The influence of nitrogen speciation on growth and toxicity of *Pseudo-nitzschia multiseries* and *P. pungens* in batch and continuous cultures

Calu Guillaume\(^1\); Martin-Jezequel Véronique\(^2\), Lefau Estelle\(^2\), Sechet Véronique\(^1\), Lassus Patrick\(^1\), Weigel Pierre\(^3\), Amzil Zouher\(^1\)

\(^1\). IFREMER, Laboratoire Phycotoxines, rue de l’Ile d’Yeu BP 21105 44311 Nantes. Zouher.amzil@ifremer.fr

\(^2\). Université de Nantes, EA 2160 Laboratoire MMS (Mer, Molécule, Santé), 2 rue de la Houssinière - B.P. 92208 44322 Nantes. Veronique.martin-jezequel@univ-nantes.fr

\(^3\). Université de Nantes, UMR CNRS U3B 6204, 2 rue de la Houssinière - B.P. 92208 44322 Nantes. Pierre.weigel@univ-nantes.fr

The amount of urea used as fertilizer in agriculture has tripled over the last four last decades. This nutrient is discharged in large quantities from rivers to coastal waters, and is used as food by many microalgae. Previous studies have shown that numerous toxic species can assimilate both organic and inorganic nitrogen. This has also been proposed by some researchers as a potential cause of the increased occurrence of some diatom species of the genus *Pseudo-nitzschia* that produce domoic acid, a neurotoxin that can contaminate shellfish. Moreover, higher domoic acid content has been reported for *P. australis* in the presence of urea. We investigated the influence of urea on the content of domoic acid in *P. multiseries* and *P. pungens*. Our results showed that, in batch cultures, *P. multiseries* contains more toxin per cell in the presence of urea. In continuous cultures, substitution of nitrates by urea increases the cellular toxin content of *P. pungens*. These findings suggest the importance of nitrogen speciation in domoic acid production by certain species of *Pseudo-nitzschia*.

Keywords : domoic acid, *Pseudo-nitzschia*, nitrate, urea.

Introduction

Domoic acid (DA) is a neurotoxic amino acid that was initially extracted from the Rhodophyte *Chondria armata*. The first evidence of DA as the cause of amnesic shellfish poisoning (ASP) was reported in 1987, following human consumption of contaminated mussels at Prince Edward Island (Wright et al., 1988). This crisis was linked with the occurrence of *Pseudo-nitzschia multiseries* blooms. Clinical symptoms of amnesic shellfish poisonings (ASP) in consumers consist of gastrointestinal distress, confusion, disorientation, memory loss and in the most severe cases, death (Mos, 2001). The threat of ASP is an important consideration as seafood demand and aquaculture activity are increasing throughout the world (Asche et al., 2008). To this end, many research and monitoring programs for the detection of *Pseudo-nitzschia* in seawater and DA in shellfish have been recently developed. In France, the first case of DA in shellfish occurred in 2001 (Amzil et al., 2001). Since that time, *Pseudo-nitzschia* has been recognized as a
cosmopolitan genus in coastal French waters, and the national monitoring network regularly detects the presence of DA in shellfish.

Nitrogen metabolism and its influence on DA production by diatoms has been a subject of recent study. A few papers have focused on this subject, mostly in *P. multiseries*, but to our knowledge, no investigations have been reported on *P. pungens*. *P. multiseries* can be grown with nitrate, urea or glutamate as a nitrogen source (Hillebrand and Sommer, 1996), and uptake rates of nitrogen, urea and ammonium have been calculated for *P. australis* (Cochlan et al., 2008) and *P. delicatissima* (Loureiro et al. 2009). It has been reported that *P. australis* produces more DA in presence of urea than with nitrate or ammonium (Howard et al., 2007); toxic strains of *P. multiseries* and *P. fraudulenta*, however, show variable DA production in the presence of urea (Thessen et al., 2009). Generally, the DA content of *Pseudo-nitzschia* cells is highest during the stationary phase of growth (Bates, 1998), but N-depleted batch cultures of *P. multiseries* present lower DA content during this phase (Bates, 1991).

