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Changes in microbial populations during co-composting of dewatered sewage sludge with pruning wastes in windrow piles

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Abstract. *Nafez AH, Nikaeen M, Hassanzadeh A, Kadkhodaei S. 2020. Changes in microbial populations during co-composting of dewatered sewage sludge with pruning wastes in windrow piles. Biodiversitas 21: 4655-4662.* The aim of this paper was to study the composting of sewage sludge (SS) and pruning waste as bulking agent with regard to abiotic factors and succession of functional microbial groups. To prepare the composting piles bulking agent and SS in volumetric ratios of 1:1 (TW1), 2:1(TW2), and 3:1(TW3) were used, and a pile of raw sludge was used as control. Samples for investigation were obtained from the composting piles at time intervals of 5-7 d. The temperature of the control pile and TW1 was just 37°C and did not reach the thermophilic phase. The number of thermophilic bacteria increased to 14 log CFU gDW^{-1} in the thermophilic phase. The *salmonella* was lost on the 47th and 19th days of the composting process, for TW2 and TW3, respectively. There was a significant correlation between mesophilic fungi with mesophilic bacteria ($r=0.942$, $P<0.001$). The majority of the microbial populations inspected revealed their maximum numbers towards the end of the thermophilic phase, with decreasing tendencies afterward. The number of evaluated microorganisms was controlled by differences in physicochemical parameters at different composting phases. The results revealed that the scheduling of the thermophilic period, as well as the microbial community and environmental situations, affects the microbial structure andTW2 had the best quality compared to other piles in achieving the best thermophilic phase and elimination of pathogenic microorganisms.

Keywords: Bulking agent, compost, sewage sludge, thermophilic, windrow

INTRODUCTION

Sewage sludge (SS) is a valuable resource as a fertilizer because of its high organic matter (OM) and nutrient content. However, raw SS is inappropriate for direct land application because of unstable OM, pathogens, weed seeds, and the problems related to preservation and transportation (Hao et al. 2019). Composting is an effective process for treatment of SS prior to land application, in which pathogens and weed seeds are destroyed and the heterogeneous OM are converted to more stable substances by the activity of different microbial groups (Villar et al. 2016a). Because of high moisture and low C/N ratio of SS it should be mixed with different bulking agents that provide sufficient porosity and absorb the additional moisture in the sludge composting matrix. If the bulking agent ratio is properly adjusted all organic wastes can be composted in a satisfactory manner and the compost obtained from SS presents a high level of maturity (Wang et al. 2018; Yuan et al. 2016).

There are numerous methods for SS composting. Zazouli and Ala reported that increasing the volumetric ratio of the bulking agent to SS, in an aerated static pile composting process, improved the temperature rising rate and shortened the composting period (Zazouli and Ala 2019). The effectiveness of the composting process is directly associated with the structure of microbial communities in turned windrows and aerated static piles, which can be influenced by temperature, pH, moisture content, and C: N ratio (Nafez et al. 2015a). In fact, composting relies upon the activity of microorganisms because of their key roles in OM degradation. Therefore, characterizing and quantifying the microbial populations could reveal the performance of the composting process, the rate of OM degradation, and the quality of product (Nafez et al. 2015b). Gómez-Silván et al. (2020) by evaluating microbial communities in a full-scale SS composting pile concluded that microbial communities could be used as an alternative for composting efficiency (Gómez-Silván et al. 2020). Wang et al. (2018) studied the factors of microbial community in SS composting with inorganic bulking agent in a cylindrical reactor and showed that microbial succession was interrelated with oxidation-reduction potential (Wang et al. 2018).

Indicator and pathogenic bacteria have also received particular attention in the process of composting. The composting process, if not properly managed, might sustain proliferation and dispersion of potentially pathogenic bacteria. Consequently, microorganisms have only recently been identified as good indicators of the stability and maturity of compost. Microbial succession of composting particularly depends on the temperature of the pile, which represents the various phases of composting (Gómez-Silván et al. 2020; Liu et al. 2018). Time-temperature approach is the most important criteria that have commonly been applied for the assessment of the microbial quality of compost. However, various studies in the literature have shown that despite meeting the appropriate time-

temperature conditions, some pathogens are able to survive in the compost (Thomas et al. 2020; Lu et al. 2020).

