Chapter-5

DISCUSSION

Population explosion, urbanization and economic disparity lead to the crunch of food resources in many parts of the world, especially in developing nations. To meet the demands of the ever growing human population, production of food has been increased by increasing the resource availability. The resources required for food production are mainly land and water, thus, agriculture is being carried out at contaminated sites and by using wastewater for irrigation (FAO, 2008; FAO *et al*., 2015; Raja *et al*., 2015). These practices lead to a buildup of contaminants in soil and among all these contaminants, heavy metals are significantly important considering their recent increase in the biosphere and the toxic effects associated with them (McBride, 2003; Roy *et al*., 2010). Accumulation of heavy metals in soil is reported to be one of the major causes for the genotoxic nature of soil. Studies have been carried out throughout the world to assess the gentoxicity of soil along with physicochemical parameters so as to determine the quality of this important component of ecosystem (Cabrera and Rodriguez, 1999; Cotelle *et al*., 1999; Mielli *et al*., 2009). Irrigation of agricultural land with wastewater is reported to increase the buildup of heavy metals in soil, thus increasing its genotoxicity (Huibers and Van Lier, 2005; Singh *et al*., 2009; Flores-Magdaleno *et al*., 2011).

Increase in heavy metals in soil leads to uptake of contaminants by food crops which enter food chain and toxic effects are seen at all trophic levels. Accumulation of heavy metals is higher in vegetables as compared to grains owing to the fleshy tissues (Islam *et al*., 2007; Peralta-Videa *et al*., 2009). Throughout the world, many studies have shown high concentration of heavy metals in vegetables growing at contaminated sites and/or those irrigated with wastewater. At many places, concentration of these metals in vegetables is found to be beyond permissible levels (Chove *et al*., 2006; Felix-Henningsen and Urushadze, 2010; Osma *et al*., 2012; Lion and Olowoyo, 2013; Hu *et al*., 2014). The hazard associated with consumption of heavy metals with food crops is measured by assessing Metal Pollution Index (MPI) and Hazard Quotient (HQ) (Sharma *et al*., 2008; Song *et al*., 2009; Sun *et al*., 2010; Huang *et al*., 2014). Levels of heavy metals in vegetables are reported to be beyond acceptable limits in many parts of the world thus representing potential health risk, these food crops can cause to the entire population (Petroczi and Naughton, 2009; Jolly *et al*., 2013; Hu *et al*., 2014). Monitoring of heavy metals in food crops growing on contaminated sites is essential to assess the probable risk of heavy metal induced toxicity to the population of the area.

Amritsar (31.64°N 74.86°E) spread across an area of 2,683 km² being part of food bowl state of India, is amongst the major producers of vegetables in the country. The average annual rainfall of Amritsar district is 541.9 mm leading to semi-arid conditions throughout the year (Govt of Punjab, 2016). The average depth of ground water table is 302-450 m and the annual draft from ground water exceeds much more than the annual replenishment of groundwater resources in the district. The net irrigated area in Amritsar district is 2170 km^2 (Central Ground Water Board, 2015). To manage the scarcity of water, irrigation in many parts of the district is carried out using wastewater. Also, the 217 MLD of domestic wastewater is discharged and there is no functional sewage treatment plant to treat the domestic wastewater (Rajya Sabha Secretariat, 2015). A network of sub-drains discharges the effluents in mainly two drains, Tung-Dhab and Municipal wastewater drains. Agriculture practices are being carried out around both these drains. Municipal wastewater drain is also used for irrigating the fields around them.

Efforts are being made to reduce the uptake of heavy metals in food crops so as to prevent their possible transfer to food chain and reduce the risk of toxicity to humans. The reduction in uptake can be achieved by reducing the mobility of heavy metals in soil. The wide array of remediation strategies and amendments are used to reduce the effect of heavy metal uptake. Soil amendments are considered to be an ideal solution to reduce the uptake by food crops grown on contaminated land. The selection of amendment is based on the availability and cost of the material. The inexpensive and easily available amendment is considered to be the best option which can help in stabilization of metal in soil (Clemente *et al*., 2015). Inorganic amendments are largely used to stabilize the soil but excessive use of inorganic amendments alter the physicochemical aspects of soil (Branzini and Zubillaga, 2012; Bauddh and Singh, 2015). Also, the biochars produced from the organic waste is known to efficiently stabilize heavy metals in soil (Jefferson, 2010; Bian *et al*., 2014). A lot of agricultural and food waste is generated per capita leading to immense problem in management of this waste. The assessment of these wastes for their metal stabilization potential can help in mitigating some of the major problems pertaining to our surrounding environment.

