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Carbon and nitrogen removal from a synthetic dairy effluent in a vertical-flow fixed bed bioreactor

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Abstract

In this study, a synthetic dairy effluent (SDE) containing high levels of COD and nitrogen was treated in a fixed-bed bioreactor (FBBR) under the influence of the variations in residence time, aeration conditions and temperature. The tests performed continuously in a single step and the same reaction vessel allowed to define a 96h cycle comprising two anoxic/aerobic phases of 48h each. Sequential anoxic and aerobic conditions improved the removal of TKN and NH_4^+ by a factor of 2.5, whereas low temperature significantly decreased the nitrogen and COD removal rate. Overall, outcomes showed a successful growth of the biomass that reached $(5.8 \pm 0.31) \times 10^6$ CFU/mL, and high removal efficiency of COD ($93.5 \pm 8.1\%$), NO_3^- ($99.2 \pm 19\%$), TKN ($87.5 \pm 16.1\%$), and NH_4^+ ($55.8 \pm 8.3\%$), and hence complied with Moroccan standards. The FBBR system, therefore, can be applied successfully to remove COD and nitrogen from wastewater.

Keywords: biological treatment, COD, fixed-bed bioreactor, heterotrophic flora, nitrogen, wastewater.

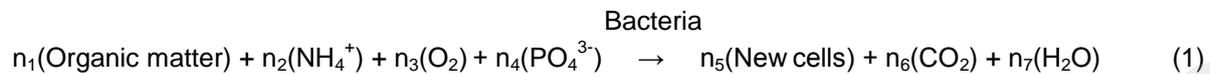
1. Introduction

Today, most Human activities whether domestic, industrial, agricultural, or touristic have not only enhanced the life quality, but also resulted in the release of wastewater pollutants on the environment and the public health. Human health risk associated with polluted water is one of the major challenges that humanity will face in the twenty-first century because of the diversity and hazardous nature of water-polluting components. There are several categories of water pollutants, i.e. nutrients (Carbon C, nitrogen N, phosphorus P), micropollutants (heavy metals, pesticides, synthetic hormones...), but also microbiological (bacteria: Fecal coliforms, fecal Streptococci, Salmonella, Vibrio cholera...; viruses: hepatitis virus...; parasites: Ascaris, Taenia...; protozoa: Giardia intestinalis, Entamoeba histolytica...) and physical (thermal pollution, suspended solids, radioactivity...). Special attention is given to the various compounds contained in liquid effluents, which are a substantial pollution source to the aquatic environment (Chen et al., 2020; Metcalf & Eddy, 2013).

The dairy industry is one of the most important activities causing water pollution due to its high demand for the resources, particularly for water: about 90 % of milk is made of water. Owing to the used raw material and the large amount of water consumed, the dairy factories negatively affect the environment by rejecting great quantities of liquid effluents with a high level of C, N, and P (Hamdani et al., 2018; Porwal et al., 2015). These three substances exist naturally at relatively low levels in aquatic environments, once released in large quantities via point source effluents they can have a negative impact on the quality of the receiving environment (malodor, asphyxia, eutrophication...), as well as on people's health and economy (OMS, 2016). To avoid these nuisances, the presence of organic matter, nitrogen, and phosphorus in raw wastewater has been regulated and their levels must be reduced to limit values established by this legislation. Organic matter removal can be provided by physico-chemical processes (oxidation, filtration, flocculation-decantation, photo-catalytic reduction...); however, this kind of treatment has a certain number of disadvantages, including a high cost, excessive sludge production, the use of chemicals agents, secondary pollutants generation, physico-chemical monitoring of the effluent (pH) and above all, only partial treatment (Crini and Lichtfouse, 2019; Hamdani et al., 2005; Porwal et al., 2015). As an indication, the costs of chemicals used in wastewater treatment plant are between 5-15% of the total project cost (Anand et al., 2012), while the typical water treatment plant produces from 0.4 to 0.8 mg of chemical sludge per mg of used coagulant (Guimaraes et al., 2016). Hamdani et al., (2005) reported that a cubic meter of dairy effluent treated by using three coagulants generates a sludge in the range of 0.98-1.38 mg of suspended matter per liter of dairy effluent depending on the chemical reagents, and require a cost from 0.01 to 0.25 USD. Van Dongen et al. (2001) reported that the nitrogen conversion process by using biological way is estimated at 1.12 to 1.68 USD/kg N removed compared to 5.6 to 14 USD/Kg N removal for a physico-chemical method. These limitations make the biological processes profitable and alternative for wastewater treatment.

Compared to physical and chemical treatment processes, biological methods are considered more attractive, economical and the best adapted alternative for removing biodegradable pollutants, because (i) their principle of treatment is similar to the natural phenomena of water self-purification, (ii) their support on a significant number of microbial species with high biodegradability of organic matter and nutrients (iii) their best pollution abatement rate, (iv) their capacity for generating much high-value-added product, i.e. water, energy, fertilizers, nutrients, and (v) their moderate cost... (Batstone et al., 2015; Crini and Lichtfouse, 2019; Kehrein et al., 2020; Samer, 2015).

In biological wastewater treatment, soluble and colloidal substances in the effluent are decomposed by the action of a variety of living organisms, i.e. plants (microalgae, aquatic vegetation...), animals (protozoa, invertebrates...), and especially purifying microorganisms which remain by far the main actors involved in the wastewater purification. The purifying microorganisms use their specific metabolism to convert carbonaceous and nitrogenous matter (C, N, P) in the liquid effluents to water, new cells, carbon dioxide, and other stables and harmless end products (Samer, 2015). This mechanism can be explained by the following global equation (1) (Wentzel et al., 2003).



Organic matter degradation involves heterotrophic bacteria, such as *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*...), which use biodegradable carbon and nutrients as a source of food and energy (Metcalf & Eddy, 2013; Samer, 2015). Based on oxygen availability, two processes can be envisaged: in the presence of oxygen, microorganisms decompose organic matter to meet their needs and produce new living cells. In the absence of oxygen, microbial growth happens when using other final electron acceptors than molecular oxygen like oxygenated mineral compounds (Batstone et al., 2015; Kehrein et al., 2020; Mauffrey, 2016; Russell, 2019). This is the case with biodenitrification, which is an anoxic process where heterotrophic bacteria will be forced to use the oxygen contained in the nitrates (nitrate and nitrite respiration) as the final acceptors of electrons during oxidation reactions of organic substances (Hamdani et al., 2018).

For the biological treatment of organic and nitrogen compounds, many studies have focused on the use of suspended biomass systems such as activated sludge and sequencing batch reactors. These systems, although commonly used for big communes, have some disadvantages: problems related to multiple ponds, recirculation of effluent and biomass, water-sludge separation, sensitivity to the load variations, high costs, maintenance... (Gurtekin, 2014; Nancharaiah and Sarvajith, 2019).

Other researchers studied the application of different configurations of fixed culture reactors in the removal of organic matters and/or nutrient during wastewater treatment, i.e. fixed bed membrane bioreactor (Subtil et al., 2014), ozone fixed bed bioreactor (Liu et al., 2017), Up flow fixed bed bioreactor (Hamdani et al, 2018), immersed bacterial bed reactor (Sylla et al., 2017), hybrid moving fixed bed biofilm reactor (Di Bella and Mannina, 2020), submerged fixed bed biofilm reactor (Schlegel and Koeser, 2009). In all studies, a mean removal efficiencies for COD and total nitrogen in the range of 70-99% and 20-92%, respectively, were obtained at hydraulic retention times in the range of 36 h to 6 months.

In Morocco, -which is experiencing a considerable delay in liquid sanitation-, activated sludge, lagoons, settlers-digesters and infiltration-percolation represent around 80% of all wastewater treatment plants; while bacterial beds are very little represented (Belloulid et al., 2018). Despite the abundance of studies on the subject, only very few investigations focused on environmental impact of Moroccan dairy industry where approximately 4.5×10^9 L of processed milk are produced annually in 2020, and 1500L of water is needed to obtain 1 kg of milk (FAO, 2011). The large majority of the water consumed is returned to the receiving environment in the form of raw effluent high in biological and chemical pollutants.

