Predictive score and probability of CTX-like toxicity in fish samples from the Official Control of Ciguatera in the Canary Islands

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ABSTRACT:

This research identifies factors associated with the contamination by ciguatoxins (CTXs) in a population of fish and proposes a predictive score of the presence of CTX-like toxicity in amberjack samples from the official control program of ciguatera in the Canary Islands of the Directorate-General (DG) Fisheries (Canary Government). Out of the 970 samples of fish studied, 177 (18.2%) samples showed CTX-like toxicity. The fish were classified according to the species, amberjack (*Seriola dumerili* and *S. rivoliana*) (n = 793), dusky grouper (*Epinephelus marginatus*) (n = 145) and wahoo (*Acanthocybium solandri*) (n = 32). The data were separated by species category and statistically examined, resulting in 137 (17.3%) amberjack and 39 (26.9%) grouper samples showing CTX-like toxicity; regarding wahoo species, only 1 toxic sample (3.1%) was found. According to fishing location the contamination rates suggested grouping the islands in four clusters; namely: {El Hierro: HI; La Gomera: LG; La Palma: LP}, {Gran Canaria: GC; Tenerife: TF}, {Fuerteventura: FU} and {Lanzarote: LZ}. For the amberjack species, the multivariate logistic regression showed the factors that maintained independent association with the outcome, which were the warm season (OR = 3.617; 95%CI = 1.249 – 10.474), the weight (per Kg, 1.102; 95%CI = 1.069 – 1.136) and the island of fish catching. A prediction score was obtained for the probability of contamination by CTX in amberjack fish samples. The area under de curve (AUC) obtained using the validation data was 0.747 (95% CI = 0.662 – 0.833). Regarding grouper species, the island of fishing was the only factor that showed significant differences associated with the presence of CTX-like toxicity. We provide herein data for a better management and prediction of ciguatera in the Canary Islands, suggesting

a review of the minimum limits of fish weight established by the Canary Government for the control program.

Keywords: ciguatoxin (CTX), predictive score, Canary Islands, amberjack, dusky grouper, wahoo.

1. INTRODUCTION

Ciguatera Fish Poisoning (CFP) is one of the most relevant seafood-borne illnesses worldwide and the most commonly reported human food poisoning related to natural marine toxins (Friedman et al., 2008; Suzuki et al., 2017). It consists of a debilitating human neuro-intoxication caused by consumption of varieties of fish species from tropical and subtropical waters, contaminated with bioaccumulated ciguatoxins (CTXs) (Meyer et al., 2016). CFP is characterized by causing gastrointestinal, neurological, and cardiovascular symptoms (Friedman et al., 2017). A range of 10 to 50 thousand people suffering from CFP annually worldwide has been estimated (EFSA, 2010). However, epidemiological data remain unreliable, given that it has been estimated that only ~ 10-20% of cases are properly diagnosed and reported (Azziz-Baumgartner et al., 2012; Laurent et al., 2005).

CFP is found endemically in tropical and subtropical waters such as the Caribbean Sea, the Indian and the Pacific Oceans (Lewis, 2006). In the 40 years that followed the discovery of CTX (Yasumoto et al., 1977), more than 400 fish species have been implicated in poisoning incidents (Tester et al., 2010), most of which are high-order carnivores (Lehane and Lewis, 2000; Lewis, 2006). In Europe, CFP and CTXs have been gaining interest in recent years due to several reported cases in European countries (e.g., France, Spain, the Netherlands, Germany, and Italy), mostly related to consumption of imported ciguateric fish or people who visited endemic areas of CFP (Caillaud et al., 2010). However, none of the current methods of analysis to determine CTX-group toxins in fish have been formally validated (EFSA, 2010).

Regarding the East Atlantic Ocean, in the Canary Archipelago CFP had not been described until 2004, when 5 people became poisoned (Pérez-Arellano et al., 2005). In 2008 two more outbreaks were reported in the Canary Islands (Boada et al., 2010) and 11 people were also affected by CFP in Madeira Archipelago (Otero et al., 2010). Therefore, for some species collected from authorized first sale points, considered a risk factor in the Canary Islands, an action protocol with the objective of making the detection of CTX prior to sale and human consumption, has been implemented since 2011 by the DG Fisheries of the Canary Government (DG of Fisheries of the Canary Government, 2018). In the last decade, several outbreaks of CFP affecting 113 people (Canary Government, 2017b) have been confirmed in the Canary Archipelago, following the consumption of subsistence and recreational harvested fish and not related to controlled fish. Additionally, since 2015, CFP has been designated a notifiable disease in the Canary Islands.

Regarding the marine biotoxins, precursors of CTXs (Gamberitoxins) are produced by benthic dinoflagellates of the genus *Gambierdiscus* (Rodríguez et al., 2017). These precursors are transferred and metabolized through the food web, as *Gambierdiscus* cells are ingested by herbivorous fish, which are then taken by piscivorous fish, both of which are finally consumed by humans. It is believed that CTXs are bioaccumulated through the trophic webs, thus, fish higher in the food web tend to contain the highest CTX concentrations (Banner et al., 1966; Dickey and Plakas, 2010). In addition, CTXs are tasteless, colourless and odourless, which increases the risk of poisoning (Friedman et l., 2008). To date, more than 29 CTX congeners have been identified and grouped according to geographic distribution: Indian CTXs (I-CTX), Caribbean CTXs (C-CTX) and most investigated, Pacific CTXs (P-CTX) according to the presence in the waters where CFP is endemic (Hamilton et al., 2002; EFSA, 2010).

Different species of *Gambierdiscus* have been isolated from water samples collected in the Canary Islands during a spatial study (Aligizaki et al., 2008; Rodríguez et al., 2017). These authors highlighted that socioeconomic impact of ciguatera on fisheries activity and public health in the Canary Islands requires further efforts to implement a faster analytical response to detect CTXs in fish samples, and multidisciplinary research to know life cycle, distribution and toxicity of *Gambierdiscus* spp.

The major goal of the present research was to study several factors which may be associated with the probability to find CTX-like toxicity in fish obtained from first sale points in the Canary Islands in order to describe the statistical significance of these associations or achieve a predictive score, if possible.

2. MATERIALS AND METHODS

2.1. Study area

The Canary Archipelago is located in the Northeastern Atlantic Ocean near Europe (2000 Km SW from the Iberian Peninsula) and North Africa (100 Km W from the Moroccan coast), FAO Major Fishing Area 34 in the subdivision 1.2 (FAO 2004).

