Heavy Metals contamination in two bioluminescent bays of Puerto Rico

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Abstract

Recent studies show that two important Bioluminesce Bays in Puerto Rico, La Parguera Bioluminescence Bay in Lajas and Puerto Mosquito Bay in Vieques have differences in the characteristic of bioluminescent. The presence of arsenic, copper, iron, lead and mercury can affect the *Pyrodinium bahamense* that lives in these bays with certain characteristics. The main purpose of this research was to evaluate the presence of heavy metals in these respectively bays. Samples were collected from four different environments in the selected bays, they were: sediments closed to the mangroves, mangroves leaves, sediments from the bottom and water column. This study indicates that the concentration of iron and copper in the two bioluminescent bays was high, arsenic, lead and mercury in most of the cases are Below Detection Limits (BDL). Iron and copper are important part of *Pyrodinium bahamense* growth because they are essential micronutrients for the formation of microorganism *phytoplankton* that the dinoflagellates consume.

Introduction

The Bioluminescence is the emission of light by microscopic organisms called *Pyrodinium bahamense* (Pyrrophyta- Dinoflagellata) (Figure 1). These dinoflagellates are a type of *phytoplankton* that absorb basic chemical elements directly from the water, take important roll in their abundance.

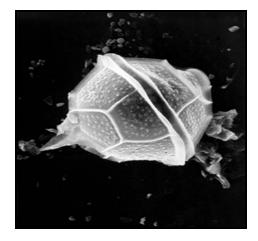


Figure1. Pyrodinium bahamense under an electron microscope. (Provided by Dr. Fernando Gilbes)

The main conditions aiding the accumulation of these organisms are: shallow basin with low tidal and narrow mouth and surrounded by mangroves. The mangroves and tidal flats export large quantities of nutrients into the bay and produce several organic substances (Figure 2). The accumulation of nutrients is also supported by a restricted flushing rate. These conditions promote the rapid growth of *Pyrodinium bahamense* populations. Alterations to these bays and or some areas of their watersheds can affect the equilibrium for a high bioluminescence. Dense blooms of dinoflagellates with extremely high bioluminescence occur sporadically in tropical latitudes around the world. In lower latitudes, as a result of many topographic and meteorological conditions, certain bioluminescent bays also exhibit persistent high concentrations of bioluminescent dinoflagellates (Walker, 1997).



Figure 2. Aerial photo of Puerto Mosquito Bay and Mangroves that surround the bay. (www. Franklyn.com)

Puerto Mosquito Bay and La Parguera Bioluminescence Bay are denominated as the most incredible bioluminescent bays of Puerto Rico, but La Parguera Bioluminescence Bay has shown a decrease in its bioluminescence by approximately 80% during recent years compared with Puerto Mosquito Bay (Walker, 1997). Previous works in San Francisco Bay, California, demonstrated that the sediment impute to the bay has been variable due to human activities. Human activities have a substantial impact on the hydrology characteristic of the bay. There are numerous potential sources of contamination. These include mining for Hg, Au, Cu; extensive agriculture use of pesticides for agriculture, untreated wastewater and variety of industrial activities (power plants, oil terminals and chemical, metal and paper facilities) (Hostetteler, 1999; van Geen and Louma, 1999).

The earth in terms of metals consists of four reservoirs: geosphere (solid earth), hydrosphere (streams, rivers, lakes groundwater and oceans), atmosphere (gases) and biosphere (living organisms). The atmosphere, biosphere and hydrosphere serve as temporary reservoirs, in which portent changes can occur for the metal. Plants and animals acquire metals by inhalation (gaseous form), ingestion (eating) and uptake of elements during plants growth. In the case of hydrosphere have been a major focus on this research, metals are added by magmatic fluids, weathering and dry and wet deposition. The transport and residence time of metals in the hydrosphere is a function of pH, oxidation – reduction and the presence clay minerals and oxhydroxides (Fe). (Eby, 2004)

