Ecology and taxonomy of potentially toxic *Pseudo-nitzschia* species in Lim Bay (north-eastern Adriatic Sea)

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Abstract

The population dynamics of *Pseudo-nitzschia* in relation to environmental factors was investigated from March 2002 to July 2008 in Lim Bay, in the north-eastern Adriatic Sea. Since shellfish and fish farming are present in the bay, domoic acid in *Mytilus galloprovincialis* was monitored, from 2005 to 2008. The principal-component analysis of environmental parameters showed that the system is mostly temperature driven. The phytoplankton community was mainly composed of diatoms. *Pseudo-nitzschia* was the dominant diatom, present in 60 % of samples on a yearly basis, with a maximum (1.6 x 106 cells L–1) contribution up to 97 % of the total diatom abundance. Morphological analysis revealed *P. manii* and potentially toxic *P. pseudodelicatissima*, *P. pungens*, *P. fraudulenta* and *P. calliantha* as the dominant species in blooms. *Pseudo-nitzschia* abundance positively correlated to temperature, phosphate and ammonia in accordance with its mass development in the summer/autumn period when fish farms had a maximum impact on the environment. Domoic acid was detected in *Mytilus galloprovincialis* in concentrations below regulatory limits, ranging from 0.097 to 0.8721 µg g-1 in five cases from April to October 2005 in Lim Bay, but so far it is not clear which of the species was responsible for DA production. This study is also the first record of *P. manii*, *P pungens* and *P. fraudulenta* species in the Adriatic Sea.

Keywords: *Pseudo-nitzschia* species*;* Domoic acid; Population dynamics, Adriatic Sea

1. Introduction

Toxin-producing diatom species of the genus *Pseudo-nitzschia* are widely distributed in oceanic and neritic phytoplankton worldwide (Hasle, 2002). Among the 32 species described in the literature so far, eleven are known to be toxic (Quiroga, 2006), producing domoic acid, a neurotoxic amino acid responsible for Amnesic Shellfish Poisoning (ASP) (Bates et al., 1998). In the Mediterranean basin, as well as in the Adriatic Sea, *Pseudo-nitzschia* is a persistent and dominant component of the phytoplankton assemblages (Socal et al., 1999; Orsini et al., 2002; Quiroga, 2006; Bosak et al., 2009; Viličić et al., 2009).

The occurrence of shellfish poisoning in the Adriatic Sea was first noted in 1989 in the northern Adriatic, which was related to diarrheic shellfish poisoning (DSP) (Della Loggia et al., 1993; Marasović et al., 1998). In Lim Bay, potentially toxic dinoflagellates are permanent constituents of the phytoplankton assemblages and have caused, so far, at least one registered DSP toxicity event (Ninčević-Gladan et al., 2008).

Toxin-producing ability may result in toxin accumulation throughout the food chain, and also impacts marine organisms, humans and ultimately the ecosystems and the economy. Therefore, this has generated considerable interest in *Pseudo-nitzschia* species also in the Adriatic Sea. Although there have been records of domoic acid (DA) production in the northern Adriatic (Marić et al., 2011), species composition, species seasonality as well as the relationship to physic-chemical parameters still remains to be elucidated. So far, *P. calliantha* Lundholm, Moestrum & Hasle in the northern, middle (Kaštela Bay, Novigrad Sea) and southern Adriatic (Bay of Kotor) and *P. delicatissima* (Cleve) Heiden in the southern part along the Italian Adriatic coast (Caroppo et al., 2005; Burić et al., 2008; Bosak et al., 2010; Moschandreou et al., 2010; Marić et al., 2011) have been identified. Investigations on the seasonality of specific species and the relation to environmental factors in the Adriatic Sea are scarce. Theaffinity of *P. calliantha* for nutrient enriched conditions, as well as for lower temperatures have been indicated due to the blooming of *P. calliantha* and *P. delicatissima* in the middle and southern Adriatic during the colder part of the year (Caroppo et al., 2005; Burić et al., 2008). Recent investigations in the northern Adriatic revealed *P.calliantha* as the dominant *Pseudo-nitzschia* species, contributing up to 70% to the total phytoplankton abundance. Its maximal appearance was in warmer part of the year, during the autumn bloom of phytoplankton, which preceded the appearance of DA in the natural mussel population of this area (Marić et al., 2011).

