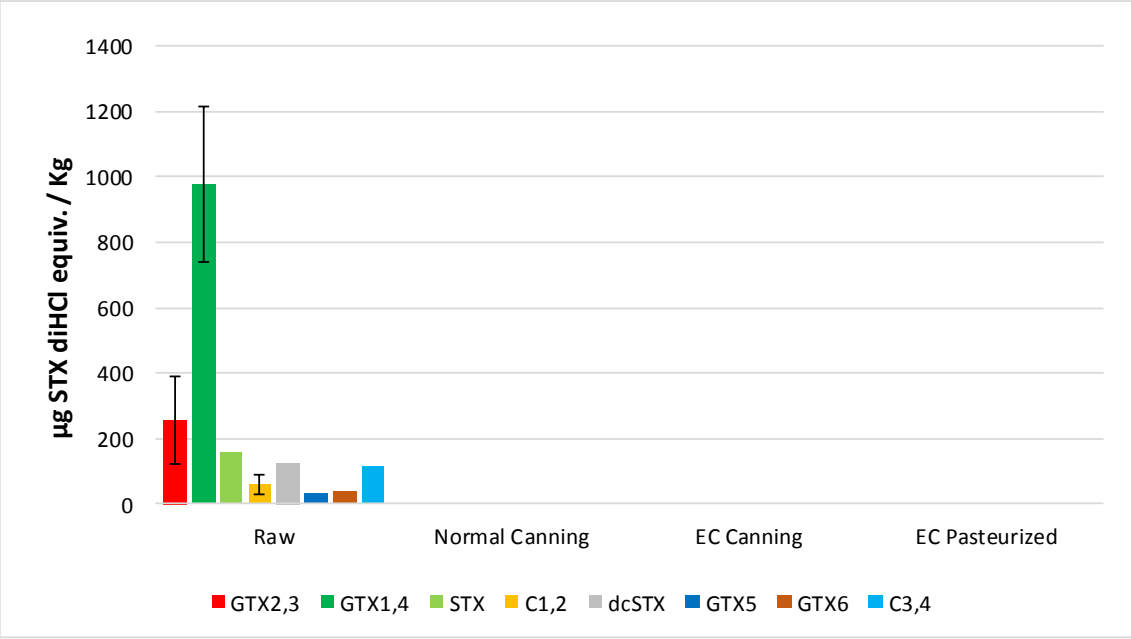


Fig 3B. PSP toxins in naturally contaminated mussels from Redondela, NW Spain. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM

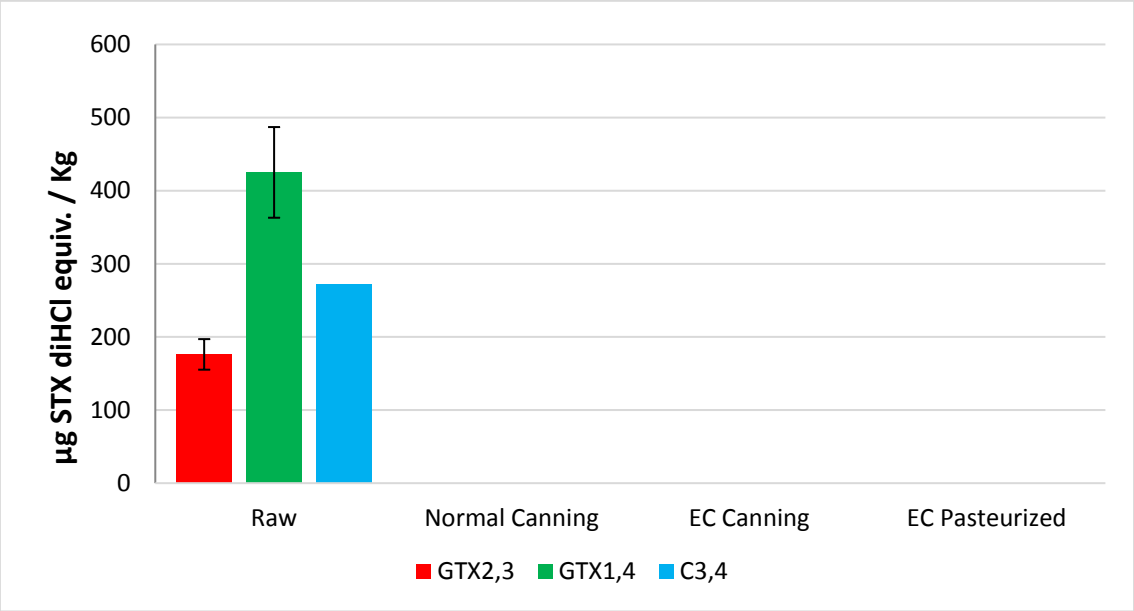


Fig 3C. Naturally contaminated mussels from Redondela, NW Spain analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM.

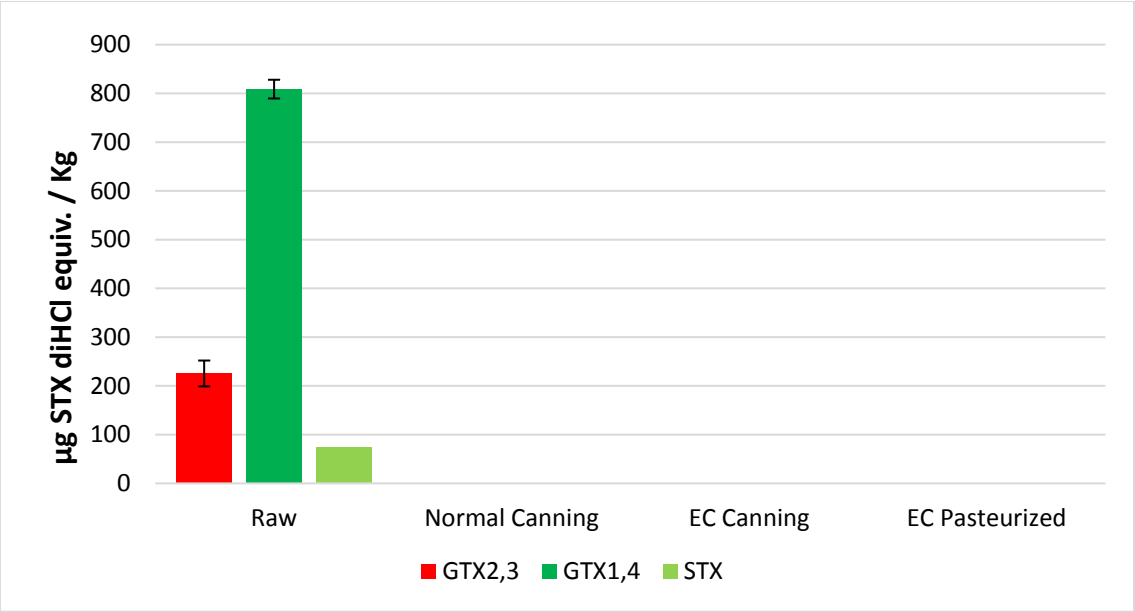


Fig 4. Naturally contaminated mussels from Vigo, NW Spain analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM.