Hence the objective of this study was to assess the performance of an immobilized biomass bed reactor to remove COD and different forms of nitrogen such as NO_3^- , TKN, and NH_4^+ from SDE in a single step and in the same reaction chamber, and under the effect of residence time (24, 48, 72, 96 h), aeration conditions (anoxic, aerobic, anoxic/anaerobic, anoxic/aerobic modes) and temperature (10, 15, 20, 30 °C).

2. Materials and Methods

2.1. Pilot description and operating conditions

Laboratory tests were conducted in a fixed-bed bioreactor, which was a rectangular stainless-steel column with dimension in cm Length \times Width \times Height = 250 \times 250 \times 950 (Fig. 1).

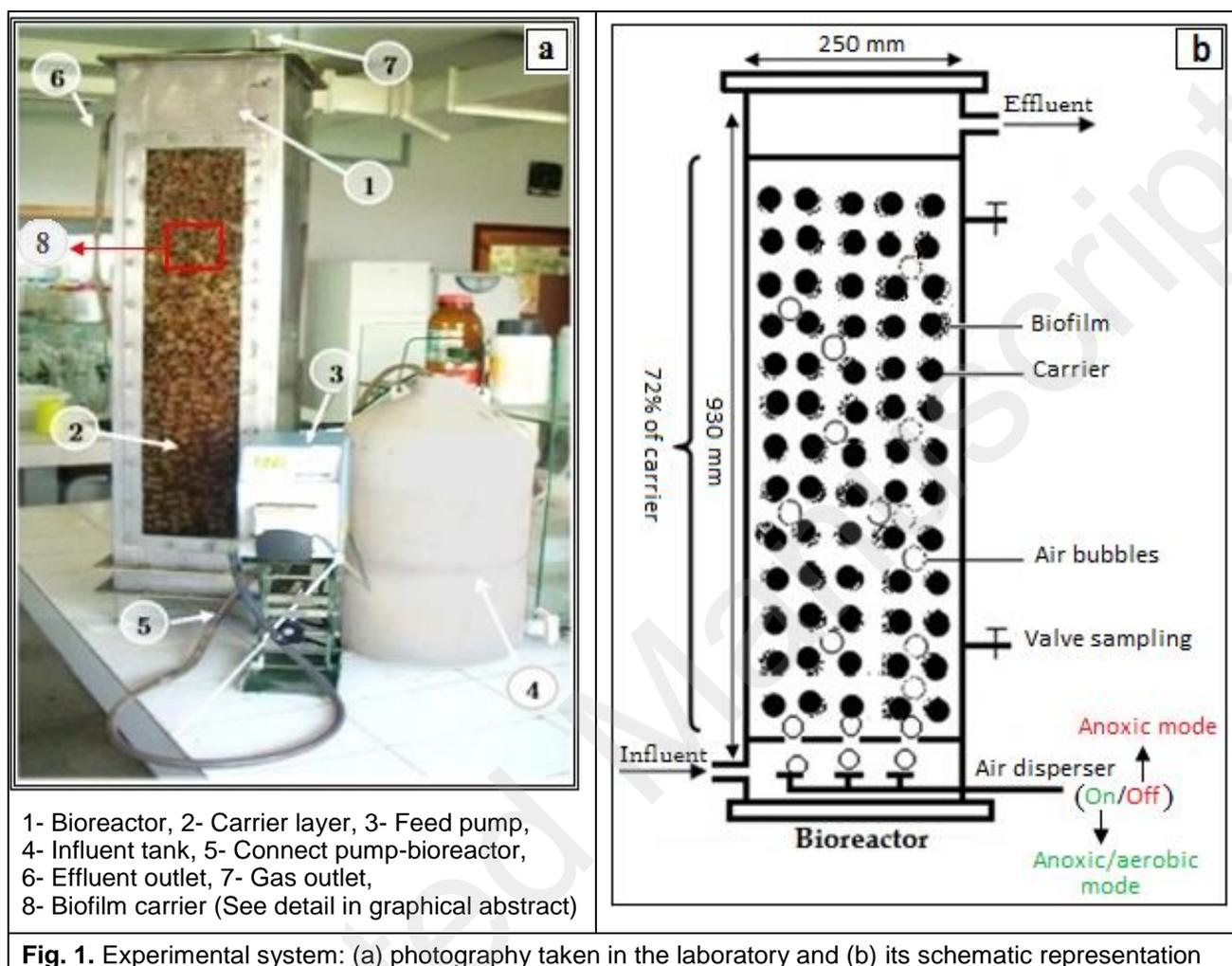


Fig. 1. Experimental system: (a) photography taken in the laboratory and (b) its schematic representation

The bioreactor was filled with food-grade plastic support used as a carrier to biomass immobilization and operated in continuous vertical flow mode. The effective liquid volume of the continuous bioreactor was approximately 60 liters while the apparent volume occupied by the carrier was 51 L with a vacuum of 44.5 L, which is equivalent to 87% of porosity. The bioreactor was equipped with a peristaltic pump "Percom N-M" for influent feeding, an aeration system ON/OFF, a valve sampling, and a pipe of 1.5 cm in diameter for the biogas outlet. To remove undesirable microbes, the column was sterilized with alcohol and then rinsed repeatedly with sterile distilled water, while the carrier was autoclaved at 105°C for 30 minutes. The main characteristics of the support material are shown in Table 1.

Table 1. Support material specifications in the bioreactor

Support material	Shape	Color	Diameter (mm)	Height (mm)	Apparent volume (L)	Vacuum (L)	Specific surface (m ² /m ³)	Porosity (%)
Plastic	Ring	White	16 x 2	15 ± 1	51	44.5	190	87

The operating conditions of the experiments are reported in Table 2.

Table 2. Bioreactor operating parameters

Parameter	Value	
	Anoxic mode	Anoxic/aerobic mode
Temperature (°C)	30 (± 5)	Variable
pH	7 (± 0.5)	7 (± 0.5)
COD/NO ₃ ⁻ Ratio	5 (± 1)	5 (± 1)
Applied load NO ₃ ⁻ (Kg/ m ³ .J)	0.536 (± 0.09)	0.536 (± 0.09)
Applied load COD (Kg/ m ³ .J)	2.62 (± 0.36)	2.62 (± 0.36)
Influent flow (l/h)	1.5	1.5
Flow direction	Up flow	Up flow
Theoretical hydraulic retention time (h)	28.7	28.7
Aeration period (h)	48	48
Anoxic period (h)	48	48
Initial biomass (CFU/mL)	[2.6 (± 0.13)] × 10 ⁷	[2.6 (± 0.13)] × 10 ⁷

Values in parenthesis indicate standard error of the mean SEM (a mean of three determinations)

2.2. Pilot inoculum, feeding and aeration

The bioreactor was inoculated properly with a bacterial laboratory suspension at approximately $(2.6 \pm 0.13) \times 10^7$ CFU/mL. These bacteria were isolated from sediments and soil sample collected in a decantation pits and storage ponds of effluents produced by a dairy industry located in El Jadida City (Morocco), purified and selected in the BIOMARE laboratory, based on their ability to remove carbon and nitrogen.

The bacterial biomass was left to grow gradually on the inert support in the presence of culture medium enriched in nitrate and organic matter, which was renewed every 48h. After four weeks, the biofilm had become visible, and a mucilaginous layer appeared on the surface of the garnishing. This duration represents the preculture time required to achieve a steady-state.

An adjustable flow rate peristaltic pump provided the bioreactor with SDE based on milk powder manufactured by Dairy industry unit located in El Jadida City (Morocco) that transited in up-flow through the plastic support previously colonized by the acclimated biomass. The recipe of the reconstituted influent was inspired by those proposed by Nopens et al. (2001) with some modifications to obtain appropriate COD/TKN/TP and COD/NO₃⁻ initial ratios. This model solution whose composition is presented in the Table 3 was prepared with distilled water and had a pH of 7 ± 0.2 .

