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Assessing Coliform Bacteria in Seawater and *Perna perna* (Linnaeus, 1758) Mussels in the Gulf of Annaba (Algeria): Influence of Seasonal Variability and Urban Development

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ABSTRACT

In recent years, there has been an increase in the number and frequency of coliform bacteria in coastal marine environments. It is therefore necessary to estimate the seawater quality that could pose a health risk to people using beaches with polluted bathing waters. Bivalve mollusks, such as mussels, are bio- indicators used to monitor environmental health due to their filter-feeding behavior, which allows them to accumulate contaminants from seawater. In this study, we focused on one mussel species, *Perna perna*, which is the most dominant species on the Algerian coast. The densities and community composition of coliform bacteria were investigated in samples collected from mussels and seawater in three sampling areas (urban, suburban and rural) in the Gulf of Annaba (northeastern Algeria) over a one-year period in 2017. Culture-based techniques were used to quantify and assess potentially pathogenic bacteria. In addition, measurements were taken of various physico-chemical parameters, including pH, water temperature, salinity, dissolved oxygen and suspended solids. High numbers of coliform bacteria, up to 4,500 MPN per 100 ml in seawater and 300,000 MPN per 100/g in mussels were detected during the summer months, representing an increase of three orders of magnitude compared to winter. Principal component analysis revealed that increased numbers of total coliform bacteria in seawater and mussels were accompanied by increased numbers of fecal indicator bacteria, suggesting that coliform growth is due to fecal contamination. As expected, the highest concentrations of coliform bacteria were observed in samples taken from the urban area, which contains a mixture of wastewater, agricultural and industrial pollutants. Biochemical analyses showed that the bacterial isolates were mainly potentially pathogenic species such as *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.* As *P. perna* mussel is an edible species, the bioaccumulation and persistence of coliform bacteria represent a serious threat to human health, highlighting the necessity to include coliform bacteria in the monitoring of seafood from coastal areas.

INTRODUCTION

Algeria’s population is heavily concentrated in the northern region of the country, particularly within the 50 to 100 km-wide coastal strip. This coastal zone, covering approximately 45,000 km²—or just 1.9% of the national territory—is home to 37% of the country's population, with a high population density of 274 inhabitants per km². The 136 coastal municipalities located along Algeria’s shoreline are among the most densely populated in the nation, accounting for around 75% of the population within the coastal provinces. This region is also characterized by intense agricultural, tourism, and industrial activities, placing significant pressure on the Algerian coastline due to rapid urbanization and development.

At the crossroads of watersheds and the sea, the coast is the receptacle for all the pollution generated by continental activities, which is transported via hydraulic or sewage systems. Wastewater contamination of coastal waters results in the presence of microbiota indicators of fecal contamination, such as viruses and enteric bacteria **(Hervio-heath *et al*., 2012)**. Their survival can be short, from a few hours to a few days, as is the case for *Salmonella* and *Shigella*, as well as *Escherichia coli*, which serves as a bioindicator of fecal contamination, with some strains being pathogenic **(Gourmelon, 2014)**. Longer survival (i.e. several days) is observed for the hepatitis A virus; the presence of the latter can be deduced from the presence of fecal *streptococci*, which show similar resistance **(Rozier, 1990)**. Such pollution, due to a high input of fecal germs into the environment, can pose a health risk to swimmers and seafood consumers. Swimming in water with high microbial contamination can lead to gastroenteritis, which may vary in severity depending on the pathogens involved. In rare cases, more serious infectious diseases, such as typhoid and cholera can result from contaminated water **(Regional Health Agency of Brittany, 2012; Loire-Brittany Water Agency, 2013)**.

Bivalve mollusks, such as mussels, are ubiquitous organisms in Mediterranean seawater. They have a complex life cycle and are highly sensitive to anthropogenic pressures. Due to their ability to filter water through their gills, mussels are valuable bioindicator species for monitoring water pollution and contamination. Mussels are highly appreciated by consumers **(China *et al*., 2003)**. They are generally found in highly anthropized coastal areas, which provide a good supply of nutrients; however, they can be a major source of contamination **(Battistini *et al*., 2020)** when the coastal environment is heavily polluted by agricultural inputs and/or wastewater **(Fiorito *et al*., 2021)**.

Coliform bacteria can be absorbed by mussels and accumulate in their flesh **(Taminiau *et al*., 2014)**. The concentration of microorganisms in shellfish tissue can thus be 10 to 100 times greater than that of the surrounding water **(Loire-Brittany Water Agency, 2013)**. The ingestion of microbial contaminants via shellfish consumption (often raw or undercooked) is implicated in the occurrence of Collective Foodborne Toxi-

Infections (CFTIs) and can lead to illnesses of varying severity, including acute gastroenteritis and hepatitis **(Purpari *et al*., 2019; Padovan *et al*., 2020)**.

In this context, the study presented here seeked to assess the quality of water and *P. perna* mussels found in different areas of the Gulf of Annaba, and determine whether bacterial contamination of both water and mollusks is reaching alarming levels likely to present a health risk to users, including swimmers and consumers. In addition, it seeked to determine the relationship of environmental factors to the abundance and composition of coliform bacteria, thereby providing an insight into the ecological dynamics of the areas studied.

MATERIALS AND METHODS

Sampling area

Situated at the extreme east of the Algerian coast, the Gulf of Annaba is a wide-open bay facing the Mediterranean Sea to the north. It extends from Cap de Garde (36° 58′ 02″ N, 7° 47′ 00″ E) in the west to Cap Rosa (36° 56′ 46″ N, 8° 14′ 13″ E) in the east. This unique coastal ecosystem receives freshwater discharges from the *Seybouse* and *Mafragh* rivers. The two watersheds, covering over 8,000km², are home to approximately 1.5×10^6 inhabitants and collectively deliver around 1.3 km³ per year of freshwater, heavily laden with various terrestrial materials and pollutants **(Ounissi *et al*., 2018)**.

