Desalination and Water Treatment
www.deswater.com

doi: 10.1080/19443994.2015.1081111

57 (2016) 15792–15805
July

Taylor & Francis
Taylor & Francis Group

Performance evaluation of stone-media pro-type pilot-scale trickling biofilter system for municipal wastewater treatment

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Received 23 June 2014; Accepted 24 July 2015

ABSTRACT

This research work was focused on the establishment and performance assessment of the locally designed pro-type pilot-scale stone-media trickling biofilter (TBF) system for the removal of pollution indicators (chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), ammonium nitrogen (NH_4-N), and pathogen indicators) from municipal wastewater under increasing environmental temperature of 20–40.5˚C for 40 d. The results indicated that removal efficiency of the parameters, COD, BOD_5, and NH_4-N, from the wastewater considerably increased from 62.4, 56.4, and 33.8%, respectively, at 20˚C on 1st day to 98.1, 98.6, and 93.5%, respectively, at 40.5˚C on 40th day of the TBF operation. The removal of pathogenic indicators from wastewater was evaluated in terms of MPN index and an average reduction of 88.8% of fecal coliforms in the effluent was recorded during the experimental period. The biofilms, which were responsible for wastewater treatment, were sampled from the top and deeper layers of stone bed of the reactor and were characterized. The *Nitrosonomas* and *Nitrobacter* sp. were identified in the deeper layers of the biofilms, while 13 bacterial strains viz. *E. coli*, *P. aeruginosa*, *E. aerogenes*, *S. typhimurium*, *P. vulgaris*, *S. dysenteriae*, *K. pnuemoniae*, *B. subtilis*, *S. aureus*, *M. luteus*, *S. epidermitus*, *S. lactis*, and *C. xerosis* were identified in the biofilm sample removed from top layer of the stone media. The overall results proved that the pilot-scale TBF has a great potential to be transferred to field scale for treating sewage for small communities in developing and underdeveloped countries even at extreme temperature conditions.

Keywords: Stone-media trickling biofilter; MPN index; Carboneous pollutants removal efficiency; Pathogenic indicators

1. Introduction

Water is crucial for all facets of life. However, about 900 million people lack access to drinking water, and an estimated 2.6 billion people lack access to basic sanitation [1]. Rapid population growth, urbanization, industrialization, unsustainable water consumption practices, and poor sanitation have immensely undermine the quality as well as quantity of water resources [2,3]. Wastewater production was estimated

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to be 60–150 L/person/d in developing countries and 500–800 L/person/d in the industrialized countries [4]. About 80–90% of all wastewater generated in developing countries is discharged directly into surface water leading to health-associated risks [5].

Like other developing countries, Pakistan is facing severe freshwater shortages [6]. The situation is aggravated further by the pollution of freshwater resources due to the discharge of untreated wastewater containing household effluent and human wastes. An estimated 2,000 million gallons (7.5708×10^9 L) of sewage/d is discharged directly to a natural drain or open agricultural land [7]. Direct and indirect input of waste from industrial, agricultural, and municipal sources is continuously deteriorating the quality of water [8]. This water contamination is affecting not only flora and fauna along with their biodiversity, but also leading to waterborne diseases and decreased agricultural productivity [9]. It was estimated that 26% of the total domestic vegetable production of Pakistan is cultivated with sewage, which when consumed by people resulted in health problems [10], and approximately, 35–40% of deaths were also reported due to waterborne diseases [11]. It is very essential to decrease the level of pathogens in wastewater before use for agriculture purposes [12].

In Pakistan, there is a severe shortage of wastewater treatment plants. Only about 12% of the urban wastewater is treated in municipal treatment plants and even these treatment plants are not performing to their expected efficiency levels due to the unavailable resources and maintenance cost [13]. This alarming situation of deteriorating freshwater quality and its growing demands for human consumption calls for effective indigenous remedies in terms of devising cost-effective wastewater treatment and water reuse systems in order to ensure public health and environment sustainability [10,14].

There are numbers of wastewater treatment processes with varying degree of effectiveness to control water pollution based on the physical and chemical removal of contaminants [15,16]. These processes offer varying degree of effectiveness besides presenting environmental and economic disadvantages [17]. However, along with physical and chemical treatment systems, biological wastewater treatment technologies have been gaining much attention in recent years. They offer low operational cost, easy handling, and have comparatively less harmful effects on the corresponding environment [18]. Depending on the composition of wastewater, generally two types of biological system have been developed: suspended and attachedgrowth [19]. Wastewater treatment using attached-growth biological process, under aerobic or anaerobic conditions, has been widely practiced [20,21]. Trickling biofilter (TBF) systems are attached-growth biological process, considered to be quite effective among all the biological treatment technologies on the basis of their low energy costs and maintenance requirements, ease of operation, and environmental compatibility. These biofilm reactors can be employed for the treatment of different industrial effluents, for the removal of carbonaceous, sulfur, and nitrogenous compounds from wastewaters [22,23]. Because of their simple design, small footprints, easy, and reliable processing [24], these systems provide an additional advantage over suspended growth systems.

This research is a step toward the establishment of a simple, efficient, and a low-cost pilot-scale TBF system for municipal wastewater treatment considered aforesaid representative situations for many developing/underdeveloped countries with varying (extreme) environmental conditions. For this purpose, the municipal wastewater treatment performance of locally designed and constructed pilot-scale stone-media TBF, was evaluated at a temperature range (20–40.5˚C) in terms of the removal of different pollution indicators (Biological oxygen demand, ammonia, pathogens, etc.). The idea is to translate this model treatment system for the treatment of wastewater in area of small residential colonies typically disconnected from the main sewer lines of main residential areas or lacking nearby wastewater treatment system.

2. Materials and methods

2.1. Developmental scheme of pilot-scale TBF system

The pilot-scale TBF was constructed near the department of Microbiology, QAU, Islamabad, Pakistan. This TBF system consisted of a feed tank of 400 gallons capacity for the storage of wastewater. It was followed by TBF containment structure of 101.6 ft^3 (2.88 m^3), made up of stainless steel (22 Gage) jacket, having an internal diameter of 4.2 ft (1.28 m), and a total height of 7.5 ft (2.28 m). The containment structure had conical shape and its dimensions are shown in the Fig. 1. It was equipped with small ports/windows on both sides for the collection of biofilm samples and for supplying air. The supporting structure for bacterial biofilm growth was stones/pebbles, with mean diameter of 0.33 ft (10 cm) and surface area of 0.342 ft^2 (0.032 m^2) were packed in the main BOD_5 of TBF leaving a head space (boat space) of 0.15 m. The filter bed has voidage of 47%.

