FOOD SAFETY

doi: 10.14252/foodsafetyfscj.2017031
Food Safety 2018; Vol. 6, No. 2, 101–106

Short Communication

Distribution of Diarrhetic Shellfish Toxins in Mussels, Scallops, and Ascidian

Ryoji Matsushima[1], Hajime Uchida[1], Ryuichi Watanabe[1], Hiroshi Oikawa[1], Yuki Kosaka[2], Toru Tanabe[3], Toshiyuki Suzuki[1]

[1]National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-8648, Japan

[2]Aomori Prefectural Industrial Technology Research Center, Fisheries Research Institute, Hiranai, Aomori 039-3381, Japan

[3]Miyagi Prefectural Fisheries Technology Research Institute, Kesennuma Fisheries Experiment Station, 107 Hajikamiiwaisaki, Kesennuma, Miyagi 988-0181, Japan

Diarrhetic shellfish toxins (DST) are a group of phycotoxins that include Okadaic acid (OA) and structurally related toxins. In Japan, the regulatory limit of DST in shellfish for human consumption is a total OA equivalent of 0.16 mg per kg of edible tissue. Distribution and individual differences of DST in scallops collected in Aomori Prefecture were investigated. Fourteen to 20 individual scallops were divided into hepatopancreas, gonads, mantles, gills, adductor muscles, and the concentrations of diarrhetic shellfish poisoning (DSP) in each tissue were quantified by LC/MS/MS after hydrolysis. The dominant toxin in the scallops was Dinophysis toxin 1 (DTX1). More than 97% of the observed DTX1 in the scallop tissue was detected in the hepatopancreas and the average level of DTX1 was higher in mussels than the scallops. The number of individual scallops or mussels required to correctly reflect the DTX1 content of a sample group was estimated by resampling. In scallops, using 10 individuals fell within ± 20% of 30 individual's average with a probability of 99.8%. On the other hand, in the blue mussel, an average of 19 individuals fell within ± 20% of 30 individual's average with 98% probability. In addition, the analysis of the DST in ascidians collected from Miyagi Prefecture was carried out. The muscles, gills, hepatopancreas and intestines were analyzed. High concentration of both DTX1 and OA were detected in the hepatopancreas after hydrolysis. Low levels of DST were detected from other tissues, indicating that DST are primarily accumulated in the hepatopancreas in the ascidians.

Key words: ascidian, Diarrhetic shellfish toxins (DST), Dinophysis toxin (DTX), mussel, Okadaic acid (OA), scallop

1. Introduction

Diarrhetic shellfish toxins (DST) are a group of phycotoxins that include Okadaic acid (OA) and structurally related toxins. In Japan, the regulatory limit of DST in shellfish for human consumption is a total OA equivalent of 0.16 mg per kg of edible tissue as described in CODEX Standards[1)]. Testing for DST using liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been officially authorized from 2015 in Japan[2)]. It is expected that the risk management of diarrhetic shellfish poisoning (DSP) using LC/MS/MS will benefit from the significantly improved sensitivity and the accuracy of LC/MS/MS compared to the conventional mouse bioassay. In addition to Japanese scallop *Patinopecten*

Received: 1 November 2018; Accepted: 2 April 2018; Published online: 31 May 2018
Corresponding author: Toshiyuki Suzuki, National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-8648, Japan (tsuzuki@affrc.go.jp)
The contents of this article reflect solely the view of the author(s).
This paper was presented at the 12th International Symposium "Toxins, Pathogens, and Foods: Challenges and Opportunities for Public Health" organized by the Toxic Microorganisms Panel of the United States-Japan Cooperative Program on Development & Utilization of Natural Resources.
Abbreviations: DSP: Diarrhetic shellfish poisoning, DST: Diarrhetic shellfish toxins, DXT: Diarrhetic shellfish toxins, DTX1: Dinophysis toxin, LC/MS/MS: liquid chromatography mass spectrometry, OA: Okadaic acid

Table 1. Concentration of DTX1 and OA in hepatopancreas of scallops

Date (2014)	May 26	Jun 2	Jun 9	Jun 30	Jul 14	Jul 22	Jul 28
Individuals	16	18	17	18	15	20	14
DTX1 (ng/g)	472	556	381	831	207	147	121
OA (ng/g)	17	14	13	28	20	7	6

Fourteen to 20 individuals were combined into each sample set used for analysis. The highest DTX1 was recorded at Jun 30.

yessoensis, which is important species for fishery industry, the distribution of DST (OA and DTX1) in the mussel *Mytilus edulis* was analyzed. Because little information is available, DST accumulation in the edible ascidian *Halocynthis roretzi* was also investigated.