This study focuses on assessing bacterial contamination in three different areas (Fig.1): (1) a suburban area located to the northwest of the city of Annaba, which is relatively sparsely populated and far from various discharges; (2) an urban area located to the east of the port of Annaba, serving as a receptacle for various domestic, agricultural, and industrial discharges; and (3) a rural area located in the eastern part of the Gulf, whose catchment area benefits from extensive vegetation cover and is sparsely populated.

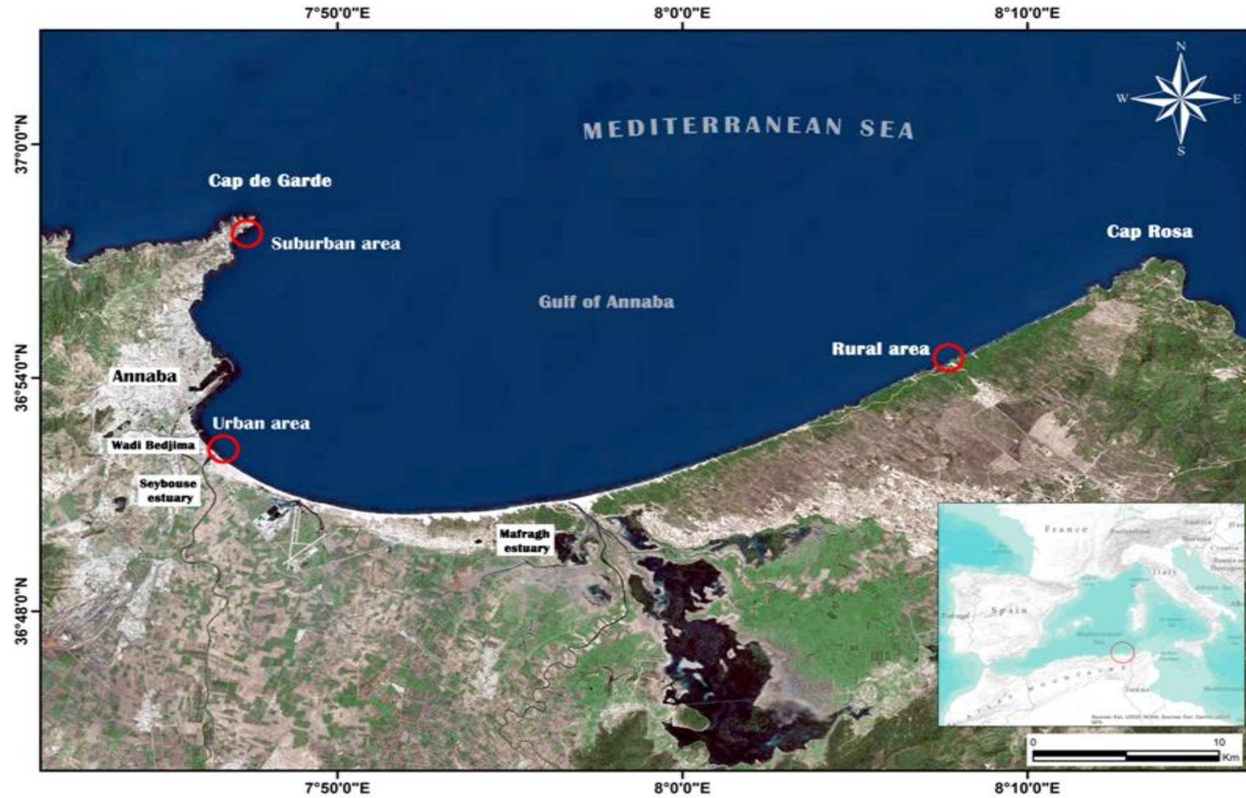

Fig. 1. Map of the Gulf of Annaba and location of the three sampling areas **(Google Earth modified, 2024)**

Sample processing and bacteriological analysis

In this study, seawater and bivalves (*P. perna* mussels) were sampled monthly at the same time between January 2017 and December 2017. For each sampling site, water samples were collected from a depth of 30 to 50cm below the water surface to avoid exposure to sunlight, using 250ml sterile glass bottles. *P. perna* mussels were harvested by hand near the water collection points, at a rate of 10 to 20 individuals, depending on their size. All samples were refrigerated in cool boxes containing freezer packs, to keep temperature close to 4°C during transportation to laboratory. In addition, during each sampling event, water temperature (WT), dissolved oxygen (DO), salinity, and pH were measured *in situ* using a multiparameter probe (WTW, Germany). Suspended solids (SS) in seawater were determined using the method described by **Aminot and Chaussepied (1983)**. In the laboratory, each mussel was opened aseptically using a sterile knife, and the soft tissues were removed, weighed and homogenized in buffered peptone water for subsequent bacteriological analysis.

Bacteriological analysis of seawater and mussels focused on fecal indicator bacteria (FIB) used as indicators of contamination by humans and other warm-blooded animals (i.e. *Escherichia coli*, fecal *streptococci)* and on potentially pathogenic bacteria isolated and identified biochemically using the commercial API (API 20E, API20NE, API Staph) identification system in accordance with the manufacturer's instructions. FIB levels are expressed in terms of the Most Probable Number (MPN) of FIB/100 g of bivalve and MPN of FIB/100 ml of seawater according to the tables of Mac Grady **(Rodier *et al*. 2009)**.

