New information about the toxicological profile of *Prorocentrum panamense* (Prorocentrales, Dinophyceae) and its global distribution

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SUMMARY

Dinoflagellates of the genera *Prorocentrum* and *Dinophysis* are known producers of toxic compounds belonging to the okadaic acid (OA) group. The ingestion of shellfish contaminated with these toxins cause a human disease named diarrheic shellfish poisoning (DSP). In this study, the first record of *Prorocentrum panamense*, a potential toxin-producer species, was reported in the Canary Islands together with its toxicological characterization. *Prorocentrum panamense* cells were collected during April 2017 from natural pools located in the Northeastern part of Gran Canaria. This new record represents an expansion of *P. panamense* distribution area, previously restricted to the Pacific Ocean, Indian Ocean, Arabian Gulf and the Caribbean, and its introduction mechanisms is discussed. Laboratory cultures of *P. panamense* were settled and toxin production was assessed in both cell pellets and culture media at four different growth phases (latency, exponential, early stationary and late stationary) implementing LC-MS/MS and neuro-2a cell-based assay (CBA). LC-MS/MS allowed the identification of OA in the fraction corresponding to the late stationary phase, and tests performed on neuro-2a cells showed, for most of the fractions, OA-like activity observable by both cell morphology changes and cell mortality. This information is fundamental for a better understanding of the genus *Prorocentrum* global distribution, its ecology and risks associated to toxic producing species.

Keywords:

Cell-based assay (CBA), central eastern Atlantic Ocean, diarrheic shellfish poisoning (DSP), okadaic acid (OA), *Prorocentrum panamense*, toxin production.

INTRODUCTION

Diarrheic shellfish poisoning (DSP) is a foodborne disease reported in several areas worldwide (Gestal *et al*. 2008). It is associated with the consumption of bivalves contaminated with toxins of the okadaic acid (OA) group, mainly produced by dinoflagellates of the genera *Prorocentrum* and *Dinophysis* (Yasumoto *et al*. 1985). Before implementing efficient monitoring programs, DSP outbreaks affected a large number of people, causing the closing of shellfish harvesting areas even for several months (Economou *et al*. 2007), resulting in dramatic losses in both the aquaculture and fisheries sectors. The toxins responsible for DSP include OA, dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2). Their mechanism of action is based on phosphatase inhibition, which can interfere with several mammalian physiological processes, such as the cell cycle regulation and the metabolism of intracellular protein, potentially causing the inflammation of the intestinal tract (i.e., abdominal pain, vomiting) and diarrhea (Yasumoto *et al*. 1985). Furthermore, OA and DTX-1 have tumor promoting activity (Fujiki *et al*. 1991). Currently, the genus *Prorocentrum* is composed of 80 species, divided among planktonic and epibenthic species (Hoppenrath *et al*. 2013). Furthermore, the taxonomic status of some species is in flux, because the *Prorocentrum* genus presents a large variety in terms of cell shape, length and width, number and shape of lateral plates and marginal pores, thus making difficult species identification with microscope techniques (Aligizaki *et al*. 2009). Recent progress in molecular techniques has improved species identification and has contributed to the clarification of taxonomy of this genus. Currently, within the genus, six planktonic and nine epibenthic species form high-biomass blooms and are considered potentially harmful (Glibert *et al*. 2012). Among the six planktonic species, only *Prorocentrum minimum* (Pavillard) Schiller has been described as potentially toxic (Grzebyk *et al*. 1998; Glibert *et al*. 2012). Instead, all the

benthic species have been described as toxic, with *Prorocentrum lima* (Ehrenberg) F.Stein being the most toxic (Moreira-González *et al*. 2018). However, since the genus *Prorocentrum* counts a huge number of species, it is probable that there may be more unidentified harmful species in addition to the currently described (Glibert *et al*. 2012). Some species of the genus *Prorocentrum* have a global distribution, such as *Prorocentrum emarginatum* Y. Fukuyo, *Prorocentrum mexicanum* Osorio-Tafall, *Prorocentrum hoffmannianum* M. A. Faust and *P. lima* (Glibert *et al*. 2012). Nevertheless, in the last years, other species that were previously considered as endemic to certain areas have been reported in other regions, exhibiting an extremely disjunct global distribution. This is the case of the epibenthic *Prorocentrum panamense* D. Grzebyk, Y. Sako & B. Berland, which was identified and described from the waters of Contadora Island on the Pacific coast of Panama in 1998, and since then, it has been recorded in La Réunion (Indian Ocean), Martinique (Caribbean Sea), Revillagigedo Islands (Mexican Pacific Ocean), and Hainan Island in the northern South China Sea (see review in Chomérat *et al*. (2019)). In the past decade, several microalgae species associated to toxin production have been newly recorded in the Canaries, and the majority of them are dinoflagellates (Fraga *et al*. 2011; Rodriguez *et al*. 2018). In fact, the particular position of the Canary Islands makes the archipelago a key point for marine transport routes (Tichavska & Tovar 2015), and its peculiar environmental conditions (Glibert *et al.* 2012) can facilitate the settlement of microalgae species introduced via marine transport (Hallegraeff 1998). In this work, the first record of *P. panamense* in the Canary Islands is reported, together with the toxicological characterization by LC-MS/MS and neuro-2a cell-based assay (CBA).. This information is crucial to better understand the global distribution, ecology and risks associated to species of the genus *Prorocentrum*.