Studies elucidating the nutrient and physical requirements, as well as the biotic interactions of *Pseudo-nitzschia* species on regional and global scales are essential for the prediction and prevention of its mass occurrence and resulting toxicity. The difficulty in monitoring these diatoms is in classifying them to the species level, which relies on ultrastructural features seen only on cleaned frustules observed with an electron microscope (Hasle and Syvertsen, 1997), or by molecular methods.

The aim of this work is to describe the population dynamics of the dominant *Pseudo-nitzschia* species and DA production in relation to environmental factors in Lim bay, north-eastern Adriatic Sea, as well as to identify presently-occurring species. An assessment of the species-specific distribution and characterisation of the physical and chemical environmental conditions is required for the prediction and management of potentially harmful *Pseudo-nitzschia* blooms.

2. Materials and methods

2.1 Study area

The Adriatic Sea is the northernmost basin in the Mediterranean. The circulation of the Adriatic is cyclonic due to the considerable freshwater input by the northern Adriatic rivers which results in the south-easterly outflowing West Adriatic Current (Orlić et al., 2006). The eastern Adriatic region is exposed to the north-westerly inflowing East Adriatic Current which brings highly saline and low-nutrient waters from the Ionian and Levantine Seas (Orlić et al., 2006). The Po river discharge and meteorological forcing are the main components triggering the alternation of stratification and mixing of the water column that strongly affects the annual dynamics of phytoplankton in the northern Adriatic (Revelante and Gilmartin, 1976; Viličić et al., 2009). Diatoms dominate the phytoplankton assemblages (both the microplankton and nanoplankton fractions) over the most of the year, while autotrophic and mixotrophic flagellates dominate in oligotrophic conditions in April-July (Viličić et al., 2009). Lim Bay, a fjord-like embayment located on the west side of the Istrian peninsula in the north-eastern Adriatic Sea is a semi-enclosed karstic area without any strong freshwater source. The freshwater inflow becomes important during heavy rainfall from September to December and with snow melting in April (Penzar et al., 2001). Some shellfish and fish farming are in operation in the inner and middle parts of the Bay (Krajnović-Ozretić et al., 2001), which is also known for providing a good spawning ground as well as a hiding place for many commercial fish (Huljev and Strohal, 1983). Comparison of the physico-chemical properties and phytoplankton dynamics between Lim Bay and other locations in the middle Adriatic Sea have indicated moderate eutrophication with a high anthropogenic influence in Lim Bay (Bosak et al., 2009).

Fig. 1

2.2 Sampling

Sampling was carried out monthly from March 2002 to July 2008 at three stations located along Lim Bay (Fig. 1). Lim Bay is 11 km long and 0.5 km wide, with a maximum depth of about 33 m in the outer part while the inner part is about 17 m deep. A total of 453 samples were collected and analyzed throughout the study period. Water samples were collected with 5 L Niskin bottles at stations the LIM1 and LIM2 stations at five depths (surface, 5m, 10m, 20 m and 2 m above the bottom) and at the LIM3 station at four depths (surface, 5m, 10m and 2 m above the bottom). At each station, water temperature and salinity were measured using a CTD probe (SBE 25 Sealogger CTD, Sea-Bird Electronics, Inc., Bellevue, Washington, USA). Dissolved oxygen concentration was determined by the Winkler titration method (Parsons et al., 1984), while the saturation percent of dissolved oxygen in each water sample was calculated (from the quotient between the measured oxygen concentration and the oxygen solubility) following the Benson and Krause equation (UNESCO, 1986). Nutrient concentrations were determined following standard methods (Ivančić and Degobbis, 1984; Parsons et al., 1984) using a Shimadzu UV-Mini 1240 spectrophotometer. Subsamples for the determination of chlorophyll (chl) *a* were filtered onto Whatman GF/C filters. Following 3 hours of extraction in 90% acetone (in the dark, with grinding), chl *a* concentrations were determined on a Turner TD-700 fluorometer (Parsons et al., 1984).