Statistical analysis

Analyses were carried out using R statistical software version 4.1.0 (R Core Team 2021). Differences in coliform bacterial abundance and environmental factors among study areas were analyzed using the non-parametric Kruskal-Wallis test, followed by a Dunn's post-hoc test due to a significant deviation from the normal distribution. In addition, we used Principal Component Analysis (PCA) and Spearman correlation to further assess the relationship between environmental factors and coliform bacteria. For PCA analysis, coliform bacteria were *Hellinger*-transformed, and $\cos^2$ values were used to estimate the quality of the representation. A high $\cos^2$ value indicates a good representation of the variable on the principal component. All statistical analyses were considered significant at $P < 0.05$.

RESULTS

Physicochemical characteristics

The physicochemical parameters recorded during the period studied are summarized in Fig. (2). Among these parameters, only DO and SS showed significant differences among areas ($P<0.05$). For both parameters, the urban area was significantly different from the suburban and rural areas (see Fig.2, D and E). DO (12.6mg/ L) and SS (0.42mg/ L) showed a similar general trend, with higher values in the winter months than in the summer ones. Conversely, the highest concentrations of WT (28°C), pH (8.9) and salinity (41.6g/ L) were observed in summer.

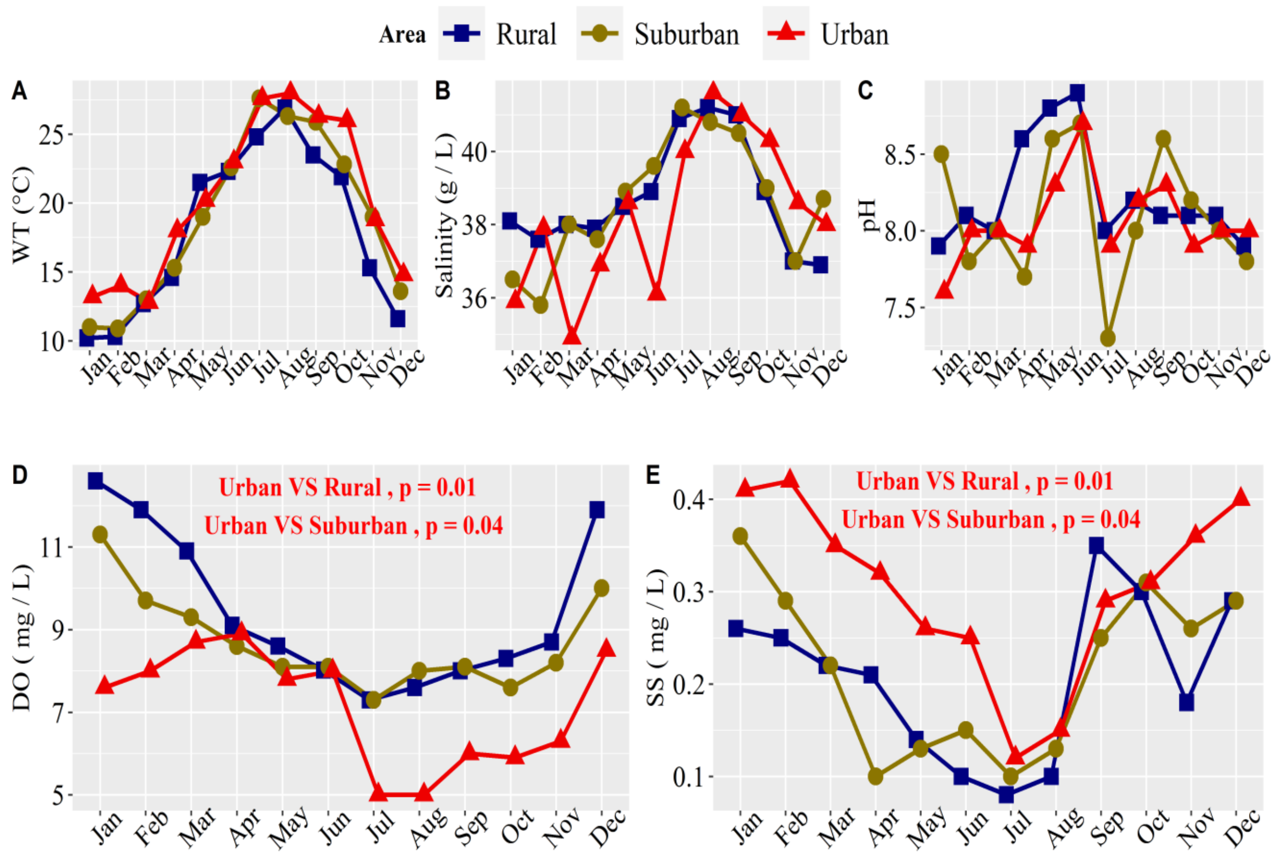

Fig. 2. Temporal variations of the physico-chemical factors across the studied areas. Differences among study areas were analyzed using the nonparametric Kruskal-Wallis test, followed by a post-hoc Dunn test

Occurrence of total coliforms, *E. coli*, and fecal *streptococci* in seawater and mussels

Figs. (3, 4) give an overview about the measured abundance of total coliforms (TC), *E. coli*, and fecal *streptococci* (FS) in seawater and mussels, respectively. As expected, the highest concentrations of total TC, *E. coli*, and FS were found in the urban area, followed by the suburban area and finally the rural area. The most significant differences among the three areas studied were systematically observed between urban and rural areas, then between urban and suburban ones (Fig.4). No significant difference was found between suburban and rural areas ($P > 0.05$). For all samples, whether seawater or mussels, FIB accumulated more in the warmer months than in the colder ones of the year. In addition, *P. perna* mussels from all areas were significantly more contaminated with FIB than seawater samples ($P<0.05$).