The main purpose of this research was to evaluate the presence of heavy metals in the water column and bottom sediments of the selected bays. Previous works conclude that the intensity of bioluminescence in bays was used an indicator of the organisms health, the brighter the light produced, the healthier the organisms and vice versa. Another objective was to determine levels of heavy metals in the mangroves habitants around Puerto Mosquito Bay and La Parguera Bioluminesce Bay. Previous works in Singapore Bay demonstrated that there is a risk from environmental pollutants especially heavy metals that are associated with diverse range of anthropogenic activities. The accumulation of heavy metals in mangrove has been reported for a number of countries including Australia, Hong Kong and Brazil (Cuong,Bayane, Wurland and others, 2005). Using the characteristic of the bays and results, the last objective of this study was to identify the possible sources of the measured metals and their relationship with the bioluminescence intensity in both bays.

Study Area

The study areas are Puerto Mosquito Bay and La Parguera Bioluminesce Bay (Figure 3). Puerto Mosquito Bay is located in the south coast of Vieques island between 18° 6' N and 62° 26' W. It has a depth of 3.9 meters in the inner zone and 1.8 meters at the mouth. It has 76.4 hectares of Mangroves surrounding the bay. Vieques has two main bioluminescent bays: Puerto Ferro and Tapón Bay. But Puerto Mosquito Bay is the biggest with .65 km² a narrow entrance and surrounded by *Rhizophora mangle* and *Avicennia germinans* (Connelly, 1993). The bottom sediments are composed by white clay. The geology of Vieques consist in volcanic rocks with carbonate sediments.

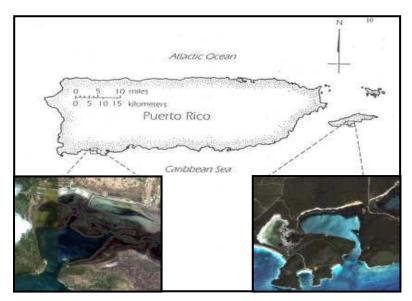


Figure 3. Study Areas (Adapted from Walker, 1997)

La Parguera Bioluminesce Bay is located at southwest of Puerto Rico between 17° 58' N and 67° 2'W and is approximately 4.5 meters deep in the inner zone and 2.3 meters in the mouth. (Seixas, 1988). The geomorphology of the area is the result of deformation of Limestone in early Cretaceous (Glynn, 1973). The bottom sediments are composed by fine mud, marine plants and mangrove leaves. (Margalef, 1961). Also the bay have high rate of evaporation because the arid conditions in the southwest of Puerto Rico.

Geology of the Area

Puerto Mosquito Bay and La Parguera Bioluminescence Bay have different geology. The geology of La Parguera Bioluminesce Bay is **Qm** - Areas containing extensive growth of mangroves. Underlain by fine sand and silt trapped by mangrove roots. **Kpl**- La Parguera Bioluminesce Bay Limestone- volcanic rock sand chert with minor serpentinite and Amphibolite ; chert clasts in the Cerro Vertero as much as 1 m; conglomerate grades upward to medium to thin bedded grayish-orange to pale yellowish brown volcaniclastic calcarenite locally containing glauconitic beds, thin beds of silicified mudstone, interbeds (to 10 m) of calcareous mudstone and sparse lenses of coarse grained light-gray bioclastic limestone which consist of skeletal debris including fragments of mollusks, solitary corals, and larger foraminifera; calcarenite grades (Figure 4).

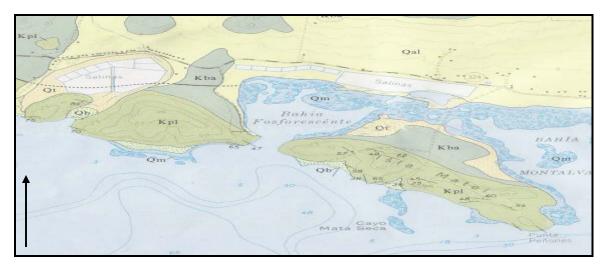


Figure 4. Geologic Map of La Parguera Bioluminescence Bay (Volckmann, 1984) (1:20,000)