Two co-occurring amberjack species present in this area were analyzed (*Seriola dumerili* and *S. rivoliana*). These predators can reach large sizes living up to 15 years (Murie and Parkyn, 2008); when the fish are mature, they migrate to coastal areas to spawn. Amberjack species are important fish stocks in the Canary Islands being the fourth and sixth most caught species in La Gomera (*Seriola dumerili*) and El Hierro (*Seriola rivoliana*), respectively (Canary Government, 2017a). Dusky grouper (*Epinephelus marginatus*) is a benthonic species that lives on the coast within 5 to 45 meters depth; it is considered a solitary fish, very territorial and sedentary (Göthel, 1992) which predates other fish, crustaceans and cephalopods (Smale, 1986); although it is a fish of relevance to human consumption in the Canary Archipelago, dusky grouper is not among the 10 most caught species in the islands (mainly represented by fish shoals) (Canary Government, 2017a), possibly due to its solitary behaviour. Wahoo is a pelagic animal and a seasonal migratory species, relatively new in the Canary Islands waters where it has settled. Normally it feeds on pelagic prey (Espino et al., 2006).

2.2. Fish sample collection

The official control protocol for CFP implemented by the Canary Government (DG of Fisheries of the Canary Government, 2018) establishes a list of certain species and weights of fish considered a risk factor. This list, based on the local experience, shows the limit weights for fish to be sampled at the authorized first sale points and investigated in the Institute of Animal Health and Food Safety (IUSA) laboratory for CTX detection (Table 1). This list has been updated in 2018.

The fish samples used in the present study were obtained between April 2016 and December 2017 from the official monitoring of CFP (DG Fisheries of the Canary Islands). Through the mentioned programme, fish samples from professional fishermen associations were sent to the IUSA laboratory of the University of Las Palmas de Gran Canaria (ULPGC) in order to determine the CTX presence in fish flesh. The laboratory received 1538 samples in the study period. However, this research only included 970 fish samples selected from the total, based on the availability of relevant information, such as fish species, weight of the specimen and the island of fishing. Fish species and weights were consistent to those established by the official control protocol, particularly amberjack (*Seriola* spp.) (n=793), dusky grouper (*Epinephelus marginatus*) (n=145) and wahoo (*Acantocybium solandri*) (n=32). Thus, the samples were classified according to the species and different data such as the year of fish catching (2016/2017), period (cold/warm), weight and fishing island.

2.3. Sample preparation and extraction of CTX

Toxin extraction was performed following the protocol proposed by Lewis in 2003 and carried out by Hossen et al. in 2015 with slight modifications according to the laboratory needs. Fish samples were first homogenized and then a portion of 10 g of flesh was cooked at 70 ^{o}C during 10 minutes. When samples reached room temperature, 30 ml of acetone was added, mixed with ultraturrax and centrifuged at 3000 xg during 5 minutes at 4 ^{o}C; this last step was repeated twice and both supernatants were pooled. The resulting acetone was filtered through a 0.45 µm of PTFE filter and evaporated with a rotary evaporator at 55 ^{o}C. The dried extract was re-suspended in methanol:water (9:1) and N-Hexane for phase separation. The upper phase of N-Hexane was discarded and the methanol phase was dried under N_2 current at 40 ^{o}C for a subsequent partition with ethanol:water (1:3) and Diethyl-Ether (DE). The DE was reserved and evaporated under N_2 current to dryness. The final residue was resuspended in 4 ml of methanol and kept at -20 ^{o}C to be used in the cellular bioassay analysis (CBA).

2.4. Neuroblastoma (neuro-2a) cell-based assay (CBA)

Neuro-2a cells (Cell line: CCL131, from ATCC, LGC Standards S.L.U., Barcelona, Spain) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 5-10% of foetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. The P-CTX-1 standard (STD) (R.J. Lewis, The Queensland University, Australia) was used as a reference toxin for the assessment of CTX-like toxicity, as C-CTX reference material is not commercially available (Soliño et al., 2015).

For the cytotoxicity assay, cells were seeded in a 96-well flat bottom microplate (200 µl/well) following the procedure of Caillaud et al in 2012 using RPMI medium supplemented with 5% of FBS at a density of 70,000 cells per well. Cells were incubated 24 h in the same mentioned conditions. Ouabain (0.1 mM) and veratridine (0.01 mM) were added to half of the seeded wells to favour cell mortality in case of the presence of CTX. In parallel to fish sample extracts testing by Neuro-2a assay, a dose-response curve with P-CTX-1 STD was always performed as an internal control, for cell response evaluation and limit of detection and quantification (LOD/LOQ) establishment. Thus, fish extracts and the STD were evaporated and resuspended in RPMI medium supplied with 5% FBS. Then, cells were exposed to flesh extract and the P-CTX-1 STD at decreasing concentrations in order to ensure the cells sensibility to CTX. Every sample extract and the STD were assayed in triplicate. After a 24 h-period of exposure to fish extracts and to P-CTX-1 standard solution, cell viability was evaluated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] and DMSO solutions. The corresponding absorbances were read at 570 nm by a multi-well spectrophotometer scanner and plotted into the Microsoft Office Excel 2016 and GraphPad Prism 7 softwares (GraphPad, San Diego, California, USA). For the interpretation of the dose-response curves, cell viabilities were related to the cell viability of the control column (cells with and without pre-treatment with ouabain/veratridine, O/V).

The assessment of fish matrix effect on the Neuro-2a assay was performed using different concentrations of several fish extracts to expose the cells with or without O/V (50 - 200 mg Tissue Equivalents (TE)/ml). Several muscle samples from grouper and amberjack showed interference with the assay with concentrations higher than 100 mg TE/ml; on the contrary extracts from muscle tissue of Wahoo species displayed interference above 80 mg TE/ml. For that reason and to unify methodology, 80 mg TE/ml was set as the maximum concentration for testing with the Neuro-2a assay.

Due to the large amount of samples used in this surveillance study, the 50% inhibition concentration (IC50, with O/V) was only assessed with the P-CTX-1 STD in ppb units (pg P-CTX-1/ml) in order to evaluate the cell response and limits. Samples extracts were tested using 1 to 4 columns (serial dilutions) of the 96-well microplate, depending on the number of samples received every week; thus, semi-quantitative estimation of the content in P-CTX-1 equivalents in fish extracts was not always possible to determine. Therefore, in the present study, a response producing less than 20% cell mortality was considered as a non-toxic effect, as other authors suggested (Caillaud et al., 2012), being the concentration of P-CTX-1 STD causing 20% inhibition of cell viability (IC20) set as the LOD and LOQ according to this concentration of fish extract used for testing. Thus, a “positive sample” was considered when the corresponding extract showed an inhibition of cell viability over this LOD value. According to the mean value of IC20 (1.359 pg P-CTX-1/ml) obtained from all dose-response curves performed with the STD in the study period and the maximum concentration of extracts set to avoid matrix effect (80 mg TE/ml), the LOD/LOQ was 0.017 ppb.

