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Domoic acid in phytoplankton net samples and shellfish from the Krka River estuary in the Central Adriatic Sea

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Abstract

This paper deals with the precise identification of species of *Pseudo-nitzschia*, focusing on those which are a potential source of domoic acid, from the Krka River estuary of the Central Adriatic Sea. Domoic acid was measured in phytoplankton net samples and shellfish collected in the winter and early spring of 2011 and 2012. Domoic acid was only detected in early March 2011, both in plankton net samples and shellfish extracts, during a *Pseudo-nitzschia* species bloom. The measured concentrations of particulate domoic acid (DA) in filtered concentrated seawater varied from 3.1-6.2 ng DA ml^{-1}. In shellfish sample DA concentration was 0.2 μg g^{-1}. Species belonging to the *Pseudo-nitzschia delicatissima* complex were more common than those from the *Pseudo-nitzschia seriata* complex. Morphological analyses by electron microscopy revealed the presence of three potentially toxic species: *P. calliantha*, *P. pseudodelicatissima* and *P. pungens,* and one non-toxic species: *P. subfraudulenta*. However, *P. calliantha* and *P. pseudodelicatissima* dominated during the March 2011 bloom. This study presents the first evaluation of particulate domoic acid along the Eastern Adriatic Sea and the first record of the presence of *P. calliantha*, *P. pseudodelicatissima*, *P. pungens* and *P. subfraudulenta* in the Krka River estuary.

Keywords: Particulate domoic acid, toxic *Pseudo-nitzschia* species, shellfish toxicity, Adriatic Sea, Krka River estuary.

Introduction

In the Adriatic Sea phytoplankton community, *Pseudo-nitzschia* species frequently occur throughout the year (Viličić *et al.,,* 1998; Viličić *et al.,,* 2009; Bosak *et al.,,* 2009; Bužančić *et al.,* 2012; Arapov, 2013). Previously, due to the limitations of light microscopy in identifying species during routine monitoring, their exact species composition was poorly known. In recent studies, during the last decade, electron microscopy and molecular methods for taxonomic identifications have greatly contributed to defining the composition of this genus in the Adriatic Sea. However, there are still unexplored areas of the Adriatic. In addition, the toxicity of the identified *Pseudo-nitzschia* species is currently largely unknown.

Six potentially toxic species of the *Pseudo-nitzschia* genus have been indentified from the Adriatic Sea: *P. calliantha* (Lundholm *et al.,* 2003), *P. delicatissima* (Caroppo *et al.,,* 2005)*, P. fraudulenta* (Ljubešić *et al.,* 2011)*, P. multistriata* (Pistocchi *et al.,* 2012)*, P. pseudodelicatissima* (Caroppo *et al.,* 2005)*, P. pungens* (Ljubešić *et al.,* 2011), as well as four species which are considered non-toxic: *P. decipiens* (Honsell *et al.,* 2008), *P. heimii* (Burić *et al.,* 2008), *P. mannii* (Ljubešić *et al.,* 2011), and *P. subfraudulenta* (Marić Pfannkuchen, 2013). Most of these species were identified in the northern part of the Adriatic Sea, except for *P. calliantha*, *P. delicatissima* and *P. subfraudulenta* which have also been confirmed in the central and southern parts (Lundholm *et al.,* 2003; Burić *et al.,* 2008; Caroppo *et al.,* 2005; Bosak *et al.,* 2010). So far, domoic acid (DA) production has only been confirmed in *P. multistriata* (Pistocchi *et al.,* 2012) and *P. delicatissima* (Penna *et al.,* 2013), which are isolated along the western coast of the Adriatic Sea.

The presence of DA in the Adriatic Sea was initially reported in 2000, in mussels collected in the Italian region, Emilia Romagna (Ciminiello *et al.,* 2005). Ciminiello *et al.* (2005) found a low concentration of DA, yet they suggested stringent monitoring of this powerful neurotoxin in the Adriatic Sea. Since then, DA has been recorded on several occasions in the Adriatic Sea. In Croatian waters, for instance, DA was first found in mussels from Istria in 2005 (Ljubešić *et al.,* 2011). Since the launching of an extensive monitoring program in 2006, DA has been detected for the first time in January 2006 in mussels from the Krka River estuary (Ujević *et al.,* 2010).

During 2006, the toxin was present in mussels from the Krka River estuary from January-March, with a maximal concentration of 6.548 μg g^{-1} found in Febru-

ary (Ujević *et al.,* 2010). Subsequently, the presence of low levels of DA was confirmed in shellfish from the Northern Adriatic coast in 2007 and 2008 (Ujević *et al.,* 2010). Since 2006, DA has been sporadically detected in Adriatic shellfish, but at levels which are below the regulatory limit (20 μg g^{-1}) (EU 2004; Pistocchi *et al.,* 2012; personal unpublished data).

Considering that DA was reported during the winter period of 2006 in the Krka River estuary and, moreover, unpublished data (personal data) for the area showed an increase in *Pseudo-nitzschia* abundance in the winter of 2011, the aim of the present study was to verify the presence of DA in plankton net and shellfish samples. In addition to toxin analyses, we aimed to define which *Pseudo-nitzschia* species were present in the phytoplankton community and were a potential source of domoic acid. The Krka River estuary is one of the most important Croatian shellfish breeding areas, with a high potential for an increase in shellfish production (Glamuzina *et al.,* 2009). Therefore, the identification of toxic *Pseudo-nitzschia* species and the determination of DA levels in shellfish and plankton net samples are a valuable contribution to our understanding of DA production in the area, providing helpful information in predicting future toxic events in this part of the Adriatic, which may help prevent possible consumer intoxication.