Samples for phytoplankton analyses were preserved in 2% (final concentration) neutralised formaldehyde solution. The cells were counted employing a Zeiss Axiovert 200 inverted microscope, using the Utermöhl (1958) method. Cells longer than 20 μm were designated as the microphytoplankton fraction while cells 2-20 μm in length were designated as the nanoplankton fraction (Sieburth et al., 1978). Cells were counted at a magnification of 400 x (1 transect) and 200 x (transects along the rest of the counting chamber base plate). Larger nanoplankton (> 5 µm) and abundant microphytoplankton (> 20 µm) were counted and identified at 400 x. At 100 x, a total count was completed for all taxa >30 µm. The minimum concentration that could be detected by this method was 20 cells L-1. For more detailed taxonomical analyses, net phytoplankton samples (53 µm mesh size) were collected during the *Pseudo-nitzschia* bloom in August and November 2007 and in February 2008.

For transmission electron microscopy (TEM) and permanent slides, *Pseudo-nitzschia* frustules were first acid-cleaned (in HNO3 and H2SO4) and washed with distilled water. The micrographs were taken with a FEI Morgagni 268D transmission electron microscope and a 515 Philips scanning electron microscope. The ultrastructure and morphometry of the valves were analysed according to the recent literature (Lundholm et al., 2003; Kaczmarska et al., 2005; Amato and Montresor, 2008; Churro et al., 2009). Permanent slides for morphometry on a light microscope were made from cleaned samples mounted in Zrax.

Domoic acid in *Mytilus galloprovincialis* was investigated at the LIM 3 station biweekly from the year 2005 to the end of research period. The method for ASP toxins, DA and *epi*-DA determination followed the protocol proposed by (Quilliam et al., 1995). For representative samples, weights of about 100 g of soft tissue were used. Homogenised samples (approximately 4 g) were extracted with 16 mL MeOH/H2O (1:1) at 10000 rpm for 5 minutes. After 40 minutes of centrifugation at 4000 rpm, 5 mL of the supernatant was filtered through a 0.45 µm membrane filter (HVHP, Millipore). Subsequent clean-up by strong anion exchange (SAX),solid phase extraction (SPE) was necessary to avoid tryptophan interference. Since DA can decompose when frozen, prepared SAX-cleaned extracts had to be analysed immediately.

The HPLC system consisted of a Varian ProSTAR 230 Solvent Delivery Module, a 310 UV/Vis Detector, a 335 Photodiode Array Detector and a 410 Autosampler. The column used was a Pinnacle II C18, 250 × 4.6 mm (Restek) with a C18 Guard Cartridge (20 × 4 mm) at a temperature of 40 ºC. Domoic acid was detected by UV absorption at a wavelength of 242 nm, which is the absorption maximum of domoic acid. The mobile phase consisted of 100 mL acetonitrile, 0.2 mL trifluoroacetic acid and up to 1000 mL deionised water. Domoic acid had a retention time of around 12.8 min.

For principal-component analysis (PCA) of the physical and chemical variables and *Pseudo-nitzschia* abundance, the statistical software package PRIMER 7 was used (Clarke & Warwick 2001). A logarithmic transformation (log (x+1)) was used on the dataset to obtain the normal distribution. A standard Spearman rank correlation using Statistica 6.0 software was used to quantify direct correlations between all parameters.

