ENVIRONMENTAL SCIENCE Renewable Resources

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Research Article Journal of Environmental Science and Renewable Resources Open Access

Assessing the Feasibility of Sewage Sludge Applications for the Cultivation of Brassica *Juncea L.*: Metal Accumulation, Growth, Biochemical and Yield Responses

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Received Date: April 26, 2018, **Accepted Date:** May 14, 2018, **Published Date:** May 25, 2018.

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Abstract

The present study was conducted to examine the suitability of sewage sludge amendment in soil for mustard (*Brassica juncea* var. Alankar) by evaluating the heavy metal accumulation, growth, biochemical and yield responses of plants grown at 0, 5, 10, 20, 40, 70 and 100% sewage sludge amendment (SSA) rates. Sewage sludge amendments modified the properties of soil by decreasing pH and increasing organic carbon, total nitrogen and heavy metals in resulting soil-sludge mixtures. Plants showed significant ($p < 0.05$) increments in root length, shoot length, plant fresh mass and plant dry mass at 10 to 40% SSA rates as compared to unamended soil, but these parameters decreased significantly at SSA rates ≥ 70% at 60 days after sowing (DAS). Biochemical parameters such as photosynthetic pigment content, proline, cysteine, protein and sugar contents in fresh leaves also showed the same trend. Yield of mustard increased by 18.90, 41.80 and 15.42% at 10, 20 and 40% SSA rates, respectively, when compared to those grown in unamended soil. Concentrations of Cd and Pb in mustard shoots were higher than the Indian safe limits of human consumption (for Cd = 1.5 mg kg^{-1} and for Pb = 2.5 mg kg^{-1}) at all amendment levels. Values of tolerance index (TI) in present study revealed that the selected cultivar is well adapted to tolerate and accumulate high quantities of heavy metals due to increased level of antioxidant cysteine and various other biochemical parameters in leaves when grown in soil amended with 5 to 40% sewage sludge. Therefore, it could be concluded that the selected cultivar if grown on sewage sludge amended soil, may act as important pathway for transfer of metals to primary plant consumers including humans. Further it may be recommended that proper guidelines should be laid for the recommended dosage of agricultural use of sewage sludge in countries which lack such guidelines.

Keywords: Sewage sludge; Mustard; Heavy metal; Proline; Cysteine; Yield

Introduction

The urbanization and industrialization with a rapid pace throughout the globe led to the release of huge amounts of solid wastes in the environment. The sewage sludge constitutes a large proportion of solid wastes. Tons of sewage sludge are generated and released in areas around densely populated and industrialized countries, eventually enhancing environmental pollution [1]. The urban population in India has soared from 210 million in 1991 to about 370 million in 2011 [2], about 73% increase from 1991 to 2011. The large scale urbanization is leading to increased production of sewage sludge in India. Its management and disposal in an environmentally sustainable manner is one of the most concerning problems of the country [3]. Sewage sludge being a good source of organic matter [4] and rich in plant nutrients such as N, P, K, S, Ca, Mg, Fe, Cu, Mn and Zn [5–7] offers a good promise for its use in agriculture [8,9]. The nutrients content of sewage sludge sustains soil fertility and the organic constituents improve soil properties [6,7,10].

Various studies indicated that application of sewage sludge to the land caused increments in the growth and production of crop plants [6,7,11]. The biochemical and physiological attributes of plants have been reported to be enhanced when grown on sludge amended soil [12]. However, the presence of high concentrations of toxic metals viz. Cd, Pb, Zn, Ni, Hg in sludge is a matter of concern as its long-term use can cause accumulation of these metals in the soil [13]. Exposure to elevated concentrations of heavy metals accumulated in plants may cause damage to the metabolic processes of plant and result in retarded growth through the production of free radicals and reactive oxygen species [14]. To repair the damage caused by reactive oxygen species, plants have evolved a complex antioxidant system which plays a pivotal role in the cellular defence strategy against oxidative stress by inducing resistance to metals and by protecting labile macromolecules. Antioxidants like cysteine and proline play an important role in detoxification of toxic metal ions [14].

Moreover, cultivation of crops on heavy metal contaminated soils can potentially result in the uptake and accumulation of these metals in the edible plant parts posing a threat to human and animal health [15]. There are social and legal concerns of uncontrolled use of sewage sludge for agriculture due to potential threats of biotransfer of heavy metals to the human food chain [16]. USEPA and EU have suggested the dosage for sewage sludge use in agriculture which is based on heavy metal and other pollutant concentrations [16], but no such recommended standard guideline is available in India.

Indian mustard (*Brassica juncea* L.) Czern has the potential to accumulate very high levels of heavy metals such as Cd, Pb and Zn in its aerial parts without showing any indications of toxicity [10,17]. Based on the above view, a pot experiment was carried out and the plants of *B. juncea* were grown in soil amended with various levels of sewage sludge in order to assess the tolerance of cultivated crop to metal contamination/stress in relation to the growth, biochemical and yield responses of the plant.

Materials and Methods

A bulk soil sample was obtained from the agricultural farm field of Aligarh Muslim University (AMU), air- dried and then divided into six equal parts. The sewage sludge was collected from the AMU Sewage Treatment Plant, which receives waste water from entire University. The sewage sludge was air-dried, finely powdered, and sieved to 2-mm mesh size before use. One part of the soil was used as unamended control, whereas in remaining five parts, 5% (5%

SS + 95% soil), 10% (10% SS + 90% soil), 20% (20% SS + 80% soil), 40% (40% SS + 60% soil), 70% (70% SS + 30% soil) and 100% (w/w) sewage sludge amendments were done. Treatments were designated as T0 for unamended control, T1 for 5%, T2 for 10%, T3 for 20%, T4 for 40%, T5 for 70% and T6 for 100% sewage sludge ratios, respectively for convenience. The soil and sludge was thoroughly mixed before filling in the pots (25 cm diameter). Each pot had a capacity to possess 6.5 kg soil in it. Twenty eight pots were prepared for different treatments and four replicates used for each treatment. All pots were filled uniformly with the soil of different amendment rates. The soil in the pots was moistened to field capacity and incubated for three weeks in order to allow the chemical degradation, biodegradation and volatilization of toxic compounds in sludge to reach equilibrium in soil. No chemical fertilization was done before and during the experiment. The characteristics and heavy metal concentrations in sewage sludge, unamended soil (garden soil) and soil amended with various levels of sewage sludge were analysed before sowing the seeds of selected crop. The pH of the soil at different treatments was measured in a soil: water suspension (1:2.5 w/v) with a pH meter (Digital pH, conductivity and temperature meter 181). Organic carbon and total nitrogen contents of the soil samples were determined by Walkley and Black's rapid titration method and Micro- Kjeldahl method, respectively.

