

# The Effects of Chemical Fertilizers on Composting process of Pistachio Waste

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## Abstract

**Purpose** A study was carried out on composting pistachio hull aided with chemical fertilizer. Recycling of pistachio wastes is one of the major agricultural way in pistachio producing country.

**Method** The collected pistachio hull was allowed to decompose either naturally through composting or with the addition of chemical fertilizers. Biological and chemical properties with organic matter were monthly measured in both composting methods. The data was analyzed in a completely randomized design. Mean values were compared using Duncan's multiple range test in SPSS software ( $p < 0.05$ ).

**Results** Increasing chemical fertilizers reduced the microbial density and diversity during the composting, the concentration of the elements in both composting methods increased over time. The EC in both methods decreased over time from 6.95 to 6.17 dSm<sup>-1</sup> in natural composting and 6.95 to 4.85 dSm<sup>-1</sup> in adding of chemical fertilizers. The C: N ratios of the produced compost was found to be lowered in adding of chemical fertilizers during composting compared with those naturally composting treatment.

**Conclusion** Addition of chemical compounds to the primary wastes could accelerate and improve the decomposition process and lead to faster composting as well as nutritional enrichment and lower C: N ratios, but it reduced microbial density and changed their diversities.

**Keywords** Pistachio waste, Chemical fertilizer, Composting, Macro-nutrients, Microbial activity, Micro-nutrients

## Introduction

Solid waste generation is inevitable throughout the globe, and it rises with the growth in the economy, population, and social development of the country

(Guerrero et al. 2013; Ebrahimi et al. 2018). An enormous amount of solid waste is produced from agricultural activities, including crop residues, weeds, leaf litter, sawdust, forest waste, and livestock waste (Kumar et al. 2012). Reuse and recycling of these organic wastes have numerous environmental, economic, and health benefits (Golchin et al. 2005; Esmaeili et al. 2020). Composting is considered as one of the most effective waste management techniques, specifically concerning to organic waste treatment (Onwosi et al. 2017; Mandpe et al. 2021). Microorganisms release a wide range of enzymes during the

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composting process, which leads to the decomposition of organic matter and product stability (Jurado et al. 2014). Microorganisms convert organic wastes into water, carbon dioxide, ammonium and humus compounds through aerobic pathway (Danon et al. 2008; Bernal et al. 2009). This action of microorganisms is useful for improving physical, chemical and biological properties of soil (Tejada et al. 2006; Qian et al. 2014; Lim et al. 2016).

Pistachio is an important agricultural crop cultivated in Iran. The annual average production of dry pistachio and its residue (dry waste) are 250 and 132-165 thousand tons, respectively. Every 3 kg of fresh pistachio consists of 1 kg dried pistachio and 2 kg wastes; In total, about 660 g of pure dry matter is obtained (Mehrnejad 2001; Hosseinifard et al. 2010). So, a huge volume of pistachio waste is produced in harvesting season every year. These materials are extremely perishable and are attacked by diverse species of microbes after disposal making it black, rotten and moldy. As a result, they cause widespread environmental pollution. Unconditional accumulation of pistachio hull wastes in the nature after harvesting has irreversible effects on the environment and consequently the health of society (Jalili et al. 2019). Burial of this waste in pistachio orchard damages the roots due to unstable compounds that can disrupt plant growth (Malakootian et al. 2014). Higher concentrations of phenolic and organic compounds in addition to solids in pistachio waste leads to major problems in its management (Demirer 2016).

The use of agricultural composts in the form of organic fertilizers is beneficial for agrarian applications (Huang et al. 2006; Gautam et al. 2010). During the

process of composting, the population of microorganisms increases and the amount of organic carbon and carbon to nitrogen (C: N) ratio gradually decreased (Golchin et al. 2005; Mandpe et al. 2020; Esmaeili et al. 2020). The concentrations of phenolic compounds are also reduced over time (Sánchez-Monedero et al. 1999; Hachicha et al. 2008). The objectives of present studies are: I. to investigate the trend of microbial population changes and II. To analyze the chemical properties of pistachio waste during the composting process in the condition of addition and non-addition of chemical fertilizers.

## Materials and methods

### Preparation and sampling

This research was carried out in station No. 2 of Pistachio Research Center located in Rafsanjan city 30.3549 ° N, 56.0027 ° E. Rafsanjan is 1.528 meters above sea level, it has a semi-desert climate, with average annual rainfall of 100 mm. This project started at the same time as the pistachio harvesting. Pistachio wastes were collected from several orchards and then dried. Sampling of primary wastes was performed. Due to the nature of the project, the primary wastes, weighing 600 kg, were divided into two equal parts of 300 kg. They were accumulated in chassis in the form of masses with a length of 2 m, a width of 1 m and height of 50 cm and covered with thick plastic. Three samples were collected, and different chemical properties (Table 1), microbial population and variety were analyzed.

**Table 1** Measured chemical properties of pistachio wastes

| C: N  | pH  | EC                 | Mn   | Cu                  | Zn   | Fe  | OC   | Mg   | Ca   | K    | P    | N    |
|-------|-----|--------------------|------|---------------------|------|-----|------|------|------|------|------|------|
|       |     | dS m <sup>-1</sup> |      | mg kg <sup>-1</sup> |      |     |      |      |      | %    |      |      |
| 20.82 | 4.9 | 6.9                | 16.3 | 12.3                | 11.7 | 448 | 40.6 | 0.32 | 1.04 | 3.19 | 0.16 | 1.95 |

One part of primary waste was composted without any additives (T1), but quantities of different chemical fertilizer (Table 2) were added to the other part and mixed thoroughly (T2). Both T1 and T2 were treated at the same location, humidity and temperature and ventilation condition.

