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# 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.



## 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;

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García et al., 2004). Chen and Chou (1998) found evidence for food chain transmission of PSP toxins from dinoflagellate *Alexandrium minutum* to carnivorous gastropod 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 incurrent and exits through the excurrent 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 (Philp 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, antihypertensive, anti-inflammatory, antithrombotic and antiarrhythmic properties (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-sulfocarbamoyl group consists of gonyautoxin 5 and 6 (GTX5,6) and N-sulfocarbamoylgonyautoxin 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 deoxydecarbamoylgonyautoxin 1–3 (doGTX1–3) toxin types (Lagos and Andrinolo, 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 (Kaštela Bay) in 1994 through 1996, based on phytoplankton cell numbers (Orhanović et al., 1996; Marasović et al., 1998; Pavela-Vrančić and Marasović, 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