After three weeks of incubation of soil mixture, ten seeds of the Indian mustard (*Brassica juncea* var. Alankar) were sown in each pot to a depth of 0.5 cm. The pots were then placed in a completely randomised design and were irrigated every day with water to maintain proper soil moisture (70-80%). To prevent leaching from pots, water retaining plates were placed below each pot to collect and reuse the percolated water. After germination, the seedlings were thinned at 4–5 leaves stage and three seedlings of equal growth and vigour per pot were allowed to grow. Plants grown in various levels of metal contaminated soil were intensely observed throughout their whole growth period for any visible toxic symptom. Toxic signs in terms of yellowing of leaves and consequently their early fall were observed in plants grown in T5 and T6 treatments.

Plant samples were collected after 30 and 60 days of sowing period. The mustard plants with intact roots were removed from the pots carefully and the adhering soil was removed by gentle washing in the bucket filled with water. Roots and above- ground parts were separated and manually cut in small pieces. The root and shoot length (cm) were measured using a meter scale. Each plant was weighed individually using electronic balance (AH220A, AHN Germany) to record their fresh mass. Dry weight of plants was recorded after drying the sample in oven at 70°C for 72 hours. After dry weight determination, the samples of shoot were ground and kept for heavy metal analysis.

Biochemical Attributes

Biochemical parameters were measured in fresh leaf samples at 30 and 60 days after sowing. The chlorophylls and carotenoids were estimated by following the method of Mackinney (1941) [18]. The levels of lipid peroxidation in root and leaf tissues were determined in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction as described by Heath and Packer [19]. Plant tissue (0.5 g) was homogenized in 5.0 ml of 0.1% (w/v) trichloroaceticacid (TCA). The homogenate was then centrifuged at 10,000 g for 5 min. 2.0 ml of the solution was then boiled for 30 min at 95°C in a water bath with 2.0 ml of 0.5 percent TBA (prepared in 20 percent TCA) and then cooled quickly on ice bath. The resulting mixture was centrifuged at 10,000 g for 15 min and absorbance of the supernatant was measured at 532 nm. The measurements were corrected for nonspecific absorbance by subtracting the absorbance at 600 nm. Proline was extracted by three percent aqueous sulfosalicylic acid and estimated by acid-ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid) methodology at the absorbance 520 nm using toluene for a reagent blank [20]. Cysteine was estimated from the perchloric acid extracts with acid ninhydrin reagent at 560 nm [21]. The protein content was determined by folin-ciocalteau reagent at 660 nm as described by Lowry, et al. (1951) [22] in a spectrophotometer (T70 UK). The total sugars were estimated by following the method proposed by Dubois, et al. (1956) [23]. 30.0 mg of fresh leaf tissue in test tubes was boiled in water bath with 10 ml of 80% ethyl alcohol (80 ml ethyl alcohol + 20 ml de-ionized water). The homogenate was centrifuged at 1500 g for 20 minutes. The supernatant was made upto 10 ml with 80% ethyl alcohol. 4.0 ml of cold anthrone reagent was added to 1.0 ml of ethanolic extract. The mixture was shaken vigorously and boiled for 10 minutes in a boiling water bath. After cooling in running tap water, the absorbance was read at 620 nm in a spectrophotometer (T70 UK). A standard curve was prepared with known amounts of glucose. The amount of total sugar was estimated with reference to a glucose standard.

Harvest Index

Harvest index was calculated by using the formula given by Donald and Hamblin (1976) [24].

$$HI = \frac{\text{Economic yield}}{\text{Biological yield}}$$

Where, economic yield refers to the seed yield and biological yield refers to the total biomass of the plant including dry weight of root, stem and seed.

Tolerance Index

Tolerance index (TI) was calculated as the mean dry weight (biomass) of a plant grown in heavy metal contaminated soil divided by the mean dry weight of the control plants [25] as

$$TI = \frac{\text{Biomass of the treated plant (g plant}^{-1}\text{ dry weight)}}{\text{Biomass of the control plant (g plant}^{-1}\text{ dry weight)}}$$

In present study, TI was calculated on the dry weight basis of plant at 60 DAS

Heavy Metal Analysis

Soil samples, collected in triplicate from each pot, were air dried, crushed and passed through a 2 mm mesh sieve. 1.0 g of soil was digested in 20 ml of triacid mixture ($HNO_3/H_2SO_4/HClO_4$, 5:1:1) at 80°C [26]. After complete digestion, the solution was allowed to cool and filtered through Whatman No. 42 filter paper and made up to a final of 50 ml with double distilled water (DDW) and analysed for Cd, Pb and Zn concentrations through Atomic Absorption Spectrophotometer (GBC, 932 plus; GBC Scientific Instruments, Braeside, Australia). The extractable (bioavailable) fraction of heavy metals in the soil was obtained by mechanical shaking of 15 g of sample with 40 ml of diethylene triamine pentaacetic acid (DTPA) extractant (0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M TEA buffered at pH 7.0) for 2 hours. The mixture was filtered through Whatman No. 42 filter paper prior to heavy metals (Cd, Pb and Zn) analysis by Atomic Absorption Spectrophotometer.

Dried shoots were powdered and sieved. 0.3 g of dried plant samples were digested in 10 ml of tri-acid mixture (HNO_3:H_2SO_4:$HClO_4$; 5:1:1) at 80°C until black fumes turned white and solution became completely clear. The digest was allowed to cool, diluted with DDW and then filtered through Whatman's No. 42 filter paper. The volume of filtrate was made to 50 ml with DDW. All chemicals used were of analytical grade and supplied by Sigma-Aldrich. Analytical quality was ensured by the analysis of certified reference materials i.e. GBW 07402 for soil, NIM-GBW10048 (celery plant) for plants. Mean recoveries from these materials were 96.7, 98.5 and 95.2% for Cd, Pb and Zn, respectively. Reagent process blanks were also digested and run in triplicate to check for process contamination.

Statistical Analysis

The experiments were conducted according to simple randomized block design. Each experiment was performed three times and each treatment was represented by four pots (replicates). Data were statistically analysed using SPSS software version 17 for window (SPSS, Chicago, IL, USA) to determine the significance at $p < 0.05$. The significance of differences among treatments was determined by Duncan's multiple range test (DMRT).