3. Results

3.1 Environmental parameters

The physico-chemical properties as well as the biological variables measured in Lim Bay over the research period are listed in Table 1. Salinity and temperature profiles showed that water column mixing events occurred throughout the year, except in the summer period (June to September) when a thermocline formed in the 5-10 m layer caused stratification.

Table 1.

Water temperatures varied from 7.8 to 27.6 °C, depending on meteorological conditions. Salinity values did not show higher oscillations and the occasional lower values (< 30), were recorded in the surface layer in the period from February to May, and were probably related to winter and early spring precipitation events.

The PCA of the environmental parameters generated five principal components, the first three had eigenvalues > 0.06 and explained 92.5 % of the variance of the original data set (Fig. 2, Table 2). The first principal component (PC1) accounted for 61.7 % of the total variance within the dataset. It was primarily related to silicate and total inorganic nitrogen. The second principal component (PC2) expressed 20 % of the variance and related mostly to temperature. On the scatter plot, two groups can be distinguished: the summer/autumn group and the winter/spring group, divided by temperature preferences (Fig. 2). Phosphate concentration was generally low, with minimum values in winter and spring and maximum in the period from September to November. The Redfield ratio was high (average value 75, maximum value 390) during the whole research period. Silicates and total inorganic nitrogen (TIN) had minimum values in the summer stratification period. The average Si:TIN ratio was 2.2, with the lowest average values in autumn (1.4), and higher average values in spring.

Figure 2.

Table 2.

* 1. *Pseudo-nitzschia* occurrence

The phytoplankton was mainly composed of diatoms (Table 1) with a dominance (maximum abundance >105 cells L–1, occurrence >20 %) of *Cerataulina pelagica* (Cleve) Hendey, *Chaetoceros socialis* Laud., *Chaetoceros* sp., *Dactyliosolen fragilissimus* (Bergon) Hasle, *Guinardia striata* (Stolter.) Hasle, *Leptocylindrus danicus* Cleve*,* *Nitzschia longissima* (Breb.) Ralfs., *Proboscia alata* (Brightw,) Sund.*,* *Pseudo-nitzschia* spp., *Rhizosolenia imbricata* Brightw.and *Thalassionema nitzschioides* Grun.. *Pseudo-nitzschia* spp. was the dominant diatom, present in 60 % of the samples on a yearly basis, with a maximum abundance (up to 1.6 x 106 cells L–1) in the period from June to October (Fig. 3). In June 2006, it contributed up to 97 % of the total diatom abundance. Its abundance had a significant positive correlation with temperature (r=0.484, p<0.05), phosphate (r=0.178, p<0.05) and ammonium (r=0.188, p<0.05).

Figure 3

3.3 DA production

Domoic acid was detected in *Mytilus galloprovincialis* at concentrations below regulatory limits, from 0.097 to 0.8721 µg g-1 on five occasions from April to October 2005 in Lim Bay. In that period, the Si:TIN ratio was from 0.6-2.9 and the Redfield ratio was from 35-327, indicating silicate and phosphate limitation. *Pseudo-nitzschia* spp. were present in that period with an abundance not higher than 5.5 x 103 cells L–1. During the rest of the research period, DA was below the detection limit of the instrument.

3.4 Morphology and ecology of individual species

Physico-chemical conditions during the August 2007, October 2007 and February 2008 blooms are listed in Table 3. All *Pseudo-nitzschia* species formed stepped colonies (Figs. 4-8). Under the light microscope, *Pseudo-nitzschia* species were distinguished based on their valve width as the *P. delicatissima* group (< 3µm) and the *P. seriata* group (> 3µm). The cell ends in girdle view were pointed and elongated and identified as the *Pseudo-nitzschia pseudodelicatissima* species complex. The August bloom of phytoplankton was dominated by *Pseudo-nitzschia pseudodelicatissima sensu lato*. The frustules were 77-98 µm long and 3.1-2.3 µm wide in valve view. The cells were linear, with 10-14 % overlap. TEM analyses revealed three species: *P. calliantha*, *P. manii* Amato & Montresor and *P. pseudodelicatissima* (Hasle) Hasle *emend*. Lundholm, Hasle & Moestrup. *P. manii* and P, *calliantha* dominanted the bloom, while *P. pseudodelicatissima* was scarce.