A wastewater distribution system made up of stainless steel had a diameter of about 3.8 ft was installed at the top of the filter (a fixed flow

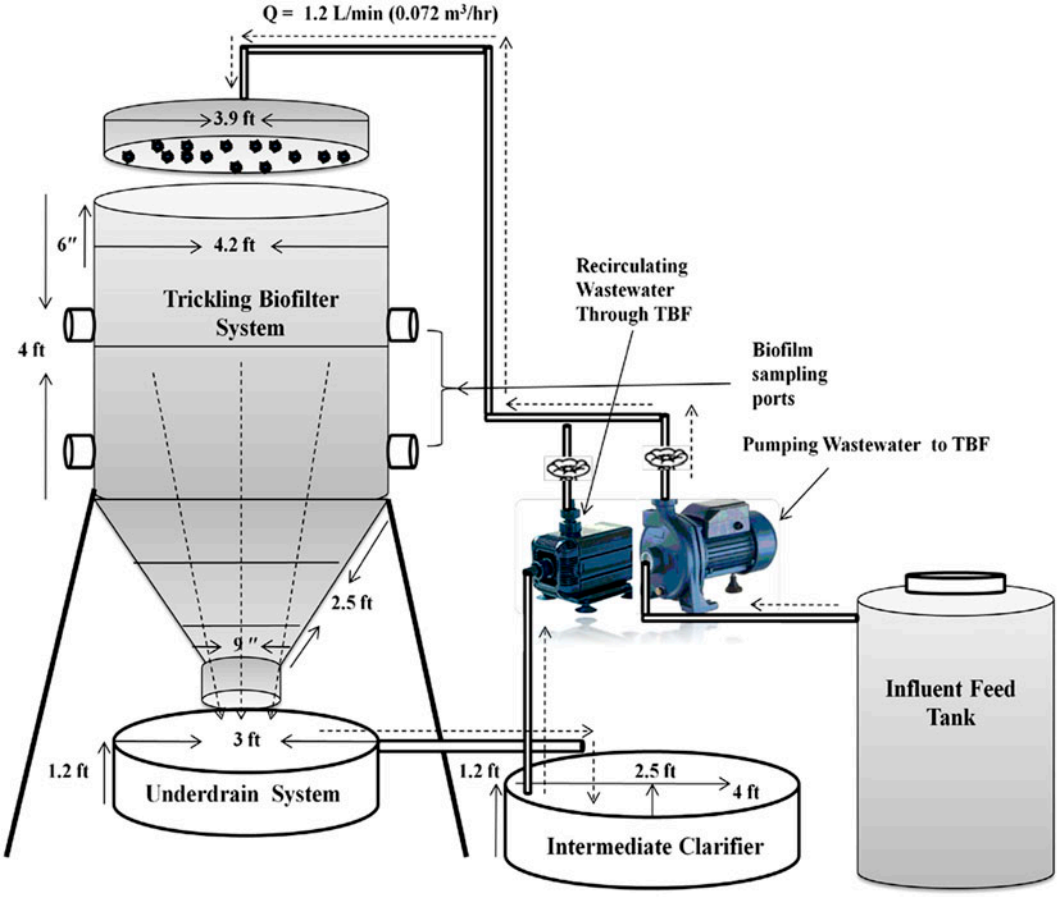

Fig. 1. A schematic of the pilot-scale TBF system used for the treatment of wastewater in the present research.

distributor). Two water pumps (Model-XXSPA and National Gold Electro Pumps) were connected to the filter wastewater distribution system through polyvinylchloride (PVC) pipe system. One of the pumps was used to connect a distribution system to a feed tank. An underdrain system (total height = 1.2 ft, diameter = 3 ft) consisted of an outlet at a height of 0.75 ft (0.23 m) from the bottom and had a capacity of 8.48 ft^3 (0.24 m^3) is located at the bottom of the filter. It was followed by a recirculation tank of elliptical shape with a height of 1.2 ft (0.36 m) and diameter (A) 2.5 and (B) 4 ft (0.76 × 1.23 m). This tank served as an intermediate as well as final clarifier and had a capacity of 9.42 ft^3 (0.27 m^3).

2.2. Operation of pilot-scale TBF system

The TBF system was used to treat 400 L/d of wastewater in a continuous recirculation flow mode to maintain a hydraulic flow rate of 1.2 L/min (Q = 0.072 m^3/h) and hydraulic loading (Q/A) of 1.35 m/d. Before the start up of wastewater treatment, stone bed of the filter was inoculated with biomass for 10 d, using activated sludge from municipal wastewater treatment plant, Islamabad, Pakistan. After 10 d of the biofilm development on the filter media of the TBF, wastewater was supplied for treatment with the help of water pump from feed tank through a wastewater distribution system at the top of the stone bed of the TBF system uniformly. After percolation through the stone media bed, the wastewater was collected by the underdrain system. Then the effluent of TBF flow into a recirculation tank which is served as an intermediate as well as final clarifier (Fig. 1), from which the effluent of the filter was continuously recirculated through the filter for 24 h by feeding to the top of the TBF via a recirculation pump. Thus, the wastewater was recirculated four times in 24 h. The final clarifier had an outlet, connected with plastic pipe to supply treated effluent to outside of the work station after 24 h of treatment in continuous mode. The samples of influent and effluent were taken from

stone-media pilot-scale TBF each day at the end of each operational phase (24 h) until 40 d from 11th April to 20th May 2013.

2.3. Characterization of biofilm on stone media in TBF

2.3.1. Sampling of biofilm

Two biofilm samples (with stones) were collected after the start up of the TBF, one from the top (for heterotrophic characterization) and other from the bottom port or window (for nitrifiers characterization) in the containment structure of the reactor. Then the stones having biofilms were washed with sterile distilled water gently in order to remove any solid particles and were further subjected for characterization of the bacterial inhabitants.

2.3.2. Isolation and identification of heterotrophs

Slime layer of biofilm on stones from top port were subjected to analysis of culturable bacteria by pure culturing techniques. This biofilm was directly streaked on nutrient agar (NA) plates and then incubated at 37˚C for 24 h. After incubation, different colonies on NA plates were observed which differed from each other on the basis of their morphology. For the isolation of pure cultures, different colonies were further subcultured on selective/differential media, viz. eosin methylene blue (EMB), MaCA, *Salmonella Shigella* agar (SSA), Pseudomonas cetrimide agar (PCA), MSA, and blood agar (BA) and then kept in incubator at 37˚C for 24 h. Finally, after incubation, identification of subcultured organisms were carried out on the basis of plate morphological characteristics (size, form, pigmentation, margins, elevation, and opacity), microscopy (shapes and Gram's staining reaction), and biochemical tests (triple sugar iron test (TSI), indole/H_2S motility test, citrate utilization test, catalase test, urease test, methyl red Voges–Proskauer test (MR-VP), and oxidase test) according to [25].

2.3.3. Isolation and identification of nitrifiers

For the isolation and subsequent identification of the nitrifiers, the stones having biofilms collected from the lower ports/widows of TBF system were washed gently thrice with distilled water and then placed in phosphate buffer and vertixed for 5–10 min. Then stones were removed immediately and the suspension of dissolved biofilms was used for enrichment of nitrifiers. The enrichment of the nitrifiers, i.e. *Nitrosomonas* sp. (ammonia-oxidizing bacteria, i.e. AOB) and *Nitrobacter* sp. (nitrite-oxidizing bacteria, i.e. NOB) were carried in a specific liquid growth media, i.e. *Nitrosomonas europaea* medium and *Nitrobacter* medium B, respectively, according to the handbook of media for environmental microbiology, second edition [26].

