Microbiological and physicochemical quality enhancement of treated wastewater using raw and chemically modified clays from Sidi Bouzid region, Tunisia

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Abstract

Environmental discharge of wastewater represents a source of chemical substances that may be pollutants (such as phosphorus, chloride, heavy metals), as well as of microorganisms of concern (such as *Escherichia coli, Salmonella typhi*), that are spread into water courses and crop soils. These facts make clear the need for improving those treatment techniques currently applied in wastewater treatment plants (WWTPs) being not effective. This study firstly evaluates the microbiological, and physicochemical quality of treated wastewaters collected from two WWTPs located in two different Tunisian cities namely Sidi Bouzid (SB) (Centre of Tunisia) and Gafsa (G) (Southern Tunisia). Then, the capacity of three raw and acid/base-activated local clays to enhance the quality of wastewaters was assessed. The results indicate that the quantities of enteric bacteria (oscillating from $1.381.10^{3}$ to $1.4.10^{8}$ CFU/100 mL), fungi (between $1.331.10^{3}$ and $1.781.10^{4}$ CFU/100 mL), as well as SARS-CoV-2 (between $4.25.10^{3}$ and $5.05.10^{5}$ CFU/100 mL) and Hepatitis A virus RNA (form $4.25.10^{3}$ to $7.4\ 10^{4}$ CFU/100 mL) detected in effluent wastewaters were not in compliance with the Tunisian standards for both studied WWTPs. Likewise for other indicators such as electrical conductivity (ranging 4.9-5.4 mS/cm), suspended matter (145-160 g/l), chemical oxygen demand (123-160 mg/l), biological oxygen demand 5 (172-195 mg/l), chloride, Total Kjeldahl nitrogen and phosphorus contents (710, 58-66 and 9.47-10.83 mg/l respectively), the registered values do not agree with the set standards established for wastewater treatment. On the other hand, the pH values fitted (oscillating from 6.86 (at G) to 7.24 (at SB) with the Tunisian standards for both WWTPs. After treatment, wastewaters showed better values for the microbiological parameters, especially for the clays designed as AM and HJ1, which eliminated 100% of viruses. In addition, when acid-activated AM clays were applied, a marked improvement in the quality of physicochemical parameters was obtained, especially for suspended matter (2 and 4 g/l for SB and G, respectively), Total Kjeldahl Nitrogen (5.2 (SB) and 6.40 (G) mg/l), phosphorus (1.01 (SB) and 0.81 (G) mg/l), where SB WWTP effluents presented a better physicochemical quality than G WWTP effluents after being treated with clays. Our results open perspectives for the possibility of efficiently using these specific clays (which are cheap and easily available) in the enhancement of the quality of treated wastewaters. This can be seen as really relevant as regards environmental and public health considerations.

Keywords

Wastewater; Clay modification; Wastewater clay treatment; Microbiological quality, Physicochemical quality

Introduction

Currently, water scarcity is becoming a growing problem, aggravated by the rapid increase in population, as well as massive pollution of rivers (Gao et al., 2008) and sea (Vikas and Dwarakish, 2015), rapid urbanization, megacity development, and increasing competition among water users, triggering concerns about both human and environmental health (Schwarzenbach et al., 2010). Every year, Tunisia loses a significant part of its hydraulic resources due to the over-evaporation in summer under the effect of high temperatures and the overexploitation of drinking water in the various areas, as well as successive periods of drought

(Ben Boubaker, H. et al., 2010). Besides, food security depends on the availability and quality of consumption water, and currently this is considered as one central problem in the world (Chahed et al., 2010). To prevent the eventual severe lack of water, several solutions can be implemented to improve the quality and the availability of the resource: (i) water basin transfer, but it is not always possible to apply due to political or environmental issues within and between countries; (ii) desalination, which is a high cost treatment and only countries who have surplus of energy are able to use this process for water recovery; and (iii) wastewater recycling, which would be the most affordable solution. The reuse of treated wastewater effluents in agriculture has several benefits such as: (i) its production is unaffected by climate conditions; (ii) it is a potential source of nutrients and therefore can improve crop yield; and (iii) the groundwater reservoir is preserved (Molinos-Senante et al., 2010).

Despite the continuous development of new systems for wastewater treatment, since the late nineteenth century, reused wastewater is still considered an important vector of organic and inorganic pollutants, which endangers human health and environmental quality (Salgot and Folch, 2018) due to the persistence of pathogens and contaminants in different environmental compartments. Pathogens such as *Escherichia coli, Salmonella typhi*, and *Vibrio cholera,* common in wastewaters, cause various diseases leading to high morbidity and even mortality, especially in low-middle income countries. Jyoti and Pandit (2001) reported that 88% of diarrhea disease cases are linked to unsafe water supply and hygiene, which results in the death of millions of children every year (Lanata et al., 2013). A growing body of evidence points to the presence of a wide range of pathogenic microorganisms in treated wastewater (El Ouali Lalami et al., 2014). In addition to bacteria, viruses are responsible as well of several diseases (Hassine et al., 2010; Brugha et al., 1999; Metcalf et al., 1995; Bosch et al., 1991). Human pathogenic viruses such as HAV and SARS-COV-2 are commonly detected in urban wastewater and some of them are responsible for a considerable proportion of waterborne diseases (Rosa et al., 2020), although the presence of SARS-COV-2 in wastewater is rather seen as indicative of the epidemiological situation of the specific airborne disease in the area. According to the World Health Organization and several seroprevalence studies, the Hepatovirus A, is considered endemic in Tunisia and its epidemiological pattern ranges between high and intermediate endemicity (Ibrahim et al., 2020; Gharbi-Khelifi et al., 2006; Letaief et al., 2005). Several studies carried out in 2020, during the pandemic, estimated the presence and evolution of the SARS-CoV-2 virus in water resources such as rivers (Guerrero-Latorre et al., 2020), affected by wastewater discharge and sewers spreading sludge (Núñez-

Delgado, 2021; Rimoldi et al., 2020; Malik, 2020). The detection of the new COVID19 coronavirus in treated wastewater effluents proved the deficiency of conventional treatments applied in WWTPs to remove these microbes (Kitajima et al., 2020; Randazzo et al., 2020; Tran et al., 2020; Jmii et al., 2021).

Wastewater treatment within a treatment plant follows several well-defined processes to eliminate pollutants (Benneni and Bouarissa, 2020). Firstly, a pre-treatment step including screening, de-sanding and oil removal is necessary to eliminate large components (Orssatto et al., 2017), followed by a first treatment consisting of extraction of suspended solids and easily decantable organic matter by physicochemical processes (Shewa and Dagnew, 2020; Arashiro et al., 2019). Secondary treatments (biological treatments) are then carried out to degrade and decompose biodegradable organic matter by the action of a wide range of microorganisms, mainly bacteria (Neveux-Guilluy, 1993). Finally, tertiary treatments improve the quality of residual wastewater and, in recent years, extraordinary efforts have been made to implement these treatments to meet the global challenges of water shortage (Rashid et al., 2021), even though they are still absent in a large number of WWTPs. These tertiary treatments include a wide variety of techniques, such as chlorination (Yang et al., 2005; Mitch and Sedlak, 2002), UV disinfection, photocatalysis (Iervolino et al., 2020), different filtration techniques (Gómez et al., 2007; Altmann et al., 2016; Ramos et al., 2016), ozonation (Nawrocki and Kasprzyk-Hordern, 2010), oxidation and physical methods such as adsorption and membrane filtration (Rashid et al,. 2021).

Adsorption is generally considered as a process of transfer of removable species from an adsorbate (liquid phase) on the surface of the adsorbent (solid phase) through physicochemical interactions (Manchisi et al., 2020). It has been recognized as one of the most effective procedures for wastewater treatment (Slatni et al., 2020; Liu et al., 2020). The percentages of pollutants removal from wastewater by adsorption can reach 99.9%, a very high level of purification which ranks adsorption among the best wastewater treatment processes according to the United States Environmental Protection Agency (US EPA) (Anil et al., 2020). The adsorption method offers significant advantages such as low cost, flexibility, design simplicity, profitability, sustainability, efficiency and preventing secondary pollutants formation (Barakan and Aghazadeh, 2021; El Ouardi et al., 2019; Foroutan et al., 2019; Singh et al., 2018). Currently, different adsorbents have been used to remove the most concentrated pollutants in municipal and industrial wastewater, including domestic waste and industrial by-products

(Crini, 2006), clays (Pineda et al., 2020; Poetsch and Lippold, 2016; Tahar et al., 2014; Errais et al., 2010), and zeolites (Tahar et al., 2014).

The treatment of WWTP effluents using natural clay as adsorbent has shown good results favoring the reuse of the treated water for agricultural irrigation (Errais et al., 2010). When compared with other physicochemical techniques such as the coagulation-flocculation method, the clay treatment appears to be more effective. This physicochemical technique based on coagulation-flocculation process transforms dissolved pollutants into a solid residue, which is certainly of a lower volume, but with higher pollutant concentration. Moreover, this conventional technique, which is generally expensive, does not always reach the standards for the re-use of the resulting sludge in the environment, which supposed a risk for environmental pollution since the sludge resulting from treatment plants is usually applied to agricultural fields as fertilizer all over the world, including Tunisia. On the other hand, the clay used as adsorbent may be re-used in brickyards, cement factories, pottery and ceramics or as an impermeable barrier in the construction of discharge sites (Errais et al., 2010).

