CHILEAN JOURNAL
of AGRICULTURAL RESEARCH

RESEARCH ARTICLE

Application of biosolid for berseem clover fertilization: Fodder characteristics and health risk assessment

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Received: 6 December 2023; Accepted: 29 February 2024, doi:10.4067/S0718-58392024000300349

ABSTRACT

Finding sustainable methods for utilizing biosolids, also known as municipal sewage sludge (SS), presents a pressing challenge in modern waste management practices. Therefore, this study aimed to evaluate the impact of SS amendment on growth, biochemical, proximate, and heavy metal bioaccumulation parameters of berseem clover (*Trifolium alexandrinum* L.) fodder crop under field conditions. *Trifolium alexandrinum* was cultivated using different rates of SS mixing (i.e., T0: 0% as control with no SS application, T1: 5%, and T2: 10%). The results obtained showed a significant ($p < 0.05$) increment in growth, biochemical, and proximate parameters of *T. alexandrinum* with an increasing SS mixing rate. The highest productivity of *T. alexandrinum* fodder (1.92 kg m^{-2} fw) was observed in the T2 treatment as compared to the control treatment. The heavy metal analysis of shoot and root parts of *T. alexandrinum* showed that the contents (mg kg^{-1}) of eight elements (Cd 0.02-0.13, Co 0.04-0.08, Cu 5.94-0.05, Cr 0.43-1.68, Fe 7.08-15.93, Ni 0.89-2.90, Mn 1.62-5.38, and Zn 3.30-7.04) increased significantly ($p < 0.05$) with SS mixing rate. The bioaccumulation factor (BAF) was below 1 except for Cu and Zn exhibiting their rapid uptake by plants from SS-treated soils. However, dietary intake modeling (DIM < 1) and health risk index (HRI < 1) studies showed that the levels of heavy metals did not exceed the permissible limits in any SS treatment. Overall, SS amendment has a positive impact on the growth, biochemical, proximate, and heavy metal characteristics of *T. alexandrinum*. Therefore, this study suggested a strategy for low-cost soil fertilization and fodder crop production which could sustainably benefit waste recycling.

Key words: Bioaccumulation, health risk studies, sewage sludge, soil fertilization, waste management.

INTRODUCTION

Sewage sludge (SS) is a semi-solid material resulting from the treatment of wastewater originated from industrial, domestic, and municipal activities. Since it accumulates numerous sources of pollutants, including bacteria, pathogens, heavy metals, dyes, and many more, it poses precarious effects on humans, plants, and animals. Sewage sludge encompasses high N content that can be nitrified and in turn, results in eutrophication if transported to surface and underground waters (Rorat et al., 2019). Sewage sludge may additionally sorb organic chemical residues, pesticides, and pharmaceuticals which may affect its leaching potential and leaching period (Styszko et al., 2022). Several methods have been adopted, like incineration, to avoid direct disposal of SS into the environment. Although previously demonstrated as an environmentally friendly trail, several countries around the globe have banned the incineration of SS due to its air pollution potential (Liang et al., 2021). Other mitigation methodologies, such as pyrolysis and anaerobic digestion, were found to be less reliable mitigation processes as they result in respective low resource recovery and high freshwater ecotoxicity (Tarpani et al., 2020). It was also noted that the thermal processing of SS would only bring benefits at high resource recovery rates.

Although it encompasses considerable heavy metal concentrations, a potential phytoremediation program can lead to minimized side effects of SS on the environment (Rostami et al., 2020; Marin and Rusanescu, 2023). Generally, soil amendment with SS can improve the soil structure, raise C sequestration, and increase essential mineral contents, i.e., N, P, and K (Zhao et al., 2023). Recent studies mentioned the role of SS in the improvement of soil microbial activity (Dhanker et al., 2021). Such activity results in improved plant growth and development, and increased remediation of environmental pollutants. Others reported a relatively long-lasting positive effect of SS on soil microbial functionality (Oszust et al., 2015). For instance, a case study on tropical soil in Brazil showed the role of SS in the suppressiveness of soil-borne plant pathogens. Sewage sludge amendment improves the oligotrophic bacterial activity in soil; this makes nutrient uptake and storage more efficient. Also, a gradual increase of humus fractions, reduction in soil acidity, and increase in soil buffering capacity are results of SS amendment. Several studies reported the role of SS in the improvement of mushrooms (*Agaricus bisporus*), horticultural crops (wheat, soybean), rice (Shan et al., 2021), and ornamental crops (marigold) (Kumat et al., 2022). All the studies concluded that SS improved the physicochemical properties of harvested crops while surpassing any environmental flaws in the mid- and long-term.

Trifolium alexandrinum L. (Fabaceae), commonly known as berseem or Egyptian clover, is an annual winter crop grown generally in subtropical regions and used as a leguminous crop (SAREP, 2023). Although originated in ancient Egypt, it has been implemented in India since the early nineteenth century. A yield of up to 22 t ha^{-1} can be obtained under suitable conditions of 550-750 mm rainfall, non-acidic and medium to heavy soils, 25-30 °C germination temperature, and 35-37 °C flowering temperature. This winter crop is a rich source of crude protein, mainly suitable and considered animal fodder. With the increase in water deficit around the globe, wastewater has become an alternative source of irrigation for cultivated crops. In the last few years, several studies investigated the impact of SS amendment on the production of protein-rich forage crops. It also results in a significant increase in yield by 1.2-2.7-fold compared to control (no SS amendment). Moreover, Cu and Zn uptake and bioaccumulation in the above parts of the crop were significantly improved.

Although it succeeded in growing in saline and drought conditions, the production of *T. alexandrinum* in soils amended with SS is still scantly investigated. Moreover, its heavy metal bioaccumulation potential under the SS amendment shows a significant knowledge gap in the literature. Therefore, this study aims to investigate the growth, yield, physicochemical properties, and heavy metal bioaccumulation in shoots and roots of *T. alexandrinum* fodder crops cultivated on soils amended with SS. Moreover, subsequent health risk studies were conducted to ensure the safe utilization of produced fodder.