Table 3. Composition of the reconstituted dairy effluent

Compounds and concentration (mg/L)	Theoretical values of chemical parameters, expressed in mg/L				
	COD	NO ₃ ⁻	NH ₄ ⁺	TKN	TP
Nutrient					
Milk powder: 750	750	00	33	45	7,5
Starch: 500	500	00	00	00	00
Yeast extract: 250	250	00	23	30	00
Chemicals					
KH ₂ PO ₄ : 20	00	00	00	00	2,7
NaH ₂ PO ₄ , 12H ₂ O: 50	00	00	00	00	4,8

NH ₄ Cl: 105	00	00	22	30	00
KNO ₃ : 500	00	306.6	69	69	00
Micro-nutrient					
CuSO ₄ , 5H ₂ O: 50	00	00	00	00	00
MnSO ₄ , H ₂ O: 100	00	00	00	00	00
ZnSO ₄ , H ₂ O: 100	00	00	00	00	00
FeCl ₃ , 6 H ₂ O: 100	00	00	00	00	00
MoO ₂₄ (NH ₄) ₆ , 4H ₂ O: 500	00	00	13.6	10.6	00
Total	1500	306.6	160.6	184.6	15

The choice of a model effluent simulating real dairy effluent was justified by the willingness to work with a water of stable composition, controllable and totally biodegradable, unlike in the case of actual raw dairy effluent, which is characterized by high variability of hourly daytime measurement for flow, temperature, pH, salinity and the pollution load. But also, to avoid technical problems related to the difficulties in taking, transporting and storing samples especially since one of the objectives was to validate the results obtained in previous work (Hamdani et al., 2018). In addition, taking into account the optimal residence time obtained during our previous work (Hamdani et al., 2018), three cycles (short, medium and long) were tested with the aim of allowing the SHF to simultaneously reduce COD and NO₃⁻ by bionitrification, which is an anoxic process. However, the abatement of the other forms of nitrogen (TKN and NH₄⁺) was researched through the creation of an aeration phase. The oxygen supply was ensured by injecting filtered air in fine bubbles from the base of the reactor to prevent detachment of biofilm and maintain de-aeration in the rest of the bioreactor. The FBBR aeration mode was controlled via the ON/OFF method, i.e. a continuous anoxic mode (OFF) and alternating cycle anoxic/aeration (ON).

The tests were performed at temperatures lower than 30°C to be close to the real conditions in the El Jadida city, where the study took place, which is characterized, - according to the climate data of the provincial meteorological Center, - by a semi-arid climate with an average temperature of 13-25°C.

2.3. Analytical and microbiological techniques

The physicochemical and microbiological analyses were determined according to the Standard Methods for the Examination of Water and Wastewater APHA (2012).

Physical and chemical analyses were carried out on the influent and the effluent with the following parameters: Temperature, pH, COD, NO₃⁻, NO₂⁻, NH₄⁺ and NTK. While, the bacteriological analysis was performed on water samples taken from only the treated effluent and focused on a qualitative and quantitative evaluation of the biomass.

Four groups of flora were searched using a basal media, e. i. Heterotrophic plate count agar -HPCA- (Difco Laboratories, USA) which contains: 3g peptone, 0.5g soluble casein, 0.2g dipotassium hydrogen phosphate, 0.05 magnesium sulphate, 0.001g ferric chloride, and 15g agar per liter of distilled water. The media was adjusted to pH 7.2 ± 0.2, incubated at 30°C for 96 to 168h depending on the targeted bacterial groups, and customized by adding selective components or modifying the cultural and incubation characteristics to select each microbial group and restrict a possible contamination by non-targeted microorganisms (Table 4).

Table 4: Types of flora and each cultivation conditions

	Addition	Cultivation & incubation characteristics
Heterotrophic denitrifying flora (HDF)	HPCA + 1 g KNO ₃	- Incubation temperature: 30°C - Incubation period: for 24 to 168h - Nitrate and nitrite reduction tests: Griess reagent and zinc powder
Aerobic heterotrophic flora (AHF)	-	Incubation at 30°C for 96h in strict aerobic
Anaerobic heterotrophic flora (ANHF)	-	Incubation on anaerobic jar under N ₂ /H ₂ atmosphere at 30°C for 96h
Aerobic mesophilic flora (AMF)	HPCA + 1g Glucose + 2.5g Yeast extract	Incubation at 30°C for 144h in strict aerobic

Three methods were used to estimate the number of bacteria:

(i) Indirect enumeration of only living cells cultivated on agar media: For the AMF, the aerobic heterotrophic flora and anaerobic heterotrophic flora, the results were expressed in number of colony-forming units (CFU) per mL of sample, and only the petri dishes whose number of colonies was between 30 and 300 were counted. However, the denitrifying flora was counted according to the Most Probable Number (MPN) method.

(ii) Direct method using epifluorescence microscope, after staining bacteria with acridine orange, which considers both living and dead cells, unlike the indirect counting method. A sample previously prepared according to the method described by Hobbie et al. (1977) is placed between a slide and a coverslip, then observed under a microscope (Olympus C×41) at ×100 and ×400 magnification.

(iii) Optical determination of total biomass concentration: The biomass specific growth rate was estimated by monitoring the absorbance (OD) periodically at 620nm. One liter of sterile nutrient broth (Difco Laboratories, Detroit, Mich.) was prepared and specifically, two media were used: uninoculated sterile media designed to blank the spectrophotometer, and the other inoculated with bacteria cells. A sample of 2 mL was taken from the bacterial culture in suspension and transferred into a clean cuvette. Then, changes in the turbidity of the second medium were recorded against the first uninoculated sterile media as the blank. For the total biomass, an absorbance of 1 was equivalent to 5.8×10^6 CFU/mL.

In order to estimate the bioactivity of the SHF in the FBBR, exogenous respirations of heterotrophic bacteria were monitored over time after adding three sources of biodegradable carbon (Milk powder, Starch, Yeast extract). The dissolved oxygen consumption rate due to the removal of biodegradable substrate was continuously measured in the bioreactor with a WTW Oxi 315i oxygen meter (Weilheim, Germany).

The SDE treatment efficiency was evaluated on grab samples collected from the bioreactor outflow, by measuring the above cited parameters. The biological treatment experiments were achieved when a steady state was almost reached, and the efficiency was analytically appreciated by monitoring the concentration evolution of the already mentioned parameters at the input or untreated effluent (C_i) and at the output or treated effluent (C_o) of the bioreactor. The abatement effectiveness (A) of these parameters (x) expressed in percentage (%) was calculated according to the equation 2.

$$A (\%) = \left[1 - \frac{C_o(x)}{C_i(x)} \right] \times 100 \quad (2)$$

2.4. Statistical analysis

The experimental data were analyzed and expressed as the standard error of the mean (SEM).

To assess the dependence between operating parameters (residence time, temperature, aeration mode) and various parameters to be removed (COD, TKN, NO_3^- , NH_4^+), statistical calculations according to the paired t-test method were carried out by using statistical software for Microsoft Excel XLSTAT 2020.4.1.1026.

3. Results and Discussion

3.1. Composition of the synthetic dairy effluent

The physical and chemical composition of the SDE used to feed the bioreactor is given in Table 3. In addition to milk powder, the reconstituted dairy effluent contained the main elements necessary for bacterial growth, notably a carbonaceous fraction (starch and yeast extract) leading according to the theoretical calculation to a COD of approximately 1500 mg/L, a mineral fraction composed of a phosphate solution, a solution of trace elements and nitrate. From this, the initial amounts of TKN, NH_4^+ , NO_3^- and TP were 184.6; 160.6; 306.6 and 15 mg/L, respectively. The respective COD/TKN/TP and COD/ NO_3^- ratios, close to 100/12/1 and 5, were in favor of a satisfactory biological and denitrifying activity of the influent. The ratios were linked to COD instead of BOD_5 because the COD was almost totally biodegradable owing to the biodegradability of the constitutive components (milk powder, starch and yeast extract). Before the biological treatment, the SDE color was milky white, but after treatment it was apparently clear and clean. This change in color might be related to the organic matter break down by the action of SHF. Similar finding was reported by Porwal et al. (2015).

