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Full Length Research Paper

Microbiological monitoring of water and *Crassostrea rhizophorae* in a mangrove ecosystem in Brazil

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Mangroves are considered as natural nurseries and classified as permanent protected areas. The study area is the municipal ecological site of Ilha do Lameirão, covering 891.83 ha of mangrove forest. This area persistently suffers from anthropic pressure. For this reason, there is the need to study and monitor the microbiological characteristics and recognition of the main factors involved in the degradation of mangroves process. This study was done in the year 2014. Samples were taken every month during dry and rainy seasons. Two areas (Maria Ortiz and Canal dos Escravos) were chosen based on the degree of their vulnerability to anthropic pressure. The results show there is a microbiological contamination both in water and *Crassostrea rhizophorae* tissues, mainly in samples taken from Maria Ortiz. According to the results, the Canal dos Escravos estuary, known as Class 1 is an environment that is able to maintain aquatic communities and fishing activities. In oyster tissues, microorganism strains that indicate the pathogens which cause diseases in humans were identified.

Key words: Environmental quality, microbiology, anthropic, pollution, oyster.

INTRODUCTION

Mangroves ecosystems maintain biodiversity offering the right conditions for feeding, reproduction and protection of several species and have an important role in nutrients and organic matter recycling (Schaeffer-Novelli, 1995). Over ecological aspects, mangroves provide direct and indirect benefits for population, especially for communities that use resources of those environments to survive (Wilson, 2002; Caffrey et al., 2016).

The transitional aquatic environment of Ecological Site Ilha do Lameirão suffers direct and indirect effects of

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water contamination; mainly because of illegal drains and domestic wastes (Moura et al., 2009). This contamination can cause the growth of fecal bacteria in the estuary waters that can exceed acceptable limits for drinking, recreation and irrigation (Brazil, 2005).

Together with mangroves root system, bivalve's mollusks of genus *Crassostrea rhizophorae,* generally known as mangrove oyster, build great communities both in the estuary edges and stuck to the substrate in the inner mangrove forest (Sroczyńska et al., 2012). Some of those mollusks are able to filter roughly 100 L of water per day (Suplicy, 2000); because they absorb toxins, pollutants and microorganisms (Corporeau et al., 2012), the concentration of biotic and abiotic elements in their tissues is a reliable indicator of environmental conditions.

Some cases of intoxication and microbiological contamination are associated with bivalves; recently, a particular attention has been given to oysters because of the habit to eat them in nature (Potasman et al., 2002; Iwamoto et al., 2010).

Concentration of pathogens in the estuary of monitoring can help you pick out certain parameters to protect public health and the environment. This study was conducted to assess the impacts of the release of domestic sewage and industrial waste under water and microbiological conditions of oysters from the mangrove forest on the island of Lameirão (EEMIL). The results of this work will provide basic information and a source of scientific knowledge on the levels of water contamination, and identify the main pathogens that cause food poisoning in oysters that are usually consumed in nature and can pose a risk to public health. The study will also help to generate a data base for fishermen, indicating the most suitable places for the collection of these organisms.

MATERIALS AND METHODS

Area of study

The Municipal Ecological Site Ilha do Lameirão (EEMIL), is placed in Vitória – Espirito Santo State - latitude 20° 14' S to 20° 17' S and longitude 40° 16' W to 40° 20' W.

Sampling points were chosen according to the class of vulnerability. The Maria Ortiz estuary (Points 1 and 2) shows a high or very high degree of anthropic pressure, mainly due to the closeness to neighborhood Maria Ortiz from that, probably, illegal drains discharge of domestic wastes (Tulli, 2007). The Canal dos Escravos estuary (Points 3 and 4) shows a low or very low degree of anthropic pressure, far from any possible drain of waste waters, and roughly 2.5 km from Points 1 and 2.

Water and oysters sampling

In the wet season samples were collected from January to March while in the dry season they were collected from June to August.

During each sampling and in each sampling Point 3 (three), water samples were manually collected, using 50 ml falcon tubes, at an approximate depth of 20 cm, in order to collect more than 20 ml of water, leaving an empty volume for a further shaking, before processing samples. Tubes filled up with samples were packed up in a thermic box for further analysis.

