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Performance assessment of stabilization ponds receiving wastewater of high organic load and feasibility of treatment using actinobacteria

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ABSTRACT

Olive mill wastewater (OMW) constitutes a major industrial and environmental problem. The uncontrolled discharging of OMW into the wastewater treatment plant has long been considered as a matter of treatment, minimization, and prevention due to the environmental effects induced by their disposal. Most of OMW treatment methods aim at organic matter destruction, hence the reduction of chemical oxygen demand (COD). In this study, bio-treatment of OMW, by a selected actinomycetes consortium in the attached growth manner, was utilized for degrading complex organic compounds. The progress on this problem is made by reducing the COD, which represents a major OMW pollutant. In this regard, Garada Wastewater Treatment Plant was firstly evaluated using principal component analysis (PCA). Secondly, microcosms study was undertaken for the reduction of organic load in raw wastewater by testing five actinomycetes isolates (19S, 32S, 56S, 62S, and 66S) for their abilities on the degradation of organics. PCA indicated that Salinity total dissolved solids, and COD of the wastewater had the greatest effect on the treatment process. The isolate *Micromonospora* sp. 19S showed the remarkable capability to degrade the organics in wastewater, reaching up to 80% at the end of 5 d in the batch experiment, whereas isolate *Micromonospora* sp. 56S achieved 70% reduction in organic load for the same duration time. Finally, the actinomycete isolates were attached to two types of carrier media in microcosm and left for 5 h to initiate biofilm formation.

Keywords: Olive mill wastewater; Bioremediation; Principal component analysis; Wastewater Treatment; Organic loads; Actinomycetes

1. Introduction

Wastewater is one of the most important environmental issues. It has a large content of pathogens and thus could result in catastrophic impacts on human health and the environment, particularly if it was disposed without treatment. Accordingly, there is a need to develop efficient wastewater management protocols to avoid hazardous impacts [1]. In this context, many approaches have been employed in wastewater treatment, including chemical, physical and biological treatment. Among these endeavors, biological treatment is considered one of the most efficient and cheapest approaches for wastewater treatment and categorized into suspended and attached growth processes. Examples of

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suspended growth systems are activated sludge systems, oxidation ditches, and aerated lagoons. Whereas, examples of attached growth systems include rotating biological reactors and trickling filters [2]. Oxidation (stabilization) ponds are one of the commonly applied suspended growth processes. These ponds can be considered as reservoirs excavated either inside or above the ground level, with the aid of earthen embankments. The process of wastewater treatment in oxidation ponds proceeds naturally depending on the following: (i) internal factors such as the mutual activity among bacteria, algae, and some other organisms, (ii) external natural factors such as sunlight, temperature, and wind. Oxidation (stabilization) ponds are classified, according to the type of activity, depth of the pond and retention time, into anaerobic pond, aerobic pond and facultative pond [3,4]. Attached growth systems are biological processes applied in the waste neutralization stage, in which the microorganisms responsible for the conversion of organic matter in the wastewater are attached to some inert solid surfaces. The first known application of biofilm technology was in the early 1880s in Wales, Great Britain when trickling filters were used for industrial wastewater treatment [5]. Many actinomycetes strains were isolated from wastewater and showed great ability to degrade organic pollutants in wastewater and to improve treated wastewater characters. This is because these microorganisms are already adapted to the habitat, highly metabolically diverse, and they can act on chemically different and toxic substrates. The application of actinomycetes as attached growth on solid carrier media could facilitate a higher degradation rate of complex organic compounds in wastewater treatment plants (WWTP). Attached growth systems were extensively studied and applied in wastewater treatment due to their obvious advantages, such as small reactor size and reduced sludge production [6].

In Egypt, North Sinai governorate, domestic WWTP suffers from frequent failure due to high organic loading, particularly related to olive mill wastewater (OMW). Olive milling is considered the main economic activity in the North Sinai governorate, Egypt. It produces over 720 m^3 of primary treated OMW per day during the milling season [7]. This load constitutes the major pollutant for domestic WWTP in the governorate.

The values of chemical oxygen demand (COD) for all treatment stages were considered high and did not match with the Egyptian standards (800–1,100 ppm) for the discharge and reuse of wastewater. The work presented here was conducted on Garada WWTP, Al-Arish, North Sinai, Egypt, which receives high organic loads from olive mill industries. It is working by stabilization pond technology, which is one of the commonly applied suspended growth processes. The results of the evaluation of Garada WWTP indicated that it was not performing efficiently and did not meet the Egyptian standards in certain wastewater parameters of particular importance such as COD, total suspended solids (TSS) and salinity. There was a gradual increase in the salinity of wastewater during the different treatment stages. Mancini et al. [8] reported that oily wastewater is characterized by high salinity levels, which limit the chances of discharge into the sewer systems. Treated wastewater with high-salinity value has an inhibitory effect on the growth of some plants [9].

In view of the aforementioned findings, the main objective of this study was to evaluate the wastewater properties affecting the performance of Garada WWTP using statistical data reduction technique along with principal component analysis (PCA). Additionally, the feasibility of using a specific actinomycete consortium, in the attached growth manner, for degrading complex organic compounds in wastewater containing excess organic load or originating from industrial sources is investigated. These objectives were achieved through the following: (i) Physicochemical and microbiological characterization of wastewater during different stages of treatment at the Garada WWTP, El-Arish, Sinai, Egypt. (ii) Evaluating the factors influencing the performance of the treatment system and organics removal, particularly the reduction of COD, using statistical methods. (iii) Investigating the biodegradation of organics, in organic-loaded wastewater, by selected actinomycetes attached on carrier media at microcosm scale.

2. Materials and methods

2.1. Wastewater samples

Wastewater samples were collected from the ponds of Garada WWTP at Al-Arish as a grab from different stages of treatment plant as shown in Fig. 1, starting from the deceleration chamber before the screens which represent the raw wastewater (influent). Garada WWTP is composed of two parallel subsequent different types of ponds (anaerobic, facultative, maturation ponds) and Zigzag canal or tank which mainly used as a contact tank for chlorination. Its design capacity reaches 60,000 $m^3 d^{-1}$ but its real capacity is 25,000 $m^3 d^{-1}$ [7]. The samples were collected in clean 1,000 ml polyethylene bottles. Samples for bacteriological investigations were collected in clean 60 ml sterile bottles and kept immediately in the icebox until transported to the laboratory for analyses, within 24 h.

2.2. Collection and composition of OMW

The seasonal extraction of olive oil usually lasts from June to January, and by the end of this season, we collected about 400 L of OMW samples. The samples were collected in 40 L-capacity containers and stored in a freezing room (–15°C). The main characteristics of OMW are listed in Table 1.

