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Isolation of *Prorocentrum lima* (Syn. *Exuviaella lima*) and diarrhetic shellfish poisoning (DSP) risk assessment in the Gulf of California, Mexico

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Abstract

A benthic toxic dinoflagellate identified as *Prorocentrum lima* (Syn. *Exuviaella lima*), and designated as strain PRL-1, was isolated from the coast of El Pardito (Coyote) Island in Baja California Sur, Mexico, after a fisherman poisoning incident involving consumption of liver from *Lutjanus colorado*, and *Mycteroperca prionura* fish. Purification and culturing was done in ES-Si medium, under 12:12 light/dark cycle (4 × 20 W cool-white fluorescent lamps), at 22 °C and constant stirring during 28 days. Whole cells were toxic to *Artemia franciscana* and its methanolic extract to mouse and to the marine yeast *Debaryomyces hansenii*. Chromatographic analysis (TLC and HPLC–MS) of such extract indicated an unusual proportion (1:2) okadaic acid (OA) and dinophysistoxin-1 (DTX-1). Estimated total toxin content by mouse bioassay (based on OA toxicity) was 19 pg/cell, a value significantly higher than that found by HPLC–MS (about 5.2 pg/cell, taking into account OA and DTX-1 only), suggesting that additional toxic components of unidentified nature are detected with the bioassay. This is the first report of a successful isolation and culturing of a toxic dinoflagellate from the Gulf of California, Mexico.

Keywords: Diarrhetic shellfish poisoning; Dinoflagellate; *Prorocentrum lima*; Okadaic acid

1. Introduction

Prorocentrum lima (Ehrenberg) Dodge (Syn. *Exuviaella lima*) is a toxic benthic, epiphytic, photosynthetic dinoflagellate found worldwide in boreal and tropical seas (Bravo et al., 2001; Hu et al., 1993, 1995; Lee et al., 1989; Maranda et al., 1999, 2000; Morton and Tindall, 1995; Murakami et al., 1982; Nakajima et al., 1981; Yasumoto et al., 1980). It is an important primary producer (Faust, 1991) that has been associated to diarrhetic shellfish poisoning (DSP) episodes in different parts of the world, although seldom in North America (Jackson et al., 1993; Marr et al., 1992; Lawrence et al., 1998; Morton et al., 1999). *P. lima* is also abundant in tropical and sub-tropical waters where ciguatera fish poisoning is considered endemic; thus, it has been suspected, but not proven, that *P. lima* toxins contribute to the ciguatera syndrome (Tindall and Morton, 1998).

In the Gulf of California, Mexico, *P. lima* has been previously detected (Licea et al., 1995) but not isolated, cultured, or analyzed with regard its toxicity. Towards these aims, after a 'DSP- or ciguatera-like' poisoning episode in August 1997 involving several fishermen, we isolated a *P. lima* strain, designated as PRL-1, from 'El Pardito (Coyote) Island', BCS, Mexico, and report here its properties and toxicity.

2. Materials and methods

The dinoflagellate *P. lima* strain PRL-1 was isolated from a rocky substrate obtained at the shoreline of El Pardito