In the majority of samples taken, TC exceeded the regulatory limits set by Algerian legislation for recreational waters (500 MPN/100ml of seawater) **(OJAR, 1993)**. Maximum TC numbers were 4600 MPN/100ml and 300,000 MPN/100g in seawater and mussels respectively. Peaks were usually observed in January, June and September, indicating seasonal variability.

For *E. coli*, the highest levels detected were 1200 MPN/100ml in seawater and 150000 MPN/100ml in mussels. Periods of high *E. coli* concentrations were observed in both seawater and mussels, starting from June until October. Finally, FS peaked at 2100 MPN/100ml in seawater and 200,000 MPN/100g in mussels. Densities began to increase in April and remained elevated until October.

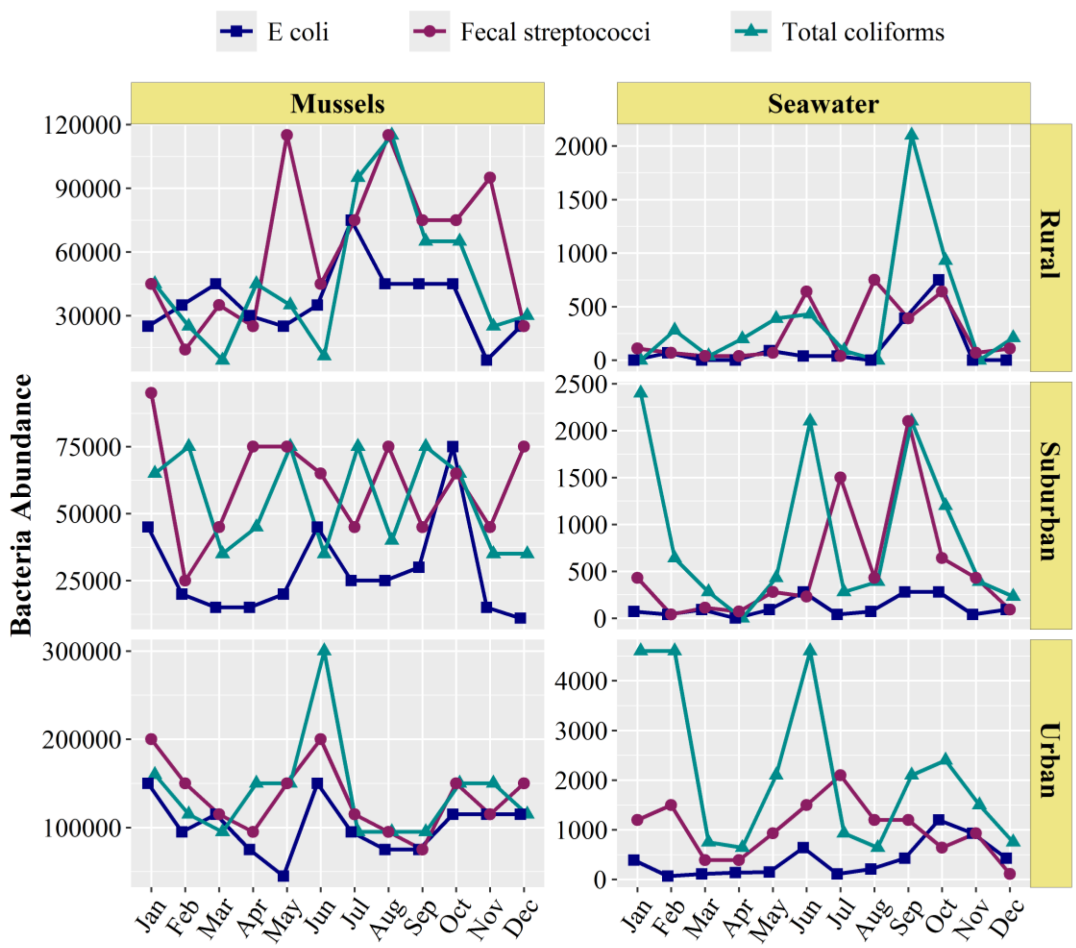

Fig. 3. Temporal variations of *E.coli*, FS, and TC in seawater and mussels across the studied areas