There is a need to find suitable low cost amendments which do not induce any toxic effects to soil and help in reducing the heavy metal uptake.

5.1. Monitoring of Soil Samples along Wastewater Drains

5.1.1. Genotoxicity assessment of soil using *Allium cepa* root chromosomal aberration assay

Many bioassays are used throughout the world to assess the genotoxicity of soil and of all the bioassays, plant bioassays have gained more popularity as they are inexpensive. The chromosomal aberration assays using plant systems like, *Allium, Vicia, Lycopersicon* and *Tradescantia* to study the genotoxic effects of soil are validated and widely documented (Kristen, 1997; Cotelle *et al*., 1999; Maluszynska and Juchimiuk, 2005). *Allium cepa* root chromosomal aberration assay has gained popularity among other higher plant bioassays as onion bulbs are easily available and have larger and lesser number of chromosomes which makes it simple for observation of chromosomal aberrations. In the present study, soil samples were collected from the agricultural fields along wastewater drains of Amritsar district and analyzed for their genotoxic potential using *Allium cepa* root chromosomal aberration assay. The samples were collected for two different seasons i.e., winter and summer. Chromosomal aberrations were classified as physiological and clastogenic aberrations depending upon the cause of anomaly. The aberrations caused due to direct impact of toxin on chromatin material, leading to chromosomal breaks, bridges or ring formation were grouped under clastogenic aberrations. The aberrations caused due to action of toxin on spindle proteins resulting in complete or partial spindle inhibition, stickiness and anomalies such as laggards, vagrants or delayed anaphases were grouped under physiological aberrations. Also, the orientation anomalies were categorized as abnormal metaphases or anaphases and were also referred as physiological aberrations.

The results revealed that the percentage aberrations caused in root tip cells of treated onion bulbs were significantly higher than the control bulbs. Physiological aberrations were more common than clastogenic aberrations in all treatments. The most common aberration was delayed anaphase in which the chromosomes are unable to separate themselves completely during anaphasic separation. The next most common aberration was found to be stickiness among all samples. No instance of a vagrant or laggard chromosome was found in squashes prepared from root tip cells of control onion bulbs but both these aberrations were observed in samples from all sites. Some of the abnormalities at metaphase and anaphase were grouped as abnormal metaphase and abnormal anaphase. These were the aberrations in which either the chromosomes were not aligned properly or there was some spatial or directional variation from the normal stage. Among clastogenic aberrations, chromatin bridges were most commonly observed. The spectrum of aberrations showed almost similar pattern for all samples from both the seasons.