3.2. Effect of the residence time on bioreactor performance

Operating in continuous anoxic mode and fed by applying loads of 0.536 ± 0.09 and 2.62 ± 0.36 $\text{Kg/m}^3 \cdot \text{J}$ in NO_3^- and COD respectively, the bioprocess provides the results shown on Fig. 2.

Fig. 2a indicates that after 24 h of treatment, the nitrate concentration in the effluent equal to 63 mg/L was higher than 45 mg/L, value recommended by the CCME (2012) related to the marine aquatic life protection against the direct toxic effects of nitrate. Biotenitrification occurred to reach its optimum (99.99%) after 48 h (Fig. 2b); it was accompanied by the reduction of COD from 1500 to 800 mg/L (24 h), 214 mg/L (48 h) and 102 mg/L (96 h).

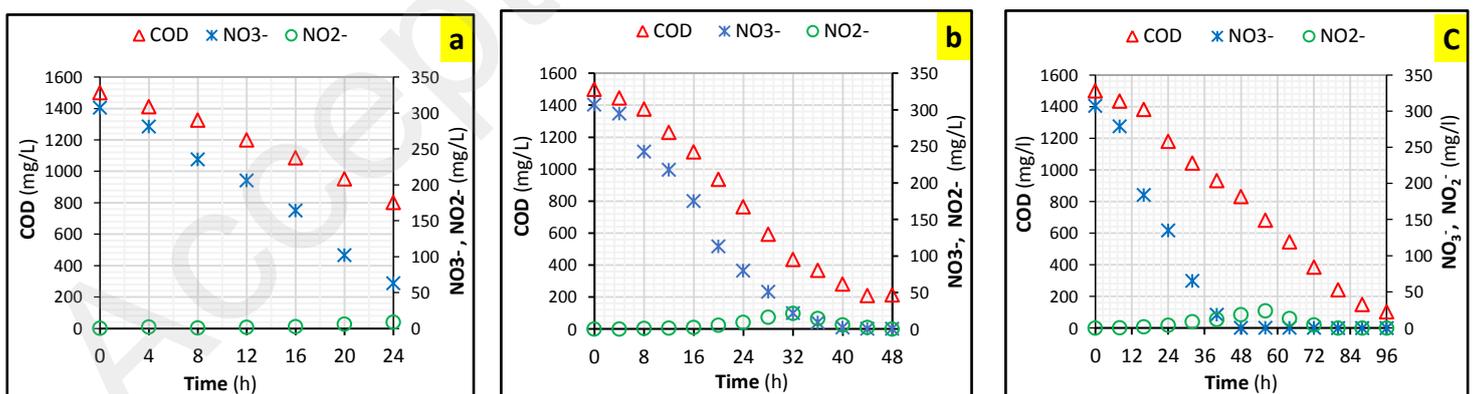


Fig. 2. Variation of nitrate and COD removal efficiency during a (a) 24 h, (b) 48 h and (c) 96 h cycle

Thus, to achieve complete biotenitrification, it was necessary to maintain contact between the nitrates, the carbon substrate and the biomass for 48h; at this moment the COD removal rate was 85.7%. It reached its maximum (93.2%) after 96h of treatment (Fig. 2c), thus meeting both the national and the international standards (120 mg/L) (BORM, 2013; Council Directive, 1991). The residence time

considered (96h) was higher than that described by Casellas (2002), who indicated that a duration of 36h is largely sufficient to guarantee a good treatment of organic and nitrogen pollution of urban wastewater.

The recorded performances appear to be comparable to those achieved for the treatment of a raw dairy waste in an anaerobic-aerobic (80:20) immersed bacterial bed: total denitrification in 72h coupled with 92% of COD removal (Hamdani et al., 2018). However, they were slightly above the results reported by Di Bella and Mannina (2020) who obtained a removal of 90% of nitrates and 93% of COD during the treatment of synthetic wastewater in a hybrid moving bed biofilm reactor with intermittent aeration. Furthermore, they were noticeably better than those obtained by Sylla et al. (2017), showing reduction of 82.2% nitrates and 71.5% COD contained in dairy effluent treated by a submerged bacterial bed, despite the longest period of treatment (42 days).

The high removal efficiencies obtained in this study suggest a high capacity to reduce nitrates and organic matter which is probably favored by:

(i) The selection of natural microbial consortia collected from dairy sludge, and well adapted to grow at the expense of the dairy influent as substrate, especially COD and N. Similar result was found by Porwal et al. (2015) who reported that the isolation of a mixed culture from dairy sludge is a key parameter to remove efficiently COD, and other parameters effectively from dairy effluent.

(ii) The use of inert carrier that offers the possibility to create a wider contact surface between the biofilm and the influent substrate. It is helpful for bacterial development and allows their clinging owing to the mucilaginous substance they secrete.

Findings from recent research demonstrated that the application of fixed cell systems offers multiple advantages in wastewater treatment overusing them in their suspended forms: higher mechanical strength, easier biomass/water separation, large increase in volumetric reaction rate, reduction in the size of reactors (Bayat et al., 2015; Martynenko and Gracheva 2003; Velkova et al., 2018). Also, Schlegel and Koeser (2009), and Ibrahim et al. (2019) added that if compared with the suspended one, the immobilized biomass increases more in density per unit bioreactor volume, develops a higher level of metabolic activity, making it possible to consume better the substrate contained in the reaction medium with both lower biomass density and shorter residence times. In this regard, Subtil et al. (2014) indicated that, compared with conventional suspended biomass (5540 ± 693 mg MLSS.L⁻¹), the attached biomass (5186 ± 435 mg MLSS.L⁻¹) to the polypropylene carrier (\varnothing :9mm, lengths: 10mm, density: 155 kg/m³, specific surface area: 330 m²/m³) showed satisfactory removal in COD (96%), total nitrogen (73%), and ammonia (98%) from domestic effluent with only 1/3 of the biomass.

Moreover, the immobilized biomass resists better against toxic compounds (acids, metals...), stress conditions (organic solvent, salinity, inhibiting elements, osmotic pressure, dehydration, self-destruction), and changes in physicochemical parameters (pH, temperature, O₂...) (Camargo et al., 2002; Muhamad et al., 2015; Velkova et al., 2018). This is important particularly in favor of wastewater purifying bacteria known for their sensitivity to pH, temperature, oxygen..., that consequently can achieve higher yields. Indeed, Ibrahim et al., (2019) reported that the application of bioreactor provided with polythene support (\varnothing :7mm, height: 10, density: 155 kg/m³, a specific surface area > 834 m²/m³) allows the attached biomass to develop better, leading to remove greater than 90% of both COD, nitrate, nitrite, and ammonia from a synthetic domestic effluent, representing about 12% more improvement if compared to the unsupported reactor.

(iii) A supply of exogenous carbon, in sufficient quantity ($\text{COD}/\text{NO}_3^- \approx 5$), easily and completely biodegradable (reconstituted and water soluble). The rate of exogenous biodenitrification ($2 \text{ mg N-NO}_3^- \text{ mgMVS}^{-1} \cdot \text{h}^{-1}$) is considered to be twice as faster, and more efficient than endogenous one ($0.8 \text{ mg N-NO}_3^- \text{ mgMVS}^{-1} \cdot \text{h}^{-1}$) for the same operating cycle (Casillas et al., 2002) and it can be up to five times according to Metcalf and Eddy (2013). On the other hand, the biodegradation of organic matter is slower if the available carbon is less rapidly biodegradable (Deronzier et al., 2001). These results are in accordance with those cited by Sylla et al. (2017) and Wang and Chu (2016), showing that biodenitrification is always coupled with the degradation of organic matter and depends on the denitrifying capacities of the bacteria, the nature of the carbonaceous substrate and the residence time.