Kba- Basaltic andesite- porphyritic basalt and andesite, phenocrysts of plagioclase and various combinations of clinopyroxene, orthopyroxene, hornblende, oxyhornblende, and rare olivine. **Qb-** Beach Deposits- Sand and minor gravel; consist of rounded fragments of shell debris, volcanic rocks, chert, and locally quartz. **Qal-** Alluvium- Clay, silt, sand and gravel in stream valleys; merges with colluvium along valleys walls and at a valley heads, form large fans which grades to the Caribbean sea, in the north east part of the area consist of sand and gravel fan deposits which grade into silt and clay of the Lajas Valley (Walkmann, 1984).

The geology in Puerto Mosquito Bay is **(QTu)** sedimentary deposits undivided marine limestone, calcarenite, sandstone, shale, marl, chalk, sand, clay and alluvial landslide beach, dune, swamp, marsh and reef deposit (Figure 5).

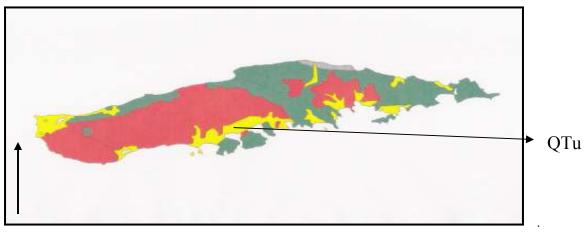


Figure 5. Geologic map of Vieques Island (USGS Maps)

Methodology

Samples of heavy metal were taken in Puerto Mosquito Bay and La Parguera Bioluminescence Bay. They were taken in 4 environments: the water column, bottom sediments, mangrove leaves, surrounding ground. The stations represented areas where we find the maximum concentration of the bioluminescent organisms. The sediments and water samples were collected during the same day in three stations; two inside the bay and near to the center and one in the narrow entrance.

The water samples were collected using a 500 ml bottle and the sediment from the bottom was collected using a sediment collector that extract sediments bottom. The mangroves leaves were collected in 4 onz. bottle (Figures 6a an 6b).



Figure 6a. La Parguera Bioluminescence Bay sample stations.



Figure 6b. Puerto Mosquito Bay samples stations.

The samples were analyzed by Environmental Quality Laboratories, Inc. (EQ lab) in Bayamón, PR. The laboratory analyzed five metals, using EPA protocols. They were mercury (Hg), lead (Pb), iron (Fe), copper (Cu), and arsenic (As). The samples were analyzed using the Inductively Coupled Plasma (ICP) method. The primary objective of ICP is to measure elements that emit specific wavelengths. ICP has the capability to identify and quantify all elements with the exception of argon (Bradford and Cook, 1997). The units provided for liquids were ppm and the method for metals was EPA 200.7, except mercury that was EPA 245.1. This last method includes analytical procedures for mercury by cold vapor atomic absorption (CVAA). This method is applied to ground, surface, sea and salt water, domestic and industrial wastes; also it applied to the determination of total mercury, organic plus inorganic. The units for solid were ppm and the method was 6010B except for mercury were EPA 7471A.



Figure 7. Sediments collector (left) and how the sediments were store (right).

Result

The study minerals have different uses for the organism for example, iron is a one of eight micronutrients in plants and it is essential for <u>chlorophyll</u> formation. Also, copper is a micronutrient and it is a component of some enzymes and vitamin A. But we have to know that also have different sources like anthropogenic activities and weathering. The EPA establishes a Maximum Concentration Level (MCL) for each metal and the sources (Table 1).

Element	Maximum Concentration Level (MCL)	Sources and Uses
Arsenic (As)	0.010 ppm	Anthropogenic activities such drainage mines, pesticides and natural sources as hydrothermal leaching or solution of arsenic-containing mineral in rocks. Erosion of natural deposits; runoff from orchards, runoff from glass & electronics production wastes
Copper (Cu)	1.3 ppm	Corrosion of household plumbing systems; erosion of natural deposits. Micronutrients in plants and it is a component of some enzymes and Vitamin A.
Iron (Fe)	0.3 ppm	Micronutrients in plants and essential for chlorophyll formation.
Lead (Pb)	0.015 ppm	Anthropogenic activities also derived from lead ore deposit and lead ore mineral is galena (PbS). Corrosion of household plumbing systems; erosion of natural deposits
Mercury (Hg)	0.002 ppm	Erosion of natural deposits; discharge from refineries and factories; runoff from landfills and croplands.