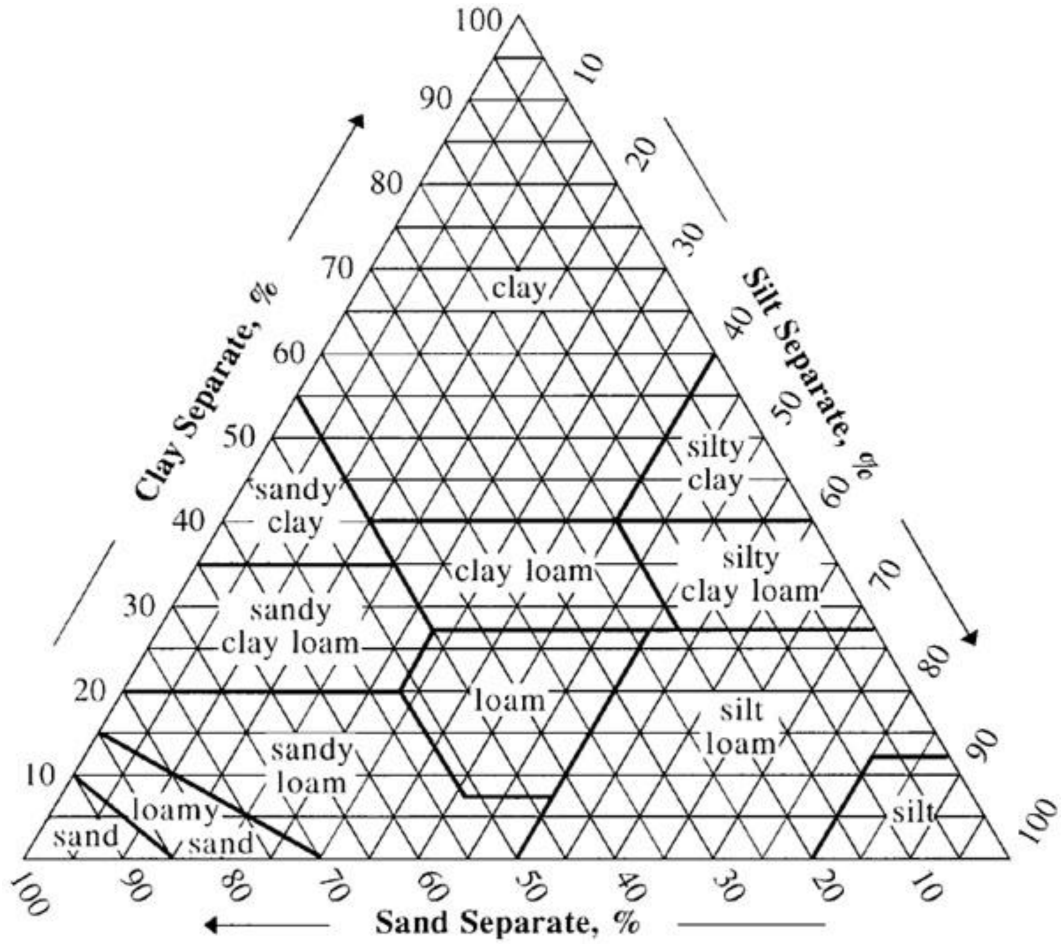
Genotoxicity of soil samples can be attributed to enormous amount of fertilizers and pesticides used during agriculture. But, we cannot ignore the site description in the present study, since all the soil samples studied are exposed to wastewater, there is a possibility of addition of heavy metals to these soils through the wastewater drains which can add to genotoxic potential of soil. In summer samples, the genotoxicity of samples from site irrigated with wastewater (Site 3) is highest amongst all other samples. Also, the site which was closer to industrial discharge (site 1) showed relatively higher percentage of chromosomal aberrations than the site farther from the industrial discharge (site 2) in samples from both the seasons. The decrease in percentage of aberrant cells in winter samples from site 3 may be because of the dilution of toxins post rainy season.

Our results are in accordance with the studies conducted across the world which have reported high percentage of chromosomal aberrations induced due to wastewater (El-Shahaby *et al*., 2003; Grisolia *et al*., 2005; Ngozi, 2008; Ukaegbu and Odeigah, 2009). Many studies in the past have reported the correlation between heavy metals and chromosomal aberrations in *Allium cepa* root chromosomal aberration assay (Fiskesjo, 1981 and 1988; Borboa and De La Torre., 1996; Steinkellner *et al*., 1998; Inceer *et al*., 2000). Presence of heavy metals in wastewater from industries and commercial usage is a common instance. In previous years, a number of studies have documented correlation between the presence of heavy metals in agricultural soil and induction of chromosomal aberrations (Ivanova *et al*., 2008; Kaur *et al*., 2014a; Soodan *et al*., 2014). Also studies have revealed that soil irrigated with wastewater has higher genotoxic potential than soil irrigated with fresh water (Song *et al*., 2007; Alam *et al*., 2009). In the present study, soil samples from agricultural fields irrigated with ground water had almost similar genotoxic potential as those irrigated with wastewater. Thus, the percolation of water from wastewater drain to the fields poses similar threat as posed by the irrigation with wastewater. Chromosomal aberrations are structural modifications which are generally caused due to DNA breaks, inhibition of DNA synthesis and replication of modified DNA (Leme and Marin-Morales, 2009). In an another report from Amritsar city, the agricultural soil samples having high concentration of heavy metals induced chromosomal aberrations in root tip cells of onion bulbs (Chahal *et al*., 2014).

Genotoxicity of soil observed in the present study can thus be attributed to the metals or toxins present in wastewater. Also, it is to be noted that the genotoxic potential of site irrigated with wastewater and sites which were in vicinity of wastewater was comparable. This shows that the metals/toxins from wastewater drain percolate to the soil causing the genotoxic effects. Like all previous reports we confirm *Allium cepa* root chromosomal aberration assay to be efficient tool in monitoring soil pollution.

5.1.2. Physicochemical analysis of soil samples

Soil texture is an important parameter as the mobility and availability of metal ions in soil largely depends on the texture of soil. Soil texture is analyzed on the basis of percentage of different sized particles in soil matrix. The different particle sizes are grouped as: sand (0.75-1.5 mm), silt (0.02-0.75 mm) and clay (0.0015-0.02 mm). In the present study, percentage of sand in soil samples from both seasons ranged from 57.01% to 59.82%, percentage of silt ranged from 0.95% to 1.12% and percentage of clay was in the range 39.24% to 41.95%. Thus, maximum percentage of particles of soil samples in the present study was sand followed by clay. The soil structural triangle as given by USDA reveals that all the samples fall in the category of sandy clay.



