

STUDY ON THE RISK OF EXPOSURE OF SEAFOOD CONSUMERS IN BULGARIA TO HYDROPHILIC MARINE TOXINS

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ABSTRACT

INTRODUCTION: Marine biotoxins can be accumulated in shellfish and in turn can lead to severe illness or chronic consequences in human shellfish consumers.

AIM: The aim of this study was to assess the levels of hydrophilic marine biotoxins in both farmed and wild mussels from the Bulgarian coast sampled in 2017 and to estimate the exposure (acute and chronic) of Bulgarian consumers to detected toxins if investigated mussels were consumed. To the group of hydrophilic marine toxins belong amnesic toxins (domoic acid, isodomoic acid) and paralytic toxins (neosaxitoxin, gonyautoxins and their decarbamoyl and *N*-sulfocarbamoyl analogs).

MATERIALS AND METHODS: The hydrophilic toxin – domoic acid (DA) was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). Paralytic toxins (saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin-1 (GTX1), gonyautoxin-2 (GTX2), gonyautoxin-3 (GTX3), gonyautoxin-4 (GTX4), gonyautoxin-5 (B1), decarbamoyl gonyautoxin-2 (dcGTX2), decarbamoyl gonyautoxin-3 (dcGTX3), decarbamoyl saxitoxin (dcSTX), *N*-sulfocarbamoyl gonyautoxin-1 (C1), *N*-sulfocarbamoyl gonyautoxin-2 (C2)) were investigated via high performance liquid chromatography with fluorescence detection (HPLC-FD).

RESULTS: Among all hydrophilic toxins investigated DA and GTX2 were detected in the studied samples. Mean domoic acid in whole mussel meat was estimated to be 0.139 mg/kg mm which is below the regulatory limit of 20 mg/kg mm. Mean GTX2 level in whole mussel meat was calculated to be 0.151 µg saxitoxin dihydrochloride equivalent (STX.2HCl eq)/kg which is far beneath the legislative limit of 800 µg STX.2HCl eq/kg mm.

Estimation of acute exposure for both detected toxins – DA and GTX2, and of chronic exposure to domoic acid showed similar results among male and female, as well as among wild and cultivated mussel consumers.

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CONCLUSION: This study showed an overall low contamination level of wild and farmed mussels with hydrophilic marine biotoxins compared to the regulatory limits. This leads to the conclusion that there is low acute and chronic exposure via consumption of contaminated mussels.

Keywords: domoic acid, gonyautoxin-2, amnesic shellfish poisoning, paralytic shellfish poisoning, mussels, Black Sea

INTRODUCTION

Seafood safety and quality is a major public health issue and its importance has been elevated to egregious levels in recent years (1).

Recently, contamination of Black Sea mussels has led to a rise in the scientific interest due to constantly increasing farming and harvesting in Bulgaria (2). Determination of chemicals with anthropogenic origin, such as persistent organic pollutants (3), heavy metals (4) in mussels from the Bulgarian coast has shown low levels of contamination assuming no risk for human health. On the other side, due to a flow of untreated household and industry sewage into the sea waters pathogenic microorganisms - *E.coli*, *Vibrio* spp., rotaviruses were registered in Bulgarian coastal waters (5). Krumova-Valcheva et al. (2017) (6) investigated 55 mussel samples from Bulgarian farms and found that almost 13% of all samples exceeded the microbiological criteria for *E.coli*, listed in Commission Regulation (EC) No 2073/2005.

Other contaminants with biogenic origin of interest are marine biotoxins. Species producing phyco toxins – toxic diatoms and dinoflagellates are steadily detected in variable biomass at the Bulgarian coast (7). Marine toxins are transferred through the food chain, and may accumulate in filter-feeding bivalves, such as mussels. If contaminated mussels are consumed by humans, certain health issues can be expected (Table 1) (8).

acid (DA) of 20 mg/kg, for paralytic shellfish toxins (PSTs) of 800 µg STX.2HCl eq/kg and for diarrhetic shellfish toxins (DSTs) of 160 µg okadaic acid (OA) eq/kg (9). This implies that seafood with levels below these values would be released on the market and shellfish consumers would be exposed to sub-regulatory toxin levels (10). However, consumption of products containing sub-regulatory levels of marine biotoxins may result in an acute intoxication or may have negative effects upon long-term consumption. Risks to human health via low level (chronic) exposure are not yet fully understood, as there are only studies on animals (11,12) or model systems (zebrafish) (13).