2.5. Statistical analysis

The statistical analysis was performed using the R package, version 3.3.1 (R Development Core Team, 2016) and IBM SPSS Statistics 23.0 software.

Univariate analysis: Categorical variables are expressed as frequencies and percentages and continuous as medians and interquartile ranges (IQR = 25th – 75th percentile). The percentages were compared, as appropriate, using the Chi-square ($\chi2$) test or the exact Fisher test, the means by the t-test and the medians by the Wilcoxon test for independent data. As usual, the statistical significance was set at *p*-value < 0.05 and the rates of contamination by the CTX were estimated by means of confidence intervals (CI) at 95% using a bootstrap method.

Multivariate analysis: In order to identify those factors that maintain independent association with the outcome, a multivariate logistic regression analysis were performed. All variables of the study were entered into the analysis and a selection based on complete enumeration algorithm (Morgan and Tatar, 1972) and Bayes information criterion (BIC) was carried out. For each one of these regressions, we evaluate the lack of fit according the BIC criteria (Schwarz, 1978). The models were summarized as coefficients (SE), *p*-values (likelihood ratio test) BIC values (for the residual models) and odds-ratio, which were estimated by 95% CI.

Receiver operating characteristics: The discriminant power of the score deduced from the logistic model was evaluated from a receiver-operating characteristic analysis (ROC). The area under the ROC curve was estimated by means of the 95% CI. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

To identify the factors associated with the contamination by CTX in the population of fish under study, data were analyzed according to the following variables:

3.1. Influence of fish species in the presence of CTX-like toxicity

Out of the 970 fish samples included in this research, 793 (81.8%) belonged to both amberjack species, 145 (14.9%) to the grouper and 32 (3.3%) to the wahoo. Overall, 228 (18.2%) of all samples exhibited measurable CTX-like toxicity.Comparing species categories, Pearson's Chi-squared ($\chi 2$) test revealed a significant difference in CTX prevalence between species of the samples tested ($p = 0.002$). Although most of the samples correspond to amberjack species, the grouper displayed the highest percentage of positive samples (26.9%), see Table 2. This observation may indicate that the chance for catching a positive grouper is two-fold higher than fishing a positive amberjack in the Canary Islands waters possibly explained by the sedentary behaviour of grouper species (Göthel 1992; Espino et al., 2006) which may continually feed in areas where *Gambierdiscus* are more abundant, allowing a continuous accumulation of the toxin. However, it must be highlighted that the fish analyzed in this study correspond to certain weights considered as risk factors for human health (Table 1) and therefore, this limitation must be taken into account before raising any conclusion. In addition, the high percentage of toxic grouper samples obtained may support the review of the lower limit of weights previously suggested for this species (see Table 1 and Figure 1). Additionally, 32 samples of wahoo species were analyzed corresponding to the 3.3% of all flesh fish studied and note that only 1 of them showed a CTX-like toxicity. Thus, this species was not considered in this analysis.

3.2. Influence of the fish weight

Bioaccumulation of CTXs in amberjack has been reported to be highly dependent on the weight of the specimen (Bravo et al., 2015). Thus, an analysis of the presence of CTX in fish against this variable is essential to fully understand the accumulation of this biotoxins through the lifetime of these animals. Accordingly, in comparison with the last mentioned reference, the present study has tripled the number of samples under investigation.

Due to the biological difference between species (Reid et al., 2016; Šegvić-Bubić et al., 2016), each species weight data were analyzed separately using Mann-Whitney (M-W)/Wilcoxon non-parametric tests (Table 2), demonstrating that the median value of weight for fish which showed CTX-like toxicity was significantly higher than the median weight of negative fish, both in amberjack (27 Kg vs. 20 Kg; $p < 0.001$) and grouper species (22.5 Kg vs. 21.1 Kg; $p = 0.013$, respectively). In both species, CTX toxicity was more frequently observed in larger specimens. The descriptive statistics of the weight of the CTX positive and negative samples according to fish species (interquartile range-IQR, median and minimum-maximum range) are summarized in Table 2. The distribution of weight data between species and CTX results is represented in the box plot diagrams below (Figure 1). Regarding wahoo samples, only one positive result was obtained, what makes the statistical analysis impossible to be performed.

It is remarkable to note the presence of positive fish with a weight close to the minimum limit established by the government of the Canary Islands (see Table 2 and Figure 1). The smallest positives amberjack and grouper species weighed 14.5 Kg and 17.4 Kg respectively, see Figure 1. This observation may justify an extension of the minimum weight proposed for analysis in these two species, mainly in grouper fish, as mentioned before. Additionally, a CFP outbreak occurring in 2016 also supports this suggestion, when two people in Tenerife Island were poisoned by the consumption of 7 Kg grouper, and the presence of CTX-like toxicity confirmed in our laboratory (Canary Government, 2017b).

3.3. Influence of the fishing island

The information of the island where the fish were caught is important to analyze due to the nature of the studied species and the possible risk of poisoning related to the location. From the selected samples, significant difference in CTX result was found between islands of fishing ($p < 0.001$). Lanzarote showed the highest contamination rate (52.9%) which was more than two fold greater than the value obtained in samples from Fuerteventura (21.0%) and Gran Canaria (17.8%) and more than three times higher than the CTX positivity showed from El Hierro (15%) and Tenerife (13.5%). The number of positive samples obtained from these Canary Islands far exceeded those resulted in the islands of La Palma (5.1%) and La Gomera (2.4%) where the lowest number of positive fish was observed (Figure 2). However, it must be considered that, under the official control program of ciguatera during the studied period, more than 300 samples received from Lanzarote were not accompanied by the necessary information, and thus, were not included in this research, which could limit accurate result from this island. These results must be considered with caution.

An in depth analysis by species, both amberjack and grouper also showed a statistical difference in CTX results between islands of capture ($p < 0.001$). For amberjack species, the number of positive samples seems to decrease from the Eastern islands to the Western islands (see Table 3 and Figure 2), thus, these rates suggested grouping the island category in four cluster; namely: {HI; LG; LP}, {GC; TF}, {FU} and {LZ}. Table 3 displays the contamination rates corresponding to each cluster.

On the contrary, the profile mentioned above was not found regarding grouper species, which showed positive results more likely linked to certain islands in particular, possibly due to its sedentary behaviour (Göthel, 1992; Espino et al., 2006). Thus, the highest number of ciguatoxic grouper was obtained in El Hierro, with a remarkable percentage of toxic samples (10 samples, 90.9%) and Lanzarote (19 samples, 41.3%). Although Fuerteventura provided the highest number of samples, only 3 were found positive to CTX (5.6%). Additionally, the low number of results from Gran Canaria and La Palma precluded any conclusion.