In recent years, wastewater treatment in Tunisia has been moving towards techniques that are more economical and more environmentally friendly. In this context, this study focuses on the use of clays to improve the microbiological and physicochemical characteristics of wastewater. Therefore, the present study aimed, firstly, to survey the presence of microorganisms indicative of pollution such as fungi, total and fecal coliforms, fecal *Staphylococcus aureus, Escherichia Coli* and *Salmonella*, as well as hepatovirus A (HAV) and SARS-CoV-2 in effluent wastewaters (WW) collected from the WWTPs of Sidi Bouzid (SB) and Gafsa (G). The quality of those effluents was evaluated by analyzing the main physicochemical parameters: pH, electric conductivity (EC), suspended matter (SM), chemical oxygen demand (COD), biological oxygen demand (BOD5), Chloride, Total Kjeldahl nitrogen content (TKN) and phosphorus content (P). Secondly, we assessed the effectiveness of three different types of local clays, both raw and after performing activation treatments with an acid, HCl (AA), and with a base, Na_2CO_3 (AB), in the amelioration of the microbiological and physicochemical quality of treated wastewaters. This alternative is included as a tertiary treatment with the aim of overcoming the deficit of conventional secondary treatment processes that guarantee a microbiological and physicochemical quality corresponding to the standards. We hypothesized that both raw and activated natural clay will adsorb pathogenic bacteria and viruses and will improve other quality parameters in wastewater, resulting in reduction of SM, turbidity, COD, BOD5, Chloride, TKN and phosphorus. All this can be considered of high relevance from the human health and environmental points of view.

Material and Methods

Clays description

All the studied sites are placed in the region of Sidi Bouzid, Tunisia (Figure 1). Three different clay samples were collected from three natural formations. The sample HJ1 was taken from the top of the El Haria formation situated in West of Jebel Jebbes El Meheri, Maknassy and the sample HJ2 was taken from the base of the Chouabine formation of Ypresian age (Lower Eocene) Maknassy. The third sample (AM) was collected from the Aleg formation at Jebel Mazzouna.

The predominant mineralogy of the clays in these formations is calcite, dolomite, quartz, gypsum, and clay minerals (smectite, illite, kaolinite, palygorskite, and sepiolite) (Mosbahi et al., 2014). In addition, they are characterized by a relatively large specific surface area, up to 50.47 m^2g^{-1} (Mosbahi et al., 2017).

Importantly, before any experiment, raw clay samples were suspended in distilled water, sieved through a 40 µm sieve to separate the impurities, and then they were oven-dried at 60 °C.

Table 1 indicates the mineralogical composition of the clays that were used in this work, with analysis carried out by means of RX diffraction (Philips PW1710 diffractometer, The Netherlands).

Description of the wastewater treatment plants (WWTPs)

Effluent wastewaters (discharge point) were collected from two Tunisian WWTPs, namely Sidi Bouzid (SB) and Gafsa (G), located in the southwest of Tunisia (Figure 2). Both WWTPs use activated sludge sanitation methods. SB has an average daily flow rate = 7,300 m^3 /day and serves 50,000 inhabitants, while the G WWTP has an average daily flow rate = 14,000 m^3 /day and serves 105,000 inhabitants.

In these wastewater treatment plants, pre-treatment begins with screening (elimination of the largest waste through a grid), then desilting (elimination of sand) and ends with de-oiling/degreasing (extraction of fats and floating matter found on the surface of the water). Additionally, the primary treatment allows the elimination of suspended solids accumulated at the bottom of the primary purification basins, by simple decantation. Subsequently, the wastewater in treatment goes to secondary treatment where oxygen-consuming organisms actuate by means of activated sludge (prolonged aeration at low load). Finally, an ultraviolet disinfection process is performed as a tertiary treatment to eliminate bacteria and viruses and even the most resistant forms such as bacterial spores or cysts, followed by micro-algae sorption. The purified water derived from both SB and G WWTPs is finally discharged into the neighboring natural environments.

Wastewater sampling

The wastewater (WW) sampling was performed 3 times in the period between March and May 2021 at the discharge point of the mentioned WWTPs. In total, the analyses were carried out for two wastewater samples corresponding to both WWTPs. The wastewater samples were collected in autoclaved (121°C for 30 minutes) glass bottles, and then conserved in plastic containers that were sterilized beforehand and kept at 4 °C upon arrival until were subjected to different treatments with clay in different experimental conditions (different types of clay tested at different concentrations as detailed below). As a control, the same wastewater samples (but without carrying out clay treatments), were used for each experiment.

Treatment of wastewater with activated clay

During the current study, we have tested the potential of both raw and activated natural clay for the purification of wastewater previously treated with activated sludge. Two types of activation have been tested: activation of the natural clay with hydrochloric acid (AA) and with sodium carbonate (AB).

The acid activation was not carried out on HJ1 and HJ2 because its carbonate content is low (HJ1) or zero (HJ2), while AM clay has a carbonate percentage of 50%. The aim of this acid treatment is to destroy the carbonate structure on the one hand and increase their porosity on the other hand. The preparation of the activated clays was performed as follow: 30 g of clay type AM was mixed with 30.33 mL of 3N hydrochloric acid (AA) and 150 mL of distilled water, and this mixture was stirred for 4 hours at 75 °C. After settling, the activated clay was washed several times with distilled water until reaching the neutralization of its pH (which was, every time, measured by using an electronic pH meter). Finally, the activated clay was dried in the oven at 60 °C (Srasra, 1989; Jarray, 1996; Komadel and Madejová, 2006).

The alkaline clay activation consisted in the dissolution of 30 g of natural clay in 300 mL of distilled water with 5 % of solid Na_2CO_3. This mixture was placed under continuous stirring for one hour at 75 °C. After decantation, the supernatant was removed using a syringe, then the resident clays were moved to the oven keeping it at 60 °C until they are dehydrated.

The treatment of wastewater samples with raw clay was performed per triplicate for each type of clay (HJ1, HJ2, and AM), using three different amounts of clay (60 g, 80 g, and 100 g) per liter of wastewater. Each clay sample was mixed with 1 L of wastewater in a beaker, stirred gently for half an hour and let to settle overnight. After decantation, the supernatant was removed with a syringe and the remaining clay was dehydrated in oven at 60 °C. Regarding the treatment of wastewater with activated clay, it was performed similarly to that carried out for the raw ones, but with only 30 g of activated clay.

Microbiological analysis

Mycological and bacteriological analysis

The microbiological analyses were performed with the aim of detecting possible contaminant germs namely fungi, fecal and total coliforms, *Staphylococcus aureus*, *Escherichia coli (E. coli)*, and *Salmonella* in WW samples. Detection and quantification of fungi was performed after cultivation and isolation of saprophytic or pathogenic yeasts and fungi on sabouraud agar, which is a classic fungi isolation medium created and named by Raymond Sabouraud in 1892. For isolation, a volume of 100 µL of each sample was inoculated on the surface of a Petri dish, and the same was done for different dilutions (10^{-1} to 10^{-10}), and then it was incubated at 25-30 °C for 3 to 5 days (Mendes et al., 1998).

Total aerobic mesophilic bacteria (TG) quantification was performed on Plate Count Agar (PCA) medium after incubation at 30 °C for 72 hours by counting the whitish colonies pushed in depth (Kacprzak et al., 2005). Furthermore, total (TC) and fecal coliforms (FC) were detected in the wastewater samples using the Violet Red Bile Lactose (VRBL) medium by counting the red colonies with a diameter greater than 0.5 mm. From the stock solutions (dilution in cascades of 10^{-1} to 10^{-10}), 1 mL of sample was transfer to an empty Petri dish using a sterile pipette, and then proceeding to inoculate deeply with approximately 20 mL of melted Plate Count Agar (PCA) yeast extract glucose agar, then making circular movements to allow the inoculum to mix with the agar. Leaving to solidify on the bench, then adding a second layer of approximately 5 mL of the same PCA agar. This double layer has a protective role against various surface contaminations. The dishes were incubated with the lid down at 30 °C for 72 hours and then read by counting the whitish colonies of TG grown in depth.

To investigate the presence of *Escherichia Coli* in our samples*,* two steps were executed: firstly, few colonies of fecal coliforms were pre-enriched in nutritive broth (peptone water) and then the standard enrichment in bromocresol purple agar (BCP) at 37 °C for 24 hours was performed (Dupray and Derrien, 1995). Similarly, *Salmonella* detection and quantification required several steps: pre-enrichment in peptone water, enrichment using specific enrichment medium: MSRV (Rappaport-Vassiliadis Semi-Solid Modified) and finally isolation of *Salmonella* colonies on *Salmonella-Shigella* (SS) Agar (Dupray and Derrien, 1995). Concerning *Staphylococcus aureus* (SA), a highly salty environment (Chapman) was used for its isolation and quantification (Porrero et al., 2014).

Virological Analysis

Virus concentration

In the current study viruses were concentrated by the adsorption-elution method using aluminum hydroxide and beef extract as described by (Jmii et al., 2021) with minor modifications. Briefly, 2 L of treated wastewater were filtered through 0.45 µm membranes (Millipore) which were latter cut and placed in 250 mL PPCO centrifuge tubes. Then 100 mL of the obtained filtrate was added to the PPCO tubes, adjusting the pH to 6.0 with $AlCl_3$ solution (0.9 N) and generating $Al(OH)_3$ precipitate (Metcalft et al., 1995), and vortexed to detach the viral particles stuck to the membranes. Afterwards, PPCO tubes were centrifuged at 2000 rpm for 5 min, and the supernatants were collected to measure virus concentration using the adsorption-elution method. Finally, the sample was centrifuged again (1700 *x* g, 20 min) and viruses were collected from the pellets, which were eluted using 3% alkaline beef extract buffer, transferred in 50 mL PPCO centrifuge tubes and shaken for 10 min at 150 rpm. Afterwards, the concentrate was recovered by centrifugation at 1900 *x* g for 30 min and the resulted pellet was re-suspended in 1 mL of phosphate-buffered saline (PBS). The obtained concentrates were aliquoted and conserved at -80 °C until use (Randazzo et al., 2020).

SARS-CoV-2 and HAV viral RNA extraction and quantification

RNA was extracted from 600 µl of the concentrate virus using the RNeas Power Water Kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA was eluted in 100 µl of RNAse-free water, aliquoted and conserved at -80 °C until use for viral RNA detection (Chomezynski and Sacchi, 1987). Analysis for SARS-CoV-2 RNA was performed using the QantiTect virus Kits (Qiagen) enabling a one-step quantitative detection of viral RNA targets.