A batch of mussels harvested during a *Gimnodinium catenatum* bloom from the South of Spain, Málaga, in Andalucía, was also processed, obtaining an important reduction of PSP toxins concentration, as expected. All the applied protocols allowed to decrease initial levels of PSP toxins below the legal limit, as shows Fig 5A. Significant differences were observed between raw and the other four thermal treatments applied in this study (t-Student, $p < 0.05$).

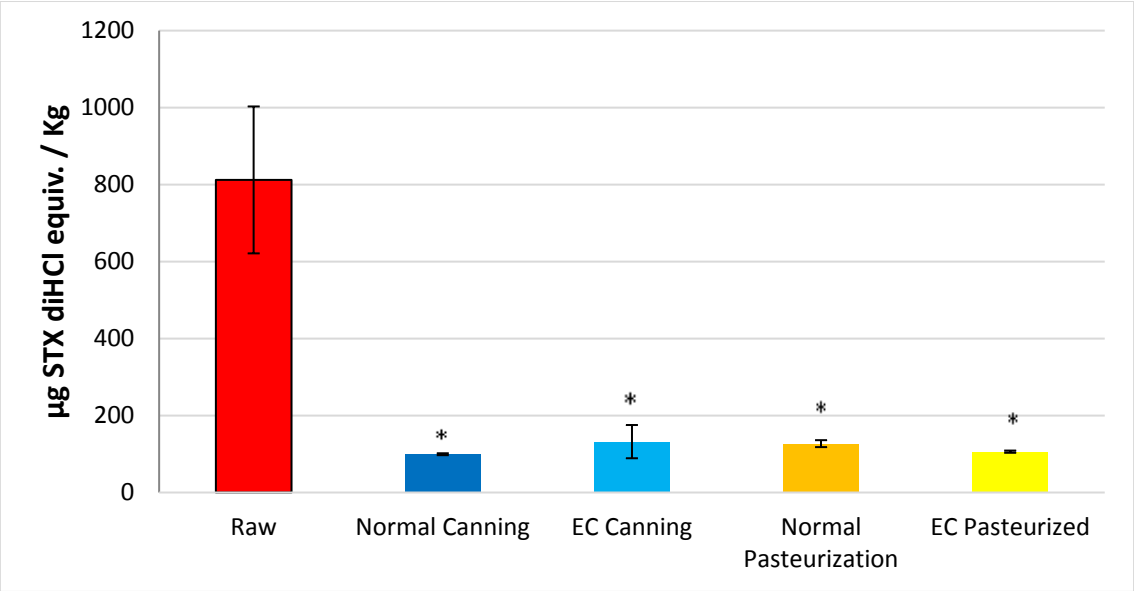


Fig 5A. Naturally contaminated mussels from Andalucía, S Spain analyzed by HPLC (total PSP l toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the four thermal treatments (t-Student, $p < 0.05$).

Fig 5B illustrates all PSP analogues identified in the samples represented in Fig 5A. Raw sample contained mostly GTX1,4 and in lower concentration GTX2,3, dcSTX, C1,2 and GTX5. All analogues except for dcSTX after the EC canning protocol showed a significant decrease ($p < 0.05$). It is remarkably that after processing dcSTX is the dominant analogue.

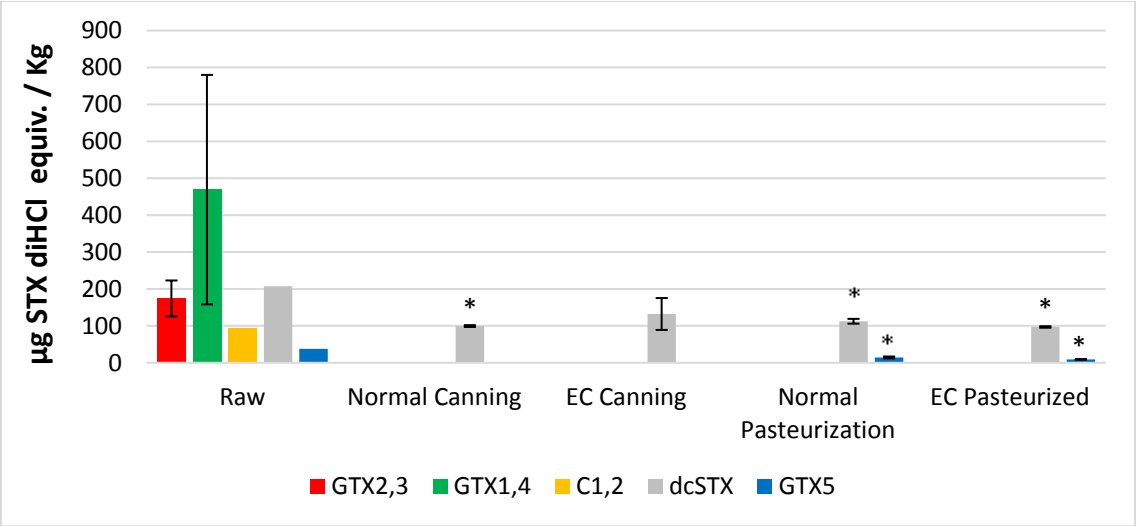


Fig 5B Naturally contaminated mussels from Andalucía, Spain. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the four thermal treatments (t-Student, $p < 0.05$).

A new batch of mussels with an extremely high concentration of PSP toxins was harvested from Portugal during a *Gymnodinium catenatum* bloom, as shows Fig 6A. In this case, mussels exposed to the toxic episode for a long time, presented a huge toxin concentration (9000 µg STX diHCl equiv/kg), exceeding more than 10 times the legal limit. Although after application of the detoxification procedure, PSP toxins concentration decreased in a significant way (t-Student, $p < 0.05$), reaching 90 % of detoxification, no safe products were attained in this case. PSP concentration in mussels elaborated with the detoxification protocol and then canned was 1054±33 µg STX equiv/kg, higher than the legal limit, whereas those samples pasteurized after the detoxification procedure showed lower PSP concentrations (783±183 µg STX diHCl equiv/kg), which was a surprising finding.

