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First report on TTX levels of the yellow spotted pufferfish (*Torquigener flavimaculosus*) in the Mediterranean Sea

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ABSTRACT

The differences of tetrodotoxin (TTX) levels in various parts of pufferfish (*Torquigener flavimaculosus*) were examined in conjunction with the seasonal and sexual variations. The TTX levels in gonads, liver, intestines, skin and muscle tissue were determined using the Q-TOF LC/MS. Instrumental analysis revealed that all examined tissues from *T. flavimaculosus* contained high TTX concentrations. TTX levels in the gonads, liver, intestines, skin and muscle tissue of pufferfish were within the range of 5.03–100.71, 7.04–106.80, 12.59–86.30, 33.95–139.88 and 15.88–86.07 (μg/g), respectively. It was determined that in all seasons, except for summer, female individuals had higher TTX levels than males. Among all seasons, the highest level of TTX was found in winter and the lowest in autumn. Consequently, *T. flavimaculosus* is a highly toxic pufferfish that is dangerous for human consumption and should not be consumed under any circumstances.

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1. Introduction

Human poisoning by marine toxins has been occurring through human history, and poisoning cases have been recorded since thousands of years (Otero, 2014). For instance, poisonous pufferfish, belonging to the Tetraodontidae family, were documented in the Ancient Chinese Pharmacopoeia compiled in 2800 BCE; thus, the warnings and recommendations associated with handling pufferfish were documented (Otero, 2014). Researches aimed at detecting and discovering unknown toxins have been going on to understand their impact and applicability on human health and ecosystem. Due to lacking toxicological data on unknown toxins, more knowledge about poisonous fish is still important for human health. The natural habitats of poisonous fish are the Pacific and Indian oceans, where tropical marine ecosystems are prominent. Societies living in these regions are familiar with poisonous fish and poisoning cases arising from the consumption of poisonous fish. However, poisonous fish were not an issue of concern in Mediterranean countries until recently. Among many species that entered the Mediterranean Sea and settled into its ecosystem, pufferfish, known for their poisonous characteristics, are of special concern (Kosker et al., 2015). Since pufferfish moved to the Mediterranean region, accidental poisoning by its consumption has happened the Mediterranean region (Eisenman et al., 2008; Bentur et al., 2008; Chamandi et al., 2009; Kheifets et al., 2012).

The majority of alien fish settling in the Mediterranean Sea are known as lessepsian species that migrate from the Red Sea through the Suez Canal. Although these species inhabiting the Mediterranean are economically valuable, some are considered harmful species (Zenetos et al., 2012). Pufferfish are not an economically significant species, and they pressurise economically valuable fish species that are commercially profitable and nutritious for human consumption (Streftaris and Zenetos, 2006; Bentur et al., 2008). Pufferfish stand out among other alien species due to their harmful effects on the economically significant fish species, fishing and public health (Streftaris and Zenetos, 2006). The majority of pufferfish species contain tetrodotoxin (TTX), which is one of the most lethal toxins (Hwang and Noguchi, 2007). Symptoms of TTX poisoning are paralysis, respiratory distress, nausea and muscle coordination disorder (Isbister et al., 2002). TTX acts by blocking the sodium channels in the neurons and has no known antidote

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(Moczydlowski, 2013). Lethal dose of TTX for humans (MLD_{50}) is around 2 mg (Hwang and Noguchi, 2007).

In recent years, incidents of pufferfish poisoning due to their toxin that causes have become frequent in social and print media in Mediterranean countries. Ten different species of pufferfish belonging to the Tetraodontidae family are present around the Mediterranean Sea (Kosker et al., 2015). Although the most prevalent pufferfish species in the Mediterranean is *Lagocephalus sceleratus,* the population of *Torquigener flavimaculosus* has also increased greatly in recent years, particularly in the eastern Mediterranean Sea. *T. flavimaculosus* is typically found in the western shores of the western Indian Ocean and is particularly widespread in the Red Sea and the shores of Kenya; it has migrated to the Mediterranean Sea in recent years through the Suez Canal (Randall, 1995). *T. flavimaculosus*, also known as the yellow-spotted pufferfish internationally, is also called the dwarf pufferfish in Turkey. It has sometimes been misclassified as *Lagocephalus hypselogenion* or *Amblyrhynchotes hypselogenion* (Randall, 1995) in some studies. Systematic naming of this species present in the western Indian Ocean was specified by Hardy and Randall (1983) as *Torquigener flavimaculosus*.