Table 3.

In October 2007, *Pseudo-nitzschia* contributed up to 90 % of the total diatom abundance and the bloom was almost exclusively formed of *P. calliantha*. In the phytoplankton assemblages in February 2008, *P. fraudulenta* (Cleve) Hasle and *P. pungens* (Grunov ex Cleve) Hasle were identified. Both species contributed equally to the total *Pseudo-nitzschia* abundance. They were present in all samples, but with a smaller contribution to the total diatom abundance (30 % on average).

Table 4.

*P. calliantha*

The cells are linear, forming stepped colonies with 12-13 % overlap (Fig. 4a). The frustules are 74-93 µm long and 0.7-2.2 µm wide in valve view (Table 4). The valve has 17-27 regularly spaced fibulae and 32-45 striae in 10 µm (Fig. 4b). The raphe is divided in the middle by a central nodule (Fig. 4c). Each stria has one row of round–squared poroids, 4-8 in 1 µm. The poroid pattern resembles a flower, the hymen being perforated with several sectors arranged in circle and often with a circle in the middle (Fig. 4e). The cingular band is perforated with striae, 2-3 poroids wide and with a varying number of poroids, the number of which decrease in a valvar direction (Fig. 4d).

*P. manii*

The cells are linear, forming stepped colonies with 10-14% overlap (Fig. 5a). The apical axis is 77-98 µm long and 1.3-1.7 µm wide in valve view. A central large interspace is present (Fig. 5d). The density of striae ranges 34-41 and the density of fibulae is 19-24 in 10 µm (Fig. 5b). Each stria contains one row of square to round poroids with a density of 4-6 in 1 µm. Poroids are split into 2-4 sectors with a radial arrangement. The first cingular band is ornamented by biseriate striae of simple poroids (Fig. 5e). Striae are four poroids high. The second and third cingular bands are less silicified, with a similar pattern of biseriate striae of simple poroids is three poroids high (Fig. 5c).

*P. pseudodelicatissima*

Overlapping cells were detected in colonies. Cells linear in valve view (Fig. 6a). The transapical axis 1.15-1.71 µm. Eccentric raphe divided in the middle by a central module (Fig. 6c, d). The density of fibulae 18-24 and the density of striae 23-40 in 10 µm. Striae with one row of oval to square poroids, with 3-5 poroids in 1 µm. Poroids divided into 2-4 parts (Fig. 6b, c).

*P. fraudulenta*

The species formed colonies with 14-17 % overlap (Fig. 7a). The valves are lanceolate with pointed ends and a central interspace. The cells are 71-101 µm long and 4-7 µm wide (Fig. 7b, c). The density of striae (19-28 in 10 µm) and fibulae (18-30 in 10 µm) is about the same. The striae are perforated by two rows of poroids (6-7 in 1 µm). Poroids are perforated with several irregular sectors (Fig. 7e). The cingular bands have striae perforated by two to three irregular rows of small poroids closed to the interstriae (Fig. 7d).

*P pungens*

The valves are linear to lanceolate, 88-105 µm long and 3.9-5.7 µm wide. The species formed colonies with 21-22 % overlap (Fig. 8a). The density of striae (8-15 in 10 µm) and fibulae (10-17 in 10 µm) is about the same (Fig. 8b, c). The striae contained two rows of poroids with a poroid density of 2-4 in 1 µm.