MATERIALS AND METHODS

Material collection and experimental site

In the present study, certified and healthy seeds of *Trifolium alexandrinum* (L.) 'Mescavi (Desi)' was procured from Ganga Seeds Pvt. Ltd., New Delhi, India. This crop is commonly known as berseem in the Northern parts of India and is considered as king of fodder. 'Mescavi (Desi)' is resistant to various pathogens and gives high yields in soils of Northern Indian states. The sewage sludge (SS) was collected from the drying bed area of a municipal sewage treatment plant located in Saliyar (29°54'05.8" N, 77°51'53.8" E), Roorkee, India. Also, pre-digested and dried SS samples were collected in 20 kg capacity polyethylene bags. The experiments were conducted in an open agricultural field located in Kulheri village of Saharanpur district (29°52'52.8" N, 77°16'17.8" E), Uttar Pradesh, India. The chosen field had no previous history of SS application. The soil class of the experimental site was clay loam (Mollisols). The average temperature and humidity of the site at the time of the beginning of experiments were 25 °C and 72%, respectively.

Experimental design and field operation

Before the beginning of experiments, the field was ploughed using a cultivator up to a depth of 5-6 cm. After that, a total of nine beds (three for each treatment) of a 3 × 5 m (width × length; the total area of bed 15 m^2) dimension were created and physically separated. Appropriate treatments of SS, i.e., control (soil with no SS mixing), T1 (5% SS), and T2 (10% SS) were given by mixing appropriate dose (relative to soil area and volume). The beds were left for 3 d for sun drying and then filled with water to a depth of 5 cm. Then, beds were left water-filled overnight, and muddy soil was created by ploughing them again using a cultivator and levelled. On the other hand, fresh seeds of *T. alexandrinum* were pre-treated by overnight soaking in a 10% NaCl solution to ensure chicory (*Cichorium intybus* L.) weed suppression. Afterward, the seeds were directly broadcasted (20 kg ha^{-1} or 30 g in each bed) evenly in all directions of the prepared beds on 15 October 2021. During the germination period, the first two irrigations were done within 10 d of showing while subsequent irrigation was done each 10 d or after each cutting. Plants were cut 3-5 cm above ground level in the early bloom stage. The first cutting was done after 40-45 d (after showing) and a total of five cuttings were done at an interval of 30 d. The experiments were terminated on 15 May 2022 and data was recorded for each cutting and treatment.

Chemical analyses

In this study, the arable soil and SS were analysed for selected physicochemical and heavy metal parameters such as pH, electrical conductivity (EC), organic matter (OM), N, P, K, Cd, Co, Cu, Cr, Fe, Ni, Mn, and Zn. For this purpose, certified reagent materials (CRM) and standard analytical methodologies were adopted as prescribed by AOAC (Latimer, 2019) and Jones (Jones, 2018). Herein, pH and EC were determined by using a microprocessor-based multi-meter (ESICO 1611, Parwanoo, India). The OM was determined by the Walkley and Black method while N was estimated using Kjeldahl's method. Similarly, P content was determined by using a double beam spectrophotometer (60 Cary, Agilent Technologies, Santa Clara, California, USA) while K was estimated using a flame photometer (ESICO 1611, ESICO International, Parwanoo, India). On the other hand, heavy metals in soil, SS, and plant samples were determined by using atomic absorption spectroscopy (AAS, Analyst 800, Agilent Technologies). In this, samples were separately oven-dried at 60 °C until a constant weight was obtained and then digested in a mixture of di-acid ($HClO_4$:HNO_3; 1:3) for 2 h at 180 °C. After digestion, the contents were adjusted to 50 mL by the addition of 3% HNO_3 solution and filtered through a filter paper (Whatman number 41). Finally, the contents of eight heavy metals (Cd, Co, Cu, Cr, Fe, Ni, Mn, and Ni) were analysed by preparing calibration curves against standard solutions.

Growth response and biochemical analyses

The *T. alexandrinum* cultivated in different SS treatments were analysed for selected growth and biochemical parameters such as the number of days taken to emergence, shoot height (cm), shoot weight (g), root length (cm), root weight (g), fodder yield (kg m^{-2}), total chlorophyll content (mg g^{-1}), carotenoids (mg g^{-1}), total phenols (mg g^{-1}), ascorbic acid (mg g^{-1}), crude protein (%), crude fibre (%), ether extract (%), and ash contents (%). In this, total chlorophyll content was estimated by taking 1 g fresh leaf sample and preparing an extract using 80% $(CH_3)_2CO$ solution followed by taking absorbance using a double beam UV-vis spectrophotometer (Cary 60, Agilent Technologies). Similarly, the content of carotenoids was also determined using the same extraction reagent and taking absorbance at 450 nm. The contents of total phenol were determined by following the Folin-Ciocalteu assay method and taking absorbance at 750 nm (dos Reis et al., 2015). Moreover, the contents of ascorbic acid (vitamin C) in *T. alexandrinum* plant samples were estimated using the colorimetric method by oxidizing the ascorbic acid into dehydroascorbic acid. The colour intensity (developed by the addition of 2,4-dinitrophenylhydrazine) was measured against a blank using a digital photo colorimeter (Alpha 03, ESICO International). On the other hand, the selected proximate parameters i.e., crude protein, crude fibre, and ether extract were estimated. Finally, the ash content in the *T. alexandrinum* plant was determined by incinerating samples in a muffle furnace at 550 °C for 1 h.

Data analyses and statistics

In the present experiment, the heavy metal bioaccumulation potential of *T. alexandrinum* plant tissue was assessed using the bioaccumulation factor (BAF) index; BAF determines the capability of a plant to accumulate heavy metals from its surrounding environments (water or soil medium). It helps in identifying hyperaccumulator plants that could be useful in the decontamination of polluted lands or indicate whether plants can accumulate potentially toxic heavy metals that could affect consumers' health. In this, Equations 1 and 2 were used for the calculation of BAF of the shoot and root parts of the *T. alexandrinum* plant grown on SS-amended soils:

$$BAF_{Shoot} = HM_{Shoot}/HM_{Soil} \quad (1)$$

$$BAF_{Root} = HM_{Root}/HM_{Soil} \quad (2)$$

where, BAF refers to bioaccumulation potential (0: none, 1: high, >1: very high) while HM refers to heavy metal concentration (mg kg^{-1} dwt) in *T. alexandrinum* plant tissues and experimental soil. Moreover, the impact of SS mixing on soil properties and *T. alexandrinum* growth, yield, biochemical, proximate, and heavy metals attributes were studied using Pearson correlation analysis. On the other hand, the risk of heavy metals accumulation in *T. alexandrinum* fodder was assessed using dietary intake modelling (DIM) and health risk index (HRI) tools. The DIM and HRI are commonly used to assess the potential health risks associated with the consumption of certain foods or diets (Ghazzal et al., 2022). For this, DIM was calculated using Equation 3:

