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Pseudo-nitzschia Peragallo (Bacillariophyceae) diversity and domoic acid accumulation in tuberculate cockles and sweet clams in M'diq Bay, Morocco

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Abstract – The diversity of *Pseudo-nitzschia* (Bacillariophyceae) and accumulation of the neurotoxin domoic acid (DA) in two types of shellfish; tuberculate cockles (*Acanthocardia tuberculata*) and sweet clams (*Challista chione*) was explored in M'diq Bay, Morocco during 2007. The highest abundances of *Pseudo-nitzschia* were found during the period from March to October, with peaks occurring in May and September. Toxin analysis showed an accumulation of domoic acid in shellfish sampled during spring and autumn. The maximum toxin concentration was 4.9 µg DA g^{-1} of the whole tissue recorded in sweet clam during spring. Using transmission electron microscopy, thirteen *Pseudo-nitzschia* species were identified, eight of which are known as producers of domoic acid: *P. multistriata, P. cuspidata, P. galaxiae, P. multiseries, P. pseudodelicatissima, P. pungens var. aveirensis, P. calliantha and P. fraudulenta*. The five non- toxic species observed were *P. subpacifica*, *P. arenysensis*, *P. dolorosa, P. subfraudulenta*, and *P. cf. caciantha.*

Key words: Diatoms, domoic acid, phytoplankton, *Pseudo-nitzschia,* shellfish, Morocco

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Introduction

The planktonic diatom genus *Pseudo-nitzschia* presently comprises 37 species (>20 µm long), several of which have a wide biogeographical distribution (HASLE 2002). Fourteen species are known to produce domoic acid (DA), a toxin that may accumulate e.g. in shellfish, and cause amnesic shellfish poisoning (ASP) in humans eating contaminated shellfish. Human intoxication with DA gives an array of gastro-intestinal and neurological symptoms such as vomiting, diarrhoea, mental confusion, memory loss (amnesia), disorientation and coma (WRIGHT et al. 1989). The first correlation between *Pseudo-nitzschia* bloom and DA contained in shellfish was confirmed in Prince Edward Island, Canada (BATES et al. 1989), where *P. multiseries* abundance exceeded 15 x 10^6 cells L^{-1} and mussels were contaminated by 790 µg of DA g^{-1} of shellfish meat. On the other hand, AMZIL et al. (2001) first reported DA in relation to *Pseudo-nitzschia* blooms on the French Mediterranean coast. Subsequently, other studies have reported detection of DA in Italy (SARNO and DAHLMANN (2000)), Greece (KANIOU- GRIGORIADOU et al. 2005, MOSCHANDREOU et al. 2010), Tunisia (INÈS et al. 2006) and Croatia (UJEVIĆ et al. 2010).

MASSUTI and MARGALEF (1950) and MARGALEF (1969) have previously described *Nitzschia delicatissima*, N. *pungens*, and *N. seriata* in the Mediterranean coasts. In the last decade, several studies were conducted on the systematics of *Pseudo-nitzschia*, and a number of species have been identified. Species identified in Spain are *P. brasiliana, P. calliantha, P. delicatissima, P. fraudulenta, P. multistriata, P. pungens, P. galaxiae, P. cacciantha, P. mannii, P. arenysensis* (QUIJANO-SCHEGGIA et al. 2008, 2010) and *P. australis* (ZAPATA et al. 2011). In Italy, *P. calliantha* and *P. delicatissima* (CAROPPO et al. 2005, ZINGONE et al. 2006), *P. galaxiae, P. multistriata* (SARNO and DAHLMANN 2000), ORSINI et al. 2002, CERINO et al. 2005, ZINGONE et al. 2006), *P. fraudulenta* (ZINGONE et al. 2006) were identified. *Pseudo-nitzschia calliantha* (SPATHARIS et al. 2007) was identified in Greece. In the southern Black Sea (BARGU et al. 2002) and in the eastern Adriatic Sea, *P. calliantha, P. fraudulenta, P. pungens, P. pseudodelicatissima, P. manii* (LJUBEŠIĆ et al. 2011, MARIĆ et al. 2011) were identified. The Maghreb coasts were also investigated and some species of *Pseudo-nitzschia* were identified such as *P. calliantha* in Tunisia (INÈS et al. 2006) and *P. calliantha* in Algeria (ILLOUL et al. 2008). In Morocco, only one study dealing with *Pseudo-nitzschia* systematics was done, by AKALLAL et al. (2002) in the Atlantic coast; seven species was identified: *P. fraudulenta, P. multiseries, P. multistriata, P. pungens var. cingulata, P. subpacifica, P. delicatissima* and *P. pseudodelicatissima*.

