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Effect of salinity and temperature on the bacterial diversity shift of anaerobic batch cultures treating abattoir wastewater

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ABSTRACT

The molecular biological analyses allowed to highlight the changes of the microflora of the batch cultures with the increase of the salinity and the temperatures. The single-strand conformation polymorphism (SSCP) patterns of the bacterial diversity at different salt concentrations (0, 20, and 40 g l^{-1}) in mesophilic (37°C) and thermophilic (55°C) conditions showed that the bacterial diversity varies depending on the culture conditions. The obtained SSCP profiles at the different salt concentrations showed that there is a greater diversity in the mesophilic than the thermophilic condition. However, the bacterial diversity richness ($1/D$) and the species evenness for the mesophilic condition for all the tested salt concentrations showed firstly the maximum diversity and secondly that the species in the sample are quite evenly distributed. The increase of the salt concentration to 20 and 40 g l^{-1} at thermophilic condition decreased the bacterial diversity due to the selection pressure caused by the elevation of salinity, which eliminated the salt-sensitive species and thus reduced the community diversity.

Keywords: Abattoir wastewater; Salinity; Bacterial community; Mesophilic; Thermophilic

1. Introduction

Abattoir wastewaters are very strong wastes containing grease, blood, feces, and recalcitrant organic matter [1,2]. The high suspended solid content in the wastewater causes severe problems, due to their insolubility which slows the rate of degradation, and its tendency to form scums. Supplementary, abattoir wastewater carries high levels of pathogenic microorganisms that may constitute a serious risk to the human and animal health [3]. The generated effluent of this wastewater was characterized by a pH ranging from 6.8 to 7.4, total solids varying from 5,060 to 5,400 mg l^{-1},

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and a total COD ranging from 5,800 to 6,100 mg l^{-1} with a conductivity of 2.9 ms cm^{-1} [4]. In addition, high salt concentrations can be found in these generated wastewaters. This high salt concentration is known to affect the metabolic activity of bacteria and reduce the microbial growth of the diverse microorganisms involved in many steps of anaerobic degradation [5,6].

Anaerobic digestion is inhibited by high salinity mainly due to the presence of cations. A sodium concentration above 10 g l^{-1} strongly inhibits methanogenesis [7–9]. In addition, the temperature has a direct effect on the physicochemical properties of all components in the digester and also affects the thermodynamics and the kinetics of the biological processes. The intensity of the activity of microorganisms and the production of methane depend on the temperature of the medium [10]. However, little is known about the dynamics and the bacterial diversity involved in the treatment of saline wastewater, although some studies have shown that the diversity of an ecosystem treating industrial saline wastewater could be similar to that of an ecosystem treating unsalted wastewater [11,12].

Although, recent advances in molecular microbial ecology allow a better understanding of the specific microorganisms that are involved in wastewaters treatment, single-strand conformation polymorphism (SSCP) offers a simple, inexpensive, and sensitive method for detecting whether or not DNA fragments are identical in sequence and hence can greatly reduce the amount of sequencing necessary. This method has been applied to study bacterial communities, e.g., in water, in the compost of organic agricultural substrate, and in anaerobic digesters [13,14]. Although the number of studies dealing with the biological treatment of hypersaline wastewater is increasing rapidly, little is known regarding the diversity of these halophilic communities. In this context, Lefebvre et al. [15] identified two dominant archaeal species in both reactors close to *Methanosaeta* sp. and *M. beijingense* which were found in all the profiles whatever the salt concentration and thus appear to be halotolerant, a fact not previously recorded

In this purpose, this study aims to investigate the effect of the salt concentration and the temperature on the bacterial community involved in the anaerobic digestion of abattoir wastewater using molecular tools based on the analysis of genomic 16S rDNA.

