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RESEARCH ARTICLE

Occurrence and distribution of fecal indicators and pathogenic bacteria in seawater and *Perna perna* mussel in the Gulf of Annaba (Southern Mediterranean)

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Abstract
The identification of fecal contamination in coastal marine ecosystems is one of the main requirements for evaluation of potential risks to human health. The objective of this study was to investigate the occurrence and distribution of fecal indicators and pathogenic bacteria in seawaters and mussels collected monthly during a period of 1 year from four different sites in Northeastern Algeria (sites S1 to S4), through biochemical and molecular analyses. Our research is the first to use molecular analysis to unambiguously identify the potentially pathogenic bacteria present in Algerian *Perna perna* mussels. The obtained results revealed that the levels of fecal indicator bacteria (FIB) from both *P. perna* and seawater samples largely exceeded the permissible limits at S2 and S3. This is mainly related to their location close to industrial and coastal activity zones, which contain a mixture of urban, agricultural, and industrial pollutants. Besides, *P. perna* collected from all sites were severalfold more contaminated by FIB than seawater samples, primarily during the warm season of the study period. Biochemical and molecular analyses showed that isolated bacteria from both seawater and mussels were mainly potentially pathogenic species such as *E. coli*, *Salmonella spp.*, *Staphylococcus spp.*, *Klebsiella spp.*, *Pseudomonas spp.*, and *Proteus spp.*

Keywords Bacterial contamination · Mediterranean coastal waters · Gulf of Annaba · Fecal indicators · *Perna perna* · Potentially pathogenic bacteria

Introduction

For many decades, the coastal marine ecosystems have been continuously threatened by several anthropogenic activities such as improper sewage disposal, urban runoff, and massive discharges of agricultural and industrial effluents (Ghozzi et al. 2017; Damak et al. 2020). Coastal waters are often the receiving environment for all kinds of wastewater discharges containing many microorganisms that are harmful to human health, especially in bathing beaches and shellfish production areas (Perkins et al. 2014). Thus, the impact on health is more than worrying, placing microbiological pollution as a major public health problem.

Due to their sessile lifestyle, resistance to environmental stressors, and efficient water clearance ability, bivalves, especially mussels, have been widely used as bioindicators of coastal pollution (Belabed et al. 2013; Jia et al. 2018; Ozkan et al. 2017). These invertebrates have the potential to accumulate large quantities of microorganisms from their surrounding waters, including opportunistic bacteria (*Aeromonas spp.*, *Vibrio spp.*, *Pseudomonas spp.*), protozoan parasites (Cryptosporidium, Giardia), and viruses (adenoviruses, hepatoviruses), as well as pathogenic bacteria (*E. coli*, Salmonella) (Ghozzi et al. 2017). They may therefore jeopardize human health, especially when they are consumed as seafood (Stabili et al. 2005; Zannella et al. 2017; Vincy et al. 2017).

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Numerous studies have reported that many serious illnesses such as acute gastroenteritis and hepatitis E virus infections are related to the presence of pathogenic microorganisms in bivalves, especially when they are eaten raw or undercooked (Le Guyader et al. 2006; O'Hara et al. 2018; Kobayashi et al. 2019; Fouillet et al. 2020). Hence, there is an urgent need for an overall assessment to predict the presence of these infectious agents related to waterborne outbreaks, and to prevent the impacts of fecal contamination on human and environmental health.

The brown mussel *Perna perna* (Linnaeus, 1758) is a bivalve mollusc belonging to the Mytilidae family. It is widely distributed in tropical and subtropical regions of the Mediterranean Sea, as well as in the Atlantic and Indian oceans (dos Santos et al. 2018; Neves et al. 2019). Because of its importance as a valuable source for human nutrition, *P. perna* is considered as one of the key aquaculture species worldwide, and the production in Brazil alone is about 18,000 tons (dos Santos et al. 2018; FAO 2019; Krampah et al. 2020). This wide geographical distribution also makes *P. perna* appropriate for determination of pollution levels, especially in regions that are not economically and technically prepared to monitor aquatic contamination through more sophisticated analysis (Sokolowski et al. 2004; Francioni et al. 2004).

In Algeria, *P. perna* mussels are harvested directly from the rocky beaches of the coastal regions where no legislation for consumption exists. This species is widely used as a bioindicator of coastal pollution to identify and classify the most suitable sites for mussel aquaculture (Belabed et al. 2013; Boudjema et al. 2014; Kadri et al. 2017; Kerdoussi et al. 2017; Abderrahmani et al. 2020). Like all North African countries, mussel aquaculture along the Algerian coasts faces important constraints such as the underdeveloped markets, low availability of good sites, lack of qualified personnel, and financial. However, universities in the Algerian cities of Oran, Mostaganem, Algiers, and Annaba are making significant efforts to develop this sector (Kara et al. 2018).

The Gulf of Annaba is one of the most valuable coastal regions of Northern Algeria, because of its great touristic and economic importance (Ouali et al. 2018). However, it is highly vulnerable to several types of pollutants, primarily related to the intensive agricultural and industrial discharges and the presence of domestic wastes, especially on the outskirts of the city where there is a high population (Soltani et al. 2012; Amri et al. 2017; Ouali et al. 2018). Other natural environmental contaminants such as the leaching of soils, animal excreta and river discharges, as well as the problems of climate change and global warming, are also likely responsible for fecal contamination in the Gulf of Annaba. According to Barreras Jr et al. 2019, rising sea surface temperatures is a consequence of the expected climate change, and this will extend the time period during which fecal bacteria can survive, which again will lead to increased bacterial load.

During recreational activities, water contaminated with fecal pollutants can pose a significant risk to human health, as many enteric pathogens are often associated with fecal matter (Oliveira et al. 2016). In case of direct or indirect contact with water, users may be exposed to a variety of waterborne diseases of ears, eyes, and skin, as well as gastrointestinal and upper respiratory illness (Maipa et al. 2001; Chávez-Díaz et al. 2020)

Despite this increasing pollution pressure, few studies have been carried out on fecal contamination and its impact on human health in the Gulf of Annaba (Kadri et al. 2015, 2017). Therefore, this study aimed to evaluate the occurrence and the distribution of fecal indicators and pathogenic bacteria in seawater and the mussel *Perna perna* samples by implementing a spatial–temporal sampling strategy, and to assess the impact of physicochemical variables on the abundance of fecal indicator bacteria (FIB). It should also be emphasized that our research is the first to use molecular analysis to identify with certainty the pathogenic bacteria present in *Perna perna* mussels collected in the Gulf of Annaba.

Materials and methods

The Gulf of Annaba is located in the northeast of Algeria. It stretches over 40 km from Cap de Garde (36°96′N, 7°79′E) in the west to Cap Rosa (36°68′N, 8°25′E) in the east. It is a heavily polluted ecosystem, due to a variety of agricultural, industrial, and urban discharges, in addition to massive domestic wastes from a large part of the city of Annaba (Abdennour et al. 2000). Four sampling sites were strategically selected for the present study, based on different potential pollution sources in these areas: S1 'Cap de Garde'; S2 'Rezgui Rachid'; S3 'Sidi Salem'; and S4 'Lahnaya' (Fig. 1). The characteristics of the selected sites are shown in Table 1.

Sampling protocol

Samples of seawater and *Perna perna* mussels were monthly and simultaneously collected at low tide at each site, in the period from January to December 2018. Low tides are generally associated with higher concentrations of fecal indicator bacteria in coastal areas. This increase of FIB levels is the result of the mobilization of sediment-associated indicator bacteria as tidal waters recede (USEPA 2010).

Water samples were obtained at a depth of 30-50 cm below the surface of the water to avoid sunlight exposure using 250-ml sterile glass bottles. *P. perna* mussels (43–110mm in length, 20–35mm in width, and 10–30mm in height) were harvested by hand near the water collecting points at a rate of 10–20 individuals (depending on size). All samples were immediately placed in a clean cooler containing ice cubes

Table 1 Characteristics of the four sampling sites

Site number	Name and coordinates of sampling site	Location in the Gulf of Annaba	Associated pollution sources	Hydrodynamic flux	Reference
S1	Cap de Garde (36°96′N, 7°79′E)	Located 7.7 km to the west of Annaba city	-Presence of bathers and fishermen in summer	High	Kadri et al. (2017)
S2	Rezgui Rachid (36°91′N, 7°76′E)	Located in a peri-urban area, on the west coast of Annaba city	-Receives urban waste from nearby houses -Receives a lot of bathing visitors during summer	Low	Belabed et al. (2013)
S3	Sidi Salem (36°86′N, 7°76′E)	Located 1 km to the east of Annaba city, close to Wadi Seybouse and Bedjima	-Receives a mixture of urban agricultural and industrial wastes -Important presence of a large colony of seabird and livestock	Low	Telailia (2014), Kadri et al. (2017)
S4	Lahnaya (36°93′N, 8°20′E)	Located in rural area, 45 km from the city of Annaba, in the National Park of EL-Kala	-Strong presence of bathers and fishermen in summer -Presence of livestock	High	Kadri et al. (2017)

(4°C) and transported to the laboratory within the following 2–4h. At each site and each month, seawater environmental variables including temperature (T), pH, salinity (Sal), and dissolved oxygen (DO) were measured *in situ* using a multiparameter probe (Multi 340i/SET-82362, WTW®, Germany). The determination of seawater suspended solids (SS) was performed as described by Aminot and Chaussepied (1983).