Microbiological analysis could provide a suitable tool for biosafety assessment in compost. Furthermore, in order to minimize risks from land application, certain biological criteria must be met in SS-derived compost. Although a large number of studies focused on microbial communities in different composting processes, there are a few studies on SS composting. Moreover, the differences in bacterial and fungal succession and their relationships with physic-chemical aspects have not been comprehensively considered. The aim of the present study was to investigate the changes in microbial population as well as the survival of pathogenic and indicator bacteria during SS composting with different proportions of pruning waste as the bulking agent. The effect of some physicochemical parameters and operational conditions on changes of microbial parameters was also determined.

MATERIALS AND METHODS

Composting process

In order to determine the microbial population during the composting, digested SS (obtained from the SS dewatering room in the South Isfahan Wastewater Treatment Plant (WWTP) (Isfahan, Iran)) was mixed with green plant wastes (a mixture of grass clippings, tree leaves and small twigs from WWTP area) at 3 proportions (1: 1=turned windrow1 (TW1), 1: 2=TW2 and 1: 3=TW3 (v: v)) and then compost ingredients were piled and composting proceeded for 12 weeks by windrow system. A pile containing SS without any addition was also used as a control (TW0). The amount of moisture content, OM content, pH, and C/N ratio of digested SS used in this study was approximately 77.8±3.1%, 52.8±3.9%, 7.6±0.4, and 11.7±0.6, respectively. The approximate dimensions of the piles were as follows: base: 2.5 m; height: 1.25 m; length: 3–4 m and they were trapezoidal in shape. Composting experiments were carried out at the uncovered composting platform and the humidity of the piles was control throughout the trial. Mechanical turning was used for aeration of windrow piles and the piles were turned every 7-10 d with a front-end loader during the composting process. Samples were collected weekly (every 5-7 d) for analyzing physicochemical and microbial parameters. Each sample was a mixture of three subsamples taken from different points along each pile. Samples were then placed in sterile bags and instantly transported to the laboratory for analyzing the physicochemical and microbial factors.

Physicochemical analysis

The temperature was measured at 3 points of the middle of each pile from different directions by a bar thermometer at the time of sampling and the average was reported as the sample temperature. The Moisture content was determined by drying the samples for 24 h at 70±5°C to consistent weight using a laboratory electrical oven (Thompson et al. 2001). The OM content was measured by determining the loss-on ignition at 550°C for 2 h in a muffle furnace and according to the OM content, the percent of organic carbon (C) was determined. Nitrogen content was measured as described by the *US Department of Agriculture and US Composting Council* (2001) (Thompson et al. 2001), and its concentration determined by DR5000 Spectrophotometer (Hach Co., USA). Consequently, C/N ratio was computed based on the concentration of total C and N. Analysis of pH was performed at compost/distilled water ratio of 1:10 (w/v) using portable pH probe (Eutech pH1500, Singapore).

Microbiological analysis

Initial suspensions for microbiological analyses were prepared by the addition of 20 g of compost to 180 ml of sterile saline solution (1:10 w/v). Then samples were diluted with sterile saline solution and a10-fold dilution series was used for analyses. In order to monitor the composting process, total and fecal coliforms, *Clostridium perfringens*, and fecal streptococcus as indicator bacteria and *Salmonella spp*. as pathogenic bacteria were analyzed using the multiple-tube fermentation technique according to the standard methods (Thompson et al. 2001; APHA 2018). Concentration of cultivable mesophilic and thermophilic bacteria, mesophilic and thermophilic fungi, and Actinomycetes were also estimated using the dilution-plating method (APHA 2018; Farhadkhani et al. 2018; Nafez et al. 2015b; Thompson et al. 2001).