In our study, we targeted the gene coding for SARS-CoV-2 nucleocapsid (gene N) and amplified two fragments of this gene latter (N1 and N2) (Kaya et al., 2022). Primers/probes were used in the RT-PCR reaction at the concentration recommended by the Centre for Disease Control (Table 2) (Kaya et al., 2022). The thermal cycling conditions were as RT at 50 °C for 20 min, preheating at 95 °C for 5 min, and 45 cycles of amplification at 95 °C for 15 s and 55 °C for 45 s. Each sample was analyzed in triplicate and every real time RT-PCR assay included negative (RNase-free water) and positive controls (SARS-CoV-2 RNA, Qiagene). The threshold value was set to 0.03 and the cycle threshold was set to 40. The standard curve was constructed using single stranded RNA fragments of SARS-CoV-2 containing the target region: gene N (Joint Research Centre, EURM-019).

Regarding the quantification of HAV RNA, QantiTect Virus Kits (Qiagen) were used in viral RNA amplification with the same reaction mixture used for SARS-CoV-2. The non-coding region 5' of viral genome was targeted and primers/probes used in HAV RNA amplification are listed in Table 1 (Legeay et al., 2000). The reaction mixture was incubated at 45 ºC during 40 min to reverse transcript HAV RNA into cDNA. Reverse transcription was followed by the DNA amplification consisting in 45 cycles of denaturation step at 94 ºC during 15 s and then an annealing step at 60 °C during 1 min. Viral load was quantified using RNA standard curve as described previously (Costa-Mattioli et al., 2002).

Physicochemical analysis

The parameters studied were hydrogen potential (pH), electrical conductivity (EC), suspended matter (SM), chemical oxygen demand (COD), biological oxygen demand (BOD5), chloride, Total Kjeldahl nitrogen content (TKN) and phosphorus concentration (P) (Letshwenyo and Veronicah, 2020). The pH and electrical conductivity (EC) of the WWTPs samples were determined using a pH meter model Istek-NeoMet and a conductivity-meter model CONSORT C831, respectively. Soluble chemical oxygen demand (COD) was determined according to the method of Knechtel (1978). The five-day biological oxygen demand (BOD5) was determined by the manometric method using a respirometer (OxiTop_ Box) as described by Zayen et al. (2010). The phosphorus concentration in aqueous media was determined according to the vanado-molybdo-phosphoric acid spectrometry method (Gouider et al., 2010). Total Kjeldahl nitrogen content (TKN) was determined as described by Kjeldahl (1883).

Statistical analysis

Statistical analysis was carried out using the SPSS 21.00 software. The objective of this statistical analysis was to compare, on one hand, the wastewater before and after the different treatments, and, on the other hand, to evaluate the difference of microorganisms concentrations among the two WWTPs, determined by analysis for independent samples, using student's T-test. Confidence levels were *: $p< 0.05$; **: $p< 0.001$.

Results

1. Qualitative and quantitative analyses of bacteria and fungi in treated wastewater samples

To assess the bacteriological quality of the wastewater treated by both WWTPs, SB and G, the presence of certain pathogenic bacteria was investigated using the bacterial culture techniques on specific culture media. We therefore looked for total bacteria (TG), total coliforms (TC), fecal coliforms (FC), fecal *Staphylococcus aureus*, *E. coli*, *Salmonella*, and fungi.

The microbiological analyses for the wastewater samples from the WWTPs have given the following results: $1.381.10^3$ and $2.29.10^3$ (CFU / 100 mL) were counted for the total germs, for SB and G, respectively, being 10^8 and $1.4.10^8$ (TC / 100 mL) for total coliforms, $1.654.10^6$ and 8.10^6 (FC / 100mL) for fecal coliforms, $1.5.10^3$ and $2.5.10^4$ (CFU / 100 mL) for fecal staphylococcus, and $1.33.10^3$ and $1.781.10^4$ (CFU / 100 mL) for fungi, respectively (Table 3). In addition, the qualitative analysis for the presence of *Staphylococcus*, *Escherichia coli* and *Salmonella* in the water discharged from the studied WWTPs was positive for all pathogens and the values of all parameters were always higher in Gafsa (G) WWTP (Table 3).

2. Treatment of wastewaters with raw clay and its effect on bacteria and fungi

During this study, we carried out the purification with natural clays of wastewater samples taken at the exit of SB and G WWTPs. Wastewater was treated with three different concentrations of raw clay (60 g, 80 g and 100 g per L of water). Our results show that microbial concentrations decreased for most parameters after the treatment (Table 3; Fig. 3).

For clay type HJ1, after adding 60 g of clay, the decrease in the percentage of total aerobic germs (TG) was around 56.6% for Sidi Bouzid WWTP samples, and around 60.7% (9.10^2) for Gafsa WWTP samples (Table 3). When 80 g of clay were added, the TG removal percentage was increased to 75.3% and 81.7% for SB and G sewage samples, respectively. Finally, when 100 g of clay were added, the microbial load reached $1.5.10^2$ CFU / 100 mL for both WWTP samples, with a concentration decrease of 89.14% for SB and 93.45% for G samples. For total coliforms (TC), a very significant reduction was detected, from 10^8 to $1.545.10^3$ TC / 100 mL, and from $1.4.10^8$ to $1.8.10^3$ TC / 100 mL when 60 g of clay were added in SB and G WWTPs, respectively (99.9 % removal efficiency). The TC amounts dropped to below detection levels when 80 g and 100 g of clay were used in both WWTPs (Fig. 3). In relation to fecal coliforms (FC), its removal efficiency rate was 99.9% for both types of wastewater with only 60 g of HJ1 clay added. Similarly, for TC, the concentration was below detection limits when 80 and 100 g of HJ1 clay were added. In addition, the results show that raw HJ1 clay was able to adsorb *Salmonella*, *E. coli* and *Staphylococcus* at any of the tested clay concentrations (Table 3). Fungi were no longer detectable in WWs when HJ1 was applied at any concentration.

As for the HJ2 type clay, the TG load in SB sewage samples treated with 60 g of natural clay decreased by 27.6% (Fig. 3). This percentage increased with the crescent quantities of clay used, reaching 49.32% and 83.35% reduction rates for 80 and 100 g of added clay, respectively. Similarly, for G wastewater samples the bigger amount of HJ2 clay used, the bigger removal efficiency was obtained. When using 60, 80 and 100 g of HJ2 the TG load was reduced by 36.69, 69.44 and 86.9%, respectively (Table 3). Additionally, we recorded a drop in the rate of total coliforms in wastewaters treated with 60 g of HJ2 clay, which went from 10^8 to 5.10^3 CT / 100 mL in SB samples, and from $1.4.10^8$ to 6.10^3 CT / 100 mL in G samples, with practically a 100% of reduction (Table 3). The number of coliforms was reduced to below detection levels when 100 g of HJ2 were used. Regarding fecal coliforms (FC), when 60 g of HJ2 clay were used, their number decreased from $1.654.10^6$ to 4.10^3 CF / 100 mL (SB samples), and from 8.10^6 to 5.10^3 CF / 100 mL for G samples (99.76 and 99.84% of reduction, respectively). When 80 g and 100 g of clay were added, FC concentration was below detection levels (Fig. 3). Regarding *Staphylococcus*, *E. coli, Salmonella* and fungi, for SB and GWW samples we have recorded a total absence (100% of removal efficiency), even with the smallest amount of HJ2 clay (Table 3).

After treating wastewater samples with natural clay type AM, the load of TGs dropped from $1.381.10^3$ to $5.3.10^2$ CFU / 100 mL, and from $2.29.10^3$ to 8.10^2 when 60 g of clay were used in the treatment of wastewaters of SB and G, respectively (61.63 and 65.07% reduction). When 80 g and 100 g of AM clay were used, 3.10^2 CFU / 100 mL (78.3% reduction) and $1.5.10^2$ CFU / 100 mL (83.14% decrease) were detected, respectively in SB samples (Table 3). For G WW samples, with 80 g and 100 g of AM clay, $3.5.10^2$ CFU / 100 mL (84.72 % reduction) and $1.5.10^2$ CFU / 100 mL (93.45% decrease) were detected, respectively. Moreover, the treatment with 60 g of AM has led to a significant decrease in TC and FC loads for samples taken from both WWTPs, with percentages of reduction higher than 99.9% in all cases. These bacterial groups were no longer detectable when wastewaters were treated with 80 g and 100 g of AM clay. In addition, this type of clay (AM) exhibited a marked capacity to adsorb *Staphylococcus* and *Salmonella* even when a low quantity of clay was used (60 g), but unlike HJ1 and HJ2, type AM clay failed to adsorb *E. coli* when 60 and 80 g of clay were added, and a higher quantity (100 g) was needed to completely remove *E. coli* from treated sewage samples (Table 3).

3. Treatment of wastewaters with activated clay and its effect on bacteria and fungi

Following the treatment of wastewater samples with activated clay, the microbial loads were significantly reduced. The treatment of wastewater with 30 g of HJ1 clay activated by Na_2CO_3 resulted in a drop in the load of total germ (TG) from $1.381.10^3$ to $0.61.10^3$ CFU/100 mL (55.83% reduction) in samples from Sidi Bouzid, and from 2.29.103 to 5.8.102 CFU/100 mL (74.68% reduction) in Gafsa samples, while for the rest of the microbial parameters the HJ1base activate clay achieved a total removal (Table 3).

The SB samples of wastewater treated with 30 g of alkaline activated HJ2 showed a significant reduction of fungi, *Salmonella, E. coli*, *Staphylococc*i and fecal coliforms, but TG (which was only reduced by 13.2%) and TC were still present. Similar results were obtained for the samples of wastewater from G treated with the alkaline activated HJ2 clay: a total absence of fungi, *Salmonella, E. coli, Staphylococci* and fecal coliforms, but presence of TC (Table 3). The number of TC in WW collected from G treatment plants and treated with 30 g of modified clay decreases by 99.9%, meanwhile TG decreased just 24.46% (Table 3).