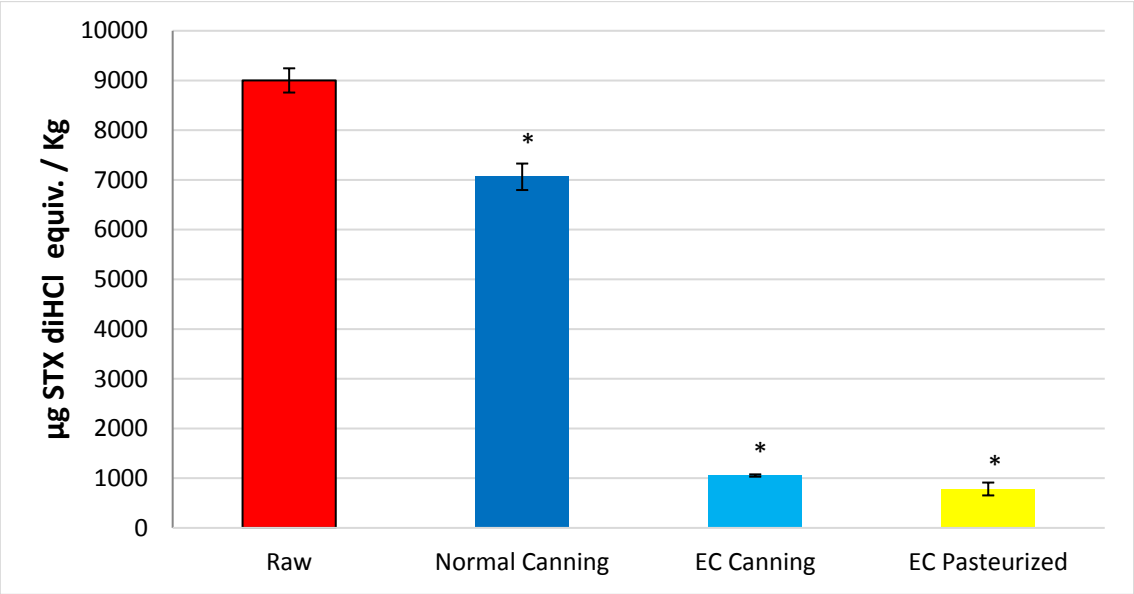


Fig 6A. Naturally contaminated mussels from Lisbon, Portugal, analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the three thermal treatments (t-Student, $p < 0.05$).

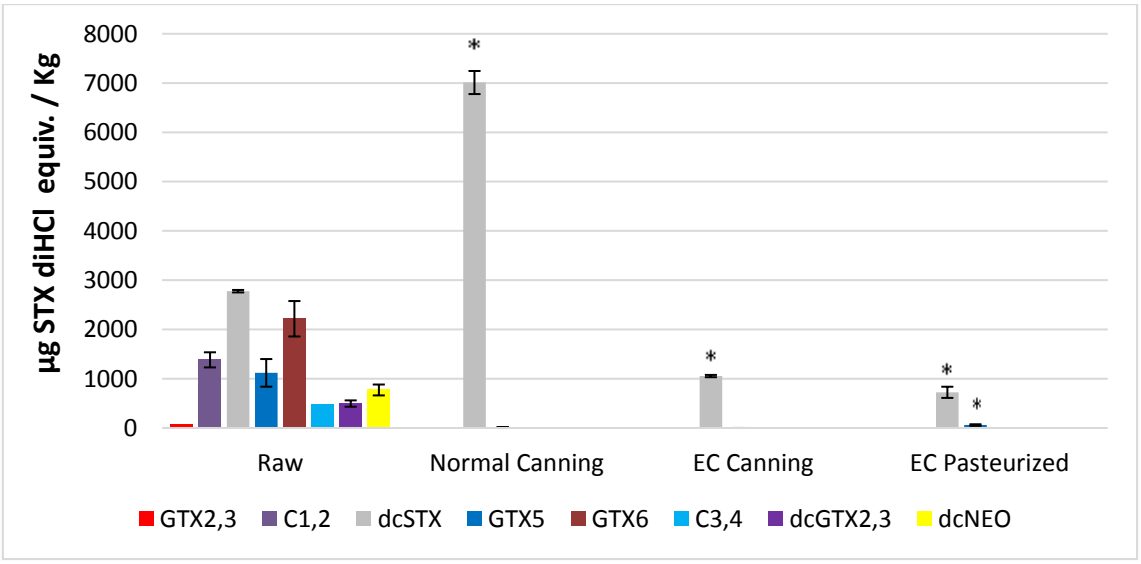


Fig 6B. Naturally contaminated mussels from Lisbon, Portugal, analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the three thermal treatments (t-Student, $p < 0.05$).

Fig 6B shows all PSP analogues identified in the samples represented in Fig 6A. The raw sample contained several toxins of the group, mainly dcSTX; GTX6; GTX5, C1,2 and dcGTX2,3. Again, dcSTX was the dominant analogue in the processed samples, even at higher levels than in the raw sample after “Normal Canning”. This fact suggests that a transformation of other toxins to dcSTX takes place due to the thermal process.

Similar results were obtained in the batch of mussels from Catalonia containing a final concentration of 4206 µg STX diHCl equiv/kg (Fig 7A). It is worth mentioning that, the same sample, after frozen storage at -20ºC for 3 weeks, showed a decrease in toxicity to 2318 µg STX diHCl equiv/kg (Fig 8A). Both treatments, the normal sterilization and the detoxification protocol, followed by sterilization or pasteurization, produced a significant decrease in PSP levels, below the legal limit (t-Student, $p < 0.05$).

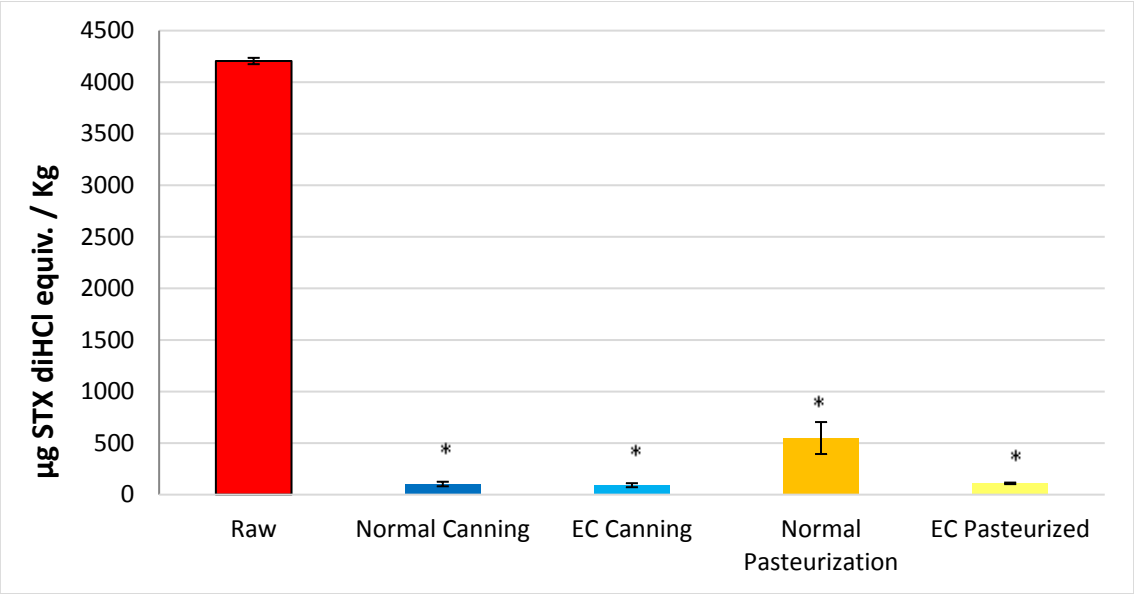


Fig 7A. Naturally contaminated mussels after controlled immersion into an area with *A minutum* in Catalonia, Spain analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the four thermal treatments (t-Student, $p < 0.05$).

The raw sample of immersed mussels coming from Catalonia contained several toxins of the group, mainly GTX1,4 and GTX2,3. STX was the dominant analogue in all four processed samples, and was not present in the raw sample. This fact suggests that a transformation to STX takes place due to the thermal process (Figure 7B).

