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# Impact of Seybouse and Bedjima Wadis Discharges on the Appearance of Antibiotic Resistant Gram-negative Bacterial Strains in the Annaba Gulf - Algeria

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**Abstract** Pathogenic microorganisms and antibiotic resistance in water are major global health and environmental concerns, impacting both human health and ecosystems. The objectives of this study are the isolation and identification of antibiotic-resistant pathogenic bacteria from wastewater discharges from wadi Bedjima (WB) which contains slaughterhouse discharges and wadi Seybouse (WS), of Annaba city. In addition to the measurements of physicochemical and microbiological parameters, pathogenic bacteria were identified by biochemical and molecular characterization and antibiotic sensitivity was studied by the disk diffusion method. Significant seasonal and spatial variations ($p \leq 0.001$) of physicochemical variables were estimated by the Kruskal-Wallis test. Fecal indicator bacteria levels at the two study sites largely exceeded the authorized limits. The highest concentrations were recorded during the warmest months of the year, namely $93x10^4$ CFU/100mL for total germs, $58x10^4$ CFU/100mL for total coliforms, $12x10^4$ CFU/100mL for fecal coliforms and $18x10^3$ CFU/100mL for fecal streptococci. Biochemical and molecular identifications showed that the bacteria isolated from the two wadis were mainly pathogenic species, including *Escherichia coli*, *Citrobacter freundii*, *Enterobacter roggenkampii*, *Pseudomonas oleovorans*, *Stutzerimonas stutzeri and Klebsiella pasteurii*. Analysis of antibiotic resistance phenotypes of isolated bacteria showed a broad presence of antibiotic-resistant bacteria in WS and WB (82.5% and 86.84%, respectively). The most frequently found resistances were against ampicillin, cefazolin, ceftazidime, cefotaxime, amoxicillin-clavulanic acid and ticarcillin.

**Keywords:** Pollution; Pathogenic Bacteria; Antibiotic Resistance;Wadis.

## Introduction

Water contamination is a serious environmental issue that harms human health and disrupts aquatic ecosystems. One of the key contributors to water pollution is microbial contamination, particularly by pathogenic microorganisms (Sabae and Rabeh, 2007). Surface water can pose significant health risks due to the potential presence of various pathogenic microorganisms, including bacteria, viruses, and protozoa. When this water is contaminated with human waste or sewage, it becomes a source of numerous waterborne diseases, such as dysentery, typhoid fever, cholera, and other gastrointestinal and respiratory illnesses. The level of sanitary risk associated with these pathogens depends on both the intended use of the water (such as for drinking, recreational activities, or bathing) and the concentration of pathogens present (Ouattara et al., 2011).

Fecal indicator bacteria (FIB) are commonly used to estimate the presence of pathogens from fecal contamination, as their abundance is often correlated to the density of these pathogens. Since FIB serve as a sign of fecal pollution, their presence can indicate the potential health risks associated with water use. Therefore, counting FIB is essential for assessing the level of microbial contamination in water (Ouattara et al., 2011; Some et al., 2021).

Several studies conducted in Algeria, particularly in the Gulf of Annaba, highlight a concerning level of degradation and significant fecal contamination of its surface waters, primarily due to increased human activity (Kadri et al., 2015; Boufafa et al., 2021).

Antibiotic residues in the environment contribute to the appearance of antibiotic resistance by exposing bacteria to these substances, allowing resistant strains to survive and spread (Gothwal and Sashidhar, 2017). According to Luque et al. (1994), natural waters contain many fecal-origin bacteria that carry genetic material coding for antibiotic resistance.

Antibiotic residues and antibiotic-resistant bacteria enter the aquatic environments, including rivers and wadis, through various routes such as industrial effluent, hospital and municipal wastewater, and agricultural runoff (Hanna et al., 2020). Wadis, in particular, serve as reservoirs for antibiotic resistance and play an important role in the spread of this resistance between different parts of the environment. These waterways can act as pathways, facilitating the transfer of antibiotic resistance between the environment,

humans, and animals (Xu et al., 2016).

Given that the surface waters of the Seybouse and Bedjima wadis in the city of Annaba are characterized by a high microbial density, intense industrial activities and extensive agriculture and livestock breeding, the aim of this study is to monitor the volution of different groups of bacteria. The analysis will be both quantitative and qualitative, with a primary focus on bacteria associated with fecal contamination. Additionally, we will assess the impact of physicochemical variables on the abundance of Gram-negative bacteria (FIB) and examine the antibiotic resistance profiles of potentially pathogenic strains in the waters.

## Materials and methods

### Description of the study sites

The sites were strategically selected based on their accessibility, location relative to urban areas, and proximity to various pollution sources. The geographic locations of the study sites are shown in Figure 1.

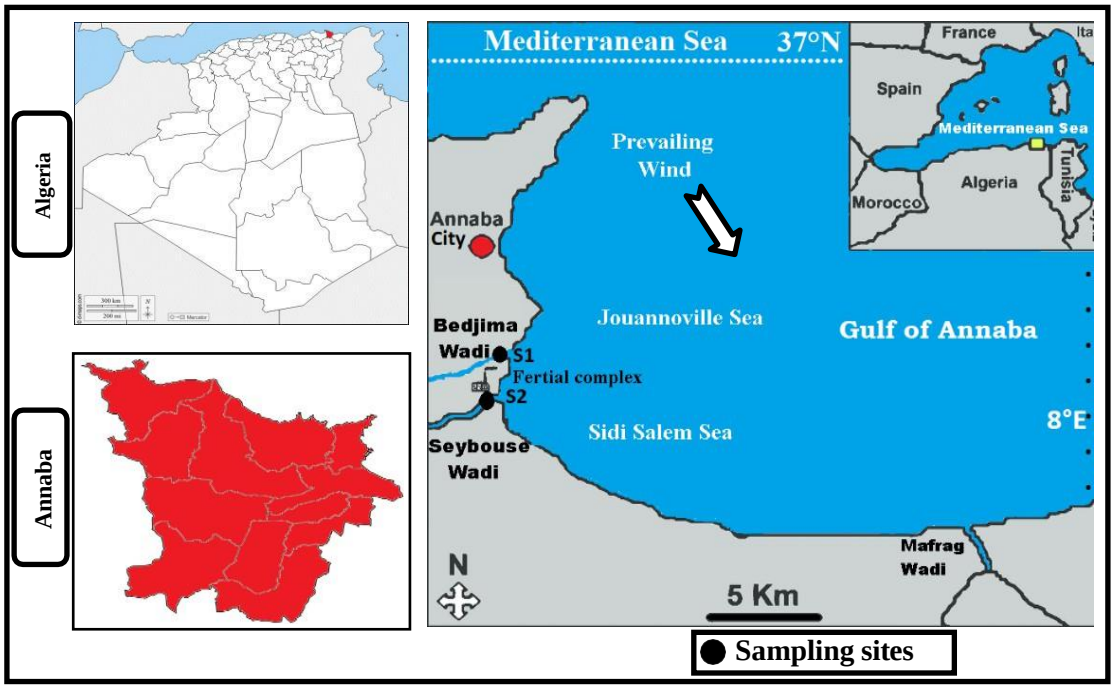


**Figure 1.** Map showing the location of the Gulf of Annaba and sampling sites

**Wadi Bedjima (S1)**: Located 100 meters before it discharges into the Joannoville Sea and 20 meters from the discharge points of a slaughterhouse, it receives all the wastewater from the western side of the town of Annaba before flowing into the coastline. **Wadi Seybouse (S2)**: Situated about 100 meters from the embouchure, it receives industrial

wastewater from Fertial factory, as well as urban and agricultural wastewater from municipalities and settlements in the catchment area.

**Samples and methods of analyses**

Water samples were collected monthly over a 12-month period, from March 2017 to February 2018, to monitor water quality and its seasonal and annual variations. Bacteriological samples were taken under rigorous aseptic conditions in sterile, labeled glass bottles with a 250 ml capacity. The samples were immediately transported from the sampling point to the laboratory in a cooler at 4°C within 2 to 4 hours.