Figure 4, 5, 6, 7, 8

1. Discussion

4.1 *Pseudo-nitzschia* diversity, abundance and temporal distribution

*Pseudo-nitzschia* species are permanent constituents of phytoplankton in the northern Adriatic Sea (Bernardi Aubry et al., 2004; Bosak et al., 2009; Viličić et al., 2009). Compared to other coastal areas like Portuguese coastal areas (Churro et al., 2009), Irish waters (Cusack et al., 2004) and the Bay of Fundy in Canada (Kaczmarska et al., 2005), species diversity is not high in Lim Bay. This could be due to year-to-year differences in the occurrence of *Pseudo-nitzschia* species. Interannual variability in species composition is readily observed in long term data sets (Dakos et al., 2009), while in this paper, we examined in detail only samples from a one-year period. In an area with high species diversity, the Aveiro coastal lagoon in Portugal, eleven different *Pseudo-nitzschia* species were reported over a four-year research period (Churro et al., 2009). On the other hand, looking at species diversity in a one-year period, no more than five species co-occurred, which supports the possible existence of additional different species in Lim Bay, which were subsequently observed in samples from further investigations (Bosak, unpublished data).

The majority of the dominant diatoms preferred nutrient-enriched conditions. *Pseudo-nitzschia* abundance was positively correlated to temperature, phosphate and ammonia, which is in accordance with its mass development in the summer/autumn period. Increase of nutrient

concentrations due to fish farming in October – November period has been reported for Middle Adriatic, showing higher concentrations of phosphate and ammonia in the upper part (0 – 20 m) of the water column (Matijević et al., 2009; Skejić et al., 2010). This is in accordance with phosphorous higher concentrations (Bosak et al., 2009) during the *Pseudo-nitzschia* outbreak in Lim Bay. The lowest values of the Si:N ratio coincided with *Pseudo-nitzschia* species blooms in our study and in other studies in the Adriatic (Penna et al., 2006; Burić et al., 2008). It may be assumed that low Si:N ratios either favour the growth of *Pseudo-nitzschia* species over other diatoms due to lower silica requirements, as has been previously indicated (Sommer, 1994; Marchetti et al., 2004), or it could be a consequence of the nutrient uptake of the bloom.

4.2 Taxonomy and ecology

*P. calliantha*

The morphological measurements of *P. calliantha* valves in Lim Bay were similar to the valves measured in the northern Adriatic (Marić et al., 2011) and generally resembled the characterisation given in the original description (Lundholm et al., 2003). Nevertheless, our measurements exceeded the ranges for *P. calliantha* reported so far (Caroppo et al., 2005; Amato et al., 2007; Spatharis et al., 2007; Quijano-Scheggia et al., 2008). The minimum width was 0.7 µm (as opposed to 1.3 µm), the maximum number of fibulae in 10 µm was 27 (as opposed to 25) and the maximum number of striae in 10 µm was 45 (as opposed to 40).

*P. calliantha* is a geographically widespread species (Lundholm et al., 2003; Quijano-Scheggia et al., 2008; Churro et al., 2009), and according to recent investigations and to this study, is a permanent constituent of phytoplankton assemblages in Adriatic Sea (Caroppo et al., 2005; Burić et al., 2008). Blooming events in both the warmer and colder parts of the year indicate its tolerance to a wide range of temperatures (Caroppo et al., 2005; Quiroga, 2006; Burić et al., 2008; Quijano-Scheggia et al., 2008). *P. calliantha* has been found to produce DA in several areas around the world as well as in cultures (Martin et al., 1990; Lundholm et al., 1997; Lundholm et al., 2003; Besiktepe et al., 2008; Sahraoui et al., 2009). During the *P. calliantha* blooms and October 2007 in Lim Bay no DA was detected. On the other hand, in the same period it bloomed in the northern Adriatic, its highest abundance preceded the appearance of DA in this area (1.315 µg DA g-1 in the tissue of *M. galloprovincialis*), indicating that northern Adriatic *P. calliantha* is able to produce DA (Marić et al., 2011). This variability in toxicity could suggest the existence of cryptic species within what is called *P. calliantha,* as has been reported before (Sahraoui et al., 2009), or this could be a consequence of different environmental conditions influencing toxin productivity. During the period of the *P. calliantha* bloom, phosphorous concentrations where twice as high as on average, suggesting that DA production in Lim Bay can be suspended because of phosphate increase, as has been recorded in batch cultures (Pan et al., 1996b).