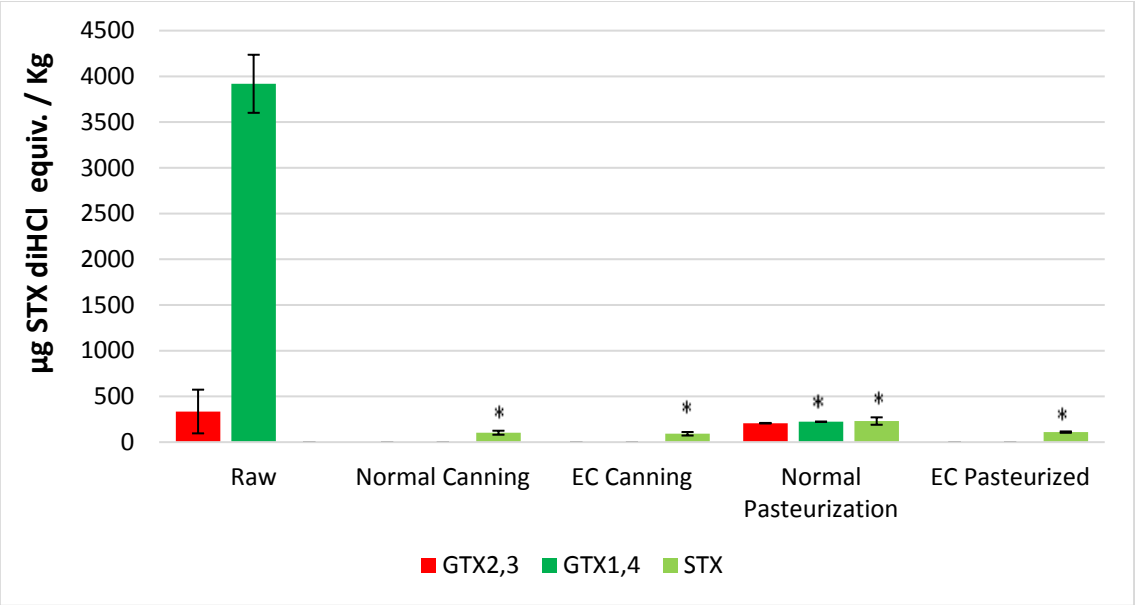


Fig 7B. PSP toxins in naturally contaminated mussels after controlled immersion into an area with *A minutum* from Catalonia, Spain, fresh sample. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the four thermal treatments (t-Student, $p < 0.05$).

Analysis of the same sample after preservation at -20ºC, showed an important decrease in total PSP toxicity in comparison with the fresh, unfrozen sample (t-Student, $p < 0.05$). From a qualitative point of view, both samples, fresh and frozen and the processed samples obtained, showed a similar behavior regarding decrease of toxins after processing (Figure 8A) and the type of toxins present, mainly GTX1,4; GTX2,3 and STX (Figure 8B). STX was again the dominant analogue in the processed samples, even at higher levels than in the raw sample after all thermal processing. This fact suggests that a transformation to STX takes place due to the canning and pasteurization process (Figure 7B and 8B).

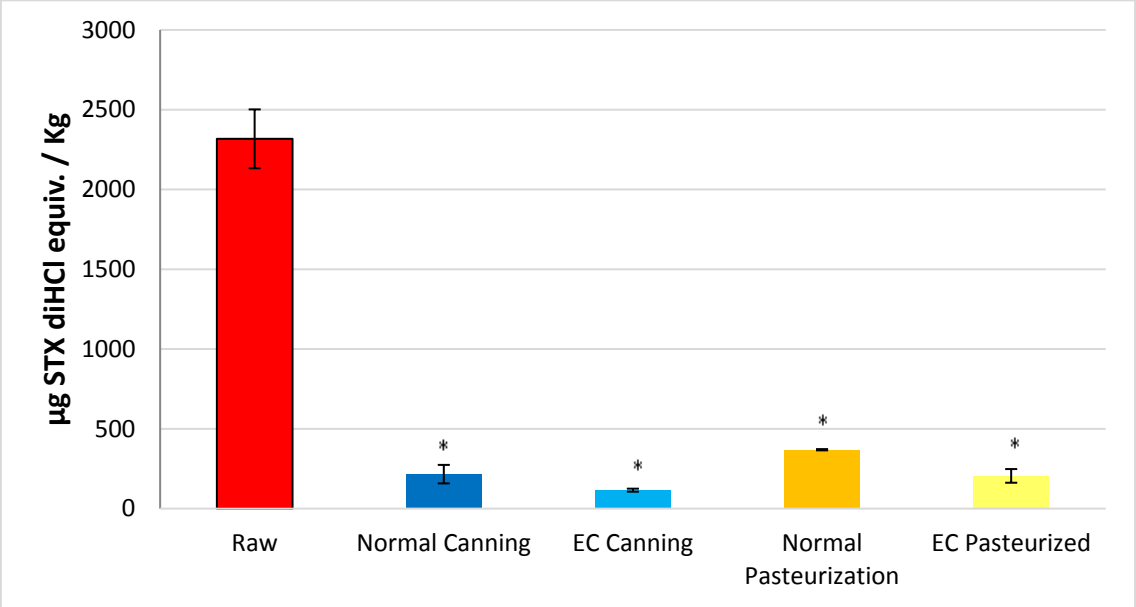


Fig 8A. Naturally contaminated mussels from Catalonia after storage at -20ºC analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw and the four thermal treatments (t-Student, $p < 0.05$).

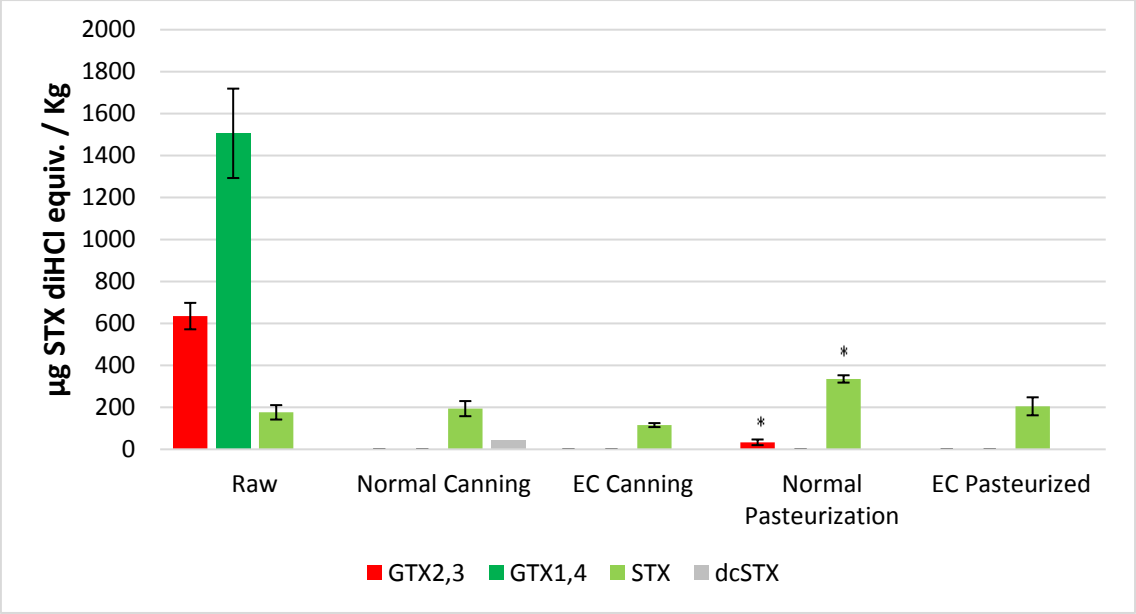


Fig 8B. PSP toxins in naturally contaminated mussels from Catalonia after storage at -20ºC. Figure shows all the PSP analogues detected in each sample, raw or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw and the four thermal treatments (t-Student, $p < 0.05$).