T. flavimaculosus is a carnivorous pufferfish species that feeds on small benthic crustaceans. It has the ability to quickly inflate its body using water or air like other pufferfish species (Golani et al., 2006). Sabour et al. (2014) indicated that they can reach a maximum length of 16 cm, while Golani et al. (2006) reported a maximum length of 11 cm. Scientific studies on this species, usually in the form of first recordings, are about weight–length relations and burrowing behaviour (Bilecenoğlu, 2005; Corsini-Foka et al., 2006; Sabour et al., 2014). Some other studies focus on toxicity and have reported that pufferfish species present in the Mediterranean Sea, which *L. sceleratus* (Katikou et al., 2009; Rodriguez et al., 2012; Kosker et al., 2016; Kirimer et al., 2016) and *L. lagocephalus* (Saoudi et al., 2008) contain TTX. However, no toxicity studies have been conducted on the TTX contents of *T. flavimaculosus*, either in the Mediterranean Sea or in its natural range of the western shores of the western Indian Ocean, around the Red Sea and Kenyan shores (Randall, 1995). Though this species was reported as harmless to humans by Fishbase (2018), there is no scientific evidence to support or dismiss this claim. To our knowledge, this is the first report on the TTX content of *T. flavimaculosus.* Therefore, in this study, it was determined the changes of the tetrodotoxin (TTX) levels in gonads, liver, intestines, skin and muscle tissue of pufferfish (*Torquigener flavimaculosus*) using Q-TOF LC/MS in conjunction with the variations that may be caused by seasonal aspects and sexual maturity.

2. Materials and methods

2.1. Tetrodotoxin standard

TTX standard was purchased from Abcam Biochemicals (Cambridge, UK). For the instrumental toxin analysis, 1 mg of standard was used. Before instrumental analysis, TTX standards were diluted using methanol containing 0.01 M acetic acid (Merck). Then, 0.05, 0.1, 0.5, 1 and 2 mg/ml standards were prepared by dilution from stock solution for use in Q-TOF LC/MS analysis to draw the standard curve and stored at −20 °C until further use.

2.2. Fish collection, measurements and identification

Pufferfish were caught in the northeastern Mediterranean Sea by commercial trawl fishing from December 2015 to October 2016. The coordinates were between 36°43′31.8″N, 34°54′27.0″E and 36°08′53.6″N, 33°39′40.7″E (Fig. 1). Fish caught from this region were transported to the lab in ice. Size–weight measurements of pufferfish for all seasons were carried out (Table 1) and genders were determined using a microscope. Only sexually mature individuals were used for the study. Among samples, the maximum weight was 37.88 g for females and 31.30 g for males, while the maximum length was 12.80 cm for females and 12.10 cm for males. For each season, 10 male and 10 female *T. flavimaculosus* individuals were selected, and the muscle tissue, gonads, liver, skin and intestines of these individuals were dissected. In each group, tissues from 10 individuals were taken and mixed for toxin extraction. After that all samples were analysed for each parts of fish.

2.3. Preparation of samples and toxin extraction

Fish were dissected to obtain some dorsal muscle (carefully avoiding the gastrointestinal tract), gonads, the entire upper and lateral skin from head to tail, intestines and the liver. Tetrodotoxin extraction from skin, liver, intestines, gonads and muscle tissues was performed according to the method of Silva et al. (2012). Eighty samples from four seasonal groups were analysed in triplicate.

For TTX extraction, 1 g of sample from each tissue of the pufferfish was used. Methanol (3 ml) containing 1% acetic acid was added to the 1 g sample. The mixture was then homogenised using the Ultra Turrax device (IKA T25 Digital Ultra Turrax, Staufen, Germany) at 7200 rpm for 10 min. Thereafter, it was kept in an ultrasonic bath (Bandelin Sonorex RK 100, Berlin, Germany) at 100 Hz for 10 min. The samples were then kept at room temperature for 15 min and centrifuged (Hettich Zentrifugen, Universal 32R, Tuttlingen, Germany) at 4500g for 20 min at 4 °C. After centrifugation, the upper phase was taken away, and 3 ml methanol with 1% acetic acid was added to the residue and the previous steps were repeated. After second centrifugation, the upper phase obtained was combined with the upper phase from the previous stage, and the mixture was completed to 7 ml. One millilitre of the extract was purified by passing it through a 500 mg/3 ml C18 solid-phase extraction (SPE) cartridge (Supelco, Bellefonte, PA, USA). The sample was eluted with 10 ml of 100% methanol and diluted with the same solvent to a final volume of 12 ml. Next, the solution was evaporated till dry, and the residue was mixed with 1 ml methanol (Clarinet, Agela Technologies, Wilmington, USA). Finally, the residue was filtered using 0.45μ membrane filters and transferred to vials for analysis.