Bacteriological analysis

For seawater samples, a volume of 100 ml was directly analyzed without any prior treatment (Rodier et al. 2009). Immediately upon return to the laboratory, the mussels were cleaned and cleared of encrusting organisms, after which they were opened with a sterile scalpel. The tissue and intravalvular liquid (25 g) were mixed and homogenized with 225-ml sterile physiological water in a sterile laboratory blender (standard NF EN ISO 6887). The levels of FIB such as total coliforms (TC) and *Escherichia coli* (EC), as well as fecal streptococci (FS), were estimated by three-tube decimal dilution using the most probable number (MPN) method (standard NF V 08-021 (1993)/ISO 7402 and NF V 08-020 (1994)/ISO 7251). All results were statistically expressed as MPN per 100 ml of the sample according to Mac Grady's tables (Rodier et al.

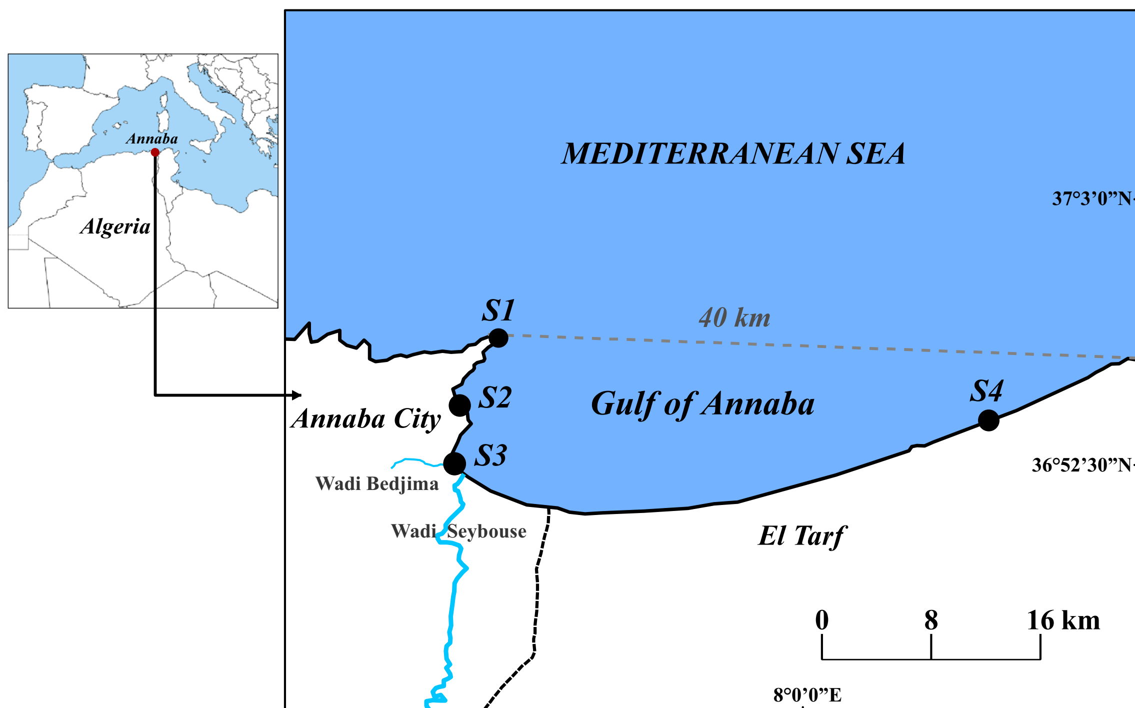

Fig. 1 Map showing the location of the Gulf of Annaba and sampling sites. The small map shows the overall location of Annaba with respect to Algeria and the Mediterranean Sea. The large map shows the exact locations of the four sampling sites (S1 to S4), which are indicated with black circles. S1, Cap de Garde; S2, Rezgui Rachid; S3, Sidi Salem; S4: Lahnaya

2009). For the isolation of potentially pathogenic bacteria, standard microbial methods were carried out (Rodier et al. 2009). Bacterial isolates were biochemically identified at the species level through Analytical Profile Index (API 20E, API20NE, API Staph) and further confirmed by 16S rRNA gene sequencing, multilocus sequence typing (MLST), and phylogenetic analysis.

DNA extraction and 16S rRNA gene amplification

Twenty-five bacterial isolated were selected, based on their potential to be human pathogens. Bacterial colonies were picked from overnight LB (Lysogeny Broth) agar plates and transferred into 1.5-ml Eppendorf tubes containing 50 μl of 1xTE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) supplemented with approximately 100 mg of 0.1 mm Zirconia beads. The tubes were incubated at 37°C for 15 min and then strongly vortexed for 3 min to disrupt the cells. The resulting bacterial lysate served as a template for the 16S rRNA gene amplification. The 25 μl PCR mixture contained 0.5 μl DNA template, 2.5 μl Dream Taq buffer (10x), 1.5 μl dNTPs (2.5 mM each), 0.5μl Dream Taq DNA polymerase (Thermo Scientific™) and 1.5 μl 10 μM of each universal primers 27F and 1492R (Table 2). PCR cycling was carried out as described by da Silva et al. (2013). Amplification products were visualized by electrophoresis on 1% agarose gel in 1x TBE buffer after staining with SYBR Safe (Invitrogen) and subsequently purified with Gene Jet Gel Extraction Kit (Thermo Scientific ™).

16S rRNA sequence analysis

The PCR-amplified regions of the 16S rRNA genes were Sanger-sequenced using primer 27F (Table 2). The obtained partial sequences of the 16S rRNA gene were compared with the GenBank NCBI database through the BLAST software, to confirm the species of the isolates. After that, a multiple sequence alignment was carried out using the Clustal X software integrated into the MEGA 7 program (Kumar et al. 2016). Finally, the phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replications.

Multilocus sequence typing analysis (MLST analysis)

To investigate the source of the detected *E. coli* strains, DNA from isolates EM3, EM18, EM97, EM102, and MM6 were subjected to PCR amplification targeting seven specific genes (*trpA*, *trpB*, *dinB*, *polB*, *putP*, *pabB*, *and icdA*) using suitable primers (Table 2), and following the same procedures used for 16S rRNA genes. The amplification program was carried out as follows: initial denaturation of 4 min at 94°C, followed by 30 cycles of 30s at 94°C, 30s at 52°C, and 2 min at 72°C, and a final extension at 72°C for 4 min. The phylogenetic tree is based on 2758 bp concatenated partial sequences of the seven genes from EM3, EM18, EM97, EM102, and MM6, as well as the equivalent loci in closely related strain. The sequences were aligned with Clustal Omega with default settings on the EBI server, and the guide-tree was visualized using iTOL (Letunic and Bork 2019; Madeira et al. 2019).

Statistical analysis

Statistical analysis was carried out with the R software version 3.1.2. Normality distribution and homogeneity of data variances were first tested by the Shapiro–Wilk test (Shapiro and Francia 1972). Since data were not normally distributed, we had to choose nonparametric tests for data analysis. First, the Spearman correlation coefficient was evaluated to investigate possible relationships between our data sets. Then, the Kruskal–Wallis test was applied to assess the intersite and intermonth comparisons. Comparisons between the groups with significant differences were identified by the Wilcoxon tests. In all tests, the significance level was set to p value < 0.05.

Finally, principal component analysis (PCA) was used as a descriptive method to characterize the four sampling sites in the study area and to assess the contribution percentage of measured environmental variables on the abundance of the fecal indicators employing the FactoMineR package. In PCA analysis, the square cosine (cos2) indicates the importance of the contribution of a component to the distance squared from the initial observation. The components with a high cos2 value contribute significantly to the total distance, and these components are therefore the important contributors (Abdi and Williams 2010).