Oysters were manually removed from roots of trees found in the mangrove forest. 6 (Six) oysters with an average size of 8 cm were collected in each point (n=90). Oysters were placed in sterilized plastic bags with hermetic lock, and then stored in a thermic box for further processing and microbiological laboratory analysis.

Microbiological analysis

Microbiological analysis of water and oyster tissues was carried out within four hours from the field sampling. The chosen method was the most probable number per milliliter (MPN/mL) based on consecutive dilutions technique (APHA, 1970). The first step of this technique is a presumptive test in which there is the identification of lactose fermenting bacteria (through the Lauryl Sulfate Tryptose Broth). The second step is a confirmative test, recognizing the presence of total coliforms (through VB broth) and / or the presence of thermotolerant coliforms, through EC broth (APHA, 1970).

For the presumptive test, aliquots of 25 g of each sample were prepared and then diluted in 250 mL of 0.1% peptone water. Serial dilution was followed in tubes containing 9 mL of 0.1% peptone water up until 10^{-5}. Then, 1 ml of each dilution was transferred into tubes, in triplicate, containing Lauryl Sulfate Tryptose broth (LTS) and these were incubated for 24 to 48 h at 37°C. After incubation, tubes showing turbidity and gas production were selected for the confirmatory test. For this step, approximately 10 µL of each tube was transferred to a tube containing Brilliant Green Bile broth (VB) and a tube containing *Escherichia coli* (EC) broth. The tubes contained VB broth were incubated for 24 to 48 h at 37°C and the tubes with EC Broth were incubated for 24 to 48 h at 45°C. The tubes with turbidity and gas production were registered to calculate the most probable number, according to the table established by APHA (Morelli and Vieira, 2003; ISO, 2015).

Oyster microbiological analysis needs before the inoculation, the preparation of a solution containing bivalves tissue: Only after that, it is possible to carry out microbiological analysis (Whitman, 2004). Preparation steps were: Valves cleaning; shell detachment; weighing 25 g of bivalves tissue; tissues liquefaction; intervalvar liquids put into sterilized blender, then inoculated in a growth medium according to most probable number (MPN) method. To detect microorganisms indicating the presence or the absence of pathogens dangerous for food sanity, like *Salmonella*, *Shigella* and other bacteria, agar *Salmonella Shigella* (agar SS) and agar MacConkey (Himedia laboratories) growth media were employed, characterized by a medium selectivity and able to isolate those genera. Although culture media makers explain how to detect pathogens presence through colony color and smell, biochemical analysis of the material are required to confirm the results. The samples were subjected to bacterial analysis in the laboratory of Microbiology and Biotechnology of the University Vila Velha (UVV).

Data were analyzed by mean and standard deviation. All sampling data points were compared each other considering different sampling stations and they were analyzed by non-parametric tests Kruskal-Wallis and student's t-test, considering significant difference in $p < 0.05$ (Zar, 1999). Values were compared with water quality standard set by the national environmental Council (CONAMA) and for the quality of oysters RDC 12/2001 of the national health surveillance agency (ANVISA), which defines criteria and microbiological standards for foodstuffs.

RESULTS

Water microbiological parameters evaluation

Total coliforms values change significantly between each sampling point and within each different season (dry and wet) (p=0.01). In the Points 1 and 2 (localized in Maria