2.4. Instrumental tetrodotoxin (TTX) analysis

Instrumental TTX analysis was performed using an Agilent brand 6545 Accurate-Mass Q-TOF LC/MS coupled with an Agilent 1260 HPLC (Agilent Technologies, Inc., Santa Clara, CA, ABD) device.

Before analysis, a standard calibration curve was created using a TTX standard. R^2 value was determined as 0.9992. Poroshell 120 HILIC (3.0 × 50 mm; 2.7 μm) column (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for analysis. The toxin was separated in the column using two different mobile phases. Mobile phase A was 20-mM ammonium acetate in distilled water (Sigma-Aldrich), and mobile phase B was 20-mM ammonium acetate in acetonitrile (Sigma-Aldrich). Analysis was completed in 8 min. TTX molecule was observed at 3.9 min. A gradient program was established as 3% mobile phase A and 97% mobile phase B in the first 2.5 min, followed by 30% mobile phase A and 70% mobile phase B for 2 min and again 3% mobile phase A and 97% mobile phase B. The column temperature was 20 °C and injection volume was 10 μl. The LC system was operated in the positive ion mode with an ESI (electrospray ionisation) interface using the parameters below: collision-activated dissociation gas, 6 psi; gas flow, 12 l/min; ion spray voltage, 3500 V; temperature, 400 °C and nebuliser pressure,

