PalArch's Journal of Archaeology of Egypt / Egyptology

Isolation, identification, cloning and phylogenetic analysis of the micro algae Prorocentrum minimum (Pavillard) J. Schiller 1933 Isolated from the Bay of Callao - Peru

[1]Carmen Ruiz, [2]Doris Esenarro, [3]Raul Mendez, [4]Elizabeth Luz Segovia Aranibar, [5]Samuel Reyna Mandujano

[1] National University Tecnologica de Lima Sur (UNTELS)
[2,3,4,5] National University Federico Villarreal UNFV -EUPG Lima, Perú
Email: [1]c.ruizh@untel.gob.pe , [2]desenarro@unfv.edu.pe, [3]rmendez@unfv.edu.pe, [4]esegovia@unfv.edu.pe, [5]sreynam@unfv.edu.pe

Carmen Ruiz, Doris Esenarro, Raul Mendez, Elizabeth Luz Segovia Aranibar, Samuel Reyna Mandujano: Isolation, identification, cloning and phylogenetic analysis of the micro algae Prorocentrum minimum (Pavillard) J. Schiller 1933 Isolated from the Bay of Callao - Peru -- Palarch's Journal Of Archaeology Of Egypt/Egyptology 17(9s). ISSN 1567-214x

Keywords: Prorocentrum minimum, Molecular Phylogeny, Cloning, Callao Bay

ABSTRACT

Harmful Algal Blooms (HAB) or Red Tides are water discolorations visible to the naked eye due to the proliferation of one or more planktonic microorganisms such as microalgae. These can reach high levels and produce adverse effects on human health, as well as cause damage to other marine organisms near the coast. For this reason, there was interest in isolating, identifying and making a phylogenetic study of the species Prorocentrum minimum found in the Bay of Callao, Peru. Several samples of seawater were taken for their subsequent morphological identification, and the technique of purification of the microalgae was carried out. At the same time, a standard of Prorocentrum minimum was acquired from the Provasoli- Guillard National Center for Culture of Marine Phytoplankton (CCMP), USA. It was carried out the growth curve for the concentration of microalgal density, later it was carried out the DNA extraction and the molecular phylogeny from the sequences of the ribosome LSU rRNA subunits of the species Prorocentrum minimum. P. minimum and P gracile were identified, which were close epibenthic species because they were more recent evolutionarily. Likewise, to confirm the presence of some marine biotoxin absence in Prorocentrum minimum culture, it was established a quantitative analysis of the animal's dose response in the mouse bioassay. It was possible to obtain the purification of Prorocentrum minimum culture and to standardize the work protocol; it was confirmed the phylogeny of microalgae species. It was not possible to obtain DSP toxin from microalgae culture.

1. Introduction

Some potentially harmful microalgae have caused fish mortalities and fish eggs, which have been associated with effects caused by incomplete decomposition of organic matter, anoxia, saturation or ulcerations of the gill apparatus and external sources of marine pollution in filtering species, mainly in natural fan-shell banks. However, it cannot be ruled out that these species may become toxic due to environmental changes or eutrophication processes.
Eutrophication is the enrichment of seawater by nutrients at a faster rate than removal, causing substantial changes in the balance of the ecological ecosystem. The water is enriched with oxygen causing the increase of phytoplankton and accelerating the addition of nutrients. For this reason, high concentrations of one or more million planktonic microalgae cells per liter are observed. The color of the seawater will depend on the microalgae, in many cases it can be; greenish, brown, reddish, orange etc. Harmful Algal Blooms (HABs) is a term adopted by the Intergovernmental Oceanographic Commission (IOC) of UNESCO and accepted internationally for any proliferation of microalgae (regardless of their concentration) perceived as a damage to public health, aquaculture, the environment and recreational activities.
In Peru, there are few specialists dedicated to the taxonomic study of harmful phytoplankton. IMARPE is a research institution, which reports through quantitative and qualitative analyses the morphological presence of microalgae present in a red tide event. The great morphological similarity between the different species of phytoplankton makes it difficult to follow them through optical microscopy. The size of the microalgae is also a limiting factor and it is necessary to use the instrumental technique of electronic microscopy to have the accuracy of the species present in the study. In order to solve this problem, the support of professionals from different research centers in the region is requested. The need to process in a minimum time the high number of complex plankton samples required for field studies, makes it essential to develop alternative techniques that are fast, specific and comfortable. Currently, the taxonomic identification at a molecular level of this phytoplankton that generates impact on the marine environment is unknown. For this reason, it is required detailed studies at molecular level, existing in this work a research initiative in the field, when carrying out the study of isolation, identification, cloning and phylogenetic analysis of the harmful algal flowering species Prorocentrum minimum. [1]
Isolations and establishments of mono-algal cultures of Prorocentrum minimum species, allowed us to carry out the taxonomic and molecular identification and presence/absence of marine biotoxins since there are abundant cells per milliliter of this organism due to the concentrations of microalgae obtained at laboratory level for its later analysis.

