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## Accumulation of Phycotoxins in the Mussel *Mytilus galloprovincialis* from the Central Adriatic Sea

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Surveys of DSP (Diarrheic Shellfish Poisoning) toxin profiles in the mussel *Mytilus galloprovincialis* from coastal waters of the central Adriatic Sea, over the years 1995 to 2001, demonstrate that incidents of shellfish toxicity in this area are dominated by the occurrence of okadaic acid (OA) and a PTX derivative, 7-*epi*-pectenotoxin-2-seco acid (7-*epi*-PTX-2SA). Toxin composition and the relative ratio of toxic components in shellfish is in correlation with the occurrence of DSP producing organisms from the *Dinophysis* species along with *Prorocentrum micans* and *Lingulodinium polyedrum*. The occurrence of *D. sacculus* shortly before the appearance of OA in shellfish implicates its possible involvement as the source of toxicity. In the central Adriatic, the infestation period generally ranges from June to August. Augmented toxin production may shift the depuration phase to September; however, the length of decontamination period is not in correlation with increased initial toxicity. The mussel *M. galloprovincialis* may retain contamination with 7-*epi*-PTX-2SA beyond the commonly recognized infestation period, extending the risk of human poisoning from consumption of seco-contaminated seafood.

**Keywords**  
central Adriatic Sea  
phycotoxins  
diarrheic shellfish poisoning (DSP)  
toxic phytoplankton

### INTRODUCTION

In the Adriatic, the recurring incidence of toxigenic dinoflagellates of the *Dinophysis*, *Prorocentrum* and *Lingulodinium* species implicates shellfish contamination with diarrheic toxins, and consequently human poisoning from consumption of intoxicated seafood. The DSP (Diarrheic Shellfish Poisoning) phenomenon arises from three structural classes of related polyether compounds: (i) okadaic acid (OA) and dinophysistoxins (DTX), (ii)

pectenotoxins (PTX), and (iii) yessotoxins (YTX). Although DSP toxicity in the Adriatic is usually ascribed to OA and DTX derivatives,<sup>1–3</sup> more recent incidents of toxicity in the central Adriatic have been associated with the occurrence of a rare PTX derivative, 7-*epi*-pectenotoxin-2-seco acid (7-*epi*-PTX-2SA).<sup>4,5</sup> In humans, OA and DTX-1 exert potent tumour promoting activity, most likely by inhibiting protein phosphatases 1 and 2A.<sup>6</sup> Oral doses of PTX-2SAs, extracted from shellfish, produce acute lesions in the duodenum and stomach of mi-

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ce,<sup>7</sup> whereas PTX-2 is known to cause severe injuries of the small intestine.<sup>8</sup> Hence, the presence of OA, PTX and related compounds in shellfish from the coastal waters of the central Adriatic has emphasized the necessity of exploring the frequency of occurrence and variability of toxin composition, the length of infestation period and the nature of causative organisms, essential in evaluating the risk of shellfish contamination and consequently human poisoning from consumption of DSP-contaminated seafood. Shellfish and seawater samples, collected from a localized site in the Kaštela Bay, were analyzed for their toxin content and composition of the phytoplankton community, respectively. The results were interpreted in terms of data obtained from surveys conducted over several years in coastal waters of the central Adriatic Sea.

## EXPERIMENTAL

### Sampling

Wild populations of the mussel *Mytilus galloprovincialis* were harvested from a localized site in the eastern part of the Kaštela Bay (Vranjic Basin), central Adriatic Sea (FI: 43°29' – 43°33', LA: 16°15' – 16°29'). Sampling was conducted over the years 1995 to 2001. Samples were collected throughout the year, from January–May and September–December once a month, and in the summer period from June–August twice a month. The mussels were collected at a depth of 50 cm, and stored at –20 °C.

### Phytoplankton Analysis

Composition of the phytoplankton community, in waters surrounding the sampling site, was determined according to Utermöhl, 1958.<sup>9</sup> Seawater samples were taken twice a month, at regular time intervals, over the years 1995 to 2001. Sampling was conducted on the surface (sample volume 250 mL) at a localized site in the central Adriatic (FI: 43°31'57", LA: 16°27'15"). The samples were preserved in 0.5 % glutaraldehyde solution. Counting and identification of the organisms was conducted in 25 mL aliquots using an Olympus IX50 inverted microscope.