SOIL TEXTURAL TRIANGLE

Source: http://www.nrcs.usda.gov/

In several reports, many different percentages of various particles have been reported. In Botswana, the agricultural soil had sand ranging from 50-82.8%, silt ranged from 7.8-18.2% and clay ranged from 9.4-35.5% (Dikinya and Areola, 2010). The agricultural soil in Peshawar, Pakistan was found to fall under the category of clay loam (Nafees and Amin, 2014). In another study of soils samples irrigated with wastewater in Baoding city, China, the percentage of sand ranged from 39.82-53.86%, silt ranged from 30.09-64.20% and clay ranged from 14.04-22.07% and groundwater irrigated soil samples had sand in the range of 45.84-49.85%, silt ranged from 36.11-40.12% and clay varied from 14.04-16.05% (Xue *et al*., 2012). In a study to estimate physicochemical and genotoxic characteristics of agricultural soil in some fields of Amritsar, it was reported that the percentage of sand varied from 55.60-74.70%, silt ranged from 0.28-0.77% and clay was in the range of 24.53-44.01% (Kaur *et al*., 2014a).

pH of soil is another essential parameter which gives the measure of hydrogen ion concentration and influences the mobility and availability of minerals in the soil. In alkaline soil, the availability of macronutrients increase while in an acidic soil, the availability of micronutrients tends to increase (Singh *et al*., 2009; Felix-Henningsen and Urushadze, 2010). Strong influence of pH on the availability of heavy metals to rice plants was observed (Zeng *et al*., 2011a). Anthropogenic activities tend to change the pH of soil thus affecting the soil toxicity. In the present study, pH of soil varied from 7.29-7.9. In a study of sewage irrigated soils in Baoding city, China, pH was found to be in the range of 7.40-8.18 (Xue *et al*., 2012). In wastewater irrigated soil samples from Glen valley, Botswana, pH ranged from 5.55 to 7.17 (Dikinya and Areola, 2010). In a previous report about agricultural soils of Amritsar city, pH of agricultural soils ranged from 8.14 to 8.25, these samples were irrigated with fresh water (Kaur *et al*., 2014a). In another study, pH of soil samples from various agricultural sites irrigated with wastewater was in the range of 7.03-8.23 (Singh *et al*., 2009). Many other studies have shown alkaline nature of agricultural soil irrigated with wastewater (Gupta *et al*., 2010; Flores-Magdaleno *et al*., 2011).

Alkalinity is the acid neutralization capacity of soil. Alkalinity of soil is the result of carbonates, bicarbonates, nitrates, phosphates, borates and sulphates. Carbonates are mainly responsible for the maximum alkalinity of soil (Katnoria *et al*., 2011). Alkalinity of soil samples in the present study ranged from 0.67-1.29 meq/100 g. In a previous study, alkalinity of soil samples from agricultural fields under rice cultivation ranged from 1.20-2.33 meq/100g (Kaur *et al*., 2014a). Alkalinity of soil samples from agricultural fields in Gwalior, India varied from 50 mg/l to 114 mg/l (Wani *et al*., 2014).

Soil is the structural unit for the life forms. The plant growth on soil depends largely on many inorganic components of soil. The nutrients like nitrates and phosphates which are essential for plant growth are fixed in soil through the biogeochemical processes and then made available to plants. Nitrogen in the form of nitrates (NO_3^-) is required by the plants. Fixation of atmospheric nitrogen through nitrogen fixing bacteria, mineralization of organic matter ($R\text{-}NH_2$) to form ammonium compounds and further nitrification leading to formation of nitrates which are required for plant growth. Apart from biological fixation, nitrogen from atmosphere also gets fixed in soil through lightening and anthropogenic activities like addition of fertilizers. World Bank in a report mentioned that irrigation using wastewater can increase the nitrate content in soil (World Bank, 2010). In the present study, the content of nitrates varied from 0.95-2.12 mg/g. It was observed that nitrate content in samples collected in winter season was higher than those collected in summer season, which can be attributed to atmospheric fixation of nitrogen during rainy season. In a study to analyze physicochemical characteristics of industrial effluent contaminated soils of Amritsar, nitrate content was found to range from 0.76-1.69 mg/g (Katnoria *et al*., 2011). In industrial wastewater irrigated soils of Lohta village, India, nitrate content varied from 14.2-32.13 mg/kg (0.014-0.032 mg/g) (Rai and Tripathi, 2008). In an agricultural fields under rice cultivation in Amritsar, nitrate content ranged from 0.004-0.016 mg/g (Kaur *et al*., 2014a). In another study of agricultural soil in Amritsar, the nitrate content ranged from 0.27-0.33 mg/kg (Chahal *et al*., 2014). In our study, the nitrate content was relatively higher than other studies from Amritsar and this can be attributed to the effect of wastewater.

