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Research Article

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High Performance Liquid Chromatography pigments formation of microalgae growth during the development of *Pseudo-nitzschia* spp. of Cyanobacteria

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ABSTRACT: *Pseudo-nitzschia* blooms from the Ri'a de Pontevedra (NW Spain) were studied by light microscopy and HPLC pigment analysis. Two main Pseudo-nitzschia blooms were registered: the first one in summer had up to 800.000 cells L' and the second in winter had up to 68.000 cells L'1. During the first bloom amnesic shellfish poisoning (ASP) was not detected and the dominant species was *P. fraudulenta*. During the second bloom ASP toxicity was detected, and the dominant species was *P. australis*. Pigment analyses from both blooms showed Chi c2 and Chi c3 as major components of the Chi c family, with Chi c\ a minor component. Although Chi c3 is usually associated with members of *Prymnesiophyceae, Pelagophyceae* and *Dinophyceae*, it has also been detected in *Pseudo- nitzschia* species as *P. fraudulenta*, *P. delicatissima*, *P. pungens* and *P. pseudodelicatissima*. However, chi c3 is not present in *P. multiseries* and *P. australis*, both able to synthesise domoic acid, the causative agent of ASP. The parallel increase of Chi c3 levels and *Pseudo- nitzschia* cell numbers (throughout the development of a quasi mono-specific blooms of *Pseudo-nitzschia* spp) can be used as preliminary information while domoic acid analysis and species identification by EM are performed.
Keywords: *Pseudo-nitzschia*, domoic acid, marine cyanobacteria

INTRODUCTION

Several species of the genus *Pseudo-nitzschia* such as *P. multiseries* and *P. australis* have been associated with ASP toxicity (Bates et al., 1989; Fritz et al., 1992). In Galician coastal waters populations of *Pseudo-nitzschia spp.* have been detected since 1994 as the causative agent of ASP toxic events, affecting many shellfish areas in the Galician Rias (Miguez et al., 1996). Due to the economic importance of aquaculture, a monitoring programme of HAB species was set up in Galician waters.

Secure taxonomic identification of Pseudo- nitzschia species requires TEM, a time consuming technique. The chemotaxonomic approach using HPLC analysis of taxon-specific pigments allows to interpret composition of phytoplankton populations, but several important markers are shared by different algal classes. In spite of it, traditional HPLC methods have ignored the value of Chi c pigments as taxonomic markers, focusing mainly to carotenoids.

In a previous work studying Chi c distribution in 30 strains of 7 *Pseudo-nitzschia* species (Zapata et al., 2000) we found three pigment types: type I, Chi c\ and Chi t'2 (*P. multiseries, P. australis*), type II, Chi cu Chi c2 and Chi c3 (*P. delicatissima, P. pseudodelicatissima, P. pungens, P. fraudulenta*), type III, Chi c2 and Chi c3 (*P. cuspidata*). Therefore, *P. australis* and *P. multiseries* most relevant species associated with ASP toxicity constituted the single Chi c3-lacking type I. We used this information to study Chi c patterns during *Pseudo- nitzs*chia blooms from the Ria de Pontevedra and Chi c3 as a marker pigment to differentiate between potentially toxic and non-toxic *Pseudo-nitzschia* blooms.

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METERIALS AND METHODS

Seawater samples were collected weekly from a station in the Ria de Pontevedra throughout the year. Sampling was based on depth integrated samples from 0-15m in order to obtain representative integrated profiles. Pigments were extracted from 1.5 L seawater, concentrated and size-fractionated by sequential filtration through a 47 mm diameter Whatman GF/D filter (nominal pore size 2.7 (im) and a Whatman GF/F filter (nominal pore size 0.7 Jim). Pigments were extracted with 95% methanol, filtered and immediately injected into a Waters Alliance HPLC equipment, including a Waters 2690 separation module and a Waters 996 diode-array detector, interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface.

HPLC pigment separation was performed using a monomeric C_8 column (Symmetry) and pyridine containing mobile-phase (Zapata et al., 2000). Chlorophylls and carotenoids were detected by diode-array spectroscopy (350-750 nm). Chlorophylls were also detected by fluorescence (Ex: 440 nm, Em: 650 nm).

Aliquots of each integrated water sample (0-15m) were preserved with Lugol's solution, phytoplankton were allowed to settle for at least 12 h followed by observations with a Nikon Diaphot TMD inverted microscope. The chamber was examined at lOOx to enumerate and identify larger and less frequent micro plankters, then 200x and 400x were used for identifying and counting smaller organisms. The identification of *Pseudo-nitzschia* species from net samples was made by light microscopy on cleaned samples following the method outlined in (Simonsen et al., 1974).

RESULTS

A comparison of *Pseudo-nitzschia* cell numbers and total diatoms in the sampled station over 20 months. During June-July, a bloom of *Pseudonitzschia spp.* was observed mainly dominated by the non-toxic *P. fraudulenta* (contïrmed by TEM). Up to 800.000 cells mL-' were present which around 90% of the total diatoms was. During December season, a toxic *Pseudo-nitzschia australis* bloom was detected (68.000 cells mL-') which was only 30% of the total diatom abundance. HPLC

Pigment chromatograms corresponding to these two Pseudo-nitzschia blooms are shown in Figs. 2A and B. During the summer bloom (Fig 2A) dominant accessory chlorophylls were ch1 c2 (0.657 pg L-l, 69 % of the total ch1 c) and ch1 c3 (0.188 pg L-l, 20 %), with lower levels of ch1 c1 (0.110 pg Le', 11 %). A ch1 c-like compound eluted close to the chl c3 peak and was identified as ch1 c-like pigment detected previously in Pseudo-nitzschia species (4).