2.3. Physico-chemical analysis

All physicochemical analysis was performed according to the standard methods for the examination of water and wastewater [10], listed in Table 2. The chemicals used in this research were purchased from Chemproha Chemproha Chemiepartner B.V. Dordrecht, Netherlands. All experiments were conducted at around 25°C ± 2°C. The biochemical oxygen demand (BOD_5) was determined following the method of 5 d BOD. COD was measured colorimetrically using silver sulfate/sulphuric acid, and closed reflux method (UV) transmittance (at 600 nm) was measured using a UV-Visible spectrophotometer with quartz cuvette having 1 cm path length. The TSS was estimated by the filtration method. The salinity was determined by calibrated salinity-conductivity

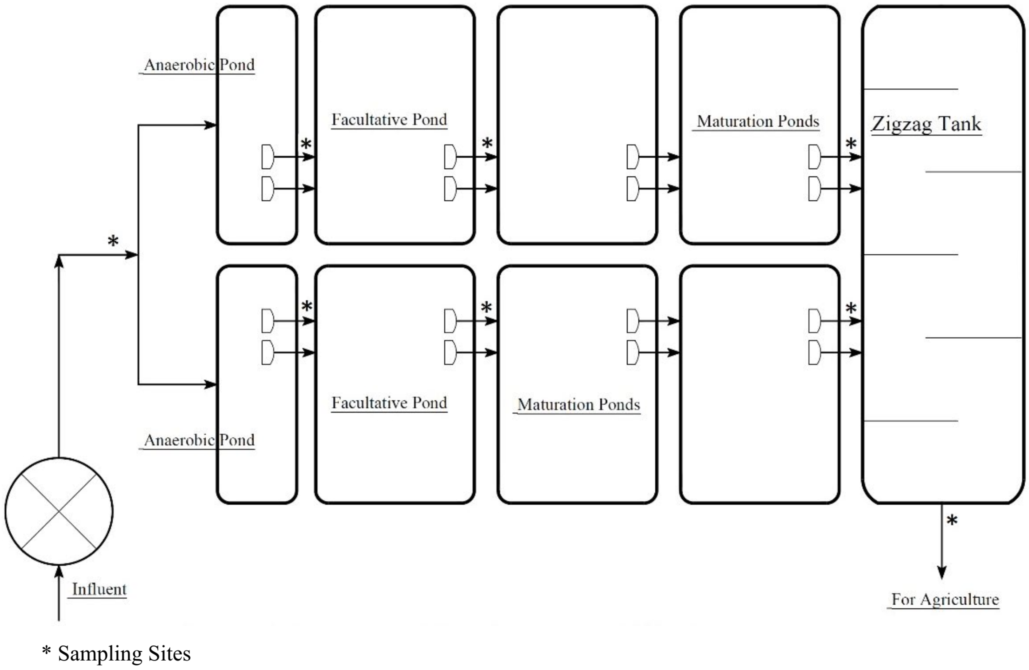

Fig. 1. Diagram of Garada waste stabilization treatment plant.

Table 1
Characteristics of olive mill wastewater. Comparing with Law No. 93 of 1962 regarding the discharge of industrial wastewater

Parameter	Concentration	Allowable limits	Units
pH	4.6–5.1	6–9.5	–
Biochemical oxygen demand	5,260	600	mg L^{-1}
Chemical oxygen demand	25,800–146,000	1,100	mg L^{-1}
Total suspended solids	12,760	800	mg L^{-1}
Oil and grease	4,230	100	mg L^{-1}
Total phenols	1,540	0.05	mg L^{-1}
Color	1,400	Not defined	TCU
Turbidity	1,264	Not defined	NTU

Table 2
Physico-chemical analysis methods of Garada waste stabilization treatment plant according to the standard methods for the examination of water and wastewater

Physico-chemical parameters	Unit	Method
pH	–	Portable calibrated pH meter (pHep®, Hanna, USA)
Temperature	°C	Calibrated conductivity/TDS and temperature meter
Total dissolved solids (TDS) mg L^{-1}/electrical conductivity (EC) S cm^{-1}	(TDS) mg L^{-1}/(EC) S cm^{-1}	(Lutron®, YK-22CT, Taiwan)
Salinity (mg L^{-1})	Mg L^{-1}	Calibrated salinity-conductivity meter (TESTO®, UK)
Turbidity	NTU	Digital Nephelometer (Orbeco-Hellige® USA)
Total suspended solids (TSS) mg L^{-1}	mg L^{-1}	APHA [10]
Biochemical oxygen demand (BOD) mg L^{-1}		
Chemical oxygen demand (COD) mg L^{-1}		
Nitrate	mg L^{-1}	Calibrated photometer (WinLab® photometer,
Ammonia		LF 2400, Windaus Labortechnik, Germany)
Phosphate		

meter (TESTO®, UK). The determination of turbidity was performed using Digital Nephelometer (Orbeco-Hellige® USA). Readymade standards were used for calibration. The turbidity was measured as nephelometric turbidity units (NTU). The temperature, total dissolved solids (TDS) and electrical conductivity (EC) of the wastewater samples were determined in-site using calibrated conductivity/TDS and temperature meter (Lutron®, YK-22CT, Taiwan). The pH of the wastewater samples was determined in-site, using a portable calibrated pH meter (pHep®, HANNA, USA). Calibration was performed using pH 4.2 and pH 7 buffers. Ammonia, nitrate, and phosphate were estimated using a calibrated photometer (WinLab® photometer, LF 2400, Windaus Labortechnik, Germany). All analyses were performed according to Standard Methods for Examination of Water and Wastewater, 18th ed. [11].

2.4. Microbiological analysis

The total heterotrophic bacteria were enumerated using nutrient agar; plates were incubated at 35°C for 48 h. Fecal coliforms were enumerated using m-FC agar; plates were incubated at 44.5°C for 24 h [11].

2.5. Microbial strains

Five actinomycete isolates, which were previously recovered from industrial WWTP [12] and showed promising enzymatic abilities to degrade complex organic polymers, were selected for the biodegradation experiments. The strains belonged to the genus *Micromonospora* and were coded: 19S, 32S, 56S, 62S, and 66S. They were provided as spore suspensions in 20% glycerol at –20°C for subsequent investigation.

2.6. The ability of the selected strains to degrade the organic contents of wastewater in a batch experiment

Batch experiment was performed to evaluate the ability of the *Micromonospora* strains to degrade the organic content of wastewater. 100 µl spore suspension from each strain (1.8×10^6 CFU mL^{-1}) was inoculated in 500 ml-conical flasks containing 200 ml of wastewater. Control flasks (inoculum-free wastewater) were included, and the experiment was performed in triplicate. The flasks were aerated by aerators and diffusers at 0.61 min^{-1}, using an air pump (Super Pump® SP-780 with two outlets, China). Samples for COD (parameter for organic content in wastewater) were taken from the control and inoculated flasks over 5 d and were measured as previously described. The removal efficiency was calculated as a normalized COD removal, i.e. measured concentration/initial concentration (C/C_0), to evaluate the ability of the selected strains to be used in this study.

2.7. Organic compounds degradation in microcosm experiments

2.7.1. Construction of percolating microcosms

Microcosms used in the biodegradation experiment consisted of vertical glass columns (4 cm diameter, 23 cm height), as shown in Fig. 2. Two carrier media were investigated in the microcosm: polyvinyl chloride (PVC, with 6–8 mm diameter) and commercial gravel (3.5–6 mm diameter). Each column was connected with a polyethylene container that received 20 L of pre-treated wastewater, provided with OMW to give 400 mg/L COD as an initial concentration, and the flow rate was regulated by using two stoppers. 500 µL (prepared from 1.8×10^6 CFU ml^{-1} stock), from each strain, was inoculated in the PVC- and gravel-columns, while the other two columns of PVC and gravel were left as controls

Fig. 2. Design of the percolating PVC and gravel microcosm.

(uninoculated with the five actinomycetes). The control columns received wastewater and the experiment was performed in triplicates. The columns were left for 5 h, to ensure the biofilm initiation, and the final flow rate was adjusted to 1.5–2 ml min^{-1}. The microcosms operated 8 h d^{-1} for 6 d, RT at 70–52.5 min (RT = volume/flow rate). The organic compounds removal efficiency was determined by measuring the COD in samples taken at a reference time (the time at which the first drop of treated wastewater obtained from the column), and at 1 d intervals, for 6 d. The removal efficiency was calculated as the ratio of COD of the sample to COD of the corresponding control (i.e.: COD_t/COD_c).