The treatments were watered to 70 % field capacity (FC) and aerated every week. The temperature of the treatments was kept between 50 to 60 °C. Treatments were sampled every month, chemical and microbial properties were analyzed. After six months, the prepared composts were collected.

**Table 2** Additional chemical fertilizers to T2

| Elements                         | N   | P   | Ca                                     | Fe                | Mn                | Zn                | Cu                |
|----------------------------------|---|---|--|-------------------|-------------------|-------------------|-------------------|
| Chemical fertilizer              | (NH <sub>4</sub> ) <sub>2</sub><br>HPO <sub>4</sub> | (NH <sub>4</sub> ) <sub>2</sub><br>HPO <sub>4</sub> | CaSO <sub>4</sub><br>2H <sub>2</sub> O | FeSO <sub>4</sub> | MnSO <sub>4</sub> | ZnSO <sub>4</sub> | CuSO <sub>4</sub> |
| Additional weight percentage (g) | 0.27  | 1.22  | 0.73                                   | 0.37              | 0.013             | 0.014             | 0.0025            |

### Microbial population

In order to study the microbial population and variety in the treatments, a 1:10 diluted compound sample with sterile distilled water was prepared and shaken for 60 min at 250 movements (round per minute). The suspension was diluted to 10<sup>-1</sup> to 10<sup>-6</sup> in two repetitions, and each repetition containing 100 µl was spread on petri dishes containing *Aspergillus flavus* and *Parasiticus* Agar (AFPA) (Gourama and Bullerman 1995), Nutrient Agar (NA), Dichloran-Glycerol 18 % (DG18), and Yeast Malt Agar (YMA) culture media. Petri dishes were stored at 30 °C for 3 to 7 days in a dark place. The number of colonies of different microorganisms, including bacteria, fungi, yeasts, molds, and actinomycetes, were counted in different culture media.

### Chemical analysis

Samples were air-dried and ground. The electrical conductivity (EC) and acidity (pH) of sample extracts were determined with the EC meter (Jenway 4520) and pH meter (Inolab WTW), respectively. The concentration of total nitrogen (N) was measured by the Kjeldahl method (Sullivan and Miller 2001). Then, 1

g of dried samples were turned to ash at 550 °C for 5 h using electric furnace (Shimifan F47). Dry ash of samples was digested by HCl 1N (Kalra 1997), diluted to 100 ml solution and were used to measure the concentration of different elements. Phosphorus (P) concentration was determined by the colorimetry method (Murphy and Riley 1962). Potassium (K) concentration was measured using a spectrophotometer (PG Instruments\_ T60UV-Visible Spectrophotometer\_ UK) (Kalra 1997). Calcium (Ca) and magnesium (Mg) concentrations were measured by the EDTA titration method (Barrows and Simpson 1962). Inductively coupled plasma mass spectrometry (ICP AE-S60-4U) was used to measure the concentration of microelements, including iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn). The organic matter (OM) percentage was determined using the difference in weight of the compost samples and their ash after burning in the furnace.

### Phenol concentration

The content of the phenolic compounds of the samples was determined using Folin-Ciocalteu reagent and based on the method of Singleton and Rossi (1965). 0.5 g of dried waste was weighed carefully and rubbed

well with 3 ml of 85 % methanol to a homogeneous solution. Samples were centrifuged at 10000 rpm for 15 minutes. 300  $\mu$ l of fluid with 1500  $\mu$ l of 10 % Folin-Ciocalteu reagent was combined, and after 5 minutes, 1200  $\mu$ l of 7 % sodium carbonate was added to them. The final solution was placed in a dark place on a shaker for 1.5 hours. The absorption of the samples was read at a wavelength of 760 nm with a spectrophotometer (A & ELAB). Gallic acid solutions were used for calibration curve from standard solutions. The amount of total phenolic compounds based on the milligram equivalent of Gallic acid in grams of dry sample was expressed.

### Statistical analysis

The data were statistically analyzed in a completely randomized design using Excel and SPSS software, version 16, and the means were compared using the Duncan multi-domain test ( $p < 0.05$ ).

## Results and discussion

### Microbial population

Microbial community structure results showed significant differences ( $p < 0.05$ ) among composting times and treatments. Overall, the total counts in enriched compost were significantly lower than non-enriched compost, with the highest and lowest densities in fourth and sixth months, respectively.