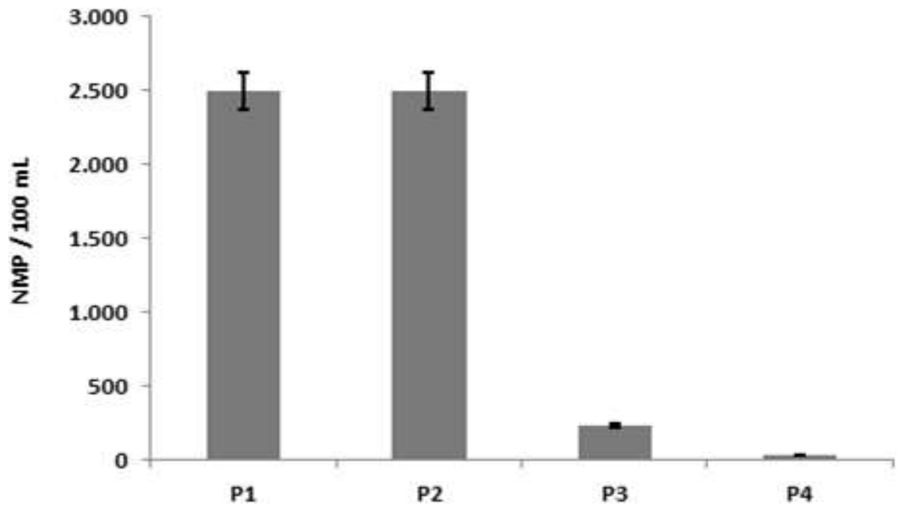

Figure 1. More probable number (MPN) of total coliforms per 100 mL in water (Y axis), in 4 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in rainy season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

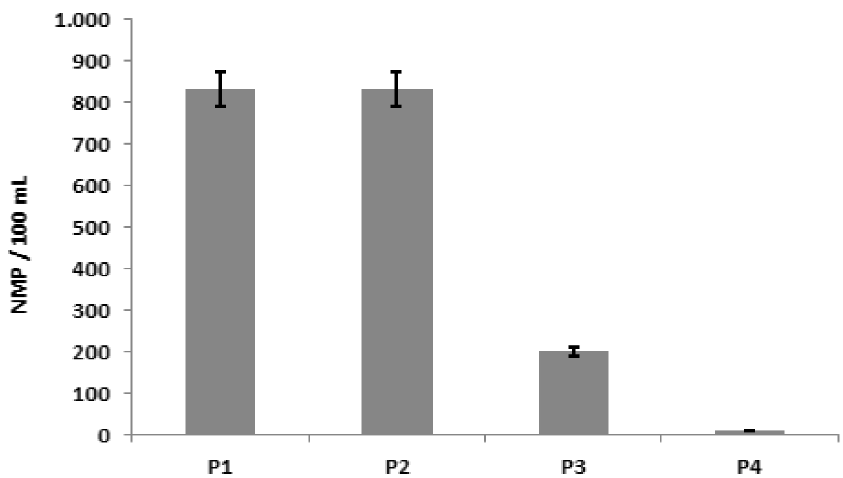

Figure 2. More probable number (MPN) of total coliforms per 100 mL in water (Y axis), in 4 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in dry season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

Ortiz area) values were greater than 2000 MNP/100 mL during the wet season and 800 MNP/100 mL during the dry season, while in the Points 3 and 4 localized in Canal dos Escravos area (characterized by a lower anthropic pressure) total coliforms values change from 210 to 28 MNP/100 mL in both seasons, respectively for Point 3 and 4 (Figures 1 and 2).

The results of thermotolerant coliforms quantification show significant changes between sampling points ($p=0.01$). During the rainy season, those values exceeded 2000 MNP/100 mL for the point 1 and remained up to 1100 MNP/100 mL in point 2 (localized in the area classified as affected by high and very high human pressure). During the dry season, these values decreased to a value over the 1100 MNP/100 mL for the point 1 and over 460 MNP/100 mL in Point 2. About Points 3 and 4 values did not change across the two seasons ($p \leq 0.95$), remaining over 210 MNP/100 mL for the Point 3 and over 28 MNP/100 mL in the Point 4 (Figures 3 and 4) but with significant differences between

