INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 5, p. 151-165, 2017

RESEARCH PAPER OPEN ACCESS

Environmental parameters and bacteriological quality of the *Perna perna* mussel (North East Algerian coast)

Kadri Skander*, Belhaoues Saber, Touati Hassen, Boufafa Mouna, Djebbari Nawel et Bensouilah Mourad

Laboratory of Ecobiology of Marine and Coastal Environments, Department of Marine Sciences, Faculty of Sciences, Badji Mokhtar University, Annaba, Algeria

Key words: Bacteria indicative of faecal contamination, North-East Algerian coast, Environmental parameters, *Perna perna* mussel.

http://dx.doi.org/10.12692/ijb/11.5.151-165 Article published on November 28, 2017

Abstract

Coastal waters are exposed to a growing sanitary problem originating from the waste water discharges related to the development of human activities; In light of the increasing use of coastal waters, we focused this study on the assessment of the level of contamination of the Perna perna mussel by bacteria indicating a faecal contamination (using colimetric method) and the effect of temperature, salinity, suspended solids and pH on the accumulation of these bacteria. Our results show that, throughout the year, all sampling mussels harboured bacteria indicating a faecal contamination; However, the high bacterial loads are recorded during the warm months and in the site of Sidi Salem. The strong presence of E. coli is observed in the mussels of Sidi Salem and Rezgui Rachid where contamination affects 100 and 91% respectively of the samples (faecal coliforms represent 50%, 41.6% and 25% respectively in Lahnaya, Cap de Garde and Laouinet). The presence of faecal streptococci is more marked in Sidi Salem where 100% of the samples of mussels contain more than 15 000 SF/100 ml of grindstone compared to only 83% in the other sites. Statistical analysis has clearly demonstrated the effects of the SS, temperature and salinity variables on the dynamics of the bacteria indicative of faecal contamination. The obtained results demonstrate the involvement of anthropogenic activity in the microbial contamination of the waters of the North East Algerian coastline and show that the mussel represents a good model for the evaluation of the bacteriological quality of coastal waters.

* **Corresponding Author:** Kadri Skander ✉ kadriskander@yahoo.fr

Introduction

Marine ecosystems are sensitive to anthropogenic pollution. According to Moore *et al.* (2013), the overall global burden of human disease caused by sewage pollution of coastal waters has been estimated at 4 million lost per person/year.

The discharge of wastewater in coastal areas is considered the main cause of degradation of bivalve molluscs growing areas (Oliveira *et al.* 2011).Bivalves such as mussels (*Perna perna*) are suspension feeders and may ingest viruses and bacteria bound to particles. Depending on several factors, including size, habitat and availability of feed organisms, an adult blue mussel (60 mm in shell length) may filter between 12 and 240 (mean 72) liter of water per day (Cranford *et al.*2011);therefore it has been proposed as a bio-samplers model for the assessment of faecal contamination in recreational waters (Roslev *et al.* 2010).Usually, bacteria including coliforms and enterococci are considered as good tools to assess the level of faecal contamination of water and shellfish (Oliveira *et al.* 2011) and they may be referred to collectively as Faecal Indicator Bacteria (FIB).

The accumulation of *E. coli* and other enteric bacteria is a physiological process regulated by many ecological factors, primarily by temperature (Kittner et Riisgård, 2005; Jozic *et al.*, 2012), but also by salinity (Rajesh *et al.*2001; Jozic *et al.* 2012), particle size (Dupuy *et al.,* 2000 ; Strohmeier*et al.* 2012)and concentration (Šolić *et al.,* 2007).Many authors have shown that concentrations of *E. coli* in bivalve molluscs growing areas depend on, among many other factors, seasonal and climatic conditions of the area (Campos *et al.* 2013b; Derolez *et al.* 2013; Lee and Silk 2013; Mignani *et al.* 2013).A possible explanation for seasonally values increase could be periodic transport of manure from farm animals, or overload of the sewage system in periods with high rainfall. A recent study has shown that rainfall can introduce large amounts of microbial contaminants, including viruses and bacteria related to human health concern, to rivers and coastal areas (Campos *et al.* 2011; Schernewski *et al.* 2014).

According to Lunestad *et al.* (2016), sampling intervals should take in consideration the information related to the variation for bacterial faecal indicators, local knowledge on possible exposure to faecal material from livestock or humans, rainfall seasons, topography of the location, as well as tidal and water current patterns.

The faecal contamination problem of Algerian coastal waters has not been deeply studied and associated health risks are not negligible in light of the increasing use of coastal waters in the last years (bathing, recreational beaches, shellfish consumption, shellfish productions sites). Studies related to bacteriological quality of coastal water have been recently conducted (Kadri *et al.* 2015; Benhalima *et al.* 2015). However, the effects of environmental factors on the accumulation of enteric bacteria in mussels (*Perna perna*) harvested on the easthern coastal areas of Algeria have not been investigated yet.

The aim of this study is to firstly assess the bacteriological quality of mussels by providing levels of total and faecal coliform in addition to levels of total and faecal streptococci, and secondly, to identify the effect of some environmental factors on the abundance of bacteria mentioned above.

Material and methods

Sampling area

The study area represents the northeastern part of the Algerian coastline which extends from the Cap de Garde in the West to Cap Segleb in the East (Fig.1). Sampling is carried out at sites located in peri-urban areas (S1 "Cap de Garde" (36°57'59.94"N-7°47'38.30"E), S2 "Rezgui Rachid" (36°55'2.68"N-7°46'4.83"E),S3 "Sidi Salem" (36°52’18.13"N-7°46’8.32"E) and sites located in rural areas (S4 "Lahnaya" [36°53'6.59"N 8°4'8.70"E], S5 "Laouinet" (36°54'26.89"N-8°30'30.68"E).

Sample processing

The bivalve *Perna perna* mussel is used as a bioindicator in this study.