**Physicochemical analyses**

Five parameters were measured *in situ,* water temperature (T°C), hydrogen potential (pH), electrical conductivity (EC) (µS/cm), dissolved oxygen (DO) (mg/L), and salinity (Sal) (g/L), by using a multi-field parameter with multiple probes (Model SX736). Suspended Solids (SS) (mg/L) concentrations were determined gravimetrically at the laboratory.

**Bacteriological analyses**

A series of analyses were carried out after serial decimal dilutions up to $10^{-5}$. Enumeration of total germs (TG) was conducted using the Plate Count Agar" medium (P.C.A.). The search for coliforms was conducted using a colimetry liquid medium, and the counting was performed using the MPN method. The detection of fecal streptococci (FS) was based on inoculation of a series of two tubes by dilution containing Rothe medium at 37°C for 24h to 48h (presumptive test). A subculture from the positive culture was then performed on Eva Litsky medium at 37 °C for 24 h (confirmatory test). According to the MacGrady table, all results were expressed in the number of germs per 100 mL (Rodier et al., 2005). The search for potentially pathogenic germs responsible for waterborne infections was conducted to evaluate the levels of possible contamination and assess their resistance to antibiotics. After the isolation on selective medium and purification, a preliminary identification of strains obtained in pure culture was based on Gram staining and oxidase reaction. The isolated bacteria were then subjected to biochemical tests, including Api 20 E and Api 20 NE (bioMérieux), to identify them at species level. Their identification was

further confirmed through 16S rRNA gene sequencing and phylogenetic analysis.

**DNA extraction and enzymatic amplification:** We selected 24 bacterial isolates based on their pathogenic potential and multiple antibiotic resistances. DNA extraction was performed from 18 to 24 hour old cultures grown on Luria Bertani agar. Molecular amplification by PCR (Polymerase Chain Reaction) was carried out using the extracted DNA as a template, with primers 27f and 1401r, as described in Batisson et al. (2009). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel to confirm the correct gene amplification, followed by purification of the PCR products.

**Sequencing analysis:** The purified products were sequenced using the Sanger method. The partial 16S rRNA genes sequences obtained were compared with the NCBI GenBank database using BLAST software, to confirm the species of isolates. A multiple sequence alignment was then performed using Clustal X software, and phylogenetic analysis was conducted with MEGA 5.0 software (Tamura et al., 2011). Finally, a phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei, 1987) and evaluated by bootstrap analysis based on 500 replicates (Felsenstein, 1958).

**Determination of antibiotic susceptibility**

The antibiotic susceptibility of the 116 bacterial isolates was determined using the disc diffusion method, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011), on Mueller- Hinton agar plates. Thirty-one antibiotics (ABs) from different groups of Gram-negative bacteria were tested. The antibiotics tested, along with their charges and abbreviations, are shown in Table 1.

Inhibition zones were interpreted as sensitive (S), intermediate sensitive (I), and resistance (R), Isolates that were intermediate or resistant were subsequently grouped into the same resistant class according to the reference to the CASFM (2014).

The multiple antibiotic resistances (MAR) index was calculated to compare the contribution of each human activity to environmental and public health risks **(**Amador et al., 2015**).** MAR index = X / (Y × Z). Where X = Number of antibiotics to which the isolated bacteria is resistant, Y = Total number of antibiotics tested, and Z= Total number of isolated bacteria. A MAR index value higher than 0.2 is observed when the isolated bacteria are resistant to a large number of antibiotics, indicating the presence of a significant contamination source of human or animal origin, where antibiotics are frequently used. In contrast, a MAR index value equal to or less than 0.2 is observed when

antibiotics are seldom or never used for human or animal treatment.

**Table 1.** List of antibiotics tested in the sensitivity study

| Antibiotic | Abbreviation | Charge (mg) |
|---|---|---|
| **Ampicillin** | AMP | 10 |
| **Penicillin** | PEN | 10 |
| **Ticarcillin** | TIC | 75 |
| **Piperacillin** | PIP | 75 |
| **Ertapenem** | ERT | 10 |
| **Imipenem** | IMP | 10 |
| **Meropenem** | MEM | 10 |
| **Aztreonam** | ATM | 30 |
| **Cefazolin** | CZN | 30 |
| **Cefoxitin** | FOX | 30 |
| **Cefotaxime** | CTX | 30 |
| **Cefepime** | FEP | 30 |
| **Ceftazidime** | CAZ | 30 |
| **Fosfomycin** | FOS | 200 |
| **Colistin** | Col | 10 |
| **Amikacin** | AKN | 30 |
| **Gentamicin** | GEN | 10 |
| **Azithromycin** | AZM | 15 |
| **Erythromycin** | ERY | 15 |
| **Tobramycin** | TOB | 10 |
| **Chloramphenicol** | CHL | 30 |
| **Tetracycline** | TET | 30 |
| **Doxycycline** | DOX | 30 |
| **Minocyclin** | MIN | 30 |
| **Nalidixicacid** | NA | 30 |
| **Ciprofloxacin** | CIP | 5 |
| **Levofloxacin** | LEV | 5 |
| **Trimethoprim-sulfamethoxazole** | SXT | 1.25+23.75 |
| **Nitrofuran** | NIT | 300 |
| **Amoxicillin clavulanic acid** | AMC | 20+10 |
| **Ticarcillin-clavulanic acid** | TC | 75+10 |

## Statistical analysis

All statistical analyses were performed using R (version 4.0.1; R Development Core Team 2020). First, we applied the Shapiro-Wilk test to check the normality condition of our variables distributions; we have selected nonparametric tests for our statistical analysis since our data are not normally distributed. To make comparisons between inter-seasons and inter-sites, we used principal component analysis (PCA, package

"factoextra"), Kruskal Wallis, and Wilcoxon test. "ggplot 2" package was used to assess boxplots, and the package "corrplot" to calculate the correlation coefficient between the different physicochemical parameters and microbiological parameters. For all tests, the significance level for differences in critical values was set to p-value 0.05.

## Results

### Physicochemical parameters

The analysis of the physicochemical parameters in Figure 2 showed that:

On a spatial scale, the temperature, pH and dissolved oxygen fluctuations did not show significant differences in inter-sites. However, on a temporal scale, the measurements of these three variables showed a significant difference ($p \leq 0.001$) between wet and warm periods (seasonal variability). So, the temperature values varied from 11°C to 29.02°C. The highest (29.02 °C) was recorded in August, while the lowest one was (11°C) detected in January 2018. Whereas, the temporal evolution of pH for two sites showed that they are slightly alkaline, where pH values vary between 5 and 8.16.

Concerning dissolved oxygen, the analysis of the obtained results showed that the extreme minimum and maximum values recorded were 0.06 mg/L (S1) and 8.13 mg/L (S2), the lowest value recorded during the warm period (summer), while the highest one was detected during the wet period for all sites.

A significant inter-site variation was estimated by the Kruskal-Wallis and Wilcoxon tests for the variables of salinity, conductivity ($p < 0.0001$), and suspended solids ($p < 0.05$).The salinity and conductivity values ranged between (0.9 - 10.2 g/L) and (1.22 - 10.64 µS/cm) respectively. In terms of SS content, the highest value (498.20 mg/L) was recorded at site 1 in the winter and the lowest was detected (52.80 mg/L) at site 2 in the summer. With reference to the maximum limit value (25 mg/L) allowed by the Algerian authorities (JORA, 2011) we note a clear exceedance of this value during the study period and at the sampling sites.