AIM

The aim of this study was to investigate the levels of hydrophilic toxins in different types of mussels (wild and farmed) collected by frequent sampling and that are often consumed in Bulgaria and to assess the acute and chronic exposure to the detected toxins for average Bulgarian male and female consumers.

MATERIALS AND METHODS

Sampling

Samples (Black Sea mussels) were collected weekly in the period March - October 2017 from the North Bulgarian Black Sea coast (Table 2) in 2017. The sampling strategy allowed covering the whole

Table 1. Classification of the main regulated marine biotoxins

| Chemical Classification | Shellfish Poisoning Type | Chemical Substances Involved in the Intoxication |
|-------------------------|---------------------------------|---|
| Hydrophilic | Amnesic shellfish poisoning | Domoic acid (DA) |
| | Paralytic shellfish poisoning | Paralytic shellfish toxins (PSTs; saxitoxin and its variants, e.g. gonyautoxin (GTXs) etc.) |
| Lipophilic | Diarrhetic shellfish poisoning | Diarrhetic shellfish toxins (DSTs; okadaic acid (OA), dinophysistoxins (DTXs)) |
| | Azaspiracid shellfish poisoning | Azaspiracids (AZAs) |

Marine biotoxins are divided into two main groups – hydrophilic and lipophilic due to their chemical character, generally causing three types of shellfish poisoning (Table 1) (8). In the EU the following regulatory limits are in force - for domoic

period of the year when mussels are collected for trade purposes and own consumption. Farmed mussels were caught directly from mussel cultivation ropes and wild mussels were harvested from natural breeding sites. Cultivated mussel samples (N=26)

were collected from a mussel farm near Kavarna and wild mussel samples (N=22) were collected from natural breeding sites near Krapets, Shabla, Kavarna, Balchik, Albena and Varna.

Table 2. Locations of sampling sites

| Sampling Site | Type of Sample |
|----------------|-------------------------|
| Krapets | Wild mussels |
| Shabla | Wild mussels |
| Kavarna | Wild/cultivated mussels |
| Balchik/Albena | Wild mussels |
| Varna/Galata | Wild mussels |

Mussel Yield

Fresh mussel samples were cleaned from algae and drained with distilled water. Cleaned mussel samples were weighted separately before shucking and after that. The edible part weight in % was obtained as follows (eq. 1):

$$\text{Edible part (\%)} = \frac{\text{mussel meat}}{\text{mussels with shells}} \times 100$$

Analytical Method

The methods used for the preparation of the samples, toxin extraction and determination were previously described in detail (14, 15). Briefly, the digestive gland (hepatopancreas) of each shucked mussel was dissected. All digestive glands were homogenized. Four grams homogenate were processed with methanol with a high-speed dispersing instrument (POLYMIX^{PT} 1200E, KINEMATIKA AG, Germany) and subsequently with hexane for DA extraction. Two grams hepatopancreas homogenate was treated with acetic acid with the same dispersing instrument for paralytic toxins extraction. An aliquot of each extract was analyzed.

Domoic acid concentration was determined and quantified via liquid chromatography tandem mass spectrometry (LC-MS/MS) on an AB-SCIEX-4000 Q Trap, triple quadrupole mass spectrometer equipped with a TurboSpray[®] interface coupled to an Agilent model 1100 LC. Chromatographic conditions included Eluent A – water, Eluent B – acetonitrile/water (95:5 v/v) by binary gradient elution, flow rate – 0.2 mL/min. The separation of DA was per-

formed on C8 column (50 x 2 mm i.d., 3 μm). In total 48 extracts were analyzed for the presence of DA.

High performance liquid chromatography with fluorescence detection (HPLC-FD) and post-column derivatization was applied for PST analysis. The PST analysis was done by reverse-phase ion-pair liquid chromatography on a LC1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PCX 2500 post-column derivatization system (Pickering Laboratories, Mountain View, CA, USA) and dual monochromator fluorescence detector (G1321A) following minor modifications of previously published methods (15). Chromatographic conditions included isocratic elution program performed by two eluents: solution of octanesulfonic acid, heptanesulfonic acid and ammonium phosphate, and solution of octanesulfonic acid and phosphoric acid. The flow rate was 1 mL/min. The separation of analytes was performed on a C18 reversed-phase column (250 mm x 4.6 mm i.d., 5 μm). Paralytic toxin levels of 17 samples were already described in our previous study (11). Additionally, 8 more mussel samples were subjected to the same method of determination.