Materials and Methods

Study area

The Krka River estuary is a highly stratified 23 km long estuary located in the central part of the Eastern Adriatic Coast (Fig. 1). It is largely narrow, except for the area around the Prokljan Lake and Šibenik harbour. The depth of the estuary gradually increases from 1-2 m in the upper reaches of the estuary to 43 m near the estuarine mouth. The estuary is characterised by a sharp halocline, which exists between the surface (brackish) and lower (marine) layer and plays an important role in biogeochemical processes (Svenssen *et al.,* 2007). It represents an accumulation layer for both freshwater and marine phytoplankton species (Viličić *et al.,* 1989) and is a site of high bacterial activity (Fuks *et al.,* 1991). The main source of nitrate and silicate is the Krka River, while phosphorus is mainly anthropogenic in origin, from the Šibenik area (Legović *et al.,* 1994). Temperature and salinity are defined as the most important indicators of river inflow that influence phytoplankton species composition and seasonal dynamics (Cetinić *et al.,* 2006). This is one of the major aquaculture areas for shellfish along the Eastern Adriatic Coast. The annual production in 2008 was 507.5 t of mussels, while the potential capacity of the entire area is estimated at 2,000 t, according to the number of registered breeding farms (Glamuzina *et al.,* 2009).

Sampling

In 2011, seawater samples were collected at station SB1 between 15th February-7th March. In 2012, sampling was conducted weekly from the beginning of February until the 20th of March at stations SB1 (43.742622° N, 15.872106° E, depth 30 m), SB2 (73.776320° N, 15.848007° E, depth 23 m) and SB3 (43.729455° N, 15.872339° E, depth 14 m) (Fig.1).

Samples for phytoplankton analyses were collected using Nansen bottles (volume 1.7 l) (at surface and 5 m depth) or by a phytoplankton net (upper diameter 35 cm, mesh size 20 μm, in the 0-7 m layer). Subsamples of each seawater sample were filtered through a Whatman GF/F filter (47 mm diameter, 0.7 μm particle retention) for toxin analyses, while the rest was preserved in a formaldehyde solution, final concentration 2%, for phytoplankton species identification.

In 2011, shellfish samples (*Mytilus galloprovincialis*) were collected for toxin analyses at station SB1 in March (1st and 7th), while during 2012 mussels were collected concurrently with the plankton samples at stations SB1, SB2 and SB3.

Water temperature and salinity were measured, using a YSI Professional Plus probe, at four depths (surface, 2 m, 5 m, 7 m) between January-March 2011 and 2012 to trace changes in environmental conditions, which could act as a trigger for domoic acid production (Ujević *et al.,* 2010).

Toxin analyses in plankton net samples

Domoic acid and epi-DA were analysed by a HPLC-UV-DAD system, using the method of Quilliam *et al.* (1995), which is extensively used worldwide for DA analysis in shellfish and fish tissue. A lower detection limit and higher sensitivity, compared to the method referenced above, were achieved by increasing the injection volume of plankton samples to 50 μl instead of the recommended 20 μl (Mafra *et al.,* 2009).

In 2011, after testing our sampling techniques, we improved the preparation of plankton samples for toxin analysis. Initially, samples were collected using Nansen bottles at surface level and 5 m below the surface. In the following week, DA was analysed in a plankton net sample, collected vertically from the 0-7 m layer. As HPLC-UV analyses did not show the presence of DA, and assuming that the abundance of *Pseudo-nitzschia* cells was still too low in our sample, the volume of filtration was increased and the plankton net was towed four consecutive times in the investigated layer on the 1st and 7th of March 2011. As a result, we obtained a more concentrated sample with a final volume of 1 l, out of which 800 ml were filtered and extracted with 16 ml of MeOH/H_2O (1:1). The filters were sonicated for 1 min and then centrifuged at 4500 rpm for 20 min. After centrifugation, 5 ml of supernatant was filtered through a 0.45 μm membrane filter (PTFE, Whatman) and cleaned by a strong

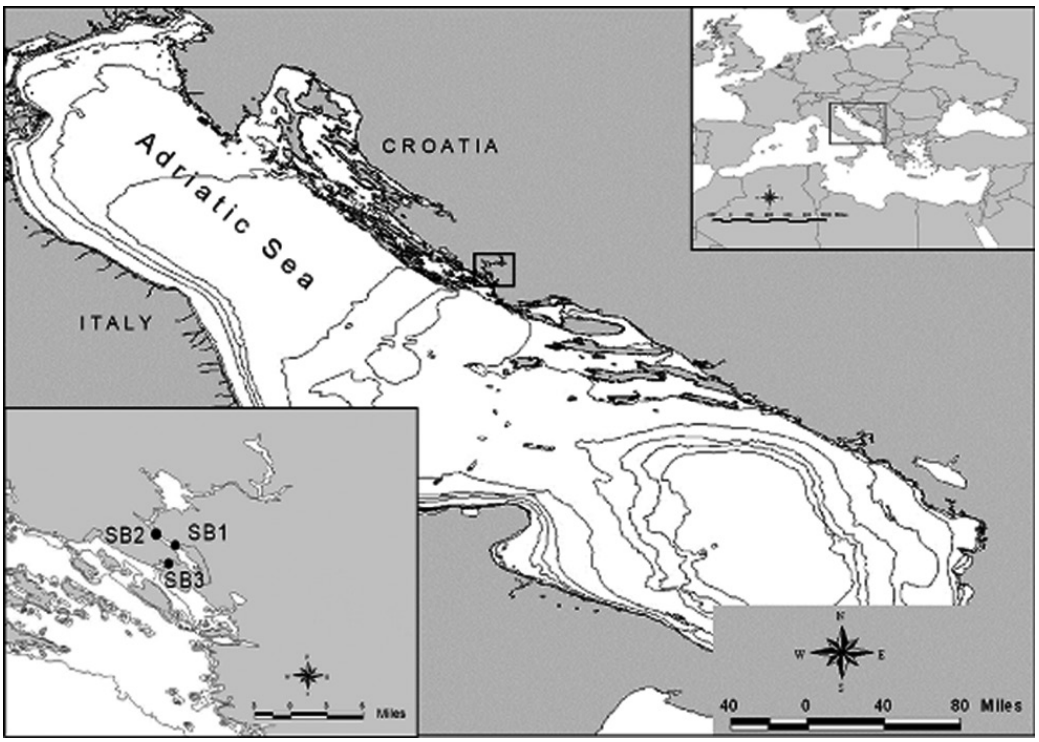

Fig. 1: Map of the study area showing the location of the sampling stations in the Krka River estuary in the Central Adriatic Sea.