In Morocco, PSP (paralytic shellfish poisoning) intoxication by mussels was first reported in November 1971 and October 1975 (ESSAID 1977). Subsequently, several intoxications by PSP toxins were recorded: in October 1982 with two deaths (BOURHILI 1984), in November 1994 with high number of incidences of human intoxication and four deaths (TALEB et al. 2001).The first human intoxication with domoic acid after ingestion of infected mussels probably dates back to 1978 in Al-Hoceima (Mediterranean coast), where patients who had eaten mussels (*Mytilus galloprovincialis*) suffered from loss of memory and disorientation. These symptoms are characteristic of DA effects on human consumers; and of other shellfish poisonings, none results in amnesiac states.

Pseudo-nitzschia blooms have frequently been observed off the western Mediterranean coast of Morocco since 2002. . Due to previous ASP episodes in and outside Morocco, it was judged important to explore *Pseudo-nitzschia* diversity in local areas such as M'diq bay, which is subjected to commercial shellfish exploitation and to gather knowledge on

species dynamics in order to highlight their effect on shellfish continuation, mainly tuberculate cockles and sweet clams.

In this regard, the present study was undertaken in M'diq Bay from January to December 2007, aiming at determining the biodiversity of *Pseudo-nitzschia*. This study deals with their seasonal diversity follow-up and abundance and identification of putative DA producers.

Material and methods

Location stations

Sampling was performed in M'diq Bay, which is located in the west Mediterranean coast of Morocco, adjacent to the Gibraltar Strait (35°43'425 N – 05°19'841 W). This sampling point is 7–10 m deep (Fig. 1). Continental inputs reach M'diq Bay through one temporal and torrential stream. This bay features important socio-economic activities, particularly tourism and fishing. Among the latter, shellfish catching is important (559 tons year^{-1}).

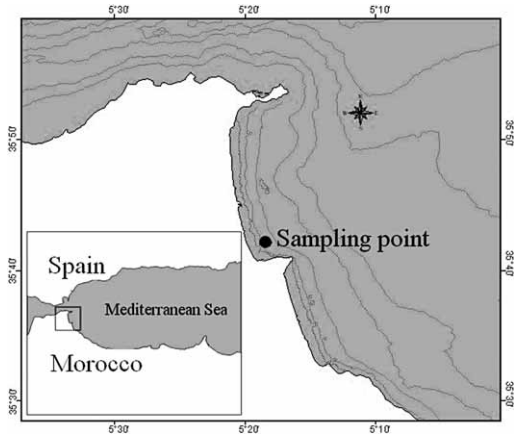


Fig. 1. Location of the sampling site in M'diq Bay (Morocco).

Seawater sampling

Seawater samples were taken on a weekly frequency basis; this was to allow a weekly estimation of *Pseudo-nitzschia* abundance. Seawater samples were taken using Nansen bottles at depths of 0.5 m. Monthly samplings of seawater were also carried out using a plankton net with a 20 µm mesh for *Pseudo-nitzschia* species identification purpose. These samples were fixed with an appropriate solution (acetic Lugol) and kept in conditions of darkness.

Phytoplankton analysis

Pseudo-nitzschia abundance was evaluated by counting using the inverteded microscope (UTERMÖHL 1958). For electron microscope analysis, about 100 specimens were examined for each sample in order to identify its species and determine its composition.

For ultra-structural examination of the frustules, the cells were rinsed by chemical oxidation (LUNDHOLM et al. 2002); ten mL of each sample was transferred into 50 mL conical tubes. $CaCO_3$ was removed by adding 1 mL 10% HCl and overnight oxidation took place after addition of 2 mL 30% H_2SO_4 and 10 mL saturated aqueous solution of $KMnO_4$, with periodic agitation. Samples were cleared by the addition of 10 mL of saturated oxalic acid and rinsed with distilled water and centrifuged (3–4 times). After removal of the supernatant, 20 µL of the obtained material was placed on a Millipore disc and left to dry. Examination of the grids was done by transmission electron microscope (TEM) using a Jeol/JEM-1011.