### Mouse Bio-assay

Analysis of DSP toxicity by the mouse bio-assay was performed according to Yasumoto *et al.*, 1985.<sup>10</sup> Toxins were extracted from mussel hepatopancreas using acetone, and after evaporation the residue was partitioned between diethyl ether and water. The organic fraction was evaporated to dryness, and the residue was dissolved in 4 mL 1 % (*v/v*) Tween 60. Aliquots (1 mL) of this solution were administered to the mice (strain BALB/C, weight limits 18–20 g) intraperitoneally. Three parallel tests were performed, and the reaction of the mice was observed during 24 hours after treatment or until death.

### Isolation of Polyether Acid

Homogenized shellfish hepatopancreases (1 g) were extracted with MeOH/H<sub>2</sub>O (80/20) containing 0.3 % (*v/v*) acetic acid. Following centrifugation, an aliquot of the supernatant was extracted with H<sub>2</sub>O/isopropyl acetate (3/8) by agitating on a Vortex mixer. The aqueous phase was extracted again with isopropyl acetate, and the combined organic fractions were evaporated to dryness.

### High-performance Liquid Chromatography Determination

Derivatization was carried out using 9-anthryldiazomethane (ADAM) in methanol, followed by separation on HPLC using fluorometric detection at  $\lambda_{ex}$ =365 nm and  $\lambda_{em}$ = 412 nm. The toxin profile was examined by comparison with standard samples of OA, DTX-1 and DTX-2 and reference material obtained from algae extracts of *Dinophysis acuta* containing PTX-2SA, OA, 7-*epi*-PTX-2SA, and DTX-2.<sup>11</sup> Toxin concentration was estimated from the peak area compared to a calibration sample containing 3.8 ng OA.

## RESULTS AND DISCUSSION

### Toxin Composition

Samples of the mussel *M. galloprovincialis* were collected during the summer of 2001 from a localized site in the eastern part of the Kaštela Bay, an area heavily polluted by domestic and industrial wastes. Mice treated with extracts from mussels collected in June 2001 revealed no shellfish intoxication, based on the mouse survival time of over 24 hours (Table I); however, HPLC analysis confirmed the presence of OA and 7-*epi*-PTX-2SA at an average concentration of 0.020 and 0.014 µg/g hepatopancreas (HP), respectively. Although orally toxic, intraperitoneally administered 7-*epi*-PTX-2SA does not exert toxicity. Mice, inoculated with an extract of the sample of July 26, 2001, displayed a survival time of 1 hour. Concentrations of OA above 0.200 µg/g mussel meat, approximately equivalent to 0.8 µg/g HP, cause mouse death within 5 hours.<sup>12</sup> Hence, the level of OA (0.132 µg/g HP) in the sample was not sufficient to account for the recorded toxicity, implying that an unidentified DSP compound might have contributed to the toxic effect. The sample of August 6, 2001 demonstrates a medial toxicity response, with an average mouse survival time of 3 hours. Again, higher toxicity than expected was recorded, taking into account the occurrence of OA at a concentration of 0.130 µg/g HP; however, HPLC analysis could not establish the nature of the causative agent. Identification of the toxic component was hampered by the limited amount of available material. In August 28, 2001 the toxin concentration was significantly reduced, as substantiated by a mouse survival time of over 24 hours.

TABLE I. DSP mouse bio-assay, toxin composition and concentration in correlation with phytoplankton composition and cell abundance. Monitoring was conducted at a localized site in the central Adriatic during 2001.

Time of sampling	Median mouse survival time / hours	Toxin composition	Toxin concentration / $\mu\text{g g}^{-1}$ HP	Phytoplankton composition	Cell abundance / cells $\text{dm}^{-3}$
April 2001				<i>D. sacculus</i>	$3.0 \times 10^3$
May 2001				<i>D. sacculus</i>	$1.5 \times 10^4$
June 16, 2001	> 24	OA 7- <i>epi</i> -PTX-2SA	0.013 0.009	<i>P. micans</i>	$1.8 \times 10^4$
June 26, 2001	> 24	OA 7- <i>epi</i> -PTX-2SA	0.026 0.019	<i>P. micans</i> <i>D. sacculus</i>	$3.3 \times 10^4$ $3.2 \times 10^2$
July 6, 2001	> 24	7- <i>epi</i> -PTX-2SA	0.006	<i>P. micans</i>	$1.2 \times 10^5$
July 26, 2001	1	OA 7- <i>epi</i> -PTX-2SA DSP-analogue?	0.132 0.028 n.d. <sup>(a)</sup>	<i>P. micans</i> <i>L. polyedrum</i>	$2.9 \times 10^5$ $2.8 \times 10^5$
August 6, 2001	3	OA 7- <i>epi</i> -PTX-2SA DSP-analogue?	0.130 0.090 n.d. <sup>(a)</sup>	<i>P. micans</i>	$8.9 \times 10^3$
August 28, 2001	> 24	OA 7- <i>epi</i> -PTX-2SA	0.017 0.003	<i>L. polyedrum</i>	$4.5 \times 10^5$

<sup>(a)</sup> n.d.: not detected.