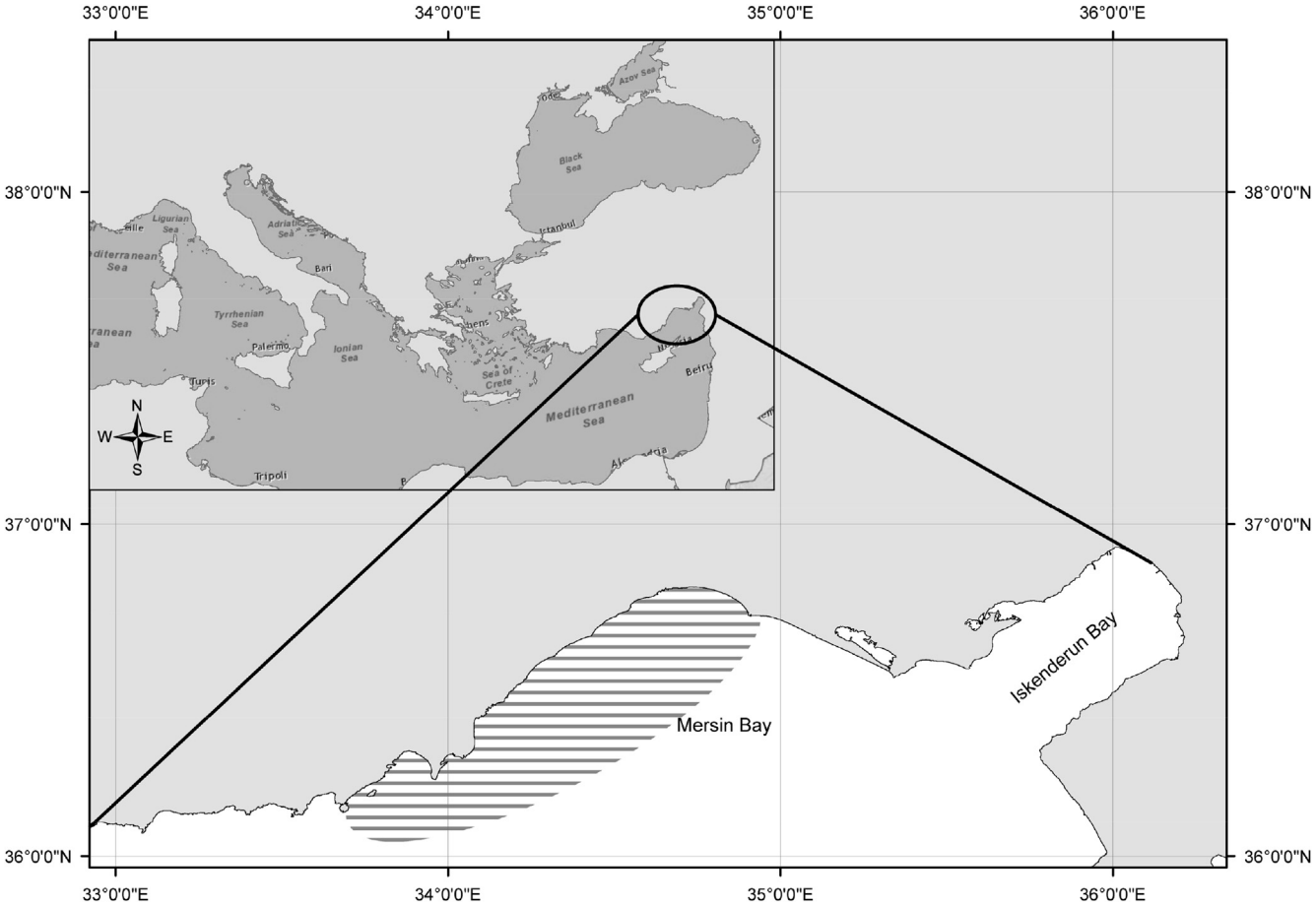

Fig. 1. *Map of the sampling location.* (The shaded area is the sampling location).

Table 1
Length and weight of pufferfish samples.

Seasons	Female				Male			
	Length (cm)		Weight (g)		Length (cm)		Weight (g)	
	Min-Max	$\overline{X}\pm S_{\overline{X}}$	Min-Max	$\overline{X}\pm S_{\overline{X}}$	Min-Max	$\overline{X}\pm S_{\overline{X}}$	Min-Max	$\overline{X}\pm S_{\overline{X}}$
Winter	8.30–12.80	10.59 ± 1.30	11.18–35.87	23.57 ± 7.61	7.40–10.40	8.98 ± 0.99	7.41–21.90	14.05 ± 4.27
Spring	9.10–12.10	10.97 ± 0.89	13.40–37.88	25.47 ± 6.23	8.60–11.80	10.42 ± 0.97	12.50–25.82	19.07 ± 4.93
Summer	8.60–10.60	9.65 ± 0.70	11.80–18.20	15.13 ± 2.31	11.80–12.10	11.94 ± 0.10	26.44–31.30	28.87 ± 1.61
Autumn	9.50–11.60	10.30 ± 0.68	15.92–28.24	21.96 ± 4.08	9.10–10.10	9.61 ± 0.38	12.81–15.57	14.20 ± 0.94

X: Mean, S_x:Standard deviation.

40 psi. The MS system was used in the MRM (multiple reaction monitoring) mode, and scans were performed at 100–1000 *m/z* range. Three protonated molecules $[M + H]^+$ at *m/z* 320, 302 and 284 corresponding to TTXs were detected. The limit of detection (LOD; S/N > 3) of the method for TTX was 0.3 μg/g. The limit of quantification (LOQ; S/N > 10) was reproducible at 0.6 μg/g of TTX. Q-TOF LC/MS analysis was repeated three times. Q-TOF LC/MS operating conditions were optimised using a TTX standard.

2.5. Statistical analyses

The results are reported as the mean ± standard deviation of these measurements. A one-way analysis of variance (ANOVA) was performed using the SPSS version 17.0 (SPSS Inc., Chicago, IL. USA), and the Duncan's multiple range test comparisons at a *p* value of <0.05 were run to determine significant differences. For all groups, statistical comparisons were done in triplicates.

3. Results and discussion

The changes of TTX levels in the muscle, liver, gonads, intestines and skin tissues of pufferfish (*T. flavimaculosus*) caught from the Mersin Bay, northeastern Mediterranean between December 2015 and October 2016 (Table 2) were investigated. The TTX levels in different tissues of male and female samples were also studied in different seasons considering seasonal variations (Figs. 2 and 3). Additionally, TTX analogues, including 4,9-anhydro TTX, were detected in the samples (Fig. 4), but their concentration was not quantified.