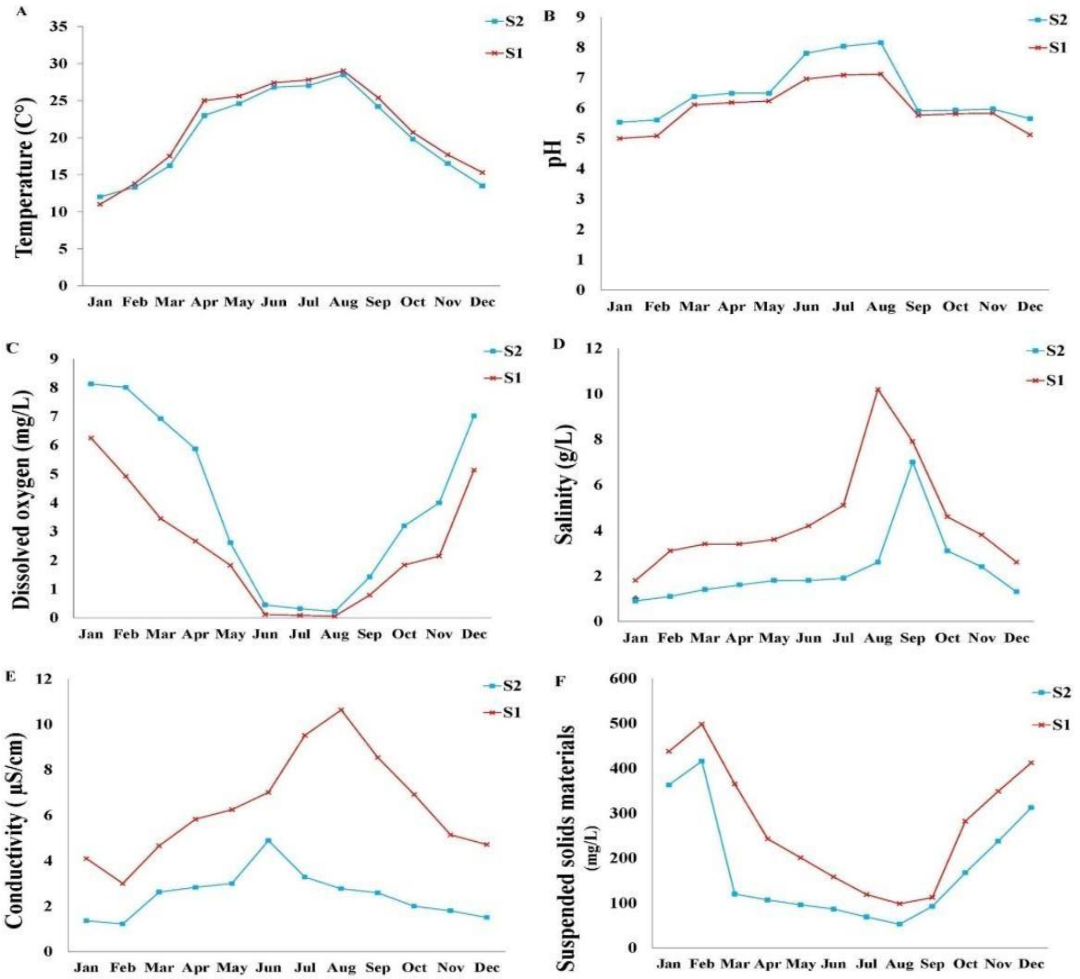


**Figure 2.** Spatio-temporal variation of the different physicochemical parameters during the study period in the two sites. ***A*** *Temperature (°C),* ***B*** *pH,* ***C*** *Dissolved oxygen (mg/L),* ***D*** *Salinity (g/L),* ***E*** *Conductivity (µS/cm), and* ***F*** *Suspended solids (mg/L). S1, Bedjima wadi; S2, Seybouse wadi*

## Bacteriological results

### Fecal contamination in the Gulf of Annaba

The spatiotemporal variation of total germs and bacteria indicative of fecal contamination in Figure 3, showed fluctuations during the study. The average TG, load was $35x10^4$ (Colony Forming Unit) CFU/100mL at S1 and $15x10^4$ CFU/100mL at S2. The minimum value ($6x10^2$ CFU/100mL) was recorded in S2 in February. While the highest one ($93x10^4$ CFU/100mL) was recorded at S1 in August.

The spatial variation of fecal coliforms (FC) results is similar to the total coliforms (TC), and at the temporal scale, the load of these variables is higher during the warmer months of the year. The maximum level ($58x10^4$ CFU/100mL) was recorded at S1 and the

minimum one ($12x10^4$ CFU/100mL) was obtained at S2. Kruskal- Wallis test showed a highly significant difference ($p < 0.0001$) for these two variables among the two study sites. Their contamination levels greatly exceeded the limits set by Algerian law, which are 500 TC/100mL, 100 FC/100mL (JORA 2011), throughout the entire study period. Concerning FS, their load was varied from 35x10 to $13x10^3$ CFU/100mL.The peaks was recorded in August, while the Wilcoxon test showed a highly significant seasonal variation($p<0.0001$).

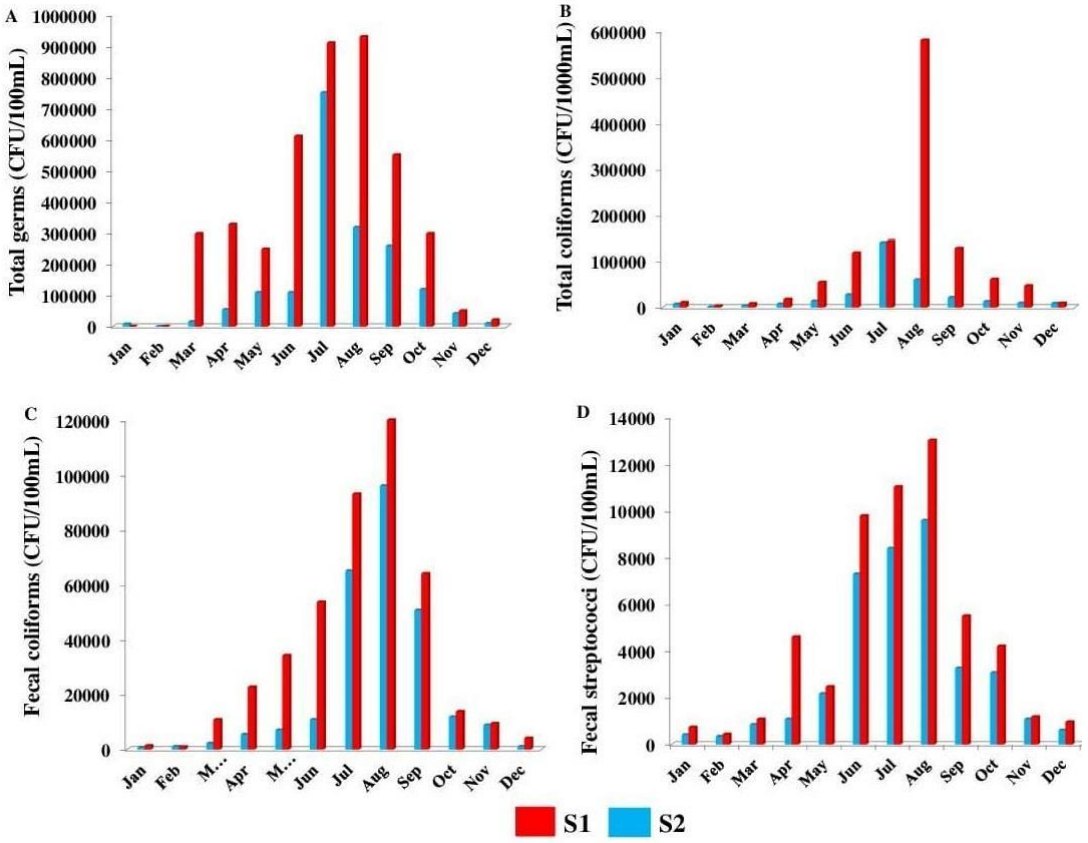


**Figure 3.** Spatio-temporal variation of total germs and fecal indicator bacteria in the two sites. ***A*** *Total germs per 100mL.* ***B*** *Total coliforms per 100mL.* ***C*** *Fecal coliforms per 100mL.* ***D*** *Fecal streptococci per 100mL*

**Abundance of potentially pathogenic bacteria**

**Biochemical characterisation of bacterial isolates:** One hundred sixteen bacteria strains were isolated from surface water samples of the Seybouse and Bedjima wadis (76 from S1 and 40 from S2), belonging to 18 genera and 39 species. The most abundant bacteria among all environmental samples was *Escherichia coli* (62.5%), followed by *Aeromonas hydrophila* (50%), *Pantoea spp* (37.5%), *Vibrio vulnificus* (33.33%), *Citrobacter freundii* (25%), *Proteus mirabilis,* and *Aeromonas sobria* (20.83%). The

relative abundance of isolates is presented in Figure 4.