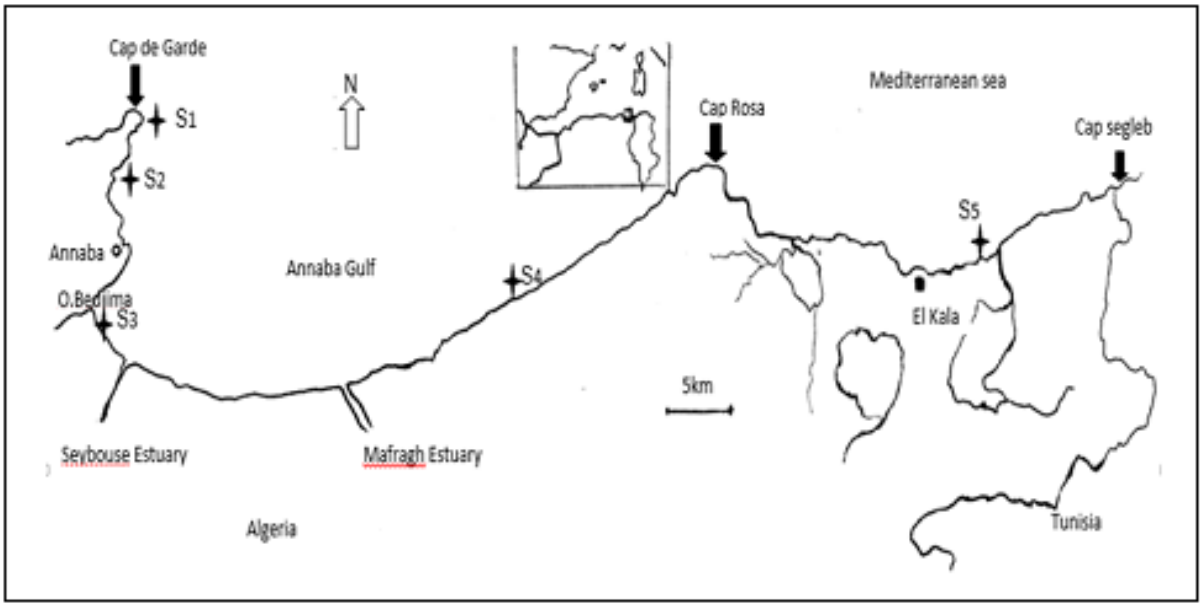

Fig. 1. Location of sampling areas.

The *Perna perna* mussel is present in the five sites mentioned above. The mussel is harvested by hand at a rate of 15-20 individuals (depending on size) per month and per site. Mussel samples are placed in clean and labeled freezer bags (date, site, species) and transported to a cooler maintained at a temperature between 4°C and 10°C (CFIA 2004) on the same day.

Bacteriological analyzes consist in collecting and counting the coliforms by liquid colimetryin triplicate: from 3 series of 3 tubes (standards V 08-020 (1994)/ISO 7251 and V 08-021 (1993)/ISO 7402) and the calculation is done using the most probable number (NPP) method according to the Mac Grady table to obtain the 95% confidence intervals for each value. As for the identification of pathogenic bacteria, the use of a biochemical mini-gallery Api 20E is recommended in accordance with standard AFNOR V 08.013.

In parallel to each sampling of the mussel, temperature (T), pH, salinity and dissolved oxygen of the water in situ were measured using a multiparameter (Consort 535).

The determination of the suspended solids in water is carried out by the application of the differential weighing method according to Aminot and Chaussepied (1983).

Statistical analysis

A principal component analysis (PCA) was carried out, using a specialized package called Facto Mine R (Husson *et al.* 2014), to characterize the structuring of the five sampling sites and to highlight the contribution of the environmental parameters measured on the abundance of the counted germs. All packages used are downloaded from the official website of CRAN (The Comprehensive R Archive Network).

Results

Total coliforms

The harvested mussels from all the Harbor sites, throughout the year, showed total coliforms(TC) levels clearly exceeding the guideline values set at 1000 germs /100 ml of ground wood (according to the European directive of 8 December 1975, taken over by the decree Executive Order No. 91-980 of 20 September 1991).

The lowest level (600 germs / 100 ml of broth) is reported in Laouinet in February (Fig.2).

Thermotolerant Coliforms

Thermotolerant coliforms are found at concentrations higher than the reference value (230 germs /100 ml of shredder according to the European decree of 23

March 1993) during 12 and 11 months of the study cycle in mussels harvested in Sidi Salem and Rezgui Rachid respectively. The samples of mussels with *E. coli* loads exceeding the guide value are 6, 5 and 3 in Lahnaya, Cap de Garde and Laouinet respectively (Fig. 3).

Total Streptococci

Mussels from all sites show a high level of contamination by total Streptococci (TS) which is usually found to be close to 140 000 germs/100 ml of groundwater and largely exceeding the guideline value (European Parliament of 23 March 1993).

Table 1. Result of presumptive tests intended for the research and identification of bacterial pathogens in bivalves (- absence, + presence).

Bacteria	*Staphylococci*				*Clostridium*						*Salmonella*					*Pseudomonas*				
	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
January	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
February	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-
March	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+
April	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+
May	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
June	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+
July	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
August	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-
September	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+
October	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-
November	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
December	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-

S1: Cap de Garde; S2: Rezgui Rachid; S3: Sidi Salem; S4: Lahnaya; S5: Laouinet.

In addition, we recorded levels of less than 3000 germs/100 ml of ground wood in January in Rezgui Rachid, Lahnaya and Laouinet (Fig. 4).

Faecal Streptococci

Mussels from all the sites generally present faecal streptococcal (FS) contents higher than the reference value fixed to 2.5 10^3 germs/100 ml of ground material (according to the European decree of 23 March 1993).

Grades of less than 2500 germs/100 ml of ground wood are recorded in January at Cap de Garde, Rezgui Rachid, Lahnaya and Laouinet.