anion exchange (SAX) (Mafra *et al.,* 2009; Quilliam *et al.,* 1995). During 2012, concentrated seawater samples were collected and prepared in the same way with a minor modification. The seawater was first filtered in the laboratory through a zooplankton net (mesh size 200 µm) and then through a GF/F filter. Size fractions of 200–0.7 µm were analysed using HPLC to verify the presence of DA. In total 26 plankton samples were analysed by HPLC, five samples in 2011 and 21 samples in 2012, respectively.

Toxin analyses in shellfish samples

In total 23 shellfish samples were analysed using HPLC, two samples in 2011 and 21 samples in 2012, respectively. Shellfish samples for toxin analyses were prepared following the protocol proposed by Quilliam *et al.* (1995). One hundred grams of soft shellfish tissue was homogenised, after which approximately 8 g was extracted in 16 ml of MeOH/H_2O (1:1) at 4000 rpm for 3 min and centrifuged at 4500 rpm for 20 min. After centrifugation, the supernatant was filtered and analysed as described above for plankton net samples.

The HPLC system consisted of a Varian ProSTAR 230 Solvent Delivery Module, a 310 UV/Vis Detector, a 335 Photodiode Array Detector (DAD) and a 410 Autosampler. The column was a Pinnacle II C18, 250 x 4.6 mm (Restek), with a C18 Guard Cartridge (20 x 4 mm) at a temperature of 40 °C and a flow rate of 1 ml min^{-1}. Domoic acid was detected by the UV detector at a wavelength of 242 nm, while the DAD detector recorded a spectrum of 220 nm to 400 nm. The mobile phase consisted of 100 ml acetonitrile, 0.2 ml trifluoroacetic acid and up to 1000 ml deionised water. The retention time of DA was around 12 min.

The limit of detection (LOD) was determined based on a 3:1 signal-to-noise ratio. The LOD for the detection of DA in shellfish (injection volume=20 µl) was 0.1025 µg DA g^{-1} of shellfish tissue, while the LOD for the detection of DA in plankton samples (injection volume=50 µl) was 0.2729 µg DA ml^{-1}. The limit of quantification (LOQ) was determined as 3 x LOD and was found to be 0.3415 µg DA g^{-1} and 0.9098 ng DA ml^{1} for the detection of DA in shellfish tissue and plankton net samples, respectively. Plankton samples were spiked to determine the recovery of DA, with an average result (n=7) of 101.92% at a concentration of 0.1979 µg DA ml^{1}.

Calibration curves were always linear, with a correlation coefficient greater than 0.99. Calibration was performed using certified DA calibration solutions (National Research Council of Canada, Halifax, Canada) prepared at four concentrations (0.25, 1.0, 2.5 and 10 µg ml^{1}).

Phytoplankton analyses

Only water samples in which DA was confirmed were further analysed to determine the phytoplankton community composition (net seawater samples collected at station SB1 on the 1st and 7th of March 2011). Phytoplankton abundance and community composition were determined using an inverted light microscope (LM) (Olympus IX50) following the Utermöhl method (1958). Subsamples of 1ml were settled in counting chambers for at least 12h. Cells were counted at a magnification of 400

x (one transect) and 200 x (half a chamber bottom). The abundance of *Pseudo-nitzschia* species was estimated for net concentrated samples and expressed as cells l^{-1} of net concentrated seawater samples.

More detailed taxonomic analyses of *Pseudo-nitzschia* species were performed under a scanning electron microscope (SEM). Phytoplankton samples, preserved in formaldehyde solution (final concentration 2%), were prepared for morphological examination by removing organic matter. In preparation for SEM, samples were treated with acids (1:1:4, sample: HNO_3: H_2SO_4), boiled for a few minutes and then washed with distilled water five times (Viličić, 2002). A drop of cleaned material was mounted on aluminium stubs, gold coated with a sputter coater (S150A Sputter coater; Edwards Ltd., Crawley, UK), and observed under a Philips 515 SEM (FEI Co.). Some of the morphological measurements were done under a light microscope (Zeiss GmbH, Oberkochen, Germany). The form of the colony and overlapping between the cells is presented in the results section and in Table 1. More accurate measurements were performed on electron micrographs, where the number of striae and fibulae were counted in 30 cells. *Pseudo-nitzschia* species identification was done by comparing ultrastructure and morphometry values with available bibliographical descriptions, e.g. Lundholm *et al.* (2003); Kaczmarska *et al.* (2005); Amato & Montresor (2008); Churro *et al.,* (2009).

Results

Environmental parameters

Sea surface temperatures ranged from 6.9 °C to 13.0 °C and from 2.6 °C to 13.2 °C in 2011 and 2012, respectively. In 2011, the minimum temperature (6.9 °C) was observed at a depth of 2 m at the end of January, while in 2012 the minimum (2.6 °C) was observed in the surface layer in mid February at station SB2. In 2011, salinity values ranged from 3.5 PSU at the surface to 38.1 PSU at 7 m depth, while in 2012 salinity values increased, ranging from 6.6 PSU at the surface to a maximum of 39.3 PSU at 7 m depth. Vertical profiles of temperature and salinity in 2011 and 2012 from station SB1 are shown in Fig. 2-5. The influence of the River Krka was more evident through the vertical salinity gradients at stations SB1 and SB2 than at SB3. The lowest surface salinity values, as well as the highest difference between surface and bottom salinity, were observed during January at all of the stations, indicating increased river runoff. Significant water column stratification was found in February 2011, in contrast to the same period in 2012 when higher surface salinity values were recorded at all of the stations. In general, in the Krka River estuary the halocline and thermocline are situated in the upper layers, between surface and 2 m depth or in the 2-5 m layer, while the water column is homogeneous below 5 m depth.