2. Material and methods

2.1. The anaerobic batch reactors preparation

To study the effect of the salt concentration and the temperature on the bacterial community involved in the anaerobic digestion, different batch reactors were performed in anaerobic bottles of 250 ml inoculated by anaerobic sludge treating abattoir wastewaters. The anaerobic sludge was collected from an upflow anaerobic filter treating abattoir wastewaters in Tunisia [4]. This anaerobic digester was operated under both mesophilic (37˚C) and thermophilic (55˚C) conditions. A sample of the sludge was taken at the end of thermophilic phase of the process. After that, enrichments cultures were prepared. Each enrichments culture contained equally 75 ml of the sludge and 75 ml of the abattoir wastewater, which were distributed in anaerobic bottles of 250 ml. The pH of all media was measured using a digital calibrated pH-meter HANNA pH 210 and was adjusted to 7.0 with a 10 M KOH solution. After the addition of different salt concentrations (0, 20 and 40 g l^{-1}), the bottles were flushed and filled with N_2/CO_2 (80/20, v/v), in order to maintain adequate anaerobic conditions and then were incubated at 37 and 55˚C for 30 d. The dynamics of the bacterial communities in each bottle was monitored by PCR–SSCP methods [16].

2.2. DNA extraction, PCR, and SSCP analysis of the sludge

DNA extractions were performed on samples of sludge collected from the mesophilic and thermophilic reactors at different salt concentrations' (0, 20, and 40 g l^{-1}). Four milliliter of samples was centrifuged at 6,000 rpm for 10 min. Pellets were resuspended in 4 ml of 4 M guanidine thiocyanate-0.1 M Tris pH 7.5 and 600 µl of N-lauroyl sarcosine 10%. 250 µl of treated samples were transferred in 2-ml tubes and stored at −20˚C.

Extraction and purification of total genomic DNA was implemented according to the protocol developed by Godon et al. [17].

Highly variable V3 regions of bacterial 16S rRNA genes were amplified by PCR using bacterial w49 (ACGGTCCAGACTCCTACGGG)—w34 (TET-TTACCGCGCTGCTGGCAC) primers [14]. Samples were treated according to the protocol previously described by Delbes et al. [18].

SSCP analyzes were performed for overall detection and to study the dynamics of bacterial community. SSCP analyses enable RNA fragments with similar size to be separate according to their configuration (secondary structure) according to the protocol of SSCP described by Delbes et al. [18]. SSCP analyses were performed on an automatic sequencer abi310 (Applied Biosystems). RNA fragment detection was done with the fluorescent w34 primer (The primer w34 is marked at 5′ end with fluorescent phosphoramidite-TET

(Applied Biosystems)). The obtained results were analyzed by GeneScan Analysis 2.0.2 software (Applied Biosystems).

2.3. Data analyses

Biological diversity can be quantified in many different ways. The two main factors taken into account when measuring diversity are richness and evenness. Richness is a measure of the number of different kinds of organisms present in a particular sample. Evenness compares the similarity of the population size of each of the present species. The reciprocal of Simpson's index (diversity richness) ($1/D$) was also used as a measure of diversity, which considers both richness and evenness widely used for ecological studies. These two parameters were calculated and interpreted as described previously by Simpson [19].

3. Results

3.1. Diversity and abundance of the bacterial communities with increasing salt concentrations

The dynamics of bacterial communities with increasing salt concentrations at mesophilic and thermophilic conditions were monitored by PCR–SSCP methods. The obtained profiles related to the domain of bacteria are shown in Fig. 1 for mesophilic condition and in Fig. 2 for thermophilic condition.

For mesophilic condition (37˚C) (Fig. 1), the obtained SSCP pattern at 0 g l^{-1} (A1) reveals the high diversity of the bacteria, with at least 40 distinguishable peaks and about 14 prominent ones. The SSCP patterns obtained at 20 g l^{-1} (A2), and this obtained at 40 g l^{-1} (A3) had 44 and 43 peaks, respectively, and about 12 prominent ones. Twenty-nine peaks of the SSCP pattern (A1) correspond to the peaks of the SSCP patterns (A2) and (A3). Eleven peaks disappeared under these conditions (peaks (5), (15 of the pattern (A2)), (20 of the pattern (A3)), (24), (26), (29), (32 of the pattern (A2)), (33), (37 of the pattern (A2)), (38), and (39)). Peaks (3) and (13) present a little decrease, and peaks (31) and (32) decreased noticeably. However, peaks (4), (6), (7), (8), (9), (10), (11), (14), (17), (25), (28), and (36) increased noticeably and became prominent. The passage from (A1) to (A2) and to (A3) is accompanied with the appearance and the outcrop of new species (peaks (a), (b), (c), (d), (e), (f), (g), (h), (i), (j), (k), (l), (m), (n), and (o)).