$$DIM = HM_{Fodder} \times DFI \times CF/ABW \quad (3)$$

where, HM is heavy metal concentration in edible fodder part (shoot; mg kg^{-1} dwt), DFI is daily food intake by farm animals (buffalo: 12.50 kg), CF is conversion factor (0.085), and ABW is the average body weight of farm animal (550 kg), respectively. Afterward, the HRI was computed by using DIM and oral reference dose (RfD) as shown in Equation 4:

$$HRI = DIM/RfD \quad (4)$$

herein, the RfD values of selected heavy metals, i.e., Cd, Co, Cu, Cr, Fe, Ni, Mn, and Ni were 0.001, 0.0003, 0.04, 0.005, 0.70, 0.02, 0.14, and 0.30 mg kg^{-1} d^{-1}, respectively (USEPA, 2010). All experiments were conducted in triplicate and a total of nine samples were collected from each treatment ($n = 3 \times 3$). The statistical differences among experimental treatments were computed using a one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). The data were analysed using OriginPro 2023b (OriginLab Corp., Northampton, Massachusetts, USA) and Microsoft Excel 2019 (Home and Student, Microsoft Corp., Redmond, Washington, USA) software packages.

RESULTS AND DISCUSSION

Effects of SS loading on soil nutrient and heavy metal properties

The physicochemical properties of sewage sludge (SS) and experimental treatments (arable soil (control), 5% SS (T1), and 10% SS (T2)) are shown in Table 1. Significant differences ($p < 0.05$) were detected between treatments as the ANOVA revealed. It is worth noting that all analysed contents were in the following decreasing order: T2 > T1 > control. More precisely, the pH of amended soils with SS varied between 7.80 ± 0.03 and 8.11 ± 0.05. It was reported that pH values above 6.5 are well recommended for forage species cultivation, which results in increased N fixation by soil bacterial populations. Our results were in this range (control) or slightly higher (T1 and T2). At such pH, the persistence and dissemination of heavy metals are relatively low and can be mitigated via phytoremediation. The determination of soil electrical conductivity (EC) is an essential step toward a healthy growing environment. It is well known that high ECs can result in salt stress, and subsequently reduced plant growth and development. Experimental treatments showed an EC level varying between 3.04 ± 0.07 and 3.55 ± 0.05 dS m^{-1}. Such values correspond to a slightly saline-soil classification, thus posing no potential salt stress. *Trifolium alexandrinum* showed moderate tolerance to salinity that could be enhanced via long-term stress-induced gene expression. Soil amendment with SS resulted in significantly higher ($p < 0.05$) organic matter (OM) (3.05 ± 0.06%-3.60 ± 0.09%) compared to the control. The amount of OM in SS used in this study was relatively higher than reported in a recent investigation (18.95 ± 3.30%). Such an increase in OM may be very helpful in the reduction of soil aggregation, and improvement of soil porosity, aeration, and water retention (Rossi and Beni, 2018). An OM of 3%-6% was attributed to productive soils as per Cornell University (Cornell University Cooperative Extension, 2023). Such a statement supports the potential benefit of SS amendment in this study; arable soil had a slightly lower OM percentage than recommended by the aforementioned study. Nitrogen content was significantly improved ($p < 0.05$) after SS amendment compared to the control (3.03 ± 0.05-4.26 ± 0.10 g kg^{-1} and 1.90 ± 0.09 g kg^{-1}, respectively). A good amelioration in the N content of agricultural soils can improve plants' greenness, CO_2 assimilation rate, and mitigation of water and salt stresses. Although it is needed for photosynthesis and N fixation, high P content can have a potential leaching risk. In the present study, SS amendment significantly improved ($p < 0.05$) P content in soil compared to the control (2.10 ± 0.04-2.95 ± 0.04 g kg^{-1}). Therefore, maintaining balanced P levels can be beneficial especially when originating from organic sources like SS rather than chemical sources. Generally, soil P and K contents act synergistically and further result in their improved assimilation by plant leaves (Setu, 2022). Such observation was also denoted by our findings. K content was significantly increased ($p < 0.05$) after SS amendment compared to the control (0.41 ± 0.03-0.56-0.02 g kg^{-1}). The improvement of soil K may be helpful in the regulation of antioxidant metabolism and growth alleviation under various environmental stresses.

Table 1. Properties of arable soil and sewage sludge used in this study. Values are mean ± standard deviation of three replicates; same letter indicates nonsignificant difference between treatment groups at $p < 0.05$; -: not applicable; *WHO (1996); [na]not available; EC: electrical conductivity.

Properties	Sewage sludge (SS)	Experimental treatments			Normal limits in arable soil*
		Arable soil (Control)	T1 (5% SS)	T2 (10% SS)	
pH	8.19 ± 0.10^c	7.42 ± 0.04^a	7.80 ± 0.03^b	8.11 ± 0.05^c	-
EC, dS m^{-1}	8.11 ± 0.12^d	3.04 ± 0.07^a	3.20 ± 0.09^{ab}	3.55 ± 0.05^c	-
Organic matter, %	21.09 ± 1.32^d	2.52 ± 0.04^a	3.05 ± 0.06^b	3.60 ± 0.09^c	-
N, g kg^{-1}	32.56 ± 2.49^d	1.90 ± 0.09^a	3.03 ± 0.05^b	4.26 ± 0.10^c	-
P, g kg^{-1}	16.25 ± 0.24^d	1.62 ± 0.10^a	2.10 ± 0.04^b	2.95 ± 0.04^c	-
K, g kg^{-1}	2.48 ± 0.04^d	0.30 ± 0.02^a	0.41 ± 0.03^b	0.56 ± 0.02^c	-
Cd, mg kg^{-1}	1.69 ± 0.03^d	0.13 ± 0.02^a	0.19 ± 0.03^{ab}	0.27 ± 0.04^c	0.80
Co, mg kg^{-1}	63.40 ± 4.95^d	9.28 ± 1.29^a	12.40 ± 1.39^{ab}	15.72 ± 1.37^{bc}	na
Cu, mg kg^{-1}	29.71 ± 1.45^d	4.98 ± 0.07^a	6.57 ± 0.40^b	7.90 ± 0.81^{bc}	36.00
Cr, mg kg^{-1}	10.46 ± 0.26^d	3.53 ± 0.15^a	4.15 ± 0.17^{ab}	4.68 ± 0.07^{bc}	100.00
Fe, mg kg^{-1}	60.73 ± 3.91^d	14.06 ± 0.31^a	17.09 ± 0.29^b	20.30 ± 0.40^c	425.50
Ni, mg kg^{-1}	41.39 ± 1.62^d	23.18 ± 0.35^a	25.31 ± 1.44^{ab}	27.58 ± 1.19^{bc}	35.00
Mn, mg kg^{-1}	19.57 ± 1.29^d	8.43 ± 0.13^a	9.46 ± 0.56^b	10.34 ± 0.15^{bc}	na
Zn, mg kg^{-1}	51.02 ± 2.77^d	4.60 ± 0.07^a	7.19 ± 0.12^b	9.63 ± 0.14^c	50.00