**Figure 4.** Relative abundance of potential pathogenic bacteria in wadis waters

Our results also revealed seasonal variation in the presence of potentially pathogenic bacteria in both wadi samples: Bedjima and Seybouse. Figure 5 and 6 show that bacterial diversity increased between April and September (the warmer months), with the highest diversity observed during the summer period at both sites.

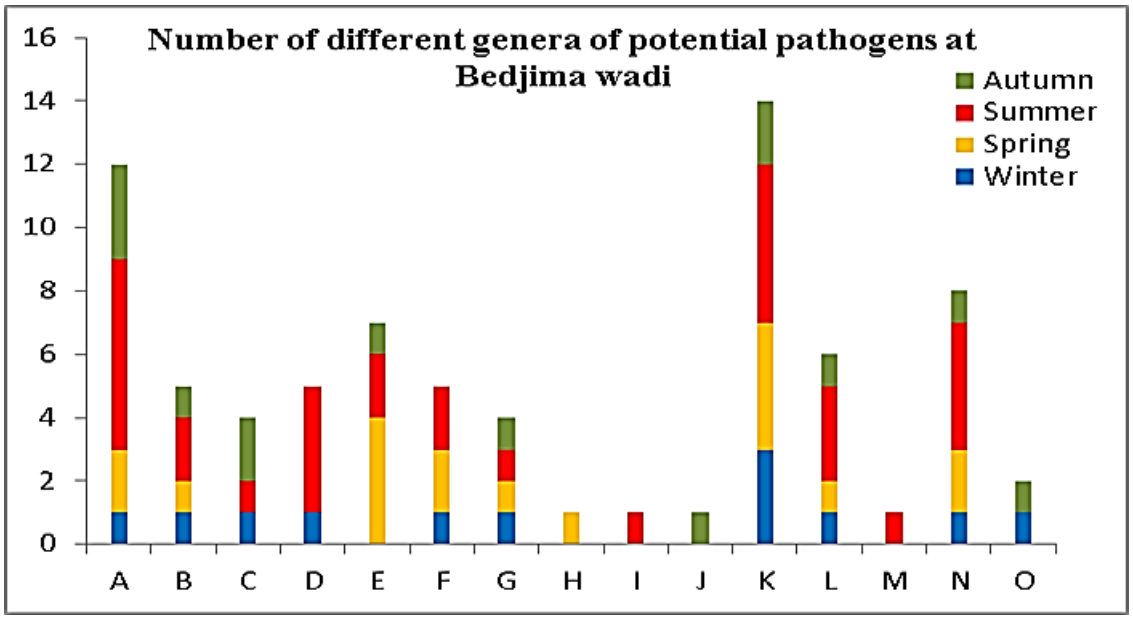


**Figure 5.** Temporal differences in the diversity of potentially pathogenic bacteria in Bedjima wadi. *A = Escherichia, B = Pantoea, C = Proteus, D = Citrobacter, E = Serratia, F = Klebsiella, G = Enterobacter, H = Shigella, I = Morganella, J= Hafnia, K = Aeromonas, L = Pseudomonas, M= Brevundimonas, N= Vibrio, O = Pasteurella*

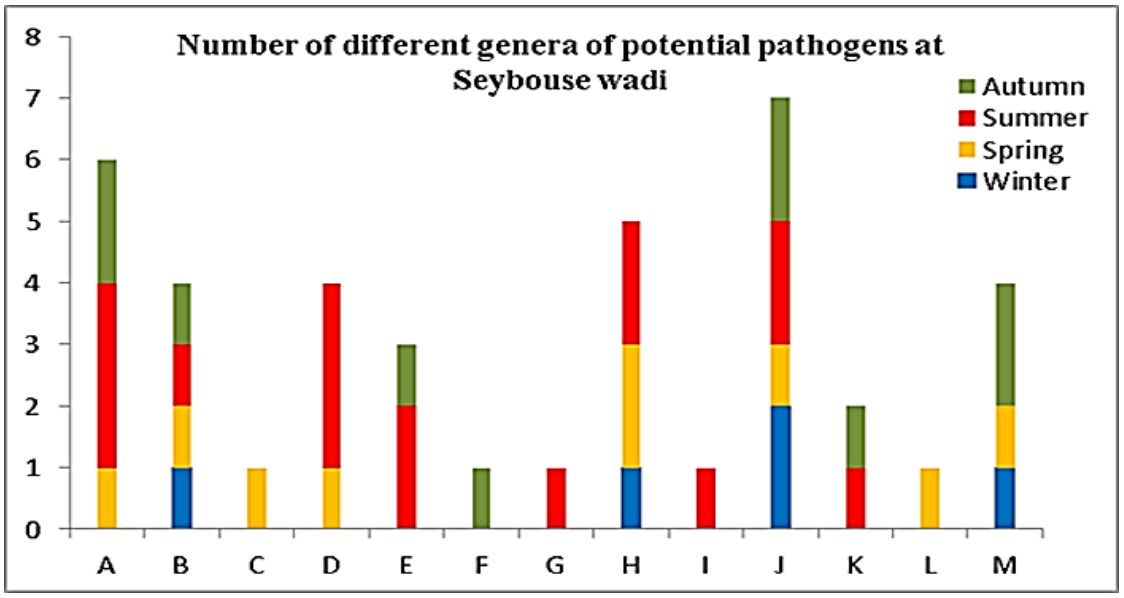


**Figure 6.** Temporal differences in the diversity of potentially pathogenic bacteria in Seybouse wadi. *A = Escherichia, B = Pantoea, C = Proteus, D = Citrobacter, E = Serratia, F = Enterobacter, G = Salmonella, H = Aeromonas, I = Pseudomonas, J = Vibrio, K = Pasteurella, L = Mannheimia, M = Burkholderia*

**Molecular identification of selected isolates:** The results of molecular identification after amplification and sequencing of PCR products are shown in Table 2. A phylogenetic tree has been generated to visualize the evolutionary position of our environmental bacteria in relation to their closest studied relatives (Figure 7). The tree has been obtained for nine species of the 24 isolates selected, the sequences presenting conflicts have been eliminated.