The AM clay activated by Na_2CO_3 showed a marked capacity for removing the majority of microorganisms from the studied WWs (fungi, *Salmonella, E. coli, Staphylococcus*, total and fecal coliforms), with the exception of TG in G, which remained present in wastewater after treatment with the modified clay ($1.1.\ 10^3$ CFU/100 mL in the samples from SB and $1.43.10^3$ CFU/100 mL in the samples from G, with respective reductions equal to 20.35 and 37.56%).

Regarding acid activation, adding 30 g of HCl activated AM was able to completely remove all the investigated germs (fungi, TC, FC, *Salmonella, E. coli, staphylococcus* and even TG), and this was recorded for all studied samples.

4. Quantitative analyses of HAV and SARS-CoV-2 in treated wastewater samples and effect of the treatment with raw and modified clays on viral content

The quantification of Hepatovirus A RNA in wastewaters revealed the presence of 2.10^3 and $7.4\ 10^4$ RNA copies/L in treated wastewater samples collected at the discharge points of Sidi Bouzid and Gafsa WWTPs, respectively (Table 3). Treatments with HJ1 or AM raw clays at different clay doses completely remove the Hepatovirus A RNA ($p< 0.001$), while they persisted ($2.5\ 10^2$ and $3.7\ 10^3$copies/L at SB and G, respectively) when 60 g of HJ2 clay was applied.

Concerning SARS-CoV-2, Sidi Bouzid wastewater samples contained $4.25.10^3$ copies/L, while Gafsa wastewater samples encompassed $5.05\ 10^5$ copies/L of SARS-CoV-2 RNA when the gene fragment N1 was targeted. Regarding the gene fragment N2, $1.17\ 10^5$ and $5.43\ 10^3$ copies/L of SARS-CoV-2 RNA were obtained for Sidi Bouzid and Gafsa samples, respectively. To note that treated sewage showed a better virological quality comparing to untreated samples. The raw clays showed a high capacity of removing (in fact 100% efficacy) *HAV* and SRAS-CoV-2 viruses in the studied WWs from both WWTPs (SB and G), with the exception of HJ2 (at 60 g), which presented a lower efficiency than HJ1 and AM in viruses' removal. After treatment with 60 g of raw HJ2 clay, the WWs of G and SB were still rich in viruses ($2.5\ 10^2$ (SB)-$3.7\ 10^3$ (G) for HAV and $1.71.10^2$ (SB)-$2.23.10^3$ (G) for the genes N1 of SRAS-COV-2). Concerning the N2 genes, they were still present only in WWs of SB ($7.90\ 10^2$). However, all these values were under the Tunisian standard NT. 106.002.

Regarding the clays activated with Na_2CO_3 and those acid-activated (AM), they showed a capacity to eliminate 100% of the viruses detected in the studied WWs.

In fact, SARS-CoV-2 RNA was absent in all samples treated with HJ1 and AM, both with raw and modified clay, even at low doses added (60 g), which proves their efficacity in adsorbing this kind of viruses (Table 2). Meanwhile, HJ2 treatment did not result in a total removal of viruses when a quantity of 60 g was applied, but it showed a total efficiency at higher quantities (80 g and 100 g) (Table 3). Therefore, the treatments used in these WWTPs are effective and showed a high efficacy in virus removal (although to a lesser extent in the case of the HJ2 treatment), which improves the virological quality of the treated wastewater.

5. Physicochemical properties of the raw WWTP wastewater and the impact of different clay treatments

The physicochemical parameters were tested on the wastewater at the outlet of the Sidi Bouzid and Gafsa WWTPs and after the application of each type of treatment, and the results obtained are reported in Table 4. When treating wastewater with three different concentrations of raw clay (60 g, 80 g, and 100 g), the values of most physicochemical parameters decreased, with the exception of pH.

The pH of untreated wastewater was oscillated between 6.86 for Gafsa and 7.24 for Sidi Bouzid. These two values are very acceptable by comparing them with the values quoted by the Tunisian standard (NT. 106.002) (6.5 <pH<8.5). The pH values in wastewater samples after the different treatments with HJ1 and HJ2 clays were in the range7.20-7.93. When AM clay is used, the pH range is slightly lower (6.98-6.73). In all cases remaining within the Tunisian standard values

The electrical conductivity (EC) recorded in wastewater collected from the GWWTP was 5.420 dS m^{-1}, which does not comply with the Tunisian standard NT.106.20 (5 dS m^{-1}) (Table 4). Regarding SBWWTP, EC was 4.9 dS m^{-1}. These last results are in line with the values adjusted by the Tunisian wastewater discharge standard. The values recorded after the treatment in SB showed an average decrease in EC, especially in the cases of treatment with 100 g of clay type HJ1, HJ2s and AM, because they were 2.3, 2.7 and 2,9 dS m^{-1}, respectively. The values recorded after the treatment in Gafsa also showed an average decrease in EC, especially in the cases of treatment with 100 g of clay type HJ1 and AM, because their EC was 2.84 dS m^{-1} and 2.89 dS m^{-1}, respectively. These values had not exceeded 5 dS m^{-1}, so they are very acceptable according to the Tunisian standard (NT. 106.002).

We detected suspended matter (SM) levels of 160 and 145 mg/L of wastewater collected from G and SB WWTPs, respectively, which do not comply with the values tolerated by the Tunisian standard NT 106-002 (SM <30 mg /L) (Table 4).

SM decreases drastically with the use of any treatment, with values ranging between 31 g L^{-1} (60 g HJ1G and 60 g HJ2G) and 2 g L^{-1} (30 g AMSB (A.A)). It is observed that when the amount of added clay increases, the reduction of SM is greater, also highlighting that the clay treatments, especially with acid, are the most effective in reducing SM (4 and 2 g L^{-1} for AMG and AMSB, respectively). Some values are close to or slightly exceed the Tunisian standard limits (30 g L^{-1}), especially in the case of some of the treatments with only 60 g of clay (Table 4).

The chemical oxygen demand (COD) of wastewater from SB and G's WWTPs was 123 and 160 mg L^{-1}, respectively, which is beyond the allowed level fixed in the Tunisian standard (125 mg L^{-1}).

The COD values for the different treatments oscillate between 83 mg L^{-1} (60 g HJ1G and 60 g HJ2G) and 38 mg L^{-1} (100 g HJ1SB and 100 g HJ1G), indicating a strong decrease for all the treatments used, the decrease being greater when more amount of clay is used. A significant decrease in COD was recorded, especially when applying the treatments with raw clays HJ1 and AM and the HCl-activated clay. The following results: 79 mg L^{-1} (TWWSB) and 83 mg L^{-1} (TWWG) were counted for the maximum COD value, which are very low compared to the COD values of untreated water and compared to the COD limit value set by the standard NT 106-002 (125 mg L^{-1}).

The BODs values in the untreated waters were 172 and 195 mg O_2 L^{-1} for SB and G, respectively, much higher than the Tunisian standard limit (30 mg O_2 L^{-1}). A strong decrease in the amount of oxygen consumed by microorganisms is observed in all treated waters, parallel to the increase in the amount of added clay, with values between 31 mg O_2 L^{-1} (60 g HJ1G and 60 g HJ2G) and 9 mg O_2 L^{-1} (30 g AMG (AA).

Basically, in untreated wastewater of both G and SB's WWTPS, the rates of Phosphorus (10.83 mg L^{-1} for G and 9.47 mg L^{-1} for SB), Chloride (710 mg L^{-1} for both G and SB) and TKN (66 mg L^{-1} for G, and 58 mg L^{-1} for SB) exceeded the limit values set by the Tunisian standard (NT. 106.002) (Table 4).

The phosphorus concentrations, that were around 10 mg L^{-1} in the raw wastewater of both WWTPs, decreased to 3.9-6.0 mg L^{-1} (for treatments with 60 g of clay), 1.9-1.2 mg L^{-1} (for treatments with 80 g of clay), and 2.0-0.3 mg L^{-1} (for 100 g of clay). After treatment with activated clay, the concentration of phosphorus varied between 1.9 -1.01 mg L^{-1} for the clays subjected to basic activation, and between 1.0-0.81 mg L^{-1} for those acid-activated. With the addition of 80 and 100 g of clay and with the acid and basic activation treatment, the values achieved were generally lower than the Tunisian standards (NT. 106.002) (<2 mg L^{-1}).

All the treatments applied reduced N concentration. When 60 g of clay are added, N concentrations are between 29 and 21 mg L^{-1} for all the clays used, while for 80 g of clay the values obtained are between 22 and 15 mg L^{-1} ,and adding 100 g the levels of TKN were between 16 and 6 mg L^{-1}. It should be noted the low values of TKN achieved when using clays activated with acid (6.4-5.2 mg L^{-1}). All the treatments applied decreased this parameter below the limit values established by Tunisian legislation (30 mg L^{-1}) (Table 4).

Regarding chloride, its concentration in the clay-treated wastewater has a wide range (16.4 to 994 mg L^{-1}) and it was sometimes higher than that recorded in the untreated wastewater, especially in activated clay treatments (Table 4). The lowest values and the most respectful of the Tunisian standard (NT. 106.002) of chloride (700 mg L^{-1}) are recorded for raw HJ1 clays, especially with the doses of 80 and 100 g of clay, contrary to other types of clays with which the treatment turns out to be ineffective in the elimination of chloride.

Globally, the results of the analyses of the physicochemical parameters of SB and G's WW treated with clays showed that the values of the physicochemical parameters recorded in the samples treated with activated clays are very close to the limit values quoted by the standard, compared to those measured in wastewater treated with 60 and 80 g of raw clay. However, when using 100 g of raw clays, we obtained values close to those achieved with 80 g of clay.