1.1 background of the investigation

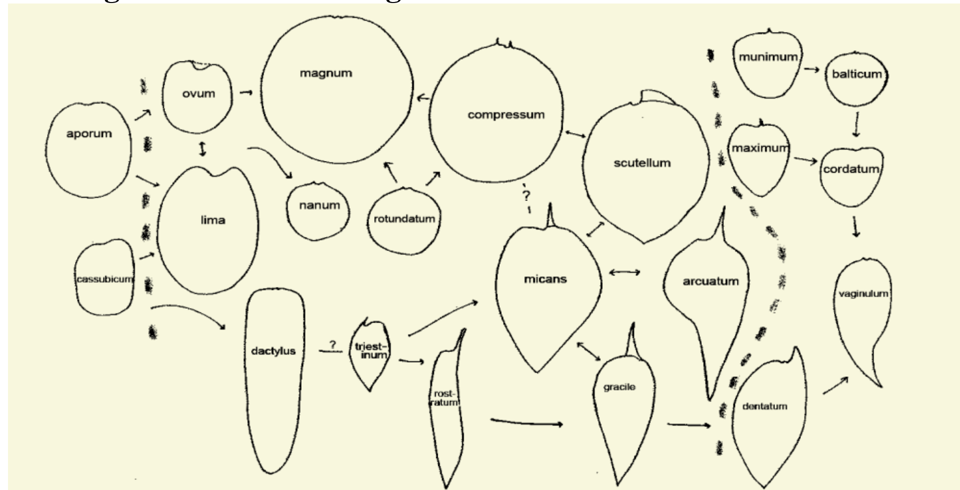

Figure 1: Phylogenetic relationships between species.

Phylogenetic relationships explore the possible relationships between Prorocentrum species, based on the evolution of the group's characters, as seen in Figure 1; the smooth shells and shells with depressions and/or spines, as well as the round and elongated cells, acquiring the apical spine increasingly longer by presenting two spines. The changes in the characteristics he proposed were not sufficient to support a comprehensive hypothesis that includes the 21 species he considered in his analysis. Therefore, and as illustrated in Figure 1, a quarter of the species, which included the segment marked with a dotted line from Prorocentrum minimum (Pavillard) J. Schiller to Prorocentrum dentatum Stein, seemed not to have a clear relationship with the other species. [2]

2. Methodology

2.1. Place of sampling

The study area was the Bay of Callao, exactly in front of the dock of the Instituto del Mar del Peru at 77°09'36" W and 12°03'36" S. It includes the Constitutional Province of Callao, as we see in Figure 2.

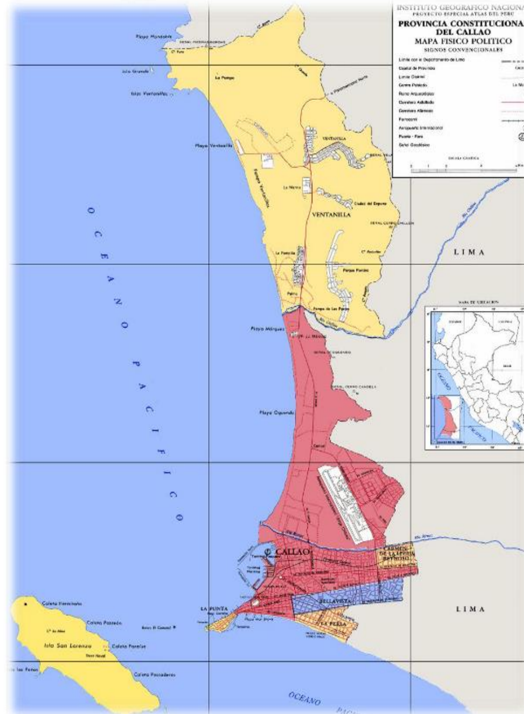

Figure 2: IMARPE's Pier Cartography. Place of sampling.