2. Materials and Methods

2-1. Bivalves and Ascidian

Scallops and mussels were collected from Nonai station of Mutsu Bay, Aomori Prefecture, Japan in 2014. Aomori Prefecture is located at the northern end of Honshu Island. Specimens of the ascidian were harvested from Iwaizaki station of Kesennuma Bay, Miyagi Prefecture, Japan in 2016.

2-2. Extraction of DSTs from Samples and Hydrolysis of Esterified DSTs

Each dissected tissue was homogenized with 9 volumes of methanol-distilled water (9:1, v/v), and the homogenates were centrifuged at 3000 rpm for 5 min[3]. Alkaline hydrolysis of OA group was carried out according to the EU harmonized standard operating procedure for lipophilic marine biotoxins in Molluscs by LC-MS/MS. Ver. 5[4]. For the hydrolysis, 125 µL of 2.5M NaOH solution was added to a 1 mL aliquot of a methanolic extract of each sample. The mixture was kept at 80°C for 30 min and neutralized with 125 µL of 2.5M HCl. The hydrolyzed samples were analyzed by LC/MS/MS without further purification.

2-3. LC/MS/MS Analysis of DSTs

Authentic standards of OA, dinophysistoxin-1 (DTX1) were prepared according to previous methods[5]. Toxins were dissolved in HPLC grade methanol to prepare the calibration standards. OA, DTX1, in sample extracts were analyzed and quantified by LC/MS/MS as reported in our previous methods[3,6]. Triplicate analysis was carried out for each sample extract. Multiple reaction monitoring (MRM) LC/MS/MS analysis for toxins was carried out using $[M-H]^-$ as the target parent ions in Q1 and particular fragment ions of each toxin in Q3, with a dwell time of 100 msec for each analog as follows. OA: *m/z* 803.5>255.3; DTX1: *m/z* 817.5>255.3. LOD of OA and DTX1 < 0.01 mg/kg.

2-4. Statistical Analyses

Statistical analysis program R with the boot (https://cran.r-project.org/web/packages/boot/index.html) and MASS (https://cran.r-project.org/web/packages/MASS/index.html) package was employed for resampling analysis. The values repeatedly calculated 10,000 times with 5-25 random sampling without overlap from 30 scallops or mussels were compared with the average value of 30 scallops or mussels. The program is a kind of bootstrap estimation that has been modified not to allow overlapping. This provides an estimate of the shape of the distribution of the mean, from which solutions are presented about how much the mean varies.

3. Results

Distribution and individual differences of DST in scallops collected from Nonai station, Mutsu Bay Aomori were investigated. The 14-20 scallops were dissected to separate the hepatopancreas, gonads, mantles, gills, and adductor muscles, and combined for each part. The concentrations of OA group in each tissue were quantified by LC/MS/MS after hydrolysis (**Table 1**).

The dominant toxin in the scallops was DTX1, the highest concentration was found on June 30 and was equivalent to about half of the regulation value. By multiplying the measured concentration by the tissue weight, the ratio of the DTX1 quantity included in each tissue was calculated. (**Table 2**). More than 97% in total amounts of DTX1 were harbored in the hepatopancreas. Regardless of DSTs amount, DSTs were hardly detected in the adductor muscles. The concentration of DTX1 included in the hepatopancreas of 30 individuals (scallop and mussel) collected at the Mutsu Bay was quantified for each individual (**Table 3**). The concentration of OA was not described due to the overall low amounts found in the individual samples. The average value of DTX 1 differs even for the same date in mussel and scallop, and the DTX1 value of mussel was higher (**Fig. 1**, **Table 3** Jun 2).

doi: 10.14252/foodsafetyfscj.2017031

Table 2. Content ratio (%) of DTX1 in each scallop tissue

	May 26	Jun 2	Jun 9	Jun 30	Jul 14	Jul 22	Jul 28
Hepatopancreas	97.1	98.5	97.5	97.8	98.2	97.8	98.6
Gonad	0.8	0.5	0.8	1.0	1.2	1.5	1.4
Mantle	1.0	0.3	0.8	0.6	0.0	0.0	0.0
Gill	1.2	0.7	0.9	0.6	0.6	0.7	0.0
Adductor muscle	0.0	0.0	0.0	0.0	0.0	0.0	0.0

In all cases, hepatopancreas contained almost of all DTX1 whether high or low.