*P. manii*

This species occurred in stepped colonies and by light microscopy is undistinguishable from *P. calliantha* and *P. pseudodelicatissima* (Lundholm et al., 2003); therefore, the August 2007 bloom was described as a *Pseudo-nitzschia delicatissima sensu lato* bloom. Summer periods were characterised by stratification and depletion of nutrients in the water column, probably not providing enough nutrients for single species domination.

The valve ultrastucture of *P. manii* is very similar to *P. calliantha* (Lundholm et al., 2003; Amato et al., 2007; Amato and Montresor, 2008) sharing the presence of uniseriate striae on the valve face, the density of poroids within the striae, and the arrangement of the poroids split into several sectors. Our measurements of *P. manii*are mainly in accordance with the original description, except for the width of the valve (1.3-1.7 µm) that corresponds more to the description of *P. calliantha* (1.4-1.8 µm for *P. calliantha,* 1.7-2.6 µm for *P. manii*)(Amato et al., 2007). Since all the other measurements do correspond to the original description, we identified the species as *P. manii.* So far, this species has been described and reported only in the Tyrrhenian Sea and this study is the first report from the Adriatic Sea for this particular species. No toxicity has been associated with this species (Amato and Montresor, 2008) to date.

*P. pseudodelicatissima*

The morphology of *P. pseudodelicatissima* is very similar to the morphology of potentially toxic *P. cuspidata* except for the shape of the valves, where *P. pseudodelicatissima* has linear valves and *P cuspidata* lanceolate valves (Lundholm et al., 2003). We identified the species as *P. pseudodelicatissima* since the linear shape of the valve and the density of striae, fibulae and poroids, as well as the width, corresponded to the original description of *P. pseudodelicatissima*. The size and number of perforated sectors within one poroid varies within and between the valves, as has been previously reported only in the Bay of Fundy (Kaczmarska et al., 2005). It has been suggested that the differences in the pattern and number of sectors in the poroids may result from individual variability in the degree of valve silification, differences in grow rate or may reflect a systematic distinction of the local populations. Since morphological, genetic and ecological variability of this species has not been fully examined (Hallegraeff, 1994; Priisholm et al., 2002; Stehr et al., 2002; Lundholm et al., 2003; Kaczmarska et al., 2005), many questions arise and further research is merited.

*P. fraudulenta* and *P. pungens*

*P. fraudulenta* also occurred in stepped colonies, with the cells overlapping by 14-17 % of their length, which is higher than recorded elsewhere (Kaczmarska et al., 2005). Morphological measurements of the valves are in accordance with the literature, but some maximum values do exceed to the reported ranges for *P. fraudulenta* (the maximum values of fibulae in 10 µm was 30, as opposed to the reported value of 22-26) (Stehr et al., 2002; Kaczmarska et al., 2005; Churro et al., 2009).

*Pseudo-nitzschia* species developing in February 2008 were identified as *P. fraudulenta* and *P. pungens*. Although *P. pungens* and *P. fraudulenta* are cosmopolitan species (Hasle and Fryxell, 1995; Hasle and Syvertsen, 1997; Hasle, 2002) this is the first record for those species for the Adriatic Sea. In the Mediterranean Sea *P. fraudulenta* is more abundant in early spring with cell density never attaining higher than 105 cells L-1 (Zingone et al., 2006; Quijano-Scheggia et al., 2008),which is in accordance with this study. In Lim Bay, it appeared mixed with *P. pungens* as was observed on the Catalan coast (Quijano-Scheggia et al., 2008).