2.2. Isolation of the microalgae

We worked inside a laminar flow chamber in aseptic conditions. A drop of the sample was deposited on a clean object slide and covered with the coverslip, being observed under the binocular microscope in the 10x and 40x phases. It was verified the morphology and swimming movement of the live microalgae. The species of interest was observed. [3]

2.3. Unialgal purification establishment

2.3.1. Isolation by pipetting and successive washings

Work was done inside the sterile laminar flow chamber in front of the burner. This technique was used to obtain a unialgal culture because the minimum Prorocentrum is greater than 10 µm.

Work was done inside the sterile laminar flow chamber in front of the burner. This technique was used to obtain a unialgal culture, to the remaining 11 holes 1 mL of Modified Guillard F2 Medium was added, then with a sterile Pasteur pipette a drop was taken from the first well transferring it to the second well by shaking it well and in this way the successive washes were continuously performed. [4]

Figure 3: Multiwell plate to the laminar flow chamber

As we see in figure 3, the fields of the washed holes and the field with less cells (1-2 microalgae) were observed under the binocular microscope, the multiwell plate was transferred to the laminar flow camera. Similarly, a rack of 13 15x150 mm test tubes with 10 mL of sterile modified F2 Guillard medium was prepared inside the chamber, to which all the well medium containing 1-2 microalgal cells was transferred with the aid of the Pasteur pipette. This same procedure was repeated with each test tube.
Incubated tubes were shaken daily at 10:00am for a period of 30 days, cell division and increase in the number of algae cells of interest were observed without any contamination. Tubes with higher staining density were selected, and the 12 tubes were concentrated in three 100 mL flasks (40 mL microalgae inoculum + 60 mL F2 Guillard medium), at the same parameters mentioned above. [5]

2.3.2. Growth curve of the micro algae Prorocentrum minimum.
The quantification of the microalgae growth density was estimated by the method of direct cell count with the Neubauer camera of 0.1 mm depth and with the help of the binocular compound microscope at 10X reporting the cell density (cell mL-1) x 104, this is an effective method to correctly evaluate the daily culture process.
Prorocentrum minimum cell counts were performed daily for a period of 30 days, the assay was carried out as follows: one control at 19 +/-2 °C and at 21+/-2 °C, the microalgal inoculum density of Prorocentrum minimum was 0.5 mL in a volume of 99.5 mL of sterile F2 Guillard medium, to have a final volume of 100 mL per flask. The objective of these trials was to observe how many days it took to concentrate the highest microgal density. The control flasks were not sampled daily for cell count, only the flasks were sampled in triplicate.

2.3.3. Minimum P. concentration for molecular analysis
From the flasks of the growth curve of P. minimum, a sample was obtained which was observed under the binocular microscope. Subsequently, a 5 mL inoculum from the microalgae plus 95 mL of F2 Guillard medium was transferred into 8 flasks of 125 mL capacity. It was incubated for 18 days in the climatic chamber conditioned at 21 °C with a photoperiod of light and manual agitation twice a day. Once the microalgal density was concentrated, it was

transferred with the help of the Pasteur pipette to test tubes. Subsequently, it was centrifuged at 5000 rpm x 10 minutes, the supernatant was removed and the sediment formed was transferred to a microtube. This procedure was carried out until sediment was concentrated in all the flasks. Finally, the microalgae cell packs were refrigerated at 3°C. [6]

2.3.4. Evaluation of the toxigenic capacity of Prorocentrum minimun

To evaluate the toxigenic capacity of P. minimun, 1.5 L of microalgae culture was concentrated, the whole culture was centrifuged at 5000 rpm for 10 minutes and 15 mL of sediment was obtained from the microalgae culture. It was worked at a temperature between 5 and 10 °C, in this way it was possible to obtain a cell pellet. 50 mL of acetone was used and the solid phase was homogenized for 2 -3 min. The supernatant was filtered through filter paper and collected in a collecting flask adaptable to the rotary evaporator. The same extraction steps were repeated twice more, using 50 mL of acetone each time. The two new filtrates from the same collecting flask were combined. The final acetone extract was evaporated in the rotavapor with water bath at a temperature of 40 ℃. It was not necessary to evaporate the waste water completely. [7]

