PSP toxins profile in ascidian *Microcosmus vulgaris* (Heller, 1877) after human poisoning in Croatia (Adriatic Sea)

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**Abstract**

Toxins known to cause Paralytic Shellfish Poisoning (PSP) syndrome in humans that can have serious economic consequences for aquaculture were determined in ascidians of the genus *Microcosmus*. Significant concentrations of toxins were confirmed in all tested samples collected from the western coast of Istria Peninsula (Adriatic Sea, Croatia) when six people were poisoned following the consumption of fresh ascidians. Several species of bivalves that were under continuous monitoring had not accumulated PSP toxins although they were exposed to the same environmental conditions over the survey period. In the present study, HPLC-FLD with pre-column oxidation of PSP toxins has been carried out to provide evidence for the first human intoxication due to consumption of PSP toxic ascidians (*Microcosmus vulgaris*, Heller, 1877) harvested from the Adriatic Sea. Qualitative analysis established the presence of six PSP toxins: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), gonyautoxins 2 and 3 (GTX2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX2,3), gonyautoxin 5 (GTX5) and N-sulfocarbamoylgonyautoxins 1 and 2 (C1,2), while quantitative analysis suggested STX and GTX2,3 as dominant toxin types and the ones that contribute the most to the overall toxicity of these samples with concentrations near the regulatory limit.

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**1. Introduction**

Research on toxicity in different marine food cycles contributes to clarifying the role of Paralytic Shellfish Poisoning (PSP) toxins in the natural marine environment and their toxic effect on marine organisms and humans (Deeds et al., 2008; Vale, 2008). Detrimental changes in the marine environment possibly synergistically caused by global climate changes, increased pollution and eutrophication (enhanced utilization of coastal waters for aquaculture, as well as agricultural, urban and industrial run offs), tourism expansion, increased shipping activity (ballast waters) and overfishing are affecting biochemical processes in wild and aquacultured organisms. PSP toxins occur naturally in cyanobacteria, that can exist as plankton (Pomati et al., 2000), marine dinoflagellates (Harada et al., 1982) and some heterotrophic bacteria (Gallacher and Smith, 1999). Nevertheless, marine biotoxins affecting filter-feeding organisms increase human health risks (García et al., 2004; Yen et al., 2004; Ujević et al., 2012) as worldwide toxic events caused by harmful phytoplankton blooms result in accumulation of phytoplankton toxins in shellfish and other aquatic organisms (Deeds et al., 2008). Ecological consequences of these events include marine trophic structure alterations where filter-feeders function as vectors for transport of phycotoxins through the food web to higher level consumers such as fish, dolphins, whales and sea birds, often with resultant animal deaths (Anderson and White, 1992; Anderson et al., 2002; Landsberg, 2002; Deeds et al., 2008; Abbott et al., 2009) and subsequent poisoning of humans (Shumway, 1990; .
García et al., 2004). Chen and Chou (1998) found evidence for food chain transmission of PSP toxins from dinoflagellate Alexandrium minutum to carnivorous gastropods through a filter-feeding bivalve, while White (1979, 1980) concluded that Atlantic herring (Clupea harengus harengus) accumulated PSP toxins through ingestion of pteropods which had grazed on Alexandrium tamarense in the Bay of Fundy (Canada) that resulted in massive fish kills. Hallegraeff (1993) mentioned that there were 2000 cases of Paralytic Shellfish Poisoning with 15% human mortality reported every year throughout the world, while Relox and Bajarias (2003) noted 2122 PSP cases with 117 human deaths between 1983 and 2002 in the Philippines. In Alaska, between 1973 and 2008 at least 204 people were affected by PSP (Trainer, 2002), but Gessner and Schloss (1996) argue that this number probably under-represents the number of people impacted by a factor of 10 or even 30 due to under-reporting or misdiagnosis.