Results and Discussion

Characteristics of Soil, Sewage Sludge and Soil-Sludge Mixtures

The sewage sludge used for soil amendment was almost neutral in pH (7.13) and had high contents of organic C and total N (Table 1). The unamended soil of the agricultural farm had high pH and low organic carbon and nitrogen contents in comparison to soil amended with sewage sludge (Table 1). The soil pH decreased significantly ($p < 0.05$) at all amendment levels except T1. The decrease in soil pH was 0.06, 0.13, 0.24, 0.37 and 0.40 units in T1, T2, T3, T4 and T5 treatments, respectively, as compared to T0 (unamended soil). The reduction in soil pH may also be ascribed to the release of humic acid as a result of biodegradation of sewage sludge rich in organic carbon. Organic C showed significant increments at all amendment ratios compared to unamended soil ($p < 0.05$), whereas enhancements in total N were significant at treatments ≥ T3 (Table 1). Higher quantities of proteinic materials in sewage increases the total N in sewage sludge [27], which in turn increased the N content in amended soils.

Sewage sludge had several fold higher heavy metal concentrations as compared to unamended soil. Concentration of Zn was highest in sewage sludge followed by Pb and Cd (Table 1). Addition of sewage sludge in soil led to higher concentrations of heavy metals in resulting mixtures as compared to unamended soil (Table 1). Total concentration was highest at T6 amendment level for all the three heavy metals studied. Total concentrations of Cd crossed the Indian permissible limits (Cd, 3-6 mg kg^{-1}) at T5 and T6 and that of Zn at T6 (Zn, 300–600 mg kg^{-1}). Whereas, total concentrations of Pb (Pb, 250-500 mg kg^{-1}) in the soil were below the Indian permissible limits on all amendments [28]. Phytoavailable (DTPA extractable) heavy metal concentrations also increased with increase in SSA rates (Table 1). The concentration of phytoavailable heavy metals was highest for Zn followed by Pb and Cd (Table 1). Successive reduction in soil pH and elevated metal concentrations with the additions of sewage sludge amendments led to more availability of heavy metals in resulting mixtures. Several previous studies have also shown increased availability of metals with decreasing pH (6,7,10,11).

Root Length and Shoot Length

Root length of 60 days old mustard plant increased significantly ($p < 0.05$) on 10 (T2) and 20% (T3) sewage sludge application rates in comparison to control (T0). The roots of mustard grown in 100% (T6) sewage sludge were highly retarded at 30 and 60 days of growth (Figure 1a). Application of 10 (T2) to 40% (T4) levels of sewage sludge increased the shoot length of mustard at 60 days growth stage and reduced significantly ($p < 0.05$) on application of 70 (T5) to 100% (T6) sewage sludge (Figure 1b). The nutrient rich sewage sludge (in lower amounts) may have promoted the growth of selected plant. Qasim, et al. (2001) [29] also found increase in shoot and root lengths of *Zea mays* grown in soil amended with lower levels of sewage sludge (1, 2 and 3 kg m^{-2}). Significant increase in plant height of dry beans grown at lower levels of sewage sludge has also been reported [30]. In contrary, Singh and Agrawal (2009) [31] found 10–20% decrease in root and shoot length of lady's finger plants grown at 20 and 40% sewage sludge application rates. These similarities and differences in the responses of varying plants are attributable to the levels of sensitivity and resistance specific to each species and cultivar. The retardation in root length and shoot length of the selected mustard plant grown in higher sewage sludge ratios (≥ 70%) may be attributed to the toxic effects of high metal concentrations in amended soil and their subsequent uptake and accumulation in plant tissues.

Plant Fresh and Dry Mass

The fresh mass and dry mass of 30 days old mustard plants increased significantly ($p < 0.05$) on treatment with 20% (T3) sewage sludge as compared to control. At 60 days of growth stage, applications of 10–40% levels of sewage sludge significantly enhanced the fresh mass and dry mass of selected cultivar (Figure 1c, 1d). In comparison to control, the dry mass of 30 days old mustard plant reduced significantly ($p < 0.05$) on application of 100% sewage sludge whereas in 60 days old plants, the significant reduction in plant dry mass was recorded on application of 70 and 100% sewage sludge (Figure 1d).

Quantification of biomass is often reliably used for monitoring the effects of various environmental factors. The increase in fresh

Parameters	T0	T1	T2	T3	T4	T5	T6
pH	7.82 ±0.03^{a}	7.76 ±0.02^{ab}	7.69 ±0.03^{b}	7.58±0.03^{c}	7.45±0.03^{d}	7.32±0.03^{e}	7.13±0.02^{f}
Org C (%)	0.70 ±0.03^{g}	0.92 ±0.03^{f}	1.25 ±0.04^{e}	1.54 ±0.04^{d}	2.26±0.03^{c}	3.53±0.05^{b}	5.68±0.05^{a}
Total N (%)	0.17 ±0.01^{e}	0.2 ±0.01^{e}	0.24 ±0.01^{de}	0.3 ±0.02^{d}	0.46±0.03^{c}	0.69±0.02^{b}	1.15±0.04^{a}
Total heavy metals (mg kg^{-1})							
Cd	0.41±0.08^{f}	0.69±0.06^{ef}	1.02±0.07^{e}	1.65±0.08^{d}	2.96±0.21^{c}	5.04±0.25^{b}	7.12±0.21^{a}
Pb	11.43±0.34^{e}	12.35±0.65e	13.58±0.78^{e}	15.79±0.60^{d}	20.46±0.90^{c}	27.18±0.90^{b}	34.15±0.85^{a}
Zn	35.53±2.46^{g}	51.17±2.61^{f}	68.59±3.51^{e}	102.12±4.91^{d}	169.52±5.55^{c}	275.15±5.98^{b}	387.38±6.64^{a}
Available heavy metals (mg kg^{-1})							
Cd	0.11±0.04^{f}	0.16±0.01^{ef}	0.27±0.03^{e}	0.41±0.06^{d}	0.65±0.04^{c}	0.9±0.03^{b}	1.23±0.07^{a}
Pb	2.15±0.13^{f}	2.37±0.15^{ef}	2.76±0.09^{de}	3.02±0.15^{cd}	3.42±0.11^{c}	4.49±0.14^{b}	5.63±0.23^{a}
Zn	3.06±0.12^{f}	4.5±0.17^{f}	7.68±0.14^{e}	11.1±0.09^{d}	19.53±0.72^{c}	27.38±0.99^{b}	35.48±0.88^{a}

Table 1: Selected properties and heavy metal concentrations (total and extractable) in unamended soil, sewage sludge and soil-sludge mixtures (mean ± SE, n = 4). Values with different superscript letters in each group are significantly different from each other at $p < 0.05$. (*T0: unamended soil; T1: 5%; T2: 10%; T3: 20%; T4: 40%; T5: 70% and T6: 100% sewage sludge*).