Phosphorus is another important nutrient for plant growth. Desorption from mineral surfaces, dissolution of phosphorus containing compounds and mineralization of organic phosphorus lead to increase in inorganic phosphorus in the form of phosphates in soil which are taken up by plants for normal growth (Cabrera and Rodriguez, 1999). Phosphorus is major limiting factor for the plant growth and is often applied in large quantities in agricultural soil in the form of phosphate fertilizers, the leaching of which can lead to eutrophication of water bodies. Phosphate content of soil in the present study ranged from 1-2.12 mg/g. In a study to analyze the effect of wastewater irrigation on soil, the available phosphorus content varied from 5.73 µg/g to 17.60 µg/g (Singh *et al*., 2009). In industrial waste contaminated soil, the phosphate content varied from 1.67-3.053 mg/g (Katnoria *et al*., 2011). Chahal *et al*. (2014) reported the content of phosphate in the range of 0.01-0.05 mg/kg in soil samples from Amritsar. Kaur *et al*. (2014a) analyzed the physicochemical parameters of soil samples from agricultural fields under rice cultivation in Amritsar district and found that the phosphate content ranged from 0.614-0.76 µg/g.

5.1.3. Elemental analysis

Calcium is an important component of soil that is required for plant growth. It is also an important component of cell wall. The amount of calcium in soil also influences the uptake of other nutrients. In the present study, range of calcium in soil varied from 7558.33 mg/kg to 13585.26 mg/kg. The common range of calcium in agricultural soil world over is 7000-500,000 mg/kg (Alloway, 2013). Content of calcium in soil is studied throughout the world to analyze the soil-metal-plant interactions (Robinson, 1999; Castaldi *et al*., 2005; Katnoria *et al*., 2008, 2011; Tiwari *et al*., 2011; Dawaki *et al*., 2013; Kaur *et al*., 2014a, 2014b; Sharma *et al*., 2014). The extractable Ca^{2+} ions in wastewater irrigated samples were found to be in the range of 105.73-704.85 µg/g (Singh *et al*., 2009). In a study of soil samples from rice fields in Amritsar, the content of calcium was found to be in the range of 53.33-90.6 mg/g (Kaur *et al*., 2014a). The content of calcium in present study is relatively less than that reported in other studies from Amritsar (Katnoria *et al*., 2008, 2011; Kaur *et al*., 2014a, 2014b).

Magnesium is another essential element for living systems and it is required for the chlorophyll production in the plants. It also aids in uptake of other minerals. In healthy soils, the ratio of Ca/Mg is required to be less than one i.e., content of magnesium should be higher than content of calcium in soil (FAO, 2016). In the present study range of magnesium in soil samples was found to be 2060.33-5481.67 mg/kg. The content of magnesium in samples studied was found to be lesser than content of calcium in them, thus increasing the possible stress of soil for plants. In previous studies content of magnesium in rice fields of Amritsar was found to be 189.3-446.50 mg/g (Kaur *et al*., 2014a). Generally, the content of magnesium in agricultural soil world over ranged from 20-10,000 mg/kg (Alloway, 2013).

Sodium is present in minerals present in earth's crust. It helps plants to concentrate carbon dioxide and also aids in normal metabolism of plants. But excess of sodium can cause toxic symptoms to plants and can also result in dehydration of plant tissues. In the present study, the content of sodium in soil samples was found to vary from 825 mg/kg to 2114 mg/kg. In another study, the extractable Na in wastewater irrigated soil samples was found to be in the range of 85-260.33 µg/g (Singh *et al*., 2009). In agricultural fields of Amritsar, the sodium content was reported to range from 0.07-0.39 mg/g (Kaur *et al*., 2014a). The content of sodium in agricultural soils irrigated with municipal waste was found to be in the range from 683-900 ppm (Pedrero *et al*., 2010). Our values were higher than other reports from Amritsar. The common range of sodium in agricultural soils throughout the globe is 750-7500 mg/kg (Alloway, 2013).