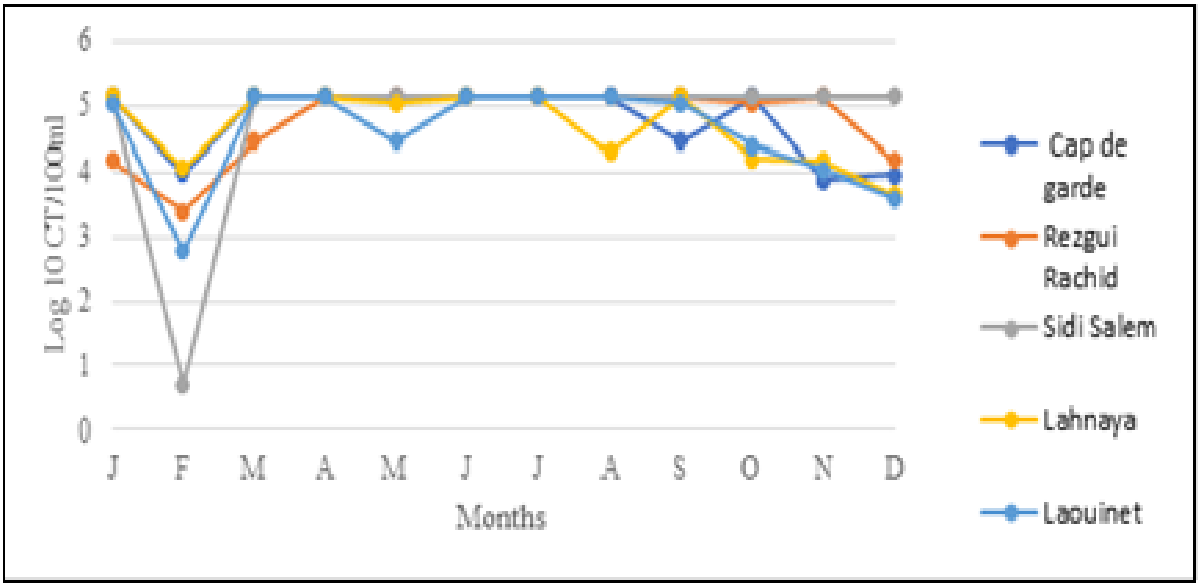

Fig. 2. Spatio-temporal variations in total Coliform contents.

Loads of the order of 600 and 1500 germs/100 ml of crushed) are noted respectively in Laouinet (in February) and Lahnaya (in May) (Fig. 5).

Pathogenic germs in mussels

The presumptive tests result to identify 4 pathogenic bacteria show the presence of *staphylococci*, *Salmonella* and *Pseudomonas* (Table 1); *Clostridium* is not present in the analyzed mussel samples. Staphylococci are present in 28% of the mussel samples; Only the mussels harvested in July, August and September show no contamination by staphylococci. Their presence is limited to two samples at Cap de Garde and Laouinet, three samples at Lahnaya and five samples at Rezgui Rachid and Sidi Salem.

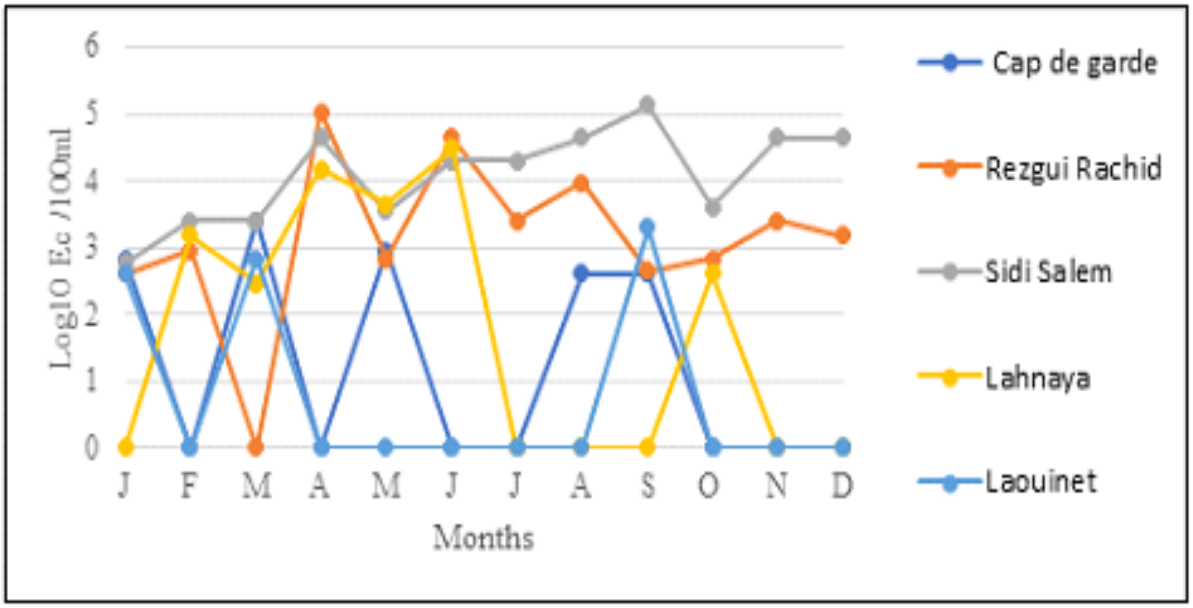

Fig. 3. Spatio-temporal variations in the contents of Thermotolerant Coliforms (*Escherichia coli*).

The presence of *Salmonella* is spread over a period of 8 months and concerns 23% of the samples analyzed. A single sample from Lahnaya and Laouinet, two samples from Cap de Garde, four samples from Rezgui Rachid and six from Sidi Salem were reported to be contaminated by salmonellae. Only mussels harvested in January, May, July and November do not show presence of *Salmonella*.