2.8. Enumeration of actinomycete counts in biofilm formed on carrier media

At the end of the experiment, the carrier medium in each column was equally separated, at 8 cm distance intervals. A weight of 5 g from each part was mixed with 50 ml sterile saline solution, shaken for 5 min to disrupt the biofilm, then serially diluted and plated on starch casein medium [13] containing cycloheximide (0.05 g L^{-1}) as an antifungal. The plates were enumerated after incubation for 7 d at 28°C. All experiments were performed in triplicates.

Isolated actinomycetes were identified by morphological, biochemical and 16s rRNA sequencing. The 16s rRNA sequencing was performed by polymerase chain reaction (PCR) amplification [14] using the following 16S rDNA primers:

* 27F: AGAGTTTGATCMTGGCTCAG
* 1522R: AAGGAGGTGATCCANCCRCA

The amplified PCR fragments were commercially sequenced (Macrogen, Inc. is a South Korea public biotechnology company. The company's headquarters are located in Seoul (Macrogen, Seoul, South Korea). in both directions using an automatic DNA sequencer. The sequences obtained were compared with those available in the GenBank database (http://www.ncbi.nlm.nih.gov) using the standard nucleotide-nucleotide BLAST program (BLASTN, http://www.ncbi.nlm.nih.gov) to ascertain their closest relatives.

2.9. Statistical analysis

PCA was performed using Minitab® 15 to deduce factors influencing wastewater characteristics at the different treatment stages of the Garada plant.

3. Results

3.1. Physical, chemical, and biological characterization of WWTP

Physical, chemical and biological characterization was conducted for each site as summarized in Table 3 and compared with the limits of Egyptian legislation: (i) law 48/1982 for discharge into underground reservoir & Nile branches/canals, (ii) the Egyptian code for reuse of treated wastewater in agricultural purposes (ECP 501-2005), [cultivation of fodder crops (sorghum), fruits trees with a crust to be used in canning and processing (lemon, mango, and olive), trees for forestation of highways and green belts around cities (casuarina and camphor)]. Wastewater stabilization in pond is achieved using physical, chemical and biochemical reactions which are significantly influenced by temperature. Thus, the rate of photosynthesis and the cellular metabolism of microorganisms are enhanced by high temperatures and retarded by low temperatures. Temperatures of the influent and effluent wastewater over the study period ranged between (20°C–29.6°C) and (19°C–32.9°C), respectively. Turbidity values showed slight elevation at the anaerobic pond then it decreased rapidly towards the zigzag canal which showed the lowest value of turbidity. As the concentration of salts in the water increases, the EC rises (the greater the salinity, the higher the conductivity). The averaged influent salinity was 3,090 mmohs cm^{-1} and showed a gradual increase during the treatment stages, reaching the greatest value in the effluent (3,994.3 mmohs cm^{-1}). Solids analysis is important in the control of biological and physical wastewater treatment processes and for assessing the compliance with regulatory agency wastewater effluent limitations. An increase of 11.13% from the influent's TDS value was recorded at the anaerobic pond. The TDS value showed a slight elevation at the facultative pond by 10.4%, and then it was reduced by 2.1% at the maturation pond. At the zigzag canal; a reduction by 4.4% in TDS value was observed. The anaerobic pond showed great efficiency in its sedimentation ability as a primary sedimentation tank where the TSS was reduced by 76.1% compared to the value at the influent. At the large area of facultative ponds, the sedimentation recorded an 80.7% reduction than that of the influent. Compared to the facultative ponds the TSS value has increased by 66% at the maturation pond. This increase was followed by a 35% reduction in the TSS value at the zigzag canal compared to the maturation pond. With pH around 7.2, influent and wastewater at anaerobic ponds (7.3 and 7.2) passed into the facultative pond (7.8) to show a slight elevation in pH value, then increased at the maturation ponds (7.9) to reach the maximum value at the zigzag tank (8.01). Ammonia is produced largely by deamination of organic nitrogen-containing compounds and by hydrolysis of urea. Ammonia concentrations can reach up to 30 mg L^{-1} in some wastewater. The high concentration of ammonia in wastewater may refer to the presence of a problem in the process of nitrification. Nitrification is a process of two-steps, for the removal of ammonia, in which ammonia is converted by autotrophic bacteria (*Nitrosomonas*) into nitrite then (*Nitrobacter*) oxidize nitrite into nitrate (APHA [10]). Garada influent was characterized by a very low concentration of ammonia (0.4 mg L^{-1}) and then its concentration began to increase in the anaerobic pond (1.7 mg L^{-1}). A sharp increase to 6.9 mg L^{-1} of ammonia was recorded in the facultative pond. Ammonia levels did not show significant changes in the subsequent stages. Nitrate is found in small amounts in fresh domestic water but in the effluent of nitrifying biological treatment plants nitrate may be found in concentrations of up to 30 mg nitrate as nitrogen/L. The highest level of nitrate was detected in the zigzag canal (21.2 mg L^{-1}), which was increased by ~ 13 times, compared to levels in the previous stages of the treatment process.

The reduction of nitrate content in anaerobic ponds was largely diminished due to the denitrification of NO_3 to N_2 gas. Denitrification is normally limited to the amount of nitrate

Table 3
Mean of physical, chemical and biological characters of wastewater, compared to the Egyptian law 48/1982 and/or ECP 501%–2005%: removal efficiency related to previous stage %: overall removal efficiency related to the influent

Parameter	Influent	Anaerobic pond	Facultative pond	Maturation pond	Zigzag canal	Max. limit
Temperature (°C)	25.02 ± 3.6	26.4 ± 5.4	25.9 ± 5.4	25.9 ± 5.7	26.3 ± 6.1	35
pH	7.3 ± 0.22	7.2 ± 0.17	7.8 ± 0.11	7.9 ± 0.18	8.01 ± 0.17	9–Jun
Turbidity (NTU)	215.1 ± 97.6	222.1 ± 184.9	89.4 ± 72.8	45.6 ± 20.9	41.9 ± 21.1	NA
EC (S cm^{-1})	4.1 ± 1.9	5.11 ± 2.04	5.3 ± 2.2	5.3 ± 2.17	5.4 ± 2.03	NA
Salinity (mmho cm^{-1})	3,090 ± 674.9	3,771.4 ± 416.9	3,821 ± 183.8	3,910 ± 229.9	3,994.3 ± 307.2	NA
TDS (mg L^{-1})	1,321.2 ± 387.1	1,458.1 ± 533.4	1,610 ± 319.8	1,576.1 ± 425.1	1,506.1 ± 543	2,000*
						800**
TSS (mg L^{-1})	175.3 ± 70.8	41.9 ± 33.4	33.8 ± 32.7	99.5 ± 198.2	60.3 ± 69.9	20*
		76.10%	80.70%	43.20%	65.60%	30**
BOD (ppm)	26.00 ± 15.4	26.00 ± 15.7	11.2 ± 4.9	17.9 ± 5.2	12.7 ± 1.9	60*
		0%	57%	31.20%	51.20%	20**
COD (ppm)	1,145.95 ± 1,175.6	1,362.6 ± 1,184.9	1,146.4 ± 1,263.5	1,171.7 ± 1,311.2	1,108.3 ± 1,243.5	80*
						30**
Ammonia (mg L^{-1})	0.4 ± 0.45	1.7 ± 0.92	6.9 ± 1.97	6.8 ± 1.77	6.8 ± 3.8	NA
Nitrate (mg L^{-1})	2.00 ± 1.03	1.6 ± 1.54	0.5 ± 0.43	2.5 ± 2.7	21.2 ± 25.1	50*
						30**
Phosphate (ppm)	3.5 ± 0.45	2.2 ± 1.36	1.00 ± 0.63	1.4 ± 0.82	0.7 ± 0.37	1**
DO (ppm)	3.2 ± 1.2	1.11 ± 0.83	2.00 ± 1.34	5.00 ± 2.8	5.2 ± 2.6	Not less than 4*
TVB (CFU ml^{-1})	1,568,571 ± 1581,986	821,428.6 ± 920,659.7	1,124,286 ± 1339,501	1,387,143 ± 14,104,934	1,024,286 ± 1186,126	NA
Fecal coliform (CFU ml^{-1})	24,971.43 ± 9,054.78	10,900 ± 7,140.9	3,542.9 ± 4,221.3	1,300 ± 1,432.6	1,185.7 ± 1,541.8	5,000*
				94.80%	95.30%	2,500**