The analysis of contaminated clams and the products obtained after processing offered similar results to those obtained in mussels. Fresh raw clams obtained from Pontevedra (NW of Spain) during an *Alexandrium* spp bloom showed a PSP concentration of 1041 ± 22 µg STX diHCl equiv/kg (Fig 9A). The same sample after frozen storage at -20ºC for 7 weeks, contained 903 ± 204 µg STX diHCl equiv/kg (data not shown). Application of both treatments: the normal sterilization procedure and the detoxification protocol followed by sterilization or pasteurization, produced a decrease in PSP levels to not detectable levels (Fig.9B).

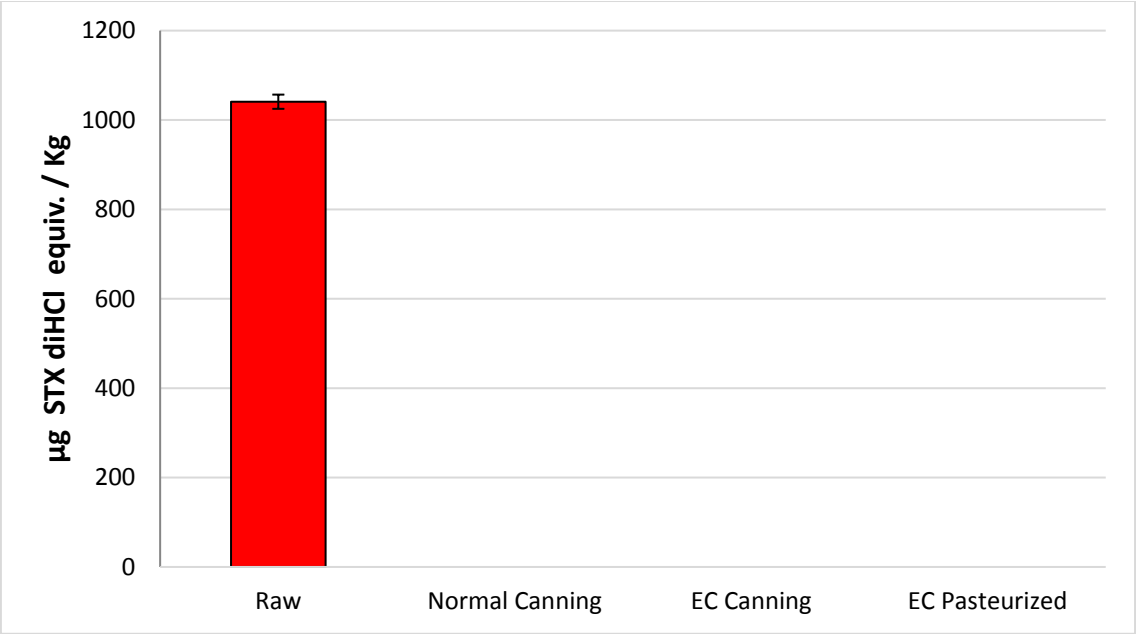
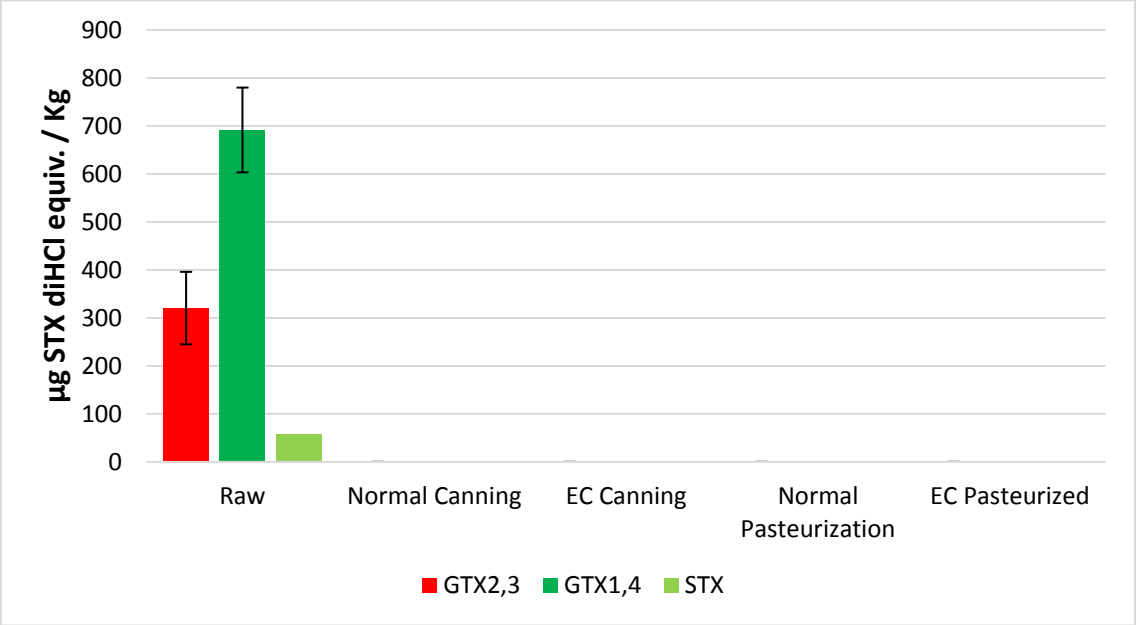


Fig 9A. PSP toxins in naturally contaminated clams from Pontevedra, NW Spain analyzed by HPLC (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM.