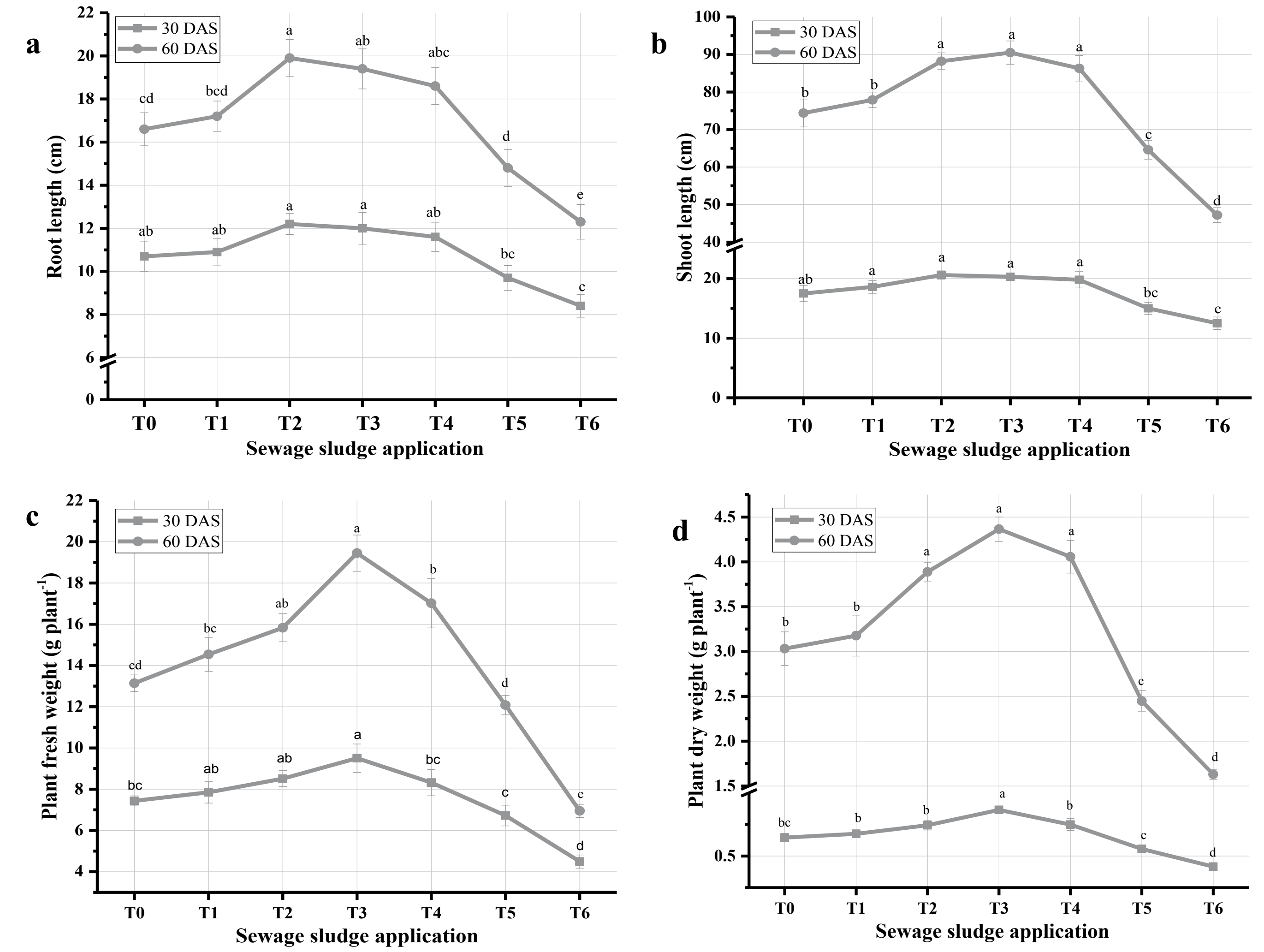

Figure 1: Effects of varying levels of soil amendments with sewage sludge on (a) root length (b) shoot length (c) Plant fresh mass (d) Plant dry mass of *Brassica juncea* cv. Alankar at 30 and 60 days after sowing (DAS). (Mean ± SE; *n* = 4). Different small letters at each growth stage show statistically significant variation at $p < 0.05$ as per DMRT. *(T0: unamended soil; T1: 5%; T2: 10%; T3: 20%; T4: 40%; T5: 70% and T6: 100% sewage sludge).*

and dry biomass of the mustard plants at lower ratios of sewage sludge may be attributed to abundant availability of nutrients through the addition of nutrient rich sewage sludge [29,32,33].

Biochemical Attributes

Chlorophyll a, Chlorophyll b and Carotenoid:

The data on chlorophyll a and chlorophyll b contents in the fresh leaf tissue of mustard are summarised in figure 2a. The chlorophyll-a content increased significantly ($p < 0.05$) in selected mustard plants on application of 20% (T3) sewage sludge at 30 DAS, whereas it showed increments on application of 10(T2)–40%(T4) sewage sludge at 60 days of plant growth. Chlorophyll a content reduced significantly ($p < 0.05$) in mustard plants grown in 100% sewage sludge at both 30 and 60 DAS (Figure 2a). The chlorophyll-b content in leaves of selected mustard plant increased significantly on applications of 10–40% sewage sludge ($p < 0.05$) at 30 and 60 days old plant, while as it reduced significantly in leaves of mustard plants grown in 100% sewage sludge (Figure 2a). Chlorophyll (chlorophyll a and b) pigments in plants play an exceptional role in the economy of the green cell. In the present study, an increase in photosynthetic pigments in the leaves of *B. juncea* on treatments T2 to T4 at both 30 and 60 days of growth stages may be attributed to the presence of various minerals and essential metal ions in the sewage sludge. Zinc maintains the chlorophyll biosynthesis by protecting protochlorophyllide reductase and inhibiting oxidation of δ- aminolevulinic acid dehydratase (ALAD) required for producing porphobilinogen, the first precursor of chlorophyll [34,35]. Zinc also maintains functioning of PSI and PSII under heavy metal stress [36]. In addition to Zn, sewage sludge also contains other mineral elements like Mn, Fe and Cu [6]. Manganese plays an important role in photolysis of water, whereas Fe acts as a cofactor for photosynthetic electron transfer and Cu is an important component of plastocyanin [37]. Thus, increase in chlorophyll biosynthesis along with appropriate functioning of photosystems and respiratory processes might have resulted in the better growth of the plants up to 40% sludge applications in soil. The findings of our results are also strengthened by the study of Singh and Sinha (2005) [12], showing a higher chlorophyll content in the leaves of *B. juncea* grown on various amendments of tannery waste. The reduction in chlorophyll content in higher amendments of sewage sludge in the present study could be due to the elevated levels of a number of toxic heavy metal ions in it and their interaction with the functional -SH group of chlorophyll synthesizing enzyme [38].