Different media were used for the enumeration of microbial groups as follows: lactose broth and brilliant green broth incubated at 37°C for 24-48 h for total coliforms and EC broth incubated at 44°C for 24-48 h for fecal coliforms. Furthermore, dextrose broth and Pfizer Selective Enterococcus (PSE) agar were incubated at 35°C for 24-48 h for fecal streptococcus in the probable and confirmatory tests, respectively. For *Clostridium perfringens* enumeration, liquid Thioglycolate and Tryptose Sulphite Cycloserine (TSC) agar, were incubated at 35°C for 24-48 h in anaerobic conditions (9–13 % carbon dioxide); moreover, Gram staining, motility, and nitrate reduction tests were performed on suspected colonies (Watkins et al. 2015).

Selenite-F, XLD agar, triple sugar iron (TSI) agar, and urea agar incubated at 37°C for 24-48 h were used for *Salmonella* spp. detection. To estimate the number of bacteria, fungi, and Actinomycetes, the relevant dilutions of samples were spread-plated in duplicate on the following media; Sabourad dextrose agar (supplied with 250 mg/L Chloramphenicol) was incubated at 25°C for 3-5 d and 45°C for 5-7 d for mesophilic and thermophilic fungi, respectively; Starch casein agar (supplied with 100 mg/L Nistatin) was incubated at 45°C for 5-7 d for Actinomycetes and Tryptic soy agar (TSA) medium (supplied with 100 mg/L Nistatin) was used for growth of mesophilic and thermophilic bacteria incubated for 24-48 h at 37°C and 45°C, respectively. All microbiological media were obtained from Merck (Darmstadt, Germany).

Data analysis

The microbial counts were changed to $\log_{10}$ CFU/g DW and $\log_{10}$ MPN/g DW for statistical analysis. ANOVA

analysis and Pearson correlation tests were performed with the SPSS program version 20.0 on physicochemical and microbial data achieved in the samples at different composting phases. Normality and homogeneity of the variances were checked using the Levene test, before ANOVA. The level of statistical significance was assumed at $P < 0.05$.

RESULTS AND DISCUSSION

Composting process and abiotic factors

The variation of physicochemical parameters is illustrated in Figure 1. This study revealed that more bulking agent ratios in the compost pile led to an earlier achievement of the thermophilic phase and the longer duration of this phase. Koolivand et al. reported that an appropriate mixing ratio could maintain and support the microbial community to present a high degradation rate of petroleum hydrocarbons (Koolivand et al. 2017). Generally, there were significant differences for almost all factors among compost from different piles, even for those coming from the same mixtures ($p<0.05$). Temperature is one of the most significant factors for assessment of composting processes, because it affects the sanitation capability and microbial activity of the process (Salgado et al. 2019). As a result of high moisture content and anaerobic conditions, the maximum temperature in TW0 and TW1 was just 37°C; consequently, these piles did not reach the thermophilic phase and their data were not shown. Moreover, the thermophilic phase in TW2 was longer than the other piles. The temperature of the piles rapidly increased and thermophilic temperatures of TW2 (60-65°C) and TW3 (55-60°C) piles remained for 14 and 24 d, respectively. These high temperatures in the thermophilic phase, which were preserved for some days to ensure that OM would become more stable and pathogenic microorganisms were inactivated (Bazrafshan et al. 2016).

The highest moisture content was observed in TW0 (78%) followed by TW1 (63%) (Data not shown). According to previous studies in the literature, one reason for the creation of anaerobic conditions in compost piles is high initial moisture content (Ma et al. 2020; Liu et al. 2018). According to (Nafez et al. 2015a), due to the high moisture content of SS, it must be mixed with dry materials that absorb the moisture and provide sufficient porosity for composting of SS. The high moisture content in composting mixtures not only prevents the formation of thermophilic phase, but also creates satisfactory conditions for survival of pathogens due to the exposure to sublethal temperatures (Villar et al. 2016b; Kadkhodaei et al. 2016). On the other hand, the moisture content of initial mixtures in TW2 and TW3 was in a range of 55–65%, which seems appropriate for composting (fig1). The moisture content of the piles decreased from 55.2-63.5% to 20.7-26.3% during composting process because of the high temperature and mechanical aeration.