MATERIALS AND METHODS

Field sampling and microalgal cultures

Macroalgae samples were collected in April 2017 from three natural rock pools in Las Salinas de Agaete (28°6'24.120"N, 15°42'40.140"W), Northwestern Gran Canaria (Fig. 1). Samples, of *ca*. 150–200 g fresh weight of macroalgae (fwm, hereafter) were collected, placed in polystyrene bottles containing 1 L of seawater, vigorously shaken for 1 min to release epiphytic microalgae, and filtered (200-µm mesh) to remove gross material. From each sample, 100 mL were fixed in 3% of Lugol's iodine solution for species identification and cell counting, and of the remaining 900 mL, 100 mL were used for cells isolation purpose. Samples were kept at room temperature and close to a natural source of light for a maximum of 3 days. Then, they were shipped to IRTA and stored in the incubator (24°C) upon laboratory procedures. In the laboratory, cells were isolated with a glass pipette under an inverted microscope (Leica, DMIL Pred LED) by the capillary method (Stein *et al.* 1973), and cultivated in untreated Nunc 24 well plate (Themo Fisher Scientific) containing filtered and autoclaved local seawater (salinity adjusted at 36 psu) supplemented with modified ES medium (Provasoli 1968). Cells were grown at 24°C under a 12:12 light/dark cycle with a photon irradiance of 110 µmol photons m^{-2} s^{-1}. Once cultures reached approximately 20–35 cell mL^{-1}, they were transferred to 28 mL round bottom glass tubes. After acclimation to laboratory conditions, four 225 cm^2 cell culture flasks (500 mL volume, vented cap) were prepared with 150 cell mL^{-1} each, from the same mother culture. Aliquots of cell cultures were stained with Calco-fluor White M2R (Sigma Aldrich, Spain) (Fritz & Triemer 1985) and identified to species level under an epifluorescence microscope (Leica, DMLB Condenser UCL). Morphological features were determined according to Hoppenrath *et al*. (2013). Microphotographs were taken with an Olympus DP-70 camera.

Molecular analysis

Molecular identification of the species was performed on genomic DNA isolated and purified from 50 mL aliquots of cultures in the stationary phase, following the phenol/chloroform procedure as described in Toldrà *et al*. (2018). The D1–D3 region of the LSU rRNA gene was amplified by polymerase chain reaction (PCR) using the primers D1R (5'-ACCCGCTGAATTTAAGCATA-3') and D2C (5'-CCTTGGTCCGTGTTTCAAGA-3') (Chomérat *et al.* 2010). PCR was performed in a 25 µL reaction containing 2.5 µL of 1 × PCR buffer, 1 µL of 2 mM $MgCl_2$, 1.5 µL of 600 µM dNTPs, 0.25 µL of each primer at a final concentration of 0.2 µM, 0.20 µL of 1 U Taq DNA polymerase (Invitrogen™, Thermo Fisher™, Massachusetts, USA), 1.25 µL of 5% dimethyl sulfoxide (DMSO), 2 µL of template DNA at a concentration of 1 ng μL^{-1}, and sterile water to a final volume of 25 µL. The PCR amplification was performed in a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, Madrid, Spain) for an initial denaturation step of 94 °C for 5 min, followed by 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min, and then a final elongation step of 72°C for 5 min. Four µL of each PCR product were separated by electrophoresis (1% TAE, 60 V), stained with ethidium bromide and checked under UV-illumination. PCR products were purified using a Thermo Scientific GeneJET PCR purification Kit (Thermo Fisher™, Massachusetts, USA) following manufacturer's instruction. The resulting purified product was sequenced in both directions at Sistemas Genómicos (LLC, Valencia, Spain). Obtained sequence was manually checked and edited using BioEdit v7.0.5.2 (Hall 1999). To assess the evolutionary relationship between the obtained sequence and *Prorocentrum* species sampled globally, we retrieved a set of 36 sequences belonging to 18 different species from GenBank (see Fig. S1). *Adenoides eludens* (Herdman) Balech was used as outgroup. Multiple sequence alignment was performed using MUSCLE algorithm implemented in

MEGA X (v10.0.5), and the phylogenetic relationships were estimated by maximum likelihood (ML) using RaxML v.8 (Stamatakis 2014) and Bayesian inference (BI) using MrBayes v.3.2.2 (Huelsenbeck & Ronquist 2001). Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ *G*, parameter = 0.5083)). Phylogenetic relationships were inferred by using the maximum likelihood method and Tamura-Nei model. The rate variation model allowed for some sites to be evolutionarily invariable. All positions containing gaps and missing data were eliminated.