Results

Physicochemical analysis of sampled water

The monthly variation in seawater environmental variables obtained throughout the sampling period is presented in Fig. 2. As expected, the annual temperature and salinity cycles showed similar seasonal fluctuations across the four study sites. Seawater temperature ranged from 10.3°C at S4 in February to 28.6°C at S2 in August, while salinity varied from 34.9 g/L at S3 in March to 41.6 g/L at S2 in August. The variations of these two parameters are primarily influenced by the climatic conditions of the area. The high values of temperature and salinity recorded at S2 (temperature: 28.6 °C; salinity: 41.61 g/L), and S3 (temperature: 28°C; salinity: 41.6 g/L) would be due to the fact that these sites are located well within the Gulf and are protected from currents. Relatively high evaporation, especially in summer, also contributed to the increase in salinity. The pH remained relatively constant and alkaline during the sampled months, with a slight

Table 2 Primers used in this study

Target gene	Primer name	Sequence (5′–3′)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	Lane (1991)
	1492R	CGGCTACCTTGTTACGACTT	
trpA	trpA-F	ATGGAACGCTACGAATCTCTGTTTGCCC	Escobar-Páramo et al. (2003)
	trpA-R	TCGCCGCTTTCATCGGTTGTACAAA	
trpB	trpB-F	ACAATGACAAGATTACTTAACCCCT	Escobar-Páramo et al. (2003)
	trpB-R	TTTCCCCCTCGTGCTTTCAAAATATC	
polB	polB-F	TGGAAAAACTCAACGCCTGGT	Bjedov et al. (2003)
	polB-R	TGGTTGGCATCAGAAAACGGC	
icdA	icdA-F	GAAAGTAAAGTAGTTGTTCCGG	Escobar-Páramo et al. (2004)
	icdA-R	GATGATCGCGTCACCAAAYTC	
putP	putB-F	GCGACGATCCTTTACACCTTTATTG	Escobar-Páramo et al. (2003)
	putB-R	CGCATCGGCCTCGGCAAAGCG	
dinB	dinB-F	TTGAGAGGTGAGCAATGCGTA	Bjedov et al. (2003)
	dinB-R	GTATACATCATAATCCCAGCAC	
pabB	pabB-F	TTTTACACTCCGGCTATGCCGATCA	Guttman and Dykhuizen (1994)
	pabB-R	GCTGCGGTTCCAGTTCGTCGATAAT	

increase in spring. An inverse relationship between dissolved oxygen and temperature vs. salinity was observed. The highest value (12.6 mg/L) of dissolved oxygen was recorded during winter at S4, while the lowest one (5 mg/L) was detected during summer at S3 and S2. Indeed, the application of Spearman's correlation test revealed a strong negative and significant correlation between dissolved oxygen and the temperature ($r = -0.84$, $p < 0.0001$) and between the same variable and the salinity ($r = -0.65$, $p < 0.0001$) (Table 3). Levels of suspended solids were lower at S1 and S4 as compared with the other two sites. The highest value (0.42mg/L) was recorded two times in February at S3 and in December at S2.

Bacteriological analysis of isolated bacteria

As shown in Fig. 3, the results of the bacteriological analysis revealed that the fecal contamination varied over time and among the four sampling sites in the Gulf of Annaba ($p < 0.05$).

The levels of all FIB (TC, EC, and FS) in seawater samples were alarmingly high at S2 and S3 (4.6×10^3 MPN/100 ml), and largely exceeded the limits defined by the Algerian law as 500 TC/100 ml, 100 EC/100 ml, and 100 FS/100 ml (JORA 1993, 2006). The minimum levels of FIB were recorded at S1 and S4 (0 MPN/100ml) (Fig. 3). The Wilcoxon test was used to examine potential differences in detected TC, EC, and FS between the four study sites. The most significant differences between the four study sites were between S3 and S4 (p=0.0004), and between S2 and S4 (p=0.0011). No statistically significant differences were found between S2 and S3 ($p > 0.05$) (Table S1).

As expected, *P. perna* mussels from all sites were several-fold more contaminated by FIB than the seawater samples. TC concentrations ranged from 9×10^3 MPN/100g at S4 to 3×10^5 MPN/100g at S3. For *E. coli*, 100% of the *P. perna* samples at S3 showed loads of more than 4.6×10^4 MPN/100g. FS was present throughout the entire study period, and the highest concentration (2.5×10^4 MPN/100g) was detected in the mussels of S2 (Fig. 3).

Table 3 Spearman's correlation matrix of the seawater quality variables in 2018. *DO*, dissolved oxygen; *Sal*, salinity; *T*, water temperature; *SS*, suspended solids; *EC*, Escherichia coli; *TC*, total coliforms; and *FS*, fecal streptococci

	T	Sal	pH	DO	SS	TC	EC
Sal	0.824***						
pH	0.181	0.185					
DO	−0.836***	−0.650***	0.008				
SS	−0.483***	−0.529***	−0.223	0.183			
TC	0.137	−0.021	0.183	−0.371**	0.565***		
EC	0.285*	0.140	0.025	−0.459**	0.507***	0.769***	
FS	0.643***	0.461***	0.119	−0.721***	−0.025	0.559***	0.453***

*$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$

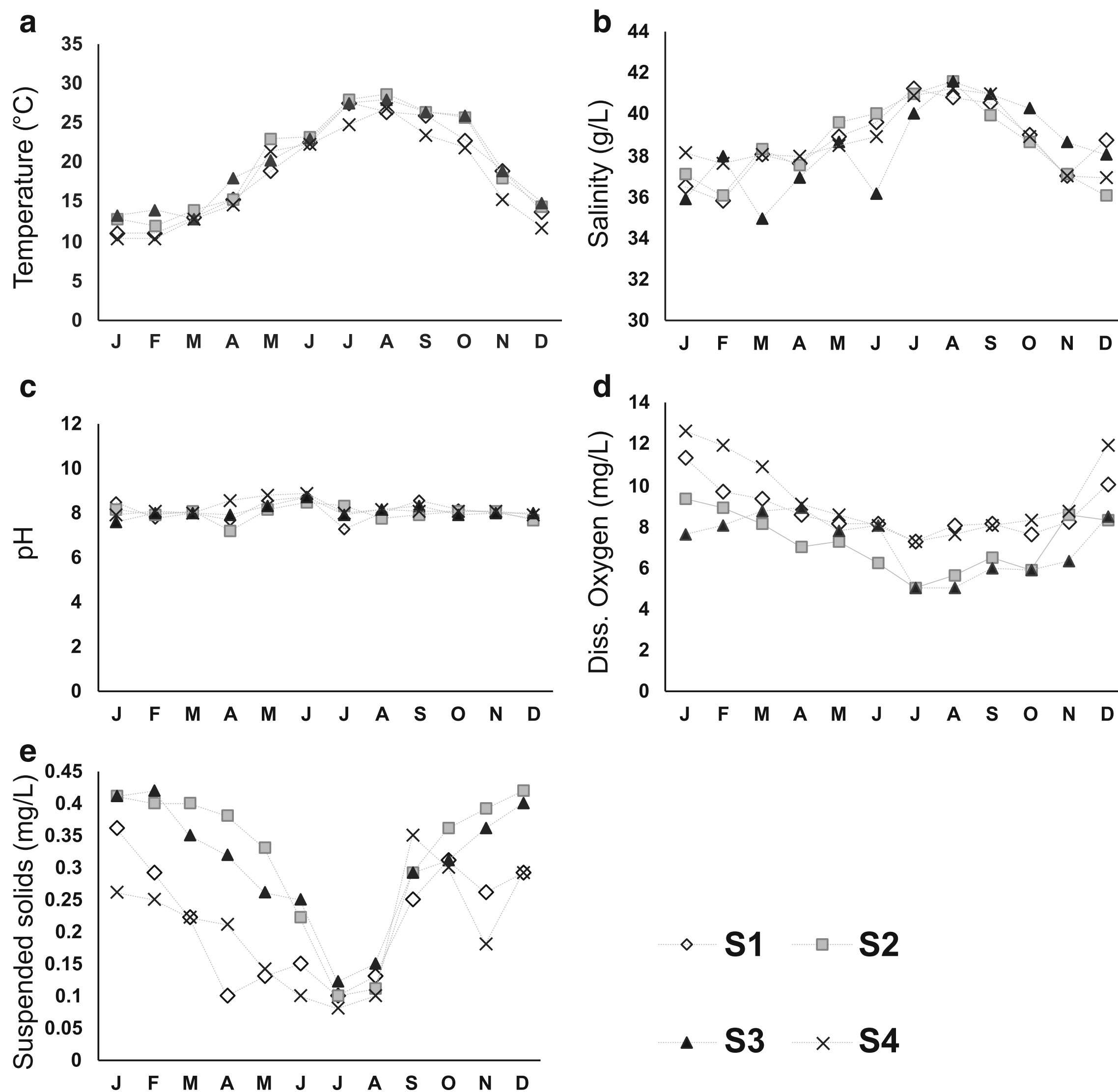

Fig. 2 Results of the physicochemical analysis of seawater samples at the four sampling sites. **a** Temperature (°C), **b** salinity (g/L), **c** dissolved oxygen (mg/L), **d** pH, and **e** suspended solids (mg/L). S1, Cap de Garde; S2, Rezgui Rachid; S3, Sidi Salem; S4, Lahnaya