Microcosmus is a solitary ascidian, a sedentary animal that remains attached for its lifetime to a hard surface on the sea floor. Water enters through the incumbent siphon. Suspended material (plankton, algal spores, bacteria and stirred-up detritus) in the water is caught in the pharynx by mucus made from the endostyle and transported to digestive tract. Since ascidians mostly prefer protected seabed areas with good water circulation (Carballo, 2000; Goodbody, 2000) it is to be expected to be easily found at shellfish farms. Most of the ascidians are found to accumulate heavy metals (Philip et al., 2003), but there is also evidence of high levels of PSP toxin accumulation in ascidians that persists for a long period of time (Sekiguchi et al., 2001). Ascidians, especially the species Microcosmus sabatieri (Roule, 1885) and Microcosmus vulgaris (Heller, 1877) are considered a delicacy in North America, Japan, Korea, Chile, Peru, New Zealand, and also in Spain, Italy and France. Common names for M. vulgaris are: ‘grooved sea squirt’ or ‘sand violet’ (English), ‘violet de sable’ (French), ‘boniato de mar’ (Spanish) and ‘limone di mare’ or ‘uovo di mare’ (Italian). Microcosmus has a high nutritional value and is a good source of vitamins, minerals (especially iodine) and long chain n-3 polyunsaturated fatty acids (PUFA) that have hypolipidemic, anti-hypertensive, anti-inflammatory, anti-thrombotic and anti-arrhythmic properties (Stamatis et al., 2008; Stamatis et al., 2008; Stamatis et al., 2007). López-Rivera et al. (2009) found the presence of Domoic acid (causative toxin of Amnesic Shellfish Poisoning) in the ascidian Pyura chilensis (Molina, 1782) in the south of Chile and call for the inclusion of these organisms in sanitation programs for marine toxins.

1.1. Paralytic Shellfish Poisoning toxins

According to EFSA (2009) at least 30 compounds belong to the Paralytic Shellfish Toxin family. These can be divided into four groups based on their side chain chemical structure. The most toxic carbamate group comprises saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 1–4 (GTX1–4); the least toxic, N-sulphocarbamoyl group consists of gonyautoxins 5 and 6 (GTX5,6) and N-sulphocarbamoylgonyautoxins 1–4 (C1–4); the decarbamoyl group with intermediate toxicity includes decarbamoylsaxitoxin (dcSTX), decarbamoylgonyautoxins 1–4 (dcGTX1–4) and decarbamoylneosaxitoxin (dcNEO); in the deoxydecarbamoyl group there are deoxydecarbamoylsaxitoxin (doSTX), deoxydecarbamoylneosaxitoxin (doNEO) and deoxydecarbamoylgonyautoxins 1–3 (doGTX1–3) toxin types (Lagos and Andrino, 2000; FAO, 2004). Specific toxicities of each analogue within the PSP group originate from different structures of the toxins. STX is the most potent within the group, while the toxicity of other congeners is compared with pure STX and expressed as STX equivalents (Shimizu, 1987). Understanding of biotoxin conversions in marine organisms is critically important since toxins of low potency in dinoflagellate cells can be converted to highly potent toxins through bacterial, enzymatic or pH mediated activity (Shimizu and Yoshioka, 1981; Sullivan et al., 1983), thereby affecting the net toxicity.

1.2. The occurrence of PSP toxic species and shellfish toxicity in the Adriatic Sea

In the spring of 1994, an A. minutum bloom caused the first recorded PSP toxicity in the Italian north-west Adriatic region (Honsell et al., 1996). There was also suspected PSP toxicity presence in the central part of the eastern Adriatic Sea (Kastela Bay) in 1994 through 1996, based on phytoplankton cell numbers (Orhanovic et al., 1996; Marasovic et al., 1998; Pavela-Vranic and Marasovic, 2004). More recently, Ujević et al. (2012) recorded and quantified PSP shellfish toxicity for the first time in Croatian shellfish farms with a few samples exceeding the regulatory limit set by the European Commission. That first PSP occurring period was represented mainly by STX and GTX2,3 in farmed mussels (Mytilus galloprovincialis) from the northern Adriatic Sea during winter and spring of 2009 (from January through April). Besides this period of toxicity, a sporadic case of dcSTX occurring in very low concentrations (below 35 μg kg⁻¹) was in late 2008 detected in shellfish from the southern Adriatic Sea (Ujević et al., 2012). For Mediterranean waters, there are more PSP toxic dinoflagellate occurrences recorded than reported PSP contaminated organisms (Ciminiello et al., 2000; Vila et al., 2001; Lilly et al., 2002; Montresor et al., 2004).