Despite of these findings, other confounding variables should be taken into consideration before any conclusion can be drawn (see section 3.4.).

In addition, it is important to emphasise that results from each island presented one or more positive individuals weighted close to the minimum control limit except for those fish caught in La Gomera.

Furthermore, the only positive wahoo obtained in this study was fished in El Hierro, representing the 4.5% of all samples analyzed from this island.

3.4. Influence of the period of fishing

Fish samples studied in the present research were received in the period from April 2016 to December 2017 and analyzed for the presence of CTX-like toxicity.

Considering the results obtained by Rodríguez et al. (2017), peaks of *Gambierdiscus* spp. cells densities were observed in the Canary Islands associated to temperatures higher than 20 ^{o}C. For this reason, time frame was divided in “cold period” (January to April) and “warm period” (May to December) in accordance with the surface seawater temperature registered in both years (NOAA, 2017), with a difference of 3^{o}C between both periods. Samples available for this research only allowed comparison of data in the warm period between both years of study. Thus, considering species separately, a significant decrease ($p < 0.001$) was observed in the percentage of positive samples of amberjack species caught within the warm period between 2016 and 2017 (31.4% and 12.1%, respectively). This finding could be explained due to the modification in the official protocol of the lower weight limit in amberjack species from 15 Kg in 2016 to

14 Kg in 2017 (Table 1) and the increasing demand for analysis with the consequent rise of samples received in the laboratory in 2017 over 2016 (573 and 220 samples, respectively, see Table 3). In contrast, grouper species maintained a similar rate of toxicity in the warm periods of both evaluated years.

Considering both years in conjunction, the amberjack species showed a rate of CTX toxicity of 12.5% (95% CI = 5.6 – 20.8) and 17.8% (95% CI = 15.0 – 20.5) in the cold and warm period, respectively, what seems to be an increase in the number of positive samples from the cold to the warm season, but no statistically significant difference (p = 0.261) was found (Table 3). In this regard, it is important to stress that in the warm period the laboratory received a considerably greater amount of samples (see table 3) what could therefore partially explain the difference of CTX toxicity rates found between seasons. Regarding grouper fish, the number of CTX-positive samples were quite similar, being 26.3% (95% CI = 10.5 - 47.4) in the cold period and 27.0% (95% CI = 19.8 - 34.9) in the warm period.

3.5. Risk gradient assessment: Predictive score of the presence of CTX-toxicity in a population of amberjack fish.

For the statistical analysis of results of samples from the amberjack species, using the training data, the multivariate logistic regression showed that the factors that maintained independent association with the outcome (contamination by CTX) were the warm season (OR = 3.617; 95%CI = 1.249 - 10.474), the weight (per Kg, 1.102; 95%CI = 1.069 - 1.136) and the island of fishing (grouping according gradient, see Table

3). It should be noted that the season did not show statistical significance in the univariate analysis (see section 3.4) but did so in the multivariate testing. This is attributable to the confounding effect of the weight, since in the warm season the weight of the fish was significantly lower than in the cold period ($p < 0.001$), as it is shown in Figure 3.

For the amberjack species, a predictive score of contamination by CTX was obtained. For this purpose, the data were randomly divided into a training data set (n = 510) and a validation data set (n = 283). The predictive score was obtained by means of the multivariate logistic analysis using the training dataset. Its discriminant power was evaluated by means of the ROC analysis using the validation dataset and was summarized as the estimated area under the ROC curve (AUC-ROC, Figure 4).

The next prediction score was then obtained from this logistic analysis:

$\mathit{Score} = 1.286 \times \text{Warm} + 0.097 \times \text{Weight} + 1.962 \times D_1 + 2.555 \times D_2 + 4.191 \times D_3$

Here, the season is a binary variable (1 = Warm; 0 = Cold) and D_1, D_2 and D_3 are the dummies variables associated with the island of fish catching (clusters) according to the design shown in table 4. Four clusters were considered: El Hierro-La Gomera-La Palma; Gran Canaria-Tenerife; Fuerteventura and Lanzarote.

The AUC obtained using the validation data was 0.747 (95% CI = 0.662 – 0.833) (Figure 4). Table 5 displayed the increasing probabilities of the contamination by the CTX according to a gradient from the cluster of western islands, in the cold season and fish with low weight to the eastern islands, in warm season and fish with high weight. Therefore, the probability of finding a positive result in a sample from amberjack is less than 1% if the sample comes from a small specimen (less than 22 Kg), from the western cluster islands {HI; LG; LP} and fished in the cold season. Additionally, as can be seen in Table 5, the change to the warm season leads to a strong increase in the probability of contamination, being over 50% when fish weighing more than 35 Kg are caught in Fuerteventura. And, in contrast, this probability reaches higher values (more than 90%) in fish caught in Lanzarote, weighting more than 41 Kg.

The decreasing gradient of presence of CTX in amberjack species observed from the eastern to the western islands is strongly consistent with the results obtained by Rodríguez et al. (2017). These authors found that the higher density of *Gambierdiscus* spp. was found in Lanzarote and Fuerteventura islands, and they also reported that *G. exentricus*, one of the most toxic *Gambierdiscus* found in the Canary Islands, was more abundant in the eastern islands compared to the western ones. This finding could be explained by the conjunction of Northwestern African upwelling and the Cold Canary Current (Sangil et al., 2012) at the bottom of these islands, along with the shallow waters of these eastern islands which provides abundance of nutrients for the growth of plankton and seaweeds (Rodríguez et al., 2017).

Regarding results obtained from grouper species, the only factor that showed significant differences associated with the presence of CTX was the island of fishing ($p < 0.001$), mentioned before in section 3.3. These results are in accordance with Dierking and Campora who analyzed samples of *Cephalopholis argus* from the

Hawaiian Islands in 2009 and found no geographic patterns in toxicity between or within islands.

Ciguateric groupers showed a different profile of distribution, as exposed above, which may be explained for a sedentary and very territorial behaviour of these fish (Reid et al., 2016). Furthermore, it must be considered the presence of an algae bloom of *G. caribaeus* occurred in October 2016 in El Hierro (Soler-Onís et al., 2016) and the rate of CTX bioaccumulation in fish tissue (Lehane and Lewis, 2000; Banner et al., 1966), what could explain that this certain island represents the highest percentage of CTX positive groupers in the Canary Archipelago (90%) in this period of study (Table 3).

Even so, the aim of the present study was not to calculate the real prevalence of CTX in fish from the Canary Islands, but to propose a predictive value of finding a positive sample according to different associated factors, such as fish species, weight, season or fishing island. Although results showed different probabilities of contamination by CTX-like toxicity between islands, none of them is free of ciguateric fish. Therefore, official monitoring should continue throughout the archipelago to ensure the food safety.