Toxin extraction

Each of the four *Prorocentrum* replicates was harvested at four different phases of the culture growth namely: latency phase (2 days, 225 cell mL^{-1}), exponential phase (14 days, 3650 cell mL^{-1}), beginning of the stationary phase (23 days, 5570 cell mL^{-1}), and end of the stationary phase (34 days, 5390 cell mL^{-1}). Pellets were obtained by splitting the entire bottle volume in 50 mL falcon tubes and centrifuging them at 3700 g for 25 min (Alegra X-15R, Beckman Coulter). The obtained supernatants from each 50 mL tube were pooled together according to the harvesting phase (the resulting fractions are referred as “culture media” from here on). Both pellet and culture media from each harvesting phase were analyzed for toxin presence. Cell pellets were extracted with 10 mL of pure methanol and sonicated for 30 minutes (three times) at an amplitude of 37%, 3 sec on/2 sec off, using a 3 mm diameter sonicator probe (Watt ultrasonic processor VCX 750, Newton, USA). Cell disruption after each sonication was evaluated under microscopy. Once solvent was evaporated, the residue was re-suspended in 500 µL of methanol, vortexed, filtered with

a 0.2 µm PTFE filter, and transferred to autosampler vials. The culture media corresponding to the four harvesting stages, instead, underwent a solid phase extraction (SPE). Briefly, the entire volume of each harvested stage (500 mL) was filtered throw an Empore C18 disk (Sigma-Aldrich, Spain) to retain the toxins, which were afterwards eluted with 10 mL of pure methanol. After solvent was evaporated, residue was re-suspended in 500 µL of methanol, vortexed, filtered with 0.2 µm PTFE filter, and transferred to autosampler vials. All samples were stored at -20ºC until toxin analysis.

LC-MS/MS analysis

Certified reference materials (CRMs) of okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), yessotoxin (YTX), homoyessotoxin (hYTX), pectenotoxin-2 (PTX-2), azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2), azaspiracid-3 (AZA-3), 13-desmethylspirolide C (SPX-1), gymnodimine A (GYM-A) and pinnatoxin G (PnTX-G) were obtained from the National Research Council of Canada (NRC, Halifax, NS, Canada). LC-MS/MS analysis of marine lipophilic toxins was performed following the method described in García-Altares *et al*. (2013). Briefly, an Agilent 1200 LC (Agilent Technologies, USA) was coupled to a 3200 QTRAP mass spectrometer (Applied Biosystems, USA) through a TurboSpray® ion source operating at atmospheric pressure. Toxins were separated on a XBridge BEH C8 2.5 µm 2.1 × 50 mm column (Waters). A binary gradient was programmed with water (mobile phase A) and acetonitrile/water (mobile phase B), both containing 6.7 mM of ammonium hydroxide All runs were carried out at 30ºC using a flow rate of 500 µL min^{-1}. The injection volume was 10 µL and the auto-sampler was set at 4ºC. All lipophilic toxins were analyzed in both negative (-ESI) and positive polarity (+ESI), selecting two product ions per toxin to allow the quantification (the most intense transition) and confirmation; identification was supported by toxin retention time and the multiple reaction monitoring (MRM) ion ratios. An

external standard calibration curve was prepared with a six-level curve, from 4 to 40 ng toxin mL^{-1} for OA. The minimum performance criteria were checked out throughout the study such as retention time deviation ± 0.2 min, peak area deviation (RSD ≤ 3.0%), linearity ($R^2 \geq 0.98$), sensitivity (individual toxin LOD should be equal or lower than $1:20^{th}$ of regulatory level), precision intra-batch ≤ 20% and inter-batch ≤ 25%. All samples were analyzed in duplicate.

Neuro-2a CBA

CBA are routinely used to study the effect of bioactive compounds. In fact, the alteration of homeostasis caused by these compounds, such as physiological cell disruption and cell mortality, can be easily observed and measured. Indeed, CBA is commonly used for the identification of marine toxins that affect food safety, including OA (see for instance Diogène *et al*. 1995; Huynh-Delerme *et al.* 2003; Cañete & Diogène 2008). Experimental conditions followed the procedures described in Cañete and Diogène (2008), with minor modifications. Briefly, for the assay on the evaluation of cells morphology changes, cultivated neuroblastoma neuro-2a cells were seeded into 96-well plates at an initial density of 40,000 cells $well^{-1}$. After 24 h, cells were exposed to a 90 mM OA standard solution (positive control), phosphate saline buffer (PBS, negative control), and the *P. panamense* culture extracts (both microalgae pellets and culture media extracts) collected at the four growing stages. Toxin standards and culture extracts were previously evaporated to remove methanol completely, and subsequently re-suspended in a 5% fetal bovine serum (FBS) medium. Then, samples were serially diluted, and initial pellet extracts exposure concentrations were 1.0×10^4 (latency phase), 1.5×10^5 (exponential phase), 2.0×10^5 (early stationary phase), and 1.8×10^5 cell equivalents mL^{-1} (late stationary phase) for the four harvested phases, respectively. Culture media extracts exposure concentration was 2.4 mL culture media equivalents mL^{-1} for the four phases.

After 4 h of exposure, the changes in cell morphology were observed under a light microscope (Nikon Eclipse TE2000-S), and cells were photographed using phase contrast. All conditions were tested in triplicate. In addition, a semi-quantitative evaluation of *P. panamense* toxicity was performed following the CBA described by Soliño *et al*. (2015). Cultivated neuro-2a cells were exposed to *P. panamense* pellets and culture media extracts collected at the four harvested phases (4 serial ½ dilutions). Initial pellet exposure concentrations for this test were 1.2×10^4 (latency phase), 1.7×10^5 (exponential phase), 2.4×10^5 (early stationary phase) and 2.1×10^5 cell equivalent/mL (late stationary phase) for the four harvested phases, respectively. Initial culture media extracts exposure concentration was 2.7 mL culture media equivalents mL^{-1} for the four phases. Cells were incubated for 24 h, and viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Manger *et al.* 1993). This compound is converted to insoluble formazan crystals by mitochondrial dehydrogenase activity. This activity can be performed only by live cells and results in a violet color that can be measured by a spectrophotometer at 570 nm. All conditions were tested in triplicate.