The microbial density varied during composting the waste. During the composting process, the microbiota varied slightly, but there was an exception for the starting substrate and mature compost (Ventorino et al. 2016). The results showed that microbial population's density rose from composting the waste, reaching a peak in the fourth month, then gradually decreased. The population of different microorganisms studied in the non-enriched compost was significantly higher than enriched compost at different sampling

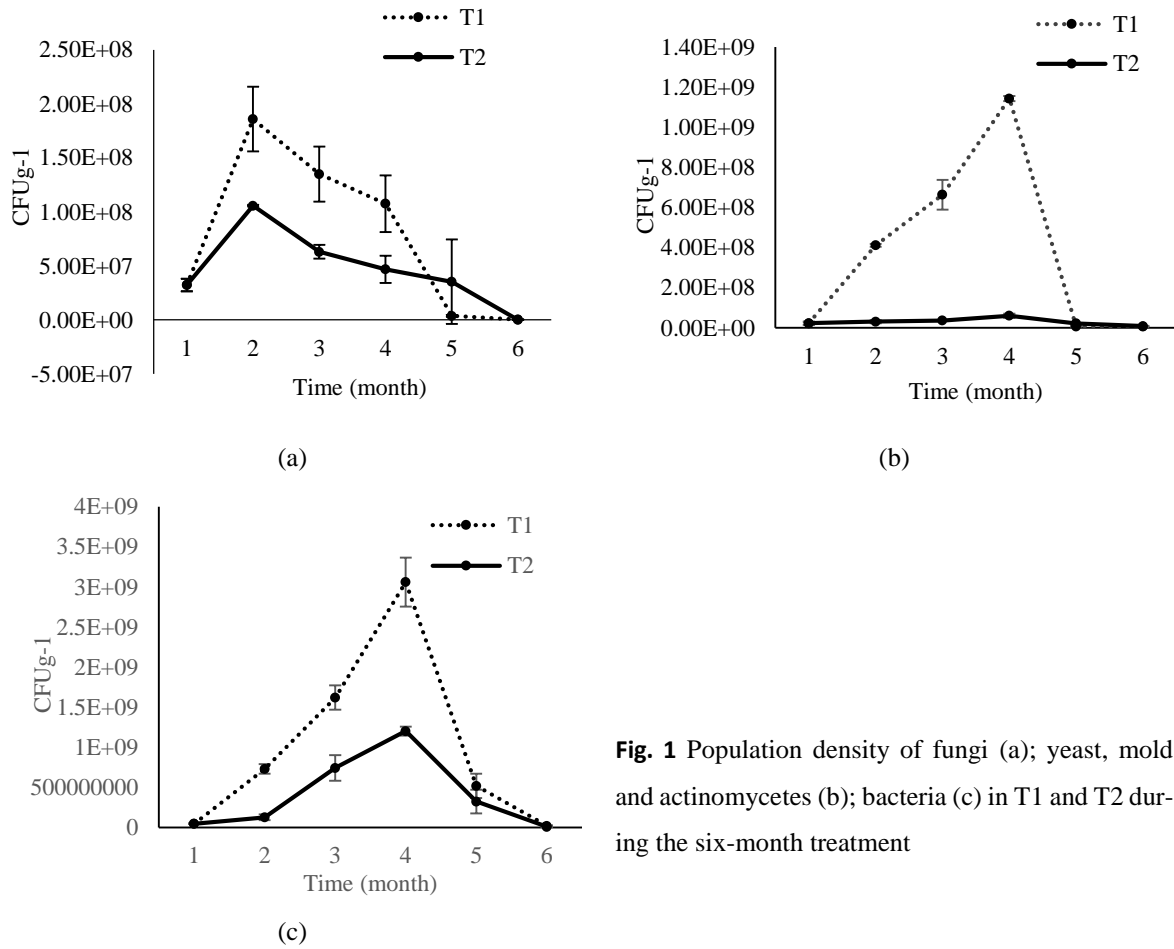
times. In non-enriched and enriched composts, the density of microbial population varied between  $2.97 \times 10^5$  to  $3.28 \times 10^9$  and  $7.2 \times 10^4$  to  $1.24 \times 10^9$  CFUg<sup>-1</sup> of dry composts. The addition of chemical compounds to organic waste in the composting process reduced the microbial activity and population (Wu et al. 2017; Wu et al. 2019). The population density of fungal, mold and bacteria varied in non-enriched compost from  $2.97 \times 10^5$  to  $2.07 \times 10^8$  (Fig. 1),  $5.06 \times 10^6$  to  $1.15 \times 10^9$  (Fig. 1) and  $1.74 \times 10^7$  to  $3.28 \times 10^9$  CFUg<sup>-1</sup> (Fig. 1) of dry compost, respectively.

In enriched compost, the density of fungal, mold and bacteria varied from  $7.2 \times 10^4$  to  $1.06 \times 10^8$ ,  $7.38 \times 10^6$  to  $6.84 \times 10^7$  and  $8.01 \times 10^6$  to  $1.24 \times 10^9$  CFUg<sup>-1</sup> of dry compost, respectively (Fig. 2). In both non-enriched and enriched composts, the highest population density belonged to bacterial species (Fig. 2). Bacteria are recognized as the most important microorganism group in terms of population and activity in the composting process. They tend to dominate the earliest composting stage because they can multiply rapidly on simple and readily available substances (Miller 1996). According to the results, in enriched compost, the population density of fungi, mold and bacteria reduced by 4 to 555%, 12 to 158%, and 1 to 76%, respectively, compared to non-enriched compost.

This indicates the highest negative impact on population densities is related to fungi, followed by molds and bacteria. The highest density of total counted microorganisms in enriched compost and non-enriched compost samples belonged to bacteria, indicating the great variety and reproduction speed of bacteria compared to other microorganisms (Dehvari and Committee 2015). The density reduction of microorganisms in enriched compost may be due to the higher temperature and osmosis pressure, as well as increasing rate of chemical degradations and toxic metabolites during the process. The increase in chemical fertilizers at the end of the first month seems to increase chemical interactions and subsequently increased the

temperature, osmotic pressure, concentration of some nutrients in the compost mass, etc. in T2 and consequently reduced the microbial population, which were consistent with the results obtained by Thilagar et al. (2016). The increase of chemical fertilizers also changed the diversity of the population of microorganisms in the studied population. In T2, the population of actinomycetes, yeasts, and molds was significantly

reduced compared to bacteria fungi. In contrast, in T1, the actinomycetes, yeasts, and molds group was the second population. According to the results, the increase in chemical fertilizers led to a decrease in microbial population and biodiversity changes in the compost mass, which can be generalized to the soil (Luo et al. 2018). This can gradually lead to reduced soil dynamics.