The increase of certain harmful algae blooms can be partly explained by human activities (Masó and Garcés, 2006) such as agriculture (fertilizer and other agricultural discharges), waste waters and consumption of fossil energies (Nixon, 1995; Heisler et al., 2008). In the last four decades, the amounts of urea used as fertilizer have increased three fold in certain agricultural regions of the world (Glibert et al., 2006), leading to large urea discharge from rivers to coastal waters. Many species of microalgae can use urea as source of nutrition (Antia et al., 1991). In the North Sea, blooms of *Phaeocystis globosa* (Prymnesiophyceae) were linked to nitrate discharge in river waters (Lancelot, 1995). In addition, the occurrence of certain paralytic shellfish poisoning (PSP) episodes has been related to increasing use of urea as a fertilizer (Glibert et al., 2006).

Each year, the French monitoring network (REPHY) detects domoic acid contamination in shellfish along north coasts of France. Thus, in order to better understand the risk of harmful diatom blooms in French coastal waters, we investigated the influence of two nitrogen sources, urea and nitrate, on the production of DA by strains of *P. multiseries* and *P. pungens* isolated from waters of the English Channel and grown in batch and continuous culture.

1. **Material and Methods**

1.1. **Strain specificities**

*P. multiseries* strain “CCL70” was isolated from the Thames estuary (U.K.) in 2007 and *P. pungens* strain “D10” from the West-Brittany coast (Bay of Crozon) in February 2008 by CEFAS Institute and Ifremer researchers. Non-axenic cultures were maintained in natural seawater (collected from the English Channel) enriched in L1 medium with 107 µM silicates (Guillard & Hargraves, 1993). Domoic acid production in both strains was previously confirmed by LC-MS-MS analysis (unpublished data).

1.2. **Batch cultures**

Experimental cultures were established in culture chambers (16°C, 12:12 photoperiod). *P. multiseries* cultures were grown in polystyrene flasks with enriched natural seawater L1 with silicates (107 µM). Experimental culture inocula were depleted of N for one week. To compare the influence of nitrate and urea, nitrogen sources where added to separate enriched batch cultures. Nitrate concentration was fixed at 441 µM and urea concentration at 220 µM.
1.3. Continuous cultures

Continuous cultures of *P. pungens* were grown in polymethylmethacrylate (PMMA) plastic (altuglass ©) 2.3 L photobioreactors, with pH regulated at 8.2, and a flow rate of 0.2 mL/min. The population was first monitored in the system as a batch culture with natural enriched seawater L1 medium with silicates (107 µM), before to switching on the pump to start the continuous culture. The system was first enriched with nitrate (441 µM), which was then replaced by urea (220 µM) on day 14.

1.4. Cell enumeration

Samples for cell quantification were fixed with Lugol iodine solution. *Pseudo-nitzschia* cells were quantified microscopically using a Nageotte counting chamber. Precision of counts was estimated statistically following Lund *et al.* (1958). The confidence interval was $p < 0.10$.

1.5. DA extraction

A 10 ml culture aliquot was centrifuged 20 min at 3600 x g. Cell culots were suspended in 50:50 v/v methanol:water, frozen at −80°C, sonicated (500W, 20 kHz), and filtered on 0.45 µm Whatman filter under ultracentrifugation (8000G, 10 min). The supernatant was filtered on 0.45 µm Whatman filter under ultracentrifugation (8000G, 10 min) to perform DA analysis in the medium. All samples were stored at 2-8°C before analysis.

1.6. DA analysis

Intracellular domoic acid was monitored by the HPLC-UV method (Lawrence *et al.*, 1991), using a Jupiter C18 (Ø = 5µm, 4.6 × 250 mm) column thermostatically controlled at 40°C; mobile phase: 90:10 H2O ; 0.1 % TFA/acetonitrile (v/v); flow rate: 1ml/min. An aliquot of 20µl of each sample was injected for a gradient of 20 min. DO was detected by UV absorbance ($\lambda$ = 242 nm). Standards were certificated by the National Research Council of Canada. (Halifax).

2. Results

Batch cultures of *P. multiseries* strain CCL 70 showed similar growth characteristics under nitrate or urea conditions, without significant differences (max. growth rate between 0.56 d⁻¹ and 0.67 d⁻¹) (Figure 1). On the other hand, growth under N-depleted conditions was very slow (max growth 0.33 d⁻¹). Intracellular DA content was detected at the stationary phase, on day 7 in urea batch culture and on day 8 in nitrate batch culture (Figure 2). Cellular DA was greater in cultures grown on urea between the 7th and the 8th days, followed by a large decrease between the samples collected on the 8th and the 9th days. However, DA levels in the culture medium showed no increase during these days (data not shown).