Table 1. Morphometry of identified *Pseudo-nitzschia* species: length (µm), width (µm), number of fibulae (N Fibulae / 10 µm) and striae (N Striae / 10 µm) in 10 µm, rows of poroids in 1 µm (R. / p).

	Length (µm)	Width (µm)	N Fibulae (10 µm)	N Striae (10 µm)	R. / p.
P. calliantha					1
Minimum	56.0	1.8	17	30	
Maximum	89.0	2.9	26	45	
Average	65.3	2.3	20.8	37.1	
Standard deviation	15.9	0.4	3.3	4.5	
P. pseudodelicatissima					1
Minimum	56.8	1.4	18	23	
Maximum	81.0	**2.9**	25	40	
Average	69.2	2.1	22.4	33.6	
Standard deviation	13.6	0.7	3.2	6.3	
P. pungens					2
Minimum	93.0	5.0	13	13	
Maximum	97.4	5.8	15	15	
Average	95.0	5.5	14.0	14.0	
Standard deviation	2.2	0.3	1.0	1.4	
P. subfraudulenta					2 to 3
Minimum	84.2	3.8	14	23	
Maximum	100.9	6.1	17	26	
Average	95.2	5.0	15.3	24.8	
Standard deviation	7.5	0.7	1.3	1.0	

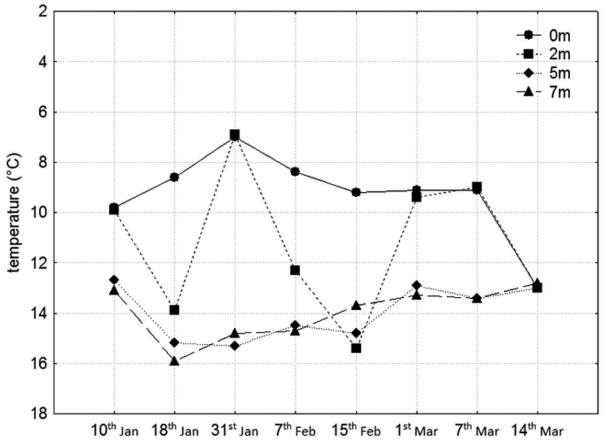

Fig. 2: The vertical temperature profile at the SB1 station during 2011.

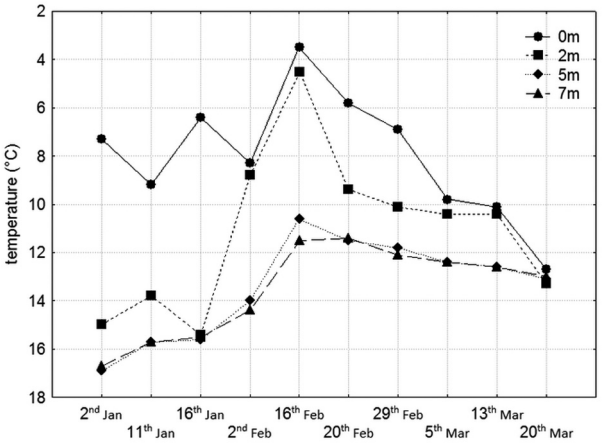

***Fig. 3*:** The vertical temperature profile at the SB1 station during 2012.

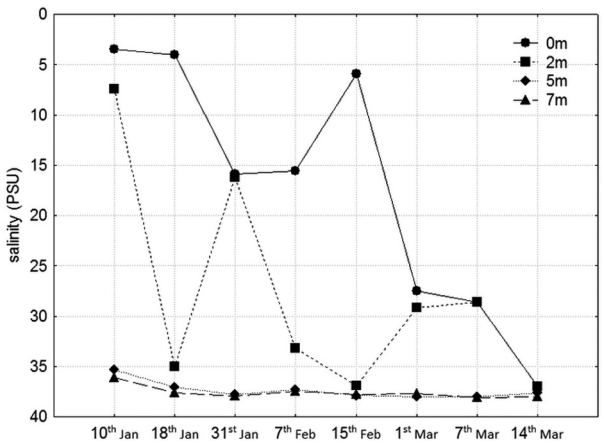

***Fig. 4*:** The vertical salinity profile at the SB1 station during 2011.

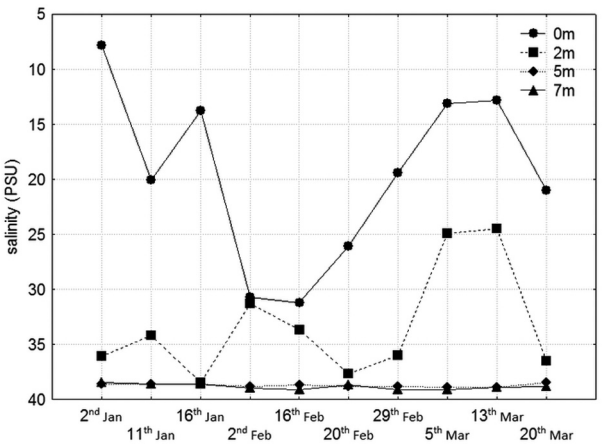

***Fig. 5*:** The vertical salinity profile at the SB1 station during 2012.

Domoic acid determination in plankton net samples and shellfish tissue

During 2011 and 2012, HPLC analyses confirmed the presence of domoic acid in the two plankton net samples collected at station SB1 on the 1st and 7th of March 2011. The concentration of particulate DA was 3.1 ng DA ml^{l} and 6.2 ng DA ml^{-1}, respectively. The UV spectrum obtained by the DAD detector confirmed the maximum absorbance at a wavelength of 242 nm. The HPLC-UV chromatograms and DAD-UV spectra for the plankton samples are presented in Fig. 6 and Fig. 7.