**Table 2.** Biochemical and molecular identification of 24 bacterial isolates selected

| Isolate code | Biochemical identification | Identification 16S rDNA |
|---|---|---|
| AHM35 | *Morganella morganii* | *Shigella flexneri* strain GBB2T |
| AHM18 | *Escherichia coli* | *Escherichia coli* strain 85QC2CO |
| AHM24 | *Escherichia coli* | *Escherichia coli* strain 3QC3O2 |
| AHM32 | *Escherichia coli* | *Escherichia coli* strain 85QC2CO |
| BLK9 | *Escherichia vulneris* | *Escherichia coli* strain A2Ɵ1 |
| AHM4 | *Citrobacter freundii* | *Citrobacter freundii* strain KCOM 1Ɵ23 |
| AHM23 | *Citrobacter freundii* | *Stutzerimonas stutzeri* strain SGAirƟ442 |
| AHM12 | *Citrobacter farmeri* | *Citrobacter sp*. S39 |
| AHM27 | *Citrobacter koseri* | *Citrobacter freundii* strain CH-GX-LA13-2Ɵ22 |
| AHM5 | *Enterobacter vulneris* | *Enterobacter roggenkampii* strain 1384Ɵ yvys |
| AHM2 | *Enterobacter cloacae* | *Citrobacter freundii* strain L75 |
| AHM34 | *Serratia marcescens* | *Citrobacter portucalensis* strain FDAARGOS_738 |
| AHM21 | *Serratia ficaria* | *Pseudomonas sp*. GDƟ 3919 |
| AHM47 | *Serratia ficaria* | *Pseudomonas putida* strain: MUFP69 |
| AHM11 | *Salmonella spp* | *Pseudomonas oleovorans* strain CGKV/78e2Ɵ13 |
| AHM31 | *Klebsiella oxytoca* | *Klebsiella pasteurii* strain KO2ƟƟ 862 |
| AHM10 | *Klebsiella pneumoniae spp pneumoniae* | *Uncultured Klebsiella sp*. clone 1-2-43 |
| AHM29.1 | *Plesiomonas shigelloides* | *Escherichia coli* strain M3 |
| BLK3 | *Pseudomonas putida* | *Pseudomonas oleovorans* JCM 13981 |
| AHM45 | *Pseudomonas luteola* | *Escherichia coli* strain Y8-2 |
| AHM13 | *Aeromonas sobria* | *Escherichia coli* strain Y8-2 |
| AHM16 | *Acinetobacter baumannii* | *Escherichia coli* strain GT8 |
| BLK1 | *Acinetobacter baumannii* | *Microbacterium imperiale* JCM 1378 |
| Ia1 | *Burkholderia cepacia* | *Pseudomonas sp*. SW3 |

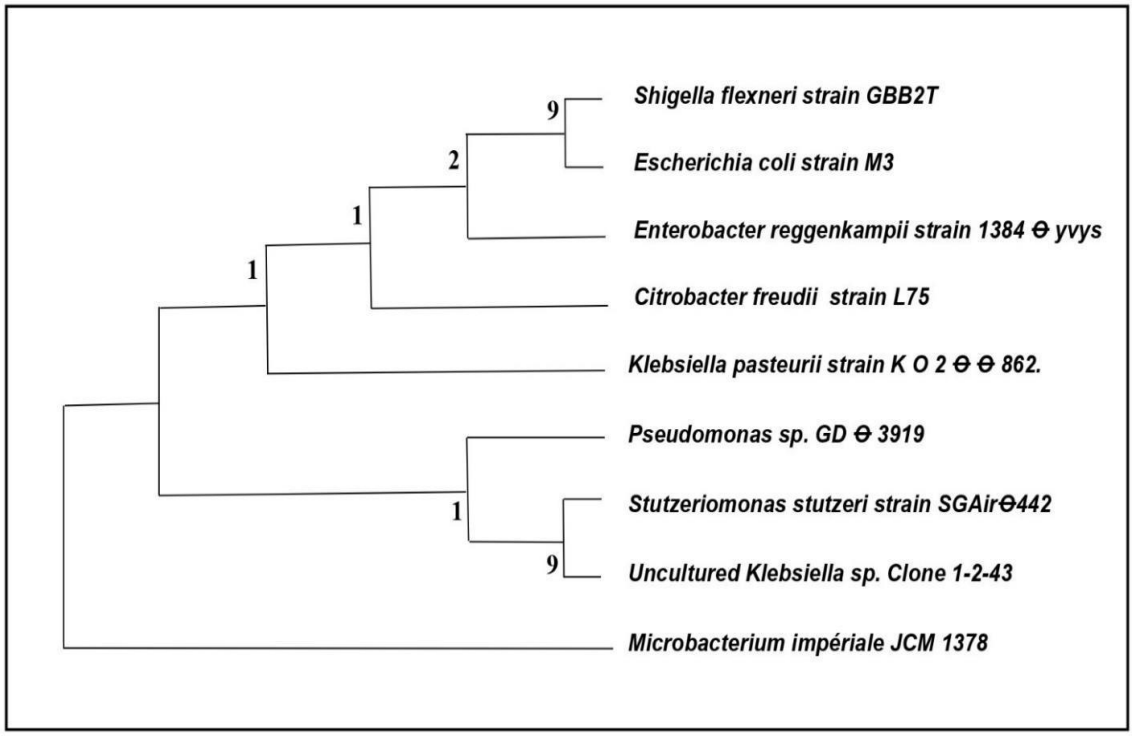


**Figure 7.** Phylogenetic tree and evolutionary relationships between the nine spices of the 24 isolates selected

**Antibiotic susceptibility testing**

The analysis of antibiotic resistance phenotypes in the waters of the two wadis revealed that most of the isolated bacteria were resistant to more than one antibiotic. The Figure 8 shows that the most frequently found resistances were to ampicillin (47.17%), followed by cefazolin (46.9%), trimethoprim-sulfamethoxazole (32.63%), cefotaxime (30.52%), amoxicillin-clavulanic acid (30.26%).

The isolated gram-negative bacterial strains from the two sites showed high percentages of resistance to certain beta-lactams, particularly in the case of strains from wadi Bedjima. More than 35% of these isolates were resistant to AMP (61.84%), CZN (51.31%), CTX (46.05 %), TCC (40.78%), CAZ (39.47 %), AMC (35.52 %) and other classes of antibiotics (non-β-lactams) us to SXT (S1: 30.26%, S2: 35%) and NIT (S1: 30.26%, S2: 17.5%). However, lower resistance frequencies were observed in the isolated strains from both sites for the antibiotics ERT, ATM, FOS, Col, NA, CIP, and CHL. The resistance to the CAZ, AKN, and GEN was lower at the S2, and no resistance was observed at the latter to MEM, AZM, LEV, MIN, DOX, TET, and TOB.

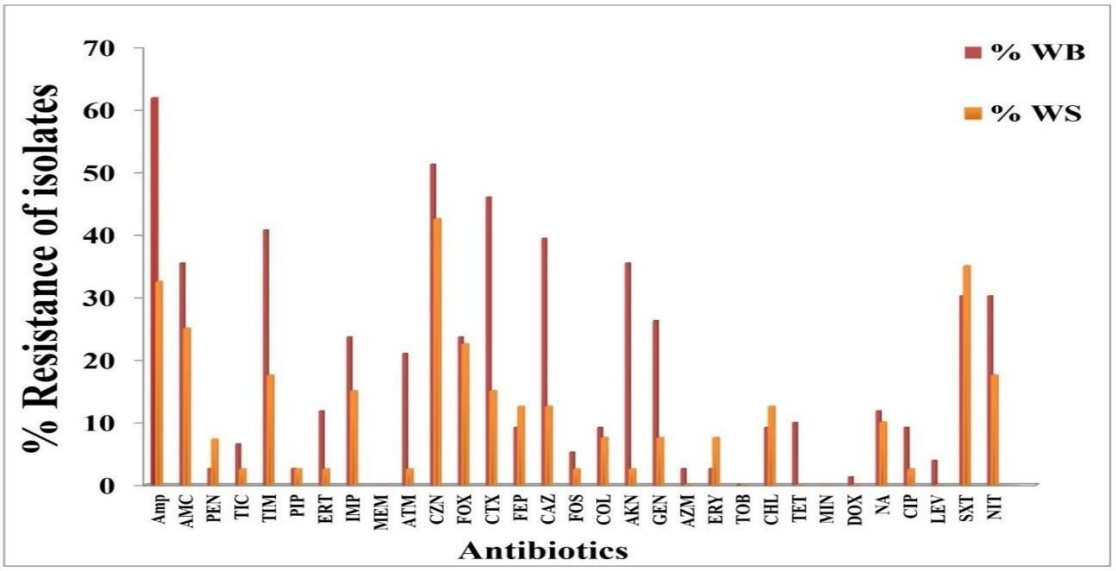


**Figure 8.** Antibacterial resistance of bacteria isolated from the waters of the two wadis. *AMP: Ampicillin; PEN: Penicillin; TIC: Ticarcillin; PIP: Piperacillin; ERT: Ertapenem; IMP: Imipenem; MEM: Meropenem; ATM: Aztreonam; CZN: Cefazolin; FOX: Cefoxitin; CTX: Cefotaxime; FEP: Cefepime; CAZ: Ceftazidime; FOS: Fosfomycin; Col: Colistin; AKN: Amikacin; GEN: Gentamicin; AZM: Azithromycin; ERY:Erythromycin; TOB: Tobramycin; CHL: Chloramphenicol; TET: Tetracycline; DOX: Doxycycline; MIN: Minocyclin; NA: Nalidixic acid; CIP: Ciprofloxacin; LEV: Levofloxacin; SXT: Trimethoprimsulfamethoxazole; NIT: Nitrofuran; AMC: Amoxicillin- clavulanic acid; TIM: Ticarcillin-clavulanic acid*

Fifty (50) different resistance patterns were observed among the 116 bacterial strains from the two wadis (Table 3). The isolated bacteria from site S1 exhibited 32 antibiotic resistance patterns, while at S2, 19 resistance patterns were noted. According to Table 3, resistance to the 3 antibiotics was the most frequent, with 8 different resistance patterns observed.