Potassium is amongst the three most important nutrients in soil. It plays an important role in photosynthesis, stomatal opening and closing and regulation of CO_2 uptake. Potassium is also important for the production of ATP (Adenosine Triphosphate) as it triggers the enzymes required for the ATP synthesis. In our samples, content of potassium in soil was found to range from 732.67-877 mg/kg. Globally, the content of potassium in agricultural soil varied from 400-30,000 mg/kg (Alloway, 2013). In a study to analyze the physicochemical parameters of soil, the content of potassium was found to be in the range of 93.49-285.52 µg/g (Singh *et al*., 2009). Katnoria *et al*. (2011) reported the content of potassium in industrial waste contaminated soil of Amritsar to be in the range of 0.09-0.13 mg/g. In agricultural soils irrigated with municipal waste in Spain and Greece, the content of K was in the range of 358-380 ppm (Pedrero *et al*., 2010). In agricultural soil of Amritsar under rice cultivation potassium varied from 0.12-0.35 mg/g (Kaur *et al*., 2014a). Content of potassium in soil samples from present study was much higher than that reported from Amritsar which could be attributed to the exposure to wastewater.

Wastewater from various sources contains many heavy metals like copper, cadmium, iron and zinc. Continuous irrigation of agricultural soil with wastewater leads to buildup of heavy metals in soil. Also, percolation of water from adjoining drains can lead to addition of metals in the agricultural soil. In the present study, we have selected three sites as explained in chapter 2. Site 1 and Site 2 are adjoining the wastewater drain but are irrigated with groundwater among which site 1 is nearest to industrial discharge points. Site 3 was also adjoining a drain but was irrigated with wastewater. Analysis of heavy metals in samples from these sites revealed high content of many toxic metals in the soil. In the present study, we estimated content of Cu, Cr, Fe, Zn, Pb, Cd, Co and Mn in the soil samples collected from all sites for two different seasons.

Copper is a trace element which tends to accumulate in upper layers of soil and is needed for the metabolic functions of plants but higher concentrations of copper in soil have been reported to be toxic to plants (Reichman, 2002). Copper is required for physiological processes like nitrogen fixation and protein metabolism (Reichman, 2002; Vinod *et al*., 2012). Anthropogenic sources contribute largely to increase copper content in soil. Many activities like use of copper containing agrochemicals and deposition of industrial and municipal waste, increase the content of copper in soil (Mwegoha and Kihampa, 2010; Machado-Estrada *et al*., 2013). In the present study, content of copper in soil samples varied from 23.9-280 mg/kg. It is to be noted that the summer sample of site 3 showed maximum concentration of copper (280 mg/kg). This site is irrigated with wastewater. While, content of copper in summer samples from site 1 and 2 was 43.4 and 58 mg/kg respectively. In winter samples, the content of copper in samples from site 1, 2 and 3 was 23.9, 59.9 and 35 mg/kg respectively. It has been reported that content of copper in sewage sludges used for agriculture vary from 50-3300 ppm and this can be a source of large scale contamination (Kabata-Pendias and Pendias, 2001). In India, desirable limit of copper concentration in agricultural soil is 135-270 mg/kg (Awashthi, 2000). Internationally, the maximum allowable concentration of copper in agricultural soil is 140 mg/kg (European Union, 2002). In the soil samples collected for the present study, content of copper in soil sample from site 3 was found to be 280 mg/kg which is above permissible limits. Comparing our results with the studies carried out throughout the world we found that in a study to assess the effect of wastewater irrigation on soil samples the content of copper was found to range from 6.33-26.38 µg/g (Singh *et al*., 2009). Another study was carried out in dry tropical area of India to compare the agricultural soil of clean water irrigated fields with wastewater irrigated fields and significantly higher concentration of copper was reported in wastewater irrigated soil as compared to clean water irrigated soil (Singh *et al*., 2010b). In Lohta village, India, a study was carried out to analyze the quality of wastewater irrigated soil and crops and the range of copper in soil was found to be 40.08-42.16 mg/kg (Rai and Tripathi, 2008). In vegetable soils from Beijing-Tianjin city cluster, the content of copper was reported to range from 13-45.2 mg/kg (Wang *et al*., 2012). In sandy clay soil samples from Harare, Zimbabwe, content of copper was found to vary between 7-145 mg/kg as a result of long term irrigation with wastewater and the content of copper in top soil was significantly higher than the content of copper in sub-soil (Mapanda *et al*., 2005). It is to be noted that our samples also fall under the sandy clay category. In another study in wastewater irrigated areas in Titagarh, West Bengal, India, the range of copper was 22-166.5 mg/kg (Gupta *et al*., 2012). In soils irrigated with river water in Addis Ababa, Ethiopia concentration of copper ranged from 25.1-51.4 mg/kg (Weldegebriel *et al*., 2012). A study was carried out to assess the effect of irrigation with treated wastewater in Varanasi, India and the content of copper in soil varied from 32.3-123.6 mg/kg (Mishra and Tripathi, 2008). A previous study, to analyze the physicochemical parameters of soil from agricultural fields of Amritsar irrigated with clean water exhibited the concentration of copper to vary from 19.2-58.1 mg/kg (Kaur *et al*., 2014a). It is to be noted that concentration of copper in all soil samples from in present study was comparable to those reported earlier throughout the world but in a sample collected during summer season from wastewater irrigated site the content of copper was found to be much above the international safe limits. Excess of copper can cause toxicity to plants and as well as can enter food chain.

