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Phytoplankton Toxins in the Central Adriatic Sea

Stjepan Orhanović,[a] *Živana Ninčević,*[b] *Ivana Marasović,*[b]* *and Maja Pavela-Vrančić*[a]

[a]*University of Split, Faculty of Natural Sciences and Arts, Department of Chemistry, N. Tesle 12, 58000 Split, Croatia.*

[b]*Institute of Oceanography and Fisheries, etalite Ivana Meštrovića 63, 58000 Split, Croatia.*

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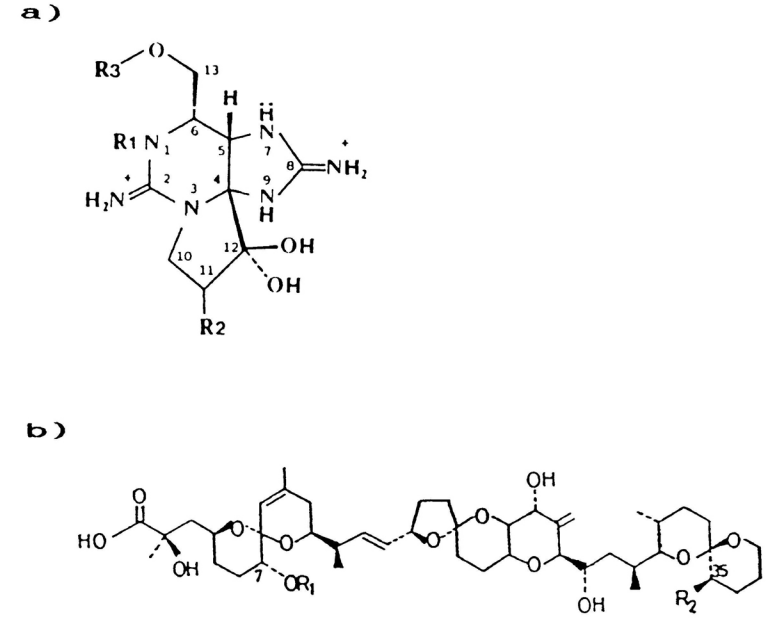
Toxin analysis of shellfish from the Central Adriatic Sea, for which no previous studies exist, have been carried out. Investigations, using the bio-assay and HPLC-directed analysis, led to identification of the toxins responsible for diarrheic shellfish poisoning (DSP). Okadaic acid (OA), a C_{38} polyether fatty acid derivative, and its 35-methyl derivative dinophysistoxin-1 (DTX-1) were identified as the principal toxin components. DSP toxins were detected in mussels sampled in the Kaštela Bay. Results are presented along with the phytoplankton structure during the outbreak of DSP toxicity.

INTRODUCTION

A wide range of phytoplankton species have been found to produce a variety of toxic metabolites responsible for shellfish poisoning and, consequently, human intoxication. It has been reported that shellfish harvested during phytoplankton blooms may cause severe human illness or even death.[1] The threat to public health has been aggravated by a recent discovery of the potential tumor promoting activity of okadaic acid (OA) and dinophysistoxin-1 (DTX-1), polyether toxins produced by dinoflagellates of genus *Dinophysis* and *Prorocentrum*.[2,3]

Poisoning by metabolites from marine algae has been classified into paralytic (PSP), diarrheic (DSP), neurotoxic (NSP) and amnesic (ASP) shell-

* Author to whom correspondence should be addressed.



Scheme 1. Chemical structures of the representative examples of phytoplankton toxins: (a) PSP: saxitoxin $R_1 = R_2 = -H$, $R_3 = -CONH_2$, neosaxitoxin $R_1 = -OH$, $R_2 = -H$, $R_3 = -CONH_2$, gonyautoxin $R_1 = -OH$, $R_2 = -OSO_3$, $R_3 = -CONH_2$, and decarbamoylsaxitoxin $R_1 = R_2 = R_3 = -H$, (b) DSP: okadaic acid $R_1 = R_2 = -H$, DTX-1 $R_1 = -H$, $R_2 = -CH_3$, and DTX-3 $R_1 = -acyl(C_{14}\text{-}C_{22})$ $R_2 = -CH_3$

fish poisoning. PSP toxins are potent neurotoxins causing paralysis and death by asphyxiation.[4] However, they are also important pharmacological tools used as sodium channel blockers.[5,6] A number of compounds structurally related to guanidine, such as saxitoxin, neosaxitoxin, gonyautoxins, and several *N*-sulfocarbamoyl compounds (Scheme 1a), have been isolated from various species of genera *Alexandrium* and *Gymnodium*. In contaminated shellfish, additional decarbamoyl derivatives could be found, arising from enzymatic and/or bacterial conversion in the mollusk's digestive system.