Table 1. Maximum Concentration Level of water content, sources and natural uses.

Four environments in La Parguera Bioluminescence Bay and Puerto Mosquito Bay was study: the water column, bottom sediments, mangrove leaves, surrounding mangroves ground. Each one has three stations and for this study we compare all station with the five metals, also each metals with the different environment station the selected bays. The concentrations of Heavy Metals in the selected bays were present in the following order: Fe>Cu>As>Pb>Hg. (Appendix 1)

Mangroves sediments stations showed higher concentration of Iron in La Parguera Bioluminescence Bay the first station 7466 ppm, second station 386 ppm and third station 4251 ppm with an average 5204 ppm compared with Puerto Mosquito Bay that had in 5456 ppm, 5975 ppm, 981 ppm and the 4137 ppm. Copper also had a significant concentration of 13.2 ppm, 5.95 ppm, 6.45 ppm with an average of 8.53 ppm in La Parguera Bioluminescence Bay. The average of 7.1 ppm in Puerto Mosquito Bay and the three stations shows 1.69 ppm, 2.39 ppm, and 2.18 ppm. Arsenic registered 5.01 ppm in La Parguera Bioluminescence Bay and Below Detention Limits (BDL) in Puerto Mosquito. Mercury showed in the first station 0.2 ppm and the others BDL in La Parguera Bioluminescence Bay instead Puerto Mosquito Bay that the station was BDL. Lead was detected in both bays also in the first station with 1.33 ppm La Parguera Bioluminescence Bay and 0.298 ppm in and Puerto Mosquito Bay, the other stations was BDL (Figure 8).

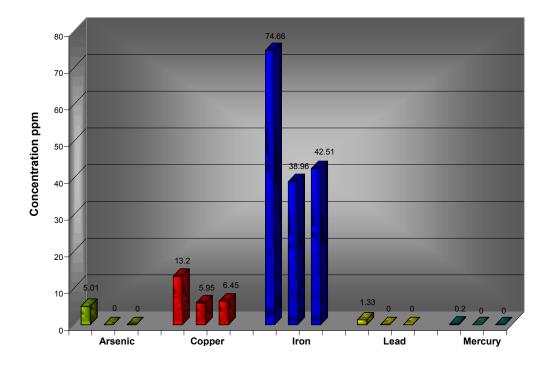
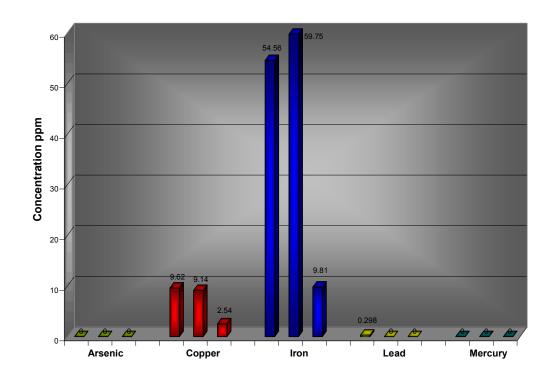


Figure 8 a and b. Comparisons Mangroves Sediments between La Parguera Bioluminescence Bay (Top) and Puerto Mosquito Bay (Bottom)



The second environment was mangroves leaves. In this case the mangroves demonstrate that the leaves absorb the nutrients of the sediments around the mangroves.

In La Parguera Bioluminescence Bay the concentration of iron was respectively 22.0 ppm, 5.70 ppm, 12.6 ppm with an average of 13.43 ppm compare with Puerto Mosquito Bay that shows 9.80 ppm, 44.0 ppm, 31.3 ppm and the average was 28.36 ppm. In the case of copper the concentration in La Parguera Bioluminescence Bay are 1.42 and Puerto Mosquito Bay has 2.08 mg/kg. The other metals lead, mercury and arsenic are Below Detection Limits (BDL) (Figure 9a and 9b).