Medium for isolation of the *Nitrosomonas* sp. contains 0.015 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2{\cdot}2H_2O$, 1.7 g $(NH_4)_2SO_4$, 1.0 mg ferric EDTA, and 1.0 mL trace element solution (composition per L). (Trace element solution was prepared by mixing 0.02 g $MnCl_2{\cdot}4H_2O$, 2.0 g $CuSO_4{\cdot}5H_2O$, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.01 g $ZnSO_4{\cdot}7H_2O$, and 0.01 g $Na_2MoO_4{\cdot}2H_2O$ in 100-mL distilled water. All the components were added to distilled/deionized water and bring volume to1.0 L and mixed thoroughly). All of these ingredients were dissolved in 1.0 L distilled water and autoclaved for 15 min at 121˚C. The pH of the medium was then adjusted to 7.5 ± 2 with sterile 50% K_2CO_3 at 25˚C.

Medium for isolation of the *Nitrobacter* sp. contains 2.0 g $MnSO_4$, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 1.0 g $NaNO_2$, 0.3 g NaCl, 5.0 g $Fe_2(SO_4)_3$, and few particles of marble chips. All of these components were dissolved in 1.0-L distilled water excluding marble chips. The medium was then autoclaved at 121˚C for 15 min at 15 psi pressure. Marble chips were wrapped in aluminum foil and autoclaved separately for 60 min at 15 psi pressure and at 121˚C. Then cool to 25˚C. These were then added into the medium aseptically. The pH of the medium was then adjusted to 7.5 ± 2.

After sterilization and cooling of the liquid media, 500 mL of each medium was poured in 1,000-mL flasks separately. Then 5 mL of the sample, i.e. biofilm suspension was added into the flasks. For maintaining dark conditions, the flasks were wrapped with aluminum foil and were then incubated in a shaking incubator with 150 rpm at 30˚C for 7 d. After 7 d of shaking incubation, 10 mL of suspension of biofilm from these flasks were used as an inoculum for freshly prepared selective growth media for both nitrifying stains of AOB and NOB, and subjected to same previous conditions. This step was repeated several times for enrichment and finally culture broth (selective liquid media having *Nitrosomonas* sp. and *Nitrobacter* sp.) were spread on selective solid media (having same composition as mentioned above and contained agar for solidification) in the Petri plates. When the mix growth stop and one kind dominant colony appeared, further purification was done by restreaking dominant isolated colonies and were identified by considering their morphological, microscopic, and biochemical characteristics [25]. Finally, both *Nitrosomonas* and *Nitrobacter* sp. were further confirmed by their activity analysis.

2.3.3.1. Determination of nitrifiers activity

2.3.3.1.1. Estimation of the NH_4 oxidation activity of enriched culture of Nitrosomonas *sp.* Activity analysis of the *Nitrosomonas* sp. was determined by measuring the strength of nitrites (NO_2^-) formed in the liquid growth medium having *Nitrosomonas* sp. as an inoculum after early identification. The *Nitrosomonas europaea* medium was prepared and then distributed in 250-mL flasks. Ammonium sulfate ($(NH_4)_2SO_4$) with varying concentrations of 5, 10, 15, and 20 mM were added to these flasks and then autoclaved. After sterilization and cooling to 25°C, these flasks having 250 mL of media amended with various concentrations of $(NH_4)_2SO_4$ as a source of ammonia were inoculated with enriched *Nitrosomonas* sp. (2.5 mL). After inoculation with the *Nitrosomonas* sp. these flasks were incubated in shaking incubator with 150 rpm at 30°C. For activity measurement, levels of NO_2^- formed from $(NH_4)_2SO_4$ by the *Nitrosomonas* sp. were determined before incubation (0 h) and periodically after incubation, i.e. after 24, 48, and 72 h by standard method 4,500 (NO_2-N) for water and wastewater [27].

2.3.3.1.2. Estimation of the NO_2^- oxidation activity of enriched culture of Nitrobacter *sp.* Activity analysis of the *Nitrobacter* sp. was determined by measuring the level of nitrates (NO_3^-) formed. For this purpose, specific medium B for growth and cultivation of *Nitrobacter* sp. was prepared, poured in 250-mL flasks, and amended with varying concentration (5, 10, 15, and 20 mM) of sodium nitrite (Na_2NO_2). It was then sterilized, cooled, and inoculated with enriched *Nitrobacter* sp. (2.5 mL). After inoculation, these flasks were incubated at 30°C in shaking incubator with 150 rpm. The level of NO_3^- formed from Na_2NO_2 was measured in these flasks before incubation and periodically after incubation, i.e. 24, 48, and 72 h in order to determine the NO^- utilization activity of the *Nitrobacter* sp. by standard method 4,500 (NO_3-N) [27].

2.4. Wastewater collection for treatment

The wastewater (400 L) was collected from sites 1 to 4 of university campus every morning for treatment using a pilot-scale TBF system (Table 1). After collection, wastewater was characterized and was given a retention time of 2–3 h in a storage tank in order to sediment the suspended solids and large particulate matters before secondary treatment in the filter. After primary sedimentation, it was transferred to feed tank of the TBF system. The quality of the raw municipal wastewater fluctuated throughout the experimental period; therefore, the quality of feed tank effluent was also unstable.

2.4.1. Wastewater characterization

2.4.1.1. Study of chemical parameters of wastewater. Various different parameters including temperature, pH, dissolved oxygen (DO), chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), ammonium nitrogen (NH_4-N), and alkalinity of influent and effluent from the pilot-scale TBF were determined by the standard methods for water and wastewater [27] . The treatment efficiency of the TBF system under environmental conditions was found by mean values of the influent and effluent of the above selected parameters.

2.4.1.2. Evaluation of the wastewater treatment efficiency of pilot-scale TBF system. The performance of the TBF system was evaluated for carboneous and nitrogenous pollutants removal by following equations:
For;

$$\text{Carboneous pollutants removal efficiency}\,(\%) = 100 \times [(\text{COD}/\text{BOD}_5)_i - (\text{COD}/\text{BOD}_5)_o]/(\text{COD}/\text{BOD}_5)_i \quad (1)$$

For;

$$\text{NH}_4^+\text{-N removal efficiency}\,(\%) = 100 \times [(\text{NH}_4 + \text{N})_i - (\text{NH}_4 + \text{N})_o]/(\text{NH}_4^+\text{-N}) \quad (2)$$

where the subscripts "i" and "o" denote inlet and outlet, respectively.

2.4.1.3. Statistical analysis of wastewater treatment efficiency of the pilot-scale TBF system. To investigate the relationships of BOD_5, COD, NH_4-N, alkalinity, pH, and pathogen indicator removal efficiency and increase in DO with days of operation and change in environmental temperature, non-parametric Spearman's correlation coefficient (rho) was calculated using PASW Statistics® 18.0 SPSS.