### Correlation between Toxin Content and Phytoplankton Composition

Along with shellfish sampling, seawater samples were taken for analysis of phytoplankton composition and cell abundance (Table I). In July and August 2001, *Prorocentrum micans* and *Lingulodinium polyedrum* dominated the phytoplankton community. As for the *Dinophysis* species, only *Dinophysis sacculus* was detected in April and May of 2001, prior to the toxic event, at an abundance of  $3.0 \times 10^5$  and  $1.5 \times 10^3$  cells  $\text{dm}^{-3}$ , respectively. DSP contamination is a phenomenon with prolonged residence of OA and DTXs in shellfish, whereas pectenotoxins seem to be more time restricted.<sup>13</sup> There is a positive correlation between the onset of mussel toxicity and the appearance of *D. sacculus*; however, the highest toxin level obtained in late July of 2001 was accompanied by an increased abundance of *L. polyedrum* and *P. micans*. The time course of 7-*epi*-PTX-2SA accumulation and loss from shellfish, in conjunction with the composition of the co-occurring phytoplankton community, revealed that temporal variations in toxin content coincide with the appearance of *P. micans*. *L. polyedrum* was recorded in July and August of 2001 at an abundance of  $2.8 \times 10^5$  and  $4.5 \times 10^5$  cells  $\text{dm}^{-3}$ , respectively. Episodes of *L. polyedrum* are a frequent occurrence in the central Adriatic.<sup>3,4,14,15</sup> The incidence of YTX in the northern Adriatic and in Galicia has been associated with a *L. polyedrum* bloom.<sup>16–18</sup> Hence, high sample toxicity,

implicating the contribution of an unidentified analogue, and the concurrent incidence of *L. polyedrum* at the time of monitoring, suggest that the presence of YTX may be suspected in this area.

To look into the observed correlation, a survey of toxin incidents in the central Adriatic over the years 1995 to 2001, in conjunction with cell abundance of co-occurring *Prorocentrum*, *Lingulodinium* and *Dinophysis* species, was carried out (Figure 1). Toxic episodes were dominated by the common DSP compound OA and the PTX derivative 7-*epi*-PTX-2SA. In 1997 and 2001, the concurrent incidence of *P. micans* and *L. polyedrum* was preceded by episodes of *D. sacculus* in April. Shellfish extracts displayed equal amounts of 7-*epi*-PTX-2SA and OA. From June to September of 1995, concomitant incidence of *P. micans* and *L. polyedrum* was recorded, displaying in late July their maximum abundance of  $3.2$  and  $5.4 \times 10^5$  cells  $\text{dm}^{-3}$ , respectively. Occurrence of *D. sacculus* was first recorded in May 1995. Coinciding with the occurrence of *P. micans* and *L. polyedrum* in July 1995, *D. sacculus* reached maximum abundance of  $4.0 \times 10^4$  cells  $\text{dm}^{-3}$ . As for toxin composition, both 7-*epi*-PTX-2SA and OA were found in shellfish extracts, with predominance of 7-*epi*-PTX-2SA. In the summer of 1996 (July–August), 7-*epi*-PTX-2SA was recorded as the only contaminant, reaching maximum concentration of  $0.62 \mu\text{g/g}$  HP in late August. *D. sacculus* and *P. micans* appeared concurrently from May until Septem-