Increasing temperature from 37 to 55˚C caused a significant change of the SSCP profiles and the decrease of the bacterial diversity. However, for the thermophilic condition (Fig. 2), the SSCP patterns at 0 g l^{-1} (B1), at 20 g l^{-1} (B2), and at 40 g l^{-1} (B3), showed the presence of 27, 25, and 21 peaks, respectively. Sixteen peaks of the SSCP pattern (B1) correspond to the peaks of the SSCP patterns (B2) and (B3). Ten peaks disappeared under these conditions. Peaks (4), (9), and (12) present a little decrease, and peaks (1) and (7) decreased. However, peaks (5), (8), and (24) increased and became prominent. The passage from (B1) to (B2) and to (B3) is accompanied with the appearance of new species (peaks (a), (b), (c), (d), (e), (f), and (g)).

The bacterial diversity richness ($1/D$) and the species evenness (Es) were used as a measure of the diversity and the abundance. For the mesophilic condition and at a salt concentration of 0 g l^{-1}, the obtained values were 25.15 and 0.62, respectively. For a salt concentration of 20 g l^{-1}, they reached 28.64 and 0.65, respectively. After increasing the salt concentration to 40 g l^{-1}, they dropped to 24.22 and 0.56, respectively. At all these salt concentrations, the obtained values of the species evenness offer toward the number of species, indicating the maximum diversity. All those of the Es offer toward to one, indicating that the species in the sample are quite evenly distributed.

As with mesophilic condition, ($1/D$) and (Es) were determined for the thermophilic condition. In fact, at a salt concentration of 0 g l^{-1}, the obtained values were, respectively, 16.84, which offers toward the value of $S = 27$, indicating the maximum diversity, and 0.62, indicating that the species in the sample are quite evenly distributed. For a salt concentration of 20 g l^{-1}, they reached 9.29 and 0.37, respectively. At a salt concentration of 40 g l^{-1}, they dropped to 7.53 and 0.35, respectively. For these two last salt concentrations, the obtained values of the diversity richness offer toward the value of one as the lowest possible value, indicating that little diversity is shown. Those of the Es offer toward to 0, indicating that only a few species are present and are dominant.

4. Discussion

The results of the SSCP patterns of bacterial 16S rRNA gene amplification products at different salt concentrations in mesophilic and thermophilic conditions showed that the bacterial diversity varies as a function of culture conditions. However, some dominant peaks for a given condition appeared, disappeared, or decreased.

The obtained SSCP profiles at the different salt concentrations showed that there is a greater diversity in mesophilic than thermophilic condition. However, the bacterial diversity richness ($1/D$) and the Es for the mesophilic condition for all tested salt concentrations showed maximum diversity and that the species