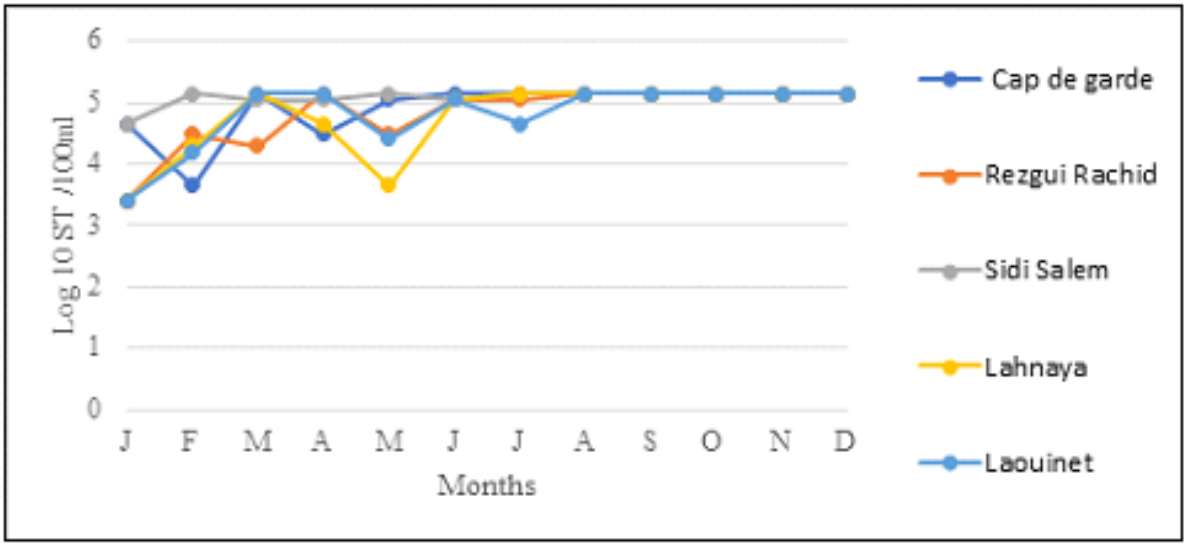

Fig. 4. Spatio-temporal variations in total *Streptococcus*.

The presence of *Pseudomonas* is noted in 41% of the mussel samples and is spread over 10 months. This bacterium is encountered, in 4 samples of the Cap de Garde, in 5 samples of Rezgui Rachid, Lahnaya and Laouinet and in 6 samples of Sidi Salem; Only the samples of mussels of January and December are spared by the *Pseudomonas*.

The confirmatory tests allowed to demonstrate the presence of *Citrobacter frundi*, *Enterobacter* and *Proteus vulgaris*; Bacteria of the genus *Salmonella*, *Pseudomonas*, and *Clostridium* appear to be absent in the mussels of all sites.

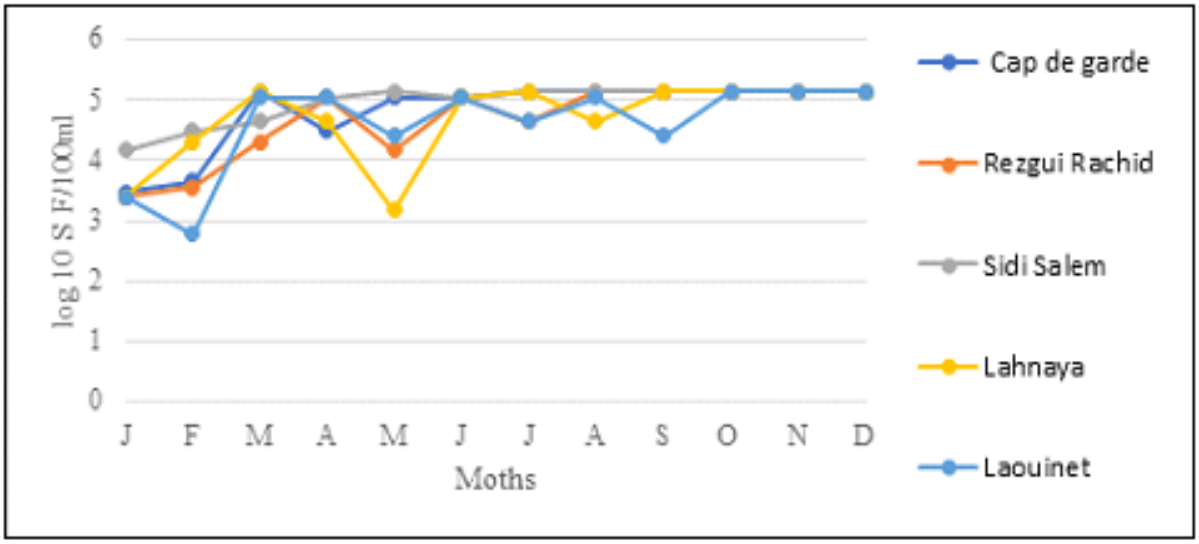

Fig. 5. Spatio-temporal variations of fecal *Streptococci*.

Characterization of the water at each site

The waters of the Cap de Garde are characterized by the highest salinity (S) and the lowest suspended solids (SS) levels; With regard to the temperature and dissolved oxygen, the values recorded are relatively low (Fig.6). In Rezgui Rachid, the salinity and water SS values show values quite similar to those found at Cap de Garde; The temperature is quite high and the contents of dissolved oxygen are low (Fig.6). It is in Sidi Salem waters that the lowest salinity and temperature but the highest SS levels are recorded; The dissolved oxygen contents are quite good (Fig.6). The waters of Lahnaya have a low salinity and temperature, the highest dissolved oxygen contents and SS levels close to those in Sidi Salem (Fig. 6). In Laouinet, the levels of SS are as high as those found in Sidi Salem; the salinity is quite high; as for dissolved oxygen and temperature, they show the lowest values (Fig. 6).

The first two main components (Plan 1-2, Fig.7) of the CPA carried out on the five environmental variables resulted in almost 71% of the information (inter-site variability). The counts of the different types of germs were used as additional quantitative variables.