Depending on the seasons of sampling (winter = December, January, and February; spring = March, April, and May; summer = June, July, and August; autumn = September, October, and November), the levels of FIB in both compartments were generally higher during the warmer months of the year. The maximum levels of TC in seawater (4.6×10^4 MPN/100ml) and *P. perna* mussels (3×10^5 MPN/g) were both recorded in summer. The highest concentrations of *E. coli* in seawater (1.2×10^4 MPN/100ml) and *P. perna* (2.5×10^4 MPN/100g) were detected in autumn, respectively. Finally, fecal contamination by FS was most pronounced in autumn in both compartments: 2.1×10^4 MPN/100ml in seawater, and 2.5×10^5 MPN/g in *P. perna* mussels (Fig. 3).

The Wilcoxon test was applied to test the significance of these differences. For TC, the most significant differences between the sampling seasons were between the autumn and winter (p=0.028), and between the autumn and spring (p=0.041). For *E. coli*, a significant difference was found between the autumn and winter (p=0.029). Finally, for FS, the most significant differences were found between the autumn and winter (p=0.023), and between the summer and winter (p=0.028) (Table S2).

Based on Spearman's correlation results, FIB-levels were highly correlated with each other ($p < 0.0001$) (Table 3). TC were found to be positively and significantly correlated with EC (r= 0.77, p<0.0001) and FS (r=0.56, p<0.0001). In addition, EC also showed positive and significant correlation with FS (r=0.45, p=0.0012)

The results of Spearman's correlation analysis between FIB and physicochemical variables are given in Table 3. According to the correlation coefficients, FS appeared to be the most correlated indicator with all environmental variables

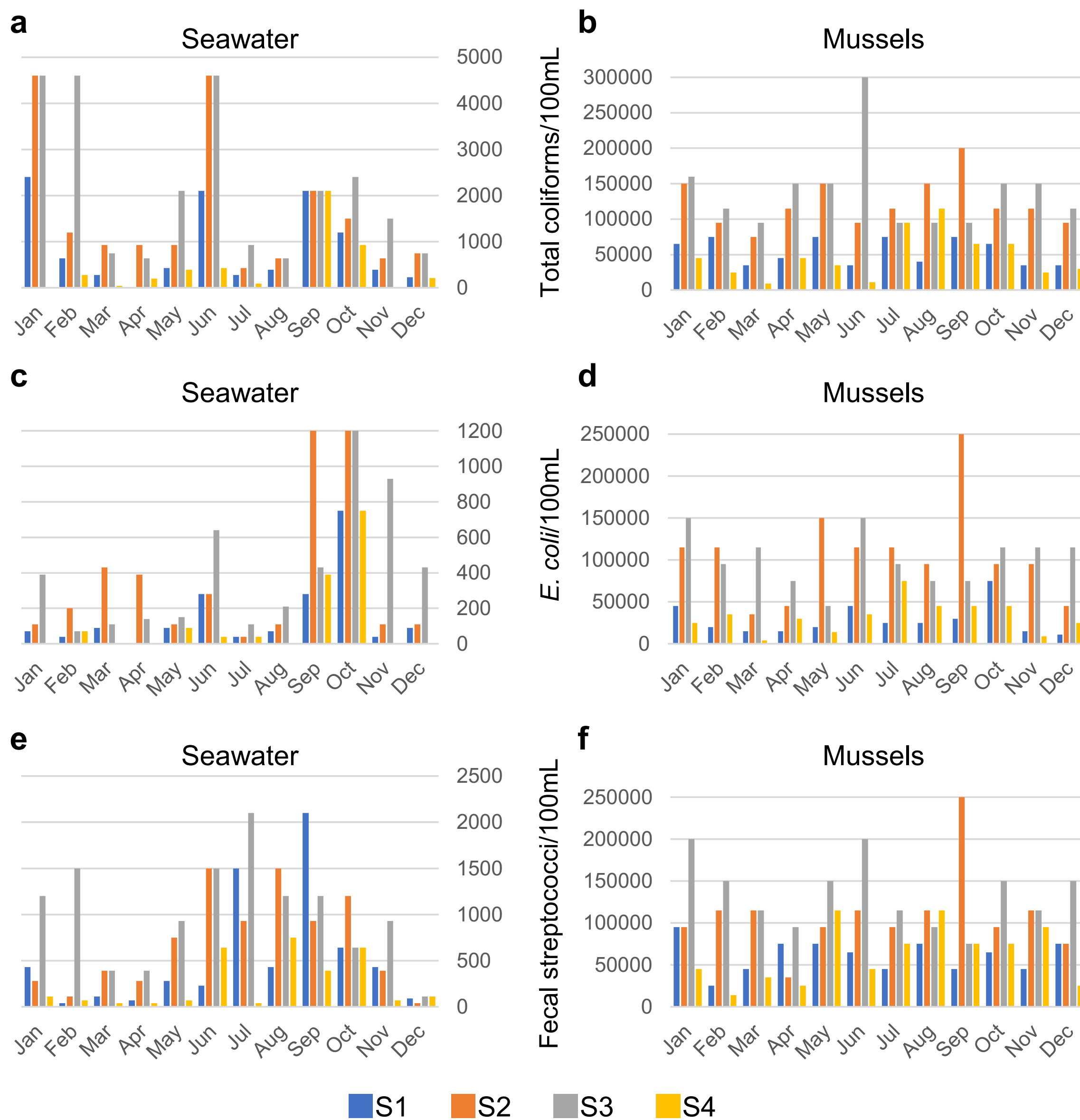

Fig. 3 Spatial and temporal variations of fecal indicator bacteria in seawater and mussels. **a** and **b** Total coliforms per 100 ml. **c** and **d** *Escherichia coli* per 100 ml. **e** and **f** Fecal streptococci per 100 ml. Note that the scales are different on each diagram. S1, Cap de Garde; S2, Rezgui Rachid; S3, Sidi Salem; S4, Lahnaya

except pH and SS: DO (r=−0.72, $p<0.0001$); temperature (r=0.64, $p<0.0001$) and salinity (r=0.46, $p<0.01$). In contrast, EC and TC were found to be positively and significantly correlated with SS (r=0.51, $p<0.001$; 0.57, $p<0.001$; respectively) and negatively correlated with DO (r=−0.46, $p<0.01$; r=−0.37, $p<0.01$; respectively).

Spatial–temporal abundance of potentially pathogenic bacteria in the Gulf of Annaba

During the entire study period, a total of 208 bacterial isolates (142 from mussels and 66 from seawater) belonging to 22 genera and 46 species were identified using biochemical tests. The most ubiquitous and abundant microorganism among all the environmental samples was *E. coli* (41.4%), followed by *Aeromonas hydrophila* (5.8%), *Klebsiella pneumoniae* (3.9%), *Pseudomonas aeruginosa* (3.4%), *Enterobacter cloacae* (2.9%), *Vibrio parahaemolyticus* (2.9%), *Burkholderia cepacia* (2.4%), *Morganella morganii* (2.4%), *Micrococcus spp* (1.9%) *Pseudomonas luteola* (1.9%), *Staphylococcus sciuri* (1.9%), *Staphylococcus xylosus* (1.9%), *Providencia rettgeri* (1.4%), *Salmonella spp* (1.4%), and *Yersinia enterolittica* (1.4%). Figure 4 shows the abundance of potentially pathogenic bacteria in both compartments (seawater and *Perna perna* samples), and in each sampling site throughout the entire study period. It only indicates