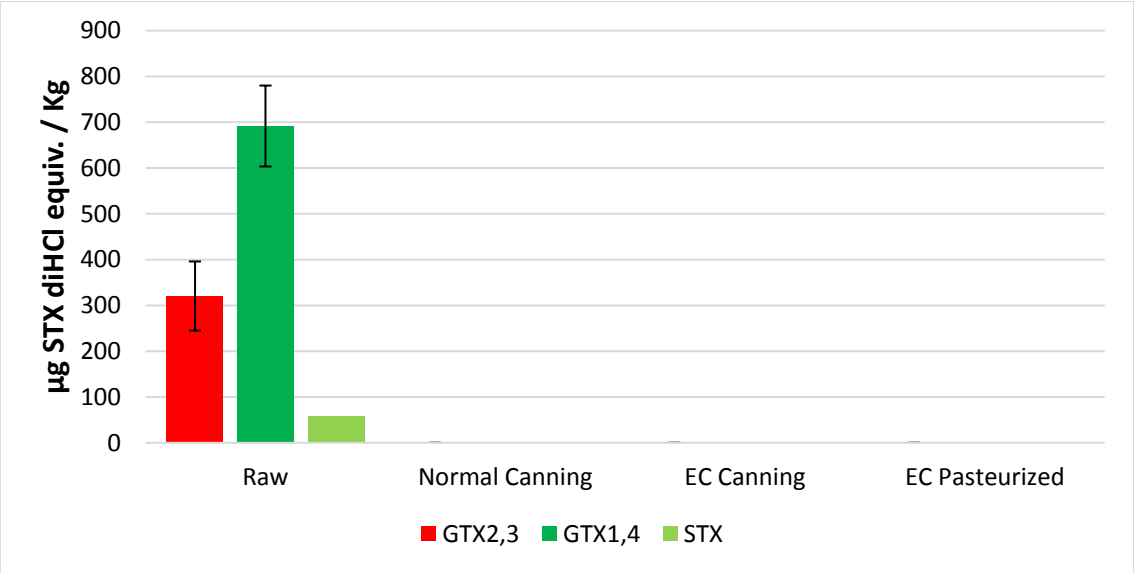


Fig 9B. Naturally contaminated clams from Pontevedra, Galicia, Spain, analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw, eviscerated or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM.

In addition to mussels and clams, scallops contaminated with PSP were harvested during a *Gimnodinium catenatum* bloom. In this species, evisceration of raw scallops reduced significantly PSP concentration, as expected (t-Student, $p < 0.05$) (Fig 10A). Evisceration was performed previously to all the thermal processes applied and reduced drastically all the congeners to non-detectable levels, in canned samples (with or without detoxification protocol). Also, it reduced to levels well below the legal limit in pasteurized samples. The different analogues detected in the raw mollusk, whole body or eviscerated, were GTX2,3; STX; C1,2; dcSTX, GTX5, and dcGTX2,3). Mostly dcSTX (327±69 µg STX diHCl equiv/kg) and, in a much lower level, GTX5 (28±9 µg STX diHCl equiv/kg) were detectable when pasteurization was carried out after a conventional pre-cooking step. In addition, only dcSTX was detectable to a lower level (144±19 µg STX

diHCl equiv/kg) when pasteurization was carried out after the detoxification protocol (Fig 10B).

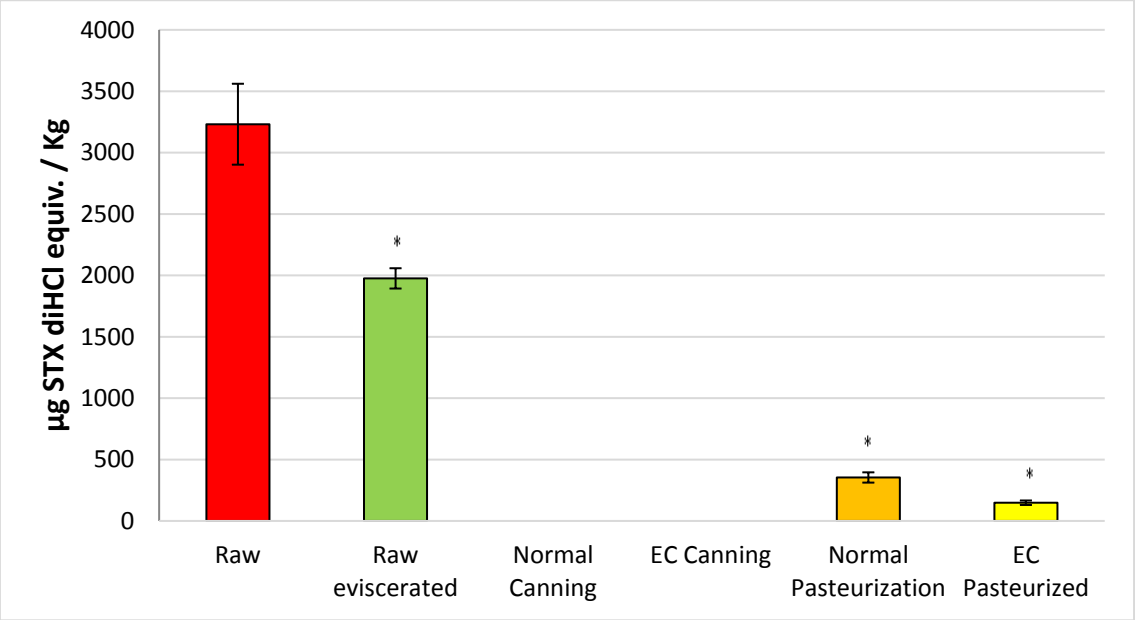


Fig 10A. Naturally contaminated scallops (*Pecten maximus*) from Marbella, Andalucía , Spain (total PSP toxicity). Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the total PSP toxicity between raw, raw eviscerated and the four thermal treatments (t-Student, $p < 0.05$).

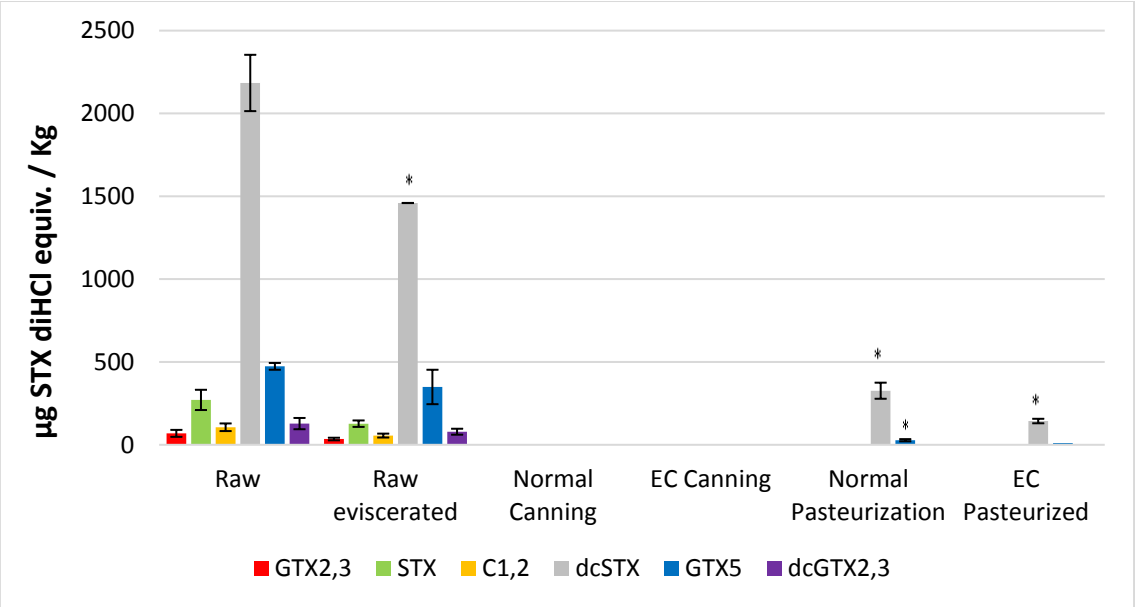


Fig 10B. Naturally contaminated scallops from Marbella, Andalucía, Spain, analyzed by HPLC. Figure shows all the PSP analogues detected in each sample, raw, eviscerated or processed under different conditions. Mean values (n=2) are represented, and vertical bars indicate the SEM. Asterisk (*) means significant differences for the toxins profile between raw, raw eviscerated and the four thermal treatments (t-Student, $p < 0.05$).