The carotenoid contents in leaves of 30 and 60 days old mustard

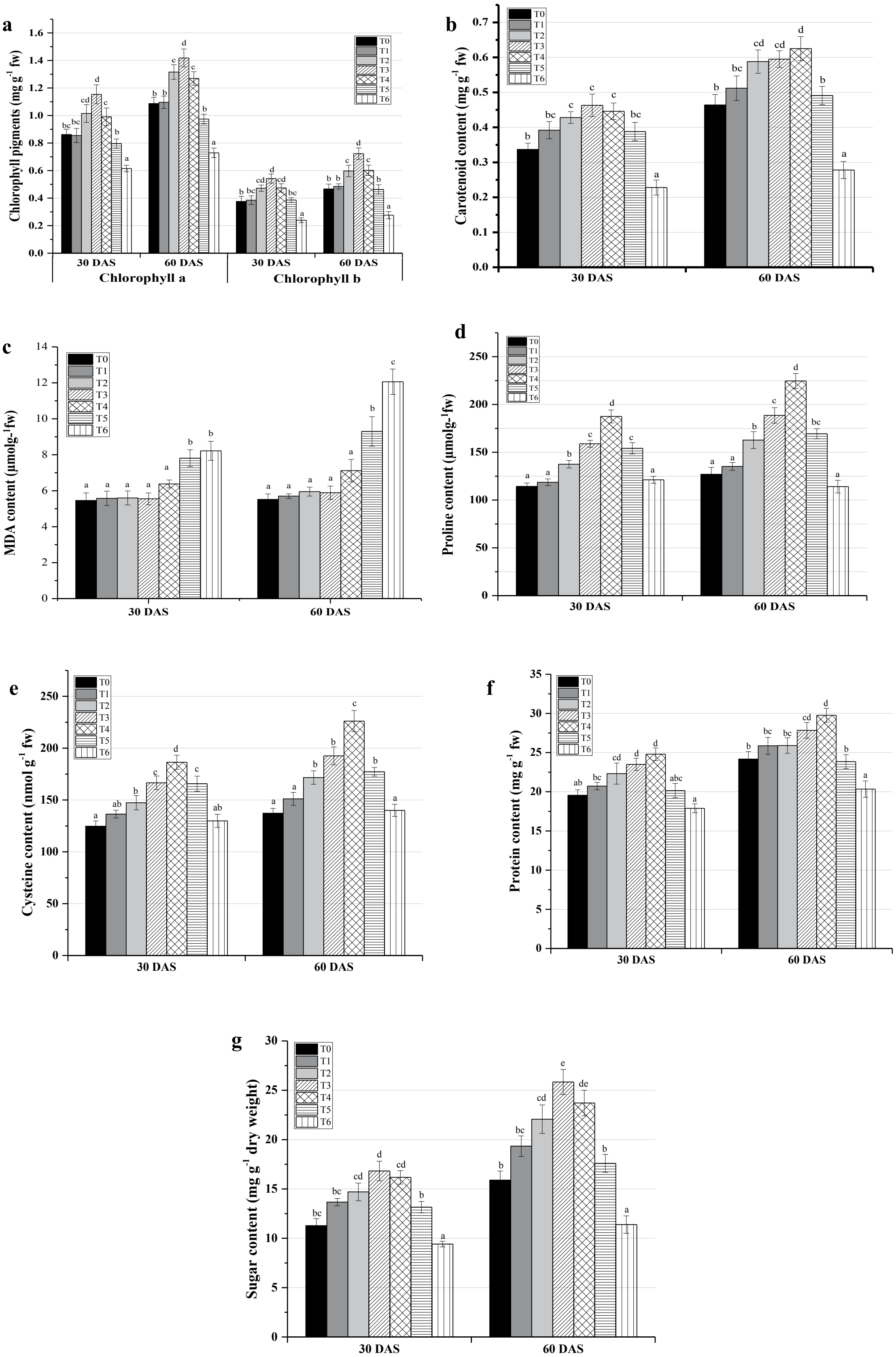

Figure 2: Effects of varying levels of soil amendments with sewage sludge on (a) chlorophyll a, b content (mg g^{-1} fw); (b) Carotenoid content (mg g^{-1} fw); (c) MDA content (µmol g^{-1} fw); (d) Proline content (µmol g^{-1} fw); (e) Cysteine content (nmol g^{-1} fw); (f) Protein content (mg g^{-1} fw); (g) Sugar content (mg g^{-1} dw) in leaves of *Brassica juncea* cv. Alankar at 30 and 60 days after sowing (DAS). (Mean ± SE; n = 4). Different small letters at each growth stage show statistically significant variation at $p < 0.05$ as per DMRT. (*T0: unamended soil; T1: 5%; T2: 10%; T3: 20%; T4: 40%; T5: 70% and T6: 100% sewage sludge*).

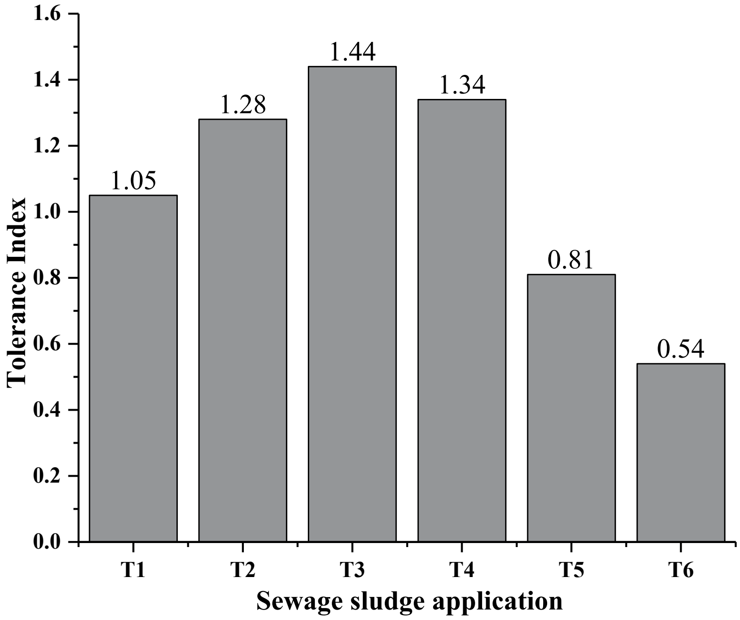

Figure 3: Tolerance index of *Brassica juncea* var. Alankar grown in soil amended with varying levels of sewage sludge. (*T0: unamended soil; T1: 5%; T2: 10%; T3: 20%; T4: 40%; T5: 70% and T6: 100% sewage sludge*).

plants increased significantly ($p < 0.05$) on application of 10 to 40% levels of sewage sludge in soil (Figure 3b). However, carotenoid content in leaves of mustard plants treated with 100% sewage sludge reduced significantly ($p < 0.05$) as compared to control (Figure 2b). The carotenoids are the accessory photosynthetic pigments, acting as nonenzymatic antioxidants and protect chlorophyll pigment by quenching the photodynamic reactions, replacing peroxidation and collapsing membrane in chloroplasts [39]. The enhancement in carotenoid content may be due to the evolved ability of the plant to counter the toxic effect of free radicals generated under metal stress [40]. The decrease in chlorophyll content on applications of higher amounts of sewage sludge may also be ascribed to the decrease in carotenoid content under excessive heavy metal stress.