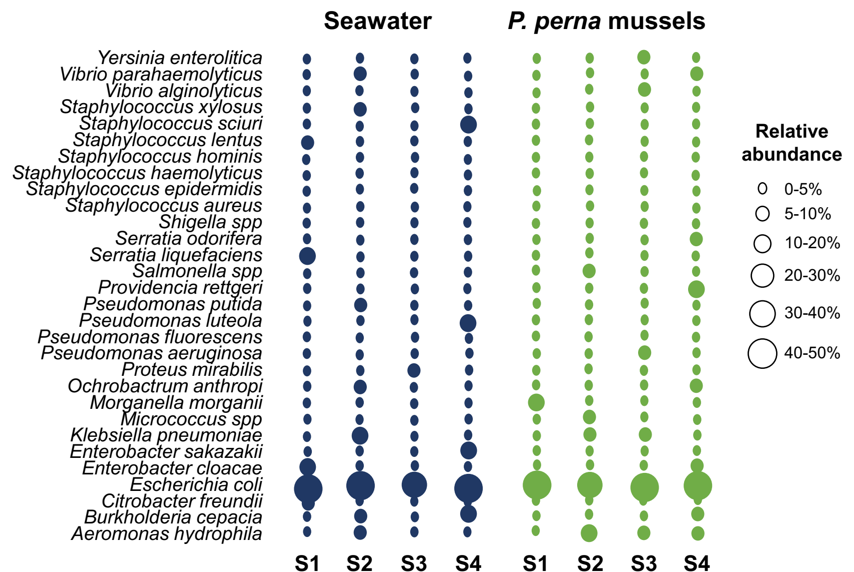

Fig. 4 Relative abundance of potential pathogenic bacteria in seawater (blue) and *Perna perna* mussels (green). (S1) Cap de Garde, (S2) Rezgui Rachid, (S3) Sidi Salem, and (S4) Lahnaya

bacteria found with more than two isolates per sample (see Table 1). In the seawater samples of S1 and especially of S4, the number of different potentially pathogenic strains did not exceed 14 whereas in S2 and S3, their number was 16 and 27, respectively. In *P. perna* samples, the number of these infectious agents was 16 in S4, 24 in S1, and 43 and 59 in S2 and S3, respectively (Table S3). The members of the family *Enterobacteriaceae* were dominant and presented the highest occurrence of all potentially pathogenic microorganisms (65.4%). This large group of bacteria was extensively found in all samples of *P. perna* mussels and its presence was most pronounced in S2 and S3 (Fig. 4; Table S3).

The results of this study also revealed a seasonal pattern in the diversity of potentially pathogenic bacteria. For both *P. perna* and seawater samples, the diversity increased during the warm-water months (between June and October), with the highest diversity of potentially pathogenic bacteria recorded in summer (June, July, August), closely followed by autumn (September, October, November) (Fig. 5).

Molecular identification of selected isolates

In addition to biochemical identification, 25 isolates of either Staphylococci or γ-proteobacteria were chosen for further identification via their 16S rRNA genes. Universal primers 27F and 1492R were used to PCR-amplify the 16S rRNA genes, and the products of approximately 1500 bp (Fig. 6) were Sanger sequenced. The 16S rRNA sequences were compared to the NCBI database, using BLAST. All sequences had between 97 and 100% identity to known bacterial species, permitting the identification of the analyzed strains (Table 4). A phylogenetic tree was generated to visualize the evolutionary placement of our environmental bacteria with respect to their closest studied relatives (Fig. 7). A main clade, with high bootstrap value (100% bootstrap), grouped 17 isolates of seven genera within the family of Enterobacteriaceae, namely, *Escherichia/Shigella spp.*, *Klebsiella spp.*, *Enterobacter spp.*, *Citrobacter spp.*, *Proteus spp.*, and *Morganella spp*. The *Staphylococci* were only represented by two isolates (BM2 and BS4) which were close to type strain *S. epidermidis* ATCC 10145^{T} (100% bootstrap). Overall, most (18/25) of the 16S rRNA gene sequence identification results matched with the genus identification using API tests (Table 4).

Multilocus sequence typing analysis (MLST)

E. coli comprised more than 40% of the isolated strains, and several individual *E. coli* isolates came from the very same environmental context (i.e., same sampling-site, sample-date, and environmental compartment). We therefore wondered whether these *E. coli* isolates were due to multiple separate contamination events or were caused by a single highly abundant *E. coli* strain which was able to thrive and outcompete other bacteria in the given condition. To test whether the five isolates (EM3, EM18, EM97, EM102, and MM6) obtained from *P. perna* mussels at S3 on the same sampling date (January 15, 2018) belonged to the same strain of *E. coli*, we PCR-amplified and Sanger-sequenced sections of seven conserved genes (*trpA*, *trpB*, *dinB*, *polB*, *putP*, *pabB* and *icdA*) (Fig. 8). A tree based on a multiple alignment of the concatenated sequences from our five strains (and the equivalent gene-sections from other *E. coli* strains) revealed that our isolates were not identical, and thus were probably not from a single contamination event. However, the isolates were closely related to each other, and slightly more distantly to *E. coli* strains K12 and SCU-103 (Fig. 9).

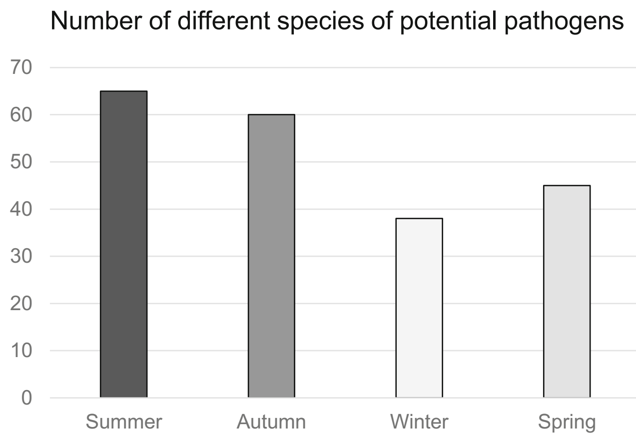

Fig. 5 Seasonal differences in the diversity of potentially pathogenic bacteria in both *P. perna* and seawater samples. The vertical axis represents the number of different potentially pathogenic strains identified from both *P. perna* and seawater samples collected from the four sampling sites combined. Summer: June, July, and August. Autumn: September, October, and November. Winter: December, January, and February. Spring: March, April, and May

Principal component analysis (PCA)

Principal component analysis (PCA) revealed that the three first main components together explain 92.4% of the total information (Fig. 10a and b). The first principal component (PC), which represents 56.3% of the variance, was the most significant component of the latter. It was mainly loaded by the Temperature (r = 0.95), Salinity (r = 0.89), Dissolved Oxygen (r = −0.77), SS (r = −0.66) and FS (r = 0.55). The second PC, representing 22.4% of the variation, was found to be positively correlated by pH (r = 0.86). The third PC, representing 13.7%, was positively correlated with TC and EC (r = 0.61 and r =0.52, respectively). According to the PCA plot a clear opposition was observed between the 12 months of sampling and the distribution of the four sites on the first two axes. S2 and S3 were strongly correlated with each other and showed maximum variations of fecal contamination during the warm months of the year, whereas S1 and S4 demonstrated lower fecal contamination variations during the cold months (Fig. S1).

Discussion

Bacteriological analyses of FIB in the Gulf of Annaba

Although our previous study detected a recent increase in bacterial contamination of the Gulf of Annaba (Hidouci et al. 2014; Kadri et al. 2015, 2017), the results of the present study revealed a highly alarming further increase of fecal contamination in the area, to a level that is higher than other coastal regions of the Mediterranean Sea (Bouhayene and Djebar 2014; Boutaib et al. 2015; Dallarés et al. 2018; Rincé et al. 2018). Much of this difference is probably due to the continuous pollution pressure in the Gulf, mainly related to anthropogenic activities, as well as rapid urbanization over the last few years. According to Inal et al. (2018), more than 40% of the Algerian population (more 19 million people) is living along the Gulfs near the largest agglomerations, of which

Table 4 Biochemical and molecular identification of 25 isolates isolated from *P. perna* mussels. *na*, not analyzed