In 2011, the concentration of domoic acid in *M. galloprovincialis* was found to be very low. In shellfish samples from the 1st of March, the concentration of DA was 0.2 μg g^{-1}, while in samples from the 7th of March the concentration of DA was below the LOD, although a very small peak was noticed on the HPLC-UV chromatogram at the same retention time as DA. The HPLC-UV chromatograms are shown in Fig 8. The concentration of DA in the shellfish samples collected during 2012 was below the LOD.

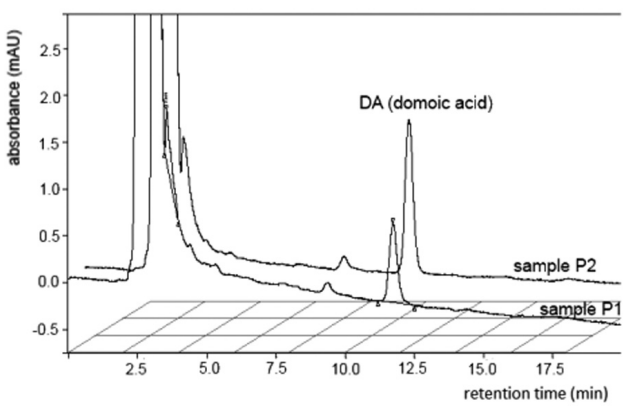

***Fig. 6*:** The HPLC-UV chromatograms, at wavelength (λ) 242 nm, for plankton extracts sampled on the 1st of March (P1) and the 7th of March 2011 (P2).

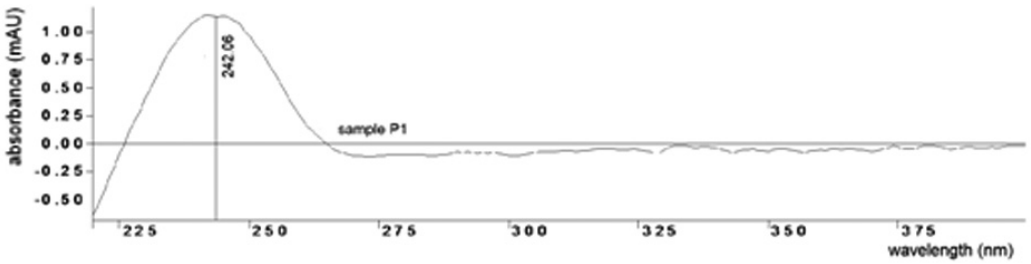

***Fig. 7*:** The UV spectrum obtained by the DAD for plankton extract sampled on the 1st of March.

Phytoplankton community composition and species identification

Seawater analysis by light microscopy showed that diatoms were dominant in the phytoplankton community in March 2011, representing more than 99.9% of the total phytoplankton abundance. Among diatoms, the contribution of *Pseudo-nitzschia* species (LM) to the total diatom abundance was not very high, representing 15.9% and 6.6% in the samples from the 1st and 7th of March, respectively. Based on their width, species belonging to the *Pseudo-nitzschia delicatissima* (sensu Hasle) complex (width<3 μm) dominated compared to the *Pseudo-nitzschia seriata* (sensu Hasle) complex (width>3 μm), contributing to 95.8% (1st March) and 92.9% (7th March) of the total *Pseudo-nitzschia* spp. abundance. The *Pseudo-nitzschia delicatissima* complex abundance was 5.1 x 10^6 cells l^{-1} in the concentrated net samples (1st March) and 2.4 x 10^6 cells l^{-1} in the concentrated net samples (7th March), while the abundance of *Pseudo-nitzschia seriata* complex was found to be similar in both plankton samples, at 2.2 x 10^5 and 1.9 x 10^5 cells l^{-1} in the net concentrated samples on the 1st of March and the 7th of March 2011, respectively.

Morphological analyses by electron microscopy revealed four species: *Pseudo-nitzschia calliantha* Lundholm, Moestrup and Hasle, *P. pseudodelicatissima* (Hasle) Hasle emend. Lundholm, Hasle & Moestrup, *P. pungens* (Grunov ex Cleve) Hasle and *P. subfraudulenta* (Hasle) Hasle.. *P. calliantha* and *P. pseudodelicatissima* dominated the bloom, while *P. pungens* and *P. subfraudulenta* were rather scarce. Data on the cell length and width and the density of the striae and fibulae for each determined *Pseudo-nitzschia* species are presented in Table 1.

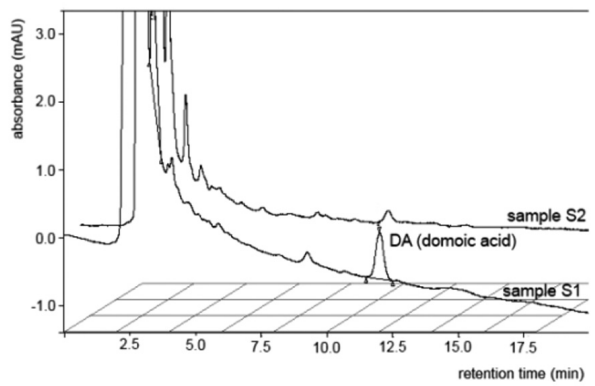

***Fig. 8*:** The HPLC-UV chromatograms, at wavelength (λ) 242 nm, for shellfish extracts sampled on the 1st of March (S1) and the 7th of March 2011 (S2).

Pseudo-nitzschia calliantha Lundholm, Moestrup & Hasle (Fig. 9A, B, C, D)

Linear cells formed stepped colonies with 12–13% overlap (Fig. 9A). The raphe was divided in the middle by a central nodule. Each stria was found to have one row of round to square poroids and the poroid pattern resembled a flower (Fig. C-D). The cingular band was perforated with striae that were 2–3 poroids wide (not shown).