Axis 1 accounts for 38.45% of the total variability; This axis is correlated negatively with the variable T ($r = -0.97$, $\cos^2 = 0.95$), total coliforms ($r = -0.92$, $\cos^2 = 0.84$), fecal coliforms ($r = -0.83$, $\cos^2 = 0.68$), total streptococci ($r = -73$, $\cos^2 = 0.54$) and fecal streptococci ($r = -0.80$; $\cos^2 = 0.64$); it is positively correlated with the SS ($r = 0.81$, $\cos^2 = 0.65$).

Axis 2 accounts for 32.41% of the total variation; it is constructed essentially by the variable S and to a lesser degree by the variable pH (respectively $r = 0.83$, $\cos^2 = 0.68$ and $r = 0.74$; $\cos^2 = 0.54$). On the negative pole of axis 2, dissolved oxygen is projected which contributes weakly to its construction ($r = -0.54$, $\cos^2 = 0.30$).

On the negative pole of axis 1 are projected sites Cap de Garde and Rezgui Rachid because their waters are

characterized by high salinity and low SS; As regards the Sidi Salem site, its waters are characterized by a the lowest salinity. The projection of the Lahnaya site on the positive pole of axis 1 is illustrated by the presence of high SS contents and a low temperature; As for Laouinet, its waters show high SS and the lowest temperature.

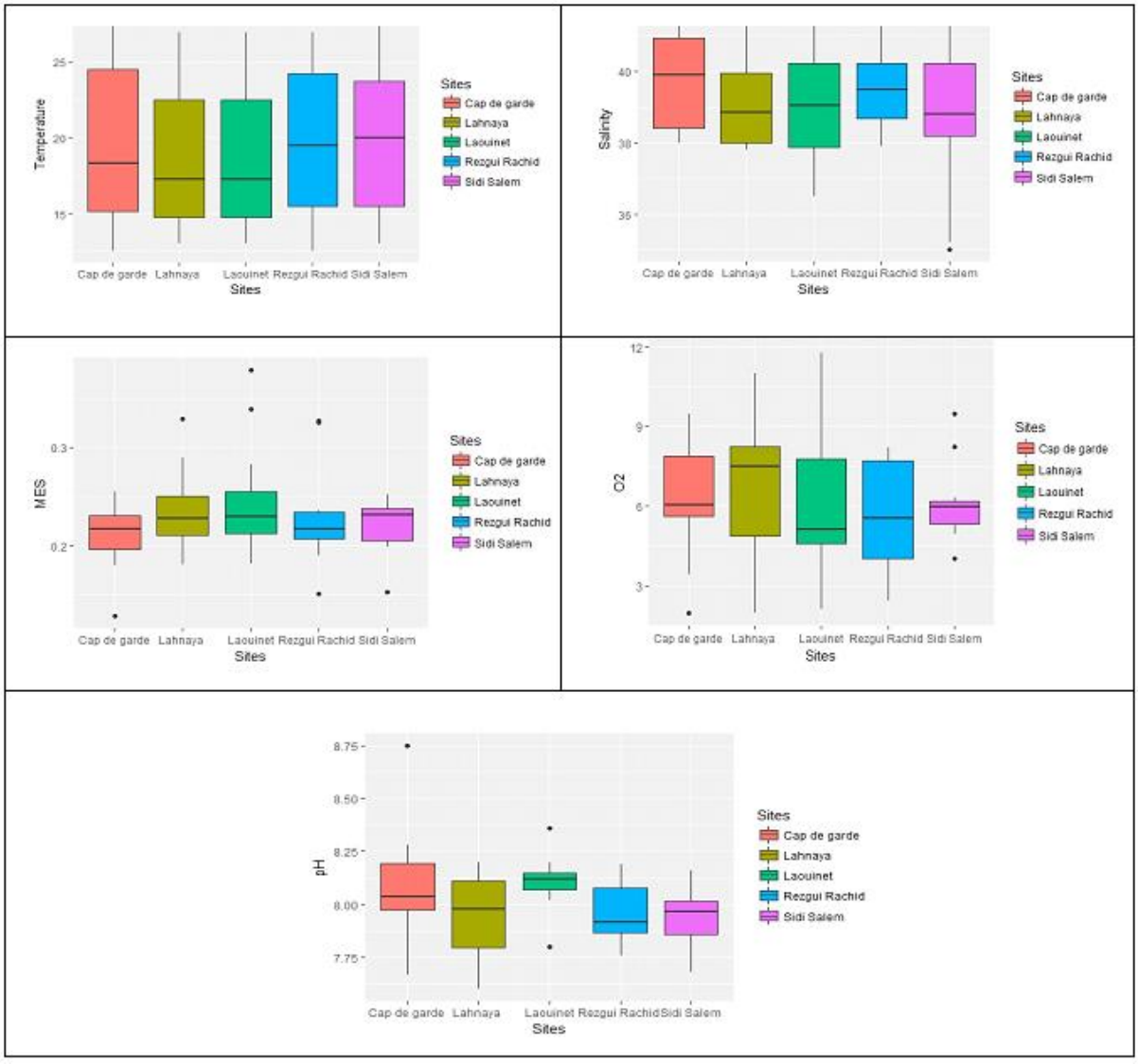

Fig. 6. Physico-chemical parameters of the Algerian North East coastal waters.

Analysis of the variation in measured environmental parameters shows existence of dissimilarities between the five sites. Depending on the Euclidian distance matrix, we can distinguish a first group including sites S4 and S5,a second group including sites S2and S1and finally a third group represented by site S3(Fig.9).

Discussion

The results of this study show that the presence of indicators of faecal contamination varies from one site to another and from one month to another depending on the inputs and the intrinsic quality of the environment.

The results of the statistical analysis reveal very highly significant differences in the distribution of *E. coli* and TC, TS and FS between sites and between months. The results of the PCA show that the first axis has clearly demonstrated the effects of the SS and temperature variables on all the bacteria indicative of fecal contamination; As for axis 2, it is essentially constructed by the variable salinity.