Consumption of shellfish contaminated with DSP causes gastrointestinal disturbances. The respective toxins are produced most frequently by dinoflagellates of genera *Dinophysis* and *Prorocentrum*.[4] Their structure resembles the structure of polyether ionophores from terrestrial microorganisms, characterized by oxolane and oxane rings.[8] This class of toxins comprises eight polyether compounds divided into two groups, acidic and neutral. The acidic group includes okadaic acid and dinophysistoxin-1 and 3 (Scheme 1b), while five polyether lactones, named pectenotoxins (PTX1-5),

constitute the neutral group.[5] Okadaic acid is a C_{38} polyether fatty acid derivative while the chemical structures of DTX-1 and DTX-3 have been identified as (S)-35-methylokadaic acid and its 7-O-acyl derivative, respectively. It has been suggested that in DTX-1 most of the backbone carbon chain is built up by direct condensation of acetate units, like in classical polyketides.[8] Pectenotoxins differ in having a longer carbon backbone (C_{40}), a C_{33} lactone ring and a novel dioxabicyclo moiety.[9] Two new toxins have been recently isolated from different strains of *Prorocentrum lima*, and their structures identified as the ester derivatives of okadaic acid, 7-hydroxy-2,4-dimethyl-hepta-2(*E*),4(*E*)-dienylokadaate and the 7-hydroxy-4-methyl-2-methylene-hept-4(*E*)-enyl ester.[10] This class of polyether toxins has received wide interest not only for their unique structure, but also for their pharmacological properties. Okadaic acid has been found to be a highly selective inhibitor of protein phosphatases and a potent tumor promoting agent.

Neurotoxic shellfish poisoning (NSP), caused by toxins produced by the dinoflagellate *Ptychodiscus brevis*, exhibits symptoms similar to PSP but in a milder form. Three classes of toxins causing neurotoxic shellfish poisoning have been established: brevetoxin A type, brevetoxin B type, and hemibrevetoxin (HemiBTX).[11] Amnesic shellfish poisoning (ASP) is a result of intoxication with domoic acid, a toxic metabolite produced by the pennate diatom *Nitzchia pungens f. multiseries*. Domoic acid is an amino acid derivative acting on the kainate receptors of the central nervous system and causing symptoms such as nausea and diarrhea, sometimes followed by confusion, disorientation, loss of memory and coma.[4] The potent neurological activity has attracted interest in pharmacological studies in recent years.

Most of the toxin producing organisms belong to dinoflagellates, although toxin producing species could be found in all other algal groups. Since phytoplankton toxins represent a serious threat to human health, as well as to fish and shellfish farming, monitoring programs have been established in many countries. Investigations of the toxin structure, their biological function, and the biosynthetic pathway are currently in progress.

Shellfish toxicity in the Adriatic Sea was first recorded in the summer of 1989. The presence of dinoflagellates of genera *Dinophysis* and *Prorocentrum* in the Northern Adriatic resulted in shellfish intoxication with DSP.[12–15] The same occurrence, although less intensive, was observed again in 1991.[16] Paralytic shellfish poisoning occurred in the waters of the Northern Adriatic in the spring of 1994 during the bloom of *Alexandrium minutum*.[17] The most alarming situation in the Central Adriatic was registered in the eutrophic Kaštela bay. Thist semi-enclosed bay is heavily polluted by land sources, both of domestic and industrial origin.[18] Since 1980, a red tide has been regularly occurring in the Kaštela Bay every summer. *Prorocentrum minimmum* and *Gonyaulax polyedra*, species, suspected of toxicity, are responsible for the red tide blooms occurring in this area.[19] Additionally, sev-

eral other known and potentially toxic phytoplankton species have been recorded in the Central Adriatic.[20] The health and economic implications of these events prompted the study of this phenomenon. Samples were collected to investigate the toxin profiles and the results interpreted in terms of the composition of the phytoplankton community.