The MAR index of the sampling sites ranged from 0.06 to 1, with the highest values observed at site S1. More than half of the MAR index values of strains were found to be equal to or more than 0.20. The highest one was exhibited by *Morganella morganii* (1), followed by *Vibrio alginolyticus* (0.71). The other strains that recorded lower values were as follows: *Pseudomonas aeroginosa* (0.63) and *Pseudomonas putida* (0.63) from S1, *Aeromonas sobria* (0.62) and *Citrobacter farmeri* (0.52) from S2, *Vibrio parahaemolyticus* (0.35), *Serratia liquefaciens* (0.34), *Citrobacter koseri* (0.31), *Enterobacter vulneris* (0.29), *Pseudomonas luteola* (0.27), *Serratia odorifera* (0.26) and *Shigella spp* (0.26) from S1. The MAR index ranged from 0.21 to 0.25 for the other bacteria strains.

**Table 3.** Resistance patterns of the bacteria strains obtained from waters of Bedjima and Seybouse wadis.

| Résistance patterns | Number of isolates having certain resistance pattern at each site | | N of isolate | N of antibiotics | Resistance bacteria |
|---|---|---|---|---|---|
| | S2 | S1 | | | |
| **TIC** | 0 | 1 | 1 | 1 | ***Pseudomonas mendocina*** |
| **CAZ** | 0 | 1 | 1 | 1 | ***Aeromonas salmonicida spp masoucida*** |
| **CHL** | 1 | 0 | 1 | 1 | ***Vibrio parahaemolytis*** |
| **CZN/FOX** | 0 | 1 | 1 | 2 | ***Klebsiella pneumoniae spp ozaenae*** |
| **CZN/AKN** | 0 | 1 | 1 | 2 | ***Klebsiella oxytoca*** |
| **CZN/SXT** | 0 | 1 | 1 | 2 | ***Hafnia alvei*** |
| **CZN/NIT** | 1 | 0 | 1 | 2 | ***Serratia marcescens*** |
| **PEN/ERY** | 1 | 0 | 1 | 2 | ***Mannheimia haemolytica*** |
| **TIC/TIM** | 0 | 1 | 1 | 2 | ***Brevundimonas vesicularis*** |
| **TIC/TIM/ IMP** | 1 | 1 | 2 | 3 | ***Pseudomonas luteola*** |
| **TIC/TIM/ATM** | 0 | 2 | 2 | 3 | ***Pseudomonas fluorescens*** |
| **AMP/TIM/CZN** | 0 | 1 | 1 | 3 | ***Serratia plymuthyca*** |
| **AMP/AMC/SXT** | 3 | 0 | 3 | 3 | ***Vibrio vulnificus*** |
| **AMP/CTX/SXT** | 3 | 0 | 3 | 3 | ***Vibrio hollisae*** |
| **AMP/CZN/GEN** | 1 | 0 | 1 | 3 | ***Citrobacter koseri*** |
| **AMC/CZN/FOX** | 1 | 0 | 1 | 3 | ***Enterobacter cloacae*** |
| **CZN/Col/NIT** | 1 | 0 | 1 | 3 | ***Salmonella spp*** |
| **CZN/Col/NIT/CTX** | 1 | 0 | 1 | 4 | ***Proteus mirabilis*** |
| **AMP/PEN/ERY/SXT** | 2 | 0 | 2 | 4 | ***Pasteurella pneumotropica*** |
| **AMP/FEP/AKN/GEN** | 0 | 1 | 1 | 4 | ***Aeromonas salmonicida spp salmonicida*** |
| **AMP/AMC/SXT/FEP/FOX** | 4 | 0 | 4 | 5 | ***Aeromonas hydrophila*** |
| **AMP/AMC/SXT/CTX/TET** | 0 | 1 | 1 | 5 | ***Vibrio alginolyticus*** |
| **AMP/SXT/CTX/TET/NA** | 0 | 2 | 2 | 5 | ***Vibrio parahaemolyticus*** |
| **TIM/CAZ/PIP/CHL/SXT** | 4 | 0 | 4 | 5 | ***Burkholderia cepacia*** |
| **TIM/CAZ/CZN/Col/NIT** | 0 | 1 | 1 | 5 | ***Shigella spp*** |
| **AMP/AMC/CTX/IMP/CZN/FOX** | 2 | 0 | 2 | 6 | ***Serratia ficaria*** |
| **AMP/AMC/CTX/TET/NA/SXT** | 0 | 5 | 5 | 6 | ***Vibrio vulnificus*** |
| **AMP/TIM/CZN/CAZ/AKN/NIT** | 0 | 1 | 1 | 6 | ***Citrobacter koseri*** |

| | | | | | |
|---|---|---|---|---|---|
| **AMP/AMC/CZN/FOX/CTX/ATM/CAZ** | 0 | 2 | 2 | 7 | ***Serratia marcescens*** |
| **AMP/TIM/CZN/FOX/CTX/ERT/GEN** | 0 | 3 | 3 | 7 | ***Klebsiella pneumoniae spp pneumoniae*** |
| **TIM/TIC/PIP/ATM/CAZ/AKN/GEN** | 0 | 1 | 1 | 7 | ***Pseudomonas aeroginosa*** |
| **TIM/PIP/ATM/CAZ/AKN/GEN/Col** | 0 | 1 | 1 | 7 | ***Pseudomonas putida*** |
| **AMP/AMC/TIM/IMP/CZN/FOX/AKN/NIT** | 2 | 0 | 2 | 8 | ***Citrobacter freundii*** |
| **AMP/AMC/PEN/AZM/ERY/CHL/DOX/SXT** | 0 | 2 | 2 | 8 | ***Pasteurella spp*** |
| **AMC/IMP/FOX/CZN/GEN/NA/SXT/NIT** | 4 | 0 | 4 | 8 | ***Pantoea spp*** |
| **AMP/AMC/TIM/IMP/FOX/CTX/CAZ/AKN/ SXT** | 0 | 2 | 2 | 9 | ***Enterobacter cloacae*** |
| **AMP/AMC/TIM/IMP/CZN/GEN/NIT/FOX/ CHL/SXT** | 1 | 0 | 1 | 10 | ***Citrobacter farmeri*** |
| **AMP/AMC/TIM/IMP/CZN/GEN/NIT/CTX/CAZ /AKN** | 0 | 2 | 2 | 10 | ***Serratia odorifera*** |
| **AMP/AMC/ERT/ATM/CTX/CAZ/SXT/FOX/ FEP/CHL** | 1 | 0 | 1 | 10 | ***Aeromonas sobria*** |
| **AMP/TIM/ERT/ATM/CTX/CAZ/SXT/CZN/ AKN/NIT** | 0 | 3 | 3 | 10 | ***Escherichia vulneris*** |
| **AMP/TIM/CZN/CTX/CAZ/FOS/NA/NIT/Col/ CIP/SXT** | 6 | 0 | 6 | 11 | ***Escherichia coli*** |
| **AMP/TIM/CZN/CTX/CAZ/FOS/NA/NIT/ATM/ AKN/GEN** | 0 | 2 | 2 | 11 | ***Enterobacter vulneris*** |
| **AMP/AMC/TIM/IMP/CZN/FOX/AKN/NIT/ GEN/NA/NIT/ATM/Col** | 0 | 4 | 4 | 12 | ***Proteus mirabilis*** |
| **AMP/AMC/TIM/IMP/CZN/FOX/AKN/NIT/ ERT/CAZ/FOS/CHL/SXT** | 0 | 2 | 2 | 13 | ***Serratia liquefaciens*** |
| **AMP/AMC/TIM/IMP/CZN/FOX/AKN/NIT/ ERT/CAZ/CTX/GEN/NA/CIP** | 0 | 9 | 9 | 14 | ***Escherichia coli*** |
| **AMP/AMC/ATM/CTX/FEP/CAZ/AKN/GEN/ CHL/FOX/TET/CIP/LEV/SXT** | 0 | 8 | 8 | 14 | ***Aeromonas hydrophila*** |
| **AMP/AMC/ATM//CTX/FEP/CAZ/AKN/GEN/ CHL/ERT/IMP/TET/CIP/LEV/SXT** | 0 | 4 | 4 | 15 | ***Aeromonas sobria*** |
| **AMP/AMC/TIM/ERT/IMP/CZN/FOX/CTX/ CAZ/AKN/GEN/NA/CIP/SXT/NIT** | 0 | 4 | 4 | 15 | ***Citrobacter freundii*** |
| **AMP/AMC/TIM/ERT/IMP//CZN/FOX/CTX/ CAZ/AKN/GEN/NA/CIP/SXT/ATM/CHL** | 0 | 5 | 5 | 16 | ***Pantoea spp*** |
| **AMP/AMC/TIM/ERT/IMP/CZN/FOX/CTX/ CAZ/AKN/GEN/NA/CIP/SXT/ATM/CHL/FOS/ Col/NIT** | 0 | 1 | 1 | 19 | ***Morganella morganii*** |
| **Number of resistance patterns** | 19 | 32 | | 50 | |