Continuous culture of *P. pungens* strain D10 was started with a high biomass (around 20 000 cells/ml) and pumps were switched on during exponential phase in order to avoid collapse of the culture (Figure 3). Cultures were at equilibrium when the biomass varied...
Figure 1. Growth of *P. multiseries* CCL70 under different N conditions (batch cultures). White diamonds: no nitrogen source. White triangles: [Urea] = 220µM. Black squares: [Nitrate] = 441 µM.

Figure 2. DA content of *P. multiseries* CCL70 under different N conditions (batch cultures). White columns: [N] = no nitrogen source. Dotted columns: [Nitrate] = 441 µM. Black columns: [Urea] = 220 µM.

by less than 5%. Biomass was slightly higher in the culture grown with nitrate (441 µM) than in that with urea (220µM). DA concentration reached a maximum steady concentration at 0.1 pg/cell with nitrate as the N source. After the transition to urea as the N source, cellular DA content increased from 0.1 pg/cell to 0.2 pg/cell, and continued to increase until the end of the experiment at day 24 in batch culture.
3. Discussion

Our results show that growth rates of *P. multiseries* strain CCL70 in batch culture are similar when grown in two different nitrogen sources, from 0.56 ± 0.06 d⁻¹ (nitrate) to 0.67 ± 0.07 d⁻¹ (urea). This has not always been the case in other studies, as Howard *et al.* (2007) reported a decrease of growth rate in *P. australis* using urea (0.52 ± 0.09 d⁻¹) as a N source in comparison with the rate of 0.89 ± 0.08 d⁻¹ obtained using nitrate. For *P. multiseries*, Thessen *et al.* (2009) provided a large dataset on intraspecific variations between strains. Their results show various growth responses with nitrate (from 0.45 d⁻¹ to 0.76 ± 0.10 d⁻¹) and urea (from 0.3 ± 0.1 d⁻¹ to 0.68 ± 0.07 d⁻¹).

In our study, the intracellular DA content of *P. multiseries* increased in stationary phase, and was higher in the urea treatment compared with the nitrate treatment during early and middle stationary phase. Maximum DA content per cell was 3.16 pg/cell with nitrate as an inorganic nitrogen source and 5.17 pg/cell with urea as an organic nitrogen source. *P. multiseries* is known for producing DA under batch conditions (Bates, 1991). Thessen *et al.* (2009) determined maximum DA content for *P. multiseries* at 0.36 pg/cell for nitrate-enriched and 1.8 pg/cell for urea-enriched medium. The results we obtained for *P. multiseries* are in agreement with the concentrations reported in the literature (Bates, 1998).

The work of Thessen *et al.* (2009) is currently the most complete comparison of growth and DA production of *Pseudo-nitzschia* species using both organic and inorganic nitrogen sources. However, Thessen *et al.* (2009) did not investigate the CCL 70 strain of *P. multiseries* in their experiments. Moreover, our experimental conditions differed from theirs, so comparison between the two studies is not easy. Our results show new data for *P. multiseries*: the high intracellular DA content found at the 8th day was not detected either in the cells or in the culture medium in the following days. This may suggest that DA was assimilated by bacteria in the culture, because *P. multiseries* CCL 70 was a non-axenic strain. Recently, epiphytic bacteria associated with the genus *Pseudo-nitzschia*
were thought to assimilate DA; however the fate of DA in bacteria is unclear (Stewart, 2008). More investigations should be done to provide a fuller understanding of our results. *P. pungens* strain D10 produces more DA when nitrate is replaced by urea under continuous culture. To our knowledge, this is the first study on nitrogen preferences for this species.

Our results are in accordance with those of other authors, and clearly demonstrate that nitrogen source can affect not only the growth, but also the production of domoic acid in *Pseudo-nitzschia* species. However, the actual links between *Pseudo-nitzschia* nitrogen metabolism and DA synthesis pathways currently are insufficiently understood to explain how nitrogen species can affect DA production. A better understanding of the role of nitrogen metabolism in domoic acid production appears to be important in our understanding of the links between agricultural urea runoff and coastal ASP events.

4. Acknowledgements

The authors thank Dr. Véronique Creach from the CEFAS Institute for having provided *P. multiseries* strain “CCL70” and Marie-Pierre Crassous from Ifremer for having provided *P. pungens* strain “D10”. This work was supported by Ifremer, the Regional Council of “Pays de la Loire”, the University of Nantes and the Centre National de la Recherche Scientifique (CNRS).

5. References


Masó M., Garcés E., 2006, Harmful microalgae blooms (HAB); problematic and conditions that induce them. Marine Pollution Bulletin 53, 620-630.