EXPERIMENTAL

Materials

Acetone, diethylether, and HCl were products of Kemika (Zagreb, Croatia). Tween 60, acetonitrile (HPLC grade), and tetrahydrofurane were purchased from Merck (Darmstadt, Germany). 4-Bromomethyl-7-metoxycoumarin was from Regis Chemical Co. (Morton Grove, Illinois). *N,N*-Diisopropylethylamine is a product of Sigma (St. Louis, MO).

Methods

Samples: Samples of wild *Mytilus galloprovincialis* used for the DSP mouse bio-assay were harvested on August 23, 1993 from a localized site in the eastern part of the Kaštela Bay during an intensive red tide bloom of *Gonyaulax polyedra* ($> 10^7$ cells dm^{-3}), then again a month after the bloom on September 20,and in 1994 during the spring diatom bloom on May 25. Samples for the HPLC analysis were collected in the late summer of 1994, on September 11, and 28. Samples of cultivated mussels were collected on July 21, on two sites in the Mali Ston Bay and tested for PSP and DSP toxicity using the mouse bio-assay.

Phytoplankton analysis: Sea samples for phytoplankton analysis were collected simultaneously with shellfish samples. In order to obtain better insight into the processes determining shellfish toxicity, an additional analysis of the phytoplankton community was performed on August 22, 1994. The analysis of the phytoplankton community in the waters surrounding the sampling sites was performed using the Utermöl method.[21] Water samples were collected with Nansen reversing bottles. Samples (250 ml) for determination of the phytoplankton concentration were preserved in a formaldehyde (2%) solution. Counting of the organisms was performed in a subsample (25 ml) after a sedimentation period of 24 hrs using an Opton inverted microscope (magnification 400 ×). Live material was analyzed using the Olympus IMT-2 microscope with a Nomarski differential interference contrast attachment.

DSP toxicity test: Shellfish hepatopancreases (20 g) were extracted with acetone and diethylether according to the method of Yasumoto *et al.*[22] Evaporated extracts were dissolved in Tween 60 (1%), followed by injection of the diluted sample (1 ml) into the standardized mouse (strain BALB/C, weight limits 18–20 g) intraperitoneally. Administration of 4 μg okadaic acid per mouse results in death after approximately 5 hours. Three parallel tests were performed, and the reaction of the mice was monitored for 24 hours or until death. A test was regarded as positive, and the shellfish as toxic for humans, if at least two mice died within 24 hours, and if the mean time of death was shorter than 5 hours. The mean time of death shorter than 24 hours and longer than 5 hours indicated the presence of the toxin, however at a level not endangering human health.

PSP toxicity test: The PSP toxins were extracted according to the method proposed by the Association of Official Analytical Chemists (A.O.A.C.). The homogenized shellfish tissue was treated by boiling in acidified water. After centrifugation, the diluted extract was injected intraperitoneally into the mouse (strain BALB/C, weight limit 19–21 g). The animal was observed for one hour. The test was performed with at least three mice, and the mean time of death was evaluated. The toxicity of the sample was determined using the table of standard values.

HPLC determination of DSP toxins: Samples of *M. galloprovincialis*, collected in the Kaštela Bay on September 11 and 28, 1994, during and after the bloom of *Gonyulax polyedra*, were examined according to the method of Hummert *et al.*,[23] based on the HPLC separation of fluorescently derivatized DSP toxins. Shellfish hepatopancreases were homogenized and extracted with dichloromethane, and subsequently purified on silica gel. The extract (1 ml) was evaporated under nitrogen to dryness. *N,N*-Diisopropylethylamine (10 ml), acetone (140 ml) and the derivatization reagent (50 ml), containing 4-bromomethyl-7-metoxycoumarin (BrMmc, 1.5 mg per 1 ml of acetone), were added to the dry sample, followed by ultrasonication (2 min) and heating for two hours at 55 °C in the dark. The sample (10–20 ml) was applied onto a Supelcosil LC-18-DB-5 μ column and eluted isocratically with acetonitrile-water-tetrahydrofurane (60:38:2) at a flow rate of 1 ml/min. Sample components were detected with a fluorescence detector at extinction and emission wavelengths of 325 and 390 nm, respectively. BrMmc-derivatives of OA and DTX-1 from commercial sources (Waco Pure Chemicals Industries, Ltd, Osaka, Japan, and Calbiochem Corporation, La Jolla, California) were employed as standards with a retention time (R_t) of 15.49 and 32.88 min for the OA and the DTX-1 derivative, respectively. The minimum detection limit was 5 ng of the toxin.