**Law 48/1982. NA: not applicable. Values exceeding standard levels are highlighted.

produced and dissolved oxygen (DO) present in wastewater. In a case like this, denitrification should take place especially in the sediment zone, anaerobic layer or during the night time when DO has been consumed for the respiration of algae and bacteria. Nitrification was the paramount nitrogen transformation; as soon as the conditions could favor its development, nitrifying bacteria rapidly multiplied and could nitrify. Afterward, nitrification was limited to the oxidation of the ammonia entering the pond. Importantly, both aeration from the maturation ponds surface and photosynthesis of algae may result in a marked increase in the oxygen concentration inside ponds thereby promoting rapid oxidation of NO_2^- to NO_3^- [15].

A reasonable concentration of DO (3.2 ppm) was recorded in the influent that passed into the anaerobic pond where it decreased to (1.1 ppm) during the treatment process. The concentrations of DO increased gradually in the subsequent treatment stages, reaching the maximum value (5.2 ppm) in the zigzag canal. Phosphorus exists in natural water and wastewater almost solely as phosphates. Phosphates also occur in bottom sediments and biological sludge, both as precipitated inorganic forms and incorporated into organic compounds. The highest value of phosphate was observed at the influent (3.5 ppm), and then it began to decrease markedly at the anaerobic and facultative ponds reaching (2.2 and 1.00 ppm), respectively. It showed a slight increase at maturation pond (1.4 ppm) and the lowest value was at the zigzag canal (0.7 ppm). The BOD values were the same for the influent and the anaerobic pond and reached the lowest values at the facultative pond (11.2 ppm), and then it showed a slight increase at the zigzag canal reaching (12.7 ppm). The COD for all treatment stages were considered high and did not match with the Egyptian standards (800–1,100 ppm) for the discharge and reuse of wastewater. The counts of Heterotrophic bacteria during the treatment stages, obviously, showed a slight reduction from the influent value to the zigzag canal, and almost reached a one-fold reduction at the end of treatment. The count of fecal coliform obviously decreased from the influence towards the zigzag canal, the highest reduction percentage was recorded in the maturation pond (94.8% reduction) and the zigzag canal (95.3% reduction) from the count in influent.

3.2. PCA for factors influencing wastewater treatment process

In the influent wastewater to WWTP, the PC1 and PC2 analysis could account for 65.8% of the total variation. It demonstrated that the physical conditions (temperature, salinity, TDS, and EC) and organic constituents (BOD, nitrate, and ammonia) were the major factors, followed by the main constituents COD. In the anaerobic pond, PC1 and PC2 could explain 68.7% of the total variation. The physical conditions (temperature, TDS, and EC) and organic constituents (nitrate, phosphate, and BOD) were the major factors, followed by the persistent constituents COD as the less important factor. In the facultative pond, PC1 and PC2 could account for 73.0% of the total variation. The physical conditions (temperature, pH, turbidity, and EC) along with organic constituents (ammonia and phosphate) were the major factors, followed by COD as the less important factor. Similarly, in the maturation pond, PC1 and PC2 results can be used to account for 75.5% of the total variation. The persistent constituents COD was the major factor, followed by the physical conditions (temperature, turbidity, and EC) and organic constituents (BOD, ammonia, and nitrate) as the less important factor. In the zigzag pond, PC1 and PC2 could explain 73.6% of the total variation. The physical conditions (temperature and DO) and the salt content (TDS and TSS) were the major factors, followed by the organic constituents (BOD, phosphate, nitrate, and ammonia) and microbial load total viable bacteria (TVB) as shown in Figs. 3 and 4.

3.3. Phylogenetic identification of actinomycete isolates

The results of sequences analysis revealed the following: (1) isolate, 32S of 1477 base pair, had a sequence with 99% similarity to *Micromonospora aurantiaca* strain R9-550, (2) isolate, 19S of 1465 base pair, had a sequence with 99% similarity to *Micromonospora* sp. L5. (3) isolate, 56S of 1491 base pair, had a sequence with 99% similarity to *Micromonospora* sp. DS3001, (4) isolate, 62S of 1413 base pair, had a sequence with 100% similarity with *Micromonospora aurantiaca* Z9-4. (5) isolate, 66S of 1465 base pair, had a sequence with 100% similarity with *Micromonospora aurantiaca* ATCC 27029. Phylogenetic tree was constructed from a multiple sequence alignment of 16S rRNA gene sequences using MEGA 4 Software as illustrated in Fig. 5.

3.4. Batch experiment for biodegradation ability of the selected strains

The five *Micromonospora* strains performed differently to degrade the organic content of wastewater between

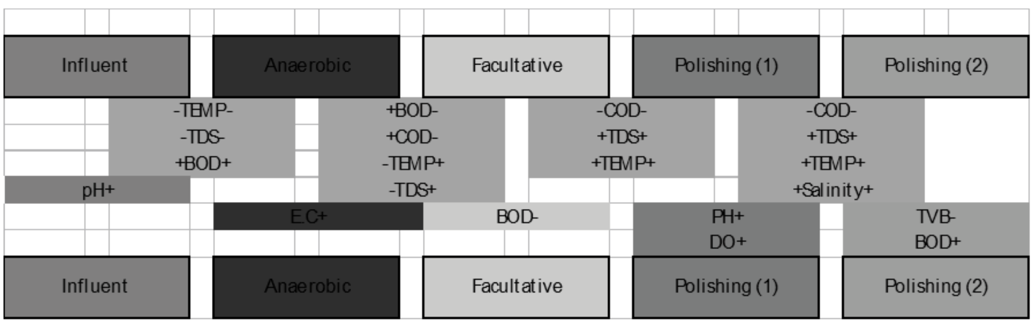

Fig. 3. Principal component analysis (PCA) summary for factors influencing wastewater treatment plant for PC1.