Domoic acid analysis

Concentrations of DA were determined by HPLC (Shimadzu 10vp type). This apparatus is composed of a SCL-10vp Controller, a LC-10ADvp Quaternary Pomp, a CTO-10vp Colonne Four, a SIL-10ADvp Autosampler, a SPD-M10Avp Photodiode Array Detector, a Vydac C18 column (250 × 4.6 mm, with 5 µm) and the Guard Cartridge (Vydac C18, 5 µm).

DA was assessed in periods of high *Pseudo-nitzschia* abundance. It is measured in the whole meat of cockles and sweet clams according to the QUILLIAM et al. (1995) protocol. Threefold analysis was performed using about 100 g of shellfish meat (ten to fifteen individuals are required to have such an amount of meat). After being shredded and homogenated, four g of meat were added to 16 mL of solvent extraction (methanol-water, 1:1) and then homogenized (Ultra-Turrax for 3 minutes at about 10,000 rpm). The homogenate was centrifuged at least at 4,000 rpm for 10 min to obtain supernatant. The later was analyzed using the following chromatographic conditions: mobile phase flow rate of 1 mL min^{-1}, detector wave length of 242 nm, injection volume of 20 µL and an oven temperature for the column of 40 °C. The determination of DA content in samples was done with a detection limit of 0.3 µg g^{-1}.

Results

Domoic acid and *Pseudo-nitzschia* abundance

During the sampling period (January to December 2007), *Pseudo-nitzschia* in M'diq Bay was present continuously in low abundance and higher abundance (ca. 10–20 cells mL^{-1}) were observed from March to November (Fig. 2). Five proliferation periods (>30 cells mL^{-1}) were recorded during this period, with two major peaks (88 cells mL^{-1} and 157 cells mL^{-1}) occurring in May and in the end of September, respectively (Fig. 2).

HPLC Analysis showed the presence of DA on five occasions (Tab. 1, Fig. 2). The highest DA levels were recorded during spring. The highest DA concentration found was 4.9 µg DA g^{-1} recorded in sweet clam in May 2007. This study reports for the first time the presence of DA in shellfish in the Mediterranean coast of Morocco. However, DA concentration never exceeded the normative threshold of 20 µg AD g^{-1} of shellfish meat (Tab. 1).

Pseudo-nitzschia diversity

Using scanning transmission electron microscopy, thirteen species were identified as toxic (Tab. 2, Fig. 3): *Pseudo-nitzschia cuspidata* (Hasle), *P. fraudulenta* (Cleve), *P.*

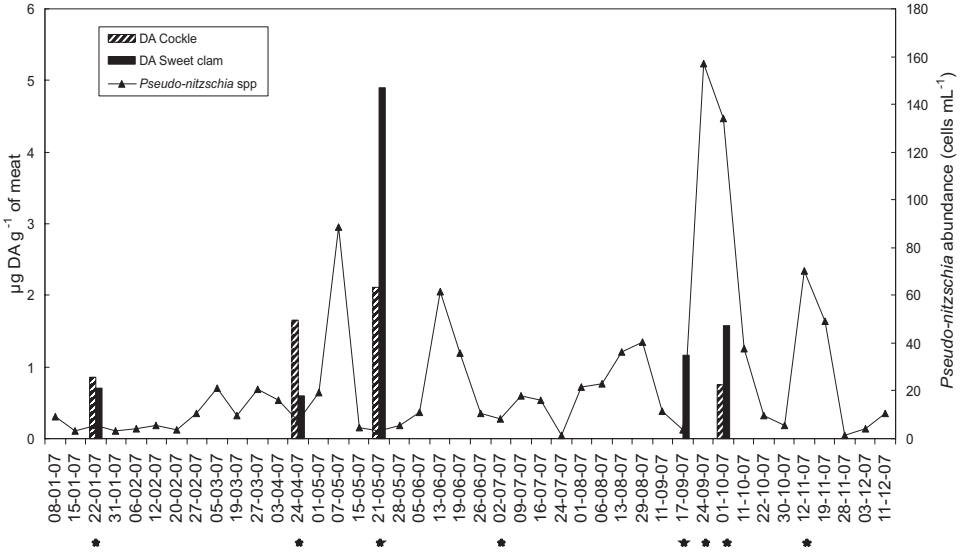


Fig. 2. Abundance of *Pseudo-nitzschia* and domoic acid (DA) concentrations in shellfish from M'diq Bay during 2007. ★ Sampling for domoic acid determination