4.3 Toxicity

Of the five species found in Lim Bay during the present study, *P pungens*, *P. fraudulenta, P. pseudodelicatissima*  and *P. calliantha* have previously been reported to be toxic elsewhere (Hasle, 2002; Stehr et al., 2002; Fryxell and Hasle, 2003; Lundholm et al., 2003; Sahraoui et al., 2009; Moschandreou et al., 2010). As DA production in Lim Bay was recorded in the year 2005, more detailed studies on the genus *Pseudo-nitzschia* were needed. Since then, further detailed taxonomical analyses of *Pseudo-nitzschia* species were performed, but so far is not clear which species is responsible for DA production in Lim Bay. The species-specific relationship, together with the environmental conditions and DA production still need to be elucidated. DA production in Lim Bay coincided with a low Si:N ratio and a high TIN:PO4 ratio, supporting previous findings that phosphate and silicate limitation enhances DA production (Pan et al., 1996a; Fehling et al., 2004; Klein et al., 2010). Phosphate is the limiting nutrient in the Adriatic Sea (Vukić-Lušić et al., 2008), but due to intensive fish farming in Lim Bay, its concentration has increased in the summer to autumn period, stimulating *Pseudo-nitzschia* growth. It can be speculated that DA production in Lim Bay might be suspended as a result of phosphate increase, as has been recorded in batch cultures (Pan et al., 1996b).

4.4 Conclusions

*Pseudo-nitzschia* spp. was the dominant diatom in Lim Bay, contributing up to 97 % of the total diatom abundance. Its positive correlation to temperature, phosphate and ammonia was in accordance with its mass development in the summer/autumn period when fish farms had a maximum impact on the environment. Presented data raise the question of increased anthropogenic influence in coastal areas and its possible negative influence on aquaculture production, fisheries and public health. Although the DA production reported in this study was below the regulatory limits, the mass and sustained development of potentially toxic species represents a risk for ASP events. Understanding the mechanisms of *Pseudo-nitzschia* species development and DA production in relation to environmental factors still remains a challenge for future research. Nevertheless, this study is the first assessment of DA productionduring *Pseudo-nitzschia* annual successions in the Adriatic Sea. We suggest the initiation of high frequency monitoring of *Pseudo-nitzschia* species and environmental factors in areas where aquaculture takes place in order to understand and manage the impact of potential harmful blooms.

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Figure captions:

Figure 1. Map showing sampling stations in the Lim bay, northern Adriatic Sea.

Figure 2. Scatterplot of the first two principal components following the PCA of environmental parameters overlaid with *Pseudo-nitzschia* spp. abundance, revealing two distinguishable groups: 1&4 spring with winter and 3&2 summer with autumn.

Figure 3. Temporal distribution of monthly mean diatom (bold line) and *Pseudo-nitzschia* spp.(solid line) abundance and silicate to total inorganic nitrogen ratio (dashed line) in the period 2004 – 2008 in the Lim bay.

Figure 4. *Pseudo-nitzschia calliantha*, field material. (a-e). Light micrograph of stepped colony in girdle view (a). Tip of the valve (b), large central interspace (c), girdle band (d) and poroid pattern (e) TEM.

Figure 5. *Pseudo-nitzschia manii,* field material. (a-e). Light micrograph of stepped colony in girdle view (a). Top of the valve (b), girdle bands (c), large central interspace (d) and poroid pattern (e) TEM.

Figure 6. *Pseudo-nitzschia pseudodelicatissima*. External view of whole valve SEM (a). Tip (b) and middle (c) of the valve TEM. Internal view of middle of the valve SEM (d).

Figure 7. *Pseudo-nitzschia fraudelenta.* Light micrograph of stepped colony in valvar view (a). Top of the valve (b), middle of the valve, central nodule (c), girdle band (d) and poroid pattern (e) TEM.

Figure 8. *Pseudo-nitzschia pungens*. Light micrograph of stepped colony in valval view (a). Tip of the valve (b) and middle of the valve (c) TEM.