Isolate code	Api identification	Best hit to 16S rRNA sequence	MLST identification
EM3	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
MM6	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
EM97	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
EM102	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
EM18	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
EM10	*Escherichia coli*	*Escherichia coli*	na
MM58	*Escherichia coli*	*Escherichia coli*	na
TCM7	*Aeromonas hydrophila*	*Proteus mirabilis*	na
MM59	*Chromobacterium violaceum*	*Pseudomonas aeruginosa*	na
SM25	*Citrobacter koseri*	*Enterobacter asburiae*	na
SM28	*Enterobacter cloacea*	*Enterobacter cloacea*	na
MM62	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	na
SM3	*Morganella morganii*	*Morganella morganii*	na
TCM19	*Ochromobacter anthropi*	*Morganella morganii*	na
IM14	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	na
MM56	*Pseudomonas fluorescens*	*Pseudomonas parafulva*	na
IM27	*Pseudomonas luteola*	*Pseudomonas aeruginosa*	na
MM2	*Pseudomonas putida*	*Pseudomonas aeruginosa*	na
SM13	*Proteus mirabilis*	*Proteus mirabilis*	na
MM39	*Salmonella choleraesuis*	*Citrobacter freundii*	na
MM23	*Salmonella spp*	*Shewanella algae*	na
SM17	*Serratia odorifera*	*Enterobacter asburiae*	na
MM27	*Shigella spp*	*Shigella dysenteriae*	na
BM2	*Staphylococcus lentus*	*Staphylocuccus epidermidis*	na
BM4	*Staphylocuccus epidermidis*	*Staphylocuccus epidermidis*	na

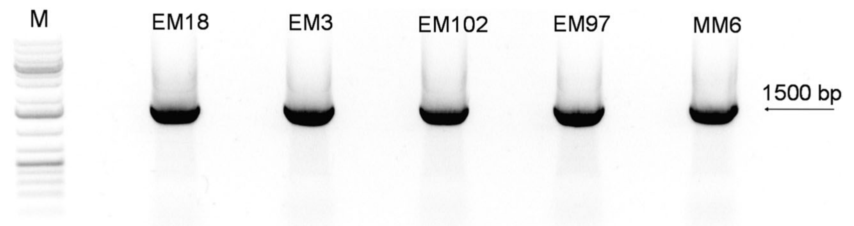

Fig. 6 PCR amplification of the 16S rRNA gene. M, DNA ladder; lanes (EM18–MM6) represent amplified product (approx. 1500bp) of *E. coli* isolates

Annaba is the third most populated, with almost 400 thousand inhabitants within the city itself (https://en.populationdata.net/countries/algeria/).

In the current study, these impacts were differently manifested depending on the local sources of pollution at each site.

The strong presence of FIB at S3 could be explained by significant urban, industrial and agricultural discharges that Wadi Seybouse (Fig. 1) drains from its catchment basin of about 6470 km^2 (ABH-CSM 1999-2000; Mebarki 2000). Further contribution to bacterial contamination at S3 presumably comes from untreated wastewater effluents from a large part of Annaba city and its outskirts, including a nearby slaughterhouse, which are discharged directly into the sea via Wadi Bedjima (Fig. 1). These contamination sources should be added to the natural contamination stemming from a large colony of seabirds and animals (Telailia 2014).

Our present results further demonstrated that the overall contamination was exceptionally high at S2, revealing that the waters of this site should be considered as unsafe for bathing in accordance with the Algerian Bathing Water executive decrees (JORA 1993, 2006). Similar to S3, these high levels of FIB were primarily due to the domestic wastewaters from nearby homes, which are discharged directly into the sea without prior treatment (Kadri et al. 2017).

The lower concentrations observed in S1 and especially S4 are presumably due to their remoteness from the city area and

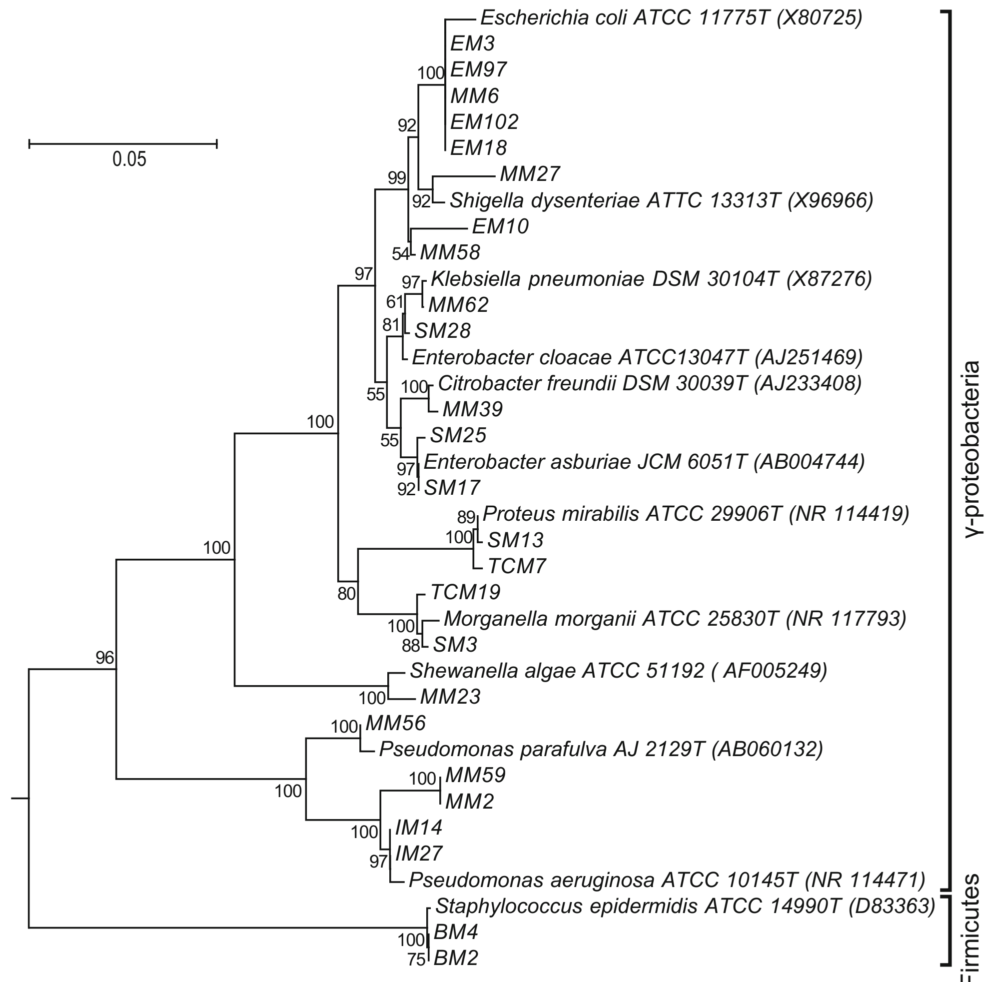

Fig. 7 Phylogeny of the 25 isolates with molecular identification. 16S rRNA sequences from the 25 selected isolates, together with the best hit from the GenBank database for each of the sequences, were compared in a Clustal X multiple sequence alignment (Kumar et al. 2016). Accession numbers of the reference sequences are in parentheses, and *Halobacterium* sp. A1T was used to root the tree

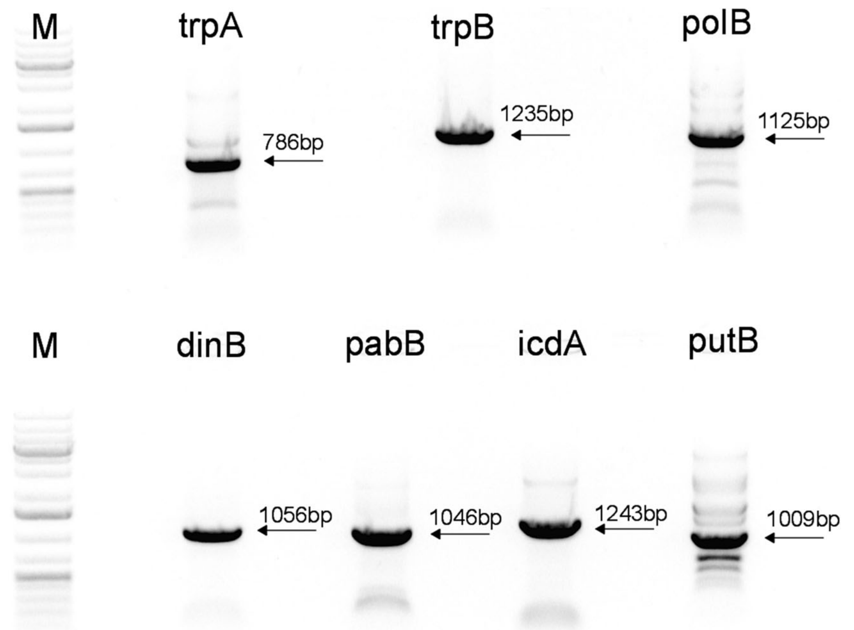

Fig. 8 PCR amplification of seven genes of *E. coli* EM97. Lanes *trpA* to *putB* represent PCR products amplified from the EM97 isolate (*E. coli*). M, DNA ladder

their strong water flux, which may contribute to the dispersion of fecal pollutants in the water column (Kadri et al. 2015). The coastal current of the Gulf of Annaba is associated with important North African upwelling processes (Arnone et al. 1990). This current permanently enters the Gulf of Annaba near Cap de Garde and flows to the east in the direction of the northeast part of the bay (Ouali et al. 2018). Thus, S1 and S4 are under the influence of dominant north-westerly winds that induce strong hydrodynamics and promote mixing of the coastal waters of the Gulf with less contaminated seawater. In contrast, S2 and S3 are characterized by relatively low hydrodynamic conditions and slow vortices (Hafsaoui et al. 2016; Ouali et al. 2018).