2.4.2. Characterization of microbiological parameter of wastewater

2.4.2.1. Determination of MPN index for fecal coliforms. For the investigation and enumeration of the fecal coliforms, samples were incubated at 35–37°C for 24–48 h in MacConkey's broth using multiple-tube technique having inverted Durham tubes. Positive tubes were subcultured on MacConkey's agar, NA, and mannitol salt agar plates and incubated at 35–37°C for 24–48 h. Negative plates were restreaked before discarding tubes. Positive isolates were

Table 1
Sewage discharge sites at Quaid-i-Azam University, Islamabad, Pakistan

Sewage collection sites	User	Strength	Sewage load (gallons/d)
Site 1	Department of Chemistry, Biological sciences, Social Sciences, and Mathematics Central Library, Administration, Cafeteria, and Mosque	7,300	146,000
Site 2	Hostel (1, 2, 3, and 4), Warden House, PIDE, Guest house, Faculty house, work shop, and Gymnasium	065	41,300
Site 3	Hostel (6, 7, 8, and 9), Bioinformatics, and New admin block	1,160	23,200
Site 4	Residential colony, School, Market, and Mosque	2,135	42,700

confirmed by microscopy, i.e. gram's staining. The MPN index (95% confidence limits) was determined from positive tubes with upper and lower limits and then final values were calculated according to the standard MPN table.

3. Results and discussions

Various fixed biofilm reactors including TBFs, biological contractors, submerged biofilters, and moving bed reactors have been used for the treatment of wastewater. Biofilm and associated microbial community structure attached to specific filtration medium proved to be an essential element of these systems for achieving desired treatment efficiencies [28,29]. Occasionally, various microbes flourishing in the biofilms fortuitously become potential biocatalysts and bioremediation agents in the removal of contaminants and pathogenic microbes [30].

Use of activated sludge for development of biofilm on support material proved to be reducing the start-up period of fixed-film wastewater treatment reactors [31]. In the present research, same strategy was employed and activated sludge was used as a seed inoculum with wastewater for the development of active biofilm on stone media of pilot-scale TBF. A mixture (1:9) of activated sludge and wastewater was continuously recirculated through the bed of the reactor and development of the biofilm was observed regularly. A well-developed, yellowish biofilm was observed on the 10th day on the growth supporting stone media, which reduced the start-up phase of the TBF operation. After development of biofilm, the samples of biofilms from the top and lower ports/windows were subjected to characterization of bacterial inhabitants by adopting simple and conventional laboratory methods. Simultaneously, wastewater treatment efficiency of the reactor was evaluated from 11th day (steady phase of the operation), over a temperature range of 20–40.5˚C (Fig. 2). The TBF was continuously operated for 24 h and then effluent was collected from the reactor for physicochemical and microbiological characterization in order to estimate its wastewater treatment efficiency. From the continuous monitoring of influent and effluent analysis, it was observed that biofilm on the stone media was able to significantly reduce contaminants and pathogen load in the wastewater (Table 2).

3.1. Characterization of biofilm on stone media in TBF

3.1.1. Isolation and identification of heterotrophs

Morphological, biochemical, and microscopic characterization of the bacterial isolates from biofilm matrix developed on stone medium revealed a diverse community of 13 different bacterial species (*E. coli, P. aeruginosa, E. aerogenes, S. typhimurium, P. vulgaris, S. dysenteriae, K. pnuemoniae, B. subtilis, S. aureus, M. luteus, S. epidermitus, S. lactis, and C. xerosis*).Out of these, six were gram-positive strains (*S. epidermitus, S. lactis, S. epidermitus, S. aureus*, and *M. luteus* were gram-positive cocci, while strains *C. xerosis* and *B. subtilis* were gram-positive rods), while, seven strains were gram negative (*E. coli, P. aerouginosa, S. typhimurium, P. vulgaris, S. dysenteriae,* and *K. pnuemoniae,*

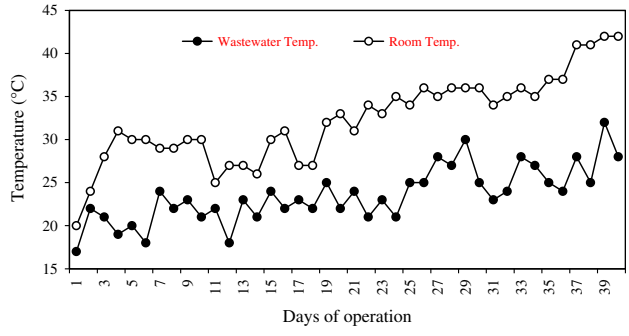

Fig. 2. Temperature of environment and wastewater (influent) during the experimental period of 40 d.

Table 2
Summary of the physicochemical characterization of the pilot-scale TBF system influent and effluent during the experimental period of 40 d

	COD (mg/L)	BOD (mg/L)	DO (mg/L)	NH_4-N (mg/L)	Alkalinity (mg/L)	pH	MPN index
Influent (range)	598.6–790	407–654	1–3.01	6.8–20.4	121–209	6.01–8.86	20 –> 1,100
Effluent (range)	12.01–295.2	6.08–232.7	5.63–8.31	0.2–12.2	19–164.5	5.85–7.17	4–220
% Efficiency (range)	62.4–98.1↓	56.4–98.6↓	58–87.6↑	17–93.5↓	8–87.4↓	2.6–27.7↓	77–98.4↓
Rho with days of operation	0.893[a]	0.998[a]	0.581[a]	0.951[a]	0.891[a]	0.457[b]	0.749[a]
Rho with temp. change	0.887[a]	0.886[a]	0.57[a]	0.835[a]	0.771[a]	0.471[b]	0.776[a]

Note: rho = Spearman's non-parametric correlation coefficient.
[a]correlation is significant at 0.01 level (2—tailed).
[b]correlation is significant at 0.05 level (2—tailed).
↓ = % decrease efficiency; ↑ = % increase efficiency.

showing scattered arrangement under microscope). Most of them were obligatory to opportunistic pathogens in nature. However, biofilm processes like TBF systems take advantage of their biotransformation capabilities such as biodegradation, biosorption, bioaccumulation, and biomineralization in the treatment of wastewater [32]. These microbial communities have been previously reported, effectively oxidizing soluble organic and nitrogenous compounds in wastewater [33,34].

3.1.2. Isolation and identification of nitrifiers (Autotrophs)

Nitrifiers are slow-growing microbes and mostly detected by molecular methods. The present research emphasized on the culturing of these bacteria in laboratory conditions by adopting enrichment technique. According to cultural, microscopic, and biochemical characteristics, the microbial strains grown on specific media (*Nitrosomonas europaea* medium and *Nitrobacter* medium B) after enrichment were identified as *Nitrosomonas* and *Nitrobacter* sp. (Table 3). The *Nitrosomonas* sp. has shown small colonies with entire elevation on the *Nitrosomonas europaea* media plates. While the *Nitrobacter* sp. formed comparatively very small colonies serrate margins. Both of these strains were gram-negative rods and have shown negative H_2S production, indole production, and catalase activity biochemical confirmatory tests. However, the *Nitrosomonas* sp. was identified by its positive MRVP reaction, citrate use, urease activity, and production of yellow coloration in TSI reaction. On the other hand, the *Nitrobacter* sp. has shown negative MRVP reaction, urease activity, positive citrate use, and oxidase activity tests. A red coloration was also noticed in its TSI test [25].