The pH values in the early stages of the process were 7.7 and 7.5 for TW2 and TW3 piles, respectively. Degradation of OM and accumulation of organic acids can decline pH at the beginning of the process (Zazouli and Ala 2019). Generally, the values of pH for both piles after the 2nd week gradually increased to a rate of 8.5 in the 5th week. The pH values of all products are within the range (6.0–8.5) suggested as suitable for land application. Abtahi et al. also stated that the maximum metabolic activity and bacterial growth occurred in a suitable pH range of 6–8 (Abtahi et al. 2020).

In our study, similar to the previous studies (Zazouli and Ala 2019; Heydari and Miraki 2016), with the degradation of OM, the C/N ratio declined continuously from 28-41 to the lowest level of 8.5-12 at the end of the process. As shown in fig 1 the OM content decreased significantly from 70% and 77.5% to 25% and 30% in TW2 and TW3, respectively. According to Koolivand et al, the addition of organic amendments supports microbial diversity and accelerates the succession of microbial populations (Koolivand et al. 2019). As the OM became more stable, the microbial activity, temperature, and OM degradation rate gradually decreased, revealing the end of the thermophilic phase.

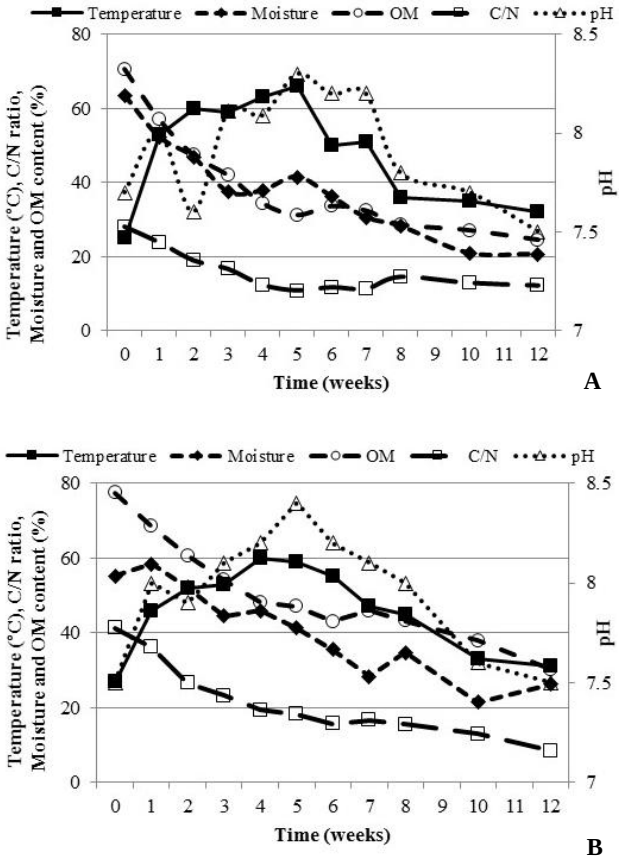

Figure 1. Variation of physicochemical parameters in TW2 (A) and TW3 (B)

Table 1. Evolution of indicator and pathogenic bacteria (n=3)