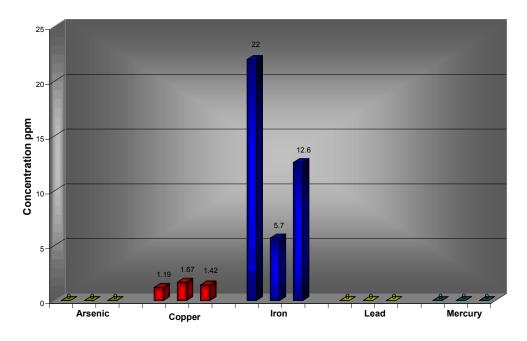
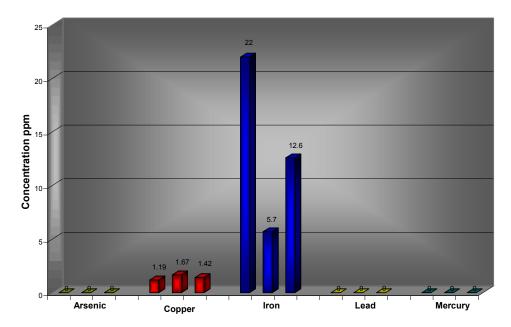


Figure 9. Comparison Mangroves Leaves between La Parguera Bioluminescence Bay (Top) and Puerto Mosquito Bay (Bottom)



The third environment is bottom sediments in La Parguera Bioluminescence Bay. Iron are present in the three stations 7136 ppm,7146 ppm,1804 ppm with an average of 5362 ppm instead Puerto Mosquito Bay than are Below Detention Limits also mercury, and lead.

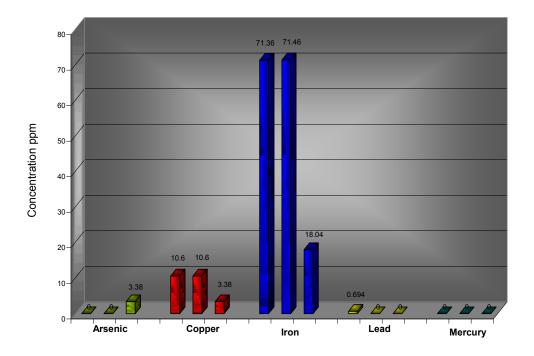
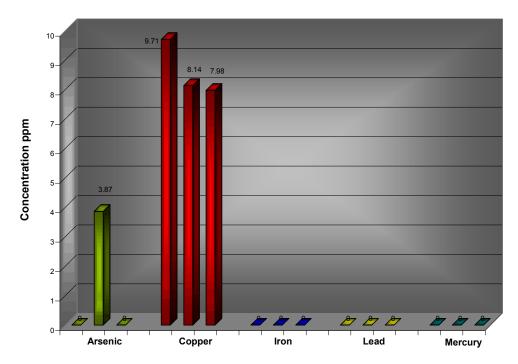


Figure 10. Comparison Bottom Sediments between La Parguera Bioluminescence Bay (Top) and Puerto Mosquito Bay (Bottom)



La Parguera Bioluminescence Bay has an average of copper 8.19 ppm and the stations has 10.6 ppm, 10.6 ppm, 3.38 ppm instead Puerto Mosquito Bay the stations shows 9.71 ppm,8.14 ppm,7.98 ppm and the average was 8.61 ppm. In La Parguera Bioluminescence Bay only the third station shows arsenic with 3.38 ppm, also Puerto Mosquito Bay show arsenic in the second station 3.87 ppm. La Parguera Bioluminescence Bay has lead in the first station with 0.694 ppm and mercury is Below Detention Limit.