Chromium is present in nature in three stable forms Cr (0), Cr (III) and Cr (IV). It is toxic to plants and reduces their germination rate and growth. There are no documented permissible limits with respect to chromium in agricultural soil in Indian Standards. As per European Union (2002), Permissible limit for chromium in agricultural soil is 150 mg/kg. In the present study, content of chromium in soil samples from all sites ranged from 22.23-38.78 mg/kg. All the samples in our study were within permissible limits. In wastewater irrigated area in Beijing-Tianjin city cluster, China, concentration of chromium in agricultural soil varied from 31.8-55.1 mg/kg (Wang *et al*., 2012). In industrial wastewater irrigated soil in Lohta village, India range of chromium was 1.06-1.4 mg/kg (Rai and Tripathi, 2008). Effect of long term irrigation using wastewater in soil of Harare, Zimbabwe was noticeable as the concentration of chromium varied from 33-225 mg/kg (Mapanda *et al*., 2005). Concentration of chromium in samples collected for present study was comparable to other reports across the globe (Jia *et al*., 2010; Ahmad *et al*., 2013; Alloway, 2013).

Iron is an essential nutrient required in traces by both plants and animals for their normal physiological functions. It is important component of various enzymes and is also involved in many metabolic reactions (Nagajyoti *et al*., 2010). There is no data on maximum allowable concentration of iron in agricultural soils in India. In the present study, content of iron in soil samples varied from 853.43-1630 mg/kg. When compared with the studies carried out world over, it was observed that in a study to estimate heavy metals in soil and medicinal plants in Karak district, Pakistan, the content of iron in soil varied from 235.53-341.90 mg/kg (Shah *et al*., 2013). A study was carried out in Vadodara, Gujarat, India to compare the soil irrigated with tube well and that irrigated with industrial effluent and results revealed the content of iron in tube well irrigated soil to be 6.32 mg/g and that in industrial effluent irrigated soil to be 278.87 mg/kg (Tiwari *et al*., 2011). In soil samples composted with urban solid wastes from Rio de Janeiro, content of iron in soil was found to be 32.6 mg/g (Jordão *et al*., 2006). Iron is taken up as ferrous (Fe^{2+}) by plants. Generally, in soil iron exists as ferric ions but is transformed to ferrous ions in anaerobic conditions (Hartley *et al*., 2004). Toxicity affects associated with excessive concentration of iron include reduction in chlorophyll and protein, apotosis and lipid peroxidation (Nagajyoti *et al*., 2010).

Zinc is an essential micronutrient important for both plants and animals to carry out various metabolic functions. It is important for the chlorophyll production and carbohydrate metabolism in plants (Machado-Estrada *et al*., 2013). It is also important for animals to manage oxidative stress and apotosis (Nagajyoti *et al*., 2010). In the present study, the content of zinc in soil samples varied from 212.25-892.2 mg/kg. In two of the three sites, content of zinc in samples collected during summer season was higher than those collected in winter. Maximum permissible limit for zinc as given by European Commission is 300 ppm (European Union, 2002). Indian standards document maximum allowable concentration in agricultural soil to be 600 mg/kg (Awashthi, 2000). Toxicity associated with higher concentration of zinc is reported for both plants and animals (Reichman, 2002; Yang *et al*., 2004). In wastewater irrigated soils from Harare, Zimbabwe, content of zinc in soil ranged from 0.5-3.4 mg/kg (Mapanda *et al*., 2005). In a study carried out near old mining area of Romania, the zinc content in soil ranged from 136.26-359.33 mg/kg (Harmanescu *et al*., 2011). In agricultural soil near Dabaoshan mine in China, content of zinc in agricultural soil varied from 176-1100 mg/kg (Zhuang *et al*., 2009). In a study carried out in India, significant increase in zinc content was observed in agricultural soils irrigated with wastewater than those irrigated with clean water (Singh *et al*., 2010b). In a previous study carried out to assess heavy metal content in rice fields of Amritsar, zinc content varied from 61.6-96.5 mg/kg (Kaur *et al*., 2014a). The content of zinc in all samples collected for present study was higher than those reported in non-polluted area. Also, summer samples of two sites (SS-2 and SS-3) had content of zinc higher than maximum allowable concentration in India.