RESULTS

Samples of *Mytilus galloprovincialis*, a shellfish with immense filtration capacity, were analyzed in an attempt to detect the presence of phytoplankton toxins in the central region of the Adriatic Sea. The shellfish were collected in the eastern part of the Kaštela Bay, an area known as very eutrophic and highly polluted.[18] Fish kills, often occurring in this area, are due to anoxia caused by the red tide bloom, followed by bacterial decomposition of accumulated organic matter.[19,20,24–28] Samples were collected in the summer of 1993 during an intensive red tide bloom of *Gonyaulax polyedra* (> 10^7 cells dm^{-3}), in 1994 during the spring diatom bloom, and in the late summer of 1994 during massive fish kills after the *Gonyaulax* bloom. Two samples were collected in unpolluted waters of the Mali Ston Bay in the summer of 1994.

The analysis was performed using the mouse bio-assay and, conclusively, high performance liquid chromatography. The results of the mouse bio-assay for the DSP toxins are presented in Table I. Shellfish samples were harvested from a localized area. The sample collected in the eastern part of the Kaštela Bay in August 1993 caused all three mice to die in an average time of 7 hrs and 20 min, showing the presence of the DSP toxin, though at a

TABLE I

The mouse bio-assay for DSP toxicity

Date	Sampling station	Mouse. No.	Time of death
23/08/1993	Kaštela Bay	1	5h 10 min
		2	7h 5 min
		3	9h 45 min
20/09/1993	Kaštela Bay	1	n.d.
		2	n.d.
		3	n.d.
25/05/1994	Kaštela Bay	1	22h 30min
		2	n.d.
		3	n.d.
21/07/1994	Mali Ston Bay (Bistrina region)	1	n.d.
		2	n.d.
		3	n.d.
21/07/1997	Mali Ston Bay	1	n.d.
		2	n.d.
		3	n.d.

n.d. = not detected by the method of Yasumoto *et al.*[22]

level not dangerous to human health. At the same time, an intensive red-tide bloom was registered. Analysis of the phytoplankton community revealed the presence of some toxic, and potentially toxic species along with a number of non-toxic algae. Table II. presents the dinoflagellate composition determined in the sea sample collected simultaneously with the DSP-positive shellfish sample. *Gonyaulax polyedra* and *Prorocentrum micans*, suspected to be toxic, accounted for 70% and 26%, respectively, of the total phytoplankton content. *Dinophysis sacculus*, a known toxic organism, was found in a number of 0.5×10^2 cells dm^{-3}.

Samples collected in the eastern part of the Kaštela Bay in September 1993 and May 1994 gave no positive results on DSP. The phytoplankton community from September 1993 was almost similar to the one recorded in August 1993, collected along with mussels displaying toxicity. On the other hand, the phytoplankton community determined in May 1994 was completely different from the one found in summer; dinoflagellates were poorly represented while diatomeas from the *Centriceae* group prevailed.

HPLC analysis was performed on samples collected after a massive fish kill registered on September 8, 1994 in the Kaštela Bay. The fish kill was caused by anoxia induced by a red tide bloom typically occurring in the

TABLE II

The dinoflagellate composition of the sea samples collected simultaneously with the DSP-positive shellfish sample

Species	Sampling date		
	23/08/1993	11/09/1994	28/09/1994
Alexandrium minutum	+	–	+
Ceratium massiliense	+	–	–
Cochlodinium sp.	–	–	+
Dinophysis sacculus	+	–	+
Diplopsalis sp.	–	–	+
Gonyaulax digitale	–	–	+
G. gracilis	–	–	+
G. polyedra	+	–	+
G. spinifera	+	–	+
Gyrodinium fusiforme	+	–	+
G. spirale	+	–	+
Noctiluca scintilans	–	+	–
Oxytoxum sp.	–	–	+
Protoperidinium depressum	+	–	+
P. diabolis	+	–	–
P.grami	–	–	+
P.steini	+	–	–
Polykrikos shwartzii	–	+	+
Prorocentrum micans	+	–	+
P. minimum	–	–	+
P. triestinum	+	–	–
Pseliodinium vaubanii	–	–	+
Pyrophacus horologicum	–	–	+
Scrippsiella trochoidea	+	–	+