Time (d)	Pile	Total coliform (log MPN gDW^{-1})	Fecal coliform (log MPN gDW^{-1})	Fecal streptococci (log MPN gDW^{-1})	*Salmonella* (MPN 4gDW^{-1})	*Clostridium perfringens* (MPN gDW^{-1})
1	TW2	8.6^{a}* (4.2)**	7.6^{a} (3.6)	4.8^{a} (1.9)	12.7^{a} (8.8)	405000^{a} (25000)
	TW3	8.6^{a} (4.3)	7.6^{a} (2.6)	4.8^{a} (2.7)	307000^{b} (15000)	10000^{b} (1300)
13	TW2	7.9^{a} (3.5)	7.6^{a} (3.3)	7.1^{b} (3.3)	9.6^{a} (7.0)	ND
	TW3	8.0^{a} (3.6)	7.6^{a} (3.9)	7.9^{b} (3.6)	4.4^{a} (2.7)	1.1^{c} (1.0)
19	TW2	7.4^{ab} (3.9)	7.4^{a} (3.0)	5.3^{a} (2.1)	3.2^{a} (2.1)	0.9^{c} (1.5)
	TW3	6.9^{b} (3.3)	6.9^{ab} (2.5)	4.7^{a} (2.0)	ND	2.6^{c} (3.3)
26	TW2	6.6^{bc} (3.0)	6.0^{b} (2.5)	5.8^{ab} (3.2)	ND	1.9^{c} (1.3)
	TW3	6.5^{bc} (3.4)	6.0^{b} (2.5)	4.9^{a} (2.1)	ND	0.8^{c}(1.1)
41	TW2	5.7^{c} (2.5)	5.6^{bc} (2.1)	6.3^{ab} (3.7)	12.7^{a}(6.5)	ND
	TW3	5.3^{cd} (2.2)	5.3^{c} (2.6)	5.8^{ab} (3.7)	ND	ND
47	TW2	8.3^{a} (3.2)	8.3^{a} (3.3)	4.3^{c} (2.3)	ND	ND
	TW3	6.6^{bc} (2.3)	6.6^{ab} (2.8)	4.1^{c} (2.0)	ND	ND
54	TW2	6.2^{c} (3.1)	4.8^{c} (2.1)	3.5^{c} (2.2)	ND	ND
	TW3	5.5^{c} (2.5)	3.5^{d} (2.1)	3.4^{c} (2.3)	ND	ND
68	TW2	4.4^{d} (2.1)	4.7^{c} (2.4)	2.5^{d} (1.4)	ND	ND
	TW3	4.9^{cd} (2.8)	3.4^{d} (1.9)	2.4^{d} (1.2)	ND	ND
83	TW2	1.6^{e} (1.9)	0.6^{e} (1.0)	2.4^{d} (1.7)	ND	ND
	TW3	ND***	ND	2.0^{d}(1.5)	ND	ND

Note: * Different letters in each column indicate significant differences in the level of 0.05 (according to Duncan's test), **the values in parentheses are standard deviation, ***Not Detected

Microbial parameters

Composting is a biological process and its quality is directly associated to the succession of microbial populations (Ince et al. 2020). The results of microbiological parameters are shown in Table 1. Statistical analysis revealed significant differences in these microbial factors for the duration of the composting process. The composting process led to significant variations in microbial population constitution as demonstrated in Figure 2.

Mesophilic and thermophilic bacteria

Table 1 represents the changes of mesophilic and thermophilic bacteria. The number of mesophilic bacteria was approximately stable during the thermophilic phase but increased to some extent after the completion of this phase. This is similar to the results of (Ince et al. 2020) and (Villar et al. 2016a) who stated a decrease in bacteria during the thermophilic phase, followed by an increase when temperatures began to decrease. In the stabilization phase, the thermophilic and mesophilic bacteria decreased to a constant level, probably due to the sharp drop in moisture and OM content and lack of biodegradable compounds.

The microbial population during the thermophilic phase was controlled by bacteria as revealed by a higher microbial count in Figure 2. In the thermophilic phase, the temperature increased to 55-60 ºC, and accordingly, the number of thermophilic bacteria also increased to their maximum levels and reached 14 log CFU g DW^{-1} (Table1). The number of mesophilic bacteria dropped after the beginning of process but (Tian et al. 2012) reported that all mesophilic microorganisms increased after the beginning of composting and then gradually decreased. Inconsistent results have been reported in the literature regarding the succession of microorganisms. Tian et al. reported that populations of all mesophilic microorganisms increased after the beginning of dairy manure and rice chaff composting and after that gradually decreased and next, when the temperature began to decrease the number of mesophilic microorganisms increased again (Tian et al. 2012). But in our study, the number of mesophilic bacteria dropped after the beginning of the composting process. Generally, during the composting process, the population of mesophilic bacteria was inversely related to the temperature, and had a direct correlation with moisture content. By reducing the moisture content of compost, all of the microbial parameters except mesophilic bacteria are decreased. It seems that the moisture reduction had no significant effect on mesophilic bacteria.