Heavy metal dispersion in the environment can both influence fauna and flora. Thus, the re-use of treated and untreated wastewater and its derivatives can result in narrow reverberations on agricultural soils and their fertility. In this study, Cd, Co, Cr, and Ni contents were significantly increased ($p < 0.05$) after 10% SS-amendment in comparison with the control (0.27 ± 0.04, 15.72 ± 1.37, 4.68 ± 0.07, and 27.58 ± 1.19 mg kg^{-1}, respectively). Soil contents of Cd, Cr, and Ni were all below the standard safe limits set by WHO (1996). Whereas, both 5% and 10% SS incorporation in soil resulted in significantly higher ($p < 0.05$) Cu, Fe, Mn, and Zn contents compared to the control. Such contents varied in the respective following ranges: 6.57 ± 0.40-7.90 ± 0.81, 17.09 ± 0.29-20.30 ± 0.40, 9.46 ± 0.56-10.34 ± 0.15, and 7.19 ± 0.12-9.63 ± 0.14 mg kg^{-1}. Soil contents of Cu, Fe, and Zn were far away from the WHO standards. The heavy metal profile showed the following decreasing trend: Ni > Fe > Co > Mn > Zn > Cu > Cr > Cd with T2 > T1 > control. Consequently, the whole physicochemical analysis of all soil treatments reveals their safety for *T. alexandrinum* cultivation.

Effects of SS loading on growth, yield, and biochemical response of berseem

The effects of SS amendment on the growth, yield, and biochemical response of *T. alexandrinum* are reported in Table 2. The days in the emergence (DE) period were significantly narrowed ($p < 0.05$) after the SS amendment in comparison with the control (42.00 ± 1.00-43.00 ± 1.00 d). However, the loading rate of SS did not show any significant impact ($p > 0.05$) on such parameters when T1 and T2 were compared. It was reported that the whole emergence period of early sown *T. alexandrinum* (September-October) can take up to 50 d, which is a longer period than observed in our investigation. A hastened DE period is highly beneficial on the productive and economic scales. The results of Pearson correlations between the physicochemical properties of soil, and the growth, yield, biochemical, and proximate composition of *T. alexandrinum* are outlined in Figure 1. It was observed that all soil physicochemical properties were strongly negatively correlated with DE ($-0.97 < r < -0.87$). Such an assumption may reveal new insights about the beneficial amendment of *T. alexandrinum* with SS. The shoot system is mainly responsible for the transport of nutrients to the various plant parts besides its role in growth, photosynthesis, and nutrient storage. Soil amendment with different SS loading rates improved significantly ($p < 0.05$) shoot height (SH) and weight (SW) in comparison with the control with varying ranges of 54.48 ± 1.24-58.77 ± 1.26 cm and 11.80 ± 0.14-12.26 ± 0.24 g fwt, respectively. Higher SH and SW mean higher biomass and leaf expansion, which may naturally result in increased photosynthesis potential. Figure 1 outlines a strong positive interrelationship between SH and SW ($r = 0.97$) besides strong positive correlations with soil physicochemical properties ($r > 0.85$). On the other hand, the root system helps in the anchoring of shoots to the soil, thereby acting as plants' stabilizer and supporter. Herein, SS amendment resulted in significantly higher ($p < 0.05$) root length (RL) and weight (RW) in comparison with the control with varying ranges of 18.04 ± 1.03-20.38 ± 1.50 cm and 2.12 ± 0.02-2.15 ± 0.04 g fwt, respectively. However, the SS loading rate did not seem to significantly affect ($p > 0.05$) those parameters when T1 and T2 were compared.

Table 2. Effect of sewage sludge (SS) amendment on growth, yield, biochemical, and proximate composition of berseem clover (*Trifolium alexandrinum*). Values are mean ± standard deviation of three replicates; same letter indicates nonsignificant difference between treatment groups at $p < 0.05$; ^: yield is average of five subsequent cuttings. fwt: Fresh weight.

	Experimental treatments		
Properties	Control	T1 (5% SS)	T2 (10% SS)
Days in emergence	46.50 ± 0.50^{c}	43.00 ± 1.00^{ab}	42.00 ± 1.00^{a}
Shoot height, cm	47.07 ± 0.68 ^{a}	54.48 ± 1.24^{b}	58.77 ± 1.26^{c}
Shoot weight, g fwt	9.15 ± 0.05^{a}	11.80 ± 0.14^{b}	12.26 ± 0.24^{c}
Root length, cm	15.84 ± 0.70^{a}	18.04 ± 1.03^{b}	20.38 ± 1.50^{bc}
Root weight, g fwt	2.01 ± 0.03^{a}	2.12 ± 0.02^{b}	2.15 ± 0.04^{b}
Fodder yield, kg/m^{-2} fwt^	1.30 ± 0.04^{a}	1.74 ± 0.08^{b}	1.92 ± 0.09^{bc}
Total chlorophyll, mg g^{-1} fwt	2.60 ± 0.08^{a}	2.80 ± 0.05^{b}	2.89 ± 0.07^{bc}
Carotenoids, mg g^{-1} fwt	0.51 ± 0.04^{a}	0.63 ± 0.04^{b}	0.67 ± 0.06^{bc}
Total phenols, mg g^{-1} fwt	0.83 ± 0.06^{a}	1.05 ± 0.08^{b}	1.12 ± 0.04^{bc}
Ascorbic acid, mg g^{-1} fwt	7.70 ± 0.25^{a}	8.46 ± 0.12^{ab}	9.05 ± 0.30^{c}
Crude protein, %	17.03 ± 1.02^{a}	19.30 ± 0.40^{ab}	19.53 ± 0.09^{b}
Crude fibre, %	24.94 ± 1.16^{a}	27.45 ± 0.85^{ab}	29.02 ± 0.16^{bc}
Ether extract, %	2.47 ± 0.06^{a}	2.54 ± 0.10^{ab}	2.59 ± 0.05^{bc}
Ash, %	6.31 ± 0.07^{a}	7.07 ± 0.05^{b}	7.32 ± 0.04^{c}