Kaštela Bay during summer. Mussels were collected on September 11, and September 28. The composition of the phytoplankton community was determined before the fish kill and shellfish sampling (August 22, 1994), and on the shellfish sampling dates (September 11 and 28, 1994). In August 1994, a red tide bloom was in progress with the species composition almost similar to the bloom from the preceding year. Again, *Gonyaulax polyedra* was the dominant species, comprising 90% of the phytoplankton community. Three days after the fish kill on September 11, only two species were detected in the sample, *Polykrikos schwartzii* and *Noctiluca scintilans*, both presumably toxic. In the sample collected on September 28, a more diverse phytoplankton community was found, with *Gonyaulax polyedra* accounting for over 20% of the organisms present. The well known DSP organism, *Dinophysis sacculus*, was also detected in the sample (1.5×10^2 cells dm^{-3}).

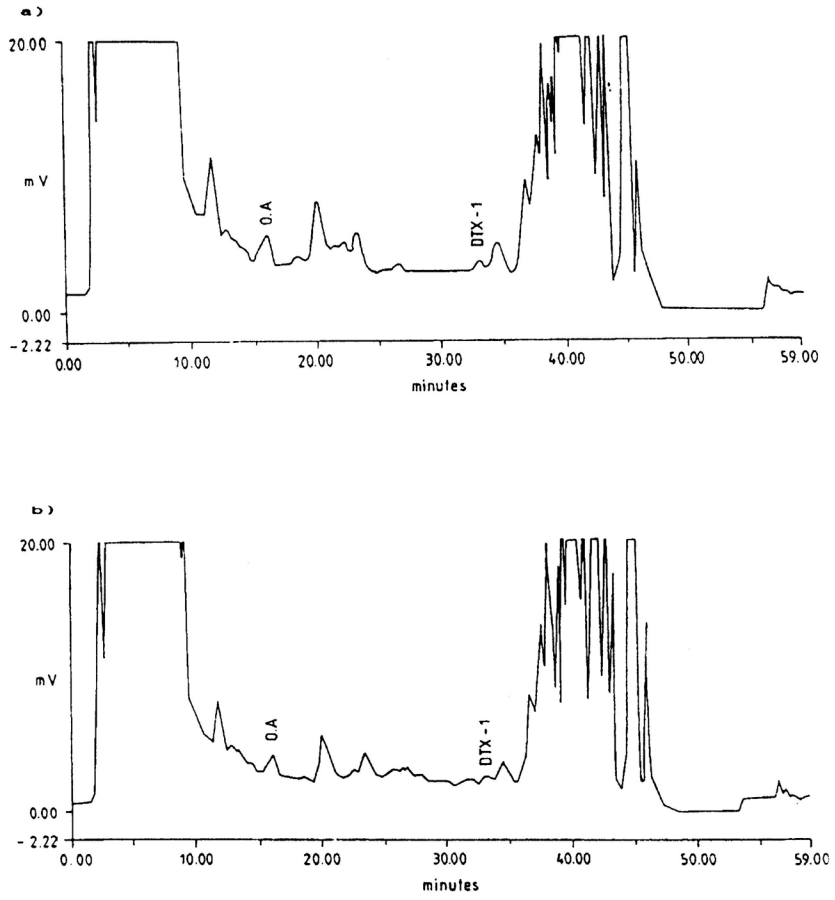


Figure 1. HPLC profiles of BrMmc derivatives derived from the hepatopancreas extracts of shellfish from the Kaštela Bay harvested on a) September 11, 1994, and b) September 28, 1994. The samples were applied onto a Supelcosil LC-18-DB-5 column and eluted isocratically with acetonitrile-water-tetrahydrofurane (60:38:2). Sample components were detected with a fluorescence detector at extinction and emission wavelengths of 325 and 390 nm, respectively.