Malondialdehyde (MDA) content: The formation of MDA content is considered as a measure of lipid peroxidation. Compared to control, it was not affected in leaves of mustard at lower amendment ratios, but enhanced significantly ($p < 0.05$) at T5 and T6 both at 30 and 60 days after sowing (Figure 2C). Maximum increases in MDA content were found in plants growing in 100% (T6) sewage sludge. MDA contents found were 50 and 118% more than their controls at 30 and 60 days of plant growth. MDA is a major cytotoxic product of lipid peroxidation which occurs due to malfunction of the scavenging system [14] and acts as an indicator of free radical production. An enhanced level of lipid peroxidation (high MDA production) has been related to the generation of toxic oxygen species [41]. Significant increase of MDA under the amendments T5 and T6 at both the stages of observations indicated the production of MDA in leaves as a biomarker to measure the level of oxidative stress. Singh and Agrawal [11] in sugar beet (*Beta vulgaris*) and Singh and Agrawal (2009) [31] in lady's finger (*Abelmoschus esculentus*) reported significantly higher levels of MDA content than control when plants were grown in soils amended with 20 and 40% SSA ratios. As *B. juncea* acts as a good metal accumulator plant [10,17], therefore it did not show higher MDA content in doses as reported by Singh and Agrawal (2007) [11] in sugar beet and Singh and Agrawal (2009) [31] in lady's finger.

Proline content: The data presented in Fig 4b show the variation in proline content in leaves of mustard grown in soil amended with varying application rates of sewage sludge. The 5% (T1) level of sewage sludge application did not cause any statistically significant change in the proline content in 30 and 60 days old plants as compared to control ($p > 0.05$). But, the sludge application rates from 10-70% (T2- T5) increased the proline content significantly ($p < 0.05$) as compared to control (Figure 2d). The maximum increase of proline content was recorded on T4 amendment with 64 and 77% more increase than the respective controls at 30 and 60 days after sowing. Proline accumulation occurs in wide range of plant species thriving under environmental stresses of varying nature [41]. The proline is also considered to have important protective roles in stressed plants [42]. Our findings indicate that relatively higher accumulation of heavy metals in the amended soil and their subsequent uptake and accumulation in mustard plants (Figure 4a,b,&c) may have affected the permeability of membranes, causing water deficit stress like condition leading to proline accumulation [43]. The main functions of metal induced proline accumulation are associated with osmoregulation and enzyme protection against dehydration. Proline also plays important roles during stress as a metal chelator and an antioxidative defense molecule [41]. Under the conditions of heavy metal stress, proline accumulation induces the formation of phytochelatins which chelate with metals and thus alleviating metal toxicity [44]. At 100% (T6) sewage sludge, the proline content diminished to a level around the control (Fig 2d). This could be due to the degradation of enzymes of proline cycle at this application rate.

Cysteine content: The cysteine content in leaves of 30 and 60 days old mustard increased significantly ($p < 0.05$) on application of 10(T2)–70%(T5) sewage sludge levels as compared to controls. The maximum increase in cysteine content was found on 40%(T4) sludge application rate with 50 and 65% more increase than their respective controls at 30 and 60 DAS (Figure 2e). Cysteine, a thiol (-SH) containing amino acid, is a key constituent of phytochelatins and metallothioneins which play an important role in metal detoxification [45]. The results of the present study are in confirmity with the findings of Vadas and Ahner (2009) [46] as they have also reported that the cysteine content increased in the roots of *Zea*