RESULTS AND DISCUSSION

Molecular identification

Isolated *Prorocentrum* cells presented the asymmetrical heart shaped form, asymmetrical lateral plates and round posterior margin, and measured 46–52 µm in length and 43–46 µm in width (mean 49 µm and 44.5 µm respectively, n = 25) (Fig. 2), as originally described by Grzebyk *et al*. (1998). Furthermore, calcofluor white stained cells showed the reticulate-foveate thecal surface with depressions that become shallower towards the plate center (Fig. 2). The internal part of this structure presents several pores, only visible in the SEM image (Hoppenrath *et al.* 2013; Luo *et al.* 2017), and it is interpreted as a

synapomorphy of the *P. panamense* group. Most importantly, cells presented a large sieve-like structure close to the thecal margin (Fig. 2A), a unique feature of *P. panamense* and *P. pseudopanamense.* Stained cells also presented the linear periflagellar area (Fig. 2B), a typical trait of the *P. panamense* species. Finally, molecular analysis showed that IRTA-SMM-17-72 (deposited in GenBank with the code MW600273) branches in a subgroup of the other *P. panamense* species (Fig. S1). It must be underlined that there are only 4 sequenced strains of *P. panamense,* three of them correspond to the same locality (i.e., Martinique Island; IFR12-210, IFR12-212 and IFR12-218), and one from South China Sea (TIO97). Thus, available data probably not represent the entire genetic variance existing in the D1–D3 region of LSU rDNA. The only species that could arise doubt about the correct species attribution of the strain described in this work is *P. pseudopanamense* Chomérat & Nézan, since studies on the SSU rDNA showed that is genetically close to *P. panamense* (Chomérat *et al.* 2011). Nevertheless, Chomérat *et al.* (2019) stated that *P. panamense* species shape is very peculiar and morphologically easy to recognize and it can be clearly distinguished from *P. pseudopanamense*, which is less asymmetrical and never heart-shaped (Hoppenrath *et al*. 2013; Chomérat *et al*. 2019).

Knowledge of the ecology of benthic *Prorocentrum* species is very limited, and this is due mostly to the difficulties to discriminate among them in benthic samples (Glibert *et al.* 2012; Hoppenrath *et al.* 2013). Species of the dinoflagellate genus *Prorocentrum* mainly occur in marine and brackish waters worldwide. Specifically, the type locality of *P. panamense* is Contadora Island, on the Pacific side of the Gulf of Panama (Grzebyk *et al.* 1998), but in the last decade its presence has been reported in La Réunion Island, Martinique Island, Revillagigedo Islands (México), China, French Polynesia and in the Arabian Gulf (Hansen *et al*. 2001; Hoppenrath *et al*. 2014; Gárate-Lizárraga & González-Armas 2017; Chomérat *et al.* 2019). Thus, its presence in the Canary Islands is the first

record of *P. panamense* in the central eastern Atlantic Ocean. *Prorocentrum panamense* exhibits an extraordinary disjunct global distribution pattern, with a low level of intraspecific genetic variation in the LSU rDNA region (Chomérat *et al.* 2019). Furthermore, the lack of previous records of this species in the Canary Islands is strongly supported by recent surveys that failed to report the presence of the species in the region (Fraga *et al.* 2011; Rodriguez *et al.* 2018). Although this absence may be due to species misidentification, from a morphological perspective this species is very peculiar and easy to recognize and identify (Chomérat *et al.* 2019). Thus, its presence in the region could have happened naturally, we consider that a human-mediated introduction may be occurred. The method of introduction is unknown, but the transport through ballast water is considered the most probable vector of introduction. Coastal ship traffic constitutes an effective introduction vector for aquatic organisms (Roy *et al.* 2012), and ballast water from shipping has been considered responsible for the introduction of several benthic dinoflagellate species in some countries (*e.g.*, Australia, Canada, Japan), sometimes with dramatic economic consequences to aquaculture, fisheries and tourism (Hallegraeff 1998; Roy *et al.* 2012). Ballast waters have been identified as a potential source for dinoflagellate species introduction (Hallegraeff & Bolch 1992), and the Canary Islands play an important role in the global marine transport. Las Palmas Port is a major logistic platform between Europe, Africa and America, with a cargo hub over 19 million tons, being a leading worldwide bunker trader (Tichavska & Tovar 2015). In addition, ballast water from oil platforms have also suggested as a potential source of marine species introduction to the Canary Islands (Brito *et al.*, 2011).