3.1.3. Assessment of nitrifying activity of bacteria

Activity analyses of the nitrifying strains were performed according to substrate consumption rates. The nitritifying species, *Nitrosomonas*, was tested for the oxidation of ammonium nitrogen (NH_4-N) to nitrites (NO_2^-) and nitratifying species *Nitrobacter* for nitrites (NO_2^-) to nitrates (NO_3^-) in specific liquid growth media, i.e. *Nitrosomonas europaea* medium and *Nitrobacter* medium B, respectively.

All the experiments for nitritifier activity characterization were conducted under very favorable growth conditions (providing oxygen by shaking at 150 rpm, optimum pH of medium of 7.5 ± 2, and temperature of 30°C). After continuous monitoring, results indicated active conversion of all concentrations of NH_4, i.e. 5, 10, 15, and 20 mM by *Nitrosomonas* sp. in specific growth medium. However, all media amended with 5, 10, and 15 mM, $(NH_4)_2SO_4$, showed maximum NO_2^- concentrations until 48 h of incubation, i.e. 43.6, 51.2, and 41.9%, respectively. After 72 h of incubation, a little increase in the NO_2^- concentrations was observed in the flasks having 5, 10, and 15 mM of $(NH_4)_2SO_4$ (48, 51.2, and 41.9%, respectively) as shown in the Fig. 3(a), (b), and (c). While the maximum concentration of NO_2^- (1.2 mg/L) was observed in the flasks with an increase in $(NH_4)_2SO_4$ (20 mM) concentration after 72 h of incubation (Fig. 3(d)). These observations clearly indicate that newly enriched *Nitrosomonas* sp. has the capacity to tolerate and utilize high NH_4 loads. These findings also correspond to various previous reports, that *Nitrosomonas europaea/eutropha* are primarily found in the environments with high NH_4 concentrations [35,36]. On the other side, investigations revealed decrease in concentrations of NO_2^- with an increase in the levels of NO_3^- in all media amended with different concentrations of Na_2NO_2 (5, 10, 15, and 20 mM) while incubated with

Table 3
Morphological, microscopic, and biochemical study of isolated strains after enrichment of nitrifiers

Isolated strains	Growth media	Morphology				Microscopy		Biochemical characterization								Identified strains
		Size	Pigment	Form	Margins	Staining	Shape	H_2S production	Indole production	MR reaction	VP reaction	citrate use	Urease activity	Catalase activity	Oxidase activity	TSI reaction	
1	*Nitrosomonas europaea* medium	Small	Nil	Irregular	Entire	–	Rods	–	–	+	+	+	+	–	–	A/A	*Nitrosomonas* sp.
2	*Nitrobacter* medium B	Very small	Nil	Rhizoid	Serrate	–	Rods	–	–	–	–	+	–	–	+	K/A	*Nitrobacter* sp.

Notes: + = Positive; − = negative; A = Acid production; K = alkaline reaction; H_2S = Sulfur reduction; K/A = Red/yellow; A/A = Yellow/yellow.

enriched *Nitribactor* sp. In case of 5 and 10 mM concentrations of Na_2NO_2 in the media, a through linear increase in the process of nitratification was observed. While overall 39.7 and 52% oxidation of NO_2^- to NO_3^- was illustrated by *Nitribactor* sp. at 5 and 10 mM concentrations of Na_2NO_2, respectively, after 72 h of incubation (Fig. 4(a) and (b)). However, an exponential increase in the concentrations of NO_3^- (51.9 and 57.4%) was found in the media having 15 and 20 mM concentrations of NO_2^- till 72 h of incubation as shown in the Fig. 4(c) and (d). These results clearly indicated physiologically active *Nitribactor* sp., having the competency of NO_2^- oxidation to NO_3^- even under higher concentrations of NO_2^- in the media. It was also reported that NO_2^- oxidation kinetics of *Nitrobacter* is higher than other nitratifiers (like *Nitrosipra*) and is likely to be dependent on available NO_2^- concentration [37].

Thus, the existence of the *Nitrosomonas* and *Nitrobacter* sp. in the deeper zones of TBF system were justified from the present research study. The distribution of these nitrifying communities could be attributed to wastewater characteristics like availability of substrates (NH_4-N) and also carbon source (from oxidation COD/BOD to CO_2) and of course the process configuration like proper aeration which, in turn, would have also affected the nitrification performance of the systems.

3.2. Wastewater treatment efficiency of pilot-scale TBF system

3.2.1. Carboneous pollutants removal

Results on carboneous material removal rates of domestic wastewater in TBF system at 20–41.5°C are presented in Figs. 5 and 6. The average COD of wastewater before treatment was recorded as 670.03 mg/L over a study period of 40 d. The results indicate that COD elimination efficiency from wastewater significantly increases with operational time (rho = 0.998; $p < 0.01$; $n = 40$) and range from 62.4 to 98.1% at flow rate of 1.2 L/min. The highest removal of COD has been recorded (98.1%) on 40th day of reactor operation, however, >90% wastewater treatment efficiency of the TBF system was observed after the 25th day. While comparatively low COD reduction (62.4%) was found on the first day of operation. A significant correlation of COD removal efficiency was noticed (rho = 0.887; $n = 40$) with a change in seasonal temperature (Fig. 5). Likewise, the average BOD_5 of wastewater influent observed over a study period of 40 d was 456.8 mg/L. Thus, the reactor operated at average BOD_5 loading rate of 0.063 kg