Subsequently, a small volume of water was added to the residual extract so that the final volume of the extract is approximately 15 mL. The extract was transferred to a decanting funnel. Any residue remaining in the collecting flask was dragged with 50 mL of diethyl ether and this ethereal extract was combined with the aqueous extract from the separating funnel. It was gently stirred by inverting the funnel several times. It was then left to stand and the separation in two phases was allowed, the aqueous fraction was separated and the ethereal fraction was reserved. The aqueous fraction was extracted twice more with 50 mL of diethyl ether. The ethereal fractions were combined and two additional washes of the ethereal extract were performed with 15 mL of water. The ethereal extract was completely evaporated. Then the aqueous fractions were combined and the water-soluble extract was evaporated. Finally, the residues were redissolved with a 60 to 1% aqueous solution of Tween, previously at a temperature of approximately 40º C.

2.3.5. Mouse bioassay:

Figure 4: Inoculation of the biotoxin in the mouse

It was inoculated 1 mL by intraperitoneal route of the liposoluble solution of the extract of the microalgae Prorocentrum minimum (Figure 20), to three mice of 19-21 g of weight and it was observed for 24 hours. The bioassay was considered positive for the presence of the toxins OA, DTXs, PTXs, and YTXs when at least 2 of the 3 mice died within 24 hours.

3. Results

3.1 Isolation of the microalgae

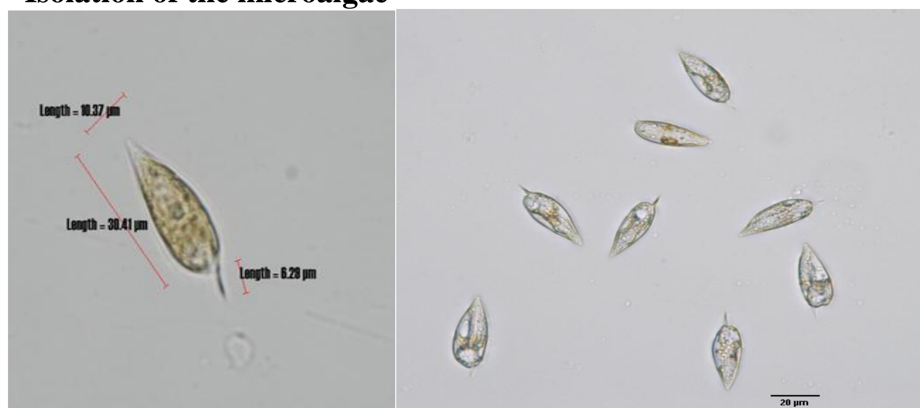

Figure 5: Morphology of Prorocentrum gracile Schutt 1895. It presents live cells.

Two strains of microalgae were isolated: Prorocentrum minimum isolated from Chucuito Beach in Callao Bay and Prorocentrum gracile isolated from Carpayo Beach also in Callao Bay. The isolation technique facilitated the work, since pure cultures were obtained from an algae unit. It is visualized in figure 5.

3.2 Growth curve of the micro algae Prorocentrum minimum, at different temperatures of 19 and 21 °C in the culture chamber.

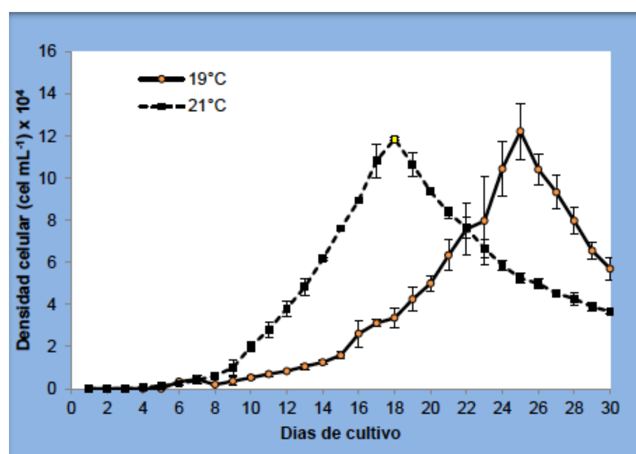

Figure 6: Growth curve of the cell density of the micro algae Prorocentrum minimum at different temperatures of 19 and 21°C

