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Yessotoxins production during the culture of *Protoceratium reticulatum* strains isolated from Galician Rias Baixas (NW Spain).

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Abstract

Yessotoxins (YTXs) production along the culture growth of three strains of the dinoflagellate *Protoceratium reticulatum* isolated from seawater of Galician Rias

Baixas, Spain was investigated. Quantification and toxin profile determination in both cells and culture medium along the growth curve were performed by liquid chromatography-mass spectrometry (LC-MS) analysis. The YTX profile was very similar among strains, the three algal strains produce mainly YTX and also some YTX analogs. Among the strains the maximum toxin production ranged between 416 and 576 ng·mL⁻¹. This is the first report about YTX production by *P. reticulatum* isolated in Galician coast, NW Spain.

Keywords: Protoceratium reticulatum, culture growth, yessotoxins, LC-MS analysis.

1. Introduction

The accumulation of biotoxins in bivalve shellfish represents a serious problem due the health risk and the serious disruptions for the aquaculture industry. Yessotoxins (YTXs) are a group of structurally disulphated polyether toxins mainly produced by the dinoflagellate *Protoceratium reticulatum* (Claparède et Lachmann) Bütschli (= *Gonyaulax grindleyi* Reinecke). YTXs when accumulated in shellfish are toxic to mice after intraperitoneal injection (Aune et al., 2002; Tubaro et al., 2003), causing false positives in the mouse bioassay for Diarrhetic Shellfish Poisoning (DSP). Despite YTXs have been found to be non diarrhoeic toxins to humans (Tubaro et al., 1998; De la Rosa et al., 2001; Alfonso et al., 2003) have been found to be potent cytotoxins (Bianchi et al., 2004; Konishi et al., 2004; Pérez-Gómez et al., 2006). With this consideration, the European Union established a maximum level permitted in shellfish of 1 mg/Kg (CEE, 2005). Known YTXs have been detected in shellfish in Japan (Murata et al., 1987),

Norway (Aesen et al., 2005), Chile, New Zealand, Italy (Ciminiello et al., 2003a) and Spain (Arévalo et al., 2004).

The producer organism of YTXs most studied, the dinoflagellate *P. reticulatum*, shows complex YTXs profiles in different countries. About 100 YTX analogs have been reported in *P. reticulatum* strains from New Zealand (Satake et al., 2006; Miles et al., 2004; 2005b; 2006b; Finch et al., 2005), Japan (Satake et al., 1999; Konishi et al., 2004; Suzuki et al., 2007), Norway (Ramstad et al., 2001; Samdal et al., 2004; 2006), Italy (Ciminiello et al., 2003b), UK, Canada (Finch et al., 2005), Spain and USA (Paz et al., 2004; 2007). In spite of the high variability in the reported YTX profile, the major toxin in *P. reticulatum* is usually YTX and, only 1a-homoYTX was found to be the main toxin in two strains from Japan (Konishi et al., 2004) and one strain from Spain (Paz et al., 2007). The amount of YTXs produced by each strain differs substantially ranging from 0 to 71 pg-cell⁻¹ (Paz et al., 2008).

The variability in YTX production and toxin profile found among *P. reticulatum* strains are mainly due to the origin of the strain (Ciminiello et al., 2003b; Eiki et al., 2005; Samdal et al., 2006). In addition, different culture conditions, growth phase, nutritional and environmental conditions have been demonstrated to influence toxin production (Guerrini et al., 2007; Mitrovic et al., 2005; Paz et al., 2006b) and also dependent of the extraction or analysis method used in the study (Eiki et al., 2005; Samdal et al., 2006; Paz et al 2008).

Although the toxin production was evaluated in several studies, reports about the monitoring of toxin production along the culture growth are scarce (Paz et al., 2004; 2006b, Guerrini et al., 2007). In the present work three strains of *P. reticulatum*, isolated in Bueu (Galician Rías Baixas, NW Spain), were cultured in laboratory with the aim of monitoring the culture growth and the YTX production along the growth curve.

Moreover the release of toxins into the culture medium and variability in YTXs profile were evaluated. It was demonstrated by LC-MS analysis that the three *P. reticulatum* strains produce YTXs during all the culture growth. Being this the first register of YTX production by *P. reticulatum* isolated in Galician coast, NW Spain.