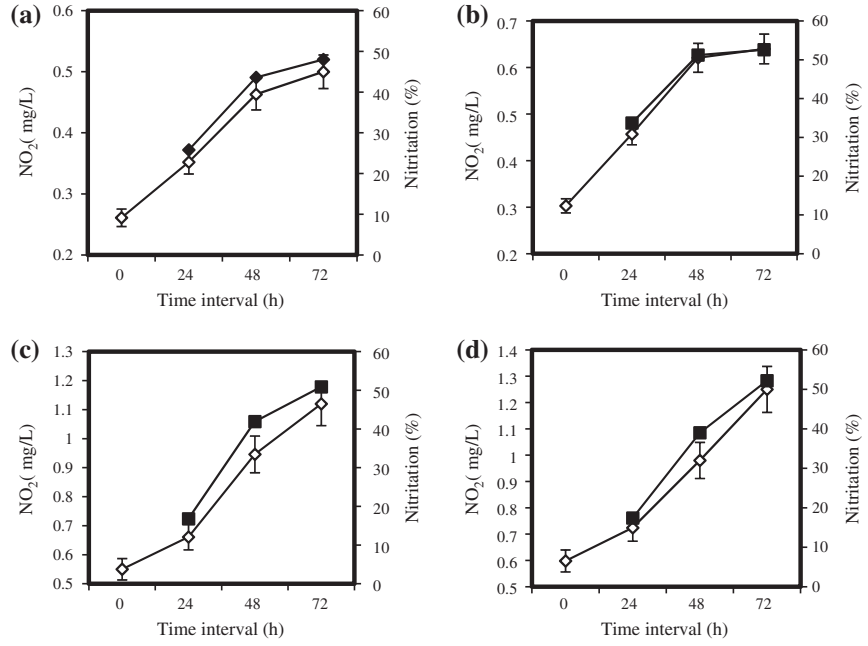

Fig. 3. Changes in nitrite (NO_2^-) concentrations with an increase in incubation time (24–72 h) in the specific liquid growth medium (*Nitrosomonas europaea* medium) amended with (a) 5 mM, (b) 10 mM, (c) 15 mM, and (d) 20 mM of ammonia nitrogen source ($(NH_4)_2SO_4$) incubated with *Nitrosomonas* sp.

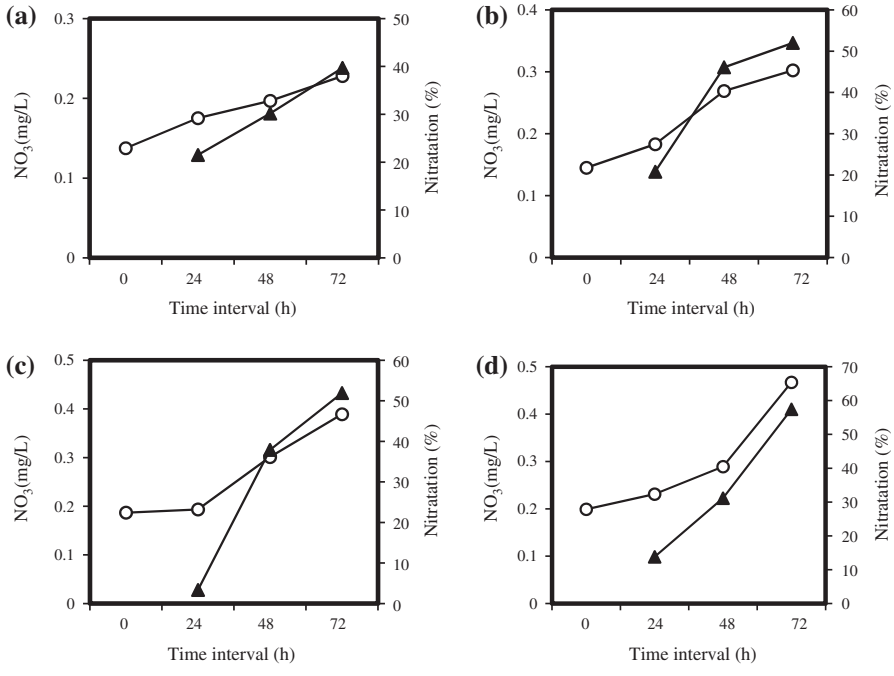

Fig. 4. Changes in nitrite (NO_3^-) concentrations with an increase in incubation time (24–72 h) in the specific liquid growth medium (*Nitrobacter* medium B) amended with (a) 5 mM, (b) 10 mM, (c) 15 mM, and (d) 20 mM of nitrite nitrogen source (Na_2NO_2) incubated with *Nitrobacter* sp.

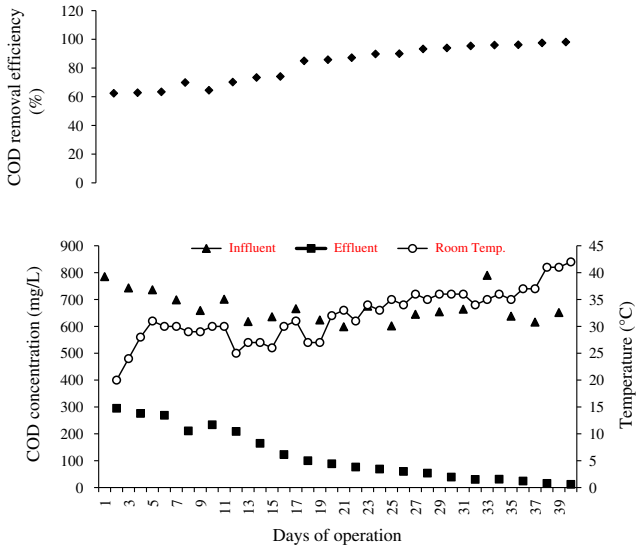

Fig. 5. Concentrations of COD in the influent and effluent and their removal efficiency (%) by pilot-scale TBF system during an experimental period of 40 d.

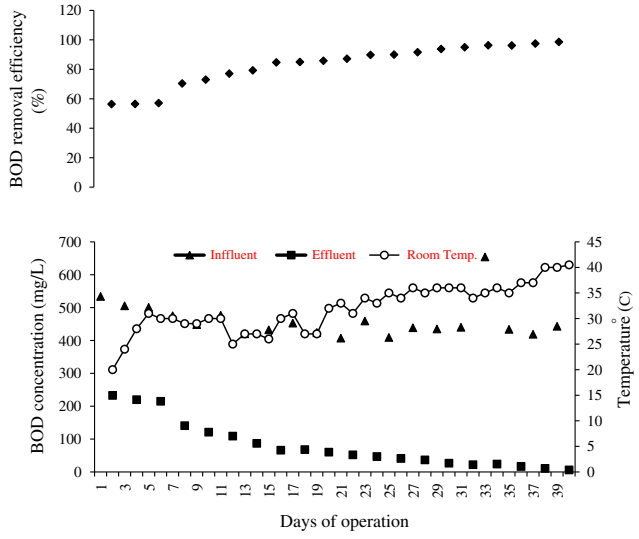

Fig. 6. Concentrations of BOD_5 in the influent and effluent and their removal efficiency (%) by pilot-scale TBF system during an experimental period of 40 d.

BOD_5/m^3 d. BOD_5 removal efficiency of the TBF with an operational time from 1st day (56.4%) to 40th day (98.6%) of operation (rho = 0.99; $p < 0.01$; $n = 40$). Data obtained from the current pilot trials also showed a significant correlation of BOD_5 removal efficiency with an increase in environmental temperature (rho = 0.886; $p < 0.01$; $n = 40$) (Fig. 6).