Results of the present study show that all analysed tissue samples from *T. flavimaculosus* had a higher TTX level than the dose considered lethal to humans according to Hwang and Noguchi

Table 2
Sexual and seasonal TTX levels in different tissues of *T. flavimaculosus* (μg/g) by Q-TOF LC/MS analysis.

Season	Sex	Muscle $\overline{X}\pm S_X$	Liver $\overline{X}\pm S_X$	Gonad $\overline{X}\pm S_X$	Skin $\overline{X}\pm S_X$	Intestine $\overline{X}\pm S_X$
Winter	Female	75.04 ± 5.59^{ax}	106.80 ± 5.56^{ax}	100.71 ± 6.36^{ax}	139.72 ± 9.40^{ax}	55.24 ± 4.86^{ay}
	Male	86.07 ± 2.05^{ax}	85.63 ± 6.14^{ax}	54.73 ± 4.49^{ay}	112.23 ± 9.23^{bx}	86.30 ± 0.84^{ax}
Spring	Female	64.06 ± 3.98^{ax}	39.94 ± 1.41^{cx}	93.78 ± 2.91^{bx}	125.99 ± 7.92^{ax}	42.35 ± 1.10^{by}
	Male	49.70 ± 1.92^{cy}	32.97 ± 1.96^{cx}	5.03 ± 0.42^{cy}	91.45 ± 6.47^{by}	50.30 ± 1.00^{bx}
Summer	Female	72.38 ± 7.57^{ax}	49.80 ± 2.31^{bx}	49.58 ± 2.73^{cx}	65.42 ± 1.57^{by}	33.54 ± 1.22^{cx}
	Male	66.95 ± 5.63^{bx}	60.53 ± 5.44^{bx}	61.05 ± 4.37^{ax}	139.88 ± 12.21^{ax}	39.67 ± 2.91^{cx}
Autumn	Female	42.07 ± 0.77^{bx}	11.62 ± 0.09^{dx}	41.49 ± 3.19^{cx}	35.19 ± 1.75^{cx}	12.59 ± 1.10^{dx}
	Male	15.88 ± 1.27^{dy}	7.04 ± 0.42^{dy}	16.27 ± 0.79^{by}	33.95 ± 2.84^{cx}	14.89 ± 2.20^{dx}

Values in same column with different letters (a,b,c and d) are significantly different ($p < 0.05$). Different letters in the same column (x,y) in every season indicate significant differences for both sexes ($p < 0.05$).
X: Mean, S_x: Standard deviation.

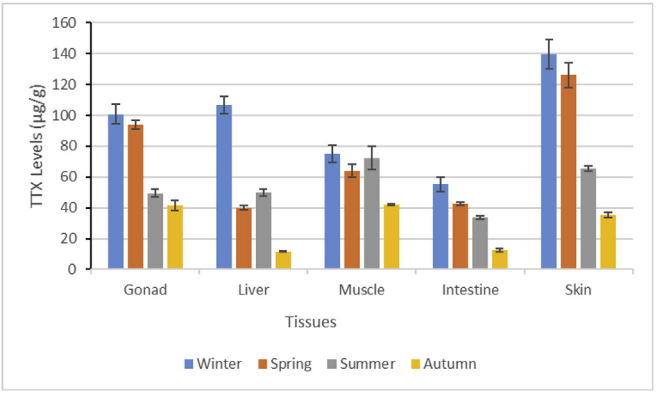

Fig. 2. TTX levels (μg/g) of female pufferfish according to tissue and season.

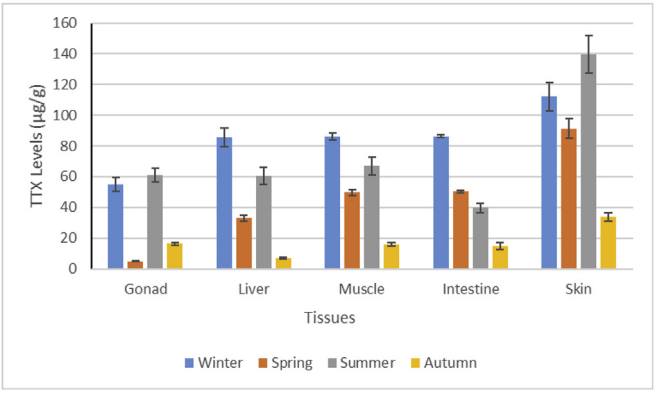

Fig. 3. TTX levels (μg/g) of male pufferfish according to tissue and season.