In figure 6, the culture of Prorocentrum minimum presented four phases in its growth which were: Induction phase; it took between six and eight days at 21 and 19 °C could be due to the size of the cell, and to the state of the inoculation, to the new conditions of culture and to the physiological adaptability.
Exponential phase: here the microalgae increased rapidly in an exponential geometric way, this phase appeared on the seventh and ninth day after the inoculum was added to the medium and lasted for several days. In the culture at 19°C the exponential phase lasted 11 days, while in the culture at 21°C the exponential phase lasted 16 days, depending on the type of microalgae.
Stationary Phase: In both cultures of Prorocentrum, it was observed a decrease in the speed of reproduction of the cells, due to Nutrient depletion of the medium caused by the growth of the crop, occurred similar to that obtained in microalgae culture.
Decline phase: it could be observed in the culture at 19 °C was notorious from day 25 to day 30, while the culture at 21 °C was perceived in the cell count from day 25 to day 30. Temperature is not a limiting factor for the development and growth of Prorocentrum minimum, since these temperatures fluctuate between 12-22 °C and salinities of 5-10 ppm. In the winter months in the Mediterranean Sea the species can predominate at temperatures of 4-28 °C. Confirming that our growth temperature of Prorocentrum minimum was within the limits of development for the microalgae. [8]

3.3 Statistical analysis.

The result of the PCR, was obtained by the combinations of primers D1R/ D2C and Perk ITS- S and the Perk ITS- A (for Prorocentrum crops). The LSU sequences obtained correspond to the species Prorocentrum minimum (2811) from the Provasoli Guillard National Center of Culture of Marine Phytoplankton Institute (CCMP) and Prorocentrum minimum (Pm5; Pm 5.1 and Pm 7), Prorocentrum gracile (Pgr), isolated from the Bay of Callao - Peru.
Each strain was sequenced with the primers D1R/ D2C approximately in the 420 bp base pair obtained from the 5' end and the 3' end of the genomic fragment. The results obtained with the LSU gene of the partial fragment ITS 1-5.8 rRNA were confirmed and ITS2 served to compare the strains *Prorocentrum minimum (2811), Pm5 and Prorocentrum gracile (Pgr). The NCBI sequences with the access number used were; Prorocentrum minimum DQ884421, Prorocentrum minimum EF517247.1, Prorocentrum minimum DQ054539.1, Prorocentrum minimum AF463409.2, Prorocentrum minimum AF512886.1, Prorocentrum minimum AF463409. 2, Prorocentrum minimum FJ939573.1, Prorocentrum minimum DQ662402.1, Prorocentrum minimum AY863005.1, Prorocentrum gracile EF517251.1, Prorocentrum gracile EF517249.1, Prorocentrum gracile EF517248.1 Prorocentrum gracile AY259165.1*

Table 1. Prorocentrum cultures used for sequencing in the present study

Species	cultivation	location	coordinates	region	ocean	sea	date	T°	Institution
P. minimum	2811	Florida, USA	27.33N 82.583W	North america	Atlantico settentrionale	Gulf of Mexico	24/08/2004	18-22	1
P. minimum	Pm5	Callao, Peru	S12°03'57.8", WO 77°09'30.4"	South america	Pacifico meridionale	Callao Bahia	05/04/2005	21	2
P. minimum	Pm7	Callao, Peru	S12°03'57.8", WO 77°09'30.4"	South america	Pacifico meridionale	Callao Bahia	06/08/2007	19	2
P. gracile	Pgr	Callao, Peru	S12°03'57.9", WO 77°09'30.5"	South america	Pacifico meridionale	Callao Bahia	05/03/2011	18	2

1. Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP).
2. Instituto del Mar del Perú (IMARPE).

3.4 Statistical analysis

The highest cell densities of the microalgae Prorocentrum minimum, were registered in the culture at 19 °C, shows in figure 5, reaching figures of 12.2 ± 0.44 x 104 cell.mL-1 on day 25. Cell density gradually increased with significant differences (P<0.05) until day 24 and 26, and then decreased. Cultures at 21°C reached their maximum cell density at day 18 (11.86 ± 0.44 x 104 cells mL-1), from which density decreased, with significant differences (P< 0.05) during days 17 and 19.