**Interrelationships between physicochemical and bacteriological parameters**

The results of the correlation coefficient analysis revealed that the bacteria (total and fecal germs) responded differently at the water parameters of the study sites. It was found that increases in T°, pH, Sal, and EC caused a highly significant increase ($P < 0.0001$) in TG, TC, FC and FS, which were correlated positively, In contrast, these bacteria were negatively correlated with the other physicochemical parameters of the water ($P< 0.0001$).

**Principal Components Analysis**

The factorial plan (Axis 1 and 2) of the principal component analysis of water physico-chemical and bacteriological properties of the two studied sites in Figure 9, showed an inertia of 64.8%. The data collected showed two periods, it seem that autumn and summer seasons values were been different then spring and winter seasons for all biotic and abiotic variables (Figure 9A). Furthermore, Figure 9B shows that all waters parameters values of site 1 (wadi Bedjima) were completely different from those of site 2 (wadi Seybouse).

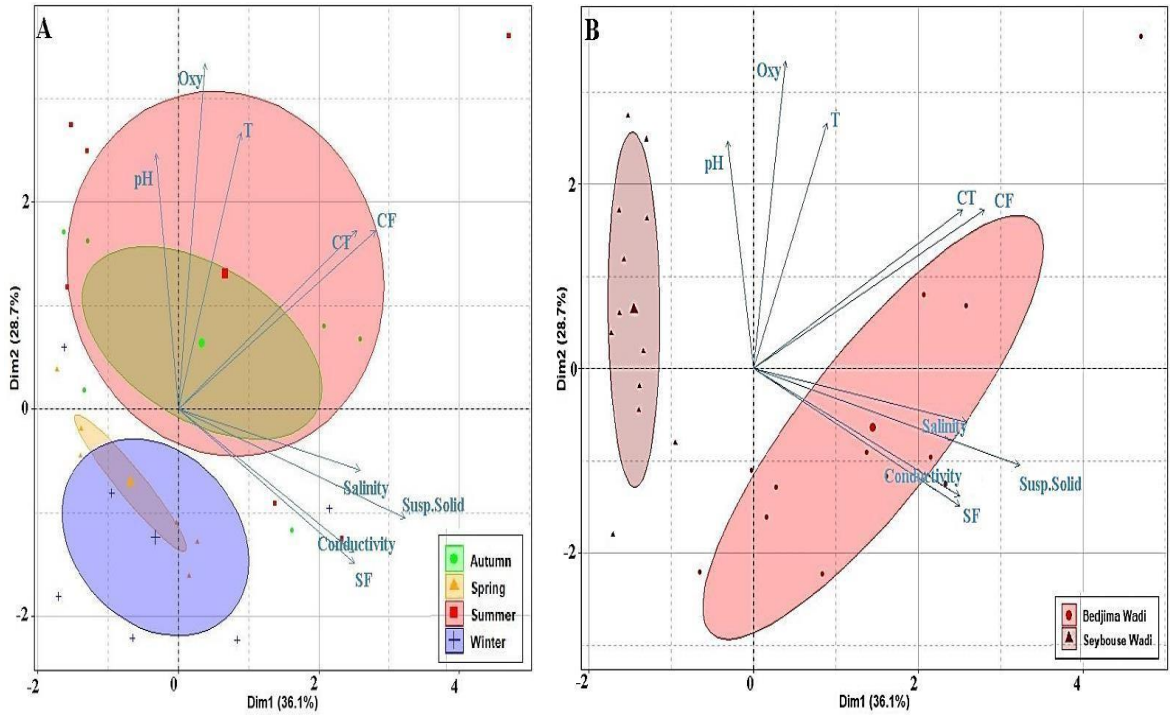


**Figure 9.** Principal component analysis (PCA) of the various variables during a study cycle from March 2017 to February 2018 at the two study sites. *(A) Correlation between the four seasons. (B) Correlation between sites*

**Discussion**

The physicochemical results obtained in this study showed that the temperatures measured throughout the study period never exceeded the 30°C limit set by the World Health Organization (2004).

Overall, the measured pH values are acceptable according to the Algerian standard recommended by JORA (2011), which is 6.5≤ pH ≥ 9. A slight decrease of this variable has been recorded for the sites during the autumn and winter. This can be explainedby the dilution of water, the effect of acidic discharges from urban and industrial areas and the bacterial activity of decomposition of the organic matter. The highest dissolved oxygen levels were recorded during the wet period. According to Makhoukh et al. (2011), cold water contains a greater quantity of dissolved oxygen than warm water.

In winter, the drop in water temperature increases its capacity to dissolve oxygen. In addition, low bacterial activity with low decomposition of organic matter as well as the mixing of strong winds promotes the enrichment of the water column in dissolved oxygen. According to Rodier et al. (2009), low values promote the development of pathogenic germs, harmful to human health and reflect a more or less clear degradation of the quality of the waters studied, with a reduction in its self-purification capacity.

A very alarming level of fecal contamination was observed in the waters of the wadis, particularly at the Bedjima wadi (S1). This is mainly due to the influence of anthropogenic activities, including untreated wastewater effluents from Annaba and its suburbs. Additionally, discharges from the nearby slaughterhouse, located just a few meters from S1, contributed to peaks in FIB (TC, FC, and FS). Our findings are consistent with those reported by Bengherbia et al. (2014). The concentration of FS was lower compared to FC and TC at both sites during the study period. These results are similar with previous studies (Aboulkacem et al., 2007; Bengherbia et al., 2014).

The low load of fecal streptococci observed in our study is likely due to the impact of some abiotic environmental factors which promote their rapid decline. In addition, some bacterial species, such as *Streptococcus bovis* and *Streptococcus equinus,* are fragile and disappear completely within 24 hours at a temperature of 10 to 20°C (Degremont, 2005).