(2007). TTX levels were measured to be in the range of 5.03 ± 0.42 and 139.88 ± 12.21 μg/g. The maximum TTX level was measured as 139.88, 106.80, 100.71, 86.30 and 86.07 μg/g in skin, liver, gonads, intestines and muscle tissue samples, respectively. Among all tissues, the highest TTX level was found in winter and summer in the skin, and the lowest TTX level was found in the spring in the gonads.

It was found that the toxicity levels were lower in female individuals than in male individuals in all samples, except for muscle tissue in the summer. In other seasons, the TTX level in female individuals was typically higher than in male individuals. This difference is believed to be caused by female individuals discharging a high amount of the toxin alongside the eggs during the breeding season. No other studies have been carried out on the TTX level of *T. flavimaculosus* in the Mediterranean Sea or anywhere else in the world. However, there are studies on the TTX level of different species belonging to the *Torquigener* genus that were not caught in same region of the Mediterranean Sea (Table 3). Ha and Sato (2011) studied the TTX levels of 30 individuals of *T. gloerfelti* pufferfish and discovered the mean TTX levels for the skin, muscle tissue, intestines, liver and gonads to be 11.86, 3.23, 2.44, 33.97 and 27.10 μg/g, respectively. Azman et al. (2014) investigated that the pufferfish identified as *T. pallimaculatus* contained 260 and 29.1 μg/g TTX levels in the liver and skin tissues, respectively; however, they did not quantify TTX levels in the muscle tissue. These results are similar to the findings of the present study. TTX levels can vary based on the species and the study area. Based on the information obtained from this study, it is thought that pufferfish identified as *T. flavimaculosus* is one of the most poisonous fish found in the Mediterranean Sea. Until now, the most poisonous fish species in the Mediterranean Sea was thought to be *Lagocephalus sceleratus*, with most toxicity studies focusing on this species. Katikou et al. (2009) reported that some tissues of *L. sceleratus*, which were caught in the shores of Greece, were not toxic while some tissues were highly toxic, with the maximum TTX level found to be 1087.80 MU/g in the gonads using mouse bioassay (MBA); 1 mouse unit (MU) was defined by Nuñez-Vazquez et al. (2000) as 0.22 μg/g. Therefore, the TTX level found by Katikou et al. (2009) using MBA is approximately 239.32 μg/g. This value was much higher than the TTX levels found in the present study. This difference might be attributable to the use of MBA, which determines not only the content of TTX individually but also that of all TTX analogues present in the sample (Campas et al., 2007). Recently, to determine TTX levels, chromatographic methods, such as LC–MS/MS and O-TOF LC/MS, have been preferred due to better accuracy and reliability. Moreover, despite using the same samples of *L. sceleratus* for determination of TTX, Katikou et al. (2009) and Rodriguez et al. (2012) found different results because they used different techniques.

Similarly, lower TTX levels have been reported in other studies using chromatographic methods. For instance, Rambla-Alegre et al. (2017) reported that the maximum TTX level of *L. sceleratus* caught from the western Mediterranean coast of Spain was found to be 21.08 μg/g in the gonads. In addition, the maximum TTX level of *L. sceleratus* caught on the shores of Turkey was reported as 52.10 and 80.00 μg/g by Kosker et al. (2016) and Acar et al. (2017), respectively. Among the studies carried out using chromatographic instruments, the current study showed that *T. flavimaculosus* contained a much higher level of TTX compared to *L. sceleratus* in the Mediterranean Sea. It can be concluded that *T. flavimaculosus* is a highly toxic pufferfish and can be very dangerous for human consumption (Table 3).