Toxin content and profile

The analysis on *P. panamense* extracts showed both the presence of toxic compounds and toxicological activity. In fact, LC-MS/MS analysis showed the presence of OA in the

285 culture media extracted from the late stationary phase (Table S1, Fig. S2). No other
286 marine lipophilic toxins were found. Thus, this is the first record of toxin production of
287 *P. panamense*. The evaluation of cell morphology changes (neuro-2a) provided additional
288 information on the toxicity of the extracts (both from microalgae pellets and culture
289 media) obtained at the four different growth phases. Okadaic acid induces cell apoptosis
290 through the disruption of the filamentous actin (F-actin) cytoskeleton, the activation of
291 caspase-3 and the collapse of the mitochondrial membrane potential. This results in a
292 change in cell morphology and substrate detachment (Diogène *et al.* 1995; Cabado *et al.*
293 2004). In this work, morphological changes including cell blebbing and detachment could
294 be observed when control cells (Fig. 3A) were compared to the cells exposed to OA (Fig.
295 3B). After 4 h of incubation with the pellets and culture media extracts, neuro-2a cells
296 showed damages in presence of the pellet extract of *P. panamense* from late stationary
297 phase (Fig. 3C) and of the culture media extract of the early stationary phase (Fig. 3D).
298 Thus, even if OA was detected with LC-MS/MS only in the culture media extract of the
299 late stationary phase, neuro-2a cell anomalies showed the presence of OA-like toxicity in
300 other two extracts, underlying the toxic capacity of the *P. panamense* strain.

301

302 CBA results (Table S1) showed cell toxicity of all pellet extracts from the different
303 growth phases, with the exception of the latency phase. The cell mortality was as follow:
304 late stationary > early stationary > exponential. Furthermore, all culture media extracts
305 induced cell mortality, with a maximum in the early stationary phase and a minimum in
306 the exponential phase (early stationary > late stationary > latency > exponential). Hence,
307 it seems that the nature and/or concentration of toxic compounds released to the media
308 may vary not only according to the number of cells, and also to the phase of the growth.
309 These results confirmed the toxicity of *P. panamense* IRTA-SMM-17-72. In some

310 treatments a concomitant mortality evaluated by the MTT assay and morphological
311 changes in cells (e.g., early stationary phase) can be observed. Nonetheless, for some
312 treatments, cell mortality has been recorded but no morphological changes were
313 observed. This may be explained by the kinetics of the effects, and the time of
314 observation. It is also possible that changes in morphology end-up in cell detachment of
315 dead cells that would reduce the MTT signal, without observable changes in morphology.
316 Our results revealed the presence of OA and its effects on neuro 2a-cell morphology and
317 viability, in particular in the stationary phases with high concentrations of *P. panamense*
318 cells. Even if there are no studies investigating the cell growth and toxin production of *P.*
319 *panamense,* a similar toxin production behavior has been observed in the stationary phase
320 of some *P. lima* cultures (Bravo *et al.* 2001; Holmes *et al.* 2001). However, the results
321 derived from the analysis of *P. lima* showed a much higher OA contents (mean of 4.74
322 pg $cell^{-1}$ in Bravo *et al.* (2001) and 15 pg $cell^{-1}$ in Holmes *et al.* (2001)) compared to the
323 ones obtained in this work (Table S1). Even though, the CBA values related to OA-like
324 activity, obtained from the sum of pellet and culture media data, gives OA concentration
325 that are comparable with the ones obtained in the analysis of *P. lima* (Table S1). Only
326 Luo *et al*. (2017) investigated the toxicity of a *P. panamense* strain from China and
327 described it as non-toxic according to LC-MS/MS. However, in their work, cells were
328 collected during the mid-exponential phase, which also in our experiment show either
329 few (only with CBA) or a total absence of toxic activity or toxins. Thus, the undetected
330 toxicity of the Chinese strain could be related to the growth phase considered. Further
331 studies are needed to characterize the toxic profile of *P. panamense,* involving strains
332 from different regions.

Conclusions

Prorocentrum panamense strain was detected and identified in samples from Gran Canaria (Canary Islands, Central Eastern Atlantic Ocean). This is the first record of this species in the Macaronesian region and underlines the expansion of the *P. panamense* distribution area. This discovery highlights the importance of monitoring programs and long-term data sets, which facilitate the new detection of species. Additionally, in this work, LC-MS/MS analysis confirmed the presence of OA, and the assay on the evaluation of neuro-2a cell morphology changes together with the viability CBA with colorimetry, identified the presence of OA-like activity in several extracts from both cell pellets and culture media, pointing this species as a possible threat for human health. This is the first toxicity report of a *P. panamense* strain. Hence, there is a need for further studies on the toxicology of several strain belonging to this species to better assess the toxin production. The identification of OA producing species out of their area of distribution can contribute to the DSP risk assessment and help in spotting future outbreaks, so limiting the economic cost associated to DSP events.

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Figure legends

Fig. 1. Global distribution of *Prorocentrum panamense* (a). Black circles indicate previous records. White circle indicates first record presented in this work. Sampling site in Gran Canaria where *P. panamense* was found (Salinas de Agaete) (b). © 2020 Google Earth vision of Salinas de Agaete (c).

Fig. 2. Cell view of *Prorocentrum panamense* after calcofluor-white staining. It is possible to observe the asymmetrical shape and reticulate-foveate thecal surface. Left thecal view (a). Arrow indicates the marginal pore. Right thecal view (b). Linear periflagellar area can be observed. Scale bar 20 µm.

Fig. 3. Morphology of neuroblasoma cells after 4 h exposure to PBS (control) (a), okadaic acid at 90 nM (b), pellet extract from late stationary phase (c), and culture media extract from early stationary phase (d). Scale bars 100 µm.

Figures

Fig. 1.