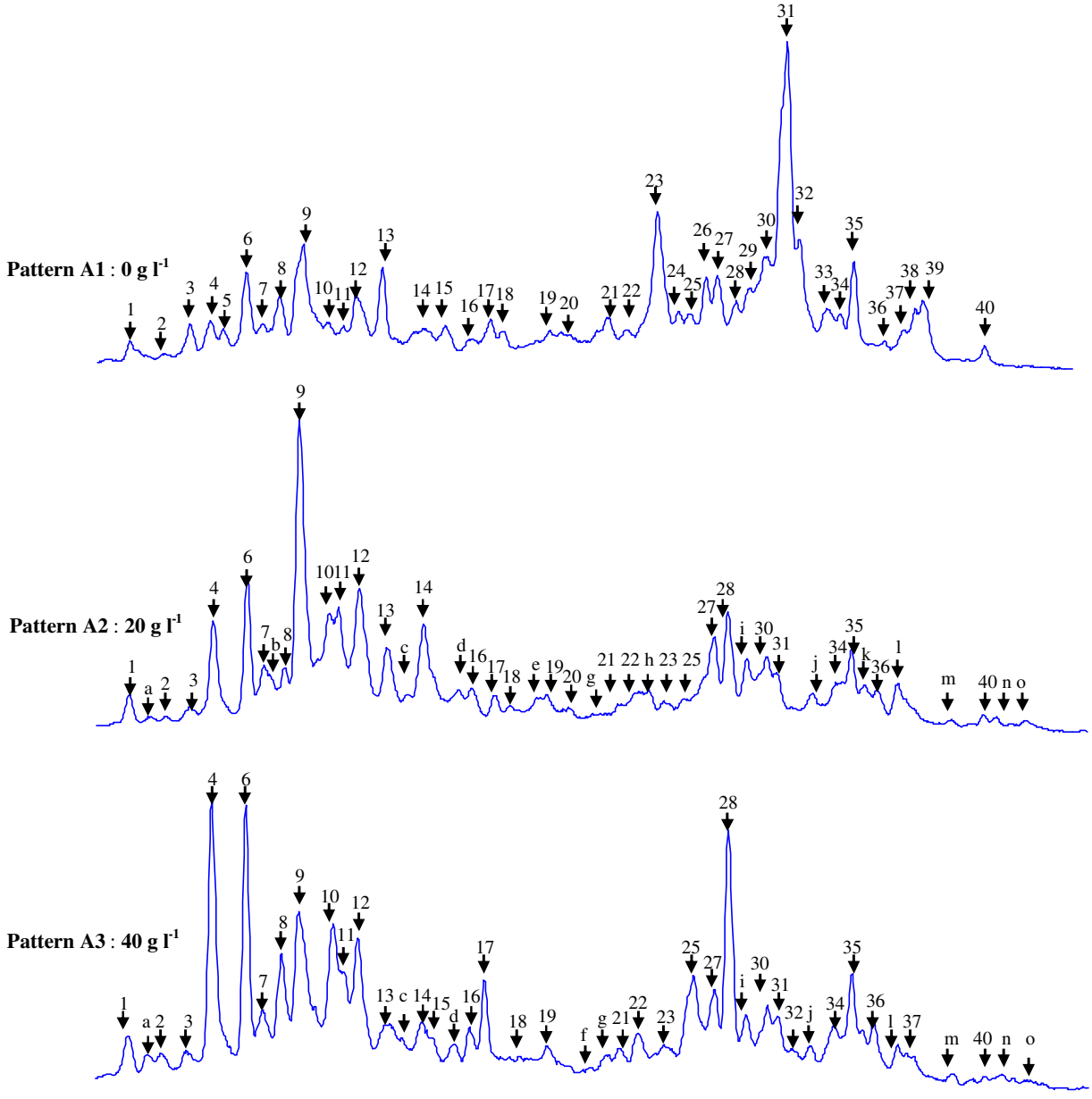

Fig. 1. Dynamics of single-strand conformation polymorphism patterns of bacterial 16S rRNA gene amplification products at different salt concentrations: the anaerobic mesophilic (37°C) sludge at 0 g l^{-1} (A1), at 20 g l^{-1} (A2), and at 40 g l^{-1} (A3).

in the sample are quite evenly distributed. In this context, Soto et al. [20], who made comparison between the biomass obtained from mesophilic and thermophilic fixed-bed anaerobic digesters, proved that the mesophilic reactor exhibited better performance than the thermophilic one, which was attributed to the more rapid adaptation of mesophilic sludges to the high salinity of the used wastewater. The obtained results proved that the bacterial community could express different degrees of tolerance to such an altered stresses of salinity. In fact, the saline wastewater usually expressed high salt stress in bacterial cells. Apparently, bacteria had to defense against high gradient gaps in osmotic pressure due to the loss of cellular water (plasmolysis) or recession of the cytoplasm [21]. Moreover, Reid et al. [22], also observed the negative effect of salt shocks (e.g. Na^+ and K^+) on sludge filterability, mainly due to the release of polysaccharides.