3.5 Sequence alignment and phylogenetic tree construction

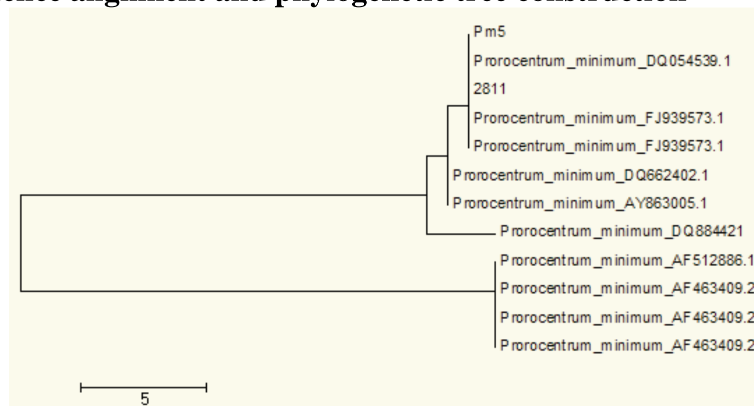

Figure 7: Molecular Phylogenetic Tree by Maximum likelihood tree method, based on Prorocentrum minimum LSU rRNA analysis

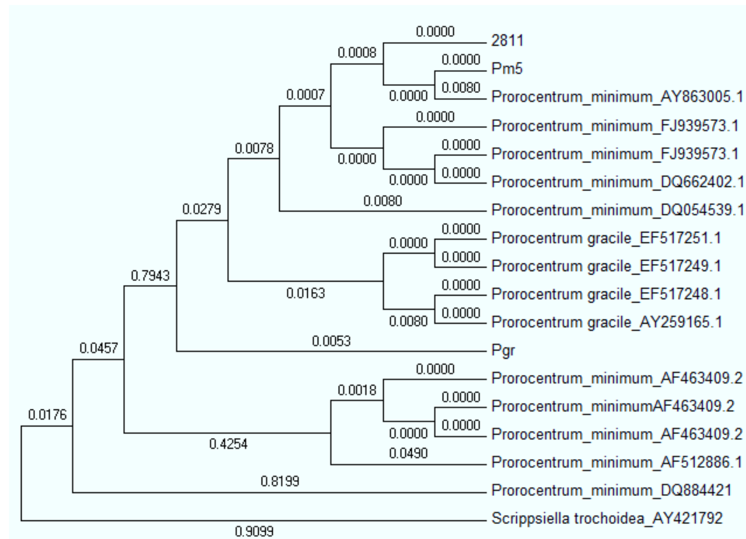

Figure 8: Molecular Phylogenetic Tree by Maximum likelihood tree method, based on LSU rRNA analysis of Prorocentrum species, using Scrippsiella thochoidea as an outgroup.

The sequences in figures 7 and 8, obtained from microalgae 2811, Pm5, and Pgr were aligned and phylogenetic trees were constructed to establish their identification and phylogenetic location, confirming the species as Prorocentrum minimum.

3.6 Results of the analysis of the mouse bioassay

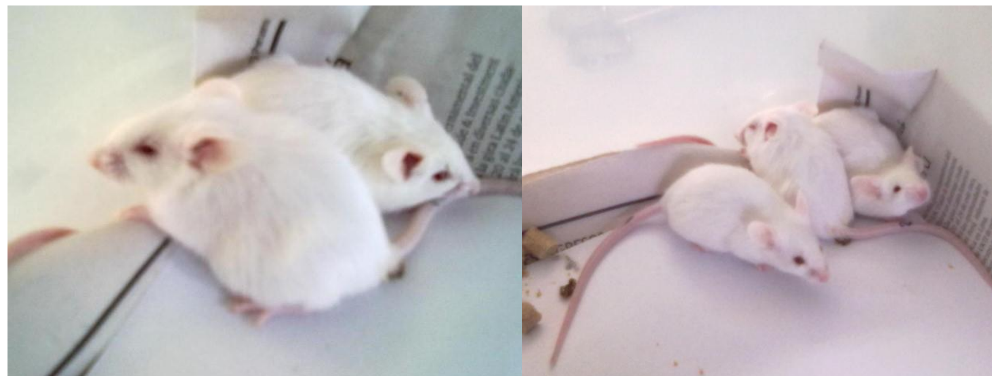

Figure 9: Mouse bioassay: A, B, C and D were observed with bristly, crestfallen and prostrate hair. D. After 72 hours, they did not die, but one of the mice was still in discomfort.

In figure 8, the symptoms of the three mice after the microalgae inoculation were; head down, with discomfort, bristly hair and prostration. Also, figure 9 show after 72 hours of observation there was no death of the rodents.