Pseudo-nitzschia pseudodelicatissima (Hasle) Hasle sensu Lundholm (Fig. 10A, B, C)

Colonies were formed with overlapping cells, while cells were linear in valvar view (Fig. 10A-B). The eccentric raphe was divided in the middle by a central nodule. Striae were perforated by one row of oval to square poroids (Fig. 10C).

Pseudo-nitzschia pungens (Grunow ex Cleve) G.R. Hasle (Fig. 11A, B, C, D)

The cells were linear to lanceolate and formed stepped colonies with almost 30% overlap (Fig. 11A) and no central nodule. The fibulae were visible in light microscopy. The striae had two rows of large poroids (Fig. 11D) positioned close to the interstriae.

Pseudo-nitzschia subfraudulenta (G.R. Hasle) G.R. Hasle (Fig. 12A, B, C, D, E)

This species also occurred in stepped colonies with up to 25% overlap between the cells. The valves were more linear in their central part than in *P. fraudulenta*. Cells were linear to fusiform, gradually tapering towards pointed ends. A large central interspace with central nodule was present (Fig. 12A-C). The striae were perforated by 2-3 rows of poroids (Fig. 12C-E). There were 4-6 poroids in 1 μm, each divided in 3-4 sectors.

Discussion

In the present study, four *Pseudo-nitzschia* species were identified: the three potentially toxic species *P. calliantha*, *P. pseudodelicatissima*, *P. pungens* and the non-toxic *P. subfraudulenta*. Our findings represent a first evaluation of the mentioned species in the Krka River

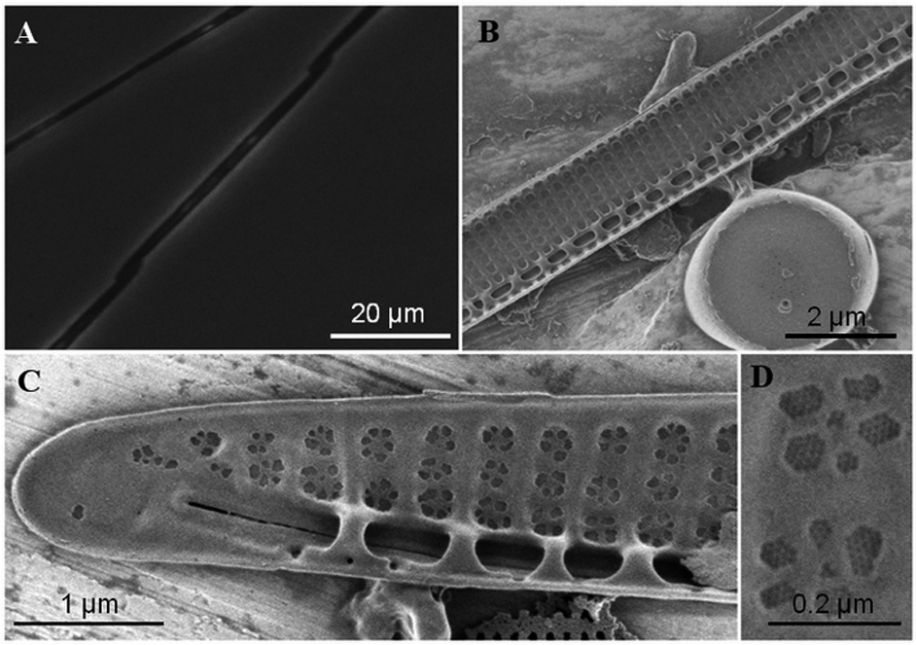

Fig. 9**:** *P. calliantha,* light micrograph of a stepped colony in valvar and girdle view (A). Middle part of the valve (B), tip of the valve (C) and the poroid pattern (D) SEM.

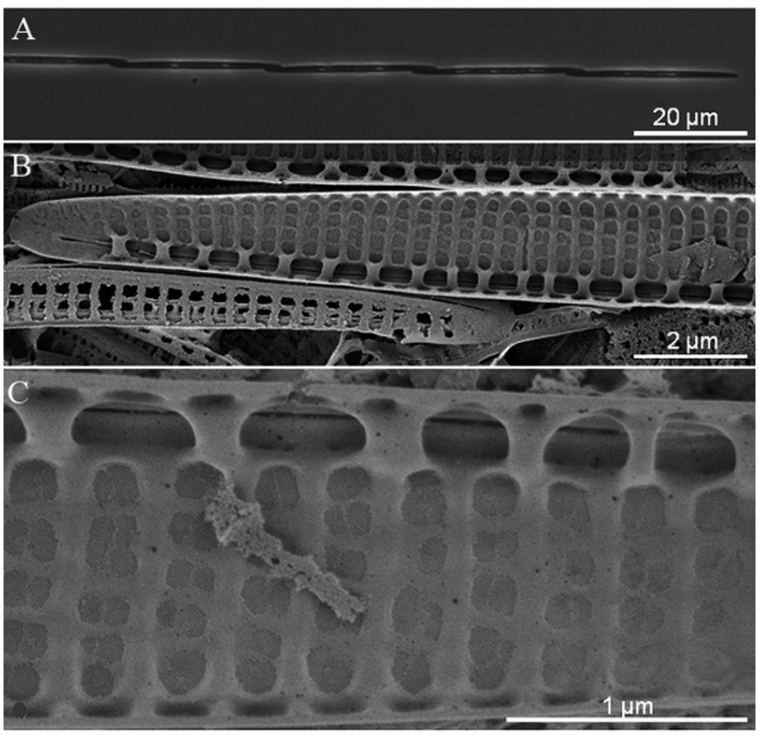

Fig. 10**:** *P. pseudodelicatissima*, light micrograph of a stepped colony in girdle view (A). Tip of the valve (B) and part of the valve with the poroid pattern (C) SEM.

estuary. Among them is *P. calliantha*, which is the most frequently found and widely distributed *Pseudo-nitzschia* species in the entire Adriatic Sea (Lundholm *et al.,* 2003; Caroppo *et al.,* 2005; Burić *et al.,* 2008; Honsell *et al.,* 2008; Bosak *et al.,* 2010; Ljubešić *et al.,* 2011; Marić *et al.,* 2011; Penna *et al.,* 2013).