Table 3. DTX1 concentration (ng/g) in hepatopancreas of 30 individual samples (scallop and mussel)

Date (2014)	May 26	Jun 2	Jun 9	Jun 16	Jul 7	Aug 8
Scallop	489 ± 127 (26%)	628 ± 168 (27%)	521 ± 110 (21%)	321 ± 82 (25%)	-	-
Mussel	-	735 ± 450 (61%)	-	-	805 ± 307 (38%)	227 ± 164 (72%)

The average value of 30 samples, standard deviation (SD) and relation ratio of SD (%)

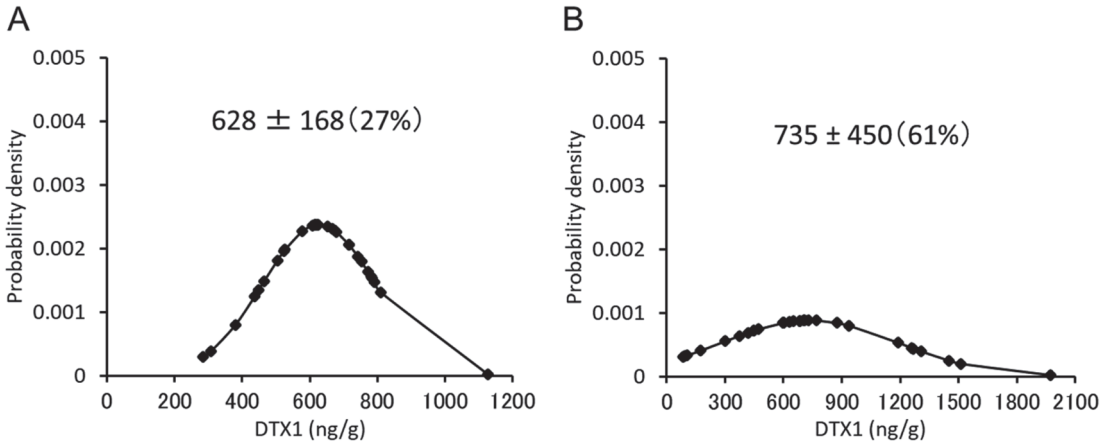

Fig. 1. The individual distribution, average and standard deviation of DTX1 at Jun 2 (A) scallop; (B) mussel.

By statistical resampling, the number of individuals that are necessary to correctly reflect the DST content of collected samples were estimated. Using the measured data set of 30 individuals at June 2nd for scallops or blue mussels, both were high and highly variable, the average value of 5 to 25 samples were calculated repeatedly by 10,000 times with random sampling without overlap (**Fig. 1**, **Table 4**). In the scallop, using 10 individuals fell within ± 20% of average value of 30 individuals with a probability of 99.8% (**Table 4** left underlined number). On the other hand, in the blue mussel, average of 19 individuals or 15 individuals fell within ± 20% of average value of 30 individuals with 98% or 90% probability, respectively (**Table 4** right underlined number).

Analysis of the DST of ascidians collected from Miyagi Prefecture was carried out when the toxification of blue mussel was confirmed at the same monitoring station (**Table 5**). The muscles, gills, hepatopancreas and intestines of ascidians were analyzed (**Fig. 2**, **Table 6**).

High concentration of DTX1 and OA were detected in the hepatopancreas after hydrolysis (**Fig. 2D**). Low levels of DST were detected from other tissues (**Fig. 2A–C**).

4. Discussion

Since there was no report that DTX2 has been detected in Japan, we analyzed OA and DTX1 in this study. In the

Table 4. The resampling analysis of DTX1 concentration in hepatopancreas of scallop and mussel