Tab. 1. Domoic acid concentration (µg DA g^{-1} of meat) in shellfish *Acanthocardia tuberculata* (tuberculate cockle) and *Callista chione* (sweet clam). ND = not detected.

Date	µg DA g^{-1} of meat (*Acanthocardia tuberculata*)	µg DA g^{-1} of meat (*Callista chione*)
2007-01-22	0.85 (±0.10)	0.71 (±0.80)
2007-04-24	1.66 (±0.19)	0.59 (±0.7)
2007-05-21	2.11 (±0.25)	4.90 (±0.58)
2007-07-02	ND	ND
2007-09-17	ND	1.16 (±0.13)
2007-09-24	ND	ND
2007-10-01	0.75 (±0.09)	1.57 (±0.18)
2007-11-12	ND	ND

multistriata (Takano), *P. pseudodelicatissima* (Hasle), *P. galaxiae (*Lundholm et Moestrup), *P. multiseries* (Hasle), *P. calliantha (*Lundholm, Moestrup et Hasle) and *P. pungens var. aveirensis (*Lundholm, Churro, Carreira et Calado). The other five non-toxic species are *P. dolorosa* (Lundholm et Moestrup), *P. arenysensis* (Quijano-Scheggia, Garcés, Lundholm), *P. subpacifica* (Hasle), *P. subfraudulenta* (Hasle), and *P. cf. caciantha* (Lundholm, Moestrup et Hasle).

The composition of *Pseudo-nitzschia* species varied greatly during the year (Fig. 4). Some species were found during a long period of the year while others appeared in specific short periods. *P. cuspidata, P. fraudulenta, P. subpacifica* and *P. arenysensis / P. delicatissima* were the most frequently recorded species. The spring and autumn periods are the

Tab. 2. Morphometric summary of *Pseudo-nitzschia* species in M'diq Bay.

Species name	Valve shape	Fibulae in 10 µm	Striae in 10 µm	Row of poroids	Poroids in 1 µm	Centrale nodule	Length (µm)	Width (µm)
P. cuspidata	lanceolate	20–24	34–41	1	5–6	+	58.5–65.3	1.6–2.3
P. subpacifuca	linear	17–20	28–32	2(3)	9–10	+	45.2–60.1	4.9–6.1
P. arenysensis/ P. delicatissima	lanceolate	20–24	36–38	2	9–12	+	39.5–45.1	1.8–2.1
P. fraudulenta	linear	21–24	21–24	2(3)	6–7	+	65.4–70.1	4.3–5.2
P. multistriata	lanceolate	26–30	38–40	2(3)	12	–	60.2–70.3	3.3–3.8
P. pseudo-delicatissima	linear	20–26	36–45	1	5–6	+	65.5–69.0	1.7–2.1
P. subfraudulenta	linear	15–18	25–26	2	6–7	+	46.3–52.1	4.2–4.9
P. multiseries	lanceolate	16–17	16–17	3(2–4)	6–7	–	80.0–85.2	2.8–3.2
P. calliantha	linear	19–21	32–36	1	5–6	+	58.2–68.1	1.7–1.9
		24–26	44–45	1	5–6	+		
P. dolorosa	lanceolate	17–20	30–37	1(2)	6	+	73.0–90.3	2.2–2.8
P. galaxiae	Lanceolate	20–25	68–70	n.d.	n.d.	+	20.0–25.0	1.3–1.6
P. pungens var. *aveirensis*	n.d.	16	16	2	4	–	n.d.	2.9–3.1
P. cf. *cacciantha*	lanceolate	18–21	33–34	1	5	+	72.0–75.0	2.6–2.7

two main seasons for proliferation of *Pseudo-nitzchia* species, particularly those known to be producing DA. Eight and nine species were identified during spring and autumn, respectively (Fig. 4). During *Pseudo-nitzschia* bloom in May, *P. arenysensis / P. delicatissima* were significantly dominant at 65% while during the *Pseudo-nitzschia* bloom in October, several species of *Pseudo-nitzschia* proliferated but there were three dominating species (*P. pseudodelicatissima*, *P. dolorosa* and *P. cuspidata*) with lower values, reaching 20%, 18% and 17% respectively.