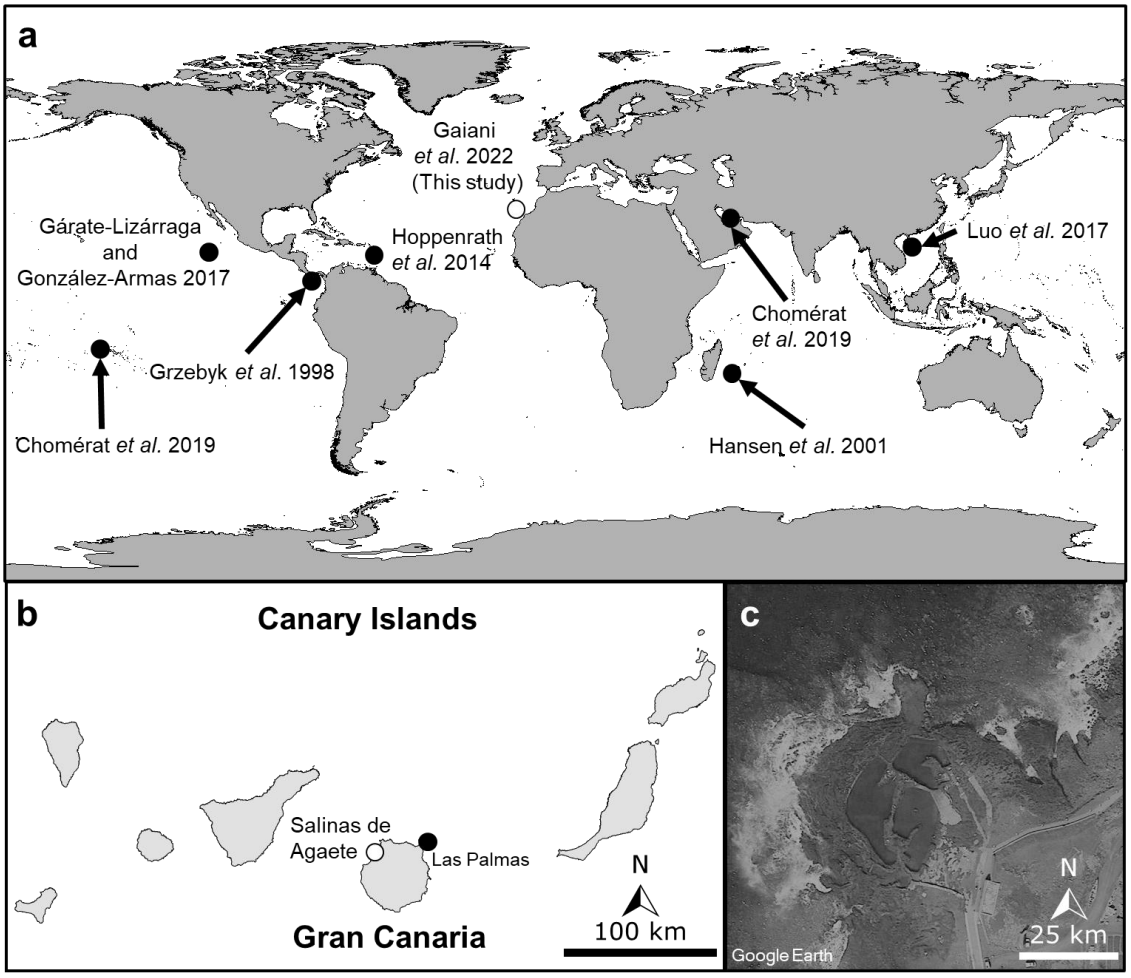


Fig. 2.