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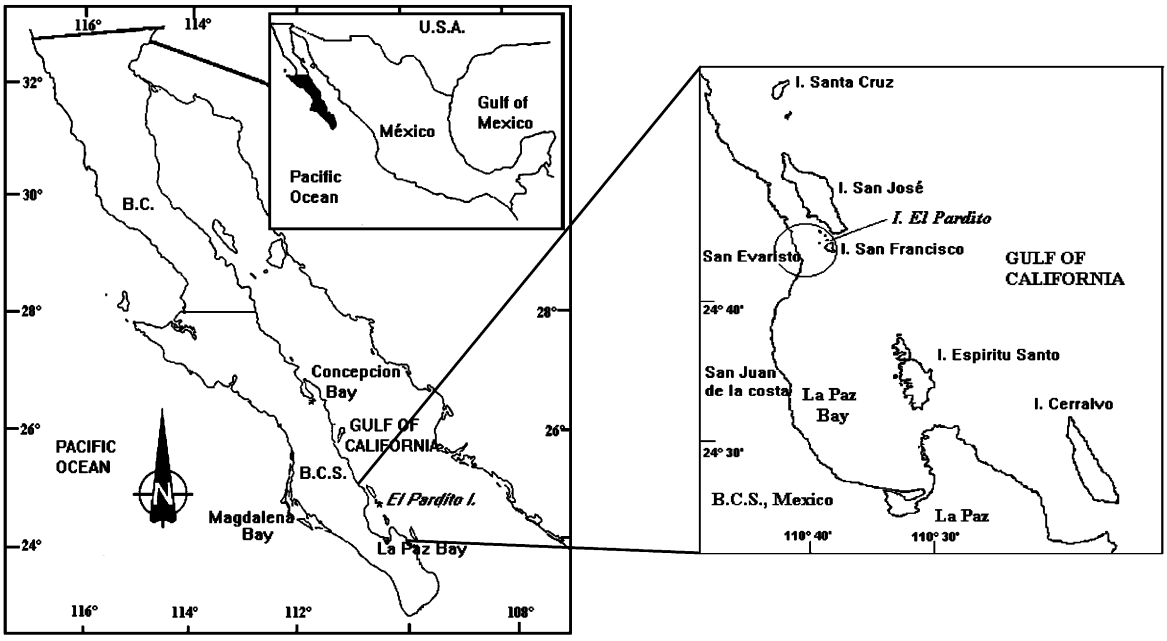


Fig. 1. El Pardito (Coyote), Baja California Sur, Mexico, site of isolation of *P. lima* strain PRL-1.

(Coyote) Island, BCS, in the Gulf of California (24°35.0′N, 110°49.6′W), Mexico (Fig. 1) by scuba diving. Its isolation and purification was done by the micropipette and serial-dilution technique, and in agar petri dishes containing ES-Si medium (Harrison et al., 1980) until obtaining a unialgal culture (Fig. 2(a)). Liquid culture growth was promoted by triplicate pouring about 6000 cells in 125 ml Erlenmeyer flasks containing 50 ml of ES-Si medium under a 12:12 light/dark cycle (4×20 W cool-white fluorescent lamps), at 22 ± 1 °C, and under constant stirring (120 rpm). Harvesting was done by centrifugation at 4000 rpm at the beginning of the stationary phase (about 28 days).

2.1. Biotoxicity assays

Whole cell toxicity was evaluated following the procedure described by Demaret et al. (1995) with *Artemia franciscana*. For this, adult animals (25 days old) obtained from the Branchiopods Laboratory of CIBNOR (Dr A. Maeda and C. Orozco MSc.) previously fed with *Saccharomyces cerevisiae*, *Chaetoceros* sp. and corn meal, were starved for 3 h in lots of 10 animals each, using 250 ml flasks containing 150 ml of filtered seawater before exposure to *P. lima* PRL-1 at a density of 2000 cells/ml. Control experiments included a normal feed (as above), or no feed at all. All the assays were run in triplicate. Survival was followed by 6 h and registered every hour.

Mouse bioassay (Lewis, 1995) for *P. lima* PRL-1 extract was done. For this, the cells from a 28 day old cell culture (50 ml) showing a cell density of 33,500 cells/ml were extracted following the procedure no. 3 recommended by Quilliam et al. (1996). After evaporation the toxins were recovered in 500 μl of methanol for testing. The antimicrobial activity of a *P. lima* PRL-1 extract obtained from 1.3×10^6 cells as above, was done using the paper-disc method introduced by Nagai et al. (1990) with the following yeasts: *Rhodotorula glutinis*, *S. cerevisiae* SKQ and 7765 and *Debaryomyces hansenii* HF1, pouring 20 μl of extract on each paper and measuring the inhibition halo after 48 h at 35 °C.

2.2. Chromatographic analysis

Thin layer chromatography (TLC) was done on a silica gel 60 F254, 250 mm, precoated plate (Merck), and using a solvent system consisting of toluene/acetone/methanol (7.5:4:1) according to Quilliam and Wright (1995). The spots obtained after application of 5, 10 and 20 μl of extract samples were detected with a UV-lamp after spraying 50% sulfuric acid–methanol solution. High performance liquid chromatography coupled to a mass spectrometry device (HPLC–MS) analysis was done with the corresponding extract obtained from 16.89×10^6 cells according to Goto et al. (2001).