**Fig. 1** Population density of fungi (a); yeast, mold and actinomycetes (b); bacteria (c) in T1 and T2 during the six-month treatment

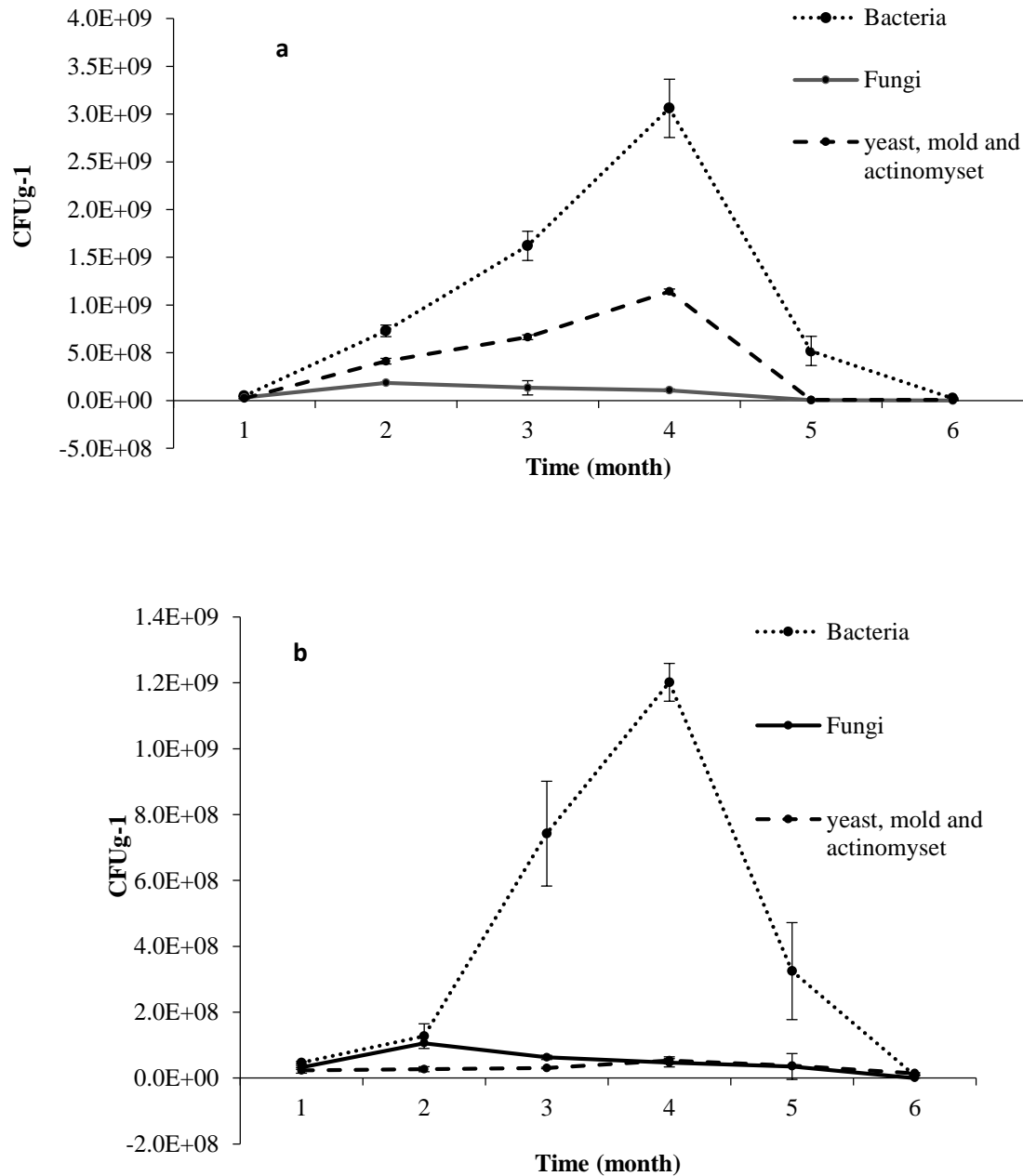
**Chemical analysis of elements**

Variation analysis of the resulted data of this study showed a significant difference ( $p < 0.05$ ) between T1 and T2 in the concentration of nutrition elements. In addition, composting process led to an increase in the amount of nutrition elements in the final composts