Besides, the denitrification was concomitant with the progressive formation of nitrite, which reached a maximum between 8.7 and 23.8 mg/L depending on the operating cycle (Figures 3a, 3b, 3c). However, this formation remained transient since the formed nitrite was completely consumed thereafter. This is in agreement with the results obtained by Pan et al. (2012) who reported that the total elimination of nitrates at pH 6.5 and 7.5 is a guarantee of the absence of the nitrites accumulation in the treated water. Furthermore, the pH values recorded in this study (between 6.5 and 9) were most likely in favor of a total elimination of the nitrite formed during the denitrification process (see Fig. 2, paragraph 3.2), reflecting a high nitrite reductase activity. Albina et al. (2019) reported that the optimal pH range for complete denitrification without nitrite accumulation is between 7.5 and 9.5. According to Cao et al. (2013), increasing pH during the denitrification process is a good sign of the reduction of nitrites because the denitrification produces alkalinity. Furthermore, Hamdani et al. (2018) indicated that a temperature of 30 °C promotes a quick and complete nitrite reduction.

Based on these results, only the operating cycle time of 96 h was therefore considered thereafter.

3.3. Temperature and pH evolution on an anoxic 96 h cycle

Temperature and pH are two factors that affect the activity of microorganisms and their living environment.

In this research, the inlet temperature was 30 ± 5 °C. Fig. 3a shows that the temperature measured at the bioreactor output remained relatively stable, around a value of 28 ± 2 °C.

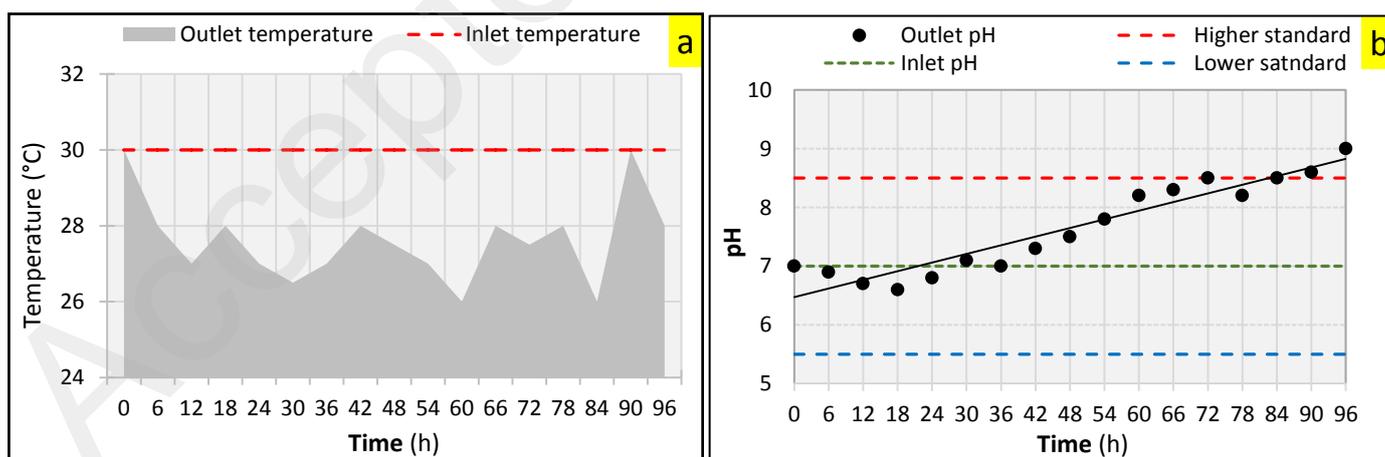
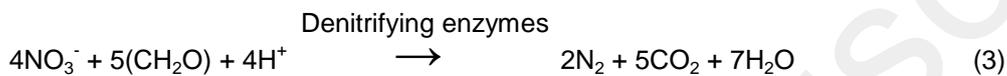


Fig. 3. Evolution over time of (a) the temperature and (b) the pH at the bioreactor outlet

At the beginning of the treatment, the temperature was supposed to increase because the bacterial growth linked to the degradation of the nutrient substrate is exothermic (Chen et al., 2020). So, this parameter seems to be more impacted by the climate than by endothermic or exothermic reactions

linked to bacterial metabolism in the reaction medium. The experiments were carried out in El Jadida city (Morocco) during the summer period when the temperature is between 25 and 30 °C, and a natural equilibrium is established between the temperature of the input water and the ambient temperature of the outside environment. Operating at room ambient temperature have the benefit to save energy consumption. The obtained values were very close to the limiting value required by the Moroccan standard (30 °C) (BORM, 2013) and considered compatible with a good biological activity. Tejaswini et al. (2019) reported that the most operated temperature range in a WWTP varies in the range of 25-30 °C.

Fig. 3b shows that the pH, initially neutral underwent a slight decline to 6.6 during the first 18h of treatment, and then tended to increase slightly until reaching 9 at the end of biodenitrification. The acidification noted at the start of denitrification was also noted by Hamdani et al. (2018). It was probably due to the accumulation of CO₂ in the reaction medium. Concerning the increase in pH, it comes from the consumption of H⁺ during the biodenitrification reactions (3) (Powers, 2005):



Overall, the pH oscillated between 6.5, value located in the pH range required by the Moroccan standard (5.5-8.5), and 9, which slightly exceeds the maximum standard set at 8.5 (BORM, 2013). The measured pH values were compatible with a good activity of SHF.

3.4. Effect of the aeration mode on the bioreactor performances

Fig. 4a shows that the COD removal rate depends on the aeration conditions. Compared to the aerobic or anoxic treatment trials where the respective abatement rates were 78 ± 6.9% and 82.1 ± 7.2%, the best yields were observed in alternating anoxia either with anaerobic (94.4 ± 11%) or aerobic (93.5 ± 8.1%) modes. COD removal was marked by a drastic decrease, from 1500 ± 370 mg/L at the inlet to 84 ± 26 mg/L during the anoxic/anaerobic mode and 98.1 ± 32 mg/L during the anoxic/aerobic period at the bioreactor outlet, respectively.

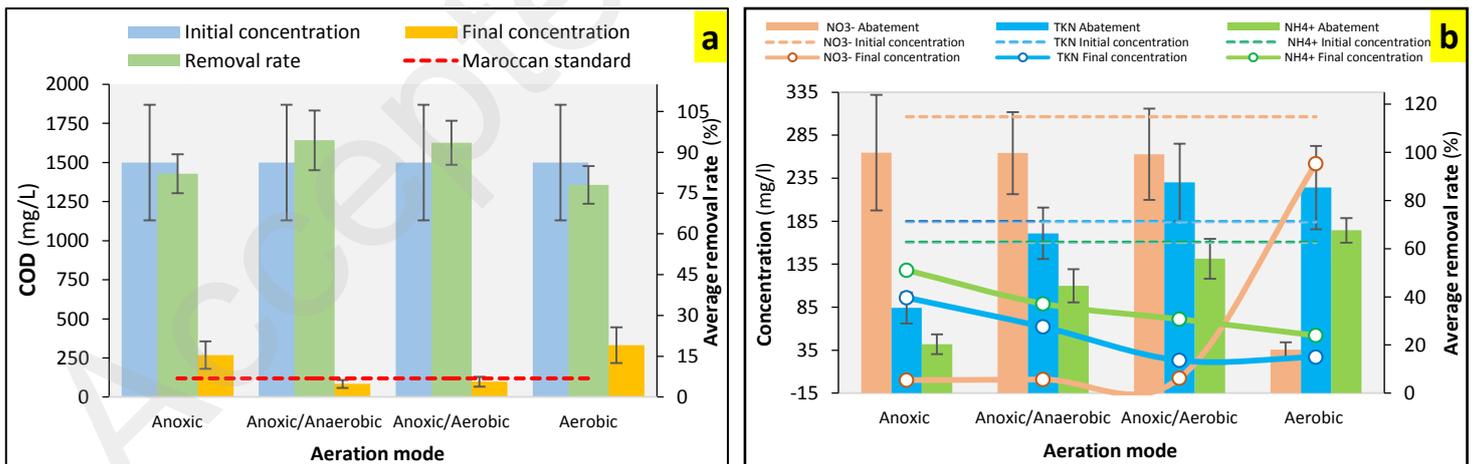


Fig. 4. (a) COD and (b) nitrogen (TKN, NO₃⁻, NH₄⁺) evolution and removal efficiency over the aeration mode