Discussion

1. Microbiological properties

The results obtained for the microbiological analyses of raw wastewaters did not comply, generally, with the values determined by the Tunisian standard NT 106-002. Therefore, the wastewater treated in the Lessouda (Sidi Bouzid) and Gafsa WWTPs is not suitable to be discharged into the environment or reused in irrigation due to its poor bacteriological quality. Statistically, the concentrations measured for the microorganisms detected were significantly higher ($p<0.05$) in the wastewater leaving the Gafsa station than those in the wastewater from Sidi Bouzid.

Correspondingly, other fecal pollution indicator bacteria were detected in wastewater treated in other WWTP located in South of France, as mentioned by Brienza et al. (2019), who indicated that the numbers of *E. coli* in the influent was 10^5, higher than that in our study, while *E. faecalis* were about 10^4 CFU/mL. Besides, Ben Salem et al. (2011) showed that *E. coli* was detected at a rate of 76.6% at entrance points and 50% at the exit points, and *Salmonella* was detected in percentages of 66.6% and 20% at the entry and exit points, respectively, in other Tunisian WWTPs located in different regions (Sousse, Monastir, Kairouan and El kef); this fact indicates that wastewater treatment did not remove all the pathogens but gave reductions of 26.6 % and 40.6% for *E. coli* and *Salmonella*, respectively.

The amount of HAV and SARS-CoV-2 in the effluents of the SB and G WWTPs is also noteworthy (Table 4). Previous studies conducted in Tunisia have revealed that the situation regarding the epidemiology of Hepatovirus A is endemic, as demonstrated by the presence of virus traces in wastewater collected from different sewage treatment plants throughout the country (Ibrahim et al., 2020; Ouardani, 2016); this situation also occurs in other parts of the world (İnat and Koluman, 2013; McCall et al., 2020; Yang et al., 2021). In a recent Tunisian paper, Hepatovirus A was detected respectively in 62% and 66% of the collected wastewater samples at El Menzeh I and Charguia I WWTPs (Ibrahim et al., 2020). Actually, HAV was detected in 38% of the water samples in Saudi Arabia, with concentrations ranging from 5.0 10^1 to 1.9 10^4 RNA copies/L of surface water (Blanco et al., 2019). To note, the latter study, performed by Blanco et al. (2019), has shown the non-effectiveness of the conventional secondary treatments carried out in the majority of WWTPs where high loads of Hepatovirus A RNA were detected in effluent wastewaters, which constitutes a potential public health issue. As tertiary treatment, UV irradiation, has been proved to be inefficient in removing completely hepatovirus A from wastewater, as reported by Ibrahim et al. (2020), which is in accordance with our results demonstrating the presence of high levels of HAV RNA in effluent wastewater where UV radiation is used. Even more, biological treatments and the tertiary treatment with UV radiation proved to be insufficient to completely eliminate the HAV viruses.

Another current and urgent issue is the presence of SARS-CoV-2 in wastewater (Rimoldi et al., 2020). The presence of viable virus in the stools has been reported, suggesting that SARS-CoV-2 can be transmitted through the oral-fecal route (Arslan et al., 2020; Heller et al., 2020; Wu et al., 2020; Tran et al., 2020) raising epidemiological and environmental concerns.

Traces of SARS-CoV-2 (its RNA) have been detected in clinical liquid discharges (hospitals) (Zhang et al., 2020), in wastewaters of planes, navy and cargo ships (Ahmed et al., 2020), in rivers (Rosa et al., 2020) and even in effluent wastewaters subjected to a secondary treatment in sewage treatments plants (Medema et al., 2020; Randazzo et al., 2020; Romero et al., 2020). Even biological treatments and the tertiary treatment with UV radiation were insufficient to completely eliminate SARS-CoV-2 from wastewaters (Bhatt et al., 2020). Viral RNA was also detected in the activated sludges used in sewage treatments plants (Arslan et al., 2020) which drives us to rethink about the ways to treat wastewaters and WWTPs residues to prevent potential virus spread. Similarly, to our results, Haramoto et al. (2020) detected $2.4.10^3$ RNA copies/L of SARS-CoV-2 in one out of 5 secondary-treated samples, while Randazzo et al. (2020) reported 2.5 10^5 RNA copies/L.

Given the inadequacy of the treatment processes currently available at the WWTPs studied, it is necessary to adopt more efficient processes. Previous studies have dealt with the assessment of the performance of natural clays and their drifts in purifying water (wastewater or domestic drinking water) as a new, more effective, and cost-effective treatment alternative (Meçabih et al., 2006). In fact, natural clay was tested to eliminate microbiological pollutants (mainly viruses and persistent bacteria), and chemical pollutants such as pharmaceuticals and personal care products (Dijaani and Amer, 2020).

The results obtained in the present study indicate that the three types of clay cantnot remove completely TG, even when a high concentration of clay was applied (100 g L^{-1}), being only 100% effective the AM acid-activated clay (Fig. 2). For TC and FC, complete removal is observed when an amount equal to or greater than 80 g of any of the clays used in this study is added. The three types of clay HJ1, HJ2 and AM were able to adsorb efficiently fungi, *Salmonella* and *Staphylococcus* even at a small amount of 60 g, while for *E. coli* AM clay is only effective when added at high doses or activated with acid or base. To sum up, according to the bacteriological analyses carried out for our wastewater samples, the activation with Na_2CO_3 was more effective for HJ1 and AM than for HJ2, since the latter was able to adsorb only five of the seven pathogens studied. Acid activated clay (AM) seems to be efficient and adsorbed all of the studied microorganisms present in the wastewater.

The adsorption process is different depending on the clay. Several studies on the antibacterial effect of green clays have shown that smectite-based clays, such as our clay samples, are characterized by strong antibacterial capacity and by completely eliminate *E. coli, S. Enterica serotype Typhimurium, P. Aeruginosa, Stapylococcus* and *M. Marinum* (Williams et al., 2004; 2008; Xia et al., 2005). Under normal conditions, illite and kaolinite adsorb organic molecules onto their external surfaces, whereas swelling clays adsorb organic compounds mainly into the interlayer space, with very little external adsorption. This is due to their relevant interior specific surface area and adsorption sites, where hydrated exchangeable cations can be replaced by organic molecules (Errais et al., 2010). In relation to carbonate minerals, several authors show their ability to adsorb different pathogens as well as to inhibit their growth. Li et al. (2022) indicate the ability of dolomite to adsorb coliform bacteria, especially when the pH is around 7.8. According to Lee et al. (2022), the presence of $CaCO_3$ can interrupt the formation of the cell walls of different pathogens, such as *E. Coli*, causing the lysis of these microorganisms. Other authors indicate that calcite decomposition causes an increase in porosity and pore size, so the presence of active sites for *E. coli* adsorption is also greater (Tong et al., 2013). All of the above would justify the greater microbial adsorption obtained in the present work in the three types of clay, since they present in their composition carbonate minerals (calcite and dolomite), as well as sepiolite, palygorskite and/or montmorillonite (Table 1).

Moreover, the results of elimination of fungi, TC, FC, *Salmonella*, *E. coli*, *Staphylococcus* and even TG by 30 g of HCl-activated AM indicate that it is more effective than raw AM, especially when only 60 g are used. The use of 30 g of AM+HCl produces results comparable to the use of 100 g of raw AM.

In relation to HAV and SARS-CoV, present in the effluents of both WWTPs, they decrease to unquantifiable values for practically all clay treatments. A study by Lipson and Stotzky (1985) showed that there are specific adsorption sites on clay for each virus population, which makes it possible to predict the behavior of viruses on clay minerals. Thus, the results of this study can explain the variation in the adsorption power of AM, HJ1 and HJ2 clays against HAV and SARS-COV-2 viruses, where HJ1 and AM are the most effective among the raw clays, which could be related to the presence of calcite and dolomite in these clays, while those minerals are absent in HJ2 (Table 1).

Thus, the difference in microbiological parameters between treated wastewater compared to untreated wastewater from G and SB was significant for all treatments, especially for samples treated with 100 g of clay. According to Pineda et al. (2020), the relevant content of silica, aluminum and ferric oxides in clays generates a biocidal environment, reducing the content of viruses, bacteria and protozoa.

In the current study, the microbiological quality of wastewater collected at the outlet of G and SB WWTPs was improved following clay treatment, to comply with the Tunisian standard NT 106 002, setting the acceptable bacterial loads in effluents wastewater, which was not the case before the clay treatment.

Several authors indicate the variable effectiveness of different natural clays, raw or physically or chemically activated, in adsorbing different types of micropollutants existing in wastewater (clinical, industrial and domestic effluents) (Heidari et al., 2022; Mohamed Amin et al., 2016; Mahouachi et al., 2020), which suggest its future use as an effective, affordable, eco-friendly and safe alternative treatment. In the context of the strong emergence of infections caused by antibiotic resistance, the use of natural clays as antibacterial and antiviral agents in municipal wastewater rich in these microorganisms may constitute a practical and economical alternative due to their adsorbent power (Benali et al., 2017).

2. Physicochemical properties

The various analyses of the physicochemical parameters of the wastewater treated in the two treatment plants G and SB revealed values that did not comply with the Tunisian standards in force NT 106-002 (CE < 50 dS m^{-1}, MES < 30 mg/L, COD < 90 mg O_2/L, BOD5 <30 mg O_2/L, phosphates < 2 mg/L, nitrates < 30 mg N/L) with the exception of pH values, which comply with the Tunisian standards. These results seem to be consistent with those obtained in other previous studies carried out in a pilot wastewater treatment plant (Ibrahim et al., 2020; Letshwenyo and Veronicah, 2020).

The results obtained after the treatment with raw clay showed a decrease in suspended matter, COD, BOD5, phosphate, nitrate and electrical conductivity, being overall slightly more effective with AM clay, and especially with HJ1, relative to HJ2. Similar conclusions were drawn from other studies conducted by Khamis et al. (2012) and Errais et al. (2010), which were focused on the removal of organic substances and chemical elements, strongly detected in wastewater, by means of clay materials of other origins.