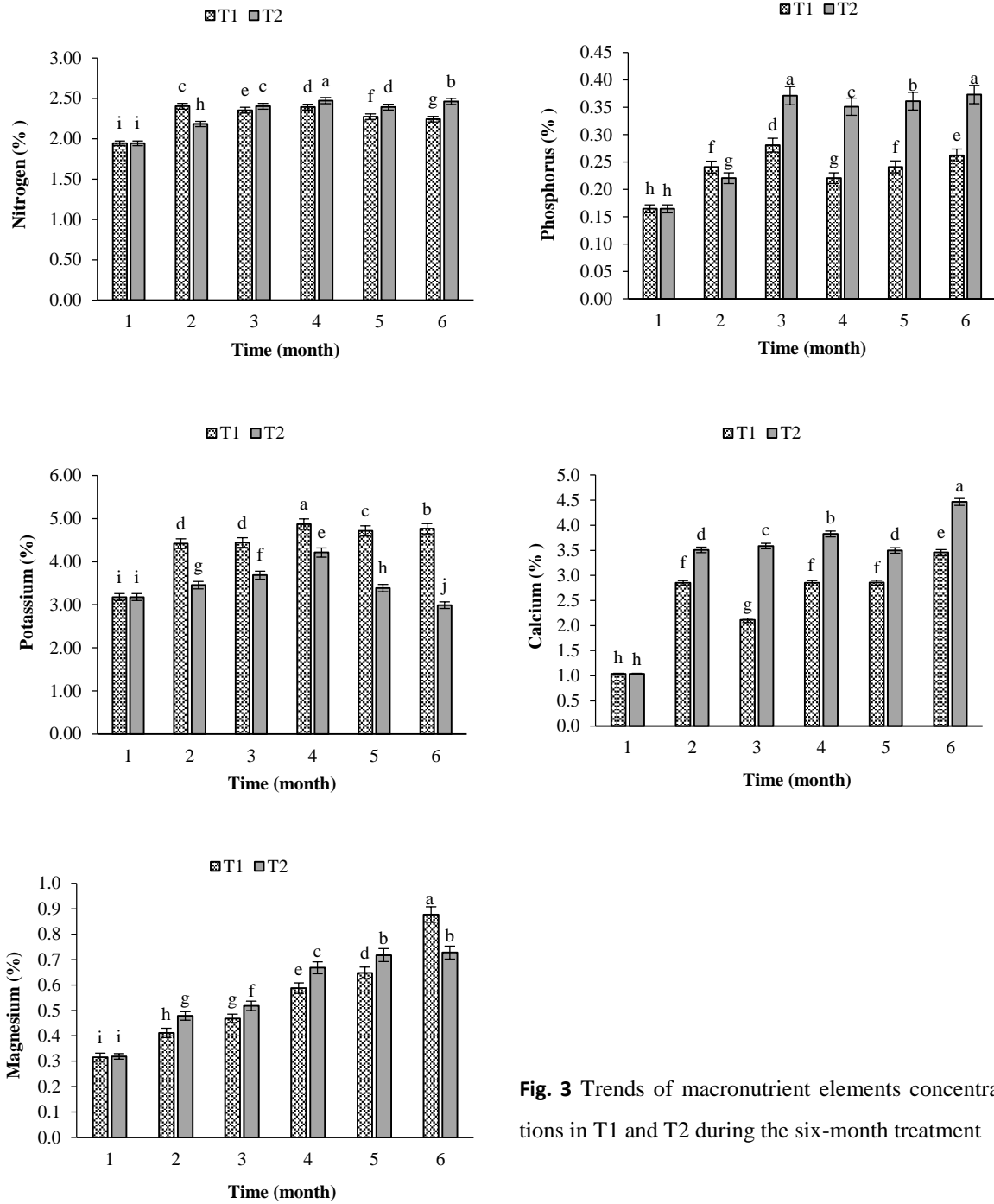
(Fig. 3). The lowest N content was observed in the primary wastes and the highest concentration in the fourth-month sample of T2 treatment (Fig. 3). The difference in nitrogen concentration in the samples of the first to sixth months in T1 was 0.3 % and in T2, 0.52 %. This means that the use of 0.27 % of nitrogen from the chemical fertilizer in the primary waste of T2 led

to a 0.25 % increase in the final nitrogen concentration in T2 compared to T1 (Fig. 3). In a study on composting of olive mill wastewater and agricultural wastes (Hachicha et al. 2008), the rate of nitrates increased by 0.16-0.42 % in final products.

According to the findings reported by various researchers (Wu et al. 2017; Li et al. 2018; Mandpe et al. 2019), addition of chemical fertilizers to the waste improved the quality, reduced ammonia emissions and increased total nitrogen content and mineral nitrogen in the final compost.



**Fig. 2** Microbial population diversity of T1 (a) and T2 (b) during the six-month treatment



**Fig. 3** Trends of macronutrient elements concentrations in T1 and T2 during the six-month treatment

The increase in total N was probably due to a concentration effect caused by weight loss of primary organic matter (Preethu et al. 2007; Santos et al. 2018). In addition, different N compounds changed the forms via mineralization and immobilization during the composting period, leading to slight emission of ammonia and subsequently variable N concentration (Santos et al. 2018).

The amount of P in the final composts was significantly ( $p < 0.05$ ) higher than the primary wastes. The addition of phosphate fertilizer to T2 at the end of the first month caused a significant increase in P concentration in the third month (Fig. 3). Also, the significant difference ( $p < 0.05$ ) in this factor between T2 and T1 continued until the end of the study.

The P concentration in the final compost of T2 was significantly higher than T1 (Fig. 3). The difference in P concentration between the samples of the first to sixth months were 0.09% in T1 and in T2, 0.2%. P concentration difference was 0.11 % between T1 and T2, which was due to the increasing 1.22 % P from diammonium phosphate fertilizer to primary wastes of T2 at the end of the first month.