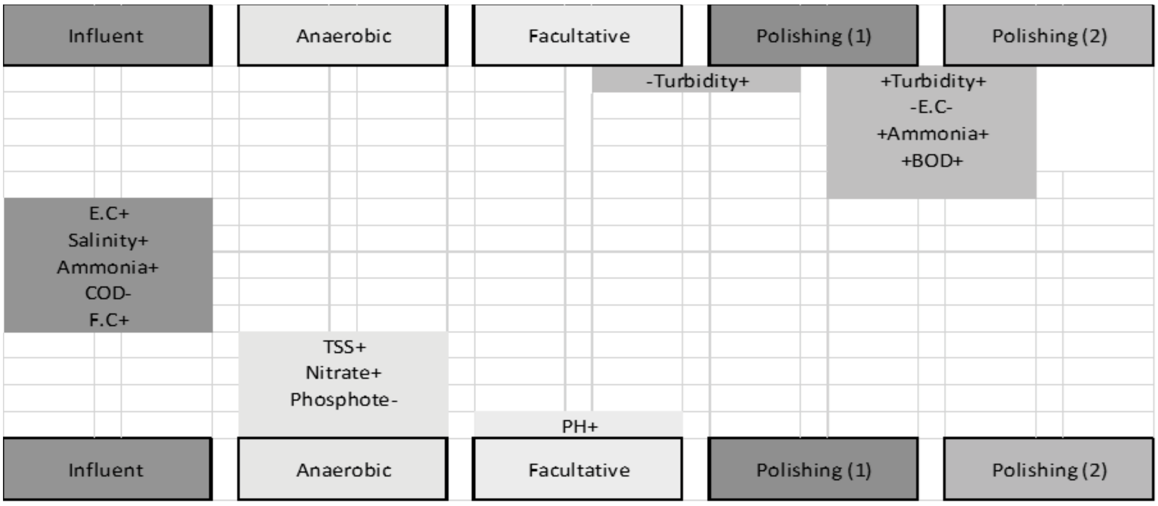

Fig. 4. Principal component analysis (PCA) summary for factors influencing wastewater treatment plant for PC2.

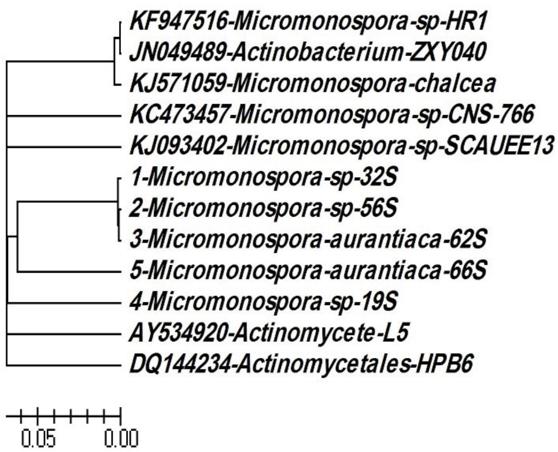

Fig. 5. Phylogenetic tree for the actinomycete isolates (Evolutionary history was inferred using the neighbor-joining method).

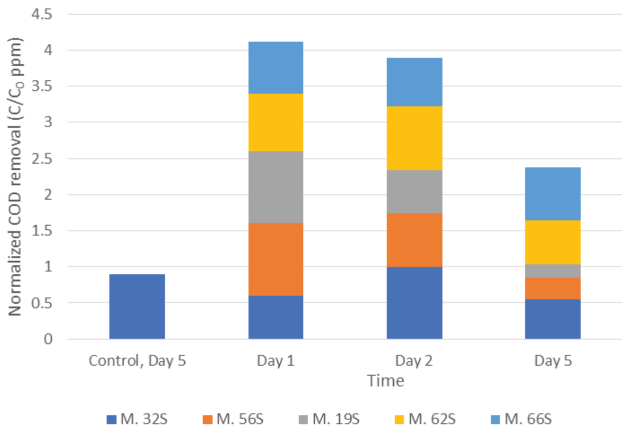

Fig. 6. Organic load reduction in wastewater by the five actinomycete strains in batch experiment.

8.6%–80% COD reduction. Species were tested to estimate their ability to degrade the organic content of wastewater in batch experiment. The results of COD obtained from this experiment were represented graphically as shown in Fig. 6.

Fig. 6 depicts that the strains showed a cumulative COD removal index 4.12–3.89 for 2 d. On the 5th day, the strains M. 56S and M. 19S showed significantly low COD removal, resulting in a decrease in the cumulative COD removal index to 2.38. However, this later value was highly significant ($p < 0.02$), compared to the control (uninoculated). Thus, the five tested strains were used, as a consortium, in the following microcosm study.

3.5. Microcosms study for organic load reduction in raw wastewater

3.5.1. Effect of carrier medium types on the efficiency of microcosm

A comparison between PVC and gravel as supporting media in the columns was studied. Fig. 7 shows the bio-removal of COD, by the consortium using both filling materials, versus time, in relation to the control columns.

On the second day of the study, the gravel column showed a 79% reduction in COD value, while the PVC column exhibited an increase in COD value by 5%. On the 6th day, further reduction (43%) in COD was recorded for the gravel column, while the PVC column showed a reduction in COD for the first time by 7%.

3.5.2. Enumeration of actinomycetes from the formed bio-film and control columns

By the end of the microcosm experiment, actinomycetes were enumerated in the biofilm that was developed in both test and control columns for PVC and gravel carrier media. Table 4 shows the numbers of actinomycetes in the columns.

4. Discussion

4.1. PCA for waste stabilization ponds treatment plant

To evaluate the most significant water quality parameters in Garada WWTP, the data were further analyzed using

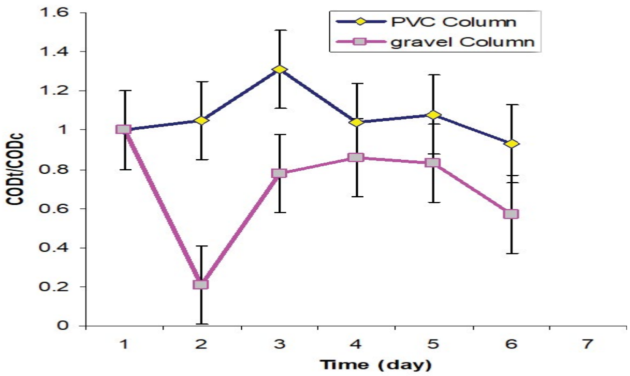

Fig. 7. Bio-removal of COD from wastewater using actinomycetes attached to PVC and gravel in microcosms experiment.

PCA. Loadings having an absolute value >0.30 were taken into consideration for evaluation of the treatment plant. Results listed in Table 5 revealed that the first three principal components (i.e. PC1, PC2, and PC3) affected the quality of the lake, with a total variability of 68.3%. As shown in Fig. 8, the first principal component (PC1: 34.1% of the variance) represented high loadings on TDS: –0.338, salinity: –0.337, ammonia: –0.320, and temperature: –0.303, and with a positive loading on BOD: 0.311. These negative loadings indicate the climate-influencing factor that is represented by the variables: TDS and salinity, ammonia, and temperature. The characteristic high temperature of this arid area (reference) results in intensive water evaporation and ultimately leads to concentrate the contents of the pond to some degree. This may increase the TDS and salinity as well as the concentration of organic matter to a point at which the osmotic balance of microorganisms in wastewater is disturbed [16].

Additionally, the groundwater wells in North Sinai are considered the main source of water for household purposes, which finally reach the WWTP causing an increase in the water salinity and TDS. There is a trend of increases in salinity from south Sinai (<2,000 mg L^{-1}) to North Sinai (>10,000 mg L^{-1}), reflecting the general flow direction (south to north) and the increasing possibilities of seawater intrusion. A similar conclusion was reached by a geo-electric survey [17]. There are some local peaks of high salinity in the central and northern parts, related to Sabkha and over-pumping areas, where the downward infiltration and upward leakage of the saline water increase groundwater salinity, respectively. The smaller salinity values are recorded in the southern and central-eastern parts, close to the recharge area by rainfall [18]. Further, the increase of wastewater temperature beyond its optimum value results in a precipitous decrease of microbial activity [19] and thus the DO is likely to be liberated to the atmosphere. Under O_2-deficient conditions, ammonia accumulates in wastewater as a result of nitrification problems. As aforementioned, nitrification is a process of ammonia removal in two-steps. Firstly, ammonia is converted into nitrite by autotrophic bacteria (*Nitrosomonas*), and in the second step, nitrite is oxidized by *Nitrobacter* into nitrate [10].