On December 16, 2008 there were six recorded cases of human poisoning (one woman and five men from Rovinj, Croatia) after consumption of fresh ascidians caught in fishing nets. Traditionally in this region the ascidians are eaten raw and are considered a delicacy. Thirty minutes after consumption, all six individuals reported the same symptoms of dizziness, vomiting, weakness in the legs and blurred vision. They were admitted to the emergency room and within a few hours all the symptoms and clinical problems had disappeared (Bašić-Palković, 2008).

2. Materials and methods

2.1. Study area and sampling frequency

During December 2008 through May 2009, 15 samples of the ascidian M. vulgaris (grooved sea squirt) were sampled at shellfish harvesting stations S1, 2 and 3 along the West Istrian coast (northern Adriatic Sea; Fig. 1) that
were suspected to be PSP toxic when six people were poisoned after their consumption (Table 1). Sampling was carried out according to the field weather conditions.

### 2.2. HPLC-FLD method

High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) method (2005.06 AOAC; Lawrence et al., 2005) includes a pre-chromatographic oxidation of PSP toxins with hydrogen peroxide and periodate solution as oxidants.

### 2.3. Chemicals, reagents and standards

Reagents and chemicals used were of HPLC grade obtained from Sigma-Aldrich, and the following solutions were prepared in deionized (DI) water: glacial acetic acid (1%, 0.1 mM and 0.1 M aq), acetonitrile (5% aq), ammonium acetate (0.01 M aq), ammonium formate (0.1 M and 0.3 M aq), disodium hydrogen phosphate (Na2HPO4, 0.3 M aq), hydrogen peroxide (10% aq), methanol, sodium chloride (0.05 M and 0.3 M aq), sodium hydroxide (0.2 M and 1 M aq), periodic acid (0.03 M aq). Mobile phases consisted of (A) 0.1 M ammonium formate and (B) 0.1 M ammonium formate in 5% acetonitrile, and 0.1 M acetic acid was used to adjust pH to 6.0. Certified standard calibration solutions for analysis of PSP toxins were obtained from the Certified Reference Materials Program (CRMP) of the Institute for Marine Bioscience (IMB), National Research Council of Canada (Halifax, Canada) and included NRC CRM: STX-e, dcSTX, NEO-c, dcNEO-b, GTX1,4-c, GTX2,3-c, dcGTX2,3-b, GTX5-b and C1,2.

### 2.4. Extraction and cleanup

Ascidians (Fig. 2a) were washed externally, opened, soft tissue (Fig. 2b) removed and subsequently homogenized (100 g) in a glass blender and frozen (−20 °C) until required for PSP analysis. Paralytic toxins were extracted following the Association of Official Analytical Chemists Official Method (AOAC) 2005.06. Aliquots (5.00 ± 0.10 g) of each sample were weighed into 50 mL polypropylene centrifuge tubes, extracted, vortexed, heated in a water bath (100 °C) for 5 min, remixed and centrifuged for 40 min at 4500 rpm (3600 × g; 20 °C). The supernatant was carefully decanted into 15 mL polypropylene tubes. The pellet was re-extracted, vortexed and centrifuged again. Two supernatants were pooled and diluted to a final volume of 10 mL with DI water. Tubes were stored at 4 °C until analysed.

The purification procedure was based on Lawrence et al. (2005). Varian, Bond Elut C-18 (500 mg/6 mL) SPE cartridges (Varian, USA) were appropriately conditioned with methanol and DI water, and used to produce a C-18 cleaned extract that should contain all PSP toxins. 1 mL of sample extract was run through an SPE C-18 cartridge, the eluate collected, then the cartridge was eluted with DI water, the eluates combined, pH corrected to 6.5 and volume adjusted to 4 mL. Aliquots were submitted to oxidation with peroxide and periodate oxidants prior to HPLC analyses. Second column purification was carried out on extracts with N-1-hydroxylated PSP toxins after C-18 cleanup. Varian Bond Elut CBA (500 mg/3 mL) ion exchange cartridges (Varian, USA) were conditioned by washing with aqueous ammonium acetate and a 2 mL aliquot of C-18 purified extract was added to each cartridge and the eluate collected. After every addition of the adequate eluent, DI water was run through the cartridge and volumes adjusted. First eluted was fraction No. 1 that, if all present, should contain C1-4 toxins. The same CBA cartridge was treated again with 0.05 M NaCl and this tube was assumed to contain fraction No. 2 with toxins: GTX1–6 and dcGTX2,3. Additional elution with 0.3 M NaCl resulted in fraction No. 3 that should contain toxins STX, dcSTX, NEO and dcNEO.