4. Conclusions

This study confirms the Canary Islands as an area of expansion of CFP endemicity and contains the first reported predictive score for the presence of CTX-like toxicity in amberjack fish samples from this area.

This work identifies the several factors associated with the probability of contamination by CTX-like toxicity of fish caught in the Canary Archipelago.

A risk gradient was obtained for amberjack, considering weight of fish, season and island of fishing, this latter being the only factor significantly associated with grouper species. The risk of contamination by CTX could not be adequately assessed for wahoo due to small sample size of this species.

Presence of CTX in amberjack from some areas seems to be highly related to the season of the year which may be related to the abundance of the most toxic *Gambierdiscus* found in the Canary Islands.

The minimum weight limits established by the official control of ciguatera in the Canary Islands for amberjack and dusky grouper need to be reviewed to safeguard consumer health.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The research was funded by Directorate-General for Fisheries of the Canarian Government, through the official control program of ciguatera in the Canary Islands, and supervised by the Public Health Service. We also thank the EuroCigua project ("Risk characterization of ciguatera food poisoning in Europe" FRAMEWORK PARTNERSHIP AGREEMENT GP/EFSA/AFSCO/2015/03) for their technical and valuable assistance, and also the REPROCAN project for their advice regarding the sea surface temperature of the canary waters. The authors also acknowledge support from CERCA Programme/Generalitat de Catalunya.

FIGURE CAPTIONS

Figure 1. CTX results by the weight variable in amberjack (box plot graph on the left) and grouper (box plot graph on the right). Line indicates the minimum limit of weight established for CTX analysis by the DG Fisheries (14 Kg and 17 Kg for amberjack and grouper species respectively). The plot represents the interquartile range (Q_3 - Q_1). Sample size is shown below the corresponding group category.

Figure 2: CTX results in all samples included in this study (left), in amberjack (middle) and grouper (right) species by the location of capture. Percentages of positive samples from the different Canary Islands are indicated in each bar graph. The Canary Islands: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro.

Figure 3. CTX results by the weight variable in cold and warm periods for amberjack (box plot graph on the left) and grouper (box plot graph on the right). Line indicates

the minimum limit of weight established for CTX analysis by the DG Fisheries (14 Kg and 17 Kg for amberjack and grouper species respectively).

Figure 4. Receiver operating characteristics for the score obtained from the logistic regression. The score obtained with the *data training* was validated using the *data validation*.