3. Results and discussion

The light microscope analysis (Nikon Optiphot-2, Japan) revealed that cultured *P. lima* PRL-1 cells grown in ES-Si medium were of oblong to ovate shape (Fig. 2(a)–(d)), with a right valve in a V-shaped depression and left valve flat or slightly curved, both located at the anterior end, as reported

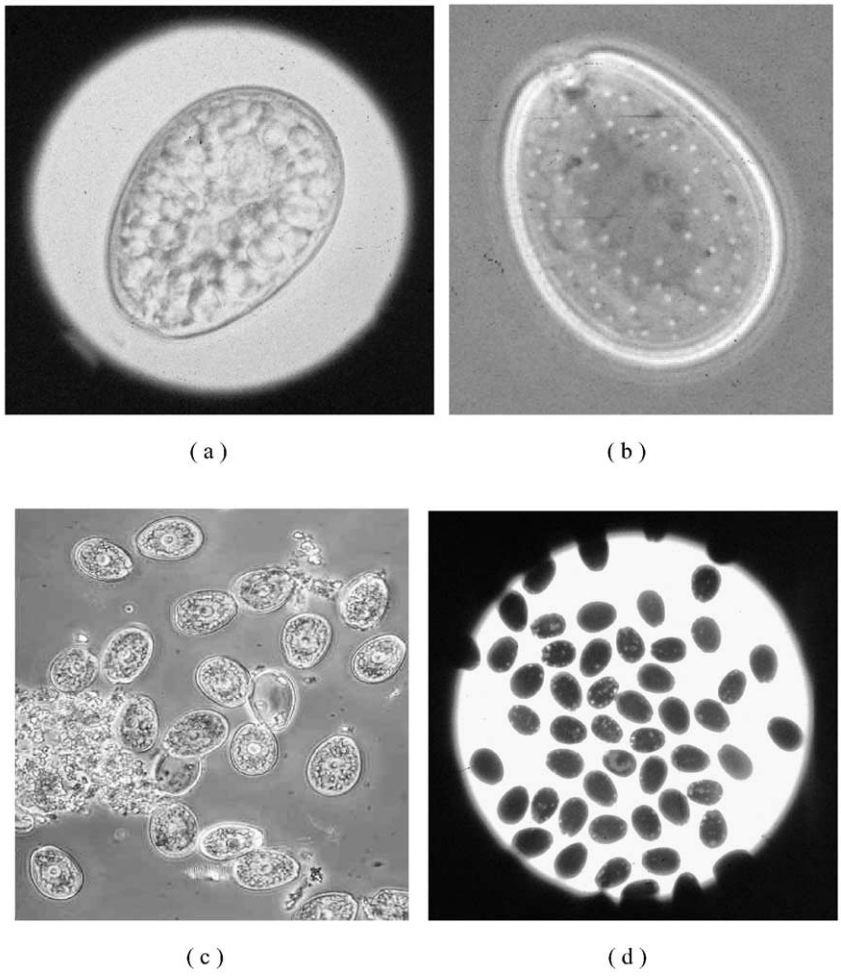
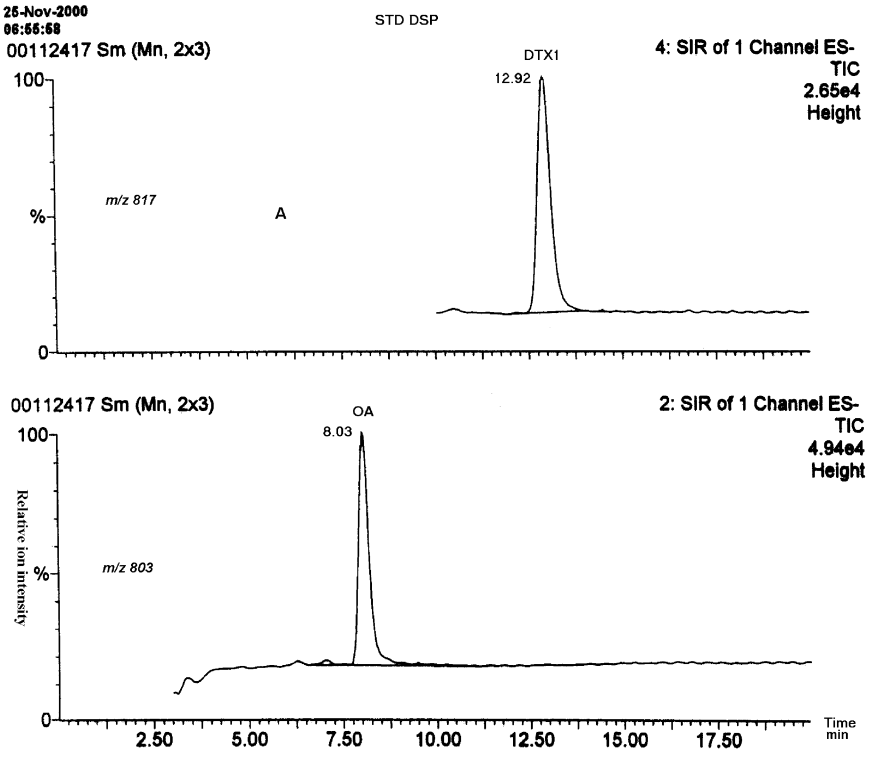