A matrix modifier was prepared from PSP-free ascidian soft tissue extract. After the SPE C-18 purification, as previously described, pH was adjusted to 6.5, filtered and left to precipitate co-extracted material for two days. Periodate and peroxide oxidation of the matrix was done to ensure the absence of toxins before being used for periodate analysis of the samples, as it enhances the fluorescent...
intensity of periodate oxidation products of N-1-hydroxylated PSP toxins (Lawrence et al., 2005). An aliquot of the extract mixed with the ascidian matrix modifier was analysed without oxidation to ensure the absence of naturally fluorescent compounds. Aliquots of all fractions were oxidized by peroxide (hydrogen peroxide) and periodate (periodic acid, ammonium formate and disodium hydrogen phosphate) solutions for HPLC pre-column analysis. Oxidative conversion of PSP toxins to fluorescent derivatives is necessary in order to perform HPLC analysis. Five point calibration curves were obtained by pre-column peroxide and periodate oxidations of four standard PSP toxin mixes (Ujević et al., 2012).

2.5. HPLC toxin analysis

The PSP ascidian extracts were analysed using a Varian ProStar 230 analytical solvent system coupled with a ProStar 363 fluorescence detector (ex. 340 nm; em. 390 nm), ProStar 410 autosampler and ProStar 500 column valve module. Separation of toxin oxidation products was carried on reversed-phase C18 column (Restek, Pinnacle II 150 × 4.6 mm, 5 μm particle size) protected by a guard cartridge Pinnacle II C18, 20 × 4.0 mm (Varian, SAD). Column temperature was kept at 30 ºC, run time 15.00 min and partial loopfill volume set to 25 μL for peroxide and 50 μL for periodate oxidized samples. Data collection and result treatment were performed with the Varian Star Chromatography software. The PSP toxin profile of ascidian samples was identified by comparing with chromatograms of standard solutions.

As the fluorescence is compound dependent it requires individual toxin calibration and Toxicity Equivalence Factors (TEFs) to calculate the total μg STX dihydrochloride equivalents per 100 g of sample. Specific toxicity factors of PSP toxins by EFSA (2009) were used for calculations. According to Lawrence’s pre-column HPLC-FLD method, toxins that co-elute are determined together (GTX1 and GTX4; GTX2 and GTX3; dcGTX2 and dcGTX3; C1 and C2) by choosing the highest toxicity factor of the two co-eluted compounds. Total PSP toxicity was calculated by summing the toxicity contribution of each quantified toxin expressed as μg STX eq. kg⁻¹. Limit of detection (LOD) was determined based on a 3:1 signal-to-noise ratio, by comparing measured signals from samples spiked with known low concentrations of PSP toxin standards with those of blank samples. Detection limits were: STX = 1.1, dcSTX = 1.9, GTX2,3 = 2.1, dcGTX2,3 = 11, GTX5 = 0.4 and C1,2 = 4.2 μg kg⁻¹.

3. Results

3.1. Toxin profile

In the present study, ascidian samples were subjected to HPLC-FLD analysis in order to detect the presence of the following PSP toxin types: STX, dcSTX, NEO, dcNEO, GTX1,4, GTX2,3, dcGTX2,3, GTX5 and C1,2.

As evident from Fig. 3, S1 had the most diverse profile of all the stations considered in this study. There were STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 and C1,2 toxins present during this period, with GTX2,3 and dcGTX2,3 as the most abundant toxins in the samples. GTX2,3 exhibited the highest concentration per sample (655.72 μg kg⁻¹), followed by GTX5 with 585.21 μg kg⁻¹. DcGTX2,3 was detected in February and April with concentrations of 389.18 μg kg⁻¹ and 348.60 μg kg⁻¹, respectively. The lowest detected concentrations were those of dcSTX, within the range of 17.30–78.11 μg kg⁻¹.