Mesophilic and thermophilic fungi

The number of mesophilic fungi was highest at the beginning of the composting process and dropped during the composting process, while the thermophilic fungi were just found in the late stage of the mesophilic phase and were not detected throughout the study. In contrast, mesophilic fungi displayed high sensitivity to temperature fluctuations and had a decreasing trend during the composting process. The fungal community was significantly higher than that in the previous literature, possibly because of organic bulking agent being used in this study. For example, in the study of Wang et al., by using the inorganic bulking agent, bacterial diversity significantly increased in the mesophilic phase and gradually decreased in the cooling phase, but fungal diversity consecutively decreased as the composting progress (Wang et al. 2018).

Table 2. Correlation between physic-chemical and microbial parameters

Parameter	Pile	Temperature	Moisture content	pH	Total coliform	Fecal coliform	Fecal streptococci	*Salmonella*
Moisture content	Tw2	0.199						
	Tw3	0.175						
pH	Tw2	0.734**	0.664**					
	Tw3	0.846**	0.336					
Total coliform	Tw2	0.291	0.413	0.510*				
	Tw3	-0.552*	0.520*	-0.216				
Fecal coliform	Tw2	-0.188	0.293	0.302	0.517*			
	Tw3	-0.395	0.337	0.075	0.853**			
Fecal streptococci	Tw2	-0.062	0.300	-0.166	0.062	-0.013		
	Tw3	-0.057	0.302	0.033	0.013	-0.048		
salmonella	Tw2	0.328	0.376	0.266	0.636*	-0.097	0.507*	
	Tw3	-.0402	0.331	0.582	0.457	-0.061	-0.092	
Clostridium perfringens	Tw2	-0.440	0.299	-0.170	0.030	-0.088	-0.133	-0.165
	Tw3	-0.578*	0.473	-0.381	0.945**	0.668**	-0.135	0.701**

Note: * and ** Correlation is significant at 0.05 and 0.01 level, respectively

As shown in Figure 2, a significant decrease in mesophilic fungi population was detected during the early thermophilic phase that is consistent with (Villar et al. 2016a; Wang et al. 2018). After the thermophilic phase, mesophilic fungi remained almost constant and finally, it increased due to temperature decline in the cooling phase. Moreover, the greatest impact of temperature on mesophilic fungi was observed in the final stages of composting that illustrates the importance of stabilization in inactivation of the fungal community. Inconsistent results have been stated in relation to the succession of fungal communities. (Jiang et al. 2017) accounted that most of the fungi were eradicated at temperatures above 50°C and recuperated below 45°C, while (Ma et al. 2019) found high numbers of fungi during the thermophilic phase.

Mesophilic fungi with temperatures and pH value were inversely related, and with moisture, ambient temperature and C/N had a direct relationship. Consequently, fungi grow better in the initial stages of composting (high C/N and moisture content, low pH, and temperature). The results underline an inhibitory effect of low moisture on the growth of mesophilic fungi. (Dastpak et al. 2017) reported that the pH value was acidic at the beginning and then the pH increased due to ammonification and consequently, the fungal growth is limited due to the pH rise.

There was a significant correlation between mesophilic fungi and mesophilic bacteria ($r=0.942$, $P<0.001$) which revealed that both of them have the same requirements. In the cooling phase, the thermophilic fungi were not detected and the number of mesophilic fungi slightly increased. In the study of (Ma et al. 2020), the number of all mesophilic microorganisms increased at the beginning of composting process due to easily available carbon and mesophilic conditions. The thermophilic and mesophilic fungi decreased in stabilization phase and reached a constant level due to the sharp drop in moisture and OM.

Actinomycetes

The Actinomycetes began to increase after the mesophilic phase and reached the highest number in active phase and the lowest number of Actinomycetes was also observed in cooling phase. Moreover, compared to total bacteria and fungi the growth of Actinomycetes was slower. According to (Varma et al. 2017), further growth of Actinomycetes in thermophilic phase is due to the making of favorable conditions for their growth. Pearson correlation showed that the temperature is effective on lowering all microbial communities, except Actinomycetes. As reported by (Liu et al. 2018; Ma et al. 2020), there is a direct correlation between moisture and the microbial community. Only Actinomycetes had an inverse correlation with the moisture content, which reveals that these microorganisms prefer the lower moisture content. On the other hand, the pH value has a direct effect on growth of Actinomycetes that could be one of the reasons for the increase of this group in final stages of composting (Figure 2).