Fucoxanthin (Fuco) constituted the major carotenoid (1.24 pg L-') and very low concentrations of *fucoxanthin acyloxy* derivatives were detected showing minor contributions by groups other than diatoms. The summer bloom of *Pseudo-nitzschia* was dominated by *P. fraudulenta*, confirmed by light microscopy and TEM.

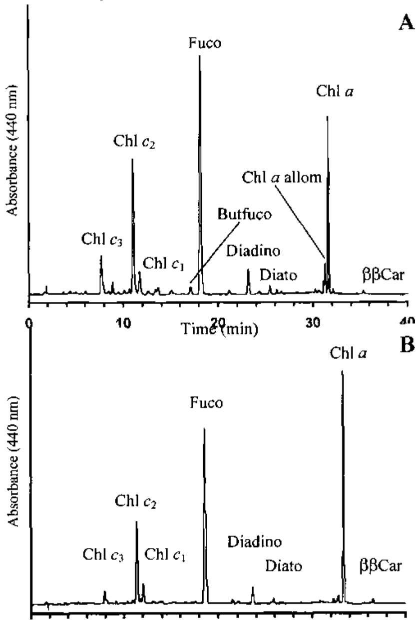

Fig. 2. HPLC Chromatograms obtained from phytoplankton samples during A) *Pseudo-nitzschia fraudulenta* bloom and B) *Pseudo-nitzschia australis* bloom.

The winter bloom (Fig. 2B) was similar in its pigment composition showing dominance of chi cj (72% of total chi c) with lower contributions of chi c3 (10%) and chi cx (18%). The expected pigment composition from *P. australis* was not reflected in the field sample due to the larger abundance of other diatoms such as *Chaetoceros socialis* and *Chaetoceros didymus*. Pigment analysis of

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cultures obtained from *Pseudo-nitzschia* isolated from this bloom (Fig. 3) revealed a chi c pattern corresponding to that previously described for *P. multiseries* and *P. australis* (4) (ch1 c3 absent).

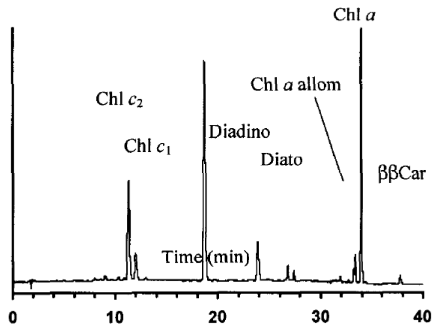

Fig. 3. Chromatogram obtained from a culture of *Pseudo-nitzschia australis* isolated Note the absence of chl c_3.

DISCUSSION

The most common diatoms found in samples from the Galician Rias include different species of *Chaetoceros, Skeletonema costatum, Leptocylindrus danicus,* etc. They have the classical pattern of diatom pigments of Chi *c*i, c_2 and Fuco as dominant components (Jeffrey et al., 1997; Stauber et al., 1988).

However, Chi c_2 and Fuco are also present in other algal classes present in field samples. Examples of these are *Cryptophyceae*, which possess Chi c_2 but can be easily identified by the carotenoid alloxanthin, some members of the class *Prymnesiophyceae* (Chi c_3 and 19'- *Hexanoyloxyfucoxanthin*), *Pelagophyceae* (Chi c_3 and 19'-Butanoyloxyfucoxanthin), etc.

As we described before, *Pseudo-nitzschia* species have shown three pigment types as based on Chi *c* pigments (4). Chi c_3 and a Chi c-like compound eluting close to this chlorophyll have been detected (in addition to the normal pigments found in diatoms) in non-toxic species, as *P. fraudulenta* and *P. delicatissima,* most commonly found in samples from the Rias. By other hand, the toxic species causing ASP events in our coast, *P. australis,* is interestingly lacking Chi c_3. In that sense, detection of Chi c_3 and Fuco during bloom episodes of *Pseudo-nitzschia* without significant levels of the fucoxanthin derivatives, can suggest that toxic *Pseudo- nitzschia* is absent while confirmation is obtained by domoic acid analysis and species identification by TEM are performed.

CONCLUSIONS

Absence or low levels of Chi c3 together with quasi-monospecific *Pseudo-nitzschia spp*. blooms indicates that dominant species are either *P. multiseries* or *P. australis.* Thus, HPLC analysis of Chi *c* pigments in samples dominated by *Pseudo-nitzschia* spp. can provide preliminary and fast information in harmful algae monitoring programmes about *Pseudo-nitzschia* blooms due to *P. multiseries* and/or *P. australis* while domoic acid analysis and TEM techniques are performed.

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CONFLICTS OF INTEREST
"The authors declare no conflict of interest".