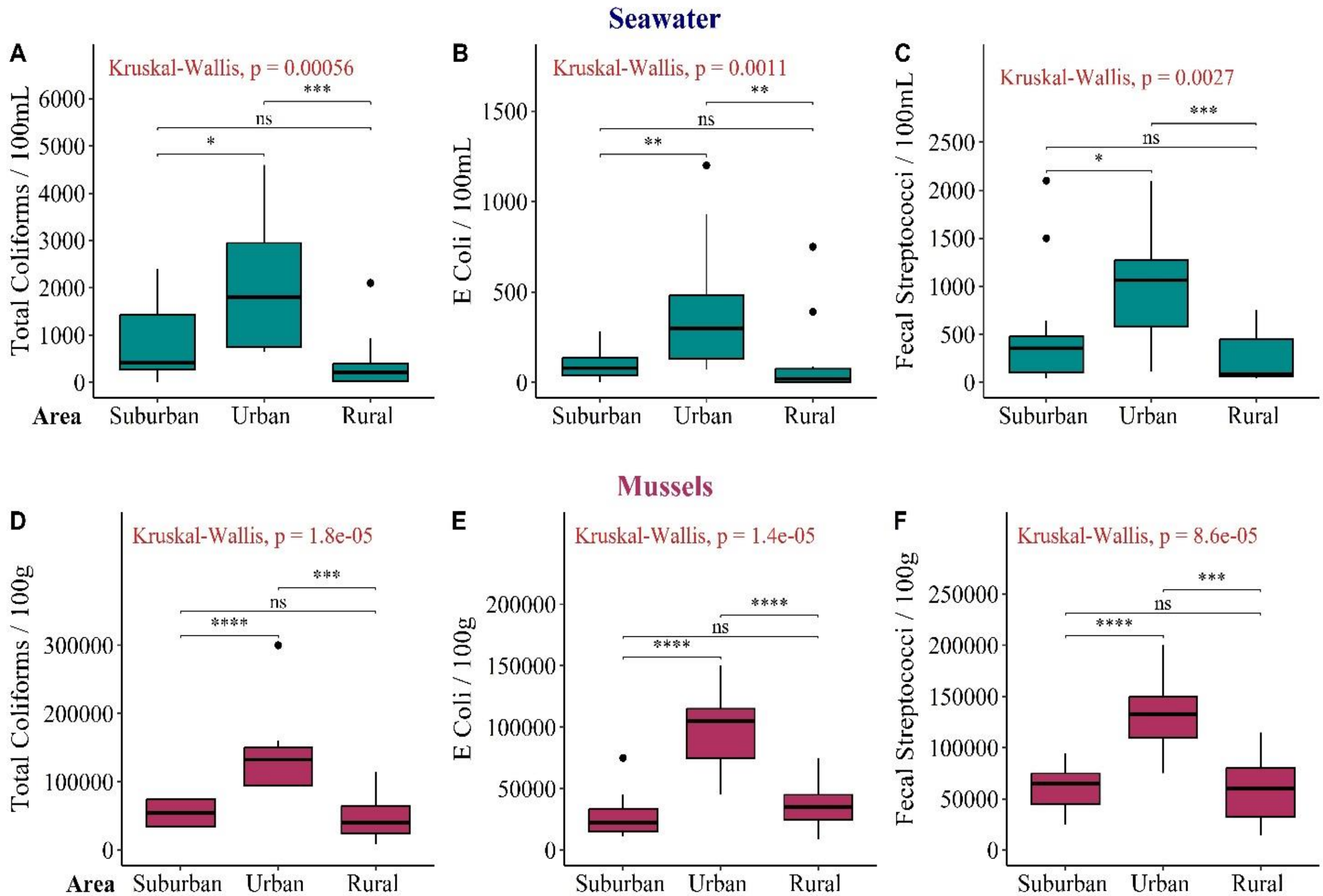

Fig. 4. Spatial variations of coliform bacteria in seawater and *P. perna* mussels. Differences among study areas were analyzed using the nonparametric Kruskal-Wallis test, followed by a post-hoc Dunn test

Identification of pathogenic bacteria

Seasonal and spatial dynamics were not only observed for the abundance of FIB, but also for the occurrence and diversity of bacterial isolates, as shown in Fig. (5). It was found that the largest number of isolates occurred in the summer. Altogether, 125 bacterial isolates belonging to 19 species were identified, of which 70.4% were isolated from mussels. The screening of the urban area revealed a total of 55 and 19 isolates in mussels and seawater respectively. The most abundant species were *Escherichia coli*, *Pseudomonas aeruginosa, Aeromonas hydrophila*, *Klebsiella pneumoniae*, and *Yersinia enterolitica*.

In the suburban area, at least 20 and 10 isolates were identified in mussels and seawater respectively. As in the urban area, *E. coli* was the most dominant. *Proteus mirabilis* in mussels and *Citrobacter freundi* in seawater were relatively present, but these genera did not dominate. From samples taken in the rural area, 21 isolates were obtained: 13 in mussels and 8 in seawater. Only the dominance of *E. coli* was notable.