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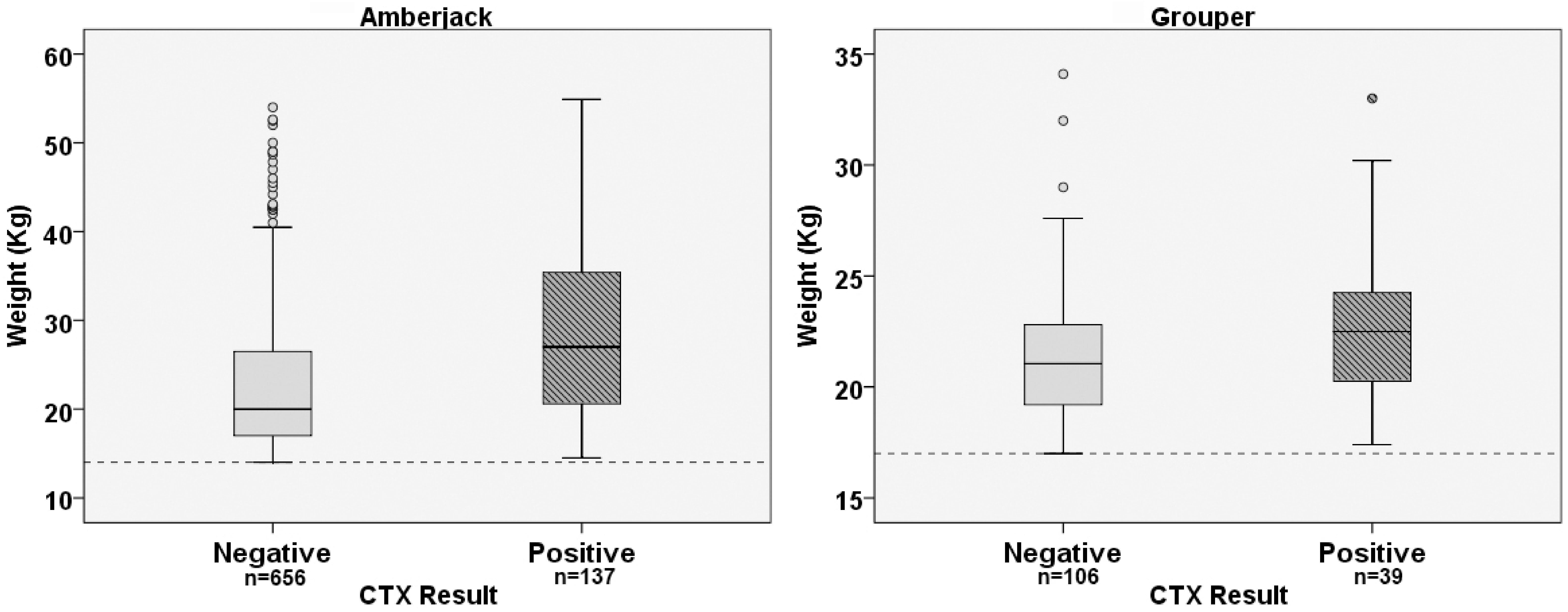
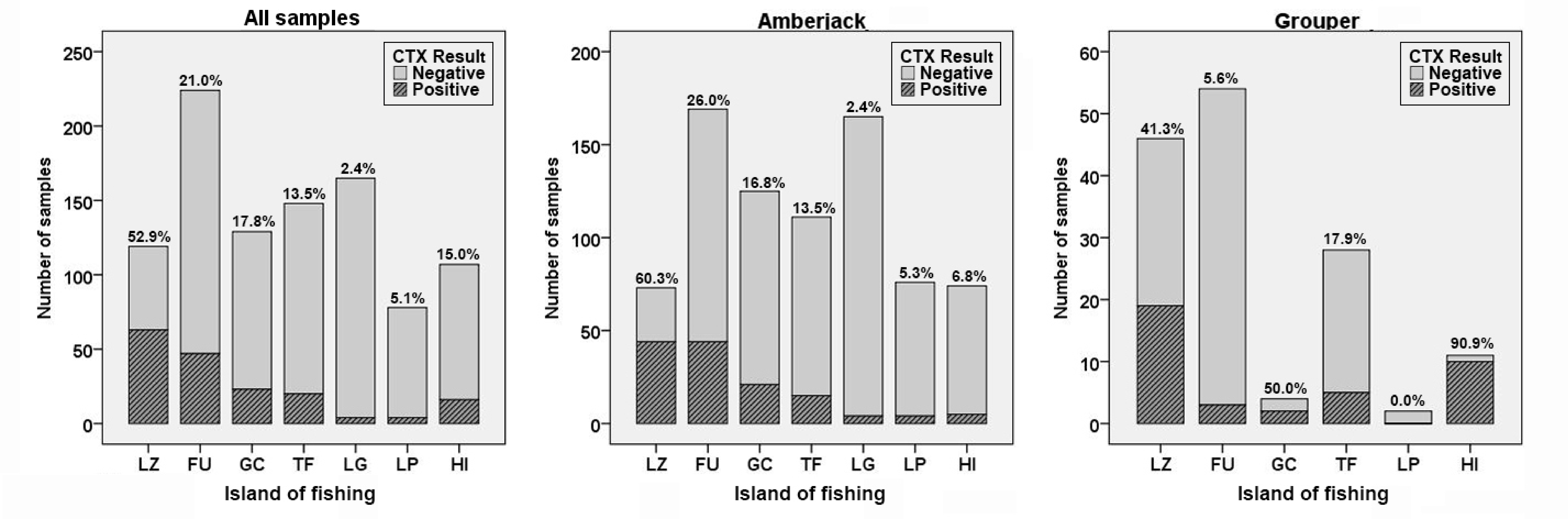
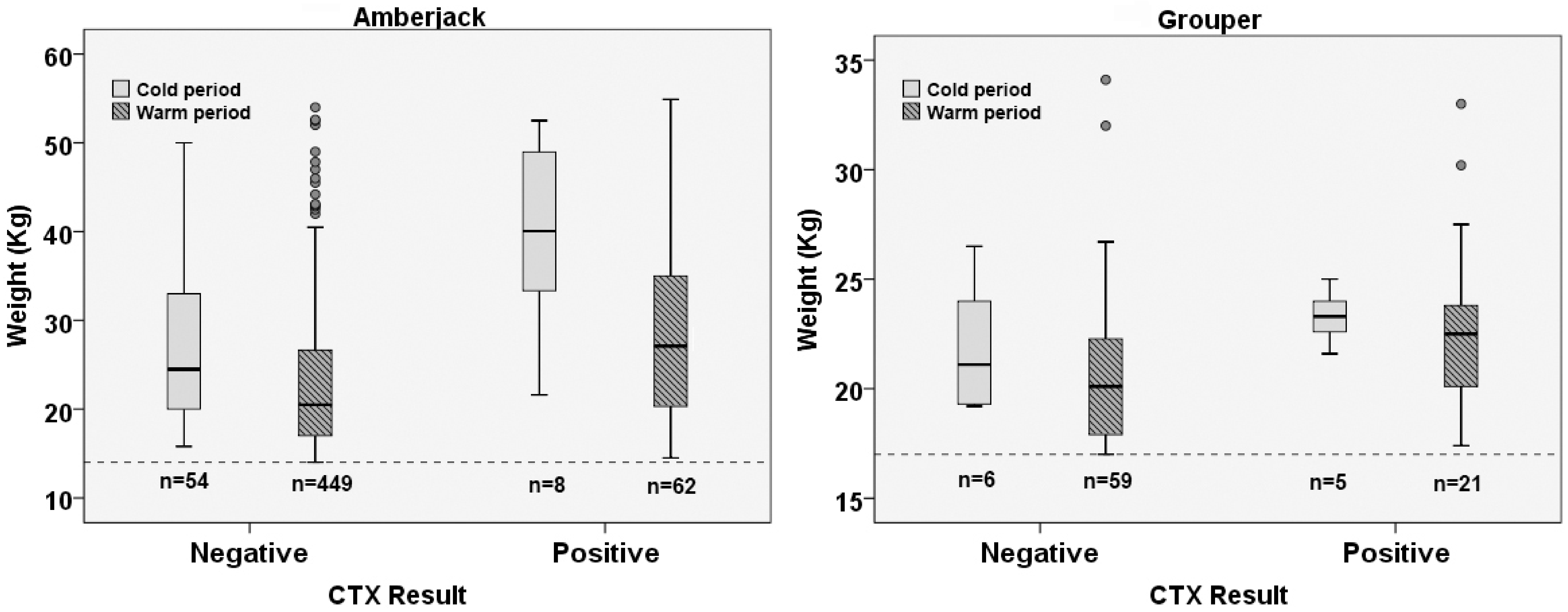
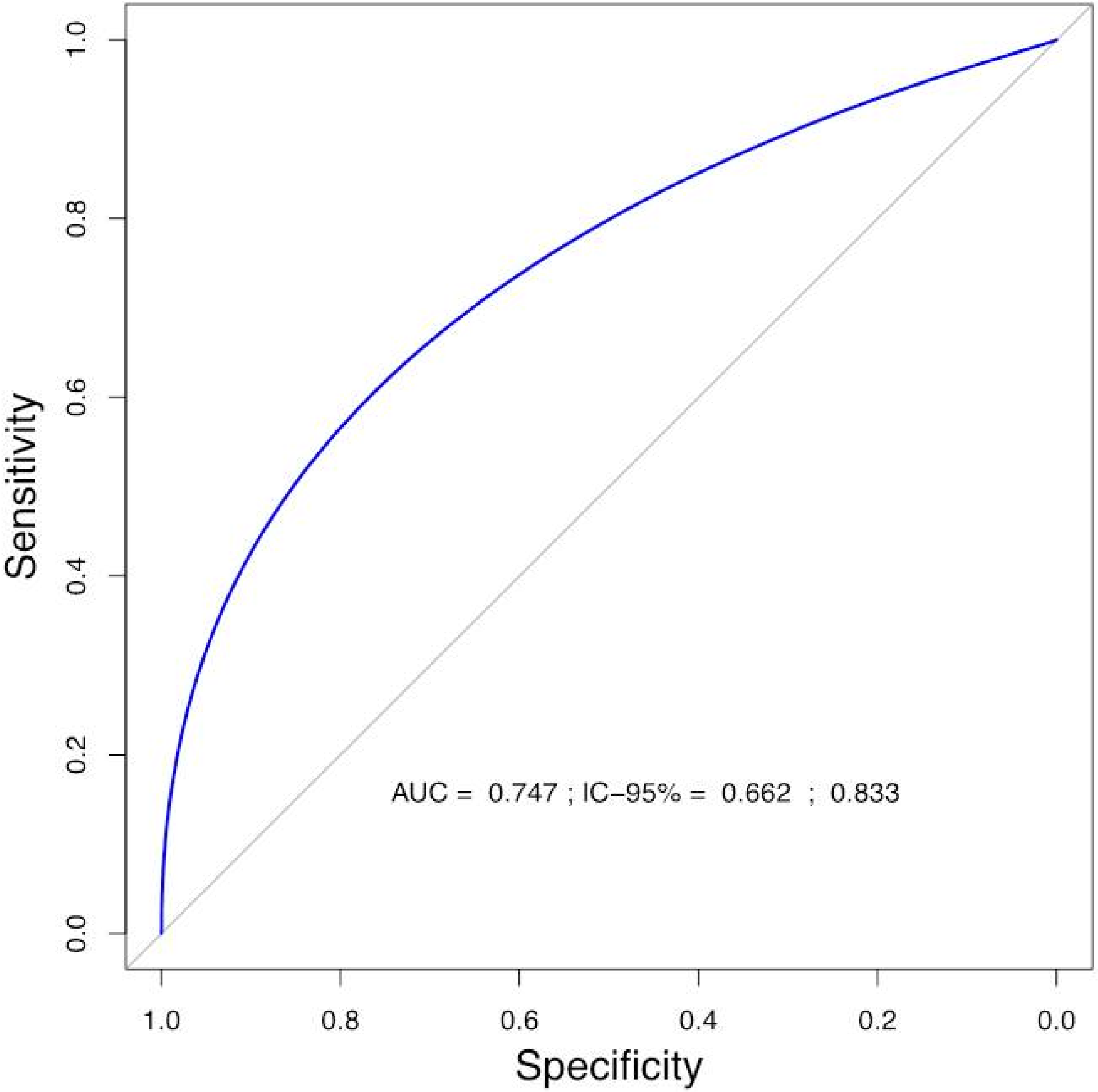