4. Discussion

The Constitutional Province of Callao, is located in the central western part of Peru, covering the central coast of the Peruvian coast. The Bay of Callao belongs to this province being the largest in this part of the continent and is considered the key to the capital of the republic due to its important seaport. The port of Callao is located at 12°LS and has a great fishing importance in Peru. It is considered a highly contaminated area due to the strong anthropogenic activity registered in the form of urban and industrial waste discharges that are introduced into the marine ecosystem. Studies conducted in the area reveal high values of nutrients, which indicates a strong biological activity and high levels of pollution leading to eutrophication. These high values of nutrients will notably favor the development of the phytoplankton community, sometimes being harmful to the marine ecosystem due to the formation of red tides. These characteristics seem to be optimal for the growth of harmful algal blooms. Being these environmental conditions favorable as it is the case of Prorocentrum minimum. During the last decade studies of benthic species of Prorocentrum have increased because some species have been shown to be toxic. [6, 9]

In Peru, Prorocentrum species should be considered as cryptogenic species (of unknown origin) since it has a worldwide distribution and is the cause of red tides in different regions of the world as it is stated. Sampling was important in the investigation because it helped us to determine the phytoplankton present in the seawater sample, the frequency of sampling was done on 2 different dates in different years, March 31 and April 28, 2005, a month and a half after the red tide event of Prorocentrum minimum in the bay of Pisco and August 06, 2007. Sampling coincided with the autumn-winter season, with salinity of 37 ppm and a temperature of 17 °C. The accentuated degradation of the environmental conditions of the coastal marine area, associated with eutrophication processes (enrichment of seawater by nutrients), pollution by anthropogenic activity and with global climate changes, favor the increase in the number of harmful algal episodes with drastic impacts on fishing and tourism resources and public health.

The filtered volume was 25 liters with a phytoplankton net of 20 µm mesh opening; sieve of 100-150 µm with clamping ring and collector in the form of a glass bottle at the end in the net

The results obtained in the Prorocentrum minimum growth curve were due to the cloning of microalgal cells in F2 Guillard medium, which is an ideal enrichment medium for all microalgae providing them with the necessary nutrients for their growth. The microalgal cultures in spite of the low temperature setbacks in the environment at night maintained a favorable development of irradiation, photoperiod of light in the culture medium. [9]

The extraction allowed to obtain the highest amount of DNA from the cells coming from the fresh culture of Prorocentrum minimum and Prorocentrum gracile. [10]

The result of the PCR, was obtained by the combinations of primers D1R/ D2C and Perk ITS- S and the Perk ITS- A (for Pm5 and Pgr crops) it was confirmed

that these species belong to the Prorocentral families and that they maintain genetically conserved regions. The tree based on the LSU rRNA analysis of Prorocentrum minimum, proposes that the species are related to each other, where it includes Pm5 and Pm2811, considered as a species complex due to their similarity.

It was concentrated the biggest quantity of microalgae and from this, it was worked the protocol of indirect determination of the possible effect of the toxin, it was negative and one of the reasons could be the very low microalgal concentration of 104 cells mL-1 and the 1.5 L of volume filtered to that density was not enough. [6, 11]

Coastal environments confirmed the potential risk to human health associated with Prorocentrum minimum and with occurrences of Bloom of this species. There is a dilemma regarding the neurotoxic components isolated from axenic cultures of Prorocentrum minimum clones isolated from the Mediterranean coasts of France. The results after 72 hours of the inoculation of the extract of the microalgae Prorocentrum minimum in mice were of discomfort, head down, bristly hair and prostration, it was not observed death of the rodents, where the toxic components were soluble in water and the blockers of the sodium channels produced neurotoxic symptoms in the mice such as convulsions, spasms with pronounced palpitations and death in a matter of minutes[6, 12].

The present study allowed isolating, identifying, cloning and molecular analysis of Prorocentrum minimum from Callao Bay, being this first task a reason for researching this cosmopolitan species in our country.

5. Conclusion

One of the main drawbacks to the sustainability of aquaculture is the presence of red tides, which can produce significant impacts on marine resources and cause economic losses. To face this problem, this thesis will contribute to provide the necessary information for the molecular phylogeny of the taxonomic diversity of the Prorocentrum minimum species of the Prorocentrales Family present in the Peruvian sea, which could lead to a possible event of algal blooms.

There are a large number of diseases that are related to imbalances in the balance of phosphorylation-dephosphorylation activity and since Protein phosphatase type 1 (PP1) and protein phosphatase type 2 (PP2A) regulate 90% of the reactions of phosphatase proteins (PSPs), selective inhibitors of these proteins have become important pharmacological tools to study the cellular processes regulated by these proteins. These include cell cycle, cell death by apoptosis (cell death used by multicellular organisms to kill damaged cells), tumor development, smooth muscle contraction and certain neurological, metabolic and respiratory disorders. Therefore, thanks to the existing biotoxins in the Algae Blooms it is possible to obtain chemical components of future commercial interest, being its main users the pharmaceutical sector.