The quality control was performed by regular analysis of procedural blanks (sample without the analyte going through all steps of the procedure with the reagents only) and certified reference materials purchased from the National Research Council, Canada.

Contamination Level

Marine toxin levels were determined in the hepatopancreas of mussels (μg/kg hp). A factor of 5, proposed by EFSA (16) (17), was applied to convert the marine toxin levels in whole mussel meat (mm) (μg/kg mm).

Mean contamination level (μg/kg) was estimated by calculation of the average contamination of all positive samples.

Mussel Consumption

A total of 40 restaurants offering 30 different dishes containing fresh mussels from the North Black Sea coast were included in the survey. Eighteen dishes were served without shells, 9 – with shells and 3 dishes were offered with or without shells in different restaurants. Mussel meat portion was calculated as an average of weight of mussel meat in dishes

containing mussels with shells and in dishes made of shucked mussels. Weight of mussels (with or without shells) used for preparation of each dish was kindly provided by the chefs in the restaurants included in the survey. Mussel meat in dishes containing mussels with shells was estimated by using the result for average mussel yield (edible part, %) (eq.1), reported in this study.

Average mussel consumption per person per day (kg mussel meat (mm)/day) was calculated for the period of mussel harvesting, i.e. excluding the days in the winter months, November until February, and using the latest published data provided by the EXACTA Research group for mussel consumption in households (kg) in Bulgaria (18) and statistical data provided by the National Statistical Institute (NSI) for the average number of members in households in Bulgaria (19).

Dietary Exposure Estimation

The assessment of human exposure to hydrophilic toxins through oral ingestion was estimated using the mean contamination level of samples.

The estimated **acute exposure** was calculated as follows (eq. 2):

$$AE = \frac{C \times P}{BW}$$

Where:

AE is mean acute exposure (µg/kg bodyweight (bw))

C is mean contamination level (µg/kg)

P is the average portion size (0.234 kg)

BW is the mean bodyweight (bw) in Bulgaria according European health interview survey (20) (67 kg for females and 81 kg for males)

The estimated **chronic exposure** was calculated according to eq. 3:

$$CE = \frac{C \times D}{BW}$$

Where:

CE is mean chronic exposure (µg/kg bw/day)

D is mean mussel consumption per day (0.005 kg /day)

P is the average portion size (0.234 kg)

BW is the mean bodyweight in Bulgaria

Chronic exposure estimation allowed calculation of hazard quotient too.

Hazard quotient was calculated as a ratio of mean CE and TDI for the certain toxin (eq. 4).

$$HQ = \frac{CE}{TDI}$$

Where:

HQ is hazard quotient, *measureless*

CE is mean chronic exposure (µg/kg bw/day)

TDI is tolerable daily intake (0.075 µg/kg bw/day for DA (16))

RESULTS

The mean calculated weight of mussel samples with shells was 1.45 kg. The mean weight of mus-

Table 3. Samples analyzed and levels of toxins detected

| Toxins Detected | Type of Samples | Number of Samples Analyzed | Number of Positive Samples | Mean Positive Contamination Level Calculated for Whole Mussel Meat | Legislative Limit |
|------------------------|-----------------|----------------------------|----------------------------|--|-----------------------|
| Domoic acid (mg/kg) | Wild | 22 | 5 | 0.141 | 20 mg/kg |
| | Cultivated | 26 | 8 | 0.137 | |
| | All | 48 | 13 | 0.139 | |
| GTX2 (µg STX.2HCl/kg) | Wild | 11 | 4 | 0.112 | 800 µg STX.2HCl eq/kg |
| | Cultivated | 14 | 5 | 0.191 | |
| | All | 25 | 9 | 0.151 | |

sel meat in the samples after shucking was estimated to be 0.44 kg. The calculated mussel yield (edible part) comprised 30% of the weight of the mean mussel sample with shells according to eq.1.

The LC/MS analysis revealed that among 48 extracts investigated, 13 were positive for DA (Table 3).

Paralytic toxin levels of 17 samples were already described in our previous study (15). GTX2 was detected in 9 samples, 5 cultivated mussel samples and 4 wild mussel samples. The additional herein described HPLC-FD analysis showed no paralytic toxins in the investigated 8 samples (Table 3).