2. Material and methods

2.1. Cultures of *P. reticulatum*

Three strains of *P. reticulatum*, VGO903, VGO904 and VGO905, were isolated from phytoplankton samples collected in seawater of Bueu (42° 21.40' N, 8° 46.42' W) (Galician Rías Baixas, NW Spain) at the end of 2006, and afterward cultured in the laboratory. The species of these Spanish strains were identified by Santiago Fraga and included in the phytoplankton culture collection at the Centro Oceanográfico in Vigo (CCVIEO).

Cultures, inoculated with 500 cells·mL⁻¹ in the exponentially growing phase of each *P. reticulatum* strain, were grown in 3 L sterile Erlenmeyer flasks containing 2 L of L1 medium without silicates (Guillard and Hargraves, 1993). Cultures were maintained at 19±1 °C, at a salinity of 30 and under an irradiance of 100-125 μmol photons·m⁻²·s⁻¹ on a 12:12 h light:darkness regime. Cultures were gently shaken once a day. For growth monitoring 5 mL aliquots were collected each 3-4 days for 28 days, fixed with Lugol's solution and cells were counted by optical microscopy in a Sedgewick-Rafter chamber (Figure 1).

In order to calculate the mean cell biovolume, were assumed to be spheres and their diameters were measured with the aid of a micrometer eyepiece using the average value between length and width, n=30.

2.2. Yessotoxins extraction and clean-up

For toxin analysis, 45 mL aliquots of each culture were harvested each 3-4 days until the culture reached the stationary/early decline phase at day 28 (Figure 1) and were filtered through 1.4 µm GF/C glass fibre filters (25 mm diameter) (Whatman, Maidstone, England). YTXs were extracted from both cells and the filtered culture medium and cleaned in solid phase (SPE), separately. For this purpose, the cells in the filter were extracted twice with MeOH. The MeOH cell extract and the filtered culture medium were respectively loaded onto different Sep-Pak C18 light cartridges (Waters U.S.A.). YTXs were eluted using 4 mL of 70 % MeOH, dried under a N₂ stream and re-suspended in 0.5 mL of MeOH for LC-MS analysis (Paz et al., 2007).

2.3. Liquid chromatography-mass spectrometry (LC-MS) analyses

For YTXs determination a LC-MS system was used. The separation column was a Xterra MS C18 5 µm (2.1 x 150 mm) cartridge at 35 °C. As mobile phase 2 mM ammonium acetate (pH 5.8) (A) and MeOH (B), in a gradient elution (40 to 30 % A in 5 min, 30 to 20 % A in 5 min, followed by 5 min with 20 % A, then 20 to 0 % A in 5 min and 0 % A for 2 min), were used. The flow rate was 200 µL·min⁻¹ and injection volume 5 µL. An ion trap mass spectrometer, Thermo Finnigan LCQ-Advantage, equipped with electrospray ionization (ESI), in negative ion mode was used. ESI was performed with a

4.5 kV spray voltage and 200 °C capillary temperature, flow 40 mL·min⁻¹ for sheath gas and 20 mL·min⁻¹ for auxiliary gas. Full scan data were acquired from m/z 300 to 2000. The most prominent signals were obtained for ion at m/z [M-H]⁻ therefore this ion was used for MS quantification.

The YTX standard used for LC-MS identification was purchased from the Institute of Environmental Science and Research Limited (New Zealand), 45-OHYTX and carboxyYTX standards were provided by Professor T. Yasumoto and the reference material G-YTXA was purified from cells of *P. reticulatum* (Souto et al., 2005). The quantification was performed using just the YTX standard, due to the lack of appropriate standards for all the YTX analogs. The assumption was made that all the analogs would give the same molar response as YTX.

3. Results and Discussion

3.1. Cultures of *P. reticulatum*

The three *P. reticulatum* strains were monitored until day 28 of culture when strains VGO903 and VGO904 reached the stationary phase, despite the VGO905 strain reached the stationary phase before (Figure 1). The cell yield ranged among strains between 18400 and 37767 cell·mL⁻¹. The lowest cell density was achieved by VGO905 strain and the highest by VGO903 strain. VGO903 and VGO904 strains showed similar growth curves with slow exponential phase and short stationary phase. Nevertheless VGO905 strain had a short exponential phase reaching the stationary phase before than the others cultures and then having a long stationary phase (Figure 1).