It was also observed that the seasonal cycle of TTX levels in *T. flavimaculosus* was found to be different than the prevailing opinions. Most researchers report that TTX molecule is a breeding

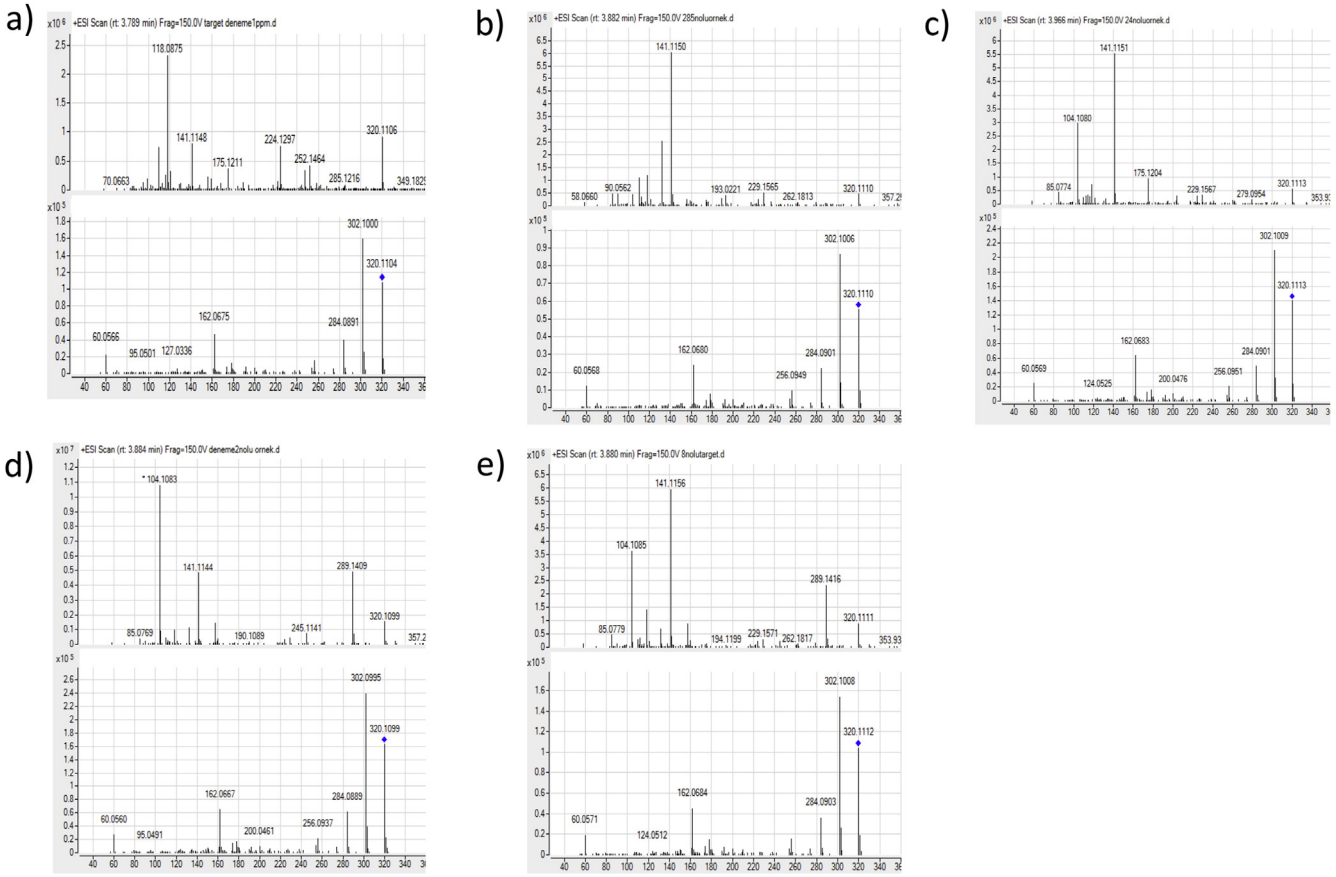

Fig. 4. Mass chromatograms of the Q-TOF LC/MS obtained MS operating in ESI mode. (a) TTX standard solution (1 μg/ml). (b) Male muscle of *T. flavimaculosus*. (c) Female liver of *T. flavimaculosus*. (d) Female gonad of *T. flavimaculosus*. (e) Female skin of *T. flavimaculosus*.

Table 3
TTX levels of *Torquigener* sp. and *Lagocephalus sceleratus* (μg/g) by various research in different tissues and regions.