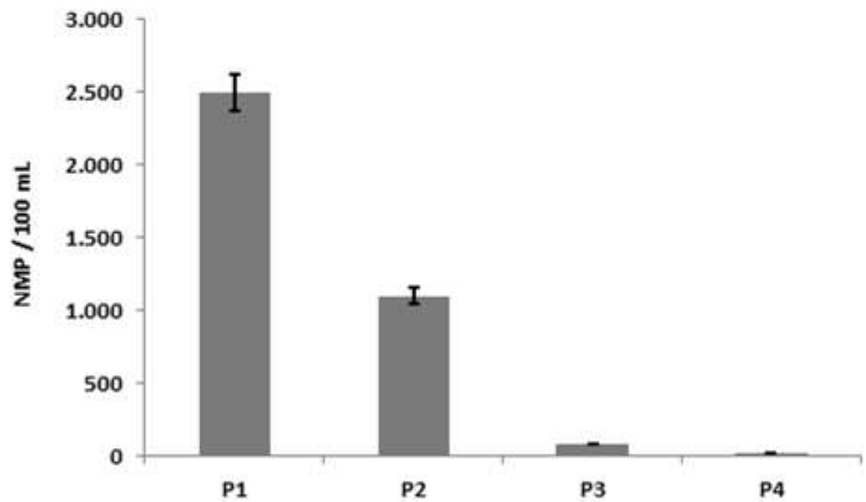

Figure 3. The most probable number (MPN) of thermotolerant coliforms per 100 mL in water (Y axis), in 04 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in rainy season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

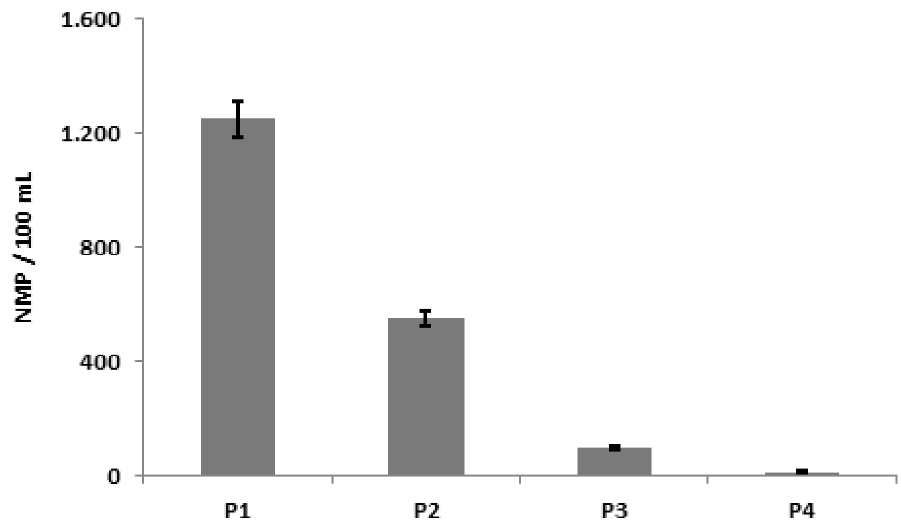

Figure 4. The most probable number (MPN) of thermotolerant coliforms per 100 mL in water (Y axis), in 04 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in dry season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

both points ($p=0.01$).

Evaluation of mangrove oyster microbiological parameters

Total coliforms values change between sampling points within the two evaluated seasons (dry and rainy) ($p \leq 0.002$). The MO point, localized in Maria Ortiz area, showed values greater than 600 MNP/100 mL during the rainy season and 1200 MNP/100 mL in the dry season (Figures 5 and 6), while in the CE point, localized in the Canal dos Escravos area (suffering a lower anthropic pressure), total coliforms values changed from 550 to 1200 MNP/100 mL in the both seasons (Figures 5 and 6).

As shown in Table 1, pathogenic strains like: *Salmonella* sp. (an amount of three strains found during