The fourth environment is water column in this case we compare the two stations inside and one outside of the bay. The two station inside has a different results in the case of La Parguera Bioluminescence Bay there are presence of copper, the first station has 0.018 ppm, 0.017 ppm and the average was 0.18 ppm iron, lead, mercury and arsenic are Below Detection Limits. Puerto Mosquito Bay has a concentration of copper 0.019 ppm and 0.019 ppm and the average was 0.019 ppm and iron 0.06 ppm and 0.09 ppm and the average was 0.019 ppm and iron 0.06 ppm and 0.09 ppm and the average was 0.075 ppm. Arsenic and mercury are Below Detention Limits (Figure 11a and 11b).

The third station outside of the bays the results in La Parguera Bioluminescence Bay was iron 0.06 ppm, copper 0.017 ppm and lead, mercury, arsenic are Below Detection Limits. In Puerto Mosquito Bay the concentration of copper was 0.02 ppm and mercury, lead, arsenic and iron are Below Detection Limits.

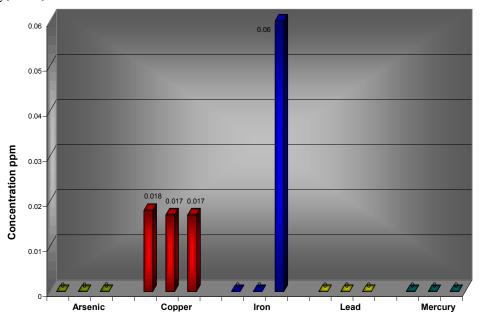


Figure 11a. Water Column Concentration in La Parguera Bioluminescent Bay

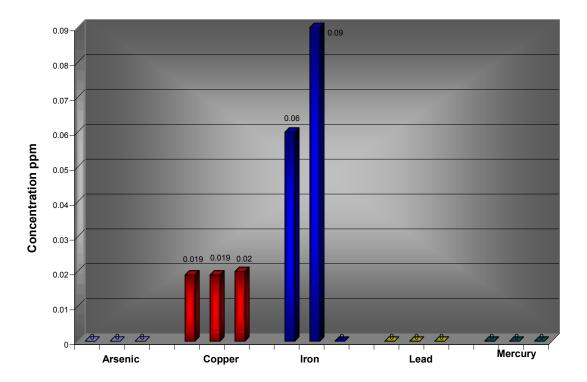


Figure 11b. Water Column Concentrations of Puerto Mosquito Bay

Discussion

Before starts the discussion of the result of this study is important to give a little explanation of how these metals interact with the environments and the organism that are present in the selected bays. The growth of marine plants is highly important since it provides the basic of the marine food chain which culminates in fish and marine mammals. During this photosynthetic process, which is known loosely as primary production the plants remove dissolved carbon dioxide and micronutrients from the water and using solar energy. The group of microscopic organism known as *phytoplankton*, accounts greatest production in the sea. Phytoplanktons are free flouting microscopic plants, which as they have only limited mobility are distributed by the ocean currents. The growth and distribution of marine plants is controlled by combination of many factors, which may by physical light, growth rate or interaction between organism and chemical and avaibility of nutrients or specific types of growth promoting substances. It is well known that the marine plants require for their healthy growth a number of trace elements, in addition to the micronutrients elements dissolved. These include iron,

manganese, zinc, copper and cobalt. Iron as a non –hem protein complex, called ferredoxin, an essential part in the reaction of photosynthesis. Copper complexes serve as co-factors in oxidation – reduction cycles. Using this fact the discussion will be focus in the interaction between metal (micronutrients) and the relationship with dinoflagellates that consume phytoplankton. (Riley and Chester1981).

The concentrations of Heavy Metals in La Parguera Bioluminescence Bay and Puerto Mosquito Bay were present in the following order: Fe>Cu>As>Pb>Hg. The fact that dinoflagellates consume the phytoplankton in the water column we can see La Parguera Bioluminescence Bay results high concentration of copper. Iron concentration does not have presence in the two stations inside the bay. The third station outside the bay has a high content of iron. This confirms the statement mention before, copper and iron is essential for dinoflagellates that produce bioluminescence. In Puerto Mosquito Bay the presence of iron was high in the two station inside the bay instead the outside station that are no presence. Suspended sediments affect the amount of light that penetrates the water column; therefore it is an important factor in the life of these organisms because of the need of light for photosynthesis.