The results of the HPLC analysis are presented in Figure 1. DSP toxins were detected in both samples. The chromatograms of the contaminated samples showed the presence of well-defined peaks whose retention time exactly matched that obtained from a standard solution of the okadaic acid (R_t 15.89 min) and dinophisistoxin-1 (R_t 32.72 min) derivative. The fluorometric determination of OA and DTX-1 was conducted by derivatization with BrMmc. The reagent quantitatively reacts with the toxin, and the resulting derivatives are sepa-

TABLE III

The mouse bio-assay for PSP toxicity

Date	Sampling station	Mouse. No.	Time of death
21/07/1994	Mali Ston Bay (Bistrina region)	1	n.d.
		2	n.d.
		3	n.d.
21/07/1997	Mali Ston Bay	1	n.d.
		2	n.d.
		3	n.d.

rable by HPLC when developed with acetonitrile-water-tetrahydrofurane. The samples contained OA as the major and DTX-1 as the minor component: 60 ng OA/g shellfish and 18.2 ng DTX-1/g shellfish were detected in the sample of September 11, 1994, while 50 ng OA/g shellfish and 18.2 ng DTX-1/g shellfish were present in the sample from September 28, 1994.

The DSP and PSP toxicity test was negative for both samples from the Mali Ston Bay collected on July 21, 1994. The results of the DSP and PSP toxicity test are presented in Table I and in Table III respectively. Phytoplankton analysis showed that penatea diatomeaes dominated in the samples; however, very few dinoflagellates were encountered.

DISCUSSION

In this paper, we describe the preliminary studies demonstrating the occurrence of shellfish intoxication by toxin producing phytoplankton species in the Central Adriatic Sea. So far, health problems due to consumption of seafood intoxicated with phytoplankton metabolites have not been registered in this area. However, the established existence of phytoplankton species known to display toxicity in other waters,[4] as well as the appearance of toxicity in the Northern Adriatic, motivated this monitoring program. Since it is the first time that such investigations have been carried out in the Central Adriatic, monitoring was restricted to potential or known harmful species. The studies on the presence of toxic compounds in shellfish were restricted to DSP intoxication. Sampling was performed on selected sites only. Samples were collected in the most polluted part of the Kaštela Bay, in an area where red tide blooms and massive fish kills occur regularly, and in the Mali Ston Bay, in waters carefully protected from pollution. The largest fish and shellfish farms on the eastern coast of the Adriatic Sea are located in the Mali Ston Bay, well known for its long shellfish farming tradition.

Routine determinations of shellfish toxicity are generally carried out by means of the standard mouse bio-assay providing the integrated value of the toxin concentration. Biological methods, with the exception of the mouse bio-assay for PSP, are all semiquantitative, with poorly defined toxin-specific symptoms. The application of biological methods may be severely limited by laws regulating the use of animals as research tools, observed by many countries. However, other analytical methods are being applied with notable success, including high performance liquid chromatography, based on the derivatization of toxins, and the subsequent chromatographic separation and determination of the fluorescent toxin derivatives. This technique allows one to quantify the levels of toxicity. However, this method is hampered by possible occurrences of non-toxic fluorescent compounds, and the existence of a wide spectra of toxic substances, requiring a large number of standard toxins for calibration.

Upon establishing the existence of toxic metabolites using the mouse bio-assay (Table I), the nature of the toxic compound characteristic of the *Gonyualax* bloom, typically occurring in the Kaštela Bay, was established by separation with HPLC (Figure 1). In both samples, OA and DTX-1 were detected, okadaic acid being the major toxic component. The results, obtained both by the mouse bio-assay and HPLC analysis, provide evidence for the occurrence of DSP toxicity in the area of the Kaštela Bay. Toxicity was recorded exclusively when the phytoplankton community was composed primarily of dinoflagellates, suggesting that some of these organisms may be responsible for it. The origin of DSP intoxication in the central Adriatic region can be traced to *D. sacculus* and the *Prorocentrum genus*, as presented in Table II.