As shown in Fig. 4b, the pilot bioprocess exposed to total anoxia during 96h of operation, allowed the removal of 99.9 ± 24% of NO₃⁻; while low abatements were observed for TKN and NH₄⁺, only 35.4 ± 6.4% and 20.3 ± 4.1% respectively. However, the implementation within the bioreactor of aeration at a rate of 50% alternating over time with anoxia significantly enhanced the elimination of these forms of

nitrogen, in addition to a total removal of nitrate: fairly low concentrations of TKN (23 ± 0.37 mg/L) and NH_4^+ (71 ± 12.2 mg/L) were recorded for the purified water. The TKN output concentration was below the Moroccan standards (40 mg/L), but slightly higher than the European Communities Directive 91/271/EEC (Council Directive, 1991) (10 to 15 mg/L). According to Sawadogo (2018), the integration of anaerobic and aerobic zones inside the same bioreactor promotes an intensive form of biodegradation of organic and nitrogen pollution

The remaining modes (aerobic, anoxic/anaerobic) led to less interesting results in terms of performance: in aerobic, the abatement rate of nitrogen was found relatively high for TKN (85.4 ± 17.3 mg/L) and NH_4^+ (67.6 ± 5.1 mg/L), but very low for NO_3^- (18 ± 3.1 mg/L). This shows significant aerobic nitrification and a slight denitrifying activity in the bioreactor. In anoxic/anaerobic mode, the nitrification, which is an aerobic process remained insufficient (44.6 ± 6.9 mg/L).

3.5. Effect of the temperature on the bioreactor performances

Fig. 5a and Fig. 5b show the COD and nitrogen concentrations (TKN, NH_4^+ and NO_3^-) measured at the input and output of the bioreactor operated in anoxic/aeration mode, at different temperatures, as well as their associated reduction rates.

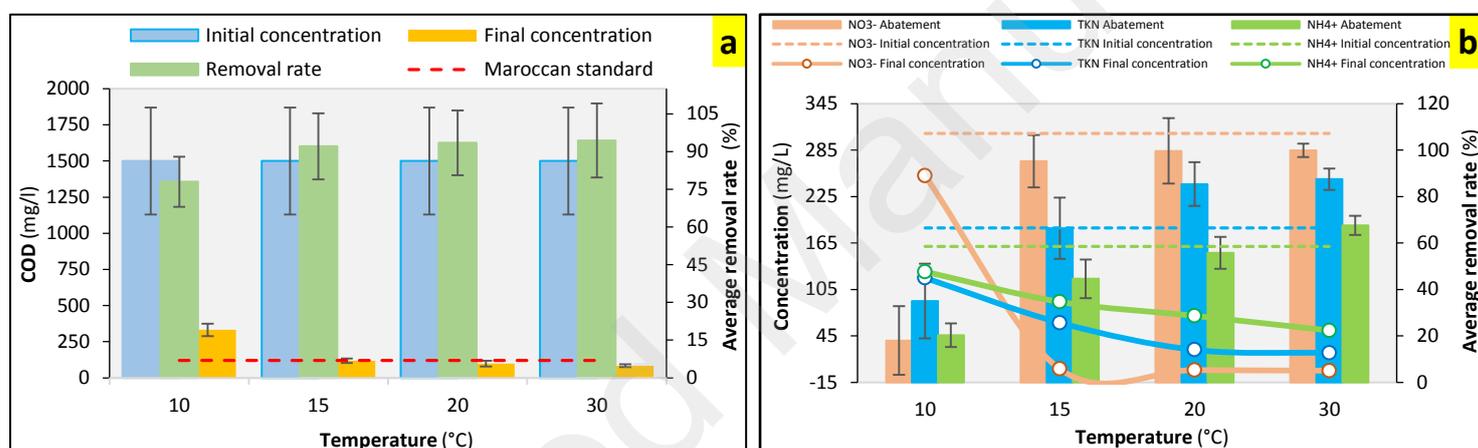


Fig. 5. (a) COD and (b) nitrogen (TKN, NO_3^- , NH_4^+) evolution and removal efficiency over the temperature

As observed in Fig. 5a, the average removal efficiencies of COD decreased as the temperature decreased from 30 °C to 10 °C. The lowest COD abatement rate ($78 \pm 10\%$) corresponded to the lowest temperature (10 °C), while the highest ($94.4 \pm 14.7\%$) was recorded at 30 °C and these high performances were almost kept at 20 °C ($93.5 \pm 12.9\%$), and even at 15 °C ($92.1 \pm 13.1\%$). At 10 °C, the effluent COD content was equal to 332 ± 42.6 mg/L, exceeding the national and international standard recommended for this parameter (120 mg/L). This result is similar and even better than that found by Lew et al. (2011) who reported 82%, 72%, 68%, and 38% COD removal at 28 °C, 20 °C, 14 °C, and 10 °C respectively. Similar trend was observed regarding nitrogen removal (Fig. 5b), since the best performances were obtained both at 30 and 20 °C for nitrates (> 99%) and TKN (> 85%) and only at 30 °C for NH_4^+ ($67.6 \pm 5.1\%$), while these yields dropped to $35.0 \pm 4.6\%$; $20.3 \pm 4.1\%$ and $18.0 \pm 2.9\%$ for TKN, NH_4^+ , and NO_3^- , respectively when the FBBR operated at 10 °C.

If compared to the other related studies, the present findings generally agree with those cited by Lotti et al. (2014) who obtained about 85% bio-denitrification in a laboratory bioreactor operated at 25 °C, which decreased below 20 °C; while Hu et al. (2013) observed 90% denitrification at 12 °C. These results clearly show that the decrease of temperature from 30 °C to 10 °C affected the biological removal

efficiency which decreased from $99.9 \pm 2.9\%$ to $18.0 \pm 2.9\%$ for NO_3^- , $67.6 \pm 5.1\%$ to $20.3 \pm 4.1\%$ for NH_4^+ , from $87.5 \pm 4.6\%$ to $35.0 \pm 4.6\%$ for TKN, and from $94.4 \pm 14.7\%$ to $78 \pm 10\%$ for COD. This represents a decrease of 82%, 70%, 60%, and 18% for NO_3^- , NH_4^+ , TKN, and COD respectively. This influence can be explained by a decrease in the metabolic activity of the purifying biomass at low temperature considered damaging for enzymes and cells, which can destabilize the reaction process (Fields, 2001). Hence, it is necessary to maintain an adequate temperature in the bioreactor. However, these results do not agree with those of Arevalo et al. (2014) who demonstrated that variation in temperature has no significant influence on the efficiency of a pilot bioreactor intended to remove COD and the nitrogen contained in domestic wastewater.

3.6. Correlative statistical analysis

The results of the statistical analysis presented in Table 5 showed that the efficiency of COD and NO_3^- removal was significantly influenced by the residence time: there was a significant difference in the removal efficiencies obtained at 24 h, 48 h, and 96 h ($p < 0.05$ and observed value $>$ critical value). Also, the efficiency of COD, TKN, NO_3^- and NH_4^+ removal in the effluent depended on the temperature because the average values of removal obtained at 10°C were different from those obtained at 15°C , 20°C , and 30°C ($p < 0.05$ and observed value $>$ critical value). However, except for the reduction of nitrate, the aeration mode had an influence on the removal of COD, TKN, and NH_4^+ ($p < 0.05$ and observed value $>$ critical value). Overall, statistical conclusions seem to be consistent with the experimental results obtained in the present study (Fig. 2a, 2b, 2c; Fig. 4a, 4b; Fig. 5a, 5b).