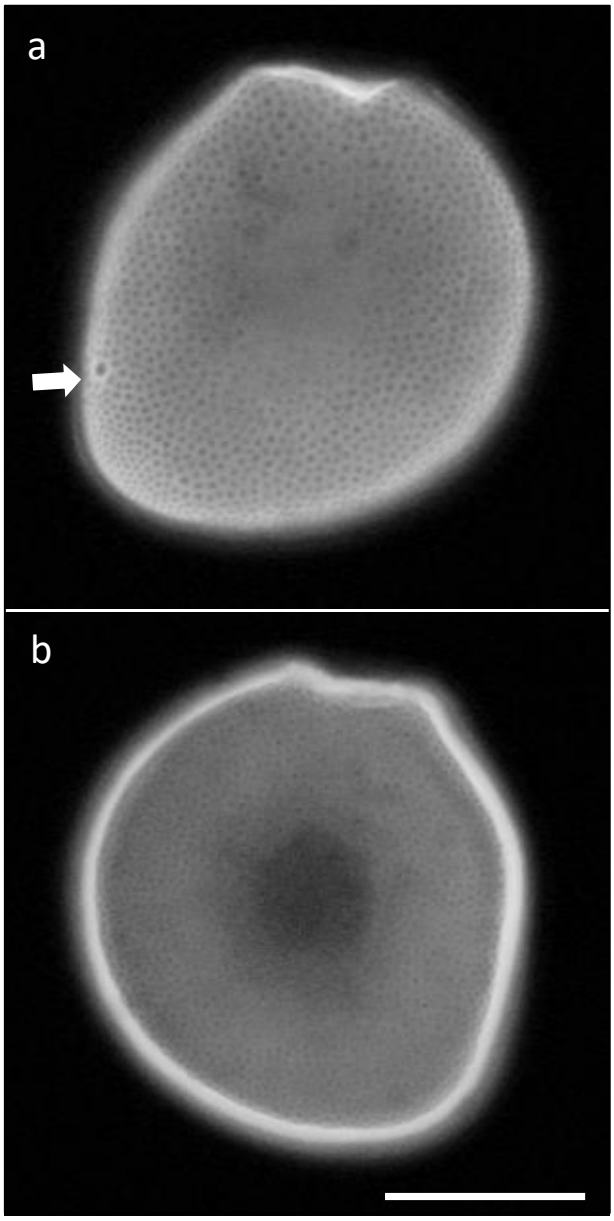
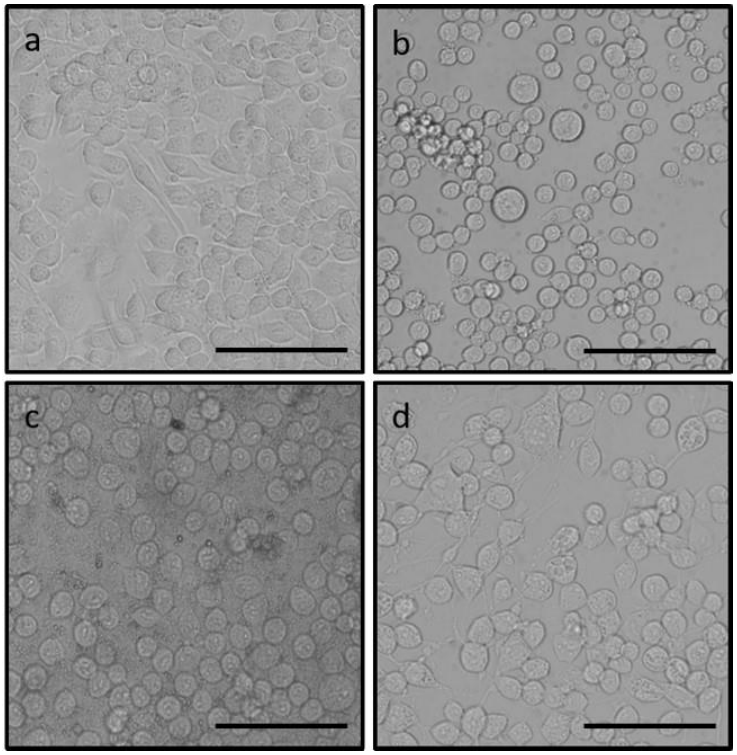


Fig. 3



Supporting information

Table S1. Comparison between CBA and LC-MS/MS toxicity results for the different culture phases.

Phase	cells/mL	Total cells	CBA			LC-MS/MS	
			Pellet OA (fg/cell)	Culture media OA (ng/mL)	Total OA (fg/cell)	Pellet OA (fg/cell)	Culture media OA (ng/L)
Latency (day 2)	225	1.13×10^5	< LOQ [a,b]	15.71 (IC_{50})[b]	43.61×10^3	< LOD [d]	< LOD [e]
Exponential (day 14)	3650	1.83×10^6	430.22 (IC_{50})[b]	7.01 (IC_{27})[b]	1.22×10^3	< LOD [d]	< LOD [e]
Early stationary (day 23)	5570	2.79×10^6	433.79 (IC_{50})[c]	36.05 (IC_{100})[c]	22.01×10^3	< LOD [d]	< LOD [e]
Late stationary (day 34)	5390	2.70×10^6	599.82 (IC_{50})[c]	22.97 (IC_{50})[b]	4.48×10^3	< LOD [d]	2.35

[a]LOQ CBA: 311-865 fg/cell in pellet, 6.2-13.8 ng/mL in culture media ; [b]no morphological affectation; [c]morphological affectation. [d]LOD LC-MS/MS in pellet : 0.14-3.64 fg/cell; [e]LOD LC-MS/MS in culture media: 0.80 ng/L