The most efficient clays to improve the physicochemical parameters of the water were HJ1 and AM (Table 4), which present in their composition montmorillonite, paligorskite, or sepiolite, as well as calcite and dolomite, while in the HJ2 clay, of these minerals, only sepiolite is present. The three previous phyllosilicates would clearly contribute to the greater efficiency of these clays, since their adsorbent properties are well known. Regarding carbonated materials, several authors point out their high efficiency in adsorbing different contaminants, being the main adsorption mechanisms ionic exchange, surface complexation, physical adsorption and, above all, precipitation processes (Khoshraftar et al., 2022; Shah et al., 2020; Ariffin et al., 2017). According to Mosbahi et al. (2017), the specific surface of HJ1 clays is relatively high, around 50.47 m^2 g^{-1}, which also explains their high efficiency in the purification of wastewater compared to raw AM and HJ2.

Electrical conductivity values recorded in the wastewater at the outlet of the stations of SB and G, are close to the Tunisian standards. In any case, these values are high and can contribute to soil degradation due to salinity (Hacini et al., 2013). The average conductivity decreased after treatment (although not significantly), what can be related to adsorption of cations and anions on the surface of the clay, or due to the constitution of oxides as previously shown by Al Bakri et al. (2011).

Regarding COD and BOD5, we note a significant reduction in their values from G's and SB's wastewaters after clay's treatment. Awad et al. (2013) found that two natural clays called Shendi and Singa reduced COD by 26.3% and 28.1%, respectively, and their combination with polyaluminium chloride (PAC) improved their COD removal efficiency by up to 70.7%. A previous study has shown a strong reduction of COD and BOD, even higher than in our study, after filtering wastewater through a micellar clay column (Khamis et al., 2012). According to these authors, the initial high BOD and COD observed in untreated waters may be due to residues of chemicals in the wastewater, which were not well removed by the secondary biological treatment.

The large amounts of chlorides contained in domestic wastewater can significantly alter the ecological balance (Apte et al., 2011). For this reason, in this study we focused on the removal of these toxic compounds. The reduction in chloride concentration was particularly significant with the HJ1 treatment. Apte et al. (2011) showed that the efficiency of a dried plant biomass of the species *Parthenium sp.* in the reduction of chlorides in wastewater by biosorption was very high, around 40%, but still lower than that obtained in our work using different doses of the HJ1 clay.

Regarding suspended organic matter (SM), there was a significant reduction of this parameter after treatment, thus meeting the Tunisian standard. Other authors also obtained a high efficiency in the removal of SM from wastewater using raw clays (Young et al., 2021) or montmorillonite clays modified with aluminum (Al) or ferric (Fe) polymeric species (Jiang et al., 2004).

In addition to the effectiveness of raw clays in improving the physicochemical conditions of these wastewaters, treatment with acid-activated or base-activated clays generally provided better results, with acid activation of AM clay being the most effective. Thus, an excellent physicochemical quality of these waters was obtained after the treatments. According to several authors studying different activated clays from Tunisia (Krupskaya et al., 2017; Komadel and Madejova, 2013), during acid activation protons are exchanged by exchange cations in the interlayer and the crystalline structure of the clay is partially dissolved, releasing some of the cations such as Mg, Al or Fe from the octahedral layer, leading to an increase in porosity, surface acidity and specific surface area in these acid-activated minerals (Krupskaya et al., 2017; Komadel and Madejova, 2013). According to Mosbahi et al. (2017), the destruction of AM carbonates following the acid attack is the origin of the improvement in the adsorbent capacity by increasing the specific surface and the active sites within the crystalline lattice of the clays. This may justify that acid activation is more effective when applied to clays with high calcite/dolomite content (HJ1 and, especially AM) (Table 1).

Regarding the basic activation of clays with similar characteristics to those of this work, Mosbahi et al. (2017) observed an increase in the specific surface area, the formation of sodium smectite clays, as well as the destruction of dolomites and the growth of zeolites. This could explain the higher adsorption obtained in these clays compared to the raw clays.

The improvement observed in the purification capacity after the activation of the clays, in comparison with the raw natural ones, has been described in other studies where starch was added (Mohamed Amin et al., 2016).

Conclusion

This work shows the results of the first study on the treatment of wastewater highly loaded with micropollutants performed by means of clays from the Maknessy-Mazzouna basin, center west of Tunisia. During this research, the effectiveness of these clays in the elimination of organic and microbiological pollutants from wastewater, as well as in improving its quality as regards physicochemical parameters, was assessed in detail.

Almost all of the high pollutant load concentrating in the effluents discharged by the two wastewater treatment plants (WWTPs) studied, one of Lessouda, Sidi bouzid and the other of Gafsa, was reduced after the treatment of the effluents using samples of natural and chemically modified clays, with removal percentages reaching up to 100%. Among the raw clays, HJ1 is the most effective in the wastewaters treatment, while AM activated by acid (HCl) proved to be the most effective among the activated clays. We have found that the acid or base activation of the clays made it possible to reduce the quantity of clays used (from 100 g to 30 g), achieving the same purification quality. This can help in preserving clay resources, that may be limited, during the implementation of a large-scale project.

In this respect, the method here used seems to be very promising, due to its simplicity and also great results in improving the microbiological quality of treated wastewaters. In the context of environmental preservation, the results obtained by this study have proven to be relevant and useful, since scaling from ‘'basic research'’ to ‘'technological application'’ requires testing its performance in real wastewater treatment plants, a comparison to commercial sorbents, regeneration, and cost evaluation.

Finally, finding new low-cost methods/tools enabling a complete elimination of microorganisms from wastewater is imperative, and it is a typical aim in developing countries, where costly resources and technologies are not sufficiently available.

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Statements and Declarations

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Author contributions

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Competing Interests

The authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

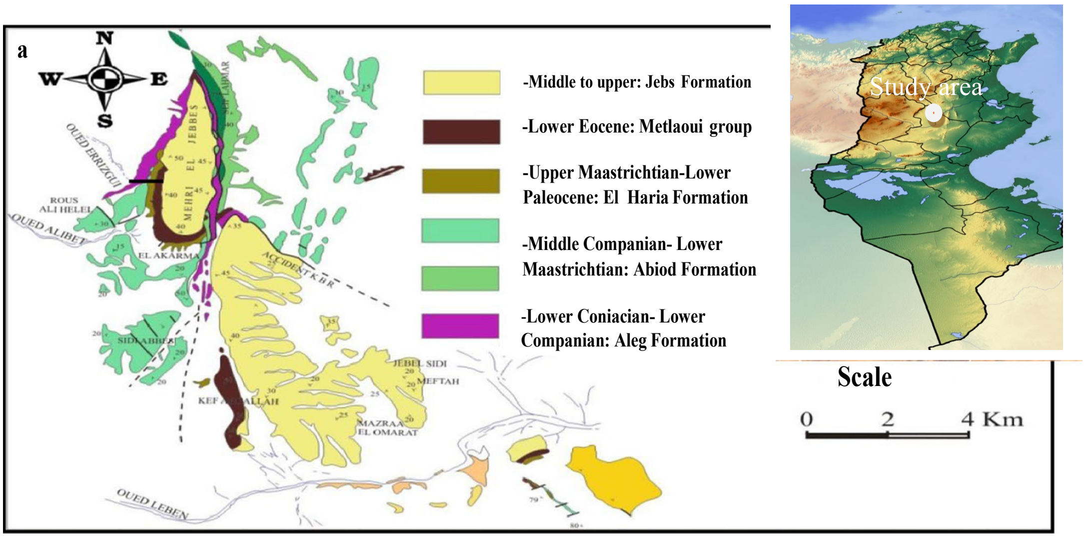


Figure 1. Location of the Maknessy-Mazzouna basin, center west of Tunisia (a) Geologic outcrops at Jebel Meheri El Jebbes showing position of lithological section (Khlifi, 2004) and (b) Location of the Meknassy-Mezzouna basin, Centerwestern Tunisia (Mosbahi et al., 2007)