In water column environment in these bays occur chemical interaction where fresh and salt waters mix, are among the most complex interactions in natural waters. Fresh water entering the ocean contains a variety of inorganic and organic particles. When mixing occurs between fresh and salt waters, there significant increase in ionic strength and adsorbed ions are often released to solution. Changes in salinity affect the equilibrium constants and changes in these constants bays role in the precipitation or resolution of metal containing solids. In addition, increase in ionic strength can lead the suspended sediments. In these case when the fresh water enters the marine environment, there often a rapid decrease in the amount of iron in solution (Nelson, 2004). The principal source of sediments is hydrology of the area that input sediments in to the bay (Figure13a and 13b).



Figure 13a. and 13b. Surface Hydrology and Elevation of La Parguera Bioluminescence Bay (left) and Puerto Mosquito Bay (right)



The accumulation of heavy metals in mangroves are present in this research and is associated with the capacity of the plants to uptake during they growth. This metals concentration we can attribute to the run off and the mangroves organic material or benthic organism. We can compare the two selected bay using two environments the mangroves ground sediments and mangroves leaves. In the obtain results we can see high concentrations of copper and iron that also present in the mangroves leaves. That confirm the factor that mangroves leaves are used as indicators of changing surface conditions for the capacity to uptake minerals and nutrients for the ground.

The bottom sediments record changing conditions also has been used to document dinoflagellates changes and activities. It important to recognize that sediment interactions can be change the sedimentary record by redistributing metals and the decomposition of organic matter as function of time. The bottom sediments in the case of La Parguera Bioluminescence Bay have iron, copper and arsenic. Puerto Mosquito Bay has high concentration of iron and shows presence of arsenic, there no explanation for this presence we recommend more studies, because there no presence in the other stations near too. Is important to recognize the geology of the area take important role for the distribution and impute of small amounts of metals by geological that may produce a detectable trace metals. Run off from the land is probably the principal sources that reach into the bay and the geology. These metals not only arrive to the bay in dissolved form also in the sediments input that become a suspend sediments. The enrichment of Fe, Cu, Pb, in the sediments of this bay was attribute to precipites formed introduced by solutions of magmatic origins also one of the sources that contribute to add iron to La Parguera Bioluminescence Bay was various combinations of clinopyroxene, orthopyroxene that can attribute to iron concentration. Natural sources as hydrothermal leaching or solution of arsenic-containing mineral in rocks, erosion of natural deposits. Lead anthropogenic activities also derived from lead ore deposit and erosion of natural deposits.

The fact that the presence of the metals that contribute bioluminescent affect positive is not a certain statement. Anthropogenic activities also contribute to reduce the bioluminescent. Houses surrounded the bay, the intensity of artificial light, dredging, the poor land planification in the near areas. Also the destruction of mangroves and the transit of boats have to affect the bay. Any other activity that may alter the drainage pattern of the mangroves or the land surrounding the bays also discharge of any type of effluents into the bays must be forbidden. Any minimum change in morphology of the shoreline, marine currents and increase levels of contamination can extinguish the bioluminescent produced by dinoflagellates.

Before do the analysis and see the result of this research we understand that the present of this heavy metal affect negative the two bays, but during this research we found that the presence of heavy metals like iron and cupper are important dinoflagellates development and are necessary for them bioluminescent. The concentration of metals depends the capacity of dinoflagellates to how they used. There no found significant differences in the metals concentration between bays, for that reason we can't conclude what causes the differences in bioluminescent. We suggest daily studies in the selected bay and use more metals that probable affect the bays.

Conclusion

In this research we evaluate the presence of heavy metals in Puerto Mosquito Bay and La Parguera Bioluminesce Bay. We collected samples from four different environments in the selected bays: sediments closed to the mangroves, mangroves leaves, sediments from the bottom, and water column. Also we determined levels of heavy metals in the mangroves habitants around Puerto Mosquito Bay and La Parguera Bioluminesce Bay. We identified the possible sources of the measured metals and their relationship with the bioluminescence intensity in both bays.