The initial mean cell biovolume was $5 \pm 1 \mu\text{m}^3 \times 10^3$ for the three *P. reticulatum* strains (Figure 2). No significant differences in cell biovolume were found between strains VGO903 and VGO904 along culture growth, but the strain VGO905 after the day 18 of culture showed an important increase in mean cell biovolume and SD reaching a final cell biovolume of $8 \pm 3 \mu\text{m}^3 \times 10^3$ (Figure 2). These data are in concordance with cell yield where VGO903 and VGO904 showed normal growth curves and VGO905 had a long stationary phase. Therefore VGO905 strain after day 18 of culture instead to increase the cell number suffers an increase in cell biovolume and high SD.

3.2. YTX production by strains VGO903, VGO904 and VGO905.

YTX was found along all the culture growth curve and reaches maximum levels at day 28 in a concentration of $416\text{--}576 \text{ ng}\cdot\text{mL}^{-1}$ depending on the strain. At the end of culture the YTX which remained in cells was $205\text{--}388 \text{ ng}\cdot\text{mL}^{-1}$ and the detected in culture growth medium $111\text{--}191 \text{ ng}\cdot\text{mL}^{-1}$ (Figure 1). The toxin quota in the cells was in general highest than in medium during all the culture. Extracellular YTX concentration showed a slight increase after day 22 of culture, when the number of cells in the culture is maximum, in the stationary phase. The presence of YTXs in the growth medium may be due to active release from cells, and also the leakage of toxins into the medium by cellular death or sample handling. Similar YTX production was found in strains VGO904 and VGO905, but in the VGO903 strain, which had the highest cell yield a higher YTX production in $\text{ng}\cdot\text{mL}^{-1}$ was detected (Figure 1). An assessment of toxin with cell biovolume was performed. Quantification of YTX production versus cellular biovolume showed similar values for all the strains along

culture growth (figure 2). The YTX production given as $\text{pg} (\times 10^{-3}) \cdot \text{Vol}^{-1} (\mu\text{m}^3 \times 10^3)$ was for all the strains 2.5-3.0 at the end of the culture growth.

3.4. YTXs profile in strains VGO903, VGO904 and VGO905.

LC-MS analysis revealed the presence of YTX and analogs in culture medium (Fig. 3) as well as in cells extracts (Fig. 4) of the three *P. reticulatum* strains studied. The three strains showed a similar toxin profile, in which YTX was the main toxin detected, together with small quantity of a large number of YTX analogs (Table 1). The toxins detected in both medium and cells of the three strains were YTX with m/z 1141 $[\text{M-H}]^-$ (10.7 min) as the main toxin, together with 41-ketoYTX at m/z 1047 (5.5 min), some unknown YTX analogs with m/z 1177 (5.2 min), m/z 1159 (8.8 min) and m/z 1157 (8.8 min) (Miles et al., 2005a; Paz et al 2007). Also the 44,55-diOHYTX at m/z 1175 (7.1 min) and 32-*O*-monoglycosylYTX at m/z 1273 (10.0 min) were detected in cells and culture growth medium of all the strains (Table 1).

Other YTX analogs were detected in a different way in cells and in culture growth medium (Table 1) such as: trinorYTX at m/z 1101 (5.5 min), 44-oxotrinorYTX at m/z 1117 (6.4 min) coeluting with the ion at m/z 1159 (Fig. 3D), 44-OHdinorYTX at m/z 1131 (6.6 min) (Miles et al., 2006a), a carboxiYTX analog at m/z 1173 (7.7 min) and 32-*O*-diglycosylYTX at m/z 1405 (9.4 min) which were only detected in culture growth medium (Figure 3). In the other hand the analog 44,55-diOH-41a-homoYTX at m/z 1189 (8.3 min) (Finch et al., 2005) was only detected in cells. Finally the 32-*O*-monoglycosylYTX at m/z 1273 (10.0 min) was mainly detected in cells (Figure 4). In the case of the ion at m/z 1047 $[\text{M-H}]^-$ (5.5 min), with the same mass as that of the three ketoYTXs (=noroxoYTX) (Miles et al., 2004), in culture medium showed a

chromatogram with three peaks at 5.4, 6.4 y 7.7 being 41-ketoYTX, 40-epi-41-ketoYTX and 41-ketoYTX-enone, respectively, nevertheless in cells only one peak was detected at 5.5 min, this retention time revealed that the ketoYTX analog detected in cells is 41-ketoYTX (Miles et al 2004). Without ruling out the possibility that these strains also contain other known and unknown YTX analogs.