Indicator and pathogenic microorganisms

One of the problems posed due to the direct use of SS in agriculture is the risk of plant and human contamination by pathogens. Large amounts of pathogenic microorganisms (e.g. bacteria, viruses, and protozoa) have initiated from the SS (Al-Gheethi et al. 2018). Table 2 indicates the effect of high temperature on the removal of pathogens. The results showed that the thermophilic phase caused a sharp decline in indicator microorganisms and pathogens which is probably due to the unfavorable condition for microorganisms during this phase. Kadkhodaei et al. (2016) reported that high temperatures decrease different microbial groups such as total and fecal coliforms. However, the type of applied waste for composting has a great effect on pathogens' existence. According to the report by Al-Gheethi et al. *Salmonella* spp., *E. coli* O157:H7, and *Shigella* sp. are considered as main health concerns which could spread the disease to the human, while *Enterobacter* spp., *Klebsiella* spp., *C. perfringens*, *S. aureus*, and *Streptococcus* spp. are insignificant concerns which are considered opportunistic

pathogenic microorganisms that cause disease in debilitated persons (Al-Gheethi et al. 2018).

At the beginning of composting, the number of total coliforms, fecal coliforms, and fecal streptococci were about 10^9, 10^8, and 10^5MPN/g DW, respectively. A significant reduction was observed in the number of fecal coliforms in thermophilic phase. The microorganisms decreased to undetectable limits during the thermophilic phase. According to previous studies, the bulking agent affects environmental factors (e.g. porosity, C/N ratio, or moisture content) and it can control the microbial community during the process (Nafez et al. 2015a; Wang et al. 2018). However, after the thermophilic phase, secondary growth of coliforms was observed in the piles.

As indicated in Table 1, in the early stages, the number of fecal streptococci increased to 7 logs MPN/g DW and 7.9 logs MPN/g DW in TW2 and TW3, respectively and after that, these bacteria decreased in both piles. (Zazouli 2019) and (Nafez et al. 2013) reported that the total coliforms have lower values in returned piles in the stability phase. Also in (Ma et al. 2019) study, the number of fecal coliforms and Streptococci in compost piles decreased from log 5 and log 2.49 to log 2.27 and log 2.12, respectively. Since the fecal streptococci are more resistant than fecal coliforms (Nafez et al. 2013), in the final compost fecal streptococci were traceable and can be used as a useful indicator for fecal contamination. Liu et al. (2018) reported that the use of fecal streptococci, as well as fecal coliforms and *C. perfringens*, could serve as an excellent method for detecting the existence of pathogens and identifying their origins related to the treatment and disposal of biosolids (Liu et al. 2018).

Table 2 shows the relationship between microbial parameters and temperature, moisture content, C/N, and pH in composting piles. Statistical analysis showed a significant relationship between total coliforms and pH for TW2. In TW3, total coliforms have a direct relationship with moisture content and have an inverse correlation with temperature. Although the correlation was just observed for total coliforms, concentration of all bacteria was affected by temperature and moisture content. (Bazrafshan et al. 2016; Kadkhodaei et al. 2016) reported that high temperatures in a composting pile reduced different microbial groups but the type of applied waste for composting has a great effect on pathogens.

As seen in Table 1, the fecal coliforms and *salmonella* decreased with increasing the temperature and finally reached an undetectable limit at the end of the process. According to the EPA guidelines, the number of fecal coliforms should be less than 1000 MPN / gDW consequently, both piles comply with the EPA's guidelines (Al-Gheethi et al. 2018; USEPA 2003).