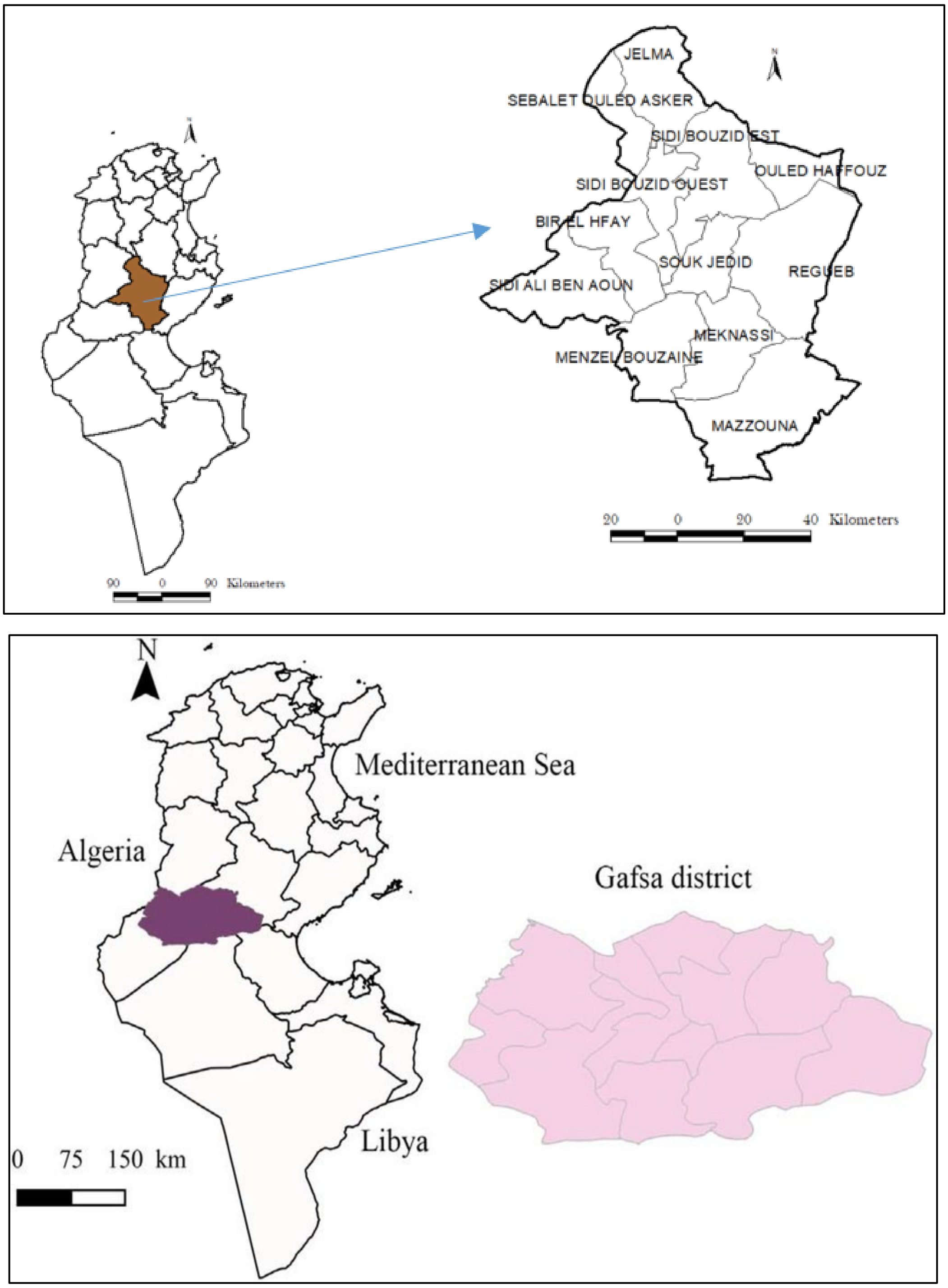
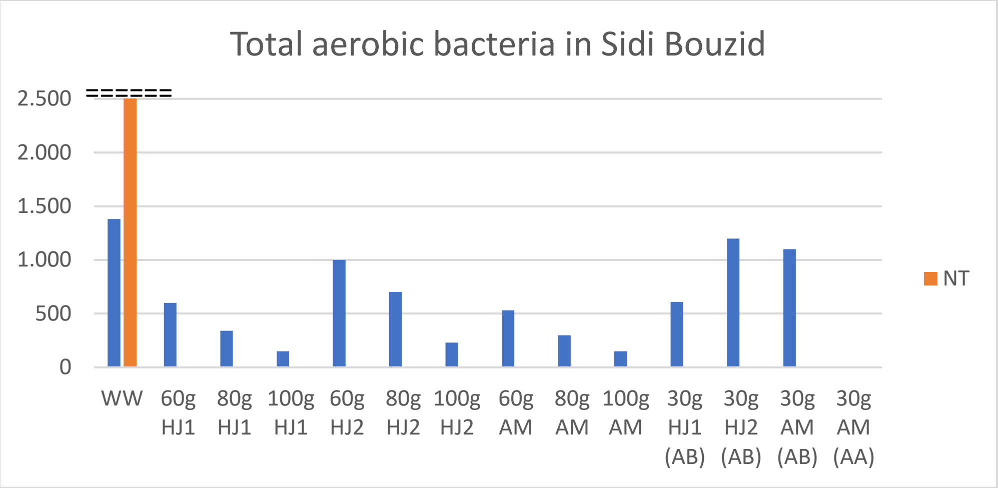
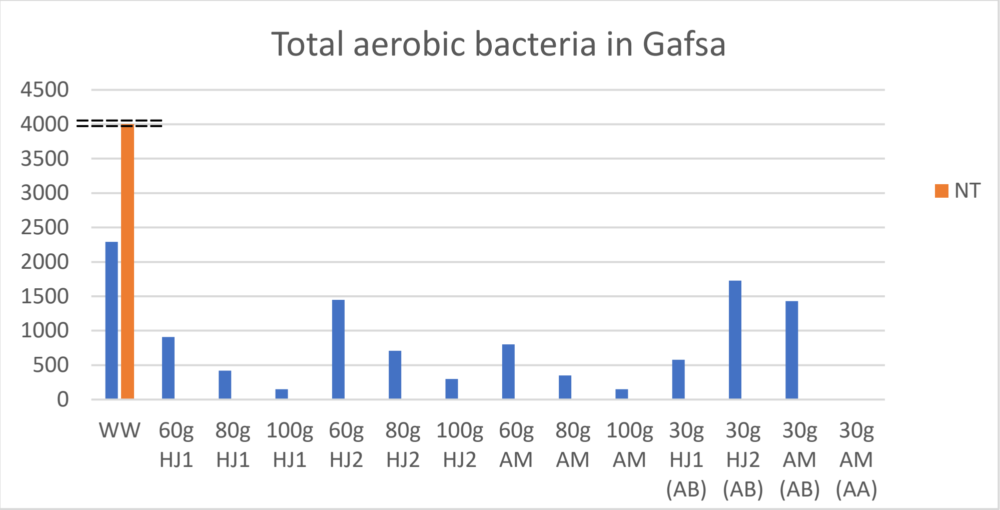
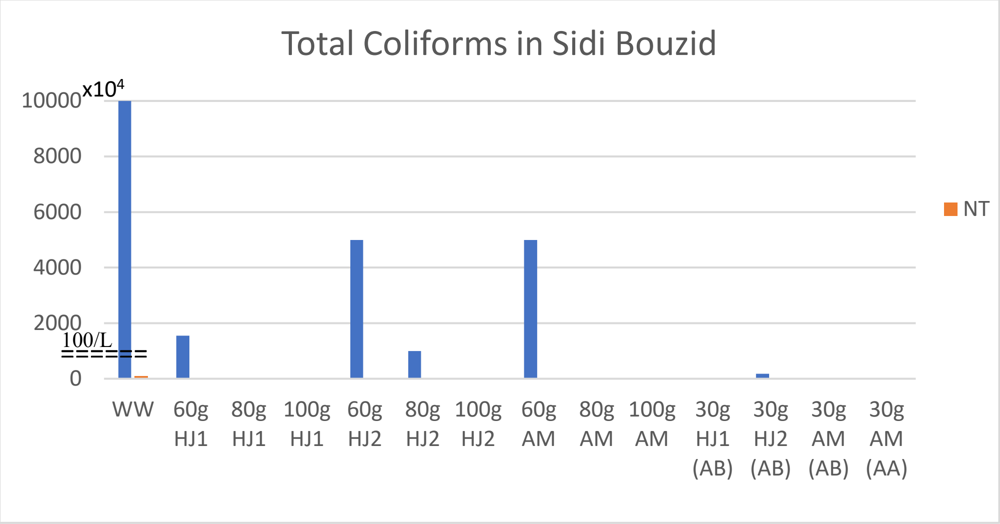


Figure 2. Geographic map of Sidi Bouzid and Gafsa (ref)


Total aerobic bacteria in Sidi Bouzid


Total aerobic bacteria in Gafsa


Total Coliforms in Sidi Bouzid

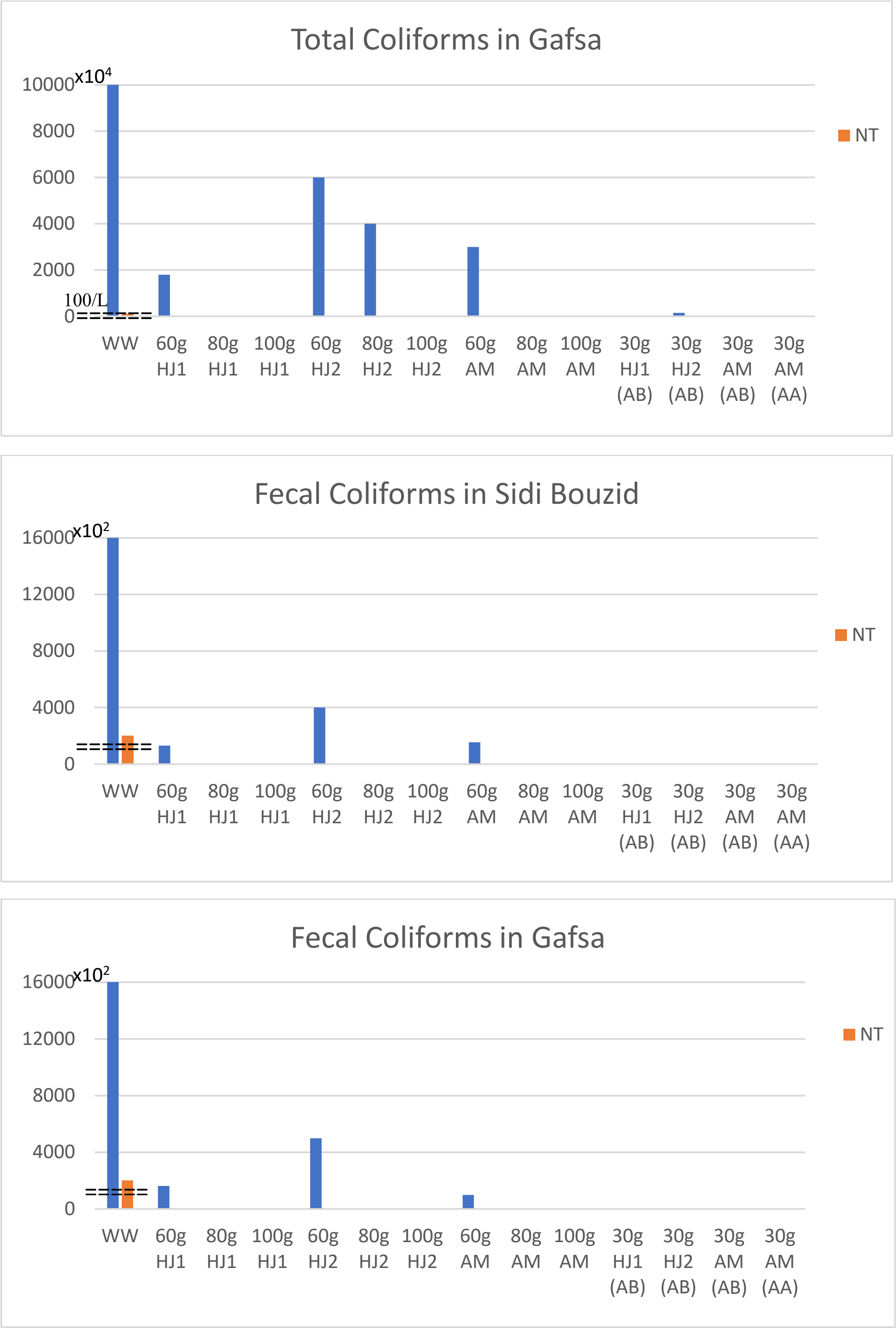


Figure 3. Bacteriological parameters of treated wastewaters from Sidi Bouzid and Gafsa WWTPs.