Fig. 4 shows the total PSP toxicity found in ascidians at station S1 calculated by summing the toxicity contribution of each quantified toxin (μg STX eq. kg⁻¹). The major contributor to PSP toxicity on this station was GTX2,3, as the least toxic representative of the most toxic carbamate group. DcGTX2,3 was the second toxicity contributor, as it is of intermediate toxicity, but was in high concentrations during this period. GTX5 has an extremely low toxicity factor, and yet it contributed to the overall toxicity because of its high concentration in one sample. C1,2 was detected only in April at a concentration of 326.20 μg kg⁻¹, and as it is an N-sulfo carbamoyl toxin, it contributed very little to the total sample toxicity. A sample from February 2009 reached (806.95 μg STX eq. kg⁻¹), the regulatory limit of 800 μg STX eq. kg⁻¹.

During the same period, ascidian samples from station S2 showed PSP toxicity with STX, dcSTX, GTX2,3 and C1,2 toxins present (Figs. 5 and 6). STX was by far the most dominant over other toxins found in the analysed samples, with sample concentrations of 193.85 μg kg⁻¹, 197.73 μg kg⁻¹ and 549.51 μg kg⁻¹, in December 2008, March and April 2009, respectively. DcSTX was present in all the analysed samples from this station, but in low concentrations, from an almost undetectable 9.90 μg kg⁻¹–63.32 μg kg⁻¹, at the end of March when it was the only toxin detected in the sample. There was only one episode of GTX2,3 presence at a concentration of 427.40 μg kg⁻¹ during March 2009, when it was found in combination with STX and dcSTX. C1,2 was detected only once at this station (353.85 μg kg⁻¹). Fig. 6 shows STX as the dominant contributor to the overall toxicity on this station, as it has the highest toxicity factor and was detected in high concentrations during the investigated period.

At station S3, STX and GTX2,3 were the two dominating derivatives with concentration ranges of 345.17–653.17 μg kg⁻¹ and 150.96–373.69 μg kg⁻¹, respectively (Fig. 7). With their relatively high concentrations and very high toxicity factors, the overall toxicity of the station was approaching the regulatory limit in this period (Fig. 8).
highest total toxicities for this station were exhibited in samples collected at the end of January and beginning of February 2009. DcSTX was always in relatively low concentrations (9.21–96.50 µg kg⁻¹). GTX5 was found in only one ascidian sample from this station in mid-December 2008, with a concentration of 41.94 µg kg⁻¹ in addition to dcSTX (9.21 µg kg⁻¹) and GTX2,3 (249.77 µg kg⁻¹) that represented the ascidian toxin profile and the amount of toxins the day after contaminated ascidians caused the moderate poisoning of six people. C1,2 was recorded only in April 2009 (258.57 µg kg⁻¹) in combination with dcSTX (96.50 µg kg⁻¹). Generally, as GTX5 and C1,2 are N-sulfocarbamoyl toxins and were in low concentrations, their toxicity contribution was negligible.

4. Discussion

The presence of PSP toxins in the northern part of the Croatian Adriatic Sea, where toxic *Alexandrium* species were only sporadically present (Boni et al., 1992; Honse et al., 1996; Ujević et al., 2012), was for the first time revealed in the ascidian *M. vulgaris*. Samples of *M. vulgaris* soft tissue from the three stations along the west coast of Istrian peninsula (Fig. 1) were analysed by AOAC Official Method 2005.06 (Lawrence et al., 2005) after six people experienced dizziness, vomiting, weakness in the legs and vision problems as a consequence of ingesting raw ascidians caught in fishing nets (Basić-Palković, 2008).

As shown in Figs. 3–8, STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 and C1,2 represent the ascidian PSP toxin profile determined in samples from the West Istrian coast (Adriatic Sea, Croatia) at stations S1, 2 and 3 during the period from December 2008 through May 2009. These toxin types were found in different combinations in analysed samples, while toxins GTX1,4, NEO and dcNEO were not detected during this period. There is also a possibility of undetected presence of C3,4 and GTX6 toxins since their standard solutions were not commercially available at the time of conducting this study. Ascidian samples harvested at station S3 the day after reported human poisoning and analysed by HPLC-FLD pre-chromatographic oxidation method exhibited toxicity (167.26 µg STX eq. kg⁻¹) five times lower than the maximum permitted level according to EU and Croatian legislatives (800 µg STX eq. kg⁻¹). Moreover, on the same harvesting locations and during the same period several shellfish species were tested by Official Method 959.08 (AOAC, 1990) for the presence of Paralytic Shellfish Poisoning (PSP) toxins, as a part of a continuous monitoring programme, and the results showed that these toxins were not found in natural populations of the following filtering bivalves: proteus scallop (*Flexopecten proteus*), Mediterranean scallop (*Pecten jacobaeus*) and European flat oyster (*Ostrea edulis*), from stations S1, 2 and 3, respectively (Ujević et al., 2012). These results may be compared to those of Sekiguchi et al. (2001) where ascidians (*Halocynthia roretzi*) accumulated about two times more toxins.