The influence of temperature on these germs is illustrated by their low presence in all sites during the winter period. The temperature plays a major role in the physiological status of bivalves because these organisms are ectothermic (Bayne, 1976). In bivalves, the accumulation of *E. coli* and other enteric bacteria is a dynamic process that, according to Kueh (1987), depends on the rate of filtration, bacteria concentration in the ambient water and filtration efficiency of the gills.

Several studies have shown that this physiological process is mainly regulated by the temperature (Haure *et al.* 1998; Kittner and Riisgård 2005; Jozic *et al.* 2012), but also by the salinity (Cole and Hepper 1954; Rajesh *et al.*2001; Jozic *et al.*2012), the size of theparticles (Tames and Drall 1955; Dupuy *et al.* 2000; Zhang *et al.*2010; Strohmeier *et al.* 2012)and the concentration of these particles (Shulte 1975; Šolić *et al.* 2007; Leight *et al.* 2016).

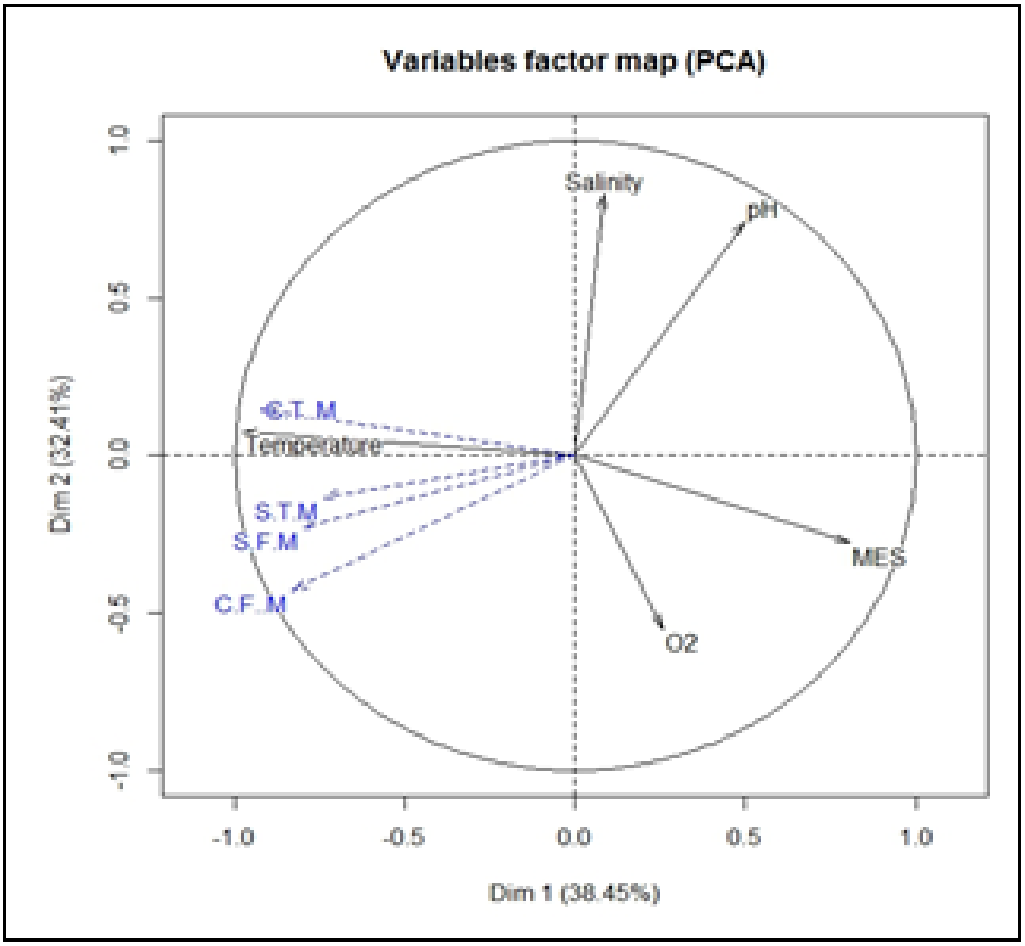

Fig. 7. Circle of correlations of environmental variables with the first two axes of the standardized ACP.

The strong presence of thermotolerant coliforms is observed in the mussels of Sidi Salem and Rezgui Rachid where contamination affects 100 and 91% respectively of the samples. In the other sites, samples of mussels harboring faecal coliforms represent 50%, 41.6% and 25% respectively in Lahnaya, Cap de Garde and Laouinet. As regards the seasonal distribution of faecal coliforms, the highest loads are recorded in spring in Rezgui Rachid and summer in Sidi Salem; It is the Sidi Salem mussels which show the highest FC loads. The relative low salinity observed in Sidi Salem and Rezgui Rachid would explain the abundance of total coliforms at their level; these results are in agreement with the observations of Bordalo *et al.* (2002) who observed that in water with low salinity there is a greater survival of the germs indicating faecal contamination.

The high bacterial loads recorded by *Perna perna* during the warm months would be related in part to the physiology of the species. Similar observations show that during the summer months, when water temperature and salinity conditions are adequate, bivalves increase their filtration capacity and thus can retain much more microorganisms (Fahim 1990; Zegmout *et al.* 2011).

According to Bernard (1989), the highest accumulations of coliforms are recorded at 17°C. These findings confirms the result of this study, because the waters of Sidi Salem are characterized by median temperatures of this order and the mussels present in this site show the highest coliform loads. Salinity would have as much influence as temperature on the indicators of faecal contamination in shallow marine waters (Mill *et al.* (2006). Still in *Mytilus edulis,* Shulte (1975) reports that at salinities of the order of 38psu, the highest filtration rates take place at the optimum temperature, which varies from 15 to 25°C. Other studies show that the highest filtration rates occur at the optimum temperature and that their decline occurs at high and low temperatures (Laing 2004; Šolić *et al.* 2007).