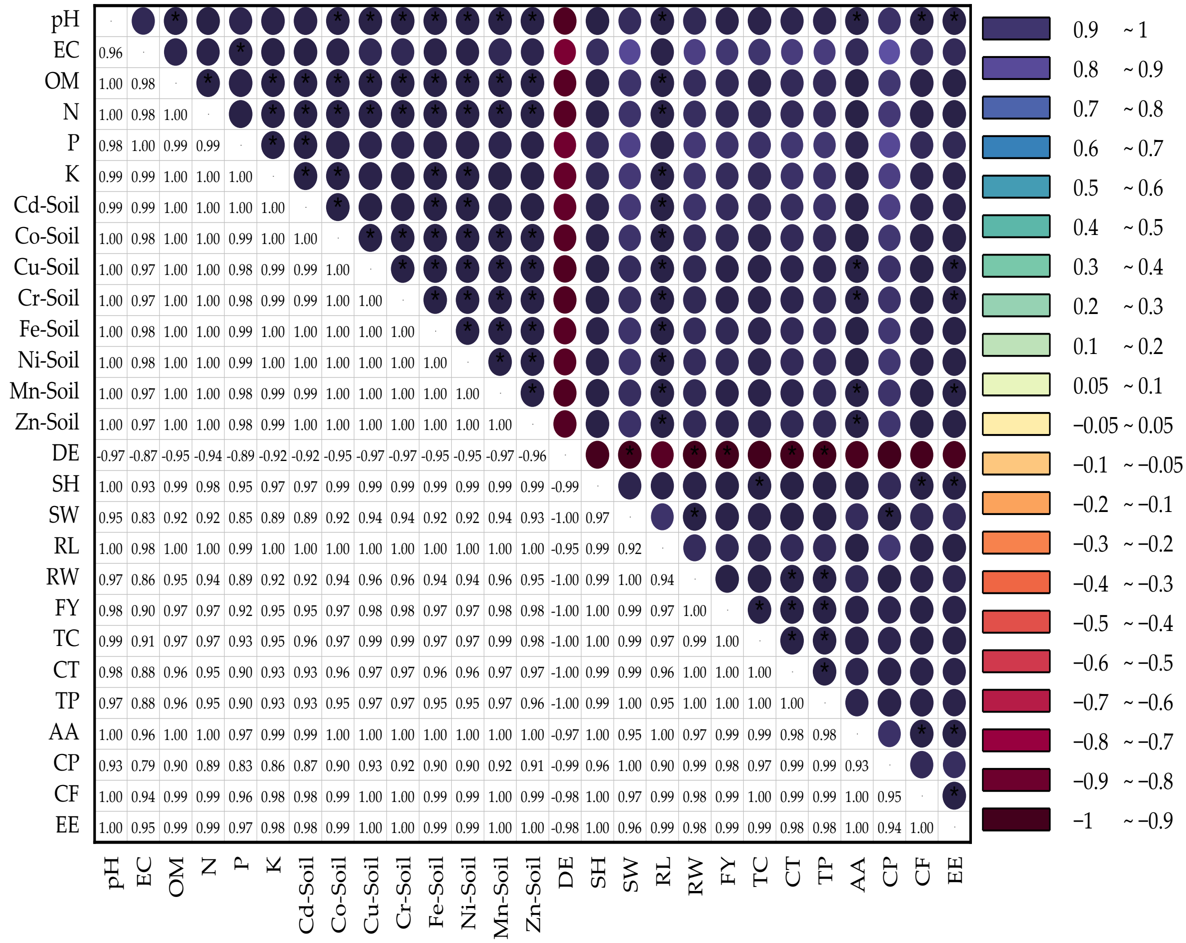

Figure 1. Pearson correlation matrix for the impact of soil characteristics on yield, biochemical, and proximate composition of berseem clover (*Trifolium alexandrinum*) grown under different treatments of sewage sludge. EC: Electrical conductivity; OM: organic matter; DE: days in emergence; SH: shoot height; SW: shoot weight; RL: root length; RW: root weight; FY: fodder yield; TC: total chlorophyll; CT: carotenoids; TP: total phenols; AA: ascorbic acid; CP: crude protein; CF: crude fibre; EE: ether extract.

The increase in RL refers to an increased soil volume exploration by roots, thereby higher water and nutrient availability for growing plants (Faye et al., 2019). Whereas, the increase in RW is a natural consequence of increased P and K in soil, which promotes root development and proliferation. Moreover, the loading rate of SS did not significantly affect ($p > 0.05$) root characteristics when T1 and T2 were compared. A strong positive interrelationship exists between RL and RW ($r = 0.94$) (Figure 1). Whereas, both RL and RW were strongly positively correlated with soil physicochemical parameters ($r > 0.86$) especially P and K ($r = 0.99$ and 1.00, respectively for RL). SS amendment significantly increased ($p < 0.05$) fodder yield (FY), total chlorophyll (TC), carotenoids (CT), and total phenols (TP) of *T. alexandrinum* plants compared to the control with varying ranges of 1.74 ± 0.08-1.92 ± 0.09 kg m^{-2} fwt, 2.80 ± 0.05-2.89 ± 0.07 mg g^{-1} fwt, 0.63 ± 0.04-0.67 ± 0.06 mg g^{-1} fwt, and 1.05 ± 0.08-1.12 ± 0.04 mg g^{-1} fwt, respectively. It is noteworthy that values obtained with T2 were not significantly higher ($p > 0.05$) than those of T1. Increased FY is a direct repercussion of increased SH and SW; such an assumption can be validated by Pearson correlations (Figure 1). Whereas, increased TC, CT, and TP are outcomes of leaf expansion and cytokinin activity. Soil amendment with 10% SS significantly increased ($p < 0.05$) ascorbic acid (AA), crude protein (CP), crude fibre (CF), and ether extract (EE) in comparison with the control.