This continuous increase in the reduction of COD/BOD_5 was attributed to the increase in temperature and to the development of efficient biofilm on stones. As one batch of the influent (400 L) recirculates through the bed of the reactor four times in 24 h, it provides organic/inorganic nutrients to the biofilm inhabitants thus resulted into the development of more active biofilms. Moreover, recirculation of wastewater during treatment improved wetting of media, avoidance of anaerobic zones, more distribution of organic load, and more accumulation of biomass as biofilm [38,39]. Further, increase in environmental temperature has a positive effect on the biofilm because most of the bacteria flourish well in the temperature range of 25–40˚C. Thus, it contributes to the removal of more pollutants from wastewater as their food [40]. This diminution of carbonaceous matter (COD/BOD_5) after continuous recirculation of wastewater through TBF system resulted in the increase of DO concentrations of effluent [41,42]. Initially, the average DO concentrations of influents to TBF system was 1.92 mg/L, indicated high COD/BOD_5 load. However, its levels showed increase in the final effluent from 1st to 40th day of operation (rho = 0.58; $p < 0.01$; $n = 40$). Finally, average values of DO obtained was 7.2 mg/L and its increase in concentrations remains within the range of 60.9–87.6% during experimental period of 40 d (Fig. 7). These results showed that increase in DO concentrations (range 5.63–8.31 mg/L) were satisfactory to consent maintenance of nitrifiers and nitrification process in the deeper layers of the TBF system, even during high ammonia nitrogen loads of wastewater. This capability of the TBF system to sustain saturated oxygen concentrations in the effluent was due to the high voidage

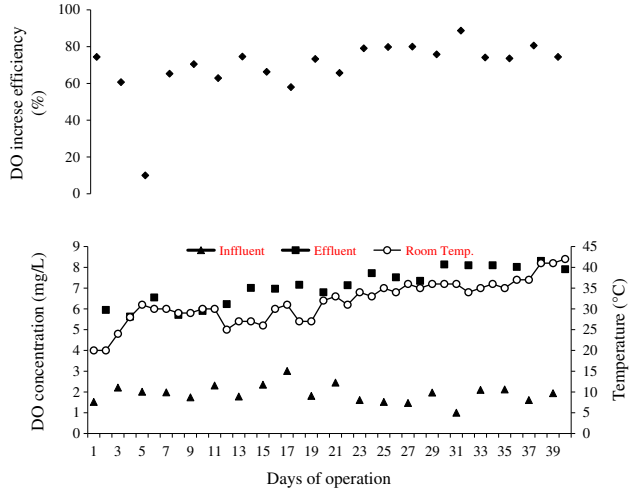

Fig. 7. Levels of DO in the influent and effluent and their increase efficiency (%) by pilot-scale TBF system during an experimental period of 40 d.

(47%) and consistent configuration of the stone filter media. The efficient passive aeration of infiltrating recirculating wastewater resulted in significant oxygenation of the incoming water during the filtration process. The remarkable oxygen transfer from the atmosphere into the bulk liquid during recirculation in the bed of the TBF constantly yielded effluent DO concentrations more than influent concentrations. It was concluded that without forced ventilation in TBF system, increase in DO, decrease in COD/BOD_5 and ammonia nitrogen concentrations were achieved with natural draft, which provided sufficient aeration.

3.2.2. Removal of nitrogenous pollutants

From the available data, it is clear that the mode of nitrogen removal in the pilot TBF system is nitrification and highly accelerated with an increase in the operational time (rho = 0.95; $p < 0.01$; $n = 40$). The average NH_4-N levels of the influent was 16.96 mg/L (0.0024 kg NH_4-N/m^3 d) and reduced by 33.8, 54.4, 76, 78.4, and 93.5% on 1st, 10th, 20th, 30th, and 40th day of treatment, respectively (Fig. 8). This increase in the rate of nitrogen removal has shown a significant association with the rise in temperature (rho = 0.83; $p < 0.01$; $n = 40$). It was previously reported that temperature has more significant effect on nitrification rates as it proliferates the growth of nitrifiers [43]. These results clearly indicate that the attached-growth systems like TBF having natural media, i.e. stones which can produce higher rate of nitrogen removal as compare to suspended-growth processes [44] and other attached-growth reactors using synthetic (sponge) media [45].

The process of nitrification is assisted under specific alkaline condition, as it requires alkaline condition for the conversion of ammonia (NH_3) to nitrates (NO_3) [46]. During the current research, regular monitoring showed that average influent alkalinity was 159.5 mg/L. Such alkaline levels are reasonably suitable for the activity of nitrifying bacteria [17]. The result indicated consumption of 7.14, 7.56, 7.88, 7.63, and 7.4 mg/L of alkalinity per mg of NH_4-N removal on 1st, 10th, 20th, 30th, and 40th day, respectively, of TBF operation (Figs. 8 and 9). This alkalinity consumption correlated well with NH_4-N removal (rho = 0.87; $p < 0.01$; $n = 40$) with an average 7.52 mg/L of total alkalinity (as $CaCO_3$) consumed for every 1 mg/L NH_4-N oxidized, which was approximately consistent with the theoretical value of 7.14 mg $CaCO_3$/L [47]. Moreover, this consumption of alkalinity during nitrification corresponded to reduction of the pH of wastewater (Fig. 10). A significant relationship between nitrification efficiency and decrease in the average pH range of influent from 7.52 to 6.62 of effluent was observed during the present study (rho = 0.73; $p < 0.05$; $n = 40$). This NH_4-N removal associated well with a decrease in pH due to the production of H^+ ions during the oxidation of ammonia [48], pH reduced by approximately 0.11 units for every 1 mg/L NH_4-N oxidized. The reduction in total alkalinity and pH provided evidence of normal biological nitrification inside the TBF. Moreover, after reduction of the pH, the

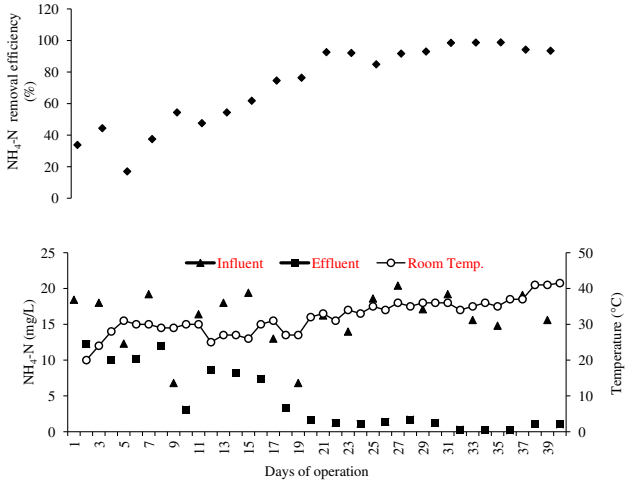

Fig. 8. Concentrations of NH_4-N in the influent and effluents and its removal efficiency (%) by pilot-scale TBF system during an experimental period of 40 d.