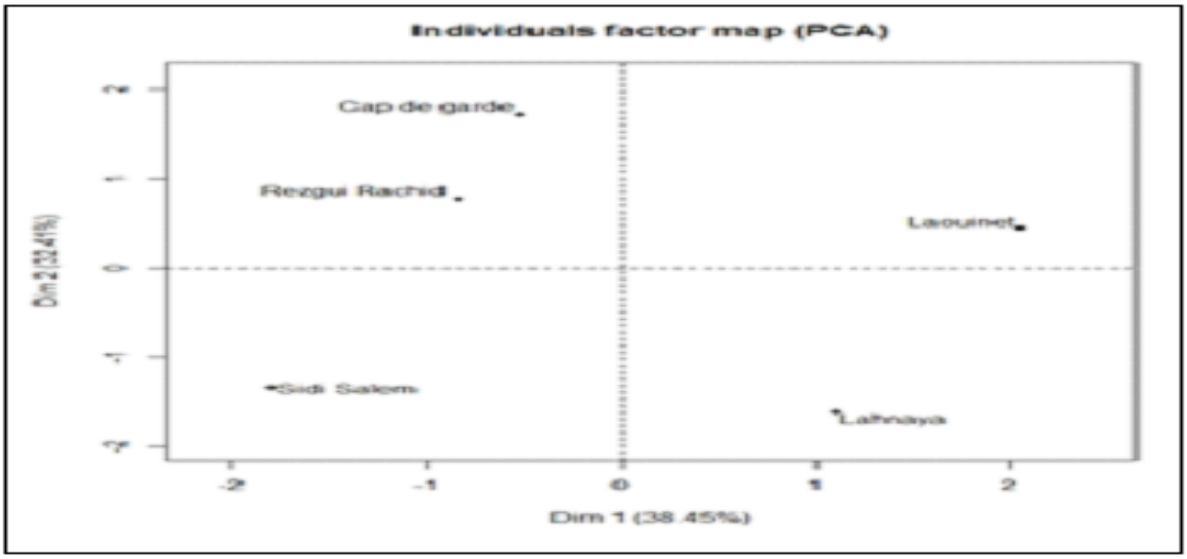

Fig. 8. Projected sites on the first two main axes of the standardized PCA.

In the *Perna viridis* mussel, in the range 10 to 32 psu, Rajesh *et al.* (2001) found a positive correlation between salinity and filtration rate. Jozic *et al.* (2012) reported the existence of significant correlations between *E. coli* concentrations in water and bivalves; these results clearly demonstrate that the retention capacity is controlled by the temperature and the salinity of the water. These same authors note that the interaction between temperature and salinity is statistically significant, suggesting that their simultaneous action would be significantly greater than when each parameter acts alone.

Study performed by Craig *et al.* (2004) demonstrated that the disappearance of *E. coli* is faster in water than in the sediment and that the small particles and the important organic matter contained in the coastal sediment would increase the survival of this germ and would promote the presence of high bacterial loads in the bivalves that accumulate them. This seems to be the case for the SS rich waters of Sidi Salem and Rezgui Rachid, which would contain large amounts of FC. It is reported that SS play a protective role in the germs that indicate fecal contamination against UV and predators; according to Yukselen *et al.* (2003), the UV is the most important factor influencing the survival of coliforms. Increased microbial load in water and sediments can also be a source of contamination of bivalves. In a study conducted in the Marennes-Oléron basin, Hearel and Prou (1980) reported that in addition to summer activity, increased bacterial load during warm months would

depend on phytoplanktonic blooms; the latter may increase the organic matter content. Many authors reported that the release of certain germs into water from sediments or plankton occurs only when the temperature of the medium is high (Gauthier and Clement 1979; Oneil *et al.*1990).

Faecal streptococci are present in all samples of mussels; Their presence is more marked in Sidi Salem where 100% of the samples of mussels contain more than 15 000 SF/100 ml of grindstone compared to only 83% in the other sites. As for the seasonal dynamics of faecal streptococci, it is similar to that of total streptococci (autumn> summer> spring> winter).

Their continued presence at very high levels, exceeding the tolerated threshold, can be explained by the resistance of these pollution indicators (Gleeson and Gray 1997) and also by evidence of possible old contamination (Noble *et al.* 2004). Many authors note that concentrations of faecal streptococci are weakly influenced by pH changes and salinity (Bennani *et al.* 2012; Chigbu *et al.* 2005; Garrido-Perrez *et al.*, 2008). These observations are in agreement with our results, which show that the streptococcal loads are as important in Cap de Garde where the salinity is the highest as in Sidi Salem where the salinity is the lowest.