Discussion

This study was conducted in the M'diq Bay during 2007. The evolution of species composition of *Pseudo-nitzschia* was studied in comparison with the local evolution of DA detection in two shellfish species: tuberculate cockle (*Acanthocardia tuberculata*) and sweet clam (*Challista chione*). This study is the first for the Mediterranean coastline of Morocco.

It has been shown that there is a continuous presence of *Pseudo-nitzschia spp.* in M'diq Bay, characterized by 5 proliferation periods (>20 cells mL^{-1}) (March to November). The lower abundances were recorded during the rainy period (December – February). Similar results were found by QUIJANO-SCHEGGA et al. (2008) on the Spanish Mediterranean coast. According to LOUREIRO et al. (2009), the *Pseudo-nitzschia* abundance recorded in Catalonia in 2007 was similar to that recorded in this study in autumn, while in April, their reported value of *Pseudo-nitzschia* abundance is lower than in the present study. Perhaps the *Pseudo-nitzschia* bloom registered on the Spanish coast during April could be conducted by ocean currents to the Moroccan coast.

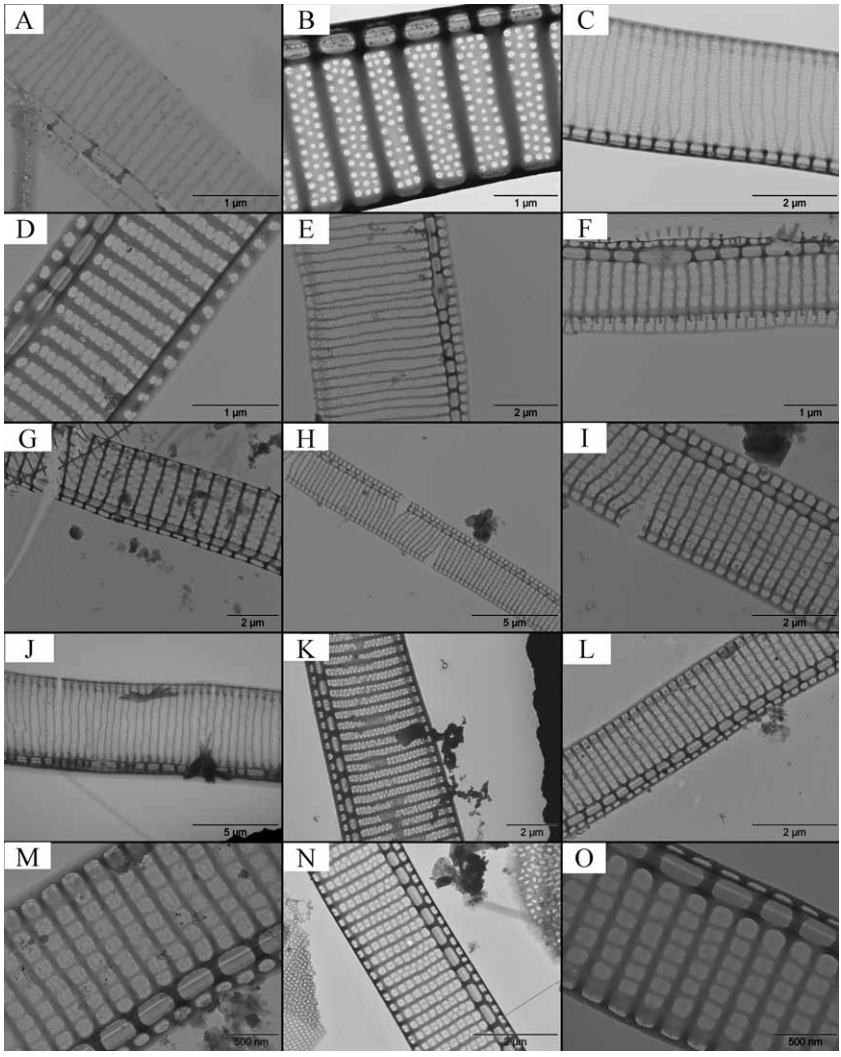


Fig. 3. Transmission electron micrographs of *Pseudo-nitzschia spp*. from M'diq Bay. A – *P. galaxiae*; B – *P. multiseries*; C – *P. multistriata*; D – *P. arenysensis*; E – *P. subpacifica*; F – *P. cuspidata*; G – *P. pungens* var. *aveirensis*; H, I – *P. cf. cacciantha*; J – *P. fraudulenta*; K – *P. subfraudulenta*; L, M – *P. calliantha*; N – *P. dolorosa*; O – *P. pseudodelicatissima*.