	Scallop							Mussel					
N	**0.1**	**1**	**5**	**95**	**99**	**99.9**	**N**	**0.1**	**1**	**5**	**95**	**99**	**99.9**
5	68.3	75.6	**82.1**	**119.0**	126.3	131.3	5	38.5	49.5	60.4	144.4	163.7	181.4
6	72.1	77.7	**84.0**	**117.0**	123.7	129.0	6	40.1	52.0	63.9	139.4	155.8	176.5
7	74.3	80.0	**85.4**	**114.9**	120.4	125.5	7	46.5	56.2	66.9	134.8	148.8	164.3
8	76.2	**81.1**	**86.5**	**113.9**	**119.0**	123.2	8	48.6	58.5	69.5	132.6	144.4	157.7
9	78.7	**82.9**	**87.6**	**112.9**	**117.6**	121.4	9	53.6	61.9	71.8	129.8	140.3	153.6
10	**80.8**	**84.4**	**88.5**	**111.6**	**116.0**	**119.6**	10	54.2	64.1	73.7	126.9	138.6	150.2
11	**81.3**	**85.5**	**89.3**	**111.0**	**114.7**	**118.4**	11	59.2	67.0	75.9	124.8	135.4	145.1
12	**82.7**	**86.1**	**89.8**	**110.4**	**114.1**	**117.4**	12	61.0	69.4	77.4	123.7	132.1	139.7
13	**84.6**	**87.4**	**90.7**	**109.4**	**112.9**	**116.0**	13	62.4	70.6	78.5	121.6	129.7	137.4
14	**85.1**	**87.8**	**91.2**	**108.6**	**112.1**	**114.9**	14	66.3	72.5	79.7	**119.9**	127.7	134.8
15	**85.4**	**88.7**	**91.7**	**108.0**	**111.1**	**114.0**	15	66.0	74.2	**81.2**	**118.9**	126.3	133.9
16	**87.1**	**89.7**	**92.5**	**107.6**	**110.3**	**113.3**	16	69.0	75.0	**82.2**	**117.5**	124.2	130.9
17	**88.1**	**90.0**	**92.8**	**107.1**	**109.7**	**112.4**	17	71.2	77.6	**83.7**	**116.5**	122.4	127.6
18	**88.5**	**90.8**	**93.2**	**106.5**	**109.1**	**111.6**	18	71.8	78.5	**84.8**	**115.1**	120.4	125.2
19	**89.4**	**91.4**	**93.6**	**106.2**	**108.4**	**110.4**	19	75.0	**80.0**	**85.6**	**113.8**	**119.2**	124.1
20	**90.2**	**92.0**	**94.0**	**105.8**	**107.8**	**110.1**	20	76.7	**81.1**	**86.7**	**112.9**	**117.3**	121.5
21	**90.4**	**92.6**	**94.5**	**105.2**	**107.2**	**109.2**	21	78.7	**82.2**	**87.4**	**111.9**	**116.0**	120.1
22	**91.5**	**93.1**	**94.9**	**104.9**	**106.6**	**108.6**	22	78.2	**83.7**	**88.4**	**110.8**	**114.5**	**118.3**
23	**92.3**	**93.5**	**95.2**	**104.5**	**106.3**	**107.5**	23	79.8	**84.7**	**89.2**	**110.1**	**113.5**	**116.6**
24	**92.9**	**94.1**	**95.7**	**104.0**	**105.5**	**107.2**	24	**82.5**	**86.1**	**90.2**	**108.8**	**111.7**	**114.0**
25	**93.7**	**94.6**	**96.1**	**103.6**	**105.0**	**106.4**	25	**83.4**	**87.3**	**91.1**	**107.9**	**110.3**	**113.0**

The percentage; each 0.1 to 99.9th percentile value takes on the average value of 30 individual data set. The N vertical columns represent extracted sample numbers 5 to 25 samples. The horizontal 0.1 to 99.9 represent each percentile. Gray back bold character and gray back white character corresponding ± 20 and ± 10% respectively.

Table 5. DTX1 concentration of mussel from Nonai station

Date (2016)	mg/kg
June 7	0.61
June 14	0.18

The DTX1 concentration in edible part of mussel.

case of scallops and mussels, it is impractical to sample 30 individuals. The results of the calculation simulated how much reasonable sampling size reflects the mean value of impractical 30 samples. Approximately 10 samples were considered to be adequate for the scallops, as the variation in DTX1 level was not large. It is assumed that a greater number of samples are necessary for the mussel, due to the larger variation in the DTX1 level. Ultimately, the required sample number will vary depending on the degree to which desired risk management level.

The concentration of DSTs in ascidians increased after hydrolysis. Thus, DSTs are accumulated in ascidians as the esterified form 7-*O*-acyl-OA/DTX1 (DTX3)[7,8], like in many bivalves[9]. Most of the DST are accumulated in the hepatopancreas of the ascidians. This portion accounts for just 5% of the edible parts and thus the risk of diarrhetic shellfish poisoning is not considered to be very high. However, there is a personal preference for consumption of hepatopancreas in Japan. To consume internal organs that have been highly contaminated will increase the risk of poisoning. Although these preliminary studies provide useful information for improving DST risk management, more detailed investigations of both contamination and consumption patterns will be required.