Reports on the occurrence of DSP have dramatically increased both in number and geographical distribution. As a result of extensive surveys of dinoflagellate populations and shellfish toxicity, it was found that *D. fortii* and *D. acuminata* are the causative organisms for intoxication by OA in Japan. Okadaic acid and DTX-1 have been identified as the principal toxin components in the shellfish from The Netherlands, France and Sweden. The Dutch and Swedish samples contained a minor component resembling DTX-3 but none of the European shellfish contained PTX.[9] Shellfish from Italy and Ireland have been found to contain only OA.[29–31] The dinoflagellate *D. acuminata* is probably responsible for OA production in most parts of Europe; however, in Norway, *D. acuta* and *D. norwegica* are suspected of being responsible for the toxin infection of shellfish. Another possible source of OA is *D. mitra*.[9] Contamination of shellfish along the west coast of Spain by toxic compounds causing diarrhea is very common, okadaic acid being the main toxin responsible for this condition.[32,10] The predominant *Dinophysis* species associated with this intoxication are *D. acuta*, *D. acuminata* and *D. sacculus*. There is evidence that *Prorocentrum lima* may be responsible for DSP in southern Portugal. It is a species with wide geographical distribution and a great potential as a toxin producer.

A serious reason for concern is the recently established tumor promoting activity of the okadaic acid which induces hyperphosphorilation of some cellular proteins by apparently inhibiting the protein phosphatase types 1 and 2A, which may eventually lead to tumor formation.[2,3] In addition, it modulates a variety of cellular functions, such as smooth muscle contraction, fatty acid biosynthesis, catecholamine synthesis and secretion.[33]

Neither of the samples from Mali Ston Bay nor the sample from the Kaštela Bay collected in the spring of 1994 showed any toxicity, which is in good agreement with the structure of the phytoplankton community determined on the sampling date in that area.

An exception, illustrating the fact that the toxin production of marine alga is not well understood yet, is the analysis of September 20, 1993, where the shellfish samples were not toxic, though the structure of the phytoplankton community was similar to the one of August 1993, displaying shellfish toxicity. An explanation may lie in the interrelationship of the factors governing phytoplankton toxicity, such as nutrient concentration, environmental factors and the growth phase. It has been reported that the concentration of phosphorus and nitrogen compounds in sea water affects the production of toxic metabolites.[34] The highest toxin production occurs in the stationary phase of growth,[35] a fact that could lead to disagreement between the results of the phytoplankton analysis and shellfish toxicity. Temperature also influences toxicity, the production of toxins being higher at lower temperatures. Toxicity could be confined to specific clones, as in the case of some PSP producers.[36] For other toxin producing organisms it is still not known whether the toxin producing ability of some species is genetically determined, the expression depending on environmental factors, or whether distinctive toxic species or clones exist whose appearance is influenced by such factors. Studies have shown that some of the toxic algae live in symbiosis with specific bacteria, suggesting the possibility that these bacteria produce or induce the production of toxic metabolites in unicellular algae. The already complex situation is even more complicated by the influence of environmental factors affecting the metabolism of plankton filtrating organisms.

Altought no PSP toxicity has been recorded in this study, the presence of the *Alexandrium minutum* species in the samples collected in the Kaštela Bay on August 23, 1993 (7.5×10^4 cells dm^{-3}) and on September 28, 1994 (3×10^4 cells dm^{-3}) requires further investigation as well as analyses of PSP toxicity.

Toxin data are useful in monitoring shellfish for public health purposes, developing an alert toxin level for shellfish, or as a natural chemical tracer of the marine environment. The results obtained so far point to the presence of DSP toxins in the Central Adriatic Sea, implying the necessity of further investigation of the still unresolved questions concerning the biosynthetic pathway, development of toxicity and its influence on the biocenosis.

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SAŽETAK

Fitoplanktonski toksini u srednjem Jadranu

Stjepan Orhanović, Živana Ninčević, Ivana Marasović i Maja Pavela-Vrančić

Prikazani su rezultati istraživanja sekundarnih metabolita dinoflagelata i njihove uloge u humanoj intoksikaciji, koja su po prvi put provedena u vodama srednjeg Jadrana. Toksičnost školjkaša ispitana je pomoću *bioassay*-a i visokotlačne tekućinske kromatografije (HPLC). Utvrđena je prisutnost toksina odgovornih za diaroričnо trovanje školjkama DSP (diarrheic shellfish poisoning). Okadaična kiselina (C_{38} polieter) i njen 35-metilni derivat, dinofizistoksin-1, identificirani su kao osnovni uzročnici toksičnosti.