As previously described, *Salmonella* was present at the initial stages, but was not found in the thermophilic phase. By increasing the temperature *Salmonella* decreased to undetectable limits in the final product. Our results revealed that *salmonella* was lost on the 47th and 19th d, for TW2 and TW3, respectively. In the study of (Ince et al. 2020), *Salmonella* completely disappeared in the first week. However, (Nafez et al. 2013) reported that *Salmonella* was not destroyed until the 21st day of composting. According to (Nafez et al. 2013), temperature from 64 to 67°C for 2-3 weeks is sufficient to inactivate *Salmonella* spp which is consistent with (Al-Gheethi et al. 2018) who reported that *Salmonella* spp. are resistant microorganisms that are readily acclimated to hostile environmental conditions. The statistical analysis showed an inverse correlation between pH and *Salmonella*. *Salmonella* grows well at a pH of 6.5–7.5 and doesn't grow at a pH value higher than 9.5.

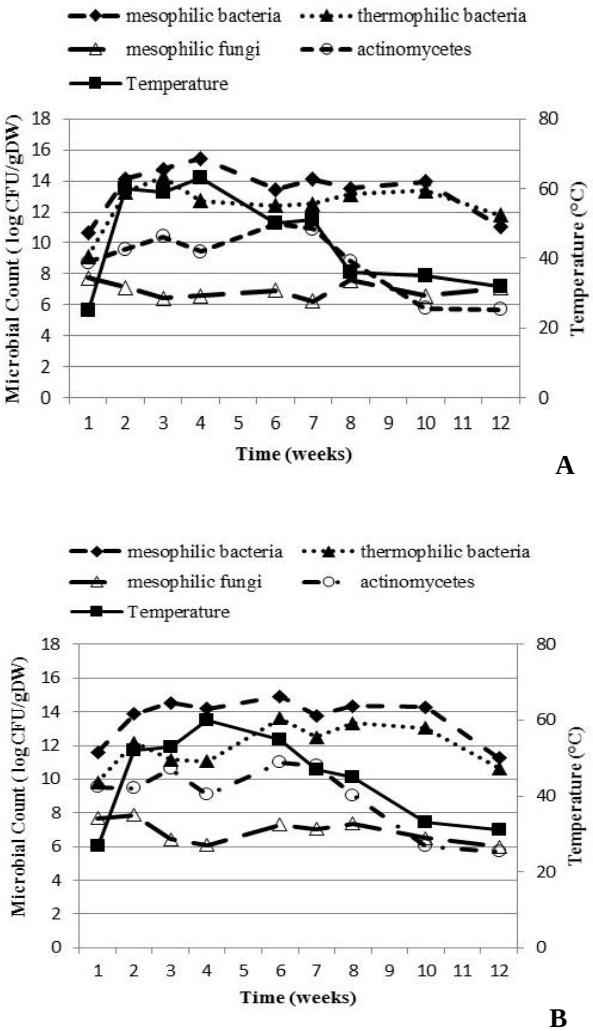

Figure 2. Trend of microbial population and temperature in TW2 (A) and TW3 (B)

This study was also showed that *Clostridium perfringens* reached an undetectable limit in the 6th week (Table 1). The study of (Lasaridi et al. 2018) showed that *Clostridium perfringens* does not grow in the final product of sludge composting. However, the study of (Grantina-Ievina and Rodze 2020) showed that the amount of this group of bacteria was high in the final product of anaerobic composting which may be due to the anaerobic conditions in this method. There was also an inverse correlation between the pH and some microbial parameters such as *Clostridium perfringens* (Table2). Vierheilig et al. (2013) stated that *C. perfringens* was not appropriate as an indicator of fecal pollution however they proposed that it could be used as a tracer for human sewage(Vierheilig et al. 2013).

The results of the physical, chemical, and microbial changes showed that TW2 had the best quality compared to the other piles in achieving the best thermophilic phase and elimination of pathogenic microorganisms. The number of fecal coliforms and *Salmonella* in the final compost of this pile reached to the undetectable limit. The results also indicated that compost product in TW3 pile was appropriate for land application from the microbial point of view. Consequently, the assessment of succession of microbial groups could be a suitable proxy in monitoring the stabilization state and pathogen removal in SS composting process.

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