The results showed that the amount of K during the composting process and in the final product in T1 was significantly higher than in T2 (Fig. 3). The increase rate in K concentration in the final product of T1 was 1.59 %, and it was -0.19 in T2. The release of the K element during the composting process up to the fourth month in T1 is seen in Fig. 3. From the fourth month, with a sharp decrease in the microbial population, the concentration of K was decreased, which could be due to a decrease in the activity of the microbial population and the conversion of this element into secondary organic compounds. The trend of changes in the K during the composting process is similar in T1 and T2 (Fig. 3), but the concentration of K in T2 was lower than in T1. The reason is the addition of other chemical fertilizers (Table 2), which did not include K-containing to primary wastes of T2, followed by an increase in the ash weight of this treatment and a decrease in K concentration compared to other elements in a given weight of dry matter, in other words, the dilution effect (Jarrell and Beverly 1981).

The amount of Ca in the final product and during the composting process in T2 was significantly ( $p < 0.05$ ) higher than in T1. The lowest and highest Ca concentrations were observed in the primary wastes and the final product of T2, respectively (Fig. 3). The amount of Ca increased 2.42 % and 3.43 % in the final products of T1 and T2, respectively. By adding 0.73% of agricultural gypsum to primary wastes of T2, the concentration of Ca in the final product of this treatment was 1% higher than T1 (Fig. 3). A significant difference ( $p < 0.05$ ) was observed between T1 and T2 in

Mg concentration. The highest Mg content in the final product of T1 was 0.88 %, while in the final product of T2, it was lower with 0.73 % (Fig. 3). The reason is probably the lack of use of Mg-containing chemical fertilizer similar to K and the dilution effect in T2 (Jarrell and Beverly 1981). There was a regular monthly increase in Mg concentration in both T1 and T2 (Fig. 3).

There was a significant difference ( $p < 0.05$ ) between T1 and T2 in Fe concentration. The lowest concentration of Fe in the raw materials was 445 mg kg<sup>-1</sup> and the highest was observed in the final product of T2, which was 3318 mg kg<sup>-1</sup> (Fig. 4). The increase in Fe concentration was 1826 mg kg<sup>-1</sup> in T1 and 2873 mg kg<sup>-1</sup> in T2 products. In other words, the addition of 0.37% of Fe from the source of Fe-containing chemical fertilizer led to an increase in the concentration of Fe by 0.15% in the final product of T2 compared to T1 (Fig. 4). The lowest Zn concentration was observed in primary wastes by 11.7 mg kg<sup>-1</sup> and the highest in the final product of T2, which was 118.6 mg kg<sup>-1</sup> (Fig. 4). Adding 0.044% Zn-containing chemical fertilizer to the primary wastes of T2 increased 91.4 mg kg<sup>-1</sup> or 0.009% Zn in the final product of T2 compared to T1. The lowest Zn concentration was observed in primary wastes by 11.7 mg kg<sup>-1</sup> and the highest in the final product of T2, which was 118.6 mg kg<sup>-1</sup> (Fig. 4). Adding 0.044 % Zn-containing chemical fertilizer to the primary wastes of T2 increased 91.4 mg kg<sup>-1</sup> or 0.009 % Zn in the final product of T2 compared to T1. The concentration of Mn, like other elements, was significantly different ( $p < 0.05$ ) between T1 and T2. The lowest Mn concentration was observed in the primary wastes, which was 16.19 mg kg<sup>-1</sup> and the highest in the final product of T2 by 183.77 mg kg<sup>-1</sup> (Fig. 4). Adding 0.013% Mn to the primary wastes of T2 increased the amount of this element in all samples of T2 during the composting process. As a result, the concentration of Mn in the final product of T2 was 0.011% higher than the final product of T1.



There was a significant difference ( $p < 0.05$ ) between T1 and T2 in the EC factor. It decreased in both final products of T1 and T2 (Fig. 5). The lowest EC (4.85 dSm<sup>-1</sup>) was observed in the final product of T2 (Fig. 5) and the highest in the samples related to the third month of composting in T1 and T2 by 0.96 and 0.88 dSm<sup>-1</sup>, respectively (Fig. 5).

Probably different elements from the organic compounds were released into soluble mineral form in the third month. Then, in the following months, by converting these substances into secondary metabolites in the body of microorganisms or conversion to secondary organic compounds, elements clattering followed by a decrease in solubility (Joffre et al. 2001).