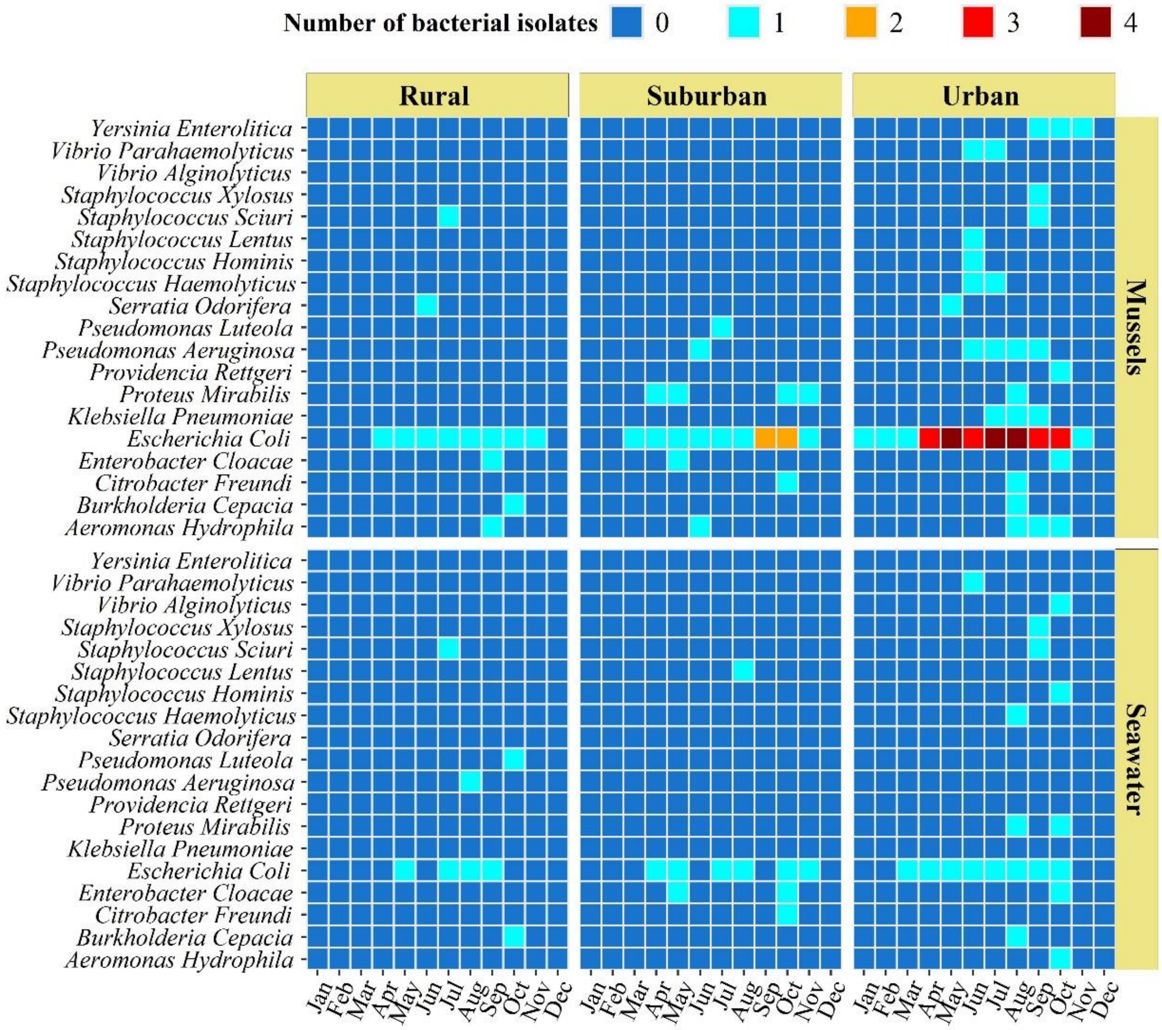

Fig. 5. Temporal distribution of bacterial isolates in seawater and *P. perna* mussels in study areas

Relationship between physicochemical factors and coliform bacteria

Spearman correlation analyses show that, for both seawater and mussels, the correlations between fecal *streptococci* (r = -0.69) and *E. coli* (r = -0.49) with dissolved oxygen are negative and significant (Fig.6). Additionally, in seawater, *E. coli* (r = 0.49) and total coliforms (r = 0.59) are positively related to suspended solids, while fecal *streptococci* are positively related to WT (r = 0.57). In mussels, total coliforms are negatively related to DO (r = -0.6).

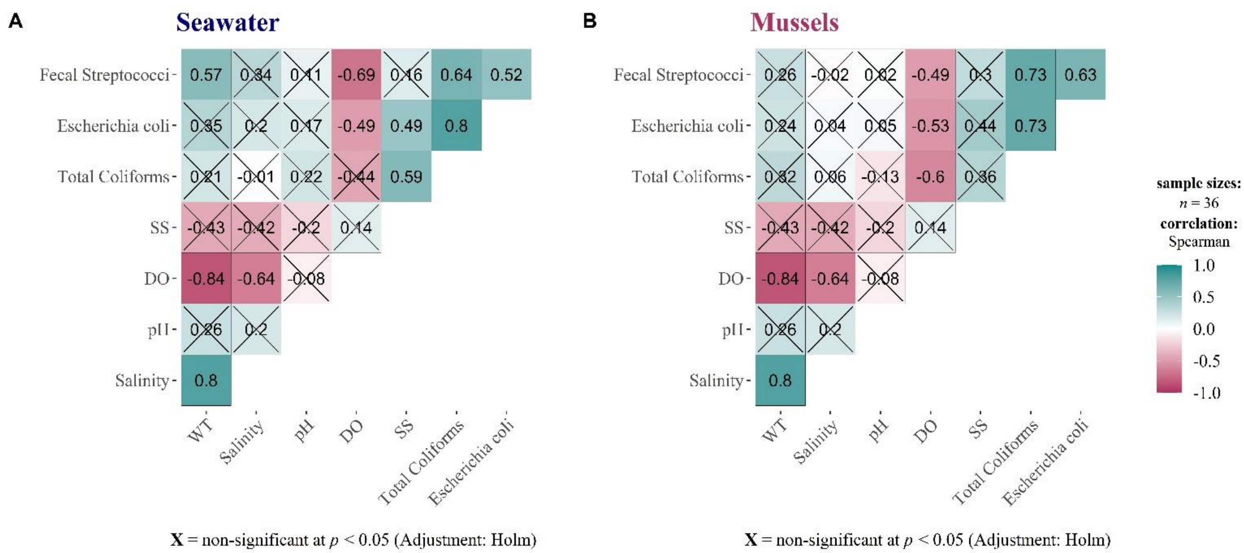

Fig. 6. Spearman correlation matrix between environmental factors and coliform bacteria in seawater and *P. perna* mussels

The results of the PCA paralleled those of the correlation analysis, and showed that the first two principal components explained 67.5% of the variation (Fig.7). The major contributors to the first axis, which explained 41.8% of the variance, were bacteriological factors including TC Mussels (r = 0.86; cos^2= 0.74), *E. coli* Mussels (r = 0.86; cos^2= 0.74), FS Mussels (r = 0.82; cos^2= 0.68), TC Seawater (r = 0.78; cos^2= 0.62), *E. coli* Seawater (r = 0.71; cos^2= 0.51), and to a lesser degree FS Seawater (r = 0.69; cos^2= 0.48). The dissolved oxygen, which was negatively correlated with the first axis, turned out to be an important factor in explaining the abundance of coliform bacteria.