![Fig. 2. Ascidian Microcosmus vulgaris; whole body (a) and soft tissue (b).](image_url)

![Fig. 3. Toxin concentrations in ascidian samples at station S1 (West Istrian area) during 2009.](image_url)
than other bivalves (scallop, mussel, oyster, short-necked clam), although they ingested equal amounts of A. tamarense. When compared to the paper of Ujević et al. (2012) that revealed the first PSP toxin profile for mussels M. galloprovincialis from the Croatian part of the Adriatic Sea, and found mussels from the West Istrian coast that were affected during the same period from January through April 2009 by STX, GTX2,3 and dcSTX, our analysis of ascidians showed a more diverse toxin profile than the mussels, but toxins were in lower concentrations. A small content of N-sulfocarbamoyl (C1,2 and GTX5) and decarbamoyl (dcGTX2,3) toxins was observed in ascidian, but not in mussel tissue (Ujević et al., 2012). C1,2 was found in April 2009 at all three stations (S1, 2 and 3) along the West Istrian coast in concentrations of 326.21 µg kg⁻¹, 353.85 µg kg⁻¹ and 258.57 µg kg⁻¹, respectively. GTX5 was detected only twice in all the analysed samples with concentrations of 41.94 µg kg⁻¹ at S3 and 585.21 µg kg⁻¹ at S1, in December 2008 and February 2009, respectively. These are the N-sulfocarbamoyl toxins that make very low contribution to the total sample toxicity because of their extremely low toxicity factor 0.01, according to EFSA (2009). Decarbamoyl dcGTX2,3 was only evident in two ascidian samples from station S1 (February and April) with relatively small contribution to the overall toxicity (relative toxicity factors of 0.2 and 0.4; EFSA, 2009; Fig. 8.), while dcSTX was detected in almost all the samples with concentrations not exceeding 96.50 µg kg⁻¹. The most dominant toxins at all three stations were GTX2,3 and STX, with concentration ranges from 150.96 to 655.72 µg kg⁻¹ and 123.85–653.17 µg kg⁻¹, respectively, that contributed the most to the overall toxicity of the ascidian samples.

A. minutum and A. tamarense have already been reported in coastal areas of the Adriatic Sea (Boni et al., 1992; Honsell et al., 1996; Ujević et al., 2012) as potential causative organisms of PSP in shellfish. It should be mentioned that Xie et al. (2013) and Chou et al. (2004) found GTX1–4 to be the only PSP toxins present, while Chang et al. (1997) discovered NeoSTX to be the dominant one, but also STX and GTX1–4 in A. minutum profiles. In the study of Asakawa et al. (1995), GTX4 and C2 are identified as major contributors to A. tamarense toxin profile from Hiroshima Bay in Japan. Kwong et al. (2006) recorded a higher content of carbamate toxins in mussels, although they ingested dinoflagellates (Alexandrium fundyense) that had N-sulfocarbamoyl as a more dominant group in their toxin profile. Although we do not suspect A. fundyense to be the causative organism for presently reported PSP toxicity, this could be one of the possible explanations for the ascidian PSP profile revealed in this study where the major toxins were car bamates (STX and GTX2,3) detected in more than half of contaminated ascidian samples (Fig. 9). However, the same culture strains of phytoplankton may exhibit different PSP profiles across both local and regional-scale spatial range, which complicates the comparison (Ichimi et al., 2002). Ciminiello et al. (1995) documented that mussels (M. galloprovincialis) from the north-west Adriatic Sea contain low levels of GTX2,3 from A. minutum. Pistocchi et al. (2012) came to the same conclusion and Abouabdellah et al. (2008) concluded the same for mussels from southern Atlantic coasts of Morocco, while Alvarez et al. (2009) noted two PSP episodes in Chile with mostly STX and GTX2,3 toxins present in shellfish tissue. However, it has to be noted that bivalves also become toxic by ingesting Gymnodinium, as well as Pyrodinium dinoflagellates. Bivalves contaminated by Gymnodinium reveal profiles with N-sulfocarbamoyl and decarbamoyl toxins: dcGTX2,3, C1,2, dcSTX and GTX5 (Rodrigues et al., 2012).