The high concentration of ammonia in wastewater may refer to the presence of a problem in the process of nitrification. This low nitrification process may be attributed to the following reasons: (1) too many organics remain in suspension, (2) inadequate contact between nitrifying organisms and ammonia in solution due to low mixed liquor suspended solids, (3) winter temperatures (mention the range: 10°C–20°C for example) cause poor seasonal performance, (4) biomass assimilated nitrogen is rereleased from sludge. Very low nitrifying bacteria populations are in ponds due primarily to the absence of physical attachment sites in the aerobic zone, although inhibition by the pond algae may also occur. The volatilization of ammonia to the atmosphere depends on the aeration, mixing, and temperature, in addition to the pH value. Ammonia volatilization is another removal

Table 5
Loading of principal components analysis for Garada waste stabilization ponds parameters

	Loading		
Variables	1	2	3
pH	–0.283	0.299	0.300
Temperature	–0.303	–0.354	0.027
EC	0.130	0.417	–0.033
Salinity	–0.337	0.011	–0.218
Turbidity	0.229	–0.243	–0.103
TDS	–0.338	–0.296	–0.111
TSS	0.058	–0.219	0.597
Ammonia	–0.320	0.241	–0.012
Nitrate	–0.187	0.086	0.146
Phosphate	0.209	–0.337	0.119
BOD	0.311	0.009	0.218
COD	0.270	0.310	–0.127
DO	–0.192	0.027	0.573
TVB	0.244	0.240	0.230
FC	0.283	–0.289	–0.022
Eigenvalues	5.16	3.48	1.65
Variance %	34.1	23.2	11
Cumulative variance %	34.1	57.2	68.3

Table 4
Counts of actinomycetes (CFU gm^{-1}) in formed biofilm of the test and control columns

Column part	Test-PVC	Control-PVC	Test-gravel	Control-gravel
Upper	<100	<100	25,300	<100
Middle	31,900	10,000	34,100	100
Lower	<100	100	20,900	<100

mechanism for nitrogen. The pKa of ammonia (where 50% of ammonia is in the gaseous NH_3 form) is 9.25.

Ammonia uptake by flora and fauna is also common and can make a significant difference in ammonia concentration during growing seasons. However, this uptake only acts as temporary storage. This is because cells senesce and deposit their stored nitrogen at the bottom of the ponds or in the downstream portion of wastewater treatment. Uptake by the flora can be the largest, temporary removal pathway depending on the season. Once the flora dies, the ponds receive a load of stored nitrogen and carbon as organic nitrogen and organic carbon. As both types of organic compounds decay, they release amino acids and dissolved organic carbon that can be used by another organism [20].

The second principal component (PC2: 23.2% of the variance) was associated with high positive loadings on EC: 0.417 and COD: 0.31, and with a negative loading on temperature: –0.354, and phosphate: –0.337. The PC2 may indicate persistent-constituents as a factor represented by EC (salt factor) and COD. Phenol in OMW is a weak acid, and then the dissolution of phenol generates an increase of EC and current. Therefore, in tests contaminated with phenol, the electrical current increased compared to tests contaminated with phenanthrene [21], while it showed an opposite trend with temperature and phosphate. The temperature of the wastewater is expected to impose a positive effect on phosphate, that is, it decreased at a lower temperature. Precipitation, which is strongly dependent on temperature and different stages of the precipitation process, maybe either aided or hindered by high or low temperatures [22].

The increase in COD concentration may be due to the presence of OMW mixed with a domestic one. OMW is generated during the production of olive oil; its treatment is a major environmental problem. The annual OMW production in North Sinai is estimated to be over 86,400 m^3/season [7]. Organics and persistent constituents could represent important factors that caused the failure of Garada WWTP to achieve acceptable wastewater quality in certain parameters. The relationship between salinity, EC and TDS arises from the fact that the conductivity is a measure of water's capability to pass electrical flow. This ability is directly related to the concentration of ions in the water [23]. These conductive ions come from dissolved salts and inorganic materials such as alkali, chlorides, sulfides and carbonate compounds [24,25].

Phosphorus removal in ponds occurs via physical mechanisms such as adsorption, coagulation, and precipitation. The uptake of phosphorus by organisms in metabolic functions as well as for storage can also contribute to its removal. The phosphorus removal ratio was 80% measured from influent to zigzag canal effluent. The removal in wastewater ponds has been reported to range from 30% to 95% [26–28]. Algae discharged in the final effluent may introduce organic phosphorus to receiving water [29]. Excessive algal "after blooms" observed in water receiving effluents have, in some cases, been attributed to nitrogen and phosphorus compounds remaining in the treated wastewater [29]. Algae, in turn, produce the O_2 necessary for the survival of aerobic bacteria. The pond reactions of biodegradation and mineralization of waste material by bacteria and the synthesis of new organic compounds in the form of algal cells can result in a pond effluent containing values higher than the acceptable level of TSS. Although this form of TSS does not contain the same constituents as the influent TSS, it does contribute to turbidity and must be removed before the effluent is discharged [29].

4.2. Cluster analysis

The score plot in Fig. 9 was applied to cluster the monitoring points along with the waste stabilization treatment plant, according to common characteristics. PC1 and PC2 clustered the dataset into three groups. Group 1 was distributed on the samples, as depicted in Fig. 8 This group (containing stations 1 and 2) is clearly recognizable in the right part of PC1. These results, characterized by high BOD and low ammonia, further confirm that PC1 clustered the data set according to the BOD and ammonia. The temperature, salinity, TDS, and ammonia had stronger negative loadings in PC1 and a positive loading on BOD, high positive loadings on COD and EC in PC2. This indicates that these zones suffered from increased COD and BOD, turbidity, phosphate, and lower ammonia levels. Group 2 (containing stations 3 and 4) was approximately distributed on the negative direction of PC1. These monitoring points had a relatively higher temperature and salinity with stronger negative loadings in PC1, lower TSS. These results, characterized by high salinity, temperature, ammonia, and TDS, attest that PC1 clustered the data set according to salinity, temperature, ammonia, and TDS. Group 3 (containing stations 3, 4, and 5) was approximately distributed on the positive direction of PC1 and PC2, with high positive loadings on COD and EC, and with a negative loading on temperature, salinity, ammonia, TDS and phosphate. These stations were characterized by a relatively lower phosphate nutrients and turbidity together with higher ammonia concentration. In contrast, seasonal changes in water quality parameters were not identified by PCA. This meant that Garada WWTP was mainly influenced by spatial variations rather than seasonal changes [30]. It was also noticed that COD had a correlation with loadings in the second principal components (i.e., PC2 and PC3).