The increase in the level of FIB during the warmer months is probably due to the high

organic load generated by liquid effluents and urban waste discharges from the city and surrounding areas located in the catchment.

regions of S1 and S2 during these months. The absence of treatment stations and low water flow limits the dispersion of fecal germs in the water column, as reported by Boufafa et al. (2021). At the same time, the loads of aerobic heterotrophic bacteria and Enterobacteriaceae increase with the rise in water temperature. The summer spike in bacterial abundance in aquatic ecosystems has also been noted by Kadri et al. (2015).

The significant fecal bacterial contamination of watercourses, especially during the summer months, may constitute a serious health risk to humans as these bacteria can cause various waterborne diseases, including gastrointestinal and upper respiratory illnesses.

The physicochemical properties of water are crucial in determining the survival, decomposition, and growth of coliform. Temperature, in particular, is considered one of the most influential factors on the abundance of these bacteria. The positive and significant correlation observed between bacterial loads and this environmental parameter is supported by the findings of Aboulkacem et al. (2007), who also demonstrated that temperature promotes bacterial proliferation.

Our results regarding the positive correlation between pH and FIB are not consistent with those of Van der Steen et al. (2000), who indicate an apparent decrease in the survival of FC in alkaline conditions and an increase in TC levels in acidic ones

The effect of bacterial adsorption by suspended matter explains the negative correlation between FIB and SS. This adsorption increases in winter, which may distort the results of bacterial enumeration in the analysis laboratory.

The isolation and identification of potentially pathogenic bacteria in the waters of the wadis revealed an abundance of species from the Enterobacteriaceae family, confirming the impact of urban effluents on water quality, particularly in the Bedjima wadi. This wadi is directly influenced by the discharges from the slaughterhouse, which contribute to a higher diversity of isolated bacterial species compared to the Seybouse wadi. The lower microbial loads in the Seybouse wadi may be attributed to the operation of the Allelik-El Bouni wastewater treatment plant located upstream of S2.

The species *E. coli* was the most abundant and may pose a potential pathogenic risk due to the production of enterotoxins, which are responsible for infantile diarrhea. Enterobacteria and non-fermenting Gram-negative bacilli, including genera such as *Aeromonas, Vibrio, Burkholderia, Pseudomonas,* and *Pasteurella*, play a major role in human infectious diseases. This significance arises from the diversity of bacterial species within these genera and their considerable impact on public health. The frequency and severity of infections, such as septicemia, meningitis, and gastroenteritis caused by pathogens like *Shigella*, *Salmonella*, *A. hydrophila*, and *V. vulnificus*, as well as typhoid fever and paratyphoid fevers (A, B, C) caused by *Salmonella* strains, illustrate the challenges in their management.

In addition to biochemical identification, 24 bacterial isolates were selected to study their taxonomy and phylogeny. More precise identification was carried out using their 16S rRNA genes. Overall, about half of the results obtained (11/24) from the 16S rRNA gene sequence were consistent with the genus identification results obtained from the API tests.

With regard to phylogenetic analysis, the optimal tree with the sum of branch length = 0.00164940 is shown in Figure 7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1958). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved g nucleotide sequences. All positions containing gaps and missing data were removed for each sequence pair. There were a total of 608 positions in the final dataset.

Antibiotic susceptibility test results of the isolated strains showed a large presence of antibiotic-resistant bacteria in the wadis. About 82.5% and 86.84% of the bacteria found in the waters of WS and WB, respectively, were MAR. These results are similar to those reported by Chitanand et al. (2008) and Wawire et al. (2013), who revealed a high prevalence of multiple antibiotic resistances in various environments.

The resistance profiles of the tested strains revealed a high prevalence of resistance to β-lactams, particularly to ampicillin and ceftazidime, (47.17 % and 46.9 %, of resistant strains, respectively). This resistance is primarily observed within the Enterobacteria group, which may be explained by the presence of naturally resistant bacteria populations

to β-lactams (Hacioglu and Dulger, 2009), or by the acquisition of high levels of resistance due to the frequent and sometimes excessive use of these antibiotics in human and animal medicine. This phenomenon of increased β-lactam resistance among environmental enteric bacteria has been documented worldwide (French, 2010). High resistance rates to β-lactams in Gram-negative bacteria have also been reported in studies by Marinescu et al. (2015) and Tao et al. (2010).

Low resistance rates were observed for amikacin (2.5%) and gentamicin (7.5%) in site 2. These results are consistent with those reported by DebMandal et al. (2011) and Poonia et al. (2014), who also found minimal resistance to these two antibiotics. This limited resistance may be attributed to the low concentration of these antibacterial compounds in the effluents of Seybouse water, which would limit bacterial exposure to levels sufficient to induce significant resistance.

A high sensitivity to tetracyclines was observed at both study sites, with resistance rates of (0%) for strain S2 and (10%) for strain S1. These results may indicate limited use of tetracycline in local agricultural practices, whether for treating animal infections, as a growth promoter, or for other veterinary applications. In contrast, higher resistance rates to tetracyclines have been reported in the studies by Amador et al. (2015), suggesting more frequent use of this antibiotic in other regions, which could lead to increased selection of resistant strains. The isolated strains that showed resistance to the highest number of antibiotics were found in the water of the Bedjima wadi, with 32 antibiotic resistance patterns. Among these, *Morganella morganii* was the most resistant strain, showing resistance to 19 antibiotics, followed by *Pantoea spp* (16ABs). In contrast, the water from the Seybouse wadi, revealed 19 resistance patterns, with *Escherichia coli* (11ABs) exhibiting the highest resistance, followed by *Citrobacter farmeri* (10ABs) and *Aeromonas sobria* (10ABs). Consistent with findings from S2, *E.coli* strains showed significant resistance to the tested antimicrobial agents (Hanna et al., 2020).

The different patterns of bacterial antibiotic resistance may result from varying selective pressures, which are most elevated at site 1. These pressures could be influenced by the environmental conditions of the wadi waters, which may favor the development of antibiotic resistance. Studies have suggested a strong relationship between antimicrobial resistance and various water quality variables, such as water depth, nutrient

concentrations, temperature, dissolved oxygen and salinity, all of which may warrant further examination (Maal-Bared et al., 2013)**.** Furthermore, the different sources of contamination such as discharges from the slaughterhouse and hospital, along with the selective pressures exerted by antibiotics used in managing bacterial infections in both animals and humans in the area, contribute to the observed different patterns of antibiotic resistance.

In our study, more than half of the MAR index values of the bacterial strains were found to be equal to or more than 0.20, suggesting that these strains have been exposed to a range of antibiotics. This finding is consistent with the results of Lamia et al. (2015), who also observed high MAR index values in strains from the Enterobacteriaceae family, the most prevalent family in our study. The highest MAR index values were recorded for *Morganella morganii* (MAR index =1), isolated from S1, and *Citrobacter farmeri* from S2 with a MAR index of 0.5. The presence of antibiotic-resistant bacteria in the waters of Seybouse and Bedjima wadis poses significant public health risks and could have ecological consequences, affecting population dynamics. Similar to findings by Lamia et al. (2015) and Fernandes et al. (2021), the contamination of the surface waters with high antibiotic loads is associated with an increase in antibiotic resistance and the dissemination of resistance genes within bacterial communities. This situation contributes to the formation of a reservoir that facilitates the transmission of resistant bacteria to humans, complicating the treatment of infectious diseases. Such findings highlight the impact of anthropogenic activities on the resistome of aquatic environments and organisms.

## Conclusions

From the results obtained, it is clear that the waters of Bedjima and Seybouse wadis are very contaminated with bacteria of fecal origin. Furthermore, the presence of pathogenic bacteria is strongly correlated with the densities of contaminating bacteria. Thus, waste from the slaughterhouse as well as fluctuations in physicochemical factors, particularly temperature, can contribute to the appearance of pathogenic bacteria multi-resistant to antibiotics in the sea. Analysis of the resistance profile of strains isolated from the two wadis revealed a high level of multi-resistance. This presence of pathogenic germs resistant to antibiotics constitutes a health risk in the more or less short term. Also, a set

of measures must be undertaken to reduce water discharges in the Mediterranean according to bacterial standards.

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