The bacterial diversity richness ($1/D$) and the Es for the thermophilic condition, showed the change of these two parameters with salt concentrations. In fact,

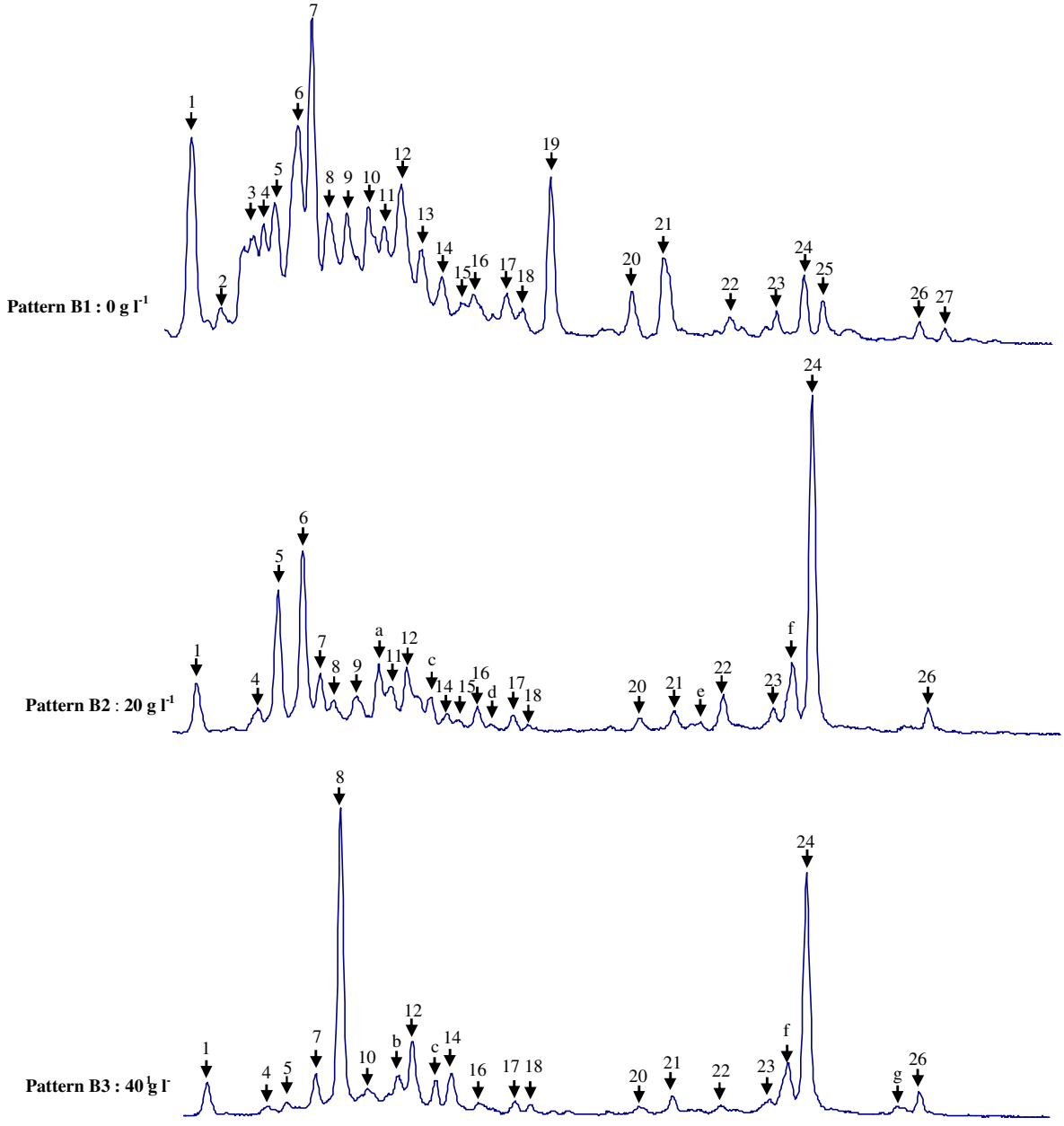

Fig. 2. Dynamics of single-strand conformation polymorphism patterns of bacterial 16S rRNA gene amplification products at different salt concentrations: the anaerobic thermophilic (55°C) sludge at 0 g l^{-1} (B1), at 20 g l^{-1} (B2), and at 40 g l^{-1} (B3).

at a salt concentration of 0 g l^{-1}, the obtained values were 16.84 and 0.62, respectively, indicating the maximum diversity and that species in the sample are quite evenly distributed. For a salt concentration of 20 and 40 g l^{-1}, the obtained values of the diversity richness indicated that little diversity is shown. Those of the Es indicated that only a few species are present and are dominant. These results were in agreement with those of Wu et al. [23], who studied the effect of the salinity on the activity, settling, and bacterial community of activated sludge in sequencing batch reactors treating synthetic saline wastewater. Their obtained results showed also that the bacterial diversity decreased with increasing the salinity and that the bacterial community structure was greatly influenced by the salinity.

For the different tested conditions, the obtained results showed that for the different SSCP profiles, some peaks disappeared, decreased, or increased noticeably, and other peaks appeared. It is thus noted

that the temperature and salt the concentration exert a selective effect on the bacterial diversity by favoring certain halophilic groups compared to others which could be involved in the degradation of abattoir wastewaters. We can therefore conclude that the increase of the salt concentration and the temperature decreased the bacterial diversity due to the selection pressure caused by the elevation of salinity, which eliminated the salt-sensitive species, and thus reduced the community diversity [24], and there is enrichment and appearance only of halotolerant microorganisms at these conditions [15]. Ke et al. [25] found that Na^+ and Cl^- were two main factors influencing the bacterial populations. In fact, the toxicities of Na^+ and Cl^- inhibited the bacterial growth. Gryndler et al. [26] found that Cl^- concentration would affect indigenous microorganism. Then, increasing Na^+ and Cl^- abated the pressure of competition of the surviving salt-tolerant microorganisms, which stimulated their growth.