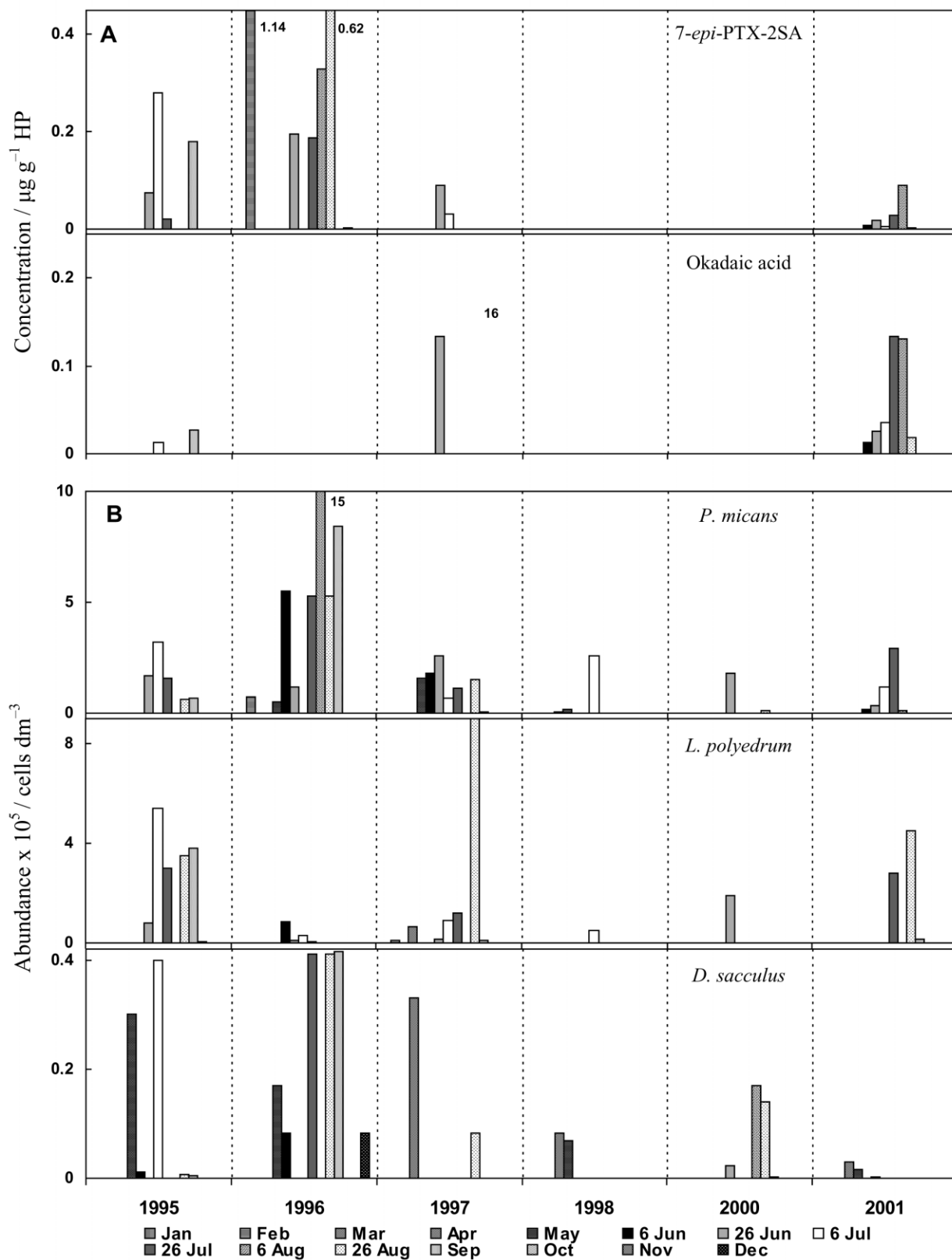


Figure 1. Toxin content in the mussel *Mytilus galloprovincialis* from the central Adriatic in correlation with phytoplankton cell abundance in surrounding seawater (1995–2001). Temporal variations in (A) 7-epi-PTX-2SA and OA concentration ( $\mu\text{g g}^{-1}$  HP), and (B) cell abundance (cells  $\text{dm}^{-3}$ ) of *P. micans*, *D. sacculus*, and *L. polyedrum*. Abundance of *P. micans* and *L. polyedrum* ranges from 0– $15 \times 10^5$  cells  $\text{dm}^{-3}$ , and for *D. sacculus* from 0– $0.5 \times 10^5$  cells  $\text{dm}^{-3}$ . Values beyond the scale are given adjacent to the corresponding bar. The time scale is distributed according to sampling intervals, once a month from January–May and from September–December, and twice a month (on the 6<sup>th</sup> and 26<sup>th</sup>) from June–August.