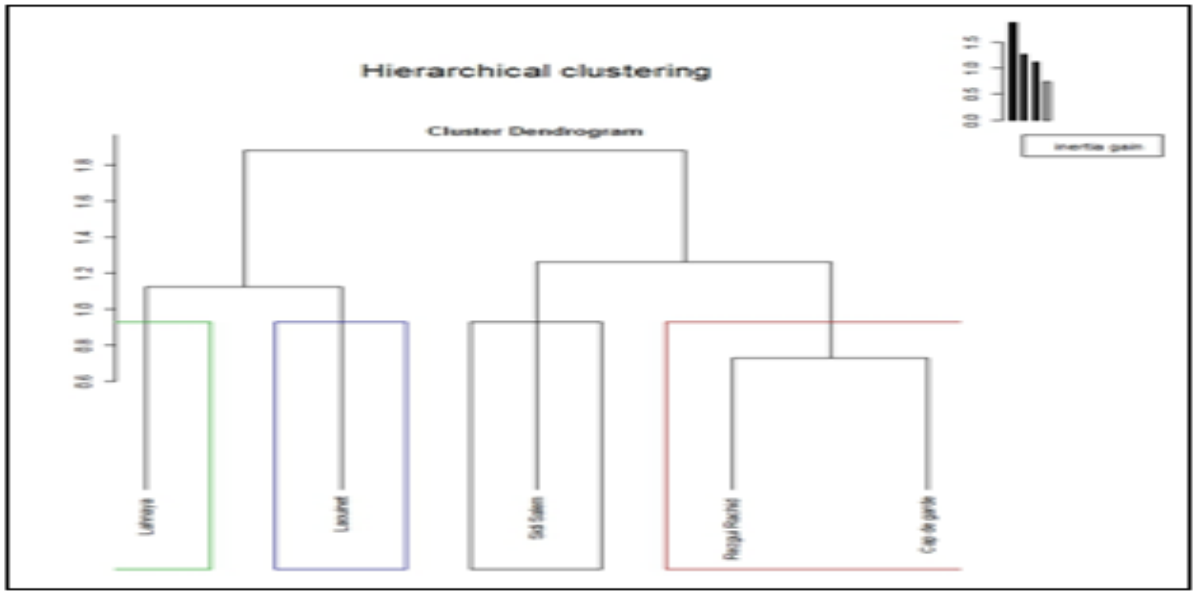

Fig. 9. Hierarchical classification of sites.

In their study of the concentration of bacteria by *Chamelia gallina,* Zegmout *et al.* (2011) showed that the different groups of microorganisms are not accumulated in the same way; Praires concentrate FS much more effectively than FC. Also, it is likely that the FC would then be easily digested by bivalves or eliminated more quickly. Several authors have reported that enterobacteria and FS are not purified at the same rate. Plusquellec *et al.* (1990) have shown that the elimination of *E. coli* and *Salmonella anatum* is almost similar whereas that of SF is slower. It has also been shown that Daphnia enzymes essentially lysed this coliba cillus (Hadas 1983).

If differences between bacteria can be observed in the captured food particles, it is likely that they also exist at the level of their residence during food transit. Geldreich, (1976) reported that SFs are characterized by their high resistance to salinity, pH and bile; Which allows them to survive a long time in the digestive tract of bivalves. According to Prieur *et al.* (1990), the bacterial population in the mussels digestive tract is quite specialized and differs from the bacterial flora of the surrounding water in young larva stages. The presence of this particular flora can be explained by the adaptation of the bacteria to the conditions encountered in the digestive tract.

The results of presumptive tests that aim to look for and identify four pathogenic bacteria show the presence of staphylococci, *Salmonella* and *Pseudomonas* and the absence of *Clostridium* in the analyzed mussel samples. However the application of confirmatory tests allowed us to demonstrate the presence of *Citrobacter frundi*, *Enterobacter* and *Proteus vulgaris*. Bacteria of the genus *Salmonella*, *Pseudomonas*, and *Clostridium* appear to be absent in the mussels of all sites.

The strongest of the contamination is noted at Sidi Salem. The presence of these germs could be explained by the high contamination of this site by other indicators of faecal pollution (coliforms and streptococci) and would testify to the filtration and accumulation power possessed by the mussels. According to Heier *et al.* (2014), in *E. coli*-contaminated samples, *E. coli* would function as an indicator organism because its charge reached 500 *E. coli* /100g. In polluted waters, Morinigo *et al.* (1990) reported a better correlation between faecal coliforms and salmonella compared to faecal streptococci. Furthermore, Efstratiou *et al.* (2009) found that the presence of *Salmonella* in marine waters is consistent with the presence of total or faecal coliforms; On the other hand, streptococci have less discriminatory power between the presence and absence of *Salmonella*.

The results of the hierarchical classification analysis reveal the existence of dissimilarities between the sites in relation to the variation of the environmental parameters measured.

Group 1 includes Lahnaya and Laouinet located in rural areas; These sites are more or less homogeneous on the factorial plane of the ACP (1,2); Their source of contamination is diffuse because it is generated by run off waters on watersheds forming an integral part of the El Kala National Park, which houses forest, pasture and domestic and wild animals. The Lahnaya and Laouinet waters show high SS and low temperature; but the salinity is higher at Laouinet than at Lahnaya.

Cap de Garde and Rezgui Rachid form Group 2; They are located in peri-urban areas and receive household waste from nearby homes. The waters of the Cap de Garde and Rezgui Rachid show values of salinity and SS close enough; On the other hand, in Rezgui Rachid the temperature is higher due to its location, compared to the current, and the importance of urban waste.

Sidi Salem represents Group 3. It is located in a peri-urban zone and its waters are characterized by the lowest salinity and temperature and the highest SS levels. This zone of the Gulf of Annaba is distinguished by the fact that it serves as a receptacle for three important point sources. The first is represented by the effluent from the fertilizer manufacturing plant (FERTIAL), which drains cooling water to the sea. The second source is the Bedjima Wadi which collects urban waste, without prior treatment, from much of the city of Annaba and the water discharged from the old slaughterhouses near the sampling point. The third source is the Seybouse Wadi which drains an important catchment area, which is home to a strong human activity (numerous agglomerations, industrial and agricultural activities). This site is also contaminated by diffuse sources in the "joanville" and "Sidi Salem" agglomerations and the large colony of seabirds on the surrounding beaches (Telailia 2014).

Conclusion

The results of this study show that the survival of indicators of faecal contamination is strongly influenced by the temperature, suspended solids and salinity. The results obtained highlight the implication of anthropic activity in the microbial contamination of the waters of the Algerian north-east coast. This study found that densities of bacteria in the tissues of mussels would reflect chronic contamination of the environment. However, the calculation of filtration and depuration rates appears to be important in decision-making on mussel farming and assessment of bacterial loads in aquaculture waters. Our results show that the mussel represents a good model for the evaluation of the bacteriological quality of coastal waters.

Acknowledgments

This work was carried out with funds allocated by the General Directorate for Scientific Research and Technological Development (DGRSDT). A special thanks to Mrs Redder Azzouz N. for translating the manuscript.

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