The AA improvement means better control of plant cell division. Whereas, increased CP and CF refer to further higher digestibility of clover by cattle (Hart et al., 2016; Fathy Abda et al, 2021). In the present study, CP and EE increase in *T. alexandrinum* plants was strongly positively correlated with increased soil pH and N content (Figure 1). Such findings contradict earlier assumptions raised by (Moorby et al., 2016). Ash content was significantly increased ($p < 0.05$) after soil amendment with SS in comparison with the control with a range of 7.07 ± 0.05%-7.32 ± 0.04%. Such improvement can be directly related to increased N levels in soil.

Heavy metal bioaccumulation in berseem

Table 3 shows the contents of eight heavy metals (Cd, Co, Cu, Cr, Fe, Ni, Mn, and Zn) in shoot and root parts of *T. alexandrinum* grown under different treatments of SS. The results showed that *T. alexandrinum* showed contents of all heavy metals in both shoot and root parts. It was observed that the heavy metal concentration in plant parts increased significantly ($p < 0.05$) with an increase in SS dose from 5% to 10% accounting lowest values in the control treatment with no SS loading. Specifically, the highest levels (mg kg^{-1} dwt) of Cd (shoot: 0.06; root: 0.13), Co (shoot: 0.06; root: 0.08), Cu (shoot: 8.97; root: 9.05), Cr (shoot: 0.70; root: 1.68), Fe (shoot: 11.01; root: 15.93), Ni (shoot: 1.67; root: 2.90), Mn (shoot: 3.03; root: 5.38), and Zn (shoot: 4.21; root: 7.04) were reported in T2 treatment. The increasing orders of heavy metals in shoot and root parts were reported as Cd < Co < Cr < Ni < Mn < Zn < Cu < Fe and Co < Cd < Cr < Ni < Mn < Zn < Cu < Fe, respectively. A higher accumulation in 10% SS treatment might be due to the increasing availability of heavy metals in cultivated soil. The levels of heavy metals in fodder (shoot parts) were reported to be within acceptable limits of FAO/WHO (2007) and Kabata-Pendias (2000) except for Mn, which exceed the safe limit (2.00 mg kg^{-1} dwt). The Pearson correlation matrix given in Figure 2 showed that all soil characteristics had a significant ($p < 0.05$) positive (> 0.63) relationship with heavy metal contents in the shoot and root parts of *T. alexandrinum*. This confirms that heavy metal bioavailability was dependent on their concentration in SS.

Table 3. Concentration of eight heavy metals in shoot and root parts of Berseem clover (*Trifolium alexandrinum*) grown under different treatments of sewage sludge (SS). Values are mean ± standard deviation of three replicates; same letter indicates nonsignificant difference between treatment groups at $p < 0.05$; [1]WHO/FAO (2007); [2]Kabata-Pendias (2000).

Heavy metals	Plant parts	Experimental treatments: Control	T1 (5% SS)	T2 (10% SS)	Normal range in fodder
		mg kg^{-1} dwt			mg kg^{-1} dwt
Cd	Shoot	0.02 ± 0.01^{a}	0.04 ± 0.02^{ab}	0.06 ± 0.02^{b}	0.21[1]
	Root	0.07 ± 0.02^{a}	0.10 ± 0.03^{ab}	0.13 ± 0.02^{b}	
Co	Shoot	0.04 ± 0.02^{a}	0.05 ± 0.01^{a}	0.06 ± 0.02^{ab}	2-10[2]
	Root	0.05 ± 0.01^{a}	0.06 ± 0.01^{ab}	0.08 ± 0.02^{ab}	
Cu	Shoot	5.94 ± 0.16^{a}	7.10 ± 0.20^{b}	8.97 ± 0.19^{c}	5-20[2]
	Root	7.06 ± 0.08^{a}	8.49 ± 0.74^{b}	9.05 ± 1.02^{bc}	
Cr	Shoot	0.43 ± 0.07^{a}	0.65 ± 0.04^{b}	0.70 ± 0.06^{b}	0.03-20[2]
	Root	0.95 ± 0.09^{a}	1.04 ± 0.10^{ab}	1.68 ± 0.07^{c}	
Fe	Shoot	7.08 ± 0.10^{a}	10.26 ± 0.83^{b}	11.01 ± 0.62^{bc}	425.00[1]
	Root	10.51 ± 0.46^{a}	13.02 ± 1.25^{b}	15.93 ± 1.47^{bc}	
Ni	Shoot	0.89 ± 0.11^{a}	1.52 ± 0.09^{b}	1.67 ± 0.14^{bc}	0.02-5.0[2]
	Root	1.17 ± 0.20^{a}	2.36 ± 0.16^{b}	2.90 ± 0.25^{c}	
Mn	Shoot	1.62 ± 0.09^{a}	2.05 ± 0.04^{b}	3.03 ± 0.18^{c}	2.00
	Root	2.91 ± 0.35^{a}	4.40 ± 0.12^{b}	5.38 ± 0.26^{c}	
Zn	Shoot	3.30 ± 0.07^{a}	3.94 ± 0.09^{b}	4.21 ± 0.15^{c}	1-100[2]
	Root	4.19 ± 0.10^{a}	6.88 ± 0.21^{b}	7.04 ± 0.50^{bc}	