The calculated mean positive levels of detected toxins in whole mussel meat are presented in Table 3. Results indicated that DA levels in wild and cultivated mussels were similar, 0.141 and 0.137 mg/kg, respectively. The average DA contamination in all mussel samples was estimated to be 0.139 mg/kg. The GTX2 levels in cultivated mussels (0.191 μg STX.2HCl eq/kg) were almost two times higher than in wild mussels (0.112 μg STX.2HCl eq/kg). The calculated mean GTX2 contamination was 0.151 μg STX.2HCl eq/kg.

Results from the restaurant survey showed that the mean mussel meat weight used for dishes without shells was 0.230 kg. The mean weight of non-shucked

mussels was estimated to be 0.790 kg. Assuming that mussel meat weight is 30% of the weight of mussels with shells, it was calculated that the mean weight of mussel meat in dishes with shell comprised 0.237 kg. The calculated average of mussel meat weight in all type of dishes was 0.234 kg.

Data on average mussel consumption for Bulgarian population is provided in Table 4. Average mussel consumption per person per day was calculated to be 0.005 kg mm/day.

Results on both acute and chronic exposure and hazard quotient for DA are presented in Table 5. Highest acute exposure was estimated for females if consuming wild mussels – 0.494 μg DA/kg and lowest for males if consuming cultivated mussels – 0.395 μg DA/kg. Mean acute exposures for both sexes if consuming both mussel types were very close to these results - 0.486 and 0.402 μg DA/kg, respectively. Results on chronic exposure showed no significant difference between sexes and by consumption of different mussel types. Average chronic exposure for males was 0.009 and for females - 0.010 $\mu\text{g}/\text{kg}$ bw per day. Respectively, the calculated hazard quotients were similar – 0.115 (males) and 0.138 (females).

Table 4. Mussel consumption in Bulgaria

| Mussel Consumption per Year (kg) (14) | Number of Households Consuming Mussels (14) | Average Number of Persons in Household (15) | Average Mussel Consumption Calculated per Person per Year (kg) | Average Mussel Consumption per Person per Day (kg mm/day) (Calculated for the Period of Fresh Mussels Harvesting) |
|---------------------------------------|---|---|--|---|
| 486 | 138 | 2.7 | 1.3 | 0.005 |

Table 5. Calculated exposure to domoic acid and hazard quotient

| Type of Sample | Calculated Acute Exposure μg DA/kg | | Calculated Chronic Exposure $\mu\text{g}/\text{kg}$ bw/day | | Calculated HQ | |
|--------------------|--|---------|---|---------|---------------------------------|---------|
| | Males | Females | Males | Females | Males | Females |
| Cultivated mussels | 0.395 | 0.478 | 0.008 | 0.010 | 0.113 | 0.136 |
| Wild mussels | 0.409 | 0.494 | 0.009 | 0.011 | 0.116 | 0.141 |
| All | 0.402 | 0.486 | 0.009 | 0.010 | 0.115 | 0.138 |
| Legislative limit | Acute Reference Dose (ARfD) 30 μg DA/kg | | Tolerable daily intake (TDI) 0.075 $\mu\text{g}/\text{kg}$ bw per day | | Hazard quotient (HQ) <1 No risk | |

Calculated acute exposure to GTX2 is presented in Table 6. Although with a very low magnitude, in general, acute exposure to GTX2 if consuming cultivated mussels was higher than if consuming wild mussels. The mean acute exposure to GTX2 of

Table 6. Calculated exposure to PSP toxins

| Type of Sample | Calculated Acute Exposure μg STX.2HCl/kg | |
|--------------------|---|---------|
| | Males | Females |
| Cultivated mussels | 0.0006 | 0.0007 |
| Wild mussels | 0.0003 | 0.0004 |
| All | 0.0004 | 0.0005 |
| Legislative limit | ARfD 0.5 μg STX.2HCl/kg | |

males (0.004 μg STX.2HCl/kg) and females (0.005 μg STX.2HCl/kg) was similar.

DISCUSSION

With the aim to characterize the exposure to hydrophilic marine biotoxins via consumption of mussels, we analyzed the phycotoxin levels in farmed and wild mussel samples by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and ion-pair chromatography with post-column derivatization (HPLC-FD). Thereafter we calculated the exposure of consumers to detected phycotoxins.