The abundance of COD presented high loading (0.310) on PC2 having only 23.2% of the variance. This suggested that COD was more important than the other effects of environmental and physiochemical factors, which could be due to the continuous disposal of OMW. The main function of anaerobic ponds is BOD removal, which can be reduced by 40%–85% [31]. The BOD values were the same for the influent and the anaerobic pond and reached the lowest values at the facultative pond (11.2 ppm), and then it showed a slight increase at the zigzag canal reaching (12.7 ppm) as displayed in Table 3. The BOD removal in anaerobic ponds is occurred by the same mechanisms implemented in other anaerobic reactors [32]. The process (as in septic tanks) depends upon the sedimentation of settleable solids followed by anaerobic digestion in the resulting sludge layer.

Facultative treatment ponds are the simplest of all waste stabilization ponds and consist of an aerobic zone close to the surface together with a deeper anaerobic zone. They are designed for BOD removal and can be used for water treatment with BOD range from 100 to 400 kg ha^{-1} d^{-1}, which is corresponding to a 10 to 40 g m^{-2} d^{-1} at temperatures above 20°C

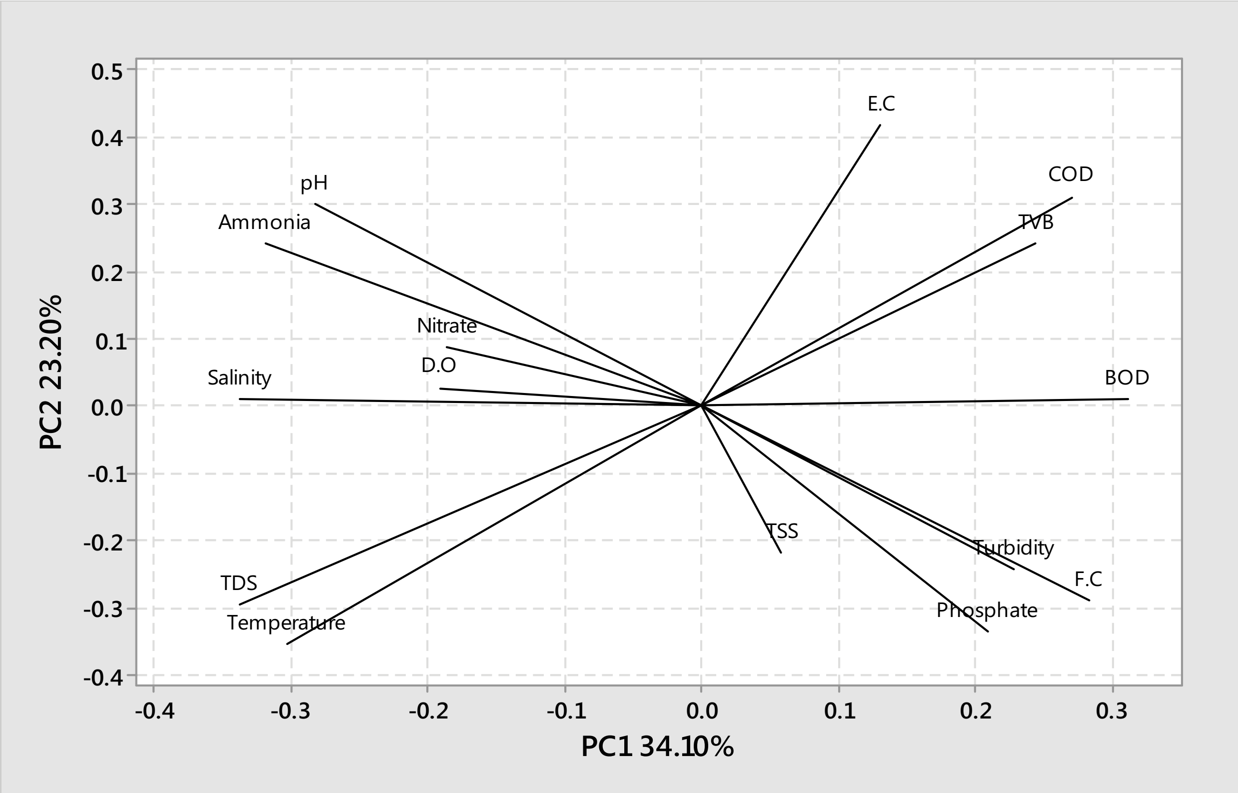

Fig. 8. Principal components analysis loading plot.

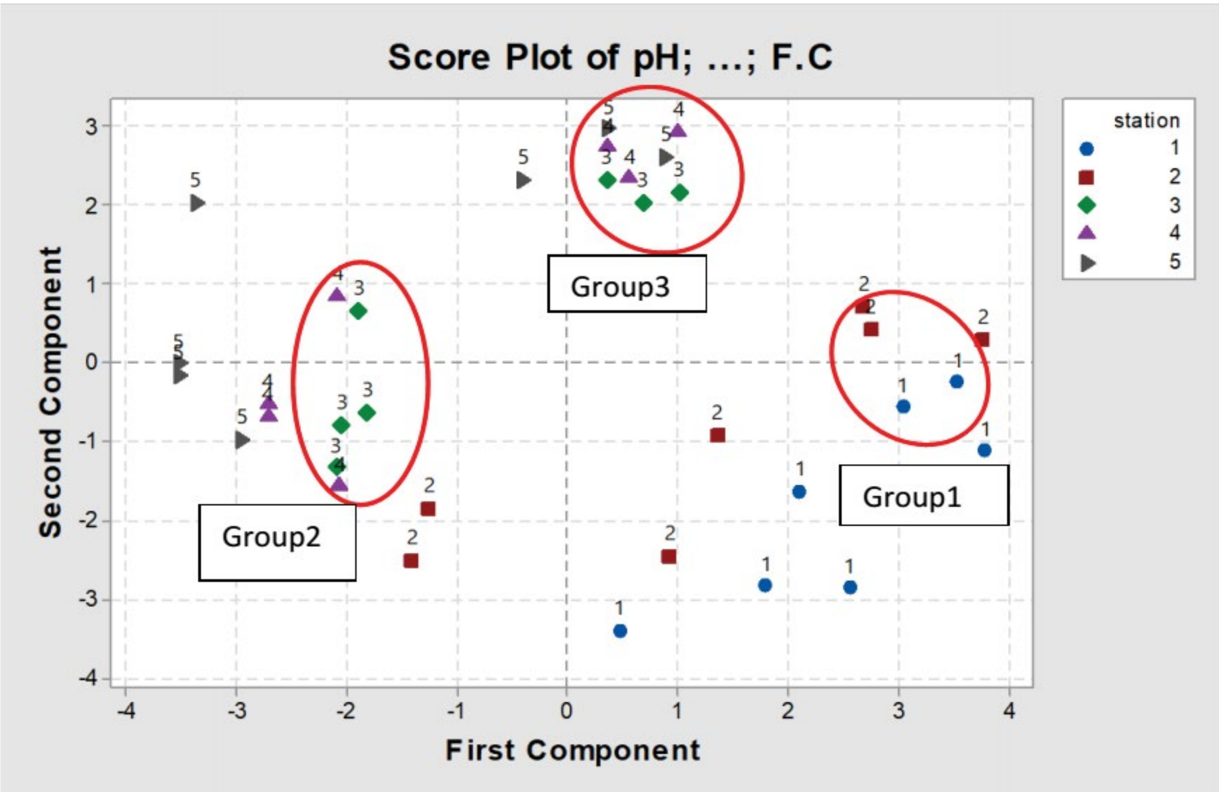

Fig. 9. Principal components analysis score plot.