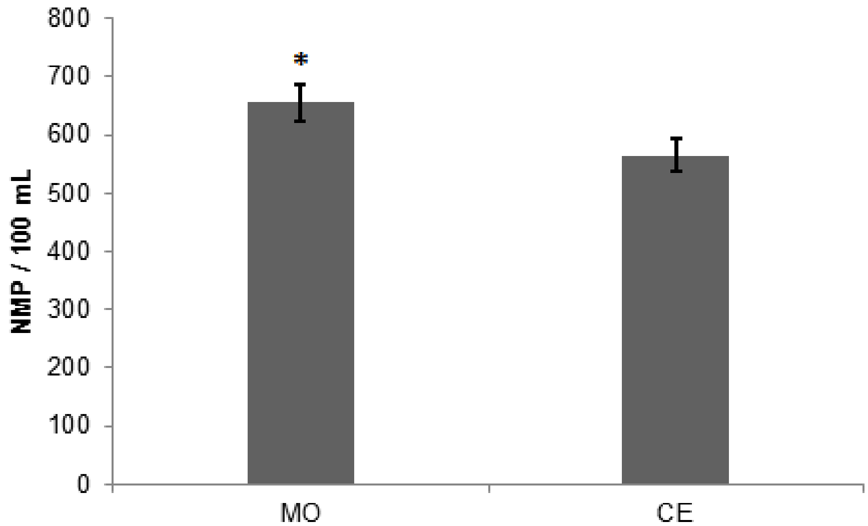

Figure 5. The most probable number (MPN) of total coliforms per 25 g of oyster tissue (Y axis), in two sampling areas (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, during the rainy season. MO corresponds to oysters collected in Maria Ortiz and CE corresponds to oysters collected in Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences accordingly to Tukey non paramentric test with a degree of confidence of 5%.

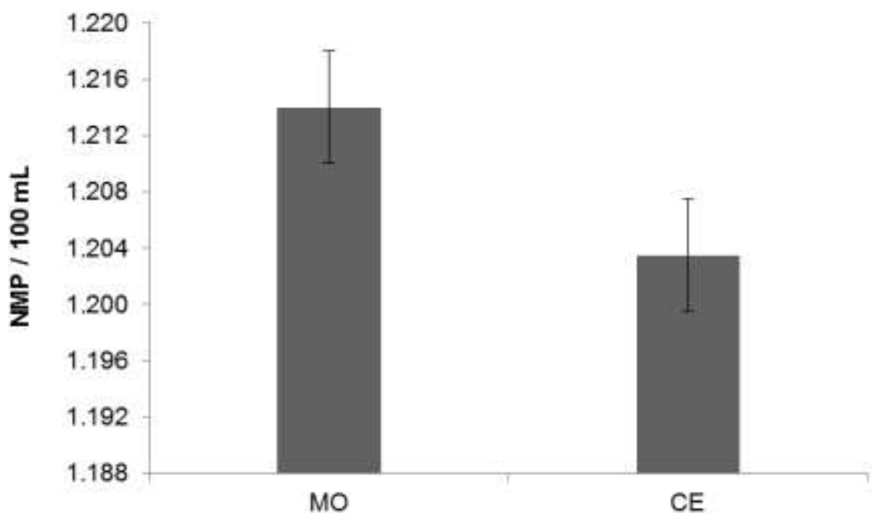

Figure 6. The most probable number (MPN) of total coliforms per 25 g of oyster tissue (Y axis), in two sampling areas (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in dry season. MO corresponds to oysters collected in Maria Ortiz and CE corresponds to oysters collected in Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences accordingly to Tukey non paramentric test with a degree of confidence of 5%.

February, April and June in MO area), *Pseudomonas* sp. (an overall of six strains found across all months in the MO area) and *E. coli* (an overall of eight strains: six found in the MO area and two in the CE area were detected). The types of bacterial were determined by serial dilution and plating of samples on differential culture media. The isolates were identified and characterized accordingly.

DISCUSSION

The CONAMA resolution nº 357/2005, characterizes the environmental classification according to the microbiological quality of the environment. The indices of total coliforms and thermotolerant found in the Canal dos Escravos are classified as Class 1 area (in which the

Table 1. Quantitative analysis for confirming presence or absence of strains with microorganisms indicating pathogens presence in oyster tissue.

Microrganism	MO	CE
Salmonella sp.	+	-
E. coli	+	+
Pseudomonas	+	-

-, Negative; +, Positive.

different uses of water are allowed for thermotolerant coliforms values not exceeding 1000 MPN/100 ml water). This class allows all activities which involve a direct use of water for protection of aquatic communities, aquaculture, fishing and supply for human consumption after standard treatment.

The values found in the estuary region of Maria Ortiz, allow you to classify it as Class 2, including the limit of 2500 MNP/100 ml. In this area in which activities are allowed sporadic or accidental direct contact with the water and the possibility of drinking water is very low as in navigation. In this estuary, the bivalves must not be collected for human consumption, mainly because it is an area that exceeds the limits allowed for the preservation of water aquatic communities and their maintenance in condition to be sold.