DA and PSP toxins belong to the group of hydrophilic marine biotoxins due to their chemical characteristics. This group of marine toxins was of a special interest because microalgae that produce them, e.g. *Pseudo-nitzschia* spp., *Alexandrium* spp. and *Gymnodinium catenatum* were detected on the Bulgarian coast (7).

We analyzed all the hydrophilic toxins currently legislated in the EU: DA and the PSP toxins including the carbamoyl toxins STX, NEO, GTX1/4 and GTX2/3, the sulfocarbamoyl toxins B1 and C1/2 and the decarbamoyl toxins dcSTX, dcNEO and dcGTX2/3.

Among them DA and GTX2 were detected in the samples, whereas all other above-mentioned toxins were below their detection limits.

In the past 10 years hydrophilic toxins were monitored in farmed mussels and detected in a

scarce number of samples (6, 21). Our results showed that 27% of all investigated samples (N = 48) were positive for DA and 36% were positive for the PSP toxin – GTX2 (N = 25) (Table 3). There was no significant difference in mean DA level in wild and cultivated mussels, while mean GTX2 level in cultivated mussels was almost 2 times higher than in wild mussels. Both mean DA and GTX2 levels were much lower than legislative limit in EU (Table 3).

Mean domoic acid content was 0.136 mg/kg mm. These results are slightly higher than data about DA levels reported by the national monitoring program on the Bulgarian North coast varying between 0.01-0.1 mg/kg mm (21). It should be taken into account that we investigated 48 mussel samples collected in one year, whereas in the national monitoring were included 5 samples from a much longer period.

In the period 2008-2009, Bouchouicha-Smida et al. (2015) (22) found similar to our results for DA levels in *Mytilus galloprovincialis* whole shellfish meat from Bizerte Lagoon (SW Mediterranean) (0.13–0.86 μg DA/g tissue).

The mean GTX2 level was 0.151 μg STX.2HCl/kg mm. This result is much lower than reported level of GTX2/3 in national monitoring on the North coast during the period 2014-2015 - 21.3 μg STX.2HCl/kg mm.

Human exposure to marine biotoxins is possible if e.g. contaminated mussels are consumed. Both acute and chronic exposures were calculated using mean values of variables - phycotoxin level, body-weight and portion size. This is reasonable assumption aiming to summarize the Bulgarian population exposure and because a person consuming mussels will not eat the same portion size containing the same level of toxin each time. On the other hand, involving mean positive phycotoxin concentration revealed worst case scenario, namely all consumed mussels contain the detected phycotoxins.

For the purposes of this study, acute exposure was defined as exceeding the ARfD (acute reference dose). Calculated mean acute exposure to DA (Table 5) showed that there was no considerable difference in exposure neither between the genders or the mussel types. In all cases the calculated exposure was up to 100 times lower than the legislative norm.

Similar conclusions were obtained by comparing the calculation for acute exposure to GTX2 (Table 5). These results suggested no risk of acute intoxication with DA and GTX2 via consumption of studied samples.

Compared to the mean Belgian population exposure to DA (0.58 µg/kg bw) via consumption of mussels (23), even the highest calculated exposure for the Bulgarian population (females, wild mussels – 0.494 µg/kg bw) was lower.

The reported mean Irish exposure to PSTs in 2016 was 0.6 µg STX.2HCl/kg, which exceeds the legislative limit of 0.5 µg STX.2HCl/kg and is around 100 times higher than the calculated in this study highest acute exposure to GTX2 (females, cultivated mussels - 0.0007 µg STX.2HCl/kg).

Chronic exposure estimation was only applicable for DA. No data on the chronic effects of paralytic toxins in animals or humans were available, so a tolerable daily intake (TDI) was not established. From the available reports on intoxications in humans ARfD was estimated and therefore only acute exposure to paralytic toxins was hereby calculated.

By definition, chronic exposure is if a population sample is exposed to low level and repetitive toxin doses intake. For households included in the survey it was calculated that their members consume 0.005 kg mm/day (Table 4). The estimated values for chronic exposure showed similar among males and females, as well as wild and cultivated mussel consumers (Table 5). Calculated chronic exposure values were about 10 times lower than the legislative threshold resulting in hazard quotients beneath the limit of 1.

CONCLUSION

This study showed an overall low contamination level of wild and farmed mussels with phycotoxins compared to the reference concentrations. This leads to the conclusion that there is low acute and chronic exposure via consumption of contaminated mussels. In general, females were assessed as a higher risk group for both acute and chronic exposure.

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