Fig. 2. Light micrography of *P. lima* PRL-1 showing cell shape 40× (a), valve pores 40× (b), a culture showing the central pyrenoid and chloroplasts of the cells and the mucus excreted 20× (c), and fluorescent spherical bodies inside the cells 10× (d).

earlier for other *P. lima* strains (Faust, 1991; Fukuyo, 1981). The valve surface appeared smooth with 56 marginal and 64 valve pores distributed over the cell surface except in the center (Fig. 2(b)). The size of cultured *P. lima* PRL-1 cells was variable, with a mean cell length of 37.4 ± 0.894 μm and mean cell width of 27.2 ± 1.095 μm (for $n = 5$), thus appearing smaller than most isolates of other parts of the world (Jackson et al., 1993; Marr et al., 1992; Morton and Tindall, 1995), but still within the range of variability observed for such species by Faust (1991) and Bouaicha et al. (2001). *P. lima* PRL-1 in ES-Si medium showed a growth rate (expressed as division per day) of 0.107 ± 0.003, which may be considered low compared to strains isolated from tropical waters showing from 0.35 to 0.20 divisions per day (Morton and Tindall, 1995) and yet, as reported by Heil et al. (1993), it was found also capable of producing mucus (Fig. 2(c)) and of reaching its maximum cell growth density (log phase) between 23 and 25 days.

Whole cell toxicity assay against *A. franciscana* showed that *P. lima* PRL-1, at the cell density tested caused brine shrimp deaths within 2 h, much more rapidly than previously reported with other *P. lima* strains (Demaret et al., 1995). Such rate of mortality appears to be the highest observed in similar experiments with this type of toxic dinoflagellates, and the high susceptibility can be explained considering that *A. franciscana* larvae are not potential grazers for *P. lima* and therefore lack a mechanism of defense towards this algae. The transfer of DSP toxins from *P. lima* to *A. franciscana* has been demonstrated and found to vary with stage development (Demaret et al., 1995). These observations may have important implications in zooplankton survival under natural conditions and, therefore, on its ecological impact.