This study indicates that the concentration of iron and copper in the two bioluminescent bays was high. Arsenic, lead and mercury in most of the cases are Below Detection Limits. Puerto Mosquito Bay has high concentration of iron and shows presence of arsenic. We don't have explanation for this presence and provenance of this arsenic, for this reason we recommend more studies. There is no presence of arsenic in the other near stations.

The geology of the area takes important role for the distribution and impute of small amounts of sediments that may produce a detectable trace metals. Run off from the land is one of the principal sources that input sediments into the bays. Iron, copper, lead, mercury and arsenic not only arrive to the bay in dissolved form also in the sediments input that become a suspend sediments. Another factor contributes to reduce of the bioluminescent in the bays, one of them are the anthropogenic activities. During this research we found that the presence of heavy metals like iron and cupper are important to dinoflagellates development and are necessary for them bioluminescent.

We don't found significant differences in the metals concentration between bays, for that reason we can't conclude what causes the differences in bioluminescent. We suggest further studies with daily samples collections in the selected bays and to add others metals that probably affect the bays environments.

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Appendix 1

Arsenic					
Station #1Station #2Station #3					
Mangrove Sediments	5.01	BDL	BDL		
Mangrove Leaves	BDL	BDL	BDL		
Bottom Sediments	BDL	BDL	3.38		
Water Column	BDL	BDL	BDL		

La Parguera Bioluminesce Bay

Copper					
Station #1 Station #2 Station #3					
Mangrove Sediments	13.2	5.95	6.45		
Mangrove Leaves	1.19	1.67	1.42		
Bottom Sediments	10.6	10.6	3.38		
Water Column	0.018	0.017	0.017		

Iron					
Station #1Station #2Station #3					
Mangrove Sediments	7466	3896	4251		
Mangrove Leaves	22.0	5.70	12.6		
Bottom Sediments	7136	7146	1804		
Water Column	BDL	BDL	0.06		

Lead					
Station #1 Station #2 Station #3					
Mangrove Sediments	1.33	BDL	BDL		
Mangrove Leaves	BDL	BDL	BDL		
Bottom Sediments	0.694	BDL	BDL		
Water Column	BDL	BDL	BDL		

Mercury				
	Station #1	Station #2	Station #3	
Mangrove Sediments	0.2	BDL	BDL	
Mangrove Leaves	BDL	BDL	BDL	
Bottom Sediments	BDL	BDL	BDL	
Water Column	BDL	BDL	BDL	

Puerto Mosquito Bay

Arsenic					
Station #1Station #2Station #3					
Mangrove Sediments	BDL	BDL	BDL		
Mangrove Leaves	BDL	BDL	BDL		
Bottom Sediments	BDL	3.87	BDL		
Water Column	BDL	BDL	BDL		

Copper					
Station #1 Station #2 Station #3					
Mangrove Sediments	9.62	9.14	2.54		
Mangrove Leaves	1.69	2.39	2.18		
Bottom Sediments	9.71	8.14	7.98		
Water Column	0.019	0.019	0.020		

Iron					
Station #1 Station #2 Station #3					
Mangrove Sediments	5456	5975	981		
Mangrove Leaves	9.80	44.0	31.3		
Bottom Sediments	BDL	BDL	BDL		
Water Column	0.06	0.09	BDL		

Lead				
	Station #1	Station #2	Station #3	
Mangrove Sediments	0.298	BDL	BDL	
Mangrove Leaves	BDL	BDL	BDL	
Bottom Sediments	BDL	BDL	BDL	
Water Column	BDL	BDL	BDL	

Mercury				
	Station #1	Station #2	Station #3	
Mangrove Sediments	BDL	BDL	BDL	
Mangrove Leaves	BDL	BDL	BDL	
Bottom Sediments	BDL	BDL	BDL	
Water Column (mg/L)	BDL	BDL	BDL	

Appendix 2