Information regarding ascidian toxicity is scarce. As ascidians are suspension-feeding organisms that ingest considerable amounts of plankton, suspended organic matter and particles mostly in the 0.5–2 µm size range (Riisgård and Larsen, 2000; Tatián et al., 2002) there is a risk of accumulating high levels of toxins in their tissues. In addition, toxic dinoflagellates can form resting cysts that, some authors argue, might contain a ten to thousand fold higher saxitoxin concentration than the mobile cells (White and Lewis, 1982). These cells sink to the bottom of the sea and accumulate there until beneficial growth conditions appear. Hamer et al. (2001) also considered a
hazard of importing toxic cysts or vegetative cells of alien organisms via ship ballast waters. Recorded toxicity during the December to May period of this study is a logical extension of enhanced mixing and resuspension of benthic organic matter that ensures higher nutrient supply during the winter months. The majority of PSP toxin contaminated ascidian samples were from the January to April period which is in agreement with periodicity found for the Mediterranean coast (Taleb et al., 2001), although Mons et al. (1998) argue that dinoflagellates develop at relatively high temperatures and abundant sunlight, therefore in Europe cases of human intoxication and mortality occur mainly between May and November. Potency of PSP toxins accumulated in filter feeding organisms is well demonstrated in the example of lethal poisoning of two fishermen in Chile, in 2002, three to four hours after consumption of contaminated ribbed mussels. These mussels reached a toxicity of 8575 µg of STX eq. kg⁻¹ in shellfish meat measured by mouse bioassay (García et al., 2004). Undoubtedly, very important is surveillance of PSP toxins in lower levels of marine food webs that are the vectors of PSP toxin transmission to higher consumers and ultimately human populations. Our investigation revealed a new vector of PSP toxins in the Adriatic Sea, suggesting that consumption of contaminated ascidians poses an additional route for causing shellfish poisoning in consumers.

5. Conclusions

PSP-contaminated ascidians from the West Istrian area of the Adriatic Sea revealed complex toxin profiles including the most potent carbamates STX and GTX2,3, less potent decarbamoyls dcGTX2,3 and dcSTX, and the least toxic N-sulfo carbamoyl group represented by GTX5 and C1,2. Six people suffered mild symptoms of PSP, as dizziness, vomiting, weakness in the legs and vision problems after eating fresh ascidians. Even though they are currently not commercially significant, ascidians are a valuable additional source of seafood. Investigations of biotoxin occurrences in different marine organisms contributes to clarifying their role and fate in the marine food web, as there may be variability of toxin composition and their metabolic transformation in different organisms producing modified forms of the toxins and possibly increasing their toxicity. There may be expected to be an increased number of toxic episodes throughout the world as red tide phytoplankton can be stimulated by burgeoning coastal pollution and aquaculture activity in coastal waters that can amplify nutrient enrichment. Nevertheless, many red tide species form resting stages, spores or cysts that can persist for a long time and may be transported by the ballast water of ships or dispersed within a region by currents, upwellings, tides and storms. Possible spreading of Paralytic Shellfish

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**Fig. 7.** Toxin concentrations in ascidian samples at station S3 (West Istrian area) during 2008–2009 sampling period. Note: 17th December 2008, is the day after human poisoning.

**Fig. 8.** Total PSP toxicity (µg STX eq. kg⁻¹) in ascidian samples from station S3.

**Fig. 9.** Mass percentage of PSP toxins in ascidian tissue according to all obtained results, STX (n = 7), GTX2,3 (n = 8), C1,2 (n = 3), dcGTX2,3 (n = 2), GTX5 (n = 2) and dcSTX (n = 13).
Poisoning to other regions of the Adriatic Sea may have serious health and economic consequences.

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Conflict of interest

The authors declare that there are no conflict of interests.

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