3.7. Biomass characteristics

3.7.1. Biomass composition

The examination of the treated water leaving the bioreactor revealed that the biomass was composed of four physiological groups; their respective number and percentage are presented in Table 6.

Table 6. Distribution of the bacterial populations in the effluent according to the counting method

Used method	Indirect counting method		Direct counting method	
	CFU/mL	%	Bacteria/mL	%
Anaerobic heterotrophic flora	$[8.1 (\pm 0.39)] \times 10^5$	40.5 (± 5.3)	$[17.6 (\pm 0.78)] \times 10^6$	41.9 ± 4.1
Heterotrophic denitrifying flora	$[5.1 (\pm 0.24)] \times 10^5$	25.5 (± 2.1)	$[9.8 (\pm 0.41)] \times 10^6$	23.3 ± 2.7
Aerobic heterotrophic flora	$[4 (\pm 0.29)] \times 10^5$	20 (± 4.6)	$[8.2 (\pm 0.45)] \times 10^6$	19.8 ± 3.8
Aerobic mesophilic flora	$[2.8 (\pm 0.15)] \times 10^5$	14 (± 1.7)	$[6.4 (\pm 0.32)] \times 10^6$	15 ± 1.2

Values in parenthesis indicate standard error of the mean SEM (a mean of three determinations)

In terms of abundance, the results show that the average concentrations of the various bacterial populations measured by direct counting with an epifluorescence microscope were in the range $(6.4 \pm 0.32) \times 10^6$ (aerobic mesophilic flora) to $(17.6 \pm 0.78) \times 10^6$ CFU/mL (anaerobic heterotrophic flora). Therefore, they were about an order of magnitude 20 times greater than those counted after cultivation on selective media which varied between $(2.8 \pm 0.15) \times 10^5$ and $(8.1 \pm 0.39) \times 10^5$ CFU/mL (Table 6). This difference between the results given by the two techniques should be most likely related to the fact that the direct methods detect all bacteria present, either alive or dead; while indirect methods underestimate the number of bacteria for two major reasons. First, they count only viable cells, and secondly, they consider that a colony comes from a single cell, which is not always true.

Table 5. Results of statistical analysis (paired two-samples t-test)

Variable 1	CV= 4.303; DF= 2; $\alpha=0.05$				CV=2.571; DF=5; $\alpha=0.05$			CV=2.201; DF=11; $\alpha=0.05$									
	Temperature				Aeration mode				Residence time								
									24h cycle		48 cycle		96h cycle				
Variable 2	COD	TKN	NH ₄ ⁺	NO ₃ ⁻	COD	TKN	NH ₄ ⁺	NO ₃ ⁻	COD	NO ₃ ⁻	NO ₂ ⁻	COD	NO ₃ ⁻	NO ₂ ⁻	COD	NO ₃ ⁻	NO ₂ ⁻
Observed value OV	141.949	11.889	8.419	463.429	16.521	11.889	8.419	2.654	3.958	3.928	2.740	5.663	6.709	3.180	5.626	9.290	3.080
p-value	<0.0001	0.007	0.014	<0.0001	0.004	0.007	0.014	0.117	0.011	0.011	0.041	0.000	<0.0001	0.009	0.000	<0.0001	0.010
OV/CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV<CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV	OV>CV

CV: critical value; DF: degree of freedom; α : alpha coefficient

Qualitatively, the most remarkable observation was the dominance of the anaerobic heterotrophic flora, which represented approximately 40.5% of the total active biomass colonizing the fixing support. In addition, the density of the aerobic heterotrophic flora was low compared to the anaerobic one. The majority of anaerobic or aerobic heterotrophic bacteria are capable of reducing nitrates to nitrites or nitrogen gas (Mauffrey, 2016). The denitrifying heterotrophic flora, characterized by its ability to use nitrate and other oxygenated mineral nitrogen compounds as the final electron acceptor, was abundant (25.5% of the total biomass). In this work, after inoculation under already favorable operating conditions of temperature, pH, C/N ratio, and hydraulic residence time, the heterogenous bacterial culture (anaerobic, aerobic, heterotrophic, denitrifier...) colonized the carrier, rapidly acclimated, proliferated and developed an immobilized biofilm because they were collected from natural sites of dairy effluent and highly adapted and specialized to grow at the expense of the organic matter and nitrogen. This seems play in favour of a better biodegradation of carbon and nitrogen pollution present in the SDE.

Hall-Stoodley et al. (2004) and Mauffrey (2016) reported that bacterial populations organized in biofilm lifestyle benefit from multiple advantages: stable development, proximity to cells, effective protection against aggressions from the living environment, and finally the emergence of new subpopulations said "persistent" more resistant than regular populations. This results in an increase in their metabolic performance, which promotes the consumption of organic and mineral substrates and improves the yield of the bioreactor. However, further bacterial analysis is needed to identify the species constituting the different groups of bacteria on the carrier media.

3.7.2. Bioactivity and growth rate determination

Fig. 6a indicates that the average biodegradation rates of organic substrate in the bioreactor were $76 \pm 11.64\%$, $53.2 \pm 7.86\%$, and $47.6 \pm 6.76\%$ for starch, milk powder, and yeast extract respectively. It is, therefore, preferable to use starch as a source of exogenous organic carbon, since its degradation is rapid by bacteria able to excrete specific enzymes responsible for carbon breakdown. The degradation of milk powder and yeast extract should then take place.

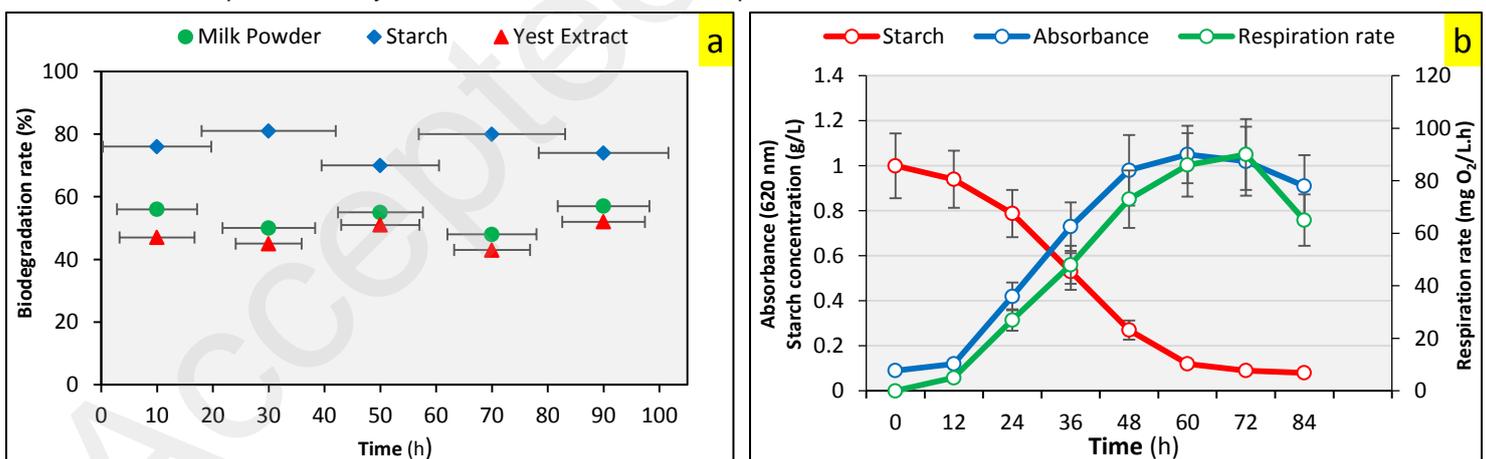


Fig. 6. Changes in (a) starch, milk powder and yeast extract removal rate, and (b) starch concentration, cell growth and respiration rate over the time at 30 ± 5 °C and pH of 7 ± 0.5

As shown in Fig 6b, the oxidation of the biodegradable substrate (starch) by heterotrophic biomass was accompanied by cell growth and respiratory activity, expressed in terms of absorbance at 620 nm and oxygen consumption respectively. After 12h of continuous cultivation, the bacterial growth was unrestricted, after which it appeared to be linear from 12h to 48h, and reached its maximum after 60h

$OD_{620} \approx 1$), which corresponds according to a calibration curve to approximately $(5.8 \pm 0.31) \times 10^6$ CFU/mL, indicating that a high bioreactor biomass concentration was attained. This is normal because the selected organic substrate (starch) is readily biodegradable by most heterotrophic populations and injected in abundance (1000 mg/L), which explains the increase of biomass concentration and the respiratory activity. This result remains less than 84h reported by Hamdani et al. (2018) who worked with a real dairy effluent rich in an organic matter less easily biodegradable if compared to reconstituted synthetic effluent containing starch, milk powder, and yeast extract.