Amberjack
Weight (Kg)
60
50
40
30
20
10
Negative
n=656
Positive
n=137
CTX Result
Grouper
Weight (Kg)
35
30
25
20
15
Negative
n=106
Positive
n=39
CTX Result



All samples
Amberjack
Grouper
CTX Result
Negative
Positive
Number of samples
Island of fishing
LZ
FU
GC
TF
LG
LP
HI
52.9%
21.0%
17.8%
13.5%
2.4%
5.1%
15.0%
60.3%
26.0%
16.8%
13.5%
2.4%
5.3%
6.8%
41.3%
5.6%
50.0%
17.9%
0.0%
90.9%



Amberjack
Cold period
Warm period
Weight (Kg)
60
50
40
30
20
10
n=54
n=449
n=8
n=62
Negative
Positive
CTX Result
Grouper
Cold period
Warm period
Weight (Kg)
35
30
25
20
15
n=6
n=59
n=5
n=21
Negative
Positive
CTX Result



AUC = 0.747 ; IC−95% = 0.662 ; 0.833
Sensitivity
Specificity
1.0
0.8
0.6
0.4
0.2
0.0

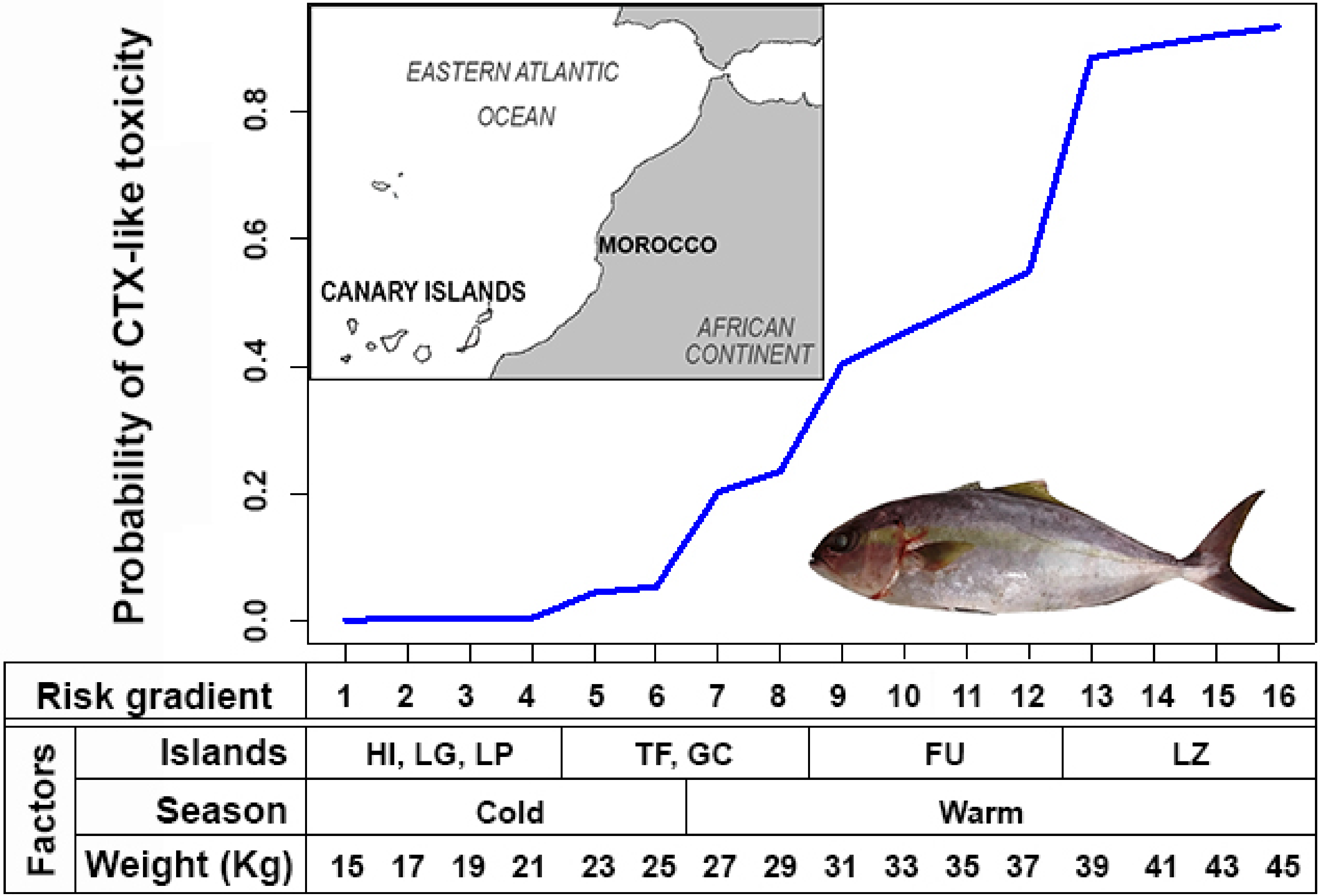

Probability of CTX-like toxicity
0.0
0.2
0.4
0.6
0.8
EASTERN ATLANTIC
OCEAN
MOROCCO
CANARY ISLANDS
AFRICAN
CONTINENT
Risk gradient
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Factors
Islands
HI, LG, LP
TF, GC
FU
LZ
Season
Cold
Warm
Weight (Kg)
15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45

Table 1. Fish species included in the present work, and weight limits established for CTX analysis by the Canary Government through the official control protocol.