- The isolation and identification of Prorocentrum minimum from the bay of Callao was achieved.
- It was possible to clone cells of Prorocentrum minimum from unialgal cultures
- It was possible to construct trees at a molecular level to establish their identification and phylogenetic location, confirming the Pm5 species as Prorocentrum minimum and Pg as Prorocentrum gracile isolated from the bay of Callao.

6. Acknowledgments

This paper has been possible to carry out as research due to the need to obtain and generate knowledge from different professionals. The authors wish to thank our university mentors for their support and guidance.

References

Berdieva, M., Kalinina, V., Lomert, E., Knyazev, N. and Skarlato, S. (2020), Life Cycle Stages and Evidence of Sexual Reproduction in the Marine Dinoflagellate Prorocentrum inimum (Dinophyceae, Prorocentrales). J. Phycol., 56: 941-952. https://doi.org/10.1111/jpy.12989, 2020,

Cohen-Fernández, E., Pedroche, F., Rodríguez,M., Álvarez,S. & Meave del Castillo,E.(2010). Molecular phylogeny of Prorocentrum (Dinoflagellata) from the Pacific Coast of Mexico based on the parsimony analysis of fragments of LSUrDNA and SSUrDNA. J. Plant Physio and Biochem 2(3), 29-37, 2010.

Garduño-Solórzano, G., Rodríguez-Palacio, M. C., Martínez-García, M., Quintanar-Zúñiga, R. E., Lozano-Ramírez, C., Campos-Contreras, J. E. & Monsalvo-Reyes, A. C. Cultivos de microalgas del Lago de Catemaco, Veracruz. Rev Latinoam Biotec Amb Algal 2(2),67-80, 2011

Hoppenrath, M., Chomérat, N., Horiguchi, T., Schweikert, M., Nagahama, Y., Murray, S. Taxonomy and phylogeny of the benthic Prorocentrum species (Dinophyceae). A proposal and review. Harmful Algae 875,1-28, 2013.

Garduño-Solórzano G., Rodríguez-Palacio M.C., Martínez-García M. et al. Rev Latin. Biotec Amb Algal 2(2):67-80, 2011.

Alcira Córdova, Pedro Amaya, Doris Esenarro, Ciro Rodriguez, Vegetable Contamination by Heavy Metal Contained in Effluents from Wastewater Plant in the Totora Community, Ayacucho –Peru, *Journal of Green Engineering, Vol. 10_7, 3484–3497.* 2020.

Tamura, K., Peterson, D., Peterson, N., Stecher, G. & Nei, M; Kumar, S. MEGA: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol and Evol. 28, 2731-2739, 2011.

Doris Esenarro, Ivana Escate, Leslie Anco, Carla Tassara, and Ciro Rodriguez. Proposal for an Ecological Research Center for the Recovery and Revaluation of Biodiversity in the Town of Quichas-Lima, Peru. ¨

International Journal of Environmental Science and Development, ISSN 2010-0264, DOI: 10.18178, Vol. 11, No. 4. 212-216, 2020

Velikova, Violeta & Larsen, Jacob, The Prorocentrum cordatum/Prorocentrum minimum taxonomic problem. Grana Vol. 38. No. 2-3. 108-112. 10.1080/00173139908559219, 2008.

Kalinina, Vera & Berdieva, Mariia & Matantseva, Olga. The role of the cytoskeleton in the ecdysis of the dinoflagellate Prorocentrum minimum. Protistology. 14. 10.21685/1680-0826-2020-14-1-6, 2020.

Alvarado, K., Esenarro, D., Rodriguez, C., y Vasquez, W. Lemna minor influence in the treatment of organic pollution of the industrial effluents. *3C Tecnología. Glosas de innovación aplicadas a la pyme, 9*(3), 77-97, 2020.

Mariano A. Sal y Rosas Julca, Doris Esenarro, Godilia Teresa Garcia, Carmen Ruiz Huamán, Jorge Breña, A Mathematical Model to determine Mass and Energy Transfer in the Palm Oil Production Process, Journal of Green Engineering (JGE) Volume-10, Issue-9, September 2020