Fig. 6b also showed that the use in this work of starch as an energy and carbon source satisfies both maximal cellular growth and respiration rates (90 mg O_2 /L.h). Degradation of organic matter and nutrient by bacteria needs dissolved oxygen as a final electron acceptor in case of aerobic treatment. When water completely devoid of oxygen (anoxic), bacteria is forced to use the oxygen contained in the nitrates (nitrate respiration). Generally, these processes can be summarized by the aerobic reaction (4) and anoxic reaction (5) (Russel, 2019).

Organics + Bacteria + Nutrients + $O_2 \rightarrow$ New cells + CO_2 + H_2O + Residual Organics + Inorganics (4)

Organics + Bacteria \rightarrow New cells + Residual organics + NH_3 + N_2 + CO_2 + H_2S + Inorganics (5)

Highly specialized bacteria play a key role in converting organic pollutants into new cells and simple end substances, and thereby producing clean water. Kargi and Pamukoglu, (2003) suggested that in the absence of purifying micro-organisms, the organic COD in wastewater must increase.

4. Conclusion

The FBBR anoxic-aerobic bioprocess produced a good quality effluent, which respects the recommended standards. The intermittent aeration was the key to achieve high COD and nitrogen removal efficiency from SDE. This investigation developed in one operating cycle and a single laboratory-scale reactor chamber constitutes the most innovative features of this work for future extrapolation on an industrial scale. This would offer a promising solution in the field of bioremediation, especially for agri-food professionals and small municipality officials in developing countries that suffer from released-raw effluents, limited financial means, lack of qualified human resources, and high-cost land.

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CRedit authorship contribution statement

Ahmed HAMDANI: Conceptualization, Methodology, Investigation, Visualization, Writing-Original draft, Writing-Reviewing and Editing. **Abdeltif AMRANE**: Visualization, Writing-Reviewing and Editing. **Imane KADER YETTEFTI**: Resource. **Mohammed MOUNTADAR**: Resource. **Omar ASSOBEI**: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision.

Conflicts of Interest

The authors declare that there is no conflict of interest whatsoever for this paper.

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Authors: Ahmed Hamdani, Abdeltif Amrane, Imane Kader Yettefti, Mohammed Mountadar, Omar Assobhei

Credit authorship contribution statement

Ahmed HAMDANI: Conceptualization, Methodology, Investigation, Visualization, Writing-Original draft, Writing-Reviewing and Editing.

Abdeltif AMRANE: Visualization, Writing-Reviewing and Editing.

Imane KADER YETTEFTI: Resource.

Mohammed MOUNTADAR: Resource.

Omar ASSOBBHEI: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision.

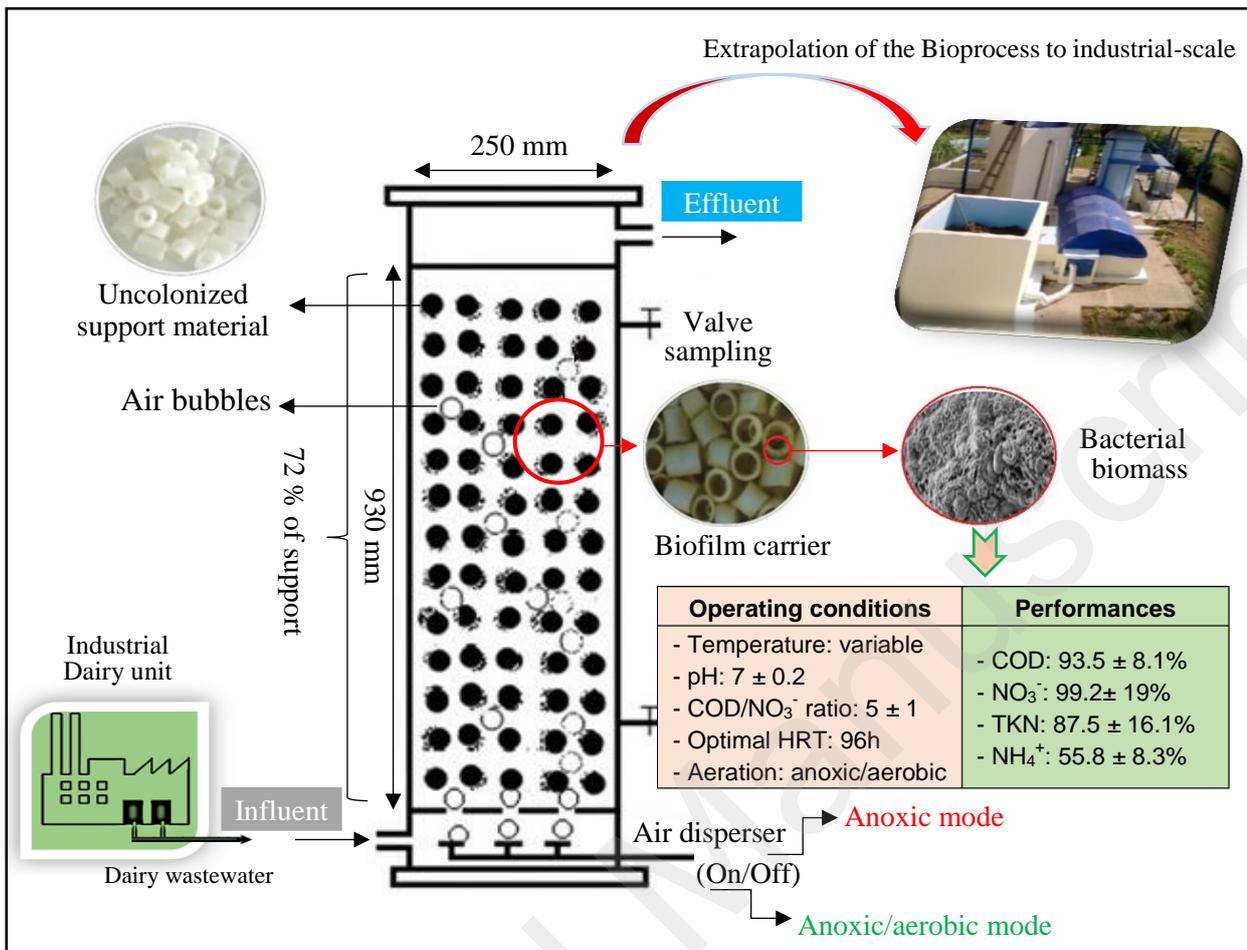
Annex 3: Declaration of interest statement

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical abstract



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Article Highlights

- ✓ *Fixed-bed bioreactor was developed for biological carbon and nitrogen removal.*
- ✓ *Treatment was conducted in one operating cycle and in a single reactor compartment.*
- ✓ *High and fast growth yields of the biomass were obtained.*
- ✓ *Anoxic treatment removed $99.9 \pm 24\%$ of nitrate, and $82.1 \pm 7.2\%$ of COD.*
- ✓ *Anoxic/aerobic mode improved both the reduction of TKN and NH_4^+ by 2.5 times.*