Table 1. Mineralogical composition of the studied clays obtained by RX diffraction

Semi-Quant (%)	**AM**	**HJ1**	**HJ2**
Kaolinite	15	30	25
Quartz	14	14	13
Sepiolite	7	14	11
Albite	11	19	14
Calcite	51	14	-
Magnetite	3	9	5
Microcline	-	-	32

Table 2. List and sequences of primers and probes used to detect HAV and SARS-CoV-2 genome

	Description	Localization	Sequence 5'→3'	Label
	2019-nCoV_N1 Forward Primer	Nucleocapsid N	*GACCCCAAAATCAGCGAAAT*	-
	2019-nCoV_N1 Reverse Primer	Nucleocapsid N	*TCTGGTTACTGCCAGTTGAATCTG*	-
	2019-nCoV_N1 Probe	Nucleocapsid N	*ACCCCGCATTACGTTTGGTGGACC*	*FAM/BHQ1*
	2019-nCoV_N2 Forward Primer	Nucleocapsid N	*TTACAAACATTGGCCGCAAA*	-
	2019-nCoV_N2 Reverse Primer	Nucleocapsid N	*GCGCGACATTCCGAAGAA*	-
	2019-nCoV_N2 Probe	Nucleocapsid N	*ACAATTTGCCCCCAGCGCTTCAG*	*FAM/BHQ1*
	HAV1-5' NCR forward primer	5' NCR	*TTTCCGGAGCCCCTCTTG*	-
	wild type reverse primers	5' NCR	*AAAGGGAAATTTAGCCTATAGCC*	-
	wild type reverse primers	5' NCR	*AAAGGGAAAATTTAGCCTATAGCC*	
	HAV-5' NCR *probe*	5' NCR	*ACTTGATACCTCACCGCCGTTTGCT*	*FAM/TAMRA*

The primers HAV2 and HAV3 differed by a single nucleotide, representing a deletion carried by some HAV strains and were used to amplify 5' non-coding region (5' NCR)

Table 3. Microbiological analysis of wastewater treated with raw and activated clays

Treatment (CFU/ 100 mL)	**TG (CFU/100 mL)**	**TC (CFU/100 mL)**	**FC (CFU/100 mL)**	***Staphylococcus* (CFU/100 mL)**	***E. coli***	***Salmonella***	**Fungi (CFU/100 mL)**	***HAV***
WW(SB)	$1.381.10^{3}$ ($<4.10^{3}$)	10^{8} (< 100/L)	$1.654.10^{6}$(2.10^{3})	$1.5.10^{3}$ (10^{3})	+ (<250)	+ (Absence)	$1.331.10^{3}$	$4.25.10^{3}$ (Absence)
WW(G)	$2.29.10^{3}$ ($<4.10^{3}$)	$1.4.10^{8}$(< 100/ L)	8.10^{6} (2.10^{3})	$2.5.10^{4}$ (10^{3})	+ (<250)	+ (Absence)	$1.781.10^{4}$	7.4 10^{4}(Absence)
60g HJ1SB	6.10^{2*}	$1.545.10^{3*}$	$1.309.10^{3*}$	0^{*}	-	-	0^{*}	NQ*
80g HJ1SB	$3.4.10^{2*}$	0^{*}	0^{*}	0^{*}	-	-	0^{*}	NQ*
100g HJ1 SB	$1.5.10^{2**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
60g HJ1G	9.10^{2*}	$1.8.10^{3*}$	$1.62.10^{3*}$	0^{*}	-	-	0^{*}	NQ*
80g HJ1G	$4.2.10^{2*}$	0^{*}	0^{*}	0^{*}	-	-	0^{*}	NQ*
100g HJ1G	$1.5.10^{2**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
60g HJ2SB	10^{3*}	5.10^{3*}	4.10^{3*}	0^{*}	-	-	0^{*}	2.5 10^{2}
80g HJ2SB	7.10^{2**}	10^{3**}	0^{**}	0^{**}	-	-	0^{**}	NQ*
100g HJ2 SB	$2.3.10^{2**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
60g HJ2G	$1.45.10^{3*}$	6.10^{3*}	5.10^{3*}	0^{*}	-	-	0^{*}	3.7 10^{3}
80g HJ2G	7.10^{2*}	4.10^{3*}	0^{*}	0^{*}	-	-	0^{*}	NQ*
100g HJ2G	3.10^{2**}	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
60g AMSB	$5.3.10^{2*}$	5.10^{3}	$1.545.10^{3*}$	0^{*}	+	+	0^{*}	NQ*
80g AMSB	3.10^{2*}	0^{*}	0^{*}	0^{*}	-	-	0^{*}	NQ*
100g AM SB	$1.5.10^{2**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
60g AM G	8.10^{2*}	3.10^{3*}	10^{3*}	0^{*}	+	-	0^{*}	NQ*
80g AM G	$3.5.10^{2*}$	0^{*}	0^{*}	0^{*}	+	-	0^{*}	NQ*
100g AMG	$1.5.10^{2**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
30g HJ1SB	$0.61.10^{3**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
30g HJ2SB	$1.2.10^{3**}$	$0.18.10^{3**}$	0^{**}	0^{**}	-	-	0^{**}	NQ**
30g AMSB	$1.1.10^{3**}$	0^{**}	0^{**}	0^{**}	-	-	0^{**}	NQ**
30g HJ1 G	$5.8.10^{2**}$	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	NQ**
30g HJ2 G	$1.73.10^{3**}$	$1.5.10^{2**}$	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	NQ**
30g AM G	$1.43.10^{3**}$	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	NQ**
30g AM SB (A.A)	0^{**}	0^{**}	0^{**}	0^{**}	0^{***}	0^{**}	0^{**}	NQ**
30g AM G (A.A)	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	0^{**}	NQ**

The significance of difference at a 5% level among means of the different microorganisms detected in untreated and clay treated wastewaters was determined by SPSS software (version 21.00), using Student's T- test. for independent sample:

** significant difference from control ($p<0.05$)*

*** very significant difference compared to the control ($p<0.001$)*

NQ. not quantifiable (<LOD)

(.). Tunisian standard NT. 106.002.

Table 4. Physicochemical parameters of the wastewater treated by the different clay samples

eatment	pH	Conductivity (dS/m)	Suspended Matter (SM) (g/L)	COD (mg O_2/L)	BOD$_5$ (mg O_2/L)	Chloride (mg/L)	Total Kjeldahl Nitrogen TKN (mg/L)	Phosphorus (mg
	7.24 (6.5-8.5)	4.9(5)	145 (30)	123 (125)	172 (30)	710 (700)	58 (30)	9.47 (2)
	6.86 (6.5-8.5)	5.420 (5)	160 (30)	160 (125)	195 (30)	710 (700)	66 (30)	10.83 (2)
	7.35	3.45	20**	65	23**	39.2**	21*	3.9
	7.4	2.9	15**	45*	17**	37.2**	18*	1.2**
	7.5	2.3	7**	38**	8**	19.2**	6**	0.8**
	7.36	2.67	31**	83	31*	532.5	29	4.02
	7.84**	3.51	17**	53*	15**	22.4**	15*	1.22**
	7.93**	2.84	8**	38**	10**	16.4**	7**	0.3**
	7.28	3.92	30**	77	29*	355	28	6
	7.3	3.1	26**	60	25*	305	22*	1.9*
	7.37	2.7	17**	49*	15**	245	14*	2*
	7.36	2.67	31**	83	31*	532.5	29	4.02
	7.51	4.20	28**	68	28*	402.5	20*	1.99**
	7.66	3.32	21**	53*	24*	332.5	16*	0.94**
	6.91	4.03	26**	78	26*	301.7	26*	4.9
	6.84	3.3	20**	53*	19**	300	16*	1.4*
	6.78*	2.9	12**	42**	10**	301.7	8**	0.9**
	6.94	3.48	26**	74	25*	639	27*	3.4
	6.97	3.91	24**	61	22**	539	18*	1.67*
	6.98	2.89	13**	45*	19**	439	10**	0.6**
	7.45**	3.1	10**	70	15**	223.65*	19*	1*
	7.82*	3.2	10**	70	17**	277.98*	21*	1.2*
	7.38	3.2	19**	79	22**	770.35	24*	1.9
	7.54	4.12	22**	82	29*	688	25*	1.89*
	7.2	4	13**	64	16**	816.5	20*	1.2**

	6.98	3.22	15^{**}	75	25^{*}	994	23^{*}	1.55^{*}
	6.82	3.2	2^{**}	40^{*}	11^{**}	674.5	5.2^{**}	1.01^{**}
	6.73	3.54	4^{**}	50^{*}	9^{**}	774.5	6.40^{**}	0.81^{**}

(.). Tunisian standard NT. 106.002.