	Species	Muscle	Gonad	Intestine	Liver	Skin	NI	Method	Region
Torquigener sp.									
This study	*T. flavimucolosus*	15.88 −86.07	5.03 −100.71	12.59 −86.30	7.04 −106.80	35.19 −139.72	80	Q-TOF LC/MS	Northeastern Mediterranean
Ha and Sato (2011)	*T. gloerfelti*	0.00–3.23	0.00 −215.14	0.00–11.99	0.79 −172.11	0.00–11.86	30	HPLC	Vietnamese
Azman et al. (2014)	*T. pallimaculatus*	–––	–––	–––	29.10	260.00	8	LC-MS/MS	East Malaysia waters
Lagocephalus sp.									
Azman et al. (2014)	*L. sceleratus*	30.00	–––	–––	24.70	0.51	8	LC-MS/MS	East Malaysia waters
El-sayed et al. (2003) [a]	*L. sceleratus*	ND–27.94	ND–165.44	ND–48.62	ND–54.12	ND–26.18	45	MBA	Northwestern Red Sea
Katikou et al. (2009) [a]	*L. sceleratus*	<1.10 −10.16	1.49 −239.32	6.31 −177.65	10.84 −87.53	<1.10–6.63	3	MBA	Aegean Sea
Rodriguez et al. (2012) [a]	*L. sceleratus*	<LOQ–3.47	0.47–46.30	1.09–37.60	4.20–44.15	<LOQ–1.40	3	LC-ESI-CID-MS/MS	Aegean Sea
Kosker et al. (2016)	*L. sceleratus*	ND–2.83	0.43–52.07	0.07–7.15	ND–46.18	0.13–3.43	16	LC-MS/MS	Northeastern Mediterranean Sea
Acar et al. (2017)	*L. sceleratus*	0.10–3.42	0.17–80.0	0.16–48.8	0.12–25.4	0.10–3.30	20	LC-MS/MS	Northeastern Mediterranean Sea
Rambla-Alegre et al. (2017)	*L. sceleratus*	0.7–0.9	20.00 −21.80	–––	2.3–4.6	1.2–1.8	1	LC-MS/MS and LC-HRMS	Western Mediterranean Sea

–––: No Data, ND: Not detected, NI: Number of individual, LOQ: Limit of quantitation.
[a] Katikou et al. (2009) and (Rodriguez et al., 2012) were used the same pufferfish samples. TTX levels reported by Katikou et al. (2009) and El-sayed et al. (2003) were converted from MU (Mouse Unit) to μg/g.

adaptation for pufferfish and that pufferfish demonstrate maximum toxicity during the breeding season in the spring and summer (El-Sayed et al., 2003; Hwang and Noguchi, 2007). However, Yu and Yu (2002) reported that pufferfish species called *Takifugu niphobles* and *Takifugu alboplumbeus* were not toxic during the breeding season. Similarly, findings from various scientific studies on *L. sceleratus* corroborate our current findings (Katikou et al., 2009; Rodriquez et al., 2012; Kosker et al., 2016). Of all seasons, Katikou et al. (2009) and Rodriguez et al. (2012) found the highest toxicity level in the samples caught in the winter season. Kosker et al. (2016) reported that pufferfish caught from the northeastern Mediterranean Sea showed a higher toxicity level in

the autumn and winter seasons. The results obtained in the current study indicated that just like the species *L. sceleratus* (Kosker et al., 2016), *T. flavimaculosus* demonstrated higher toxicity in the winter, which is not a breeding season. It is thought that higher TTX levels in the winter and autumn are specific to the fish in the Mediterranean Sea. For this reason, it is recommended that not only the toxin levels of pufferfish but also the different biological characteristics of *T. flavimaculosus* and other pufferfish species that spread every day through the Mediterranean Sea should be further studied in detail.

4. Conclusion

The highest TTX levels were found to be in the skin, liver, gonads, intestines and muscle tissue samples, in decreasing order. TTX levels of *T. flavimaculosus* caught from the Mediterranean Sea were higher in the winter and summer seasons. The results of this study indicate that *T. flavimaculosus* is a toxic pufferfish in the Mediterranean Sea and that its consumption poses the risk of severe poisoning. However, there is no legislation for the fishing of this species in some Mediterranean countries (Anonymous, 2016). Authorities should consider the risk posed by this species and establish the necessary regulatory action for its toxin. More studies should be carried out to decrease the negative effects of pufferfish on local fish species, fishing and public health.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2018.04.018.

Conflicts of interest

The authors declare that they have no competing interests.

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