The ion at m/z 1157 $[M-H]^-$ (8.8 min), with the same mass than 45-OHYTX but the retention time did not show coincidence with 45-OHYTX standard. This suggests the presence of a YTX analog different to 45-OHYTX (Paz et al 2007). Moreover most studies have indicated that 45-OHYTX is produced only by metabolism in shellfish and a series of four YTX analogs with m/z 1157 $[M-H]^-$, and different to 45-OHYTX, have also been found by Miles et al (2005), however structures have not been determined yet.

For ions at m/z 1273 $[M-H]^-$ (10.0 min) and m/z 1141 $[M-H]^-$ (10.7 min) the retention time matches with the G-YTX A (=32-*O*-monoglycosylYTX) and YTX standards, respectively (Souto et al., 2005; Paz et al., 2006a).

3.5. Percentage of toxins in the strains

The calculated contribution percentage of YTX analogs to the total toxin production was very low ranging between 0.01 and 1.19 % depending on the analog. In extensive the presence of analogs was more important in the culture medium than in cells. For the strain VGO903 the YTX was practically the only toxin detected in both cells and culture medium, being the 99 % of total YTXs. In the case of VGO904 and VGO905 strains the YTX which remained inside the cells and also the released to culture growth medium ranged from 97.53 to 99.92 % of total toxin (Table 1).

1 This low contribution percentage of YTX analogs to total toxin yield was also found in
2 other strains in previous studies ranging between 0.22-3.95 % (Paz et al., 2007). In a
3 Norwegian strain the most abundant analog, trinorYTX, was a 3.8 % of the total toxin
4 production, and for the other analogs was 1-2 % (Samdal et al., 2006).

7 **4. Conclusions.**

8
9 In summary, it was demonstrated by LC-MS analysis that the three *P. reticulatum*
10 strains, isolated in Bueu (Galician Rias Baixas, NW Spain), produce YTXs during all
11 the culture growth. Important amount of toxins were found in the culture medium
12 mainly at the end of the culture growth. The three strains of *P. reticulatum* showed a
13 small variability in YTXs profile and YTX yield in cells and culture medium during
14 culture growth. Toxin profile of *P. reticulatum* determined by LC-MS appeared to be,
15 definitively, more complex than that previously determined. Finally, *P. reticulatum*
16 have been detected previously in the Mediterranean coast of Spain (Paz et al 2007), but
17 there are not references about the presence of *P. reticulatum* in Galician Rias Baixas,
18 Atlantic coast of Spain. Being this the first register of YTX production by *P.*
19 *reticulatum* isolated in Galician coast.

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Figure captions

Figure 1. YTX production, in $\text{ng}\cdot\text{mL}^{-1}$, in cells and culture medium during the culture growth of the Galician *Protoceratium reticulatum* strains Bueu (Galician Rias Baixas, NW Spain).

Figure 2. YTX production in $\text{pg} (\times 10^{-3})\cdot\text{Biovolume}^{-1}$ (given as $\text{Mean}\pm\text{SD Vol } \mu\text{m}^3 \times 10^3$) in cells and in culture medium during the culture growth curve of the three *P. reticulatum* strains from Bueu (Galician Rias Baixas, NW Spain).

Figure 3. Selected ion chromatograms and mass spectra after negative LC-MS analysis of culture medium extract of the *P. reticulatum* strains at day 28 of culture. Detected ions at m/z $[\text{M-H}]^-$ were: A) 1177 for a YTX analog, B) 1047 for noroxoYTX-enone, C) 1101 for trinatorYTX, D) 1159 for a YTX analog and 1117 for 44-oxotrinorYTX, E) 1131 for 44-OHdinatorYTX, F) 1175 for 44,55 diOHYTX, G) 1173 for a YTX analog, H) 1157 for a 45-OHYTX analog, I) 1405 for 32-*O*-diglycosylYTX, J) 1273 for 32-*O*-monoglycosylYTX, K) 1141 for YTX.

Figure 3 (*Continued*)

Figure 4. Selected ion chromatograms and mass spectra after negative LC-MS analysis of cell extract of *P. reticulatum* VGO903, VGO904 or VGO905 strains at day 28 of culture. Detected ions at m/z $[\text{M-H}]^-$ were: A) 1177 for a YTX analog, B) 1047 for noroxoYTX-enone, C) 1159 for a YTX analog, D) 1175 for 44,55 diOHYTX, E) 1189 44,55 diOH-41a-homoYTX, F) 1157 for a 45-OHYTX analog, G) 1173 for a YTX analog and H) 1141 for YTX.

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Fig 2

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