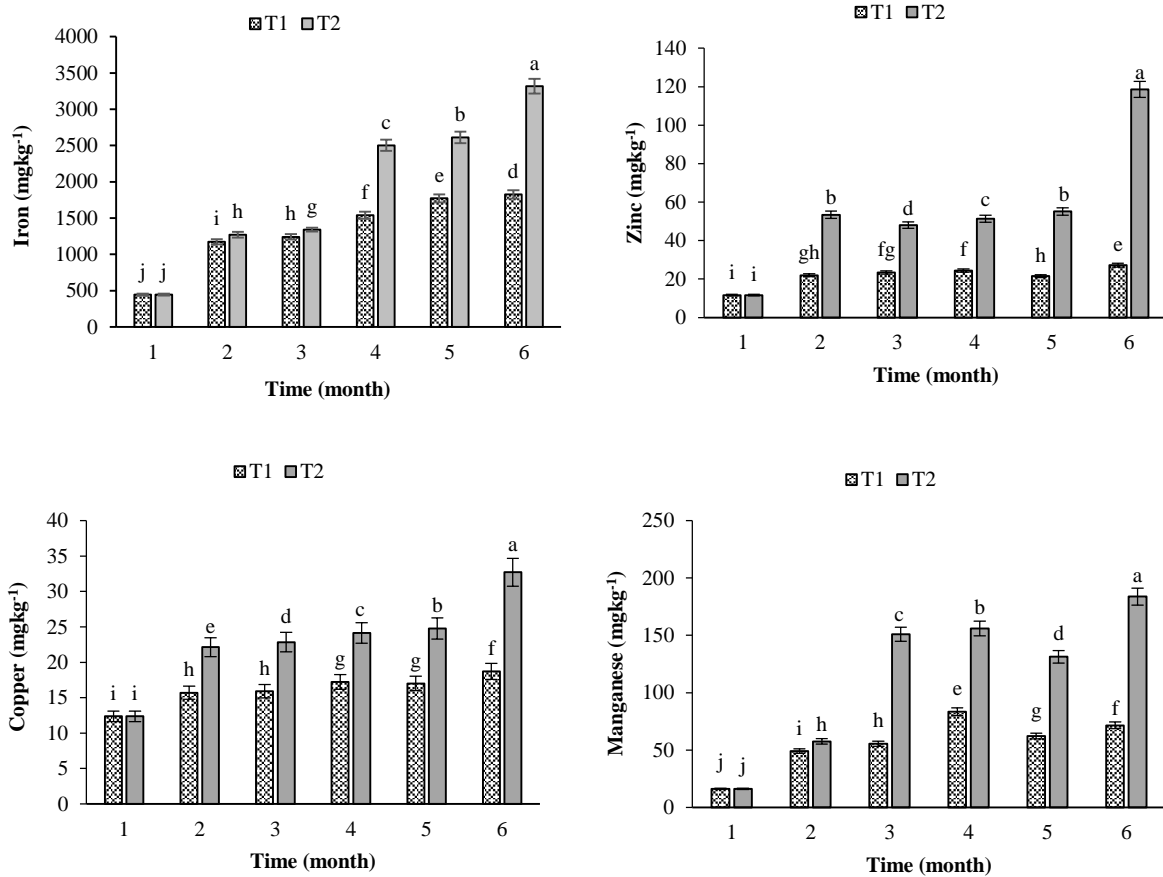


Fig. 4 Trends of micronutrient elements concentrations in T1 and T2 during the six-month treatment

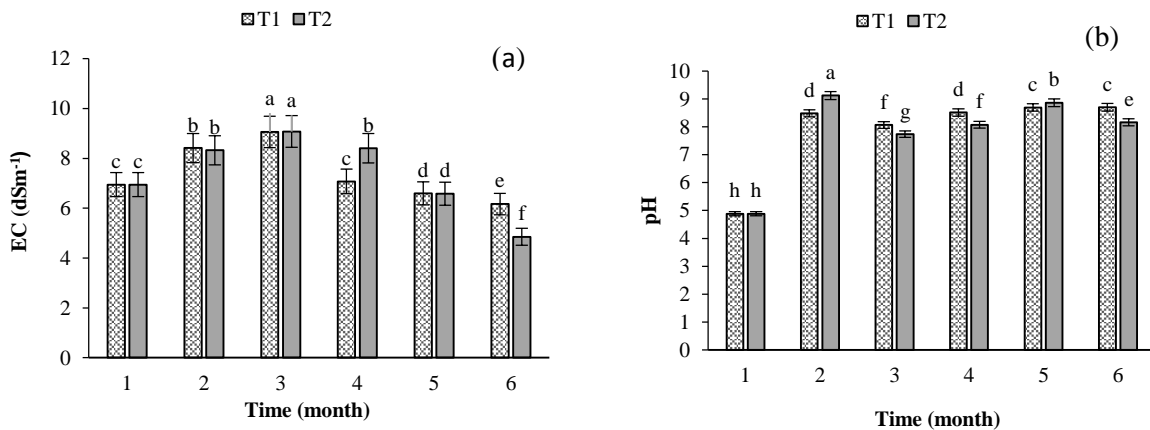
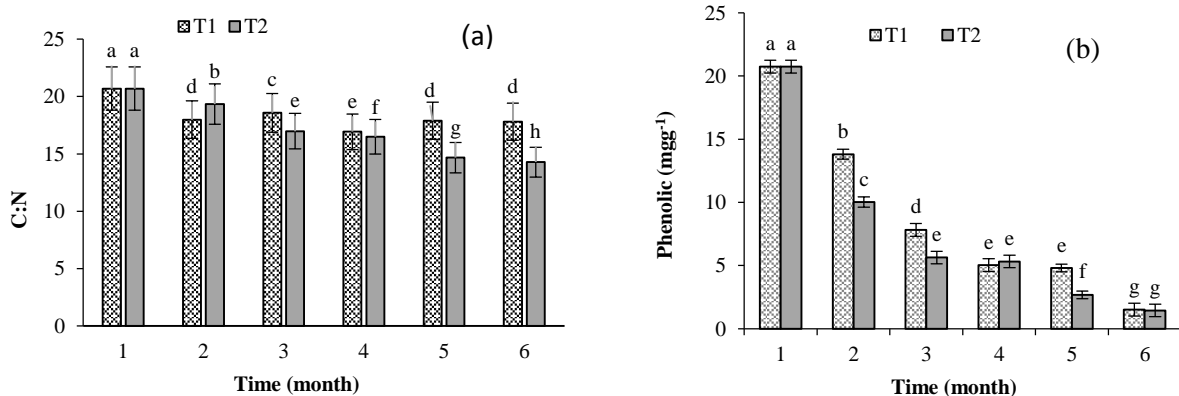