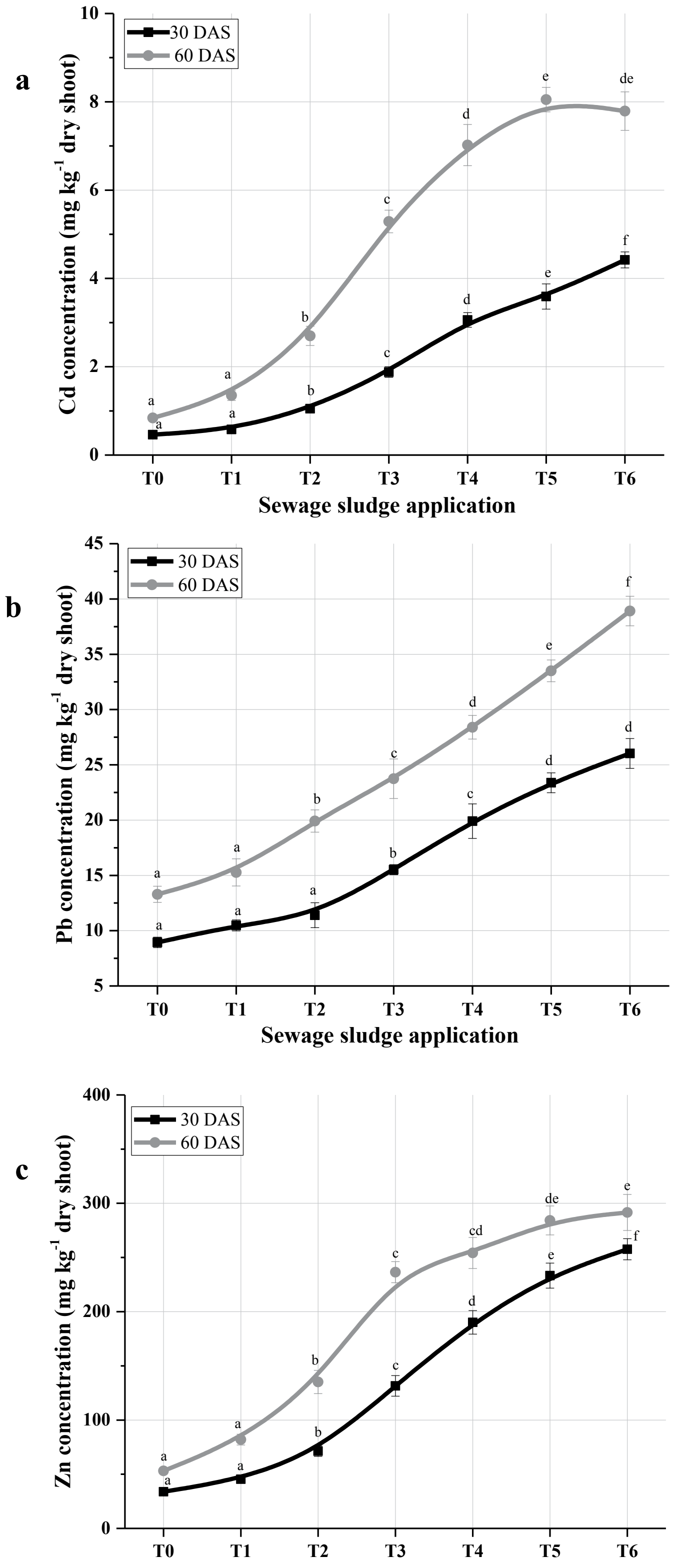

Figure 4: Heavy metal accumulation (mg kg^{-1} dry weight; mean ± SE; n = 4) in shoots of *Brassica juncea* var. Alankar grown in soil amended with varying rates of sewage sludge (a) Cadmium (b) Lead (c) Zinc. Different small letters show statistically significant variation at each growth stage (at $p < 0.05$ as per DMRT). (*T0: unamended soil; T1: 5%; T2: 10%; T3: 20%; T4: 40%; T5: 70% and T6: 100% sewage sludge*).

mays at a low level of Cd followed by a decrease with an increase in metal concentration. Increased levels of cysteine contents in leaves of mustard correspond to the level of tolerance exhibited by sludge-treated plants. However, the decrease in cysteine content in the plants under higher amendments of sewage sludge was probably due to decreased activities of sulfate reduction enzymes, ATP sulfurylase, and adenosine 5-phosphosulfate sulfotransferase under metal stress [47].

Protein content: The protein content in leaves of 30 days old mustard increased significantly ($p < 0.05$) as compared to control when treated with 10(T2)–40%(T4) sewage sludge application rates, whereas at 60 DAS, protein content increased on addition of 20 (T3) and 40% (T4) sludge application rates (Figure 2f). The highest content of proteins was recorded on amendment of soil with 40% (T4) sewage sludge with 27 and 23% more increase than their controls at 30 and 60 days of plant growth, respectively. The increase in total protein content in the leaves of selected mustard on application of sewage sludge upto 40% might be another defense strategy of metal stressed plants to cope up with the toxicity. It appears that high metal accumulation in tissues of selected mustard plants might have triggered the synthesis of heavy metal detoxifying low molecular weight proteins like phytochelatins and metallothioneins [45], which is evident from the increase in cysteine content (Figure 2e). The cysteine containing proteins have higher affinity to bind with heavy metals and detoxify them [44]. The total protein content reportedly enhanced in *Corchorus, Eruca, Raphanus, Daucus, Lactuca*, *Spinacia* and *Triticum* grown in sewage sludge amended soils [46,31]. Higher concentrations of sewage sludge (≥ 70%) reduced the total protein content in present study (Figure 2f) possibly due to increased protease activities leading to break down of soluble proteins.

Sugar content: The analysis of our data revealed that sugar content in leaves of 30 days old mustard increased significantly ($p < 0.05$) on 20% (T3) level of sewage sludge. However, application of 10 (T2)–40% (T4) levels of sewage sludge increased the sugar content in leaves of 60 days old mustard plants ($p < 0.05$). The mustard plants grown in 100% (T6) sewage sludge registered the lowest sugar content ($p < 0.05$) in leaves at both the sampling periods (Figure 2g). The increase in sugar content in present study may be due to corresponding increase in chlorophyll contents (Figure 2a), leading to higher photosynthetic activity. Singh and Sinha (2005) [12] reported the similar results of sugar content in *B. juncea* grown in soil amended with various ratios of tannery sludge. Sugar contents in leaves of sweet potato also showed increments when grown in soil amended with sewage sludge [48]. The decrease in sugar content in the leaves of selected mustard plant at 100% (T6) ratio of sewage sludge (Figure 2g) may be due to inhibition in synthesis of photosynthetic by-products in plants [12] caused by reduced chlorophyll contents (Figure 2a) at this amendment level.

Yield attributes: Application of 5 (T1) to 70% (T5) sewage sludge application rates did not have any statistically significant impact on the number of seeds per siliqua ($p > 0.05$), but 100% (T6) sewage sludge level reduced the seed number per siliqua significantly ($p < 0.05$) as compared to control. Mustard plants treated with 20% (T3) sewage sludge had significantly ($p < 0.05$) highest number of siliquae per plant as compared to control (Table 2). Seed weight per plant increased significantly ($p < 0.05$) in mustard plants grown in 10% (T2)–40% (T4) sludge amended soils (Table 2). Seed weight in mustard plants increased by 18.90, 41.80 and 15.42% at 10 (T2), 20 (T3) and 40% (T4) SSA rates, respectively, as compared to those grown in unamended soil. The yield attributes (siliquae per plant, seed weight per plant) significantly ($p < 0.05$) reduced in mustard plants when grown in 70% (T5) -100% (T6) levels of sewage sludge (Table 2). Similar results were also found by several other scientists. The yield of *Helianthus annus* [49], *Abelmoschus esculentus* L. cv. Varsha uphar [31] and *Oryza sativa* [6] were also found to increase on application of 1.4 kg m^{-2}, 2.51 kg m^{-2} and 12 kg m^{-2} sewage sludge, respectively. The rise in the productivity due to the sludge amendment in soil is attributed to the increase in nutrient availability to the plants [11]. But at higher doses of sewage sludge, the availability of the macronutrients might have been outplayed by the availability of heavy metals in the soil which caused reduction in the yield. The decrease in yield of the selected plant at higher applications of sewage sludge may also be ascribed to high uptake and accumulation of heavy metals in aerial parts of the plant (Figure 4a,b,c). In contrary to the present findings, Singh and Agrawal (2007) [11] reported significant reduction in the yield of *Beta vulgaris* grown in soil amended with 40% sewage sludge. Increase in yield even at elevated accumulation of heavy metals in present study could be due to relatively higher tolerance of selected mustard cultivar to the elevated levels of accumulated heavy metals.