The high values of coliforms in Maria Ortiz can be related with the influence of anthropogenic pressure on the ecosystem. Human activities involving improper discharge of domestic waste are prevalent in coastal areas, negatively affecting the quality of water (Edun and Efiuvwevwere, 2012). High values of thermotolerant coliforms are often found in low quality organic matter enriched environments (Kolm and Miquelante, 2011). Evangelista-Barreto et al. (2014) assessed the microbiological characteristics of water and oysters from estuary of Maragogipe, Bahia, where they performed the analyses of total coliforms count, thermotolerants and isolation of *E. coli*. By comparing their results with the 357/2005 of CONAMA resolution allows the authors concluded that the environment in which the oysters were being drawn was with a high degree of contamination.

Severe or punctual pollution is a kind of environmental degradation and negatively affects the environmental and human health, reducing the human lifespan and exposing people to potentially dangerous pathogens. Health and quality of life are strictly related to the environment, since it is impossible to show conditions of good health, living in a degraded and polluted environmental plan (Komeily and Srinivasan, 2015).

Oysters as a bioindicator is efficient, being sensitive to the parameters evaluated through microbiological analysis of water from the estuary. The National Agency, in resolution Nº 12 of 2001 does not indicate parameters about the group of total coliforms and nor has assessment criteria for shellfish consumed in nature, considering only bivalve molluscs cooked or industrially chilled or frozen, that establishes a limit for thermotolerant coliform of 5×10/g. In accordance with the results presented in this work, Vieira et al. (2008) was able to assess the microbiological quality of the water habitat of oyster, the Pacoti River estuary, and succeeded in isolating 29 strains of *E. coli* samples collected. The presence of *E. coli* in fresh foods indicates fecal contamination and demonstrates environmental contamination by sewage drainage system.

In this study three strains of bacteria of the genus Salmonella were isolated, all from the region of Maria Ortiz, a fact very worrying, since the legislation requires total absence of this bacterium to any random sample of 25 g. Lee and Younger (2003) analyzed the United Kingdom coast oysters and realized that the presence of *Salmonella* was greatly influenced by the collection location. The influence varied according to the disposal of sewage and the type of agriculture performed in the region. These factors influenced directly the amount of bacteria present in the marine environment and therefore in oysters.

Bacteria of the genus *Salmonella* are Gram-negative bacteria and can be used as indicators of faecal contamination in samples of *Crassostrea rhizophorae* (Silva et al., 2004), and the consumption of these fresh oysters can be a serious health hazard, being responsible for cases of salmonellosis and colibacillosis (caused by *E.coli*) in the human population (Sanchez-Vargas et al., 2012).

Salmonella is a bacterium widely distributed in nature, being the main reservoir for these bacteria in the intestinal tract of humans and warm-blooded animals and cold. Molluscs and crustaceans are among the animals which pollute more easily with this type of bacteria (Rampersad et al., 1999). These molluscs are not capable of being used for human consumption from the point of view of health when compared to microbiological standards established by RDC n° 12 (Brazil, 2001). In America and Southeast Asia, salmonella is a common cause of gastrointestinal disease and accounts for about 1.4 million cases of infections annually (Ponce et al., 2008). These pathogens, as well as heavy metals and other toxic compounds can be bioaccumulated in the tissues of these organisms and be biomagnified along the trophic chain (Mok et al., 2015). Machado et al. (2001) suggest that the determination ofthermotolerant coliforms in soft tissues and intravalvular liquid, to assess the quality of the molluscs, produced for commercial purposes.

Conclusion

The high average of total coliforms and thermotolerant organism in the region of Maria Ortiz demonstrated that the level of proximity to urban centers and the degree of degradation is directly related to the microbiological quality of the environment, jeopardizing the life of the ecosystem. The results obtained from the analysis of the water support with the results found in the analysis of the tissue of oysters, which were found in pathogens microorganisms collected at the nearest region bivalve. The definition of management measures of harvesting areas of oysters and other bivalves can be optimized through data like this that allow the evaluation of environmental quality by using microrganisms indicators of brackish waters.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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