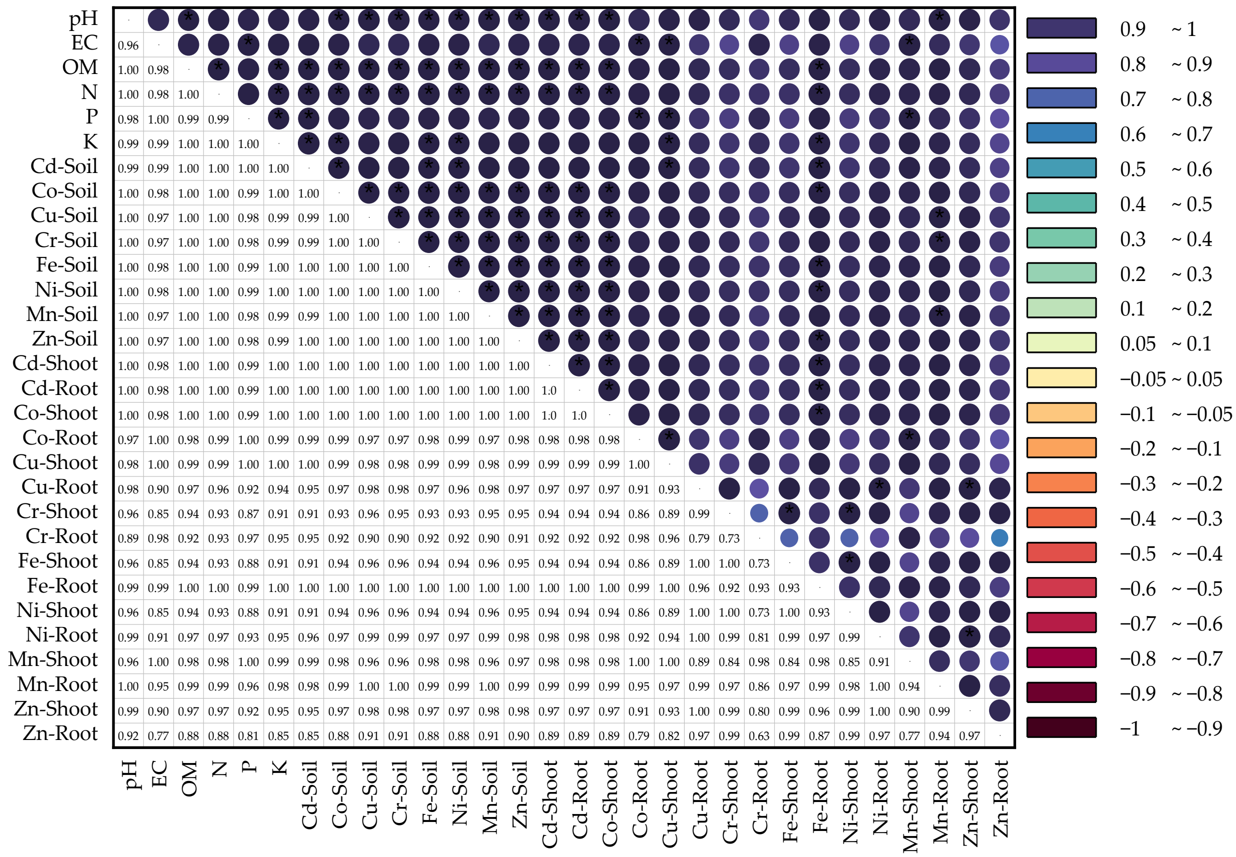

Figure 2. Pearson correlation matrix for the impact of soil characteristics on eight heavy metals in shoot and root parts of berseem clover (*Trifolium alexandrinum*) grown under different treatments of sewage sludge. EC: Electrical conductivity; OM: organic matter.

Like other plants, fodder crops including *T. alexandrinum* can accumulate heavy metals from soil which is governed by several intrinsic and extrinsic factors such as soil pH, OM, bioavailability, type of irrigation source, environmental factors, etc. SS application can accelerate heavy metal bioavailability in soil which results in their increased uptake by plants. However, a controlled SS application has appeared as beneficial for several crops such as pepper (*Piper nigrum*) (Pascual et al., 2010), wheat (Wydro et al., 2022), *Jatropha curcas* (Ahmadpouret al., 2010), etc. On the other hand, Figure 3 shows BAF values of selected heavy metals in the shoot and root parts of *T. alexandrinum* grown under different SS treatments. The BAF values higher than 1 indicate that the plant has a strong capability to accumulate these heavy metals from the soil. The results showed that BAF values of selected heavy metals did not exceed 1 except for Cu in both shoot and root parts. However, Zn in the root parts of *T. alexandrinum* approached the level of almost 1. The BAF values were in line with the dose of SS applied to soil, i.e., increased from control to 10%.

Other studies have reported similar findings, with higher BAF values for heavy metals in crops grown in contaminated soils. Out of them, Altaf et al. (2008) reported the accumulation of five heavy metals (Cr, Cd, Cu, Zn, and Ni) in root nodules of *T. alexandrinum* crop irrigated with treated tannery effluent. They found that the highest accumulation was found for Cr which is in line with the results of the current study. However, controlled use of such fertilization supplies could minimize the risk of hazardous metal accumulation by plants. Therefore, it is important to monitor the heavy metal content of both SS and cultivated crops to prevent their excessive accumulation in the food chain.

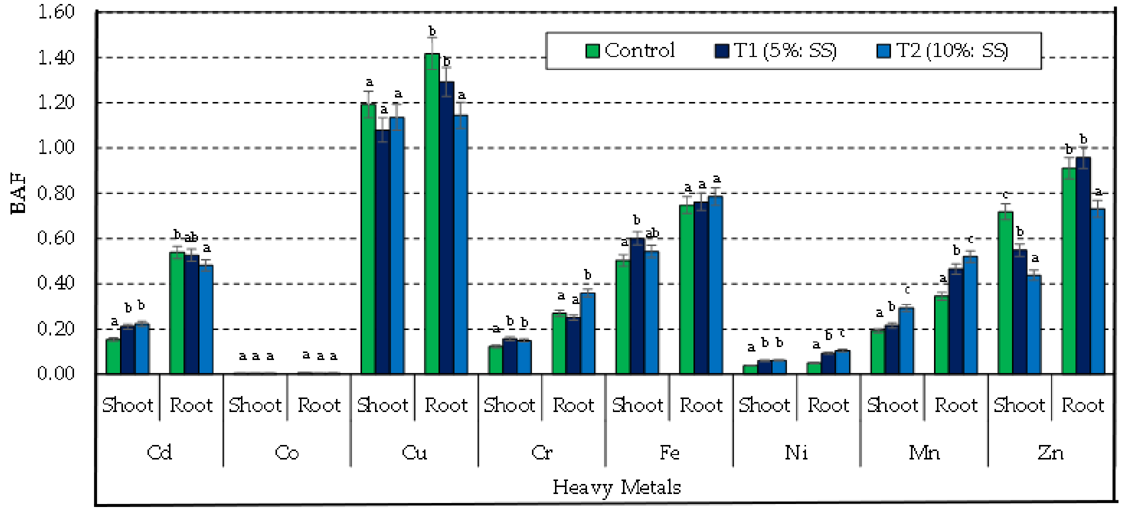

Figure 3. Bioaccumulation factor (BAF) of eight heavy metals in shoot and root parts of berseem clover (*Trifolium alexandrinum*) grown under different treatments of sewage sludge (SS). Different letters indicate significant difference among treatment groups based on Tukey's mean comparison test at $p < 0.05$).