doi: 10.14252/foodsafetyfscj.2017031

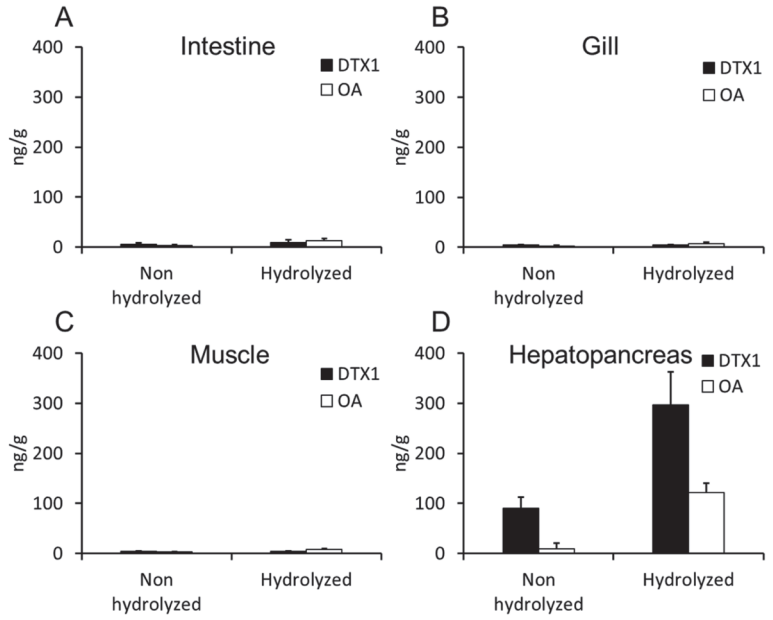

Fig. 2. DTX1 and OA concentration in ascidian tissues (A) intestine; (B) gill; (C) muscle; (D) hepatopancreas.

Table 6. The tissues and weight (g) of ascidian samples at June 13

Sample	Muscle	Gill	Hepatopancreas	Intestin
1	56.8	4.4	2.8	5.0
2	49.5	2.7	3.0	2.5
3	56.2	2.9	2.5	2.5
4	34.8	1.5	1.8	1.5
5	53.6	2.4	3.3	2.7
6	45.7	1.4	1.9	2.3
7	42.2	1.5	2.8	2.0
8	36.2	2.0	1.6	1.8
9	27.8	1.9	1.7	1.9
10	50.3	2.4	2.6	1.5

Numbers in the table represent weights of tissues.

Acknowledgments

The series of studies was conducted and supported by Ministry of Agriculture, Forestry and Fisheries.

Conflict of interest

The authors have no conflict of interest.

References

1. CAC (2008b) Codex Standard for liver and raw bivalve molluscs (CODEX STAN 292-2008). Available at: http://www.fao.org/input/download/standards/11109/CXS_292e_2015.pdf.
2. Guidelines for Risk Management of Shellfish Toxins in Bivalves (2015). Available at: http://www.maff.go.jp/j/syouan/tikusui/gyokai/g_kenko/busitu/pdf/150306_kaidoku_guide.pdf.

3. Suzuki T, Jin T, Shirota Y, Mitsuya T, Okumura Y, Kamiyama T. Quantification of lipophilic toxins associated with diarrhetic shellfish poisoning in Japanese bivalves by liquid chromatography-mass spectrometry and comparison with mouse bioassay. *Fisheries Science*. 2005; **71**: 1370–1378. doi:10.1111/j.1444-2906.2005.01104.x
4. EU-Harmonized Standard Operation Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS Version 5. Available at: http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/laboratorios/LNRBM/ARCHIVO2EU-Harmonised-SOP-LIPO-LCMSMS_Version5.pdf.
5. Goto H, Igarashi T, Sekiguchi R, et al. A Japanese project for production and distribution of shellfish toxins as calibrants for HPLC analysis. In *Proc. VIII International Conference on Harmful Algae*, ed. Reguera B, Blanco J, Fernández M.L, Wyatt T. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO: Vigo, Spain,1998: pp. 216–219.
6. Suzuki T, Miyazono A, Baba K, Sugawara R, Kamiyama T. LC–MS/MS analysis of okadaic acid analogues and other lipophilic toxins in single-cell isolates of several *Dinophysis* species collected in Hokkaido, Japan. *Harmful Algae*. 2009; **8**: 233–238. doi:10.1016/j.hal.2008.06.001
7. UNESKO *Manual on harmful marine microalgae*. Hallegraeff G.M, Anderson D.M, Cembella A.D, ed. IOC Manuals and Guides. 1995. No 33.
8. Wright JLC. Dealing with seafood toxins: present approaches and future options. *Food Research International*. 1995; **28**: 347–358. doi:10.1016/0963-9969(95)00001-3
9. Suzuki T, Ota H, Yamasaki M. Direct evidence of transformation of dinophysistoxin-1 to 7-O-acyl-dinophysistoxin-1 (dinophysistoxin-3) in the scallop *Patinopecten yessoensis*. *Toxicon*. 1999; **37**: 187–198. PMID:9920491, doi:10.1016/S0041-0101(98)00182-2