The morphological measurements of the *P. calliantha* found in the Krka River estuary generally resembled the characterisation given in its original description (Lundholm *et al.,* 2003), only exceeding the range of the maximum number of striae (45 rather than 40), which is similar to that found by Ljubešić *et al.* (2011). The morphological characteristics of *P. pseudodelicatissima* generally corresponded to its original description, but we found a wider transapical axis, 2.9 µm rather than the 2.5 µm reported by Ferrario *et al.* (2002). In *P. pun-*

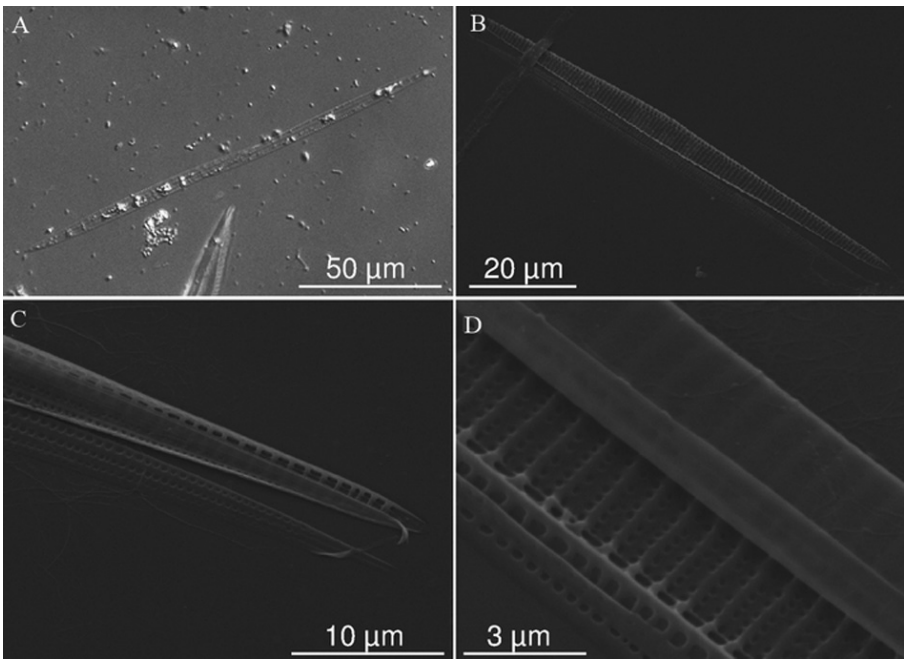

Fig. 11*: *P. pungens, light micrograph of a stepped colony in girdle view with visible interstriae (A). External view of whole valve (B), tip of the valve with the girdle bands (C) and the middle of the valve with the poroid pattern (D) SEM.

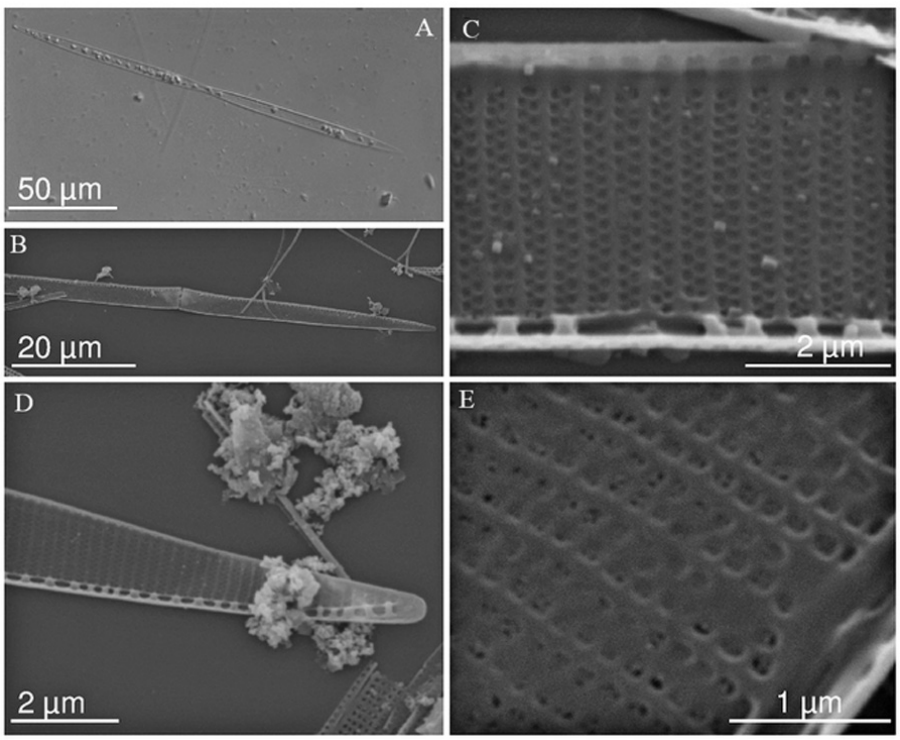

Fig. 12*: *P. subfradulenta, light micrograph of a stepped colony in valvar view (A). SEM valve overview (B), the middle of the valve with central interspace (C), tip of the valve (D) and the poroid pattern (E) SEM.

gens, the morphological measurements were in accordance with those reported in the literature (Ljubešić *et al.,* 2011 and references given within), except for a wider transapical axis of 5.8 µm. This increased width possibly indicates recent sexual reproduction (Holtermann *et al.,* 2010). In *P. subfraudulenta,* measurements of the density of the striae and fibulae were in accordance to the species description, but we found a transapical axis width of 3.8 µm, which is smaller than previously reported. The number of striae was higher than reported by Bosak *et al.* (2009) in the Kotor Bay. These three *Pseudo-nitzschia* species have already been reported in Croatian waters, but only in northern regions. The presence of *P. pseudo-delicatissima* was reported in Lim Bay (Ljubešić *et al.,*

2011) and in the Velebit and Pag channels (Šupraha *et al.,* 2011), while *P. pungens* and *P. subfraudulenta* have been found only in Lim Bay (Ljubešić *et al.,* 2011; Bosak *et al.,* 2009). In the other parts of the Adriatic Sea, *P. pseudodelicatissima* and *P. subfraudulenta* have been found in Kotor Bay (Bosak *et al.,* 2010) and *P. pungens* in the north-west (Penna *et al.,* 2013).