Species	***Latin name***	**Weight* (Kg)**
Amberjack	*Seriola spp.*	15/14**
Wahoo	*Acanthocybium solandri*	35
Dusky grouper	*Epinephelus marginatus*	17

* A particular fish is sampled if weight is equal to or greater than this value

** The minimum weight regarding amberjack was decreased from 15 to 14 Kg in 2017 by DG Fisheries of the Canary Government, to better adjust the risk of CTX detection for this species

Table 2. Percentages of CTX positive and negative samples according to fish species and the corresponding fish weights, expressed as the mean and median values (Kg).

Species		Presence of CTX-like toxicity		Total
		Negative	Positive	
Amberjack (14 kg)*	Number of samples	656	137	793
	% of samples	82.7%	17.3%	
	Mean weight ± SD	22.77 ± 7.8	28.9 ± 10.3	
	Median weight (min-max)	20.0 (14-54)	27.0 (14.5-54.9)	
Grouper (17 kg)*	Number of samples	106	39	141
	% (Species samples)	73.1%	26.9%	
	Mean weight ± SD	21.4 ± 3.2	22.7 ± 3.3	
	Median weight (min-max)	21.1 (17.0-34.1)	22.5 (17.4-33.0)	
Wahoo (35 kg)*	Number of samples	31	1	32
	% (Species samples)	96.9%	3.1%	
	Mean weight ± SD	39.5 ± 6.0	40.0	
	Median weight (min-max)	37 (28.0-58.0)	40.0	
TOTAL SAMPLES	Number of samples	793	177	970
	% (Total samples)	81.8%	18.2%	

SD, standard deviation. Min, minimum weight. Max, maximum weight.
*Minimum weight limits established for CTX analysis by the Canary Government through the official control protocol.

Table 3. Rates of contamination by CTX according to the considered factors

Factor	Species					
	Amberjack (n = 793)			Dusky grouper (n = 145)		
	N	Crude rate * (95% CI)	P	N	Crude rate * (95% CI)	P
Year			< 0.001			0.555
2016	220	30.5 (24.5 - 36.8)		54	24.1 (13.0 - 37.0)	
2017	573	12.2 (9.6 - 15.0)		91	28.6 (19.8 - 37.4)	
Period			0.261			0.951
Cold	72	12.5 (5.6 - 20.8)		19	26.3 (10.5 - 47.4)	
Warm	721	17.8 (15.0 - 20.5)		126	27.0 (19.8 - 34.9)	
Fishing Island			< 0.001			< 0.001
LZ	73	60.3 (49.3 - 71.2)		46	41.3 (27.1 - 55.5)	
FU	169	26.0 (19.5 - 32.5)		54	5.6 (-0.6 - 11.7)	
GC	125	16.8 (10.4 - 23.2)		4	50 (1 - 99)	
TF	111	13.5 (7.2 - 19.8)		28	17.9 (3.7 - 32.0)	
LG	165	2.4 (0.6 - 4.8)		0	-	
LP	76	5.3 (1.3 - 10.5)		2	-	
HI	74	6.8 (2.7 - 13.5)		11	90.9 (73.9 - 107.9)	
Gradient			< 0.001			< 0.001
HI; LG; LP	315	4.1 (2.2 - 6.3)		13	76.9 (53.8 - 100)	
GC; TF	236	15.3 (11.0 - 19.9)		32	21.9 (9.4 - 37.5)	
FU	169	26.0 (19.5 - 33.1)		54	5.6 (0 - 13)	
LZ	73	60.3 (47.9 - 71.2)		46	41.3 (28.3 - 54.3)	
Island Orientation			< 0.001			0.099
Eastern	367	29.7 (25.1 - 34.6)		104	23.1 (15.4 - 31.7)	
Western	426	6.6 (4.2 - 9.2)		41	36.6 (19.5 - 51.2)	

Fishing Island: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro. (*) Toxin

Table 4. Design of the dummies variables associated with the island of fishing (clusters).

Cluster	D_1	D_2	D_3
El Hierro, La Gomera, La Palma	0	0	0
Gran Canaria, Tenerife	1	0	0
Fuerteventura	0	1	0
Lanzarote	0	0	1

1 = warm season; 0 = cold season.

Table 5. Probabilities of contamination by CTX in amberjack species according to a gradient of risk (from the western islands in the cold season with low weight of fish to eastern islands in warm season and specimens with high weight). Study period (2016-2017)

Gradient	Islands	Period	Weight (Kg)	Probability-CTX (95% CI)*
1	HI, LG, LP	Cold	15	0.31 (0.07 ; 1.31)
2	HI, LG, LP	Cold	17	0.37 (0.09 ; 1.55)
3	HI, LG, LP	Cold	19	0.45 (0.11 ; 1.83)
4	HI, LG, LP	Cold	21	0.55 (0.14 ; 2.17)
5	TF, GC	Cold	23	4.55 (1.48 ; 13.12)
6	TF, GC	Cold	25	5.47 (1.82 ; 15.31)
7	TF, GC	Warm	27	20.3 (13.8 ; 28.8)**
8	TF, GC	Warm	29	23.6 (16.1 ; 33.2)
9	FU	Warm	31	40.4 (29.0 ; 52.9)
10	FU	Warm	33	45.2 (32.8 ; 58.2)
11	FU	Warm	35	50.0 (36.6 ; 63.4)
12	FU	Warm	37	54.8 (40.5 ; 68.4)
13	LZ	Warm	39	88.3 (77.7 ; 94.3)
14	LZ	Warm	41	90.2 (80.3 ; 95.4)
15	LZ	Warm	43	91.8 (82.6 ; 96.3)
16	LZ	Warm	45	93.1 (84.7 ; 97.1)

(*) The probabilities of the presence of CTX-like toxicity are expressed as percentages
(**) The change to the warm season leads to a strong increase in the probability of contamination
The Canary Islands: LZ, Lanzarote; FU, Fuerteventura; GC, Gran Canaria; TF, Tenerife; LG, La Gomera; LP, La Palma; HI, El Hierro.