4 Discussion

Some studies have been conducted to reduce or eliminate PSP toxins in mollusks and other invertebrates. The influence of thermal processing in naturally contaminated bivalves has already been studied by our group and by other authors, finding PSP detoxification in shellfish after application of high temperatures (Berenguer et al., 1993) (Lawrence et al., 1994; Reboreda et al., 2010; Vieites et al., 1999).

In this study, an approved thermal procedure to decrease PSP toxins in the giant cockle, *Acanthocardia tuberculata*, (EC, 1996), was evaluated on mussels, clams and scallops naturally contaminated with PSP toxins. We applied the heat

treatment (so called “detoxification procedure”) that consists on several cleaning and cooking steps, as establishes the European legislation but with certain modifications (EC, 1996). Mainly, in this legislated procedure, the edible parts are separated from the non-edible parts. In the case of mussels, the digestive viscera constitute 30 % of the total tissue weight (FAO, 2004), and removing it is not realistic regarding the commercial practices by the industry for this species (as well as for clams), while it is accepted for scallops. Hence, we did eviscerate the scallops, since this is a common practice.

A standard canning or pasteurization, as usually performed in an industrial situation, without previous washes and cooking, was carried out to compare the results with those obtained after application of the “detoxification procedure”.

Different batches of mussels, clams and scallops containing PSP levels above the regulated limit (800 µg STX equiv/kg) and coming from several areas of Spain and Portugal, were thermally processed. In our hands, scallops, clams and almost all mussels unless one batch, underwent an important detoxification process, leading to fulfill PSP legislated limit. Only one sample of mussels, with the highest concentration, 9000 µg STX diHCl equiv/kg, more than 10 times the legal concentration, (Fig 6), showed a statistically significant decrease in total PSP toxicity after processing, but in a minor extent for normal canning, with a decrease of 22% in total PSP toxicity. Otherwise, the rest of mollusk batches processed along this study, either after standard canning or pasteurization, even without the application of the “detoxification procedure”, reached levels of detoxification higher than 85 %. This was a much unexpected finding. These results suggest that in some circumstances, if concentrations of toxins in shellfish are not very elevated, it is not necessary to apply the “detoxification

procedure", since a normal canning or pasteurization seems sufficient to reduce PSP levels. Nevertheless, in our opinion, detoxification of mussels depends not only on the initial concentration, but also on the exposure time to toxins and the seawater conditions as well. So, the detoxification procedure should be applied in all cases. In contrast, when shellfish are contaminated with high levels (>5300 µg STX diHCl equiv/kg), even the "detoxification procedure" application is not enough to reduce PSP levels below the legislated limit. Taking this into account, a threshold level should be established in mussels if a detoxification legislative proposal is expected. Based on data found in this work, with detoxification levels of 85%, a maximum level of 5300 µg STX diHCl equiv/kg is proposed to be established for the application of this procedure. Nevertheless, in order to obtain a higher amount of data to support this proposed limit, further work would be necessary. The highest level allowed in the European Legislation authorized for the harvesting of *A. tuberculata* is 3000 µg STX diHCl equiv/kg, if the product is intended to the canning industry, and analytical control of each batch, which "must not contain a PSP level detectable by the bioassay method after the application of this heat treatment" is mandatory (EC, 1996). The analytical control of produced batches should be maintained if the present detoxification procedure was extended to other molluscan species. In addition to this Decision, applied in Spain for the giant cockle, a legislation in Canada allows canning of soft shell clams and mussels with levels between 800 and 1600 µg STX equiv/kg (Fernández et al., 2003). Also, butter clams containing 3000 to 5000 µg STX equiv/kg may be commercialized after removing the entire siphon, whereas butter clams containing 800 to 3000 µg STX equiv/kg may be marketed

after removing the distal half of the siphon (Fernández, 1998), cited in (FAO, 2004).

A preliminary article shows that the standard canning process resulted in a significant and reproducible reduction of PSP toxicity in mussel meat (> 50%), decreasing toxin levels under the limit of detection (Vieites et al., 1999). In this paper, authors observed that the decrease of toxicity was not dependent on toxin levels of raw material, although it is worth to mention that raw samples in this work contained PSP toxin levels below 4000 µg STX diHCl equiv/kg. These results are in good agreement with ours, since only when the concentrations of PSP toxins are very high (9000 µg STX diHCl equiv/kg), we find that the traditional canning did not allow detoxifying mussels below the legal limit. A subsequent work confirmed the detoxification after canning, although no very relevant results were obtained in that study due to the unavailability of highly PSP contaminated shellfish (Reboreda et al., 2010); in this study a mollusk type-dependent decrease in toxicity after freezing was observed, and the conclusion was that PSP toxins leaked with thawing water.

In lobsters, boiling or steaming reduced toxicity by approximately 65% compared to values obtained in the raw samples (Lawrence et al., 1994).

Our results suggest that thermal treatment induces chemical modifications of toxins, changing the toxic potency of processed shellfish, converting toxins into more or less toxic analogues, as occurs by biotransformation or metabolic transformations in shellfish (Reis Costa et al., 2018).

In the present study, GTX1,4 was the dominant detected toxin in raw samples of mussels, except for the most toxic sample from Portugal where a mixture of analogues was identified and dcSTX and GTX6 were the major toxins with

similar levels. Among the different PSP toxins, dcSTX was the major analogue obtained in processed samples, including the one with the highest PSP concentration except for the mussel from Catalonia where the major analogues obtained in the processed samples was STX. GTX1 and GTX4 together are less toxic (TEF=1 and 0.7, respectively) than dcSTX (TEF=1) (EFSA, 2009). This was taken into account for the calculation of total PSP toxicity since HPLC results were expressed as STX diHCl equiv/kg.

More studies will be necessary to assess if toxins in cooked mussels and scallops are removed through chemical decomposition, leached out during the loss of water or transferred to packing medium.

Regardless of these possibilities, our study demonstrates that processing of PSP contaminated shellfish, significantly reduces the PSP toxicity and industrial processes may be a solution in shellfish harvesting areas affected by intense and frequent PSP closures. The proposed method has been already implemented in the transformation of *Acanthorcardia tuberculatum* and should be easily transferred to other bivalve transforming facilities.

5 Conclusions

In conclusion, an efficient and inexpensive “detoxification procedure” can be applied in PSP contaminated mussels, clams and scallops to decrease PSP toxins below the legal limit (800 µg STX diHCl equiv/kg). However, a maximum threshold level in raw material should be previously established to define if the processing will efficiently reduce PSP toxins below the legal limit. Based on our data, 5300 µg STX diHCl equiv/kg would be the highest level. Although it is still necessary that the industry should proceed with quality controls of the final

product to ensure that it responds to the legal requirements and levels of PSP toxins are safe, in the same way as stated in the reference legislation for *Acanthocardia tuberculatum*.