ber at an average abundance of  $4.15$  and  $7.3 \times 10^5$  cells  $\text{dm}^{-3}$ , respectively. *L. polyedrum* occurred prior to the toxic event, displaying maximum abundance of  $8.3 \times 10^4$  cells  $\text{dm}^{-3}$  in early June. All *P. micans* blooms persisted for at least three months. Occasional variations in toxin concentrations, at equal dinoflagellate abundance in surrounding waters, may be explained by a difference in seawater temperature.<sup>19</sup> A similar relationship between DSP and different phytoplankton species from the *Dinophysis* and *Prorocentrum* genus was proposed in the description of a DSP outbreak in the northern Adriatic in 1989 and 1990.<sup>20</sup> There was a high positive correlation between mussel toxicity and the appearance of the *Dinophysis* species *D. fortii* and *D. acuminata*; however, the highest toxin level obtained in late September 1989 was accompanied by an increased abundance of *L. polyedrum* and species from the *Prorocentrum* genus, *P. micans* and *P. minimum*.

The first occurrence of PTX-2SA and its isomer 7-*epi*-PTX-2SA in Europe was associated with the Irish species *D. acuta*.<sup>11</sup> Incidence of PTX-2SAs in shellfish from Spain was related to *D. acuta* and *D. caudata*,<sup>21</sup> and in Portugal to *D. acuta* and *D. fortii*.<sup>13</sup> It was shown that PTX-2SAs may arise from bioconversion of PTX-2 in mussel tissue.<sup>13,22</sup> Plankton samples off the Spanish coast, mainly composed of *Dinophysis* species with prevalence of *D. acuta*, illustrate a toxin profile dominated by OA, DTX-2 and PTX-2.<sup>23</sup> Generation of PTX-2 in the northern Adriatic area was identified with *D. fortii*.<sup>24</sup> Although DSP toxins can remain at high levels within shellfish tissues for considerable periods of time after contamination by toxic phytoplankton has ceased,<sup>25</sup> reports from the Mediterranean have shown clear relationships between the presence of *D. fortii* and the content of total toxins in mussel digestive glands.<sup>26</sup> Incidents of potentially toxic *Dinophysis* species, *D. fortii* (400 cells  $\text{dm}^{-3}$ ) and *D. caudata* (200 cells  $\text{dm}^{-3}$ ) were recorded in September 1995, preceding the occurrence of 7-*epi*-PTX-2SA in February 1996, and *D. acuta* (300 cells  $\text{dm}^{-3}$ ) was found in May 1996 before the lengthy 7-*epi*-PTX-2SA episode characterizing the summer season of 1996, but no correlation between temporal toxin accumulation in shellfish and cell abundance of the respective species could be established.

The most frequent DSP causative organism in Europe is *D. acuminata*. It has traditionally been regarded as a northern species commonly found in coastal waters of the North Atlantic and Pacific Oceans. Shellfish intoxication with DSP toxins in the Netherlands has been associated with *D. acuminata*, *P. redfieldii* and *P. micans*,<sup>27,28</sup> in Ireland with *D. acuta*, *D. acuminata*, *P. lima* and *P. concavum*, and in Scandinavia with *D. acuta*, *D. acuminata*, *D. norvegica*, *P. minimum*, *P. lima* and *P. micans*.<sup>29,30</sup> The appearance of *D. acuminata*, even at low densities such as 200 cells  $\text{dm}^{-3}$ , can cause intoxication

of shellfish that is sufficient to affect humans.<sup>31</sup> *D. sacculus* has been linked to DSP occurrences along the Mediterranean and Atlantic European coasts.<sup>32–34</sup> DSP incidents in France were associated with *D. acuminata*, *D. sacculus* and *P. lima*,<sup>28,29</sup> in Portugal with *D. acuta*, *D. sacculus*, *D. acuminata*, *D. caudata* and *P. lima*, in Spain with *D. acuminata* and *D. acuta*,<sup>23,35</sup> and in the Adriatic with *D. sacculus*, *D. acuminata*, *D. tripos*, *D. caudata* and *D. fortii*.<sup>15</sup> In Italy, *D. fortii* has been identified as the organism chiefly responsible for transmitting OA to shellfish.<sup>24</sup> Studies of *D. sacculus* Stein, a species with variable morphology, have clarified its taxonomic relationship to *D. acuminata* Claparede and Lachman, merging different morphotypes under the same taxon.<sup>36</sup> Sequence comparisons indicated 88 % similarity of *D. sacculus* to *P. micans*, which is consistent with a 24S rRNA sequence analysis showing a close relationship of *D. acuminata* to *P. micans*.<sup>37</sup> Toxicity of specific *Dinophysis* species varies spatially and temporally, and the abundance needed to contaminate shellfish is highly variable. Data obtained from investigations conducted in Portuguese waters exclude *D. acuminata* from contributing to contamination with pectenotoxins.<sup>13</sup> Monitoring carried out in Port Phillip Bay, Victoria, established the predominance of PTX-2SA, whereas OA and DTX were absent or formed only a small component of the DSP present.<sup>38</sup> *D. acuminata* was found at regular intervals, usually in fluctuating but low numbers; however, not enough data was available to draw a correlation between the toxin content and phytoplankton composition. The time needed for shellfish to become toxic depends not only on the presence of toxic algae but also on the relative abundance of the accompanying non-toxic species.<sup>39</sup> Our data indicate that toxin composition and the relative proportion of toxic components in shellfish may be associated with the incidence of *Dinophysis* species along with *P. micans* and *L. polyedrum*. Since *D. sacculus* was found to produce OA,<sup>36,40,41</sup> and has been linked to DSP incidences in southern Europe,<sup>32–34</sup> the occurrence of *D. sacculus* shortly before the appearance of OA in shellfish implicates its possible involvement as the source of toxicity.