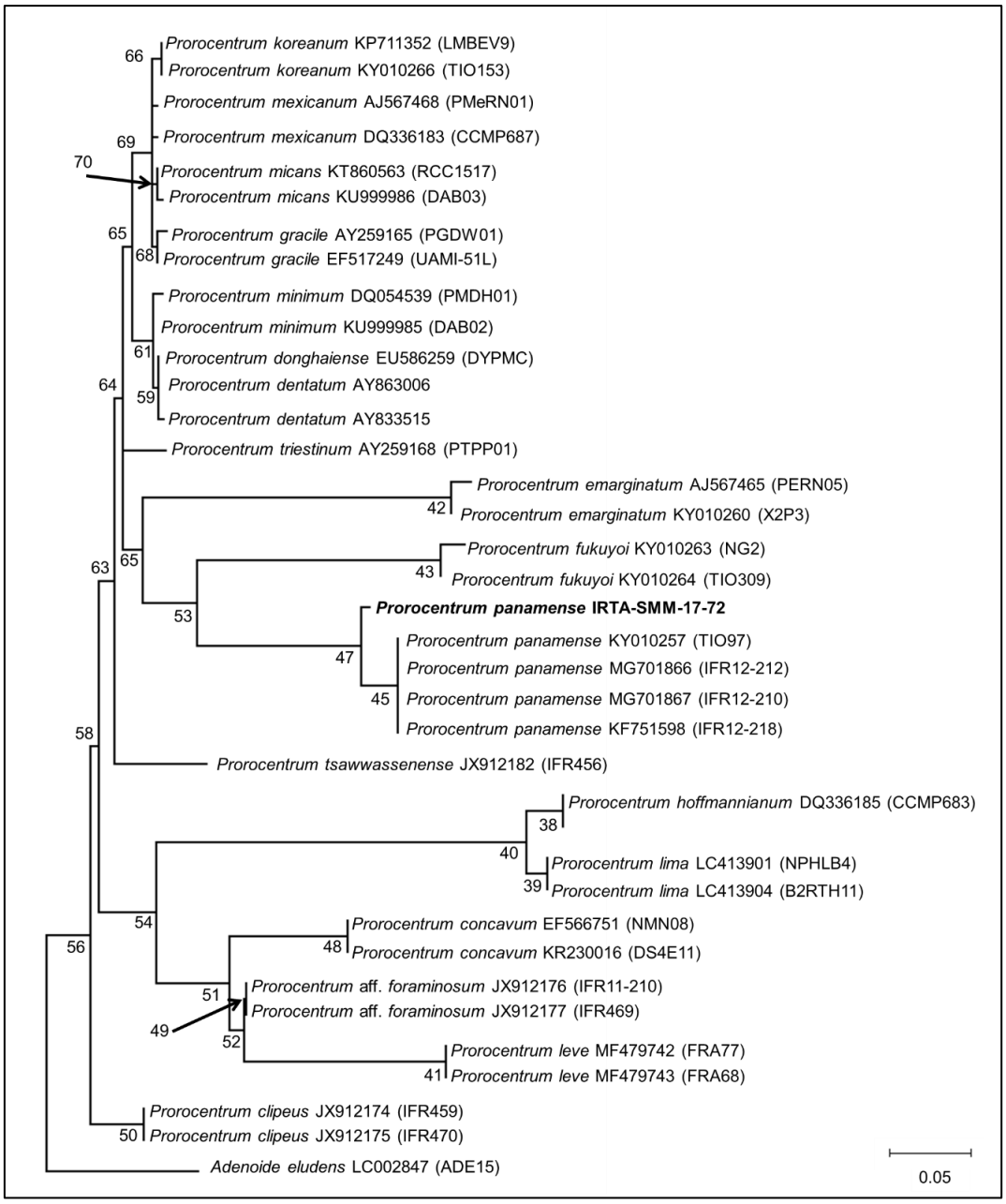


Fig. S1. Phylogenetic analysis of LSU 28S rDNA gene of the available *Prorocentrum* sequences (GenBank) and *P. panamanse* collected in this study (in bold). The tree with the highest log likelihood is shown (-2071.16). Values at nodes are bootstrap values obtained by the maximum likelihood method. Bootstrap values less than 30 % are not shown.

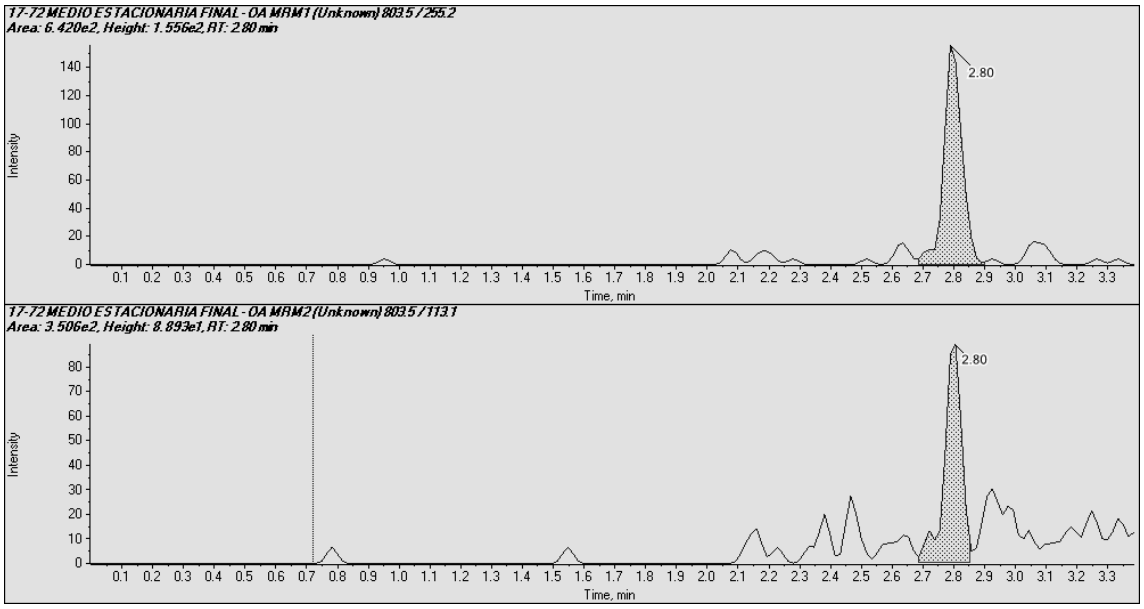


Fig. S2. Multiple reaction monitoring chromatogram of transitions monitored obtained following the analysis of OA in the late stationary culture media by LC-MS/MS.