[33]. The FPs can efficiently remove ~80%–95% of the BOD_5 [31], which implies an overall removal of 95% from the two ponds (aerated pond and facultative Pond). Whereas anaerobic and facultative ponds are designed for BOD removal; maturation or polishing ponds are essentially designed for pathogen removal and retaining suspended stabilized solids [32,34]. The size and number of maturation ponds depend on the required bacteriological quality of the final effluent. The principal mechanisms for fecal bacterial removal in facultative and maturation ponds are hydraulic retention time, temperature, high pH (>9), and high light intensity. Viruses and microorganisms get also removed. This type of pond is also effective for removing the majority of nitrogen and phosphorus from the effluent if used in combination with algae and/or fish harvesting [35]. Statistical analysis indicated that salt concentrations, thermal effect, organics, and persistent constituents could represent the important factors that caused the failure of Garada WWTP to achieve acceptable wastewater quality in certain parameters. Salinity is a measure of the content of salts in water.

The performance of biological treatment processes for saline wastewater is usually low due to the adverse effects of salt on microbial flora. Due to the scarcity in the potable water resources, the North Sinai governorate depends on the amounts of drinking water that come from the drinking-water production plant in El-Kantara east (Morrashaha) 150 km west of Al-Arish city. However, the groundwater wells are considered the main source of water for household purposes which finally reach the WWTP, causing an increase in the water salinity. According to the data obtained from the company of potable water and sanitary drainage at North and South Sinai (one of Holding Company for Water and Waste Water companies) for representative groundwater wells in the city of Al-Arish, the values of TDS ranged in between 2,900–4,600 ppm. Also, as a result of the presence of a combined sewer system, the domestic wastewater and industrial one (especially OMW) reach together to the treatment plant, thereby causing a marked increase in salinity and organic load. The PCA showed that temperature is an important factor that influences the treatment process during wastewater treatment stages.

Temperature is important in the design and operation of biological processes in treatment facilities [36]. In principle, the higher the temperature, the higher the microbial activity until an optimum temperature is reached. Further increase of the temperature beyond its optimum value caused a precipitous decrease of microbial activity [19]. At the same time, aerobic bacteria consume oxygen at a higher rate, creating conditions that are likely to result in the appearance of anaerobic patches at different points in the pond [16]. Garada treatment plant locates in an area characterized by a desert climate at daytime with considerably high temperature, which drops markedly at night. A sudden increase in temperature may adversely affect facultative pond efficiency in the following way: bacterial activity is quickly stimulated, growth is also enhanced and the oxygen uptake rate increases. If this higher uptake is not compensated by a higher oxygen production, anaerobic conditions may arise, the effluent may become turbid with releasing of anaerobic odors. Evaporation as a factor should also be considered, intense evaporation may affect the ecological balance in stabilization ponds through the increase in the concentration of solids. It may also cause an undesirable reduction in the depth of water and affects retention time. In hot arid regions, evaporation may exceed 15 mm d^{-1}. Such intense water evaporation can concentrate the contents of a pond to some degree, with the possibility of increasing the salinity and concentration of organic matter to a point at which the osmotic balance of the cells of the aquatic microorganisms is disturbed [16]. According to PCA, the Garada plant seems to be suffering from high organic and persistent compounds load which are represented in BOD and COD. The geometry of ponds and nature of wastewater play essential role in the efficiency of the treatment process, where the anaerobic ponds, which are considered as primary sedimentation tank, are responsible for stabilization of solids and depends on the depth of the pond. Due to the shortage of ponds cleaning practices (sludge removal), retention time and the ability to stabilize solids will be affected.

The presence of large amounts of sludge in the anaerobic pond shorten the optimized depth of ponds and thus decreases the retention time required for the treatment process, solid stabilization, settlement of the most suspended solids, removal of some pathogenic agents, and breakage down of organic materials by anaerobic bacteria, in terms of BOD_5 (5 d BOD). Therefore, the anaerobic pond effluent will not have a standard 40%–60% reduction in concentration from that in the raw influent. Taken together, all previous findings may explain the significant effect of organic factor (BOD and ammonia) and persistent constituents factor (COD and phosphate) on the treatment process and the increase in organic load and nutrients availability that pass into the facultative ponds.

4.3. Biodegradation of organics by Micromonospora consortium

The strains *Micromonospora* sp. 19S and *Micromonospora* sp. 56S showed a strong ability to degrade organic load in wastewater COD by 80% and 70%, respectively, thereby reflecting their high efficiency in organic load removal from wastewater. These results are very close to those obtained from applying actinomycetes in Beni Suef WWTP [37] and in a good agreement with the reported results that the average COD removal efficiency at up-flow anaerobic sludge blanket reactor was 80%–86% [38]. The observed high ability of *Micromonospora* sp. 56S and *Micromonospora* sp. 19S (80% and 70% COD reduction, respectively) could be attributed to their enzymatic abilities as reported by El-Shatoury et al. [39] who found out that both isolates were particularly effective in the production of lipid and carbohydrate degrading enzymes. *Micromonospora* species are particularly important in the recycling of organic carbon and are able to degrade complex polymers. Also, in some contaminated sites, they represent the dominant group among the degraders [40].

Significantly, the investigated actinomycetes consortium in this study showed high reduction in COD, on the second day of experiment in the gravel column, which may be due to the degradation of readily available nutrients (the soluble and easy to degrade ones) by both the actinomycetes consortium and other heterotrophic microorganisms that existed in the wastewater. Gravel as a filter media showed a strong ability to form a well-established biofilm and reduce COD better than PVC. The extent of microbial colonization appears to increase as the surface roughness increases, due to the higher surface area on rougher surfaces [41]. The counts of actinomycetes, which were recorded on the formed biofilms on both filling materials, confirmed that the roughness and multi surfaces of gravel enhanced the actinomycetes colonization and the formation of biofilm, while the PVC which was characterized by its smooth surface and less roughness showed less colonization by actinomycetes. Rough surfaces with large pores are reported to provide an increased surface area for microbial attachment [42]. In addition, maximum attachment depends upon high surface free energy or wettability of surfaces. Surfaces with high surfaces free energies such as silica sand and gravel are more hydrophilic. These surfaces generally show greater bacterial attachment than hydrophobic surfaces with low surface energy such as PVC, Teflon and Buna-N rubber [43].

By introducing new wastewater containing olive mill wastes to the microcosms, each cycle represents an extra load of persistent and toxic organics that may adversely affect the metabolic activities of native heterotrophs in the wastewater. The situation is different for actinomycetes; in the presence of resistant polymers, such as lignocellulose residues and aromatic compounds, actinomycetes can grow slowly, but still better than other microorganisms [44]. This may explain the high reduction in COD that occurred after 6 d of the experiment. Many studies have reported the ability of actinomycetes to adapt to local and seasonal fluctuations of nutrient concentration and to utilize a wide range of complex and resistant polymers [45]. The gravel was a significantly efficient carrier medium compared to PVC.

5. Conclusion

Firstly, the wastewater characterization showed an elevation in COD values which represents the persistent organics in wastewater at all sites of the plant. It may be due to OMW discharging into the WWTP after mixing with domestic wastewater in the combined sewer system. The analyses revealed also an increase in TSS values for influent, maturation ponds and zigzag tanks in addition to the increase of fecal coliform group numbers in influent and anaerobic ponds.

Secondly, a consortium of five *Micromonospora* strains, attached to gravel and PVC in microcosms, accomplished high organic load for 6 d. The results revealed that the formed biofilm on the gravel had the superiority in organic load reduction compared to that on the PVC.

Finally, the combined findings revealed that the actinomycete attached growth approach is a promising technology that could efficiently contribute to the removal of persistent and complex organic pollutants from wastewater.

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