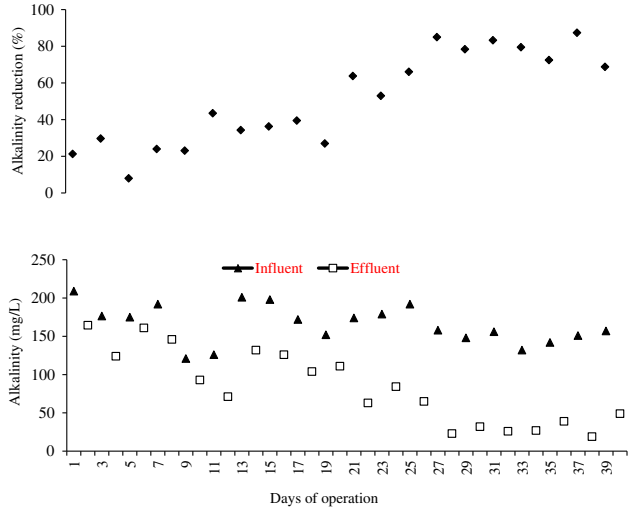

Fig. 9. Concentrations of alkalinity (as Ca_2CO_3) in the influent and effluents and its removal efficiency (%) during an experimental period of 40 d by pilot-scale TBF system.

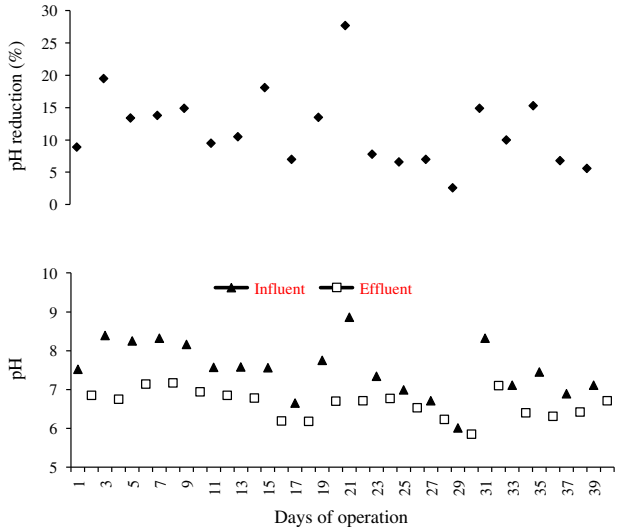

Fig. 10. Levels of pH in the influent and effluents and its percentage decrease (%) during an experimental period of 40 d by pilot-scale TBF system.

nitrification process continues as a biofilm system on a chalk filter media and has shown nitrification at low pH of 5 equal to those predictable at pH > 7 [49]. It was contrary to the kinetics of conventional nitrification, which suggested an optimum pH of 7.2–7.5 for nitrification. Thus, supporting the proposition that once acclimatized nitrifying biomass is constrained by alkalinity and not by low pH. In the present research, the identified ammonia and nitrite oxidizers were reported as *Nitrosonomas* and *Nitrobacter* sp. both are normally found in nitrifying systems working at alkaline pH, which suggests that individual strains are capable of acclimatizing to slightly low pH.

3.2.3. Removal of pathogens

For the quantitative analysis of pathogenic indicators in wastewater before and after treatment, the spread plate and MPN techniques were used. MPN index of fecal coliforms was carried out using MacConkey's broth (95% confidence limit for various combinations of positive and negative results). Before treatment, the untreated wastewater showed MPN index of 210 on 1st day and >1,100 on all other days of operation (10th, 20th, 30th, and 40th day influents). However, after 24 h of treatment in the TBF, its value dropped to 35, 220, 75, 80, and 43 on 1st, 10th, 20th, 30th, 39th, and 40th day, respectively, (average 88.8% reduction) (Fig. 11). These results clearly indicated a significant decrease in the strength of bacterial population from first to last day of the TBF operation (rho = 0.75; $p < 0.01$; $n = 20$) and with an increase in the seasonal temperature (rho = 0.78; $p < 0.01$; $n = 20$). One likely reason of the higher performance of the pilot-scale TBF could be the greater retention time in the bed of the reactor, which resulted in the retention of pathogenic micro-organisms (present in waste influent) on filter media by adsorption. After adsorption on the surface of stone media, they become a part of biofilms and participated in the removal of organic and inorganic pollutants from wastewater stream. Also, the physical factors such as retention time, porosity, and active volume were found in the reactor to be the most important factors affecting fecal coliform removal. The removal efficiency of pathogenic indicators has also been directly linked with removal of organic/inorganic (BOD_5/COD) pollutants (rho = 0.76; $n = 20$) in the reactor and indirectly linked with parameters such as DO (rho = 0.52; $n = 20$), pH (rho = 0.61; $n = 20$), and temperature (rho = 0.78; $p < 0.01$; $n = 20$) [50].

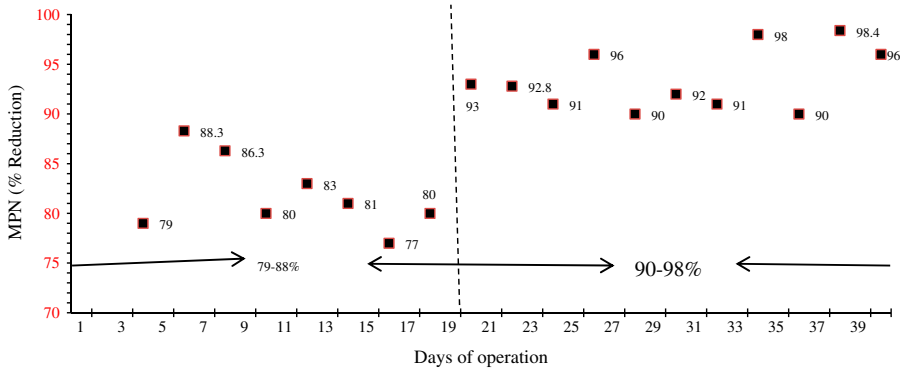

Fig. 11. MPN index (% reduction) of fecal coliforms in the effluent of pilot-scale TBF system during 40 d operation.

4. Conclusions

From the 40 d research study on the pilot-scale TBF wastewater treatment system, the results indicated that the reactor started wastewater treatment on the 10th day and its efficiency of treating wastewater increased with operational time. Maximum removal efficiency of COD, BOD_5, NH_4-N, and pathogen indicators from wastewater was shown by the reactor on the 40th day of operation. The processes of nitrification is also confirmed by the decrease in the pH and alkalinity of treated effluent, the key argument being that the efficient oxygen transfer afforded by the media design is sufficient to satisfy heterotrophs and autotrophs oxygen demand simultaneously. This is further supported by the identification of 13 heterotrophic strains, *Nitrosomonas* sp. and *Nitrobacter* sp., from the biofilms on the filter media.

The experiments here described were conducted in a pilot TBF system of small dimensions inside a work station facility. They have been protected along the whole experimental period against many external conditions that could interfere in the process such as direct exposition to sunlight, extreme variations of temperature, exposition to rain, and among others. Thus, a similar experimental work in an open field and using larger biofilters would be the natural next step for further investigations. In order to lower the cost of the installation of TBF systems, it would be desirable that the filling medium/packing media could be self-sustainable, so the walls of the filter could be of no structural nature. An investigation on efficiency as a function of the hydraulic loading rate is also recommended, besides the treatment capacity as a function of the organic loading rate and possible effects of recirculation.

Acknowledgments

Authors sincerely acknowledge the Higher Education Commission of Pakistan (HEC, Pakistan) for providing financial assistance for this research work.

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