The second axis, which explained 25.7% of the variance, was positively correlated with SS (r = 0.75; cos^2= 0.57), but negatively correlated with salinity (r = -0.87; cos^2= 0.76) and WT (r = -0.86; cos^2= 0.73). Water temperature showed a certain relationship with FS seawater. The differences among the areas were also visible and clearly individualized in the PCA (Fig. 7A). The coliform bacteria were mainly detected in the urban area, followed by the suburban area and the rural area, which is in line with the results obtained previously through the Kruskal-Wallis analyses.

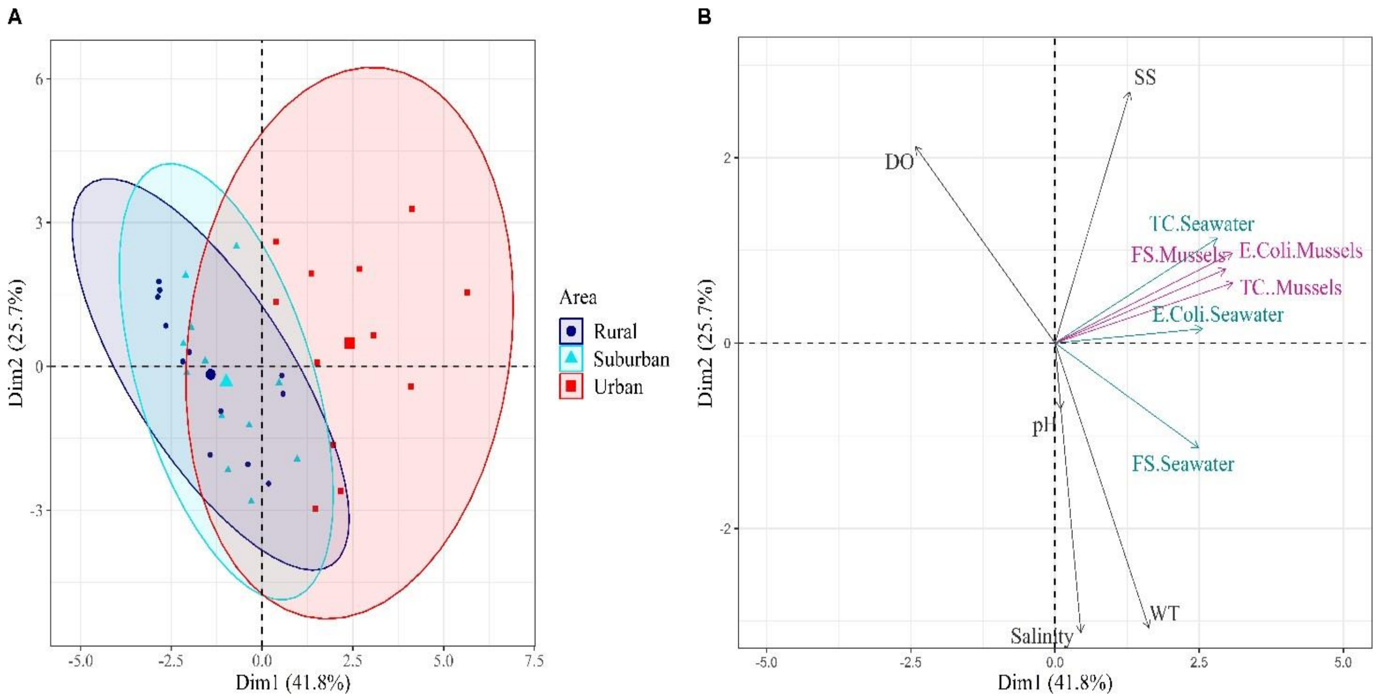

Fig. 7. Principal component analysis between environmental factors and coliform bacteria in seawater and *P. perna* mussels. TC: Total coliforms, FS: Fecal *streptococci*

DISCUSSION

Mediterranean coastal areas are rich in biodiversity, and the conservation and sustainable use of these ecosystems depend on water quality. Health indicators of coastal environment can be defined as chlorophyll-*a*, dissolved oxygen, suspended solids, nitrate, pH, ammonium, total phosphorous, and fecal coliform bacteria **(Kucuksezgin *et al*., 2019)**. In this study, the temporal dynamics of coliform bacteria, with a particular focus on fecal indicator bacteria (FIB) in three areas of the Mediterranean coast of northeastern Algeria were investigated. The three areas were chosen for their different exposure to urban development **(Kadri *et al*., 2017)**.

Studies carried out in Mediterranean coastal waters suggest a lower level of bacterial pollution during the summer season than in other seasons, which is probably linked to the increase in daylight and solar radiation during the summer, both of which affect bacterial mortality **(Maipa *et al*., 2001)**. These findings are inconsistent with our results, which indicate that FIB densities and the number of bacterial isolates were higher in the warmer months. PCA (Fig. 7) and Spearman correlation (Fig. 6) revealed that high numbers of total coliform bacteria in seawater and mussels were accompanied by increased numbers of fecal indicator bacteria. Thus, it can be assumed that coliform growth is due to fecal contamination.