ACKNOWLEDGEMENTS

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement no. 773400 (SEAFOODTOMORROW). This output reflects the views only of the author(s), and the European Union cannot be held responsible for any use which may be made of the information contained therein.

The authors acknowledge the Departament d'Agricultura, Ramaderia, Pesca i Alimentació (DARP) of the Generalitat de Catalunya for financing of the Shellfish Harvesting Areas Monitoring Program. We also appreciate the support of the CERCA program of the Generalitat de Catalunya. The authors acknowledge Margarita Fernández-Tejedor for her assistance in the mussel exposure to a toxic bloom of the dinoflagellate *Alexandrium minutum.*

REFERENCES

Álvarez, G., Díaz, P.A., Godoy, M., Araya, M., Ganuza, I., Pino, R., Álvarez, F., Rengel, J., Hernández, C., Uribe, E., Blanco, J., 2019. Paralytic Shellfish Toxins in Surf Clams *Mesodesma donacium* during a Large Bloom of *Alexandrium catenella* Dinoflagellates Associated to an Intense Shellfish Mass Mortality. Toxins (Basel) 11, 188.

AOAC, 2005. Official Method 2005.06. Paralytic Shellfish Poisoning Toxins in Shellfish. Prechromatographic Oxidation and Liquid Chromatography with fluorescence detection. First Action 2005. J AOAC INT 88, 1714.

Barbosa, V., Santos, M., Anacleto, P., Maulvault, A.L., Pousao-Ferreira, P., Costa, P.R., Marques, A., 2019. Paralytic Shellfish Toxins and Ocean Warming: Bioaccumulation and Ecotoxicological Responses in Juvenile Gilthead Seabream (Sparus aurata). Toxins (Basel) 11.

Ben-Gigirey, B., Rodriguez-Velasco, M.L., Gago-Martinez, A., 2012. Extension of the validation of AOAC Official Method 2005.06 for dc-GTX2,3: interlaboratory study. J AOAC INT 95, 111-121.

Berenguer, J.A., Gonzalez, L., Jimenez, I., Legarda, T.M., Olmedo, J.B., Burdaspal, P.A., 1993. The effect of commercial processing on the paralytic shellfish poison (PSP) content of naturally-contaminated Acanthocardia tuberculatum L. Food Additives & Contaminants 10, 217-230.

Braga, A.C., Camacho, C., Marques, A., Gago-Martinez, A., Pacheco, M., Costa, P.R., 2018. Combined effects of warming and acidification on accumulation and elimination dynamics of

paralytic shellfish toxins in mussels Mytilus galloprovincialis. Environmental research 164, 647-654.
EC, 1996. Commission Decision of 18 January 1996 establishing the conditions for the harvesting and processing of certain bivalve molluscs coming from areas where the paralytic shellfish poison level exceeds the limit laid down by Council Directive 91/492/EEC (96/77/EC). Off. J. Eur. Communities L 15, 46-47.
EC, 2004. REGULATION (EC) No 853/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 laying down specific hygiene rules for food of animal origin. Off. J. Eur. Communities L 226, 55-205.
EFSA, E.F.S.A., 2009. Marine biotoxins in shellfish – Saxitoxin group. EFSA Journal 7, 1019.
EURLMB, 2019. AOAC 2005.06 Standard Operating Procedure. Simplified version for the screening and semi-quantitation of PSP toxins. Avaliable at http://www.aecosan.msssi.gob.es/en/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/SOP_AOAC_2005_06_semi.pdf.
Fabre, A., Lacerot, G., de Paiva, R.R., Soares, M.C.S., de Magalhães, V.F., Bonilla, S., 2017. South American PSP toxin-producing Cylindrospermopsis raciborskii (Cyanobacteria) decreases clearance rates of cladocerans more than copepods. Hydrobiologia 785, 61-69.
FAO, 2004. Marine Biotoxins. In FAO Food and Nutrition Paper 80. p. 295. Available at http://www.fao.org/3/y5486e/y5486e00.htm in: F.a.A. Organization (Ed.), Rome, Italy.
Fernández, M.L., 1998. Phycotoxins: Regulatory Limits and Effects on Trade. , in: M. Miraglia, H.P.v. Egmond, C. Brera, J. Gilbert (Eds.), Mycotoxins and Phycotoxins – Developments in Chemistry, Toxicology and Food Safety. Alaken, Inc., Fort Collins, Colorado, pp. 503-516.
Fernández, M.L., Shumway, S.E., J., B., 2003. Managementof shellfish resources., in: H. M., A.A. D., A.D. M. (Eds.), Manual on Harmful Marine Microalgae. UNESCO Publishing, Paris, pp. 657-692.
Gracia Villalobos, L., Santinelli, N.H., Sastre, A.V., Marino, G., Almandoz, G.O., 2019. Spatiotemporal distribution of paralytic shellfish poisoning (PSP) toxins in shellfish from Argentine Patagonian coast. Heliyon 5, e01979.
Hatfield, R.G., Punn, R., Algoet, M., Turner, A.D., 2016. A Rapid Method for the Analysis of Paralytic Shellfish Toxins Utilizing Standard Pressure HPLC: Refinement of AOAC 2005.06. Journal of AOAC International 99, 475-480.
Lawrence, J.F., Maher, M., Watson-Wright, W., 1994. Effect of cooking on the concentration of toxins associated with paralytic shellfish poison in lobster hepatopancreas. Toxicon 32, 57-64.
Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J AOAC INT 88, 1714-1732.
Lee, R., Lovatelli, A., Ababouch, L., 2008. Bivalve Depuration: Fundamental and Practical Aspects. FAO. Fisheries Technical Paper No 511. Rome, FAO. , 139.
Pitois, F., Fastner, J., Pagotto, C., Dechesne, M., 2018. Multi-Toxin Occurrences in Ten French Water Resource Reservoirs. Toxins (Basel) 10.
Reboreda, A., Lago, J., Chapela, M.-J., Vieites, J.M., Botana, L.M., Alfonso, A., Cabado, A.G., 2010. Decrease of marine toxin content in bivalves by industrial processes. Toxicon 55, 235-243.
Reis Costa, P., Braga, A.C., Turner, A.D., 2018. Accumulation and Elimination Dynamics of the Hydroxybenzoate Saxitoxin Analogues in Mussels Mytilus galloprovincialis Exposed to the Toxic Marine Dinoflagellate *Gymnodinium catenatum*. Toxins (Basel) 10, 428.
Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., Lees, D.N., 2009. Refinement and extension of AOAC Method 2005.06 to include additional toxins in mussels: single-laboratory validation. J AOAC INT 92, 190-207.
Vieites, J.M., Botana, L.M., Vieytes, M.R., Leira, F.J., 1999. Canning Process that Diminishes Paralytic Shellfish Poison in Naturally Contaminated Mussels (*Mytilus galloprovincialis*). Journal of Food Protection 62, 515-519.