For *P. lima* PRL-1 extract, the mouse bioassay showed typical symptoms for DSP toxins such as diarrhea, hind limb paralysis, dyspnoea, respiratory failure, and death after 1 h.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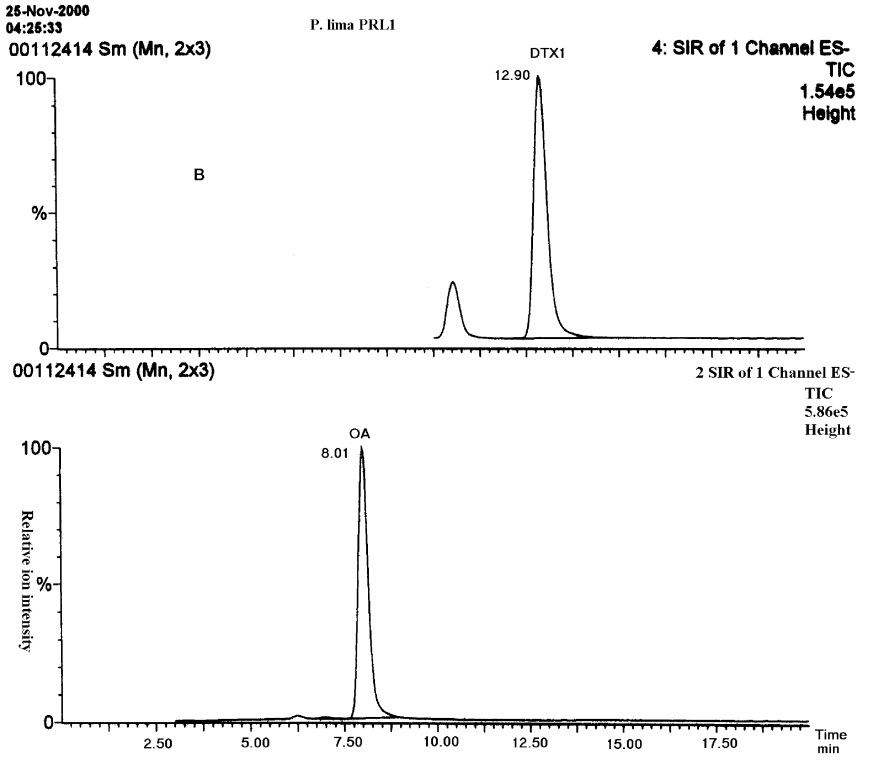
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P. lima PRL1
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2 SIR of 1 Channel ES-
TIC
5.86e5
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8.01
Relative ion intensity
Time
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The estimated total toxin concentration by this method was 19 pg/cell, which appears to be higher than previously reported for other *P. lima* species (Jackson et al., 1993; Sohet et al., 1995). Also, the yeast-killing activity of PRL-1 extract against *Rhodotorula glutinis*, *D. hansenii* and *S. cerevisiae* was assayed. In this case, only the marine yeast *D. hansenii* showed an inhibition halo of about 0.5 mm which is an interesting finding because *D. hansenii* appears to be quite resistant to disinfectant agents (Ramírez-Orozco et al., 2001). Fungicidal, and in general anti-microbial activity of dinoflagellate toxins is an issue of interest for developing new strategies of therapy and preventive infections (Nagai et al., 1990; Quod et al., 1995). It has been found that okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are powerful growth inhibitors of *Aspergillus niger* and *Penicillium funiculosum*, but not of Gram-positive bacteria such as *Bacillus megaterium* and *Staphylococcus aureus*, or the Gram-negative *Escherichia coli* (Nagai et al., 1990). The yeast-killing activity of *P. lima* remains unclear: Nagai et al. (1990) reported activity against *Candida albicans*, but Quod et al. (1995) did not. In our case, comparing against various yeast species we found that *P. lima* PRL-1 extract inhibited only *D. hansenii*, a yeast that can be found in marine environments (Ochoa et al., 1995). In general, the biological role of *P. lima* toxins is not well understood; yet, it has been assumed that they may play a defensive role against predators, infectious agents, or, perhaps as allelopathic agents for dominance purposes (Aguilera et al., 1997; Sugg and Van Dolah, 1999; Windust et al., 1996).