It is important to note that S1 and S4 are often visited by bathers in summer (just like S2), which is a possible explanation for the increasing levels of FIB during this period of the year (Kadri et al. 2017). This is due to the direct or indirect discharge of waste from toilets and tourist resorts located along the coast, the absence of water treatment plant, as well the lack of awareness among tourists regarding solid waste and wastewater collection and disposal (Torres-Bejarano et al. 2018).

According to several studies, fecal contamination at bathing beaches can be hazardous to humans because many pathogenic bacteria could be ingested during recreational water activities leading to various waterborne diseases (Marion et al. 2010; Santhiya et al. 2011; Arnold et al. 2016). The governments should therefore develop solutions to minimize the risks associated with the use of contaminated recreational water such as, the installation of wastewater treatment plants, prohibiting direct industrial and agricultural discharges, improving public awareness, and developing control measures by means of quality criteria (Kacar and Omuzbuken 2017).

Fecal contamination in *Perna perna* mussels

Data obtained showed that the levels of FIB were even higher in *P. perna* than in the surrounding seawaters during the entire study period. This is consistent with reports from other coastal regions worldwide, suggesting that this strong accumulation capacity is mainly related to the filter-feeding behavior of these sentinel organisms, which make them one of the best bioindicators of fecal pollution in coastal waters, even when pollutants are at low concentrations (Stabili et al. 2005;

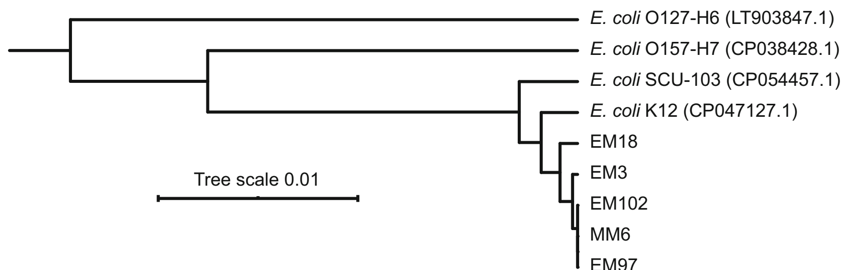

Fig. 9 The *E. coli* strains isolated from S3 are similar but not identical. Phylogenetic tree showing the distances between the five analyzed *E. coli* strains from S3, compared to *E. coli* strains from the NCBI database (accession numbers in parentheses). The tree was rooted using *Salmonella enterica*

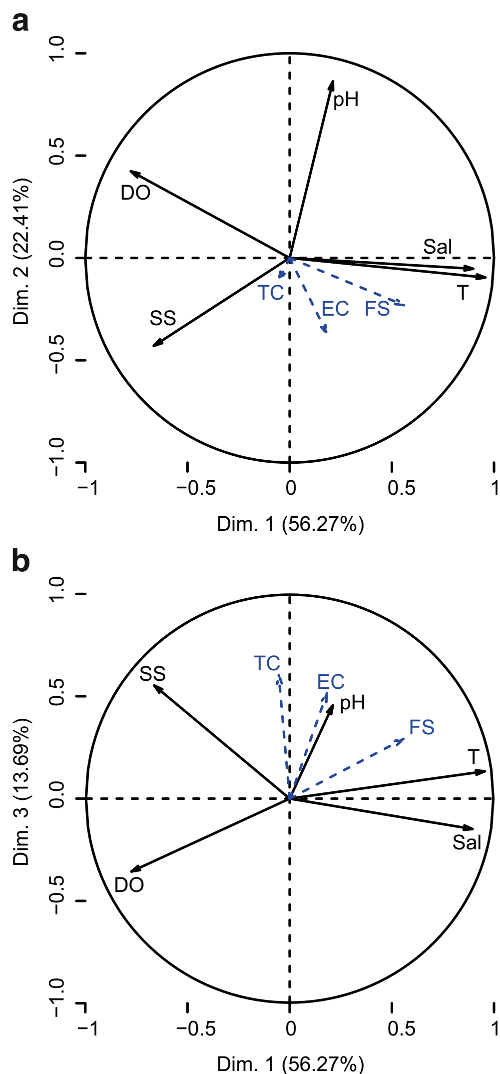

Fig. 10 Principal component analysis performed on data from seawater samples. **a** Correlation of environmental variables with the two first axes of the standard PCA. **b** Correlation of environmental variables with the first and third axes of the standard PCA. The main variables are indicated with solid arrows, and the supplemental variables are indicated with dotted arrows. DO, dissolved oxygen; Sal, salinity; T, water temperature; SS, suspended solids; EC, *Escherichia coli*; TC, total coliforms; and FS, fecal streptococci

Martinez and Oliveira 2010; Jayme et al. 2016; Bozcal and Dagdeviren 2020). It has furthermore been demonstrated that the mussels were contaminated with *E. coli* for a long period of time after a brief exposure to fecally contaminated water (Ho et al. 2000).

Similar to the study conducted by Stabili et al. (2005) and Cavallo et al. (2008) in the Northern Ionian Sea of Italy, the bacterial community of *P. perna* mussels from all sites in our study was very similar to that of surrounding waters but with higher abundance (Fig. 4). The levels of intestinal indicators in all sampling sites were well above the permissible limits recommended according to Regulation (854/2004/EC) of 29 April 2014 for human consumption, which recommends less than 230 *E. coli*/100g. Thus, the mussels inhabiting the Gulf of Annaba would be unfit for direct consumption.

FS were present in mussel and seawater samples throughout the entire sampling period with concentrations higher than *E. coli* in almost all samples, and this is also in agreement with previous reports (Tiefenthaler et al. 2009; Zegmout et al. 2011; Kadri et al. 2017; Islam et al. 2017). These FIB are known to have a better survival period than thermotolerant coliforms in surface waters, as well as in the digestive tract of bivalves (Geldreich and Litsky 1976, Noble et al. 2004).

Influence of environmental variables on fecal indicators in the Gulf of Annaba

In addition to anthropogenic activities, the survival was also influenced by a multitude of natural variables, among them; the seasonal variations of temperature were very likely responsible for the observed differences in bacterial concentrations in our samples. Indeed, our results demonstrated that elevated bacterial loads during the warmer months of the year (between June and October) were associated with maximum FIB rates in both compartments. Another probable reason for the high levels of FIB in *P. perna* in this period is probably the physiology of this sentinel species. Burge et al. (2016) have indicated that elevated temperatures promote an increase in the filtration rates in mussels and, therefore, they can retain more microorganisms from the surrounding waters. According to the Intergovernmental Panel on Climate Change (IPCC) Fifth Assessment Report (2013), Algeria will experience an increase in temperatures between 1°C and 3.7°C over the next few years. Consequently, this may extend the time period during which FIB can persist in the water (Barreras Jr et al. 2019). This hypothesis was supported by the significant and positive correlations between *E. coli* and FS, and the temperature revealed by Spearman's correlation test ($p < 0.05$) and PCA analysis in the current study, all of which is consistent with other studies (Koirala et al. 2008; Gutiérrez-Cacciabue et al. 2014; Abia et al. 2015; Islam et al. 2017).

In addition to elevated temperatures, summer also brings a large number of bathers to the beaches, which increase the influx of bacterial contaminants. On top of that, the presence of FIBs is affected by the local hydrodynamic patterns, which are calmer during summer, thus reducing the dispersion of fecal bacteria in the water column (Lacaze 1996).

In our study, only FS showed a significant positive correlation ($p < 0.001$) with salinity (Table 3). These FIB are known for their high resistance to harsh environmental stressors and tolerance to high concentrations to salt, making them powerful indicators of fecal contamination in seawater (Byappanahalli et al. 2012). Conversely, DO show the strongest correlations with all groups of FIB, mainly due to the bacterial degradation of detritus which consumes a lot of

oxygen. This biodegradation was more important with the increase in temperature in summer (5 mg/L) (Fig. 2), especially in highly contaminated sites, which receive massive quantities of domestic discharges and industrial effluents. These findings are in agreement with those of a recent study by Chávez-Díaz et al. (2020), which found negative correlations between DO and FIB. The latter were found to be positively correlated with SS, which, according to the literature, play a protective role for intestinal bacteria against solar radiation and predators (Walters et al. 2014; Kadri et al. 2017). This appears to be the case for the SS-rich waters of S2 and S3.