DIM and HRI studies of heavy metals in berseem

Consumption of meat, milk, and other products from animals fed contaminated fodder may endanger human health. Health risk studies for heavy metals in fodder are essential to ensure that the consumption of contaminated fodder does not pose a risk to animal or human health. This aids in identifying heavy metal levels in fodder that can lead to harmful levels of heavy metals in animal products, as well as providing advice for acceptable consumption levels. In this study, DIM and HRI tools were employed in order to understand the dietary risk of heavy metal intake by animals (buffalo) from *T. alexandrinum* cultivated on SS-amended soils. Table 4 depicts the calculated DIM and HRI values of heavy metals in the shoot and root parts of *T. alexandrinum*. The DIM values were higher in the case of root parts as compared to the shoot. However, the shoot parts of *T. alexandrinum* are mostly used as fodder by farmers as compared to the root parts. The DIM values were highest in 10% SS treatment followed by 5% and control. Similarly, the values of HRI for eight heavy metals were below the threshold limit of 1 in 10% SS treatment. The higher HRI values (> 1) show health risks to animals fed with contaminated *T. alexandrinum* fodder. However, both 5% and 10% SS treatment showed nonsignificant health risk associated with heavy metal exposure. Therefore, it is suggested that an SS application of up to 10% mixing rate could be considered a feasible dose with no health hazard to consumers.

Previously, Bhatti et al. (2017) studied the health risk of Cr, Cu, Co, and Pb in grain and straw (fodder) of wheat grown around rivers and found that DIM and hazard quotient (HQ), and hazard index (HI) values were significantly higher near contaminated sites. Similarly, Ghazzal et al. (2022) studied health risks associated with the intake of three forage crops (*Z. mays*, *Sorghum bicolor*, and *T. alexandrinum*) in the Punjab region of Pakistan. They found that the Cr contents were above the permissible limits suggesting serious health hazards. The HRI values were maximum in the *Z. mays* crop followed by *S. bicolor*, and *T. alexandrinum*, respectively. Because these heavy metals could enter the food chain through the consumption of animal products or meat that was fed with contaminated fodder crops, it is a must to monitor their levels and health risks. Thus, this study suggests a strategy for low-cost soil fertilization and fodder crop production which could sustainably benefit waste recycling.

Table 4. Dietary intake modeling (DIM) and health risk index (HRI) values of heavy metals in berseem clover (*Trifolium alexandrinum*) grown under different treatments of sewage sludge (SS). Values are mean ± standard deviation of three replicates.

Heavy metals	Plant parts	DIM			HRI		
		Control	T1 (5% SS)	T2 (10% SS)	Control	T1 (5% SS)	T2 (10% SS)
Cd	Shoot	0.0001	0.0001	0.0002	0.0386	0.0773	0.1159
	Root	0.0001	0.0002	0.0003	0.1352	0.1932	0.2511
Co	Shoot	0.0001	0.0001	0.0001	0.2576	0.3220	0.3864
	Root	0.0001	0.0001	0.0002	0.3220	0.3864	0.5152
Cu	Shoot	0.0115	0.0137	0.0173	0.2869	0.3429	0.4332
	Root	0.0136	0.0164	0.0175	0.3410	0.4100	0.4371
Cr	Shoot	0.0008	0.0013	0.0014	0.1661	0.2511	0.2705
	Root	0.0018	0.0020	0.0032	0.3670	0.4018	0.6491
Fe	Shoot	0.0137	0.0198	0.0213	0.0195	0.0283	0.0304
	Root	0.0203	0.0252	0.0308	0.0290	0.0359	0.0440
Ni	Shoot	0.0017	0.0029	0.0032	0.0860	0.1468	0.1613
	Root	0.0023	0.0046	0.0056	0.1130	0.2280	0.2801
Mn	Shoot	0.0031	0.0040	0.0059	0.0224	0.0283	0.0418
	Root	0.0056	0.0085	0.0104	0.0402	0.0607	0.0742
Zn	Shoot	0.0064	0.0076	0.0081	0.0213	0.0254	0.0271
	Root	0.0081	0.0133	0.0136	0.0270	0.0443	0.0453

CONCLUSIONS

The findings of this study demonstrated that municipal sewage sludge (SS) had a significant positive impact on the growth, biochemical, and proximate parameters of *Trifolium alexandrinum*. The growth and productivity parameters were found to increase with increasing rate of SS mixing (0%, 5%, and 10%) out of which 10% SS treatment showed highest values for all parameters. However, a higher SS mixing application showed accelerated bioaccumulation of selected heavy metals (Cd, Co, Cu, Cr, Fe, Ni, Mn, and Ni) in both the shoot and root parts of *T. alexandrinum*. The contents of heavy metals were below the permissible limits except for Mn. The health risk studies further suggested that there was no health hazard to animals feeding *T. alexandrinum* fodder grown on SS-amended soils up to a 10% mixing rate. However, proper management practices, such as limiting the application rate of SS and monitoring soil quality, should be taken into consideration to reduce the risks associated with heavy metal accumulation in crops.

Author contributions

Conceptualization: S.K.A., E.A.A-S., P.K., E.M.E. Methodology: S.K.A., E.A.A-S., P.K., E.M.E. Software: I.Š., Ž.A., S.A.F., B.A. Validation: I.Š., Ž.A., S.A.F., B.A. Formal analysis: P.K. Investigation: P.K. Resources: P.K. Data curation: I.Š., Ž.A., S.A.F., B.A. Writing-original draft: P.K., S.A.F. Writing-review & editing: I.Š., Ž.A., S.A.F., B.A. Visualization: S.A.F., B.A. Supervision: E.M.E. Project administration: S.K.A., E.A.A-S., E.M.E. Funding acquisition: S.K.A., E.A.A-S., P.K., E.M.E. All co-authors reviewed the final version and approved the manuscript before submission.

Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through Large Groups Project under grant number (R.G.P.2/242/44).

The authors are grateful to their host institutes for providing the necessary facilities to conduct this study. This is joint work by the members of the Sustainable Agro-Environment International Research Group (SAEIRG).

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