We can conclude that the salt is required for microbial growth and, consequently, affects specific growth rate like any other nutrient. While moderate concentrations stimulate microbial growth, excessive amounts slow down the growth, and even higher concentrations can cause severe inhibition or toxicity [2,27]. However, at low concentrations, sodium is essential for methanogens, probably because of its role in the formation of adenosine triphosphate or in the oxidation of NADH. McCarty [28] reported that sodium concentrations in the range of 100–200 mg l^{-1} may be beneficial for the growth of mesophilic anaerobes. According to Kugelman and Chin [29], the optimal sodium concentration for mesophilic aceticlastic methanogens in waste treatment processes was 230 mg Na^+ l^{-1}. The optimal growth conditions for mesophilic hydrogenotrophic methanogens reportedly occurred at 350 mg Na^+ l^{-1}. At high concentrations, the salts could readily affect the activity of microorganisms and interfere with their metabolism. The level of inhibition depends on the concentration of sodium ions. An early study reported that sodium concentrations ranging from 3,500 to 5,500 mg l^{-1} may be moderately inhibitory and 8,000 mg l^{-1} to be strongly inhibitory to methanogens at mesophilic temperatures [2].

5. Conclusion

Salinity and temperature are important key environmental parameters that influence the bacterial communities. In this study, the results showed that the temperature and the salt concentration exert a selective effect on the bacterial diversity by favoring certain halophilic groups which could be involved in the degradation of abattoir wastewaters. The increase of the salt concentration and the temperature decreased the bacterial diversity due to the selection pressure caused by the elevation of salinity.

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