Major toxin components of *P. lima* strain PRL-1 by TLC, were identified as acidic DSP toxins and diol-esters with their corresponding spots at Rf 0.43 and 0.80, respectively (Murakami et al., 1982). HPLC–MS (Goto et al., 2001) analysis, confirmed further the presence of OA and DTX-1 (Fig. 3). HPLC–MS showed also an unusual proportion (1:2) of OA versus DTX-1 in *P. lima* PRL-1, which compares to some strains isolated from NW Spain (Bravo et al., 2001; Lee et al., 1989), and to *P. faustiae* in this respect (Morton, 1998), but not with those reported also by Bravo et al. (2001) or by Jackson et al. (1993), Lee et al. (1989) and Quilliam et al. (1996), with *P. lima* cultures showing the opposite, or even very similar proportions (Marr et al., 1992). As pointed out before, such variations may depend on origin (Bravo et al., 2001; Lee et al., 1989; Sechet et al., 1998), and culture conditions (McLachlan et al., 1994; Yasumoto et al., 1987). The quantitative analysis of *P. lima* PRL-1 toxins by HPLC–MS yielded rather low values as compared to the mouse bioassay suggesting the presence of other toxins not detected by the chromatographic method. As a matter of fact, prorocentrolide (Torigoe et al., 1988), and the soluble DTX4 and DTX5 toxins, are not observed with the HPLC–MS technique employed in this study (Goto et al., 2001). Yet, this method is the most advanced and complete available for the quantitative determination of most DSP-associated toxins.

The risk of a DSP–*P. lima* related event is specially elevated in warm regions where the high water temperature may favor a rise in production and variability of toxins (Jackson et al., 1993). Also the substrate, sediment, salinity, and light intensity may determine the overall expression of *P. lima* toxicity (Morton and Norris, 1990). The intoxication risk is increased by re-suspension, due for example to a sudden upwelling caused by climate conditions such as 'El Niño' or during the hurricane season. Moreover, suspended and ingested *P. lima* cells are still viable after passing through scallop's gut, which may determine its long-term survival, growth and dissemination (Bauder and Cembella, 2000). Thus, to prevent a DSP outbreak in tropical and sub-tropical regions, monitoring of *P. lima* in the water body becomes important, especially in areas where marine aquaculture is carried out.

It appears that, as occurs in bivalve mollusks (Bauder et al., 2001), toxin retention in fish is also more important in gonadal tissue and viscera. The fish poisoning incident that led to this study suggests that popular edible fish species such as groupers and snappers may become rather toxic because their liver and viscera depurate slowly. El Pardito Island, BCS, is an area with high primary productivity and abundance of shellfish and fish, being an attractive site for sport fishing practice (Ramírez-Rodríguez, 1997). It has been reported that only when the catch is eaten together with its viscera, the symptoms of 'ciguatera' or DSP are induced. We have been unable to detect any DSP-related toxin in extracts obtained from the viscera of various fish belonging to *Lutjanus colorado* and *Mycteroperca prionura* collected in the area, although their lipo- and hydro-soluble extracts showed biological activity in the mouse bioassay (not shown). This would indicate that the poisoning in question may be quite different than the classic ciguatera syndrome observed in different incidents elsewhere. The intoxicated fishermen (five individuals) of El Pardito Island, showed the classical manifestations of a neurotoxin ingestion such as diarrhea, nausea, eye, muscle and abdominal pain, headache, numbness, vomiting, weakness, pruritus, desquamation, hyperesthesia, lip and tongue paralysis, and in one case convulsion, 4–12 h after fish consumption. All these symptoms lasted a few days. Yet, as mentioned earlier, although the liver extract showed positive for liposoluble toxins in the mouse bioassay, the HPLC analysis did not reveal the presence of OA or any of its derivatives. As a matter of fact, only one report describing the presence of OA in barracuda implicated in ciguatera poisoning in the Caribbean exists (Gamboa et al., 1992). Hence, it remains to be demonstrated if other toxic benthic dinoflagellates, such as

Fig. 3. Chromatographic profile of okadaic acid (OA) and dinophysistoxin-1 (DTX-1) standards (A), and or *P. lima* PRL-1 extract (B), by HPLC–MS.

Gambierdiscus toxicus, in addition to *P. lima*, are also present in El Pardito Island and how they are involved in fish toxicity. Lehane and Lewis (2000) recently reviewed the conditions that describe a ciguateric-risk area and confirm the now well accepted notion that, although its symptoms may be modulated by other toxins produced by different benthic organisms, only *G. toxicus* can produce ciguatera (Holmes and Lewis, 1994). Therefore, at this time the area of Isla El Pardito may still be considered both a DSP and a ciguatera-risk zone and work is in progress to determine if they may occur simultaneously or in alternate episodes.

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