By comparing the three areas using the Kruskal-Wallis test, it could be observed that the highest concentrations of total coliform bacteria, *E. coli*, and fecal *streptococci* were systematically observed in samples taken in the urban one (Fig. 4). This can be explained by the significantly high concentrations of suspended solids (SS) and the significantly low concentrations of DO observed in this area (Fig. 2). The higher suspended solids and FIB levels detected in the urban area indicate poor flushing and dilution of fecal contamination in this area. **Walters *et al.* (2014)** suggest that SS play a protective role for FIB against UV radiation and predators. The high concentrations of SS in the urban area were due to industrial and agricultural waste transported by the *Bedjima* and *Seybouse* wadis; to this must be added waste generated by the "Fertial" complex, specialized in the manufacture of mineral fertilizers (i.e., phosphate fertilizers, urea, and ammonium nitrate) and waste from the port of the city of Annaba.

Fecal *streptococci* in both seawater and mussels were significantly and positively correlated with WT (Fig. 6), suggesting that high temperature is suitable for their growth and persistence. *Enterococci* are known to be highly resistant to aggressive environmental stressors and are therefore considered to be the best indicators of the quality of recreational marine waters **(Byappanahalli *et al*., 2012)**. Their detection indicates fecal contamination of the water and therefore the possible presence of pathogenic bacteria, viruses or fecal protozoa **(Wade *et al.*, 2006)**.

In the same areas, in 2009, *E. coli* abundances in seawater ranged from 0 to 1400 MPN/100ml **(Kadri *et al.*, 2015)** versus 0 to 4600 MPN/10ml in the current study. Similarly, fecal *streptococci* recorded in 2009 did not exceed 230 MPN/100 ml **(Kadri *et al.*, 2015)**, whereas in the current study they ranged from 40 to 2100 MPN/100ml, indicating a dramatic deterioration in water quality over the past decade leading to a significant impact on this ecosystem and on the bacteriological quality of the mussels.

Our results also show that in all the areas studied, FIB densities were significantly higher in *P. perna* mussels than in the surrounding waters, as these filter-feeding organisms are capable of concentrating microorganisms, even when they are in low concentrations **(Stabili *et al*, 2005; Martinez & Oliveira, 2010; Jayme *et al*., 2016)**. **Bighiu *et al.* (2019)** evaluated the bacterial accumulation capacity of mussels in the laboratory and reported that the concentration of *E. coli* and *enterococci* was 132 times higher than in water samples. High temperatures, particularly during the spawning season, promote an increase in the filtration rate of mussels, leading to greater retention of microorganisms present in the surrounding water **(Le Bec *et al*., 2002; Burge *et al.*, 2016)**.

During the summer season, not only the densities of FIB changed entirely, but also the diversity and occurrence of bacterial isolates (i.e. pathogenic bacteria). The pattern between seawater and mussels was similar. Several studies have shown that pathogenic bacteria are influenced by seasonal changes, especially the warm season **(Maipa *et al.*, 2001; Reitter *et al.*, 2021)**. Regardless of the area, *E. coli* was largely dominant (Fig. 5), accounting for 48.64% of the bacterial isolates identified in mussels, and even higher in seawater (53.40%). With the exception of December, *E. coli* in mussels is detectable in the urban area during the whole year, with a high number of isolates between April and October.

According to **Campos *et al.* (2013)**, the contamination of the coastal environment by *E.coli* results from three main sources of fecal contamination, primarily originating from upstream watersheds: (1) urban sources, characterized mainly by wastewater from abattoirs, sewage treatment plants, and domestic discharges; (2) agricultural sources, characterized by manure from grazing animals and livestock effluents; this source increases in wet weather due to runoff from farm buildings and fields where effluents are spread **(Blaustein *et al.*, 2016)**; and (3) environmental sources, characterized by the presence of wild animals. Wild animals present in forests and uncultivated land contribute to environmental contamination by *E. coli* **(Goto & Yan, 2011)**. In addition to the predominance of *E. coli*, we noted the relative presence of *Pseudomonas aeruginosa*, *Yersinia enterolitica*, *Aeromonas hydrophila* and *Klebsiella pneumoniae* in the mussels.

The bacterium *A. hydrophila* is recognized as a reliable biomarker for assessing stress and pollution in aquatic environments **(Leung *et al.*, 1995)**. *A. hydrophila* has been identified as the causative agent of various symptoms related to gastroenteritis, systemic infections, and bacterial endocarditis in humans and other species. *P. aeruginosa*, *Klebsiella* spp., and *Enterobacter* spp. are classified as emerging multidrug-resistant organisms and are linked to diarrhea, intra-abdominal infections, and nosocomial infections, particularly in immunocompromised patients **(Streeter & Katouli, 2016)**. This implies that consumption of mussels harvested near the Gulf of Annaba, particularly in the urban area, poses a considerable risk to human health. Strict compliance with industrial water and wastewater treatment protocols will help protect these ecologically important coastal areas.

CONCLUSION

This study describes the environmental and bacteriological state of the Gulf of Annaba, assessed simultaneously in mussels and the seawater in which they live, in areas subject to different levels of anthropogenic pressure. We detected seasonal differences in the number of coliform bacteria and isolates of potentially pathogenic bacteria. PCA analysis indicated that coliform proliferation was due to fecal contamination and that the latter are associated with high levels of suspended solids. The bacterial community was largely dominated by *E. coli*. The results of this study clearly indicate that consumption of mussels harvested close to urban area, where deterioration of mussel microbiological quality is more likely, can present high risks to human health. Therefore, corrective measures must be taken quickly to improve water quality and mitigate risks to human health. Proposed measures may include upgrading wastewater treatment plants, implementing pollution prevention and remediation programs, and enhancing the protection of riparian vegetation, among others.

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