Harvest index (HI) of mustard increased significantly ($p < 0.05$) on applications of 20 (T3) and 40% (T4) sewage sludge rates as compared to control (Table 2). At higher ratios (≥ 70%) of sewage sludge applications, the reduced HI corresponded with decrease in chlorophyll and carotenoid contents and higher accumulation of heavy metals as recorded in the present study (Table 2, Figure 2a,b; 4a,b,c). Togay, et al. [30] reported over 40% increase in HI of *Phaseolus vulgaris* grown at 6 kg m^{-2} sewage sludge application rate. The tolerance index (TI) above 1.0 signifies resistance and below 1.0 susceptibility. The tolerance index on 70% (T5) and 100% (T6) application rates of sewage sludge were less than 1.0, indicating that the selected cultivar of mustard was susceptible to higher amounts of sewage sludge applications (Figure 3). The tolerance of plants was higher (TI > 1) on addition of 5 (T1)–40% (T4) sewage sludge in the soil (Figure 3), suggesting that the selected cultivar showed positive response upto treatment T4.

Heavy metal accumulation in mustard shoot: The heavy metals accumulation in shoots of mustard treated with varying levels of sewage sludge was also determined at 30 and 60 days after sowing. Concentration of Cd in mustard shoots increased with levels of sewage sludge applied in soil and plateaued between 40% (T4) and 100% (T6) sewage sludge ratios at 60 DAS (Figure 4a). The concentrations of Pb in shoots at the observed growth stages of mustard plants increased almost linearly with the increase in sewage sludge application rates (Figure 4b). The concentration of Zn in mustard shoots followed a sigmoid pattern with increasing levels of sewage sludge in soil, indicating that the Zn concentration

Amendments	Seeds siliqua^{-1}	Siliquae plant^{-1}	Seed weight (g plant^{-1})	Harvest Index
T0	12±0.41^{ab}	77±2.35^{b}	5.55±0.15^{c}	0.39±0.01^{b}
T1	12.25±0.75^{ab}	78±2.48^{b}	5.95±0.16^{cd}	0.41±0.01^{ab}
T2	12.5±0.50^{a}	80.5±2.47^{ab}	6.60±0.30^{b}	0.42±0.02^{ab}
T3	13±0.41^{a}	87±2.86^{a}	7.87±0.33^{a}	0.45±0.01^{a}
T4	12±1.08^{ab}	81±2.38^{ab}	6.40±0.21^{b}	0.44±0.01^{a}
T5	10.25±0.75^{bc}	65±2.86^{c}	4.62±0.19^{d}	0.39±0.01^{b}
T6	8.5±0.29^{c}	51±3.16^{d}	2.85±0.09^{e}	0.35±0.02^{c}

Table 2: Effects of varying levels of soil amendments with sewage sludge on various yield attributes of *B. juncea* var. Alankar. (Mean ± SE; n = 4). Different superscripts in each parameter show statistically significant variation at $p < 0.05$ as per DMRT. (T0: unamended soil; T1: 5%; T2: 10%; T3: 20%; T4: 40%; T5: 70% and T6: 100% sewage sludge).

decreased with the increase in sludge amendment rates (Figure 4c). McBride, et al. [50] suggested that certain plant mechanisms render the decrease in uptake at high metal loadings. These mechanisms include exclusion of metals, limited translocation from root to shoot and saturation of the metal transport channels at high metal concentrations [51]. Chaney and Ryan [52] reported that higher rates of biosolids application increased metal adsorption capacity of soil in addition to metal concentration due to which the metal availability is levelled off at high biosolids application rate. Among the three studied heavy metals, accumulation of Zn in shoot was more pronounced than the Cd and Pb. The movement of Zn from root to shoot is facilitated by proteins of ZIP (SLC39) and CDF/ZnT (SLC30) families [53]. Moreover, P-type ATPase transporters have been identified in a wide range of plants which are implicated in the transport of a range of essential metals like Cu^{2+} and Zn^{2+} across cell membranes [54]. Thus, the higher accumulation of Zn than other two metals in shoot can be explained by the presence of specific transporting mechanisms for the essential metals like Zn, which are lacking for non-essential elements.

Moreover, our results revealed that Cd concentrations in shoots of mustard at varying levels of sludge amendments ranged between 0.46 to 4.42 and 0.84 to 7.79 mg kg^{-1} dry weight at 30 and 60 days of plant growth, respectively (Figure 4a). Lead concentration expressed on a dry weight basis in shoots of *B. juncea* grown in different sludge amendments, reached values of 8.94 to 26.03 and 13.29 to 38.91 mg kg^{-1} dry weight at 30 and 60 DAS, respectively (Fig 4b). Our results signify a possible health risk if the shoot material of *B. juncea* was used for human consumption or eaten by animals, due to the amounts of Cd and Pb in shoot tissue exceeding Indian permissible limits {for Cd = 1.5 mg kg^{-1} and Pb = 2.5 mg kg^{-1}; [26]} at most of the treatments. The Zn concentrations also exceeded the threshold amounts for animal feeds [> 60 mg kg^{-1}; [55]] in most of the amendment levels at both the sampling periods, indicating contamination and a possibility of health risk if consumed.

Conclusion

Sewage sludge amendment modified the soil properties as its addition increased organic C, N and heavy metals in soil. Plants grown in soil amended with 5 (T1) to 40% (T4) sewage sludge ratios showed positive response by increasing the growth characteristics and seed yield due to higher nutrient availability in resulting sludge-soil mixtures. The present study clearly indicates that varying amendment levels of sewage sludge in soil increased the concentrations of Cd, Pb and Zn in resulting soil-sludge mixtures as compared to the unamended soil. The amounts of these three heavy metals also increased in the shoots of mustard when grown in sewage sludge-amended soil. Despite the positive responses shown by selected cultivar of mustard in terms of growth and yield, the concentrations of Cd, Pb and Zn in its shoots were above the permissible limits of Indian standard on most of sludge amendment levels. Such higher amount of accumulation of heavy metals in edible parts such as shoots is a major concern due to risk to human health at higher consumption rate. It is evident from the values of TI in present study that despite the accumulation of high amounts of heavy metals in the shoot, the selected cultivar is well adapted to the elevated levels of accumulated heavy metals due to increased level of antioxidant (cysteine) and various other biochemical parameters like proline, protein in leaves when grown in soil amended with 5 to 40% sewage sludge. Despite the accumulation of higher amounts of heavy metals, no any visual toxicological symptoms were evident in the present study except at the higher amendment levels.

Conflict of Interest

No conflict of interest to disclose.

Acknowledgments

Authors are very thankful to the chairman, Department of Botany, Aligarh Muslim University, Aligarh, for allowing to avail necessary facilities during this research work.

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Received Date: April 26, 2018, **Accepted Date:** May 14, 2018, **Published Date:** May 25, 2018.

Citation: DarMI, Naikoo MI, Khan FA, Green ID (2018) Assessing the Feasibility of Sewage Sludge Applications for the Cultivation of Brassica *Juncea L.*: Metal Accumulation, Growth, Biochemical and Yield Responses. J Envir Sci Renew Res 1(1): 104.