In this study, the highest *Pseudo-nitzschia* abundance and DA concentration occurred in May and the end of September; both periods showing a transition between cold and warm seasons. Environmental condition changes seem likely to have boosted *Pseudo-nitzschia* development. It is possible that the M'diq bay area experienced an increase in nutrients during these periods. Several studies suggested that *Pseudo-nitzschia* blooms are associated with cool and high nutrient waters (TRAINER et al. 2000). *Pseudo-nitzschia* abundance has a positive correlation with water temperature, phosphate and ammonium (LJUBEŠIĆ et al. 2011).

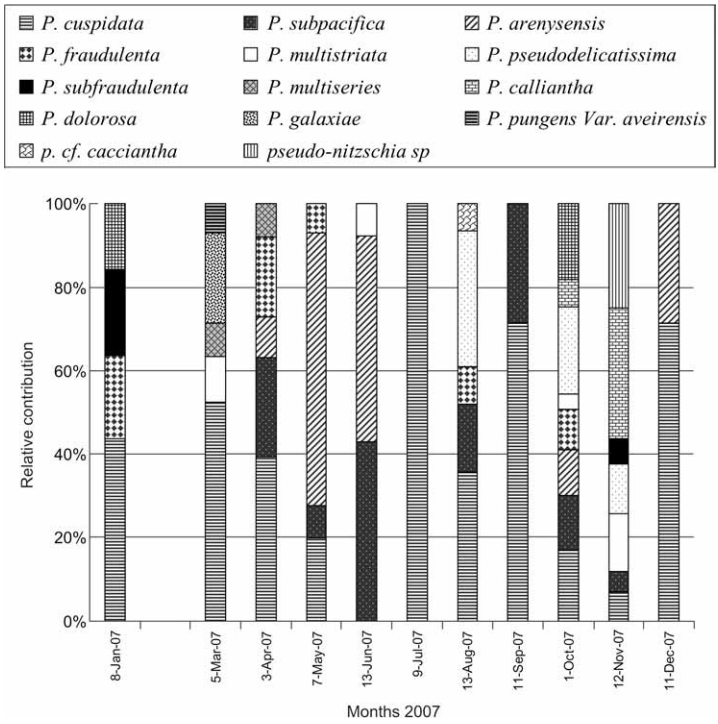


Fig. 4. Relative contributions of *Pseudo-nitzschia* species in M'diq Bay in the period January to December 2007.

In spring bloom (88 cells mL^{-1}), we recorded DA presence in both the two studied shellfish species, showing a high value in sweet clam (4.9 µg DA g^{-1}) and low value in tuberculate cockle (2.11 µg DA g^{-1}). This variation of DA contamination between these two shellfish species could be related to their different biological characteristics; it seems that their specific receptors for DA could be different and have varying affinities for DA. The same explanation was given by SAGOU et al. (2005) for contamination by PSP toxins. During spring bloom, we observed four dominant *Pseudo-nitzschia* species: *P. arenysensis* (65%*)* and *P. cuspidata* (19 %) as well as two species with lower abundance*: P. fraudulenta* (7%) and *P. subpacifica* (9%) (Fig. 4).