#### *Infestation Period*

DSP toxins are readily accumulated by shellfish; however, little is known about their retention time. In some coastal areas of the northern Adriatic the infestation period ranges from May to November, in Japan and the Atlantic coasts of Spain and France from April to September, though it may vary locally, whereas in Scandinavia mussels were shown to cause DSP events even in October.<sup>42</sup> Detoxification of mussels was shown to follow a biphasic pattern: first a rapid toxin release, followed by a more gradual toxin loss.<sup>22</sup> The pattern of contamination and decontamination is specific to the shellfish spe-

cies and does not appear to depend on the type of dinoflagellate toxin. In the central Adriatic, the mussel *M. galloprovincialis* accumulates toxic substances from June until August, and after that it rapidly deparates the toxin. By the end of August 2001, the OA concentration decreased to 14.2 % of the maximum value recorded in late July, whereas only 3.3 % of the 7-*epi*-PTX-2SA level was still present. Clearance rates for the removal of DSP toxin from shellfish may be affected by interrelated factors such as shellfish feeding, temperature, salinity, level of non-toxic algae, fluorescence and light transmission.<sup>43</sup> In the case of augmented toxin production, as in 1996, depuration was shifted to September; however, the length of the decontamination period did not correlate with increased initial toxicity. In October 1996, only traces of 7-*epi*-PTX-2SA were found in shellfish, corresponding to 0.5 % of the highest concentration measured in August. The rate of detoxification is highly dependent on the site of toxin storage. Toxins in the gastrointestinal tract (*e.g.*, *Mytilus*) are eliminated much more readily than toxins bound in tissues.<sup>44</sup> Long intoxication periods may lead to chemical modification, as exemplified by the transformation of OA into its acyl-derivatives.<sup>35</sup>

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## SAŽETAK

### Fikotoksini u školjkašu *Mytilus galloprovincialis* is srednjeg Jadrana

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Pregled sastava toksina DSP (Diarrheic Shellfish Poisoning) u školjkašu *Mytilus galloprovincialis* u priobalnim vodama srednjeg Jadrana, za razdoblje od 1995. do 2001. godine, pokazuje da su glavni uzročnici toksičnosti školjkaša u ovom području okadaična kiselina (OA) i derivat PTX-a 7-*epi*-pektenotoksin-2-seko kiselina (7-*epi*-PTX-2SA). Sastav toksina i relativni udio toksičnih komponenti u školjkašima u korelaciji su s pojavom toksičnih vrsta iz roda *Dinophysis* zajedno s *Prorocentrum micans* i *Lingulodinium polyedrum*. Cvjetanje vrste *Dinophysis sacculus*, neposredno prije pojave OA u uzorcima školjki, upućuje na mogući doprinos ove vrste intoksikaciji školjkaša. U srednjem Jadranu, vrijeme zadržavanja toksina u tkivu školjkaša traje od lipnja do kolovoza. Povećana proizvodnja toksina može fazu pročišćavanja školjkaša odgoditi i do rujna, međutim duljina trajanja dekontaminacije nije u korelaciji s razinom početne toksičnosti. Produljeno vrijeme intoksikacije školjkaša *M. galloprovincialis* povećava rizik od trovanja prilikom konzumacije morskih plodova kontaminiranih seko-kiselinom.