Fig. 5 Trend of EC (a) and pH (b) in T1 and T2 during the six-month treatment

There was a significant difference ( $p < 0.05$ ) between T1 and T2 in pH. The lowest pH belonged to the primary wastes (Fig. 5). Addition of chemical fertilizers to primary wastes of T2 at the end of the first month of composting increased the pH in this treatment. The highest pH of 9.12 was observed at the second-month sample of T2 (Fig. 5). During the composting process, there was no trend in decreasing or increasing pH in the samples, but it was lower in the final product of T2, 8.16 than T1, 8.70 (Fig. 5). Changes of pH during the composting process were due to the decomposition of complex organic compounds into a series of simpler compounds that temporarily changing the acidity of the compounds. However, buffering nature of humic substances led to pH stabilization. The acidity of the waste can control the speed of the composting process and the release of elements in the compost by controlling the microbial populations and their level of activity. The findings of the present study confirmed the results reported by other researchers (Preethu et al. 2007; Hachicha et al. 2008).

One of the most important indicators of composting is the C: N ratio (Sefidkar et al. 2014). The degree of maturation and stabilization of organic matter is a factor in compost quality for agronomical use. C: N ratio

is one of the traditional parameters of humification substances used to assess compost maturity and stability (Jurado et al. 2014). The C: N ratio in T1 and T2 decreased during the composting process (Fig. 6). The highest and lowest rates of this factor were observed in the primary wastes by 20.68 and the final product of T2 by 14.28 (Fig. 6). In T2, the reduction trend was based on regular composting months, but in T1, no complete order was observed in this ratio trend during the sampling months (Fig. 6). The difference in C: N ratio between T1 and T2 was 3.52 (Fig. 6). This research has shown that adding chemical fertilizers despite the declining microbial population leads to a faster reduction in C: N resulting in faster decomposition of raw materials. During the composting process, chemical bonds between organic matters break down by microorganisms and primary matter is converted into compost, water, carbon dioxide, heat or energy (Ziaee et al. 2012). So, the final product of composting showed a reducing trend in organic carbon content. The reduction in the total weight of the initial wastes during composting yield to increasing the N content. This change in organic carbon and nitrogen is reflected in the C: N ratio (Nicolardot et al. 2001; Preethu et al. 2007).



**Fig. 6** Trend of C: N ratio (a) and phenol concentration (b) in T1 and T2 during the six-month treatment

Sagdeeva et al. (2018) investigated the effect of mineral complexes on the rate of organic matter composting process in two thermal, thermophilic and mesophilic diets. They found that the use of mineral compounds accelerated the decomposition process of organic substances in both conditions. The researchers looked at the effects of adding various organic, inorganic and biological compounds to the process of compost and vermicompost production (Barthod et al. 2018). Their results showed that the addition of different compounds to the primary wastes affected acidity, temperature and overall decomposition. They found that adding organic, inorganic and biologic compounds improved the quality of the final product, reduced nitrogen leaching, and improved plant access to the nutrients in the final product (Barthod et al. 2018). The amount of nitrogen added to T2 was 0.3 %, which did not affect C: N changes. Therefore, it seems that adding chemical compounds to the primary wastes not only increased the rate of decomposition and composting but also led to an increase in the concentration of nutrients in the final product. Adding calcium bentonite to an organic fertilizer at the beginning of the decomposition process led to an increase in soluble carbon dioxide, total nitrogen, phosphorus, nitrate-nitrogen, decreased organic matter, conversion of ammonium nitrogen and significant immobilization of copper and zinc (Wang et al. 2016). The positive results of mineral use to increasing the humic acid concentration by 10.87 % to 17.3 % in the final compost compared to the control in the composting process of organic matter have been confirmed by some researchers (Yu et al. 2019). The concentration of all elements increased during the composting process in both treatments; this is the result of microorganism's activity which released the structural elements in the organic compounds of primary wastes and a reduction in the total weight of the primary wastes during composting yielded an increase in the content of nutrition elements in the final products (Preethu et al. 2007; Liu et al.

2014). In T2, the concentration of added elements as chemical fertilizer increased significantly compared to T1.

### Phenol concentration

It was observed that total phenol content in the treatments decreased over time. The phenol content in the primary wastes was the highest at 20.75 mgg<sup>-1</sup> in dry matter and the lowest in the final products of T1 and T2 by 1.52 mg g<sup>-1</sup> and 1.45 mgg<sup>-1</sup>, respectively (Fig. 6). The reduction in total phenol in T2 was higher than in T1, but its rate did not differ significantly in the final products (Fig. 6). Thus, reduction of phenolic compounds during the composting process happened (Garcia et al. 1992; Baddi et al. 2004). These compounds were degraded, released, and involved in biosynthetic pathways and led to humic substances formation.

### Conclusion

The present study showed that the concentrations of nutrients were increased in the final product of pistachio hull composting; however, the concentration of phenol, C: N ratio and EC were found to be reduced. The microbial population grew rapidly until the fourth month of composting and then declined rapidly until the end of the composting process. The addition of chemical compounds to the primary wastes accelerated the decomposition process and led to a faster composting as well as nutritional enrichment, but it reduced microbial population and changed their diversity.

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## Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with this study.

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