Eight identified species (Tab. 2, Fig. 3) are considered producers of DA (according to LUNDHOLM et al. 2011). Domoic acid recorded in shellfish could be related to *P. cuspidata and P. fraudulenta*, as they are known to be DA producers (RHODES et al. 1996, RHODES 1998, TRAINER et al. 2009, QUIJANO-SCHEGGIA et al. 2010). For the other two species, *P. subpacifica* is not known to be a DA producer while the case of *P. Arenysensis* requires some clarification. *Pseudo-nitzschia arenysensis* is quite similar to *P. delicatissima*; it is not possible to distinguish between them using microscopic observation, only by genetic analysis (QUIJANO-SCHEGGIA et al. 2008). Moreover, *P. delicatissima* is deemed to be toxic but *P. arenysensis* is not. Thus, the 65% of spring bloom, apparently taken by *P. arenysensis*, could be also composed partially or totally of *P. delicatissima*, which could produce DA and likely infect shellfish.

In the autumn bloom, we recorded a *Pseudo-nitzschia* abundance reaching 157 x 10^3 cells.L^{-1} and composed of 8 *Pseudo-nitzschia* species, such as: *P. pseudodelicatissima* (20%), *P. dolorosa* (18%), *P. cuspidata* (17%), *P. subpacifica* (13%), *P.* arenysensis (10%), *P. fraudulenta* (9%), *P. calliantha* (6%) and *P. multistriata* (3%). Among these species, *P. dolorosa* has never been recognized as a DA producer while *P. pseudodelicatissima* (MARTIN et al. 1990, PAN et al. 2001, AMZIL et al. 2001), *P. multistriata* (RHODES et al. 2000, SARNO and DAHLMANN 2000, AMATO et al. 2010, QUIJANO-SCHEGGIA et al. 2010) and *P. calliantha* (BESIKTEPE et al. 2008, ALVAREZ et al. 2009, QUIJANO-SCHEGGIA et al. 2010) are already known to be DA producers.

Even if *Pseudo-nitzschia* bloom in autumn was the highest, DA concentration in both the two studied shellfish species was low and lower than that recorded in spring bloom: 1.57 µg DA g^{-1} in sweet clam and 0.75 µg DA g^{-1} in tuberculate cockle. This difference of contamination between spring and autumn could be explained by both the difference in environmental conditions during spring and autumn periods and the differences in *Pseudo-nitzschia* species composing blooms. The production of DA is dependent on the concentration of nutrients (PAN et al. 1996, KLEIN et al. 2010).

The DA detected in tuberculate cockle and sweet clam was due to the presence of some toxic species such as *P. multistriata, P. cuspidata, P. galaxiae, P. multiseries, P. pseudodelicatissima, P. pungens var. aveirensis, P. calliantha* and *P. fraudulenta*. In the present study, the DA level measured in spring and autumn blooms did not exceed the normative threshold of 20 µg DA g^{-1} of shellfish meat. In the literature, several blooms of *Pseudo-nitzschia* resulting in shellfish contamination by DA were studied. Taking into account the available literature on the Mediterranean Sea, the results of the present study, particularly in terms of shellfish DA concentrations, are not sufficient to suggest there is a danger to public health.

During 2007, a large diversity of *Pseudo-nitzschia* species was observed in M'diq Bay. Some of them have already been identified in Mediterranean waters while others are described for the first time: *P. cuspidata, P. multiseries, P. subpacifica* and *P. subfraudulenta*.

Conclusion

The present study has shown that there is a seasonal succession of thirteen species of *Pseudo-nitzschia* all the year round. Some of them have already been identified in Mediterranean waters while others are described for the first time: *P. cuspidata, P. multiseries, P. subpacifica* and *P. subfraudulenta.*

The highest abundance of *Pseudo-nitzschia* species was recorded in spring and autumn. Some of them are known to be producers of a large quantity of DA. During spring and autumn seasons, DA concentration in the two shellfish species studied, tuberculate cockle (*Acanthocardia tuberculata*) and sweet clam (*Callista cheone*), was higher in the latter than in the former. In M'diq bay, these two seasons have to be considered a potentially dangerous period for ASP events. However, much more work needs undertaking for the mechanisms of *Pseudo-nitzschia* species development in relationship with local environmental conditions to be understood. This study is in fact the first attempt at an assessment of *Pseudo-nitzschia* species succession and domoic acid production on the Moroccan Mediterranean Coast.

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