Numerous studies have indicated that FIB can be used as surrogates to estimate the possible presence of pathogenic microorganisms, especially when they are found at high levels (Wilkes et al. 2011; Shoults and Ashbolt 2018).

Abundance of potentially pathogenic bacteria in the Gulf of Annaba

For all samples in the study, it was consistently Proteobacteria which was the most dominant phylum (88/208, 46%). Within this phylum the most frequently isolated family was *Enterobacteriaceae*, which indicated that domestic wastes, especially from the most polluted sites are most likely the primary source of pollution in the Gulf of Annaba since enteric bacteria are mainly derived from the excrement of warm-blooded animals, including humans (Poharkar et al. 2017). The enteric bacteria, *Salmonella spp.*, *Shigella spp.* and, *E. coli* are major causes of human gastrointestinal tract infections, with 1.7 billion cases of human diarrhea recorded each year, primarily caused by pathogenic strains of *E. coli* (Yang et al. 2017), and 450 per 100,000 children in India and Pakistan contracting enteric fever caused by *Salmonella typhi* (Sánchez-Vargas et al. 2011; Neogi et al. 2014).

Environmental bacteria such as *Pseudomonas spp.*, *Aeromonas spp.*, and *Shewanella spp.* were also identified in both environmental compartments. Species of the genus *Aeromonas* are widely isolated from aquatic environments and frequently reported to cause waterborne and seafood infections (gastroenteritis and septicemia) (Chopra and Houston 1999; Joseph et al. 2013; Hamid et al. 2016). *Pseudomonas spp.* are ubiquitous microorganisms found in marine shellfish and recreational waters (Maravić et al. 2018; Goh et al. 2019). These opportunistic pathogens are frequently multidrug-resistant and associated with diarrhea, intraabdominal and nosocomial infections, particularly in immune-compromised patients (Morrissey et al. 2013; Streeter and Katouli 2016). The results of biochemical identification also revealed the detection of different species of the genus *Vibrio* in the four sampling sites, of which *V. paraheamolyticus* was the most isolated microorganism. *Vibrio.* spp are waterborne bacteria naturally found in estuarine and coastal environments. Yet, certain species can be pathogenic to humans and marine organisms such as bivalves (Eggermont et al. 2017; Rincé et al. 2018; Bozcal and Dagdeviren 2020). In August 2018, the Algerian Ministry of Health reported a cholera outbreak in Blida and five other regions (Algiers, Tipaza, Bouira, Médéa, and Ain Defla) in the north of the country. This devastating and strictly human epidemic caused mainly by *V. cholerae* O1 or O139 can cause severe dehydrating diarrhea and even death (Feldhusen 2000). In the Gulf of Annaba, *V. cholerae* was found in S3 mussels in the same period as the outbreak, classifying this site as the area of highest risk.

In addition to Proteobacteria, 24 isolates of the genus *Staphyloccocus* were also detected during the study period. These potentially pathogenic bacteria, including *S. aureus*, are well-known causative agents of several human diseases, such as skin rashes, pneumonia, ear and eye infections, endocarditis, and meningitis (Schets et al. 2020; Yaghoubzadeh et al. 2020). According to Pomykała et al. (2012), some coagulase-positive staphylococci are common seafood pathogens and may pose a significant risk to human health through improper consumption of bivalve mollusks. Furthermore, methicillin-resistant *S. aureus* (MRSA), which is one of the most harmful pathogens to human health, has also been frequently detected on several recreational beaches in the United States (Abdelzaher et al. 2010; Levin-Edens et al. 2012; Plano et al. 2013; Thapaliya et al. 2017).

Biochemical and molecular identification of bacteria

Strains of *Enterobacteriaceae* are known to be difficult to distinguish by conventional methods (Nhung et al. 2007; Hamdi et al. 2017). Besides, the use of biochemical identification alone can be problematic as some new taxa may not be included in available databases (Janda and Abbott 2002). For this reason, additional molecular identification targeting the 16S rRNA gene was performed on 25 strains, including two Gram-positive bacteria isolated from *P. perna* mussels of S3. In general, the biochemical identification at the genus level was confirmed in 72% of the cases by the 16S rRNA gene sequencing (Table 4). All strains exhibited more than 97% sequence similarities with their matching sequences retrieved from the GenBank database.

The ribosomal 16S rRNA gene has highly conserved regions in all bacterial cells, interspersed with nine hypervariable stretches of sequences (named V1–V9), and is a molecular fingerprint for bacterial identification and taxonomic classification (Benga et al. 2014; Jo et al. 2016; Monticelli et al. 2019). Sequencing of the 16S ribosomal RNA (rRNA) gene to study bacterial taxonomy and phylogeny has been the most widely used technique for several reasons: the detection and identification of fastidious, noncultured, and slow-growing bacterial pathogens, as well as novel isolates, its presence in all bacteria, and also its large size (approximately 1500 bp) which contains statistically valid and relevant

sequence information (Patel 2001; Clarridge 2004; Janda and Abbott 2007). Despite its accuracy, sequence analysis of the 16S rRNA gene has disadvantages when comparing closely related species, where the16S rRNA genes might not be sufficiently different to distinguish the two species. It is therefore often desirable to complement the 16S rRNA analysis with multilocus sequence typing (MLST). This technique examines multiple protein coding genes, which are more susceptible to genomic drift than the 16S rRNA gene. For some species, there are even standardized MLST approaches, which can be compared directly to public MLST databases (Sabat et al. 2013). However, the main disadvantage of this technique is its relatively high coast, since it requires more PCR and Sanger DNA sequencing reactions per analyzed strain (Boers et al. 2012; Sabat et al. 2013).

For the strains (EM3, EM18, EM97, EM102, and MM6) identified as *E. coli* using API tests and 16S rRNA gene sequencing, MLST was performed to further understand their phylogenetic relationships. Interestingly, the results indicate that our isolates were very similar to each other but nevertheless distinct, and therefore did not belong to the same strain of *E. coli* (Fig. 8). This suggests that they came from a variety of separate human and animal sources of fecal contamination, since *E. coli* is mainly found in the fecal wastes of warm-blooded mammals (Poharkar et al. 2017). Therefore, the use of Microbial Source Tracking techniques to identify both human and animal specific markers in future studies will be an important tool for understanding the origin of fecal pollution in the Gulf of Annaba, and for assessing the associated health risks related to the presence of pathogenic microorganisms.

To summarize, this study indicated that identification systems based on molecular techniques provide the means for accurate identification of potentially pathogenic bacteria in coastal waters, and are rapid when the required tools are available and the techniques have been established in the analyzing laboratory. In contrast, biochemical analyses are simpler to use, have an acceptable level of accuracy, and are less expensive compared with molecular techniques, but these techniques are time consuming, and their results are not always easy to interpret (Gracias and McKillip 2004; Russell et al. 1997). It is therefore prudent to use molecular identification when the results from biochemical identification are ambiguous.

Conclusion

The results obtained in this study allowed assessing the presence and distribution of fecal indicators and potentially pathogenic bacteria in *Perna perna* and seawater samples from the Gulf of Annaba. The bacteriological concentrations in *P. perna* were significantly higher than in seawater samples, and were well above the permitted limits for human consumption and recreational water in Sidi Salem (S3) and Rezgui Rachid (S2). Discharges from Wadi Seybouse and Wadi Bedjima, as well as anthropogenic activities, are likely to be important factors in the fecal contamination in the Gulf of Annaba. Additionally, the bacteriological quality of the water was influenced by multiple physico-chemical variables such as temperature, salinity, and dissolved oxygen, combined with the local hydrodynamic conditions. The survival and the presence of infectious agents in *P. perna* are a matter of great concern with regard to epidemic diseases that they may occur when mussels are consumed by humans. Therefore, the implementation of necessary measures should be carried out, especially in highly polluted sites in order to protect environmental resources and human health.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-13978-4.

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Author contribution MBo performed the experiments. MBo, SK, and MBe analyzed the environmental sampling data. PR analyzed the MLST data. MBo, SK, PR, and MBe wrote the paper.

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Data availability The datasets used and analyzed during the current study are available, upon reasonable request, from Mouna Boufafa (email: mouna_boufafa@yahoo.fr), the corresponding author.

Declarations

No ethical approval was required for this study.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare that they have no competing interests.

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