The present study is the first examination of DA in phytoplankton net samples from the Eastern Adriatic Sea. The species *P. calliantha* and *P. pseudodelicatissima* dominate in the samples (contributing more than 90% to the total *Pseudo-nitzschia* spp. abundance) when compared to *P. pungens* and *P. subfraudulenta*. Therefore, we assume that *P. calliantha* and *P. pseudodelicatissima* are the source of the DA.

Worldwide, toxin analyses of field phytoplankton samples are scarce compared to analyses carried out on laboratory cultures. The reported concentrations of particulate domoic acid, related to the different abundance of *P. pseudodelicatissima* in seawater, are 14 264 ng l^{-1} (abundance 0.87±0.26 x 10^4 cells l^{-1}) and 30 ng l^{-1} (abundance 2.3 x 10^7 cells l^{-1}) (Marchetti *et al.,* 2004; Trainer *et al.,* 2007). Much higher values of particulate domoic acid are associated with the presence of *P. australis* (Schnetzer *et al.,* 2007; García-Mendoza *et al.,* 2009; Klein *et al.,* 2010), while lower concentrations are associated with *P. brasiliana* in Tunisia (Sahraoui *et al.,* 2012). In the Adriatic Sea, toxin analyses conducted on *P. calliantha* strains in field samples from the Gulf of Trieste (Northern Adriatic Sea) showed no domoic acid (Honsell *et al.,* 2008), while a bloom of *P. calliantha*, detected along the north-eastern coast in 2007, was related to and co-occurred with DA in shellfish (Marić *et al.,* 2011). However, as the authors (Marić *et al.,* 2011) themselves suggest, there is a need to further investigate the possible link between the toxin found in the shellfish and the presence of *P. calliantha* in the seawater.

DA production has only been confirmed in two species of *Pseudo-nitzschia* found in the Adriatic Sea, *P. multistriata* and *P. delicatissima*, based on toxin analyses carried out on laboratory cultures. The other species analysed, including *P. fraudulenta*, *P. calliantha* and *P. pungens*, did not produce DA (Pistocchi *et al.,* 2012; Penna *et al.,* 2013). However, in other areas of the World Ocean DA production has been confirmed, based on analyses of laboratory cultures, for *P. calliantha* (Martin *et al.,* 1990; Lundholm *et al.,* 1997; Sahraoui *et al.,* 2006; Besiktepe *et al.,* 2008; Trainer *et al.,* 2012), *P. pseudodelicatissima* (Moschandreou *et al.,* 2010) and *P. punges* (Trainer *et al.,* 2012). In general, *P. calliantha* and *P. pseudodelicatissima* are classified as low DA producing species (Trainer *et al.,* 2012).

An increase in the abundance of species belonging to the *Pseudo-nitzschia delicatissima* complex in the Krka River estuary during winter is consistent with previous reports of a high abundance of *Pseudo-nitzschia* spp. in this area during this period (Ujević *et al.,* 2010; Bužančić *et al.,* 2012). Moreover, a negative correlation between *P. calliantha* and water temperature was documented in the Zrmanja River estuary by Burić *et al.,* (2008) and on the south-western coast of the Adriatic Sea by Caroppo *et al.* (2005).

Our research has reconfirmed the contamination of shellfish with DA in the Krka River estuary during winter. Domoic acid was found at a lower concentration than previously reported by Ujević *et al.* (2010) and far below the regulatory limit of 20 μg g^{-1} (EU 2004). In their study, Ujević.*et al.* (2010) reported an accumulation of domoic acid as a result of a *Pseudo-nitzschia* spp. bloom which occurred after a rainfall event. At that time, the lowest mean monthly temperature for February from the eight years period (2001-2008) was recorded. In our study, during 2011, when DA was found, a decrease in water temperature at the surface and at a depth of 2 m was registered at the end of January. At that time, the sea temperature at 2 m decreased by 7 °C (from 13.9 to 6.9 °C) over a period of 10 days at the SB1 station.

Here, we present the results of preliminary research related to the DA production and toxicity of *Pseudo-nitzschia* species in the Krka River estuary in the Central Adriatic Sea. DA was found in plankton net samples and in shellfish samples. Although the concentrations of DA found in shellfish were very low, 0.2 μg g^{-1}, this indicates that the emergence of DA recurs in winter and early spring under the influence of low temperatures. Species belonging to the *Pseudo-nitzschia delicatissima* complex (*P. calliantha* and *P. pseudodelicatissima*) dominated over the *Pseudo-nitzschia seriata* complex (*P. pungens* and *P. subfraudulenta*), contributing to more than 90% of the total *Pseudo-nitzschia* spp. abundance. Given that three potentially toxic *Pseudo-nitzschia* species were found concurrently, it was not possible to distinguish which is the source(s) of the domoic acid. Further field and laboratory studies with cell cultures of *Pseudo-nitzschia* are necessary to clarify which species is the source(s) of the domoic acid in the Krka River estuary, as well as to determine the ecological conditions that act as triggers or enhance the production of DA in this area.

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