Lead is a toxic metal and is also considered as potential carcinogen by USEPA. In the present study, concentration of lead was below detection limits. Considering the dilutions we used for the soil samples in the present study, the instrument could detect the concentration as low as 0.1 mg/kg of soil. In a study, carried out to compare soils from tube well irrigated fields with industrial effluent irrigated fields in Vadodara, Gujarat, it was observed that content of lead in tube well irrigated fields was below detection limits, while in industrial effluent irrigated field was 13.12 mg/kg (Tiwari *et al*., 2011). In sewage-irrigated soils in suburbs of Baoding City, China, content of lead varied from 26.11-44.01 (Xue *et al*., 2012). Content of lead in soil near industrial areas of Dhaka, Bangladesh was found to be 39.14 mg/kg (Ahmad and Goni, 2010). Maximum allowable concentration of lead in agricultural soil is 300 mg/kg (European Union, 2002). As per Indian standards the safe limit for content of lead in agricultural soil is 500 mg/kg (Awashthi, 2000).

Cadmium is amongst the most toxic metals and is also considered as potential carcinogen. Genotoxic and mutagenic effects of cadmium are widely reported (Seregin and Ivanov, 2001; Wang *et al*., 2004; Depault *et al*., 2006; Kalaji and Loboda, 2007; Nagajyoti *et al*., 2010; Kwankua *et al*., 2012). Maximum allowable concentration of cadmium in agricultural soil as given by European Commission is 3 mg/kg (European Union, 2002). As per Indian standards, the concentration of cadmium up to 6 mg/kg is considered to be safe (Awashthi, 2000). In the present study, concentration of cadmium varied from 0.61-3.6 mg/kg. Maximum concentration of cadmium was found in summer sample of site irrigated with wastewater (SS-3). The concentration of cadmium in samples during present study was comparable to the concentrations reported across the world. In agricultural soil near old mining area, Romania, cadmium concentration varied from 0.4-2 mg/kg (Harmanescu *et al*., 2011). In paddy fields around Dabaoshan mine, China, concentration of cadmium ranged from 3-5.5 mg/kg (Zhuang *et al*., 2009). A study was carried out around Dinapur sewage treatment plant, Varanasi, to compare the effects of irrigation using wastewater on heavy metal concentration of soil and crops with those irrigated with clean water. The study revealed that concentration of cadmium in soil irrigated with clean water ranged from 0.81-2.62 mg/kg while cadmium content in soils irrigated with waste water varied from 1.92-4.53 mg/kg (Singh *et al*., 2010b). In an earlier study on agricultural soils of paddy fields of Amritsar, concentration of cadmium was found to be in range of 9.70-30 mg/kg (Kaur *et al*., 2014a). All the samples in the present study had cadmium concentration within the permissible limits as per Indian standards.

Cobalt is another naturally occurring essential micronutrient. It is present in the earth's crust in the form of ores. It is also a component of Vitamin B12 (Nagajyoti *et al*., 2010). Excess of cobalt is known to be phytotoxic, effects on shoot growth, translocation of other important minerals and water potential have been observed

(Chatterjee and Chatterjee, 2000; Kukier *et al*., 2004; Li *et al*., 2004; Bakkaus *et al*., 2005). There is no data on maximum allowable concentration for cobalt in India or by European Commission. In the present study, concentration of cobalt in agricultural soil samples varied from 14-27.2 mg/kg. The range of cobalt in agricultural soils worldwide is 0.1-70 mg/kg (Alloway, 2013). Concentration of cobalt reported in the present study was comparable to the levels of cobalt in agricultural soil reported worldwide. Among the polluted sites, range of cobalt in soil samples around industrial area of Islamabad, Pakistan, varied from 7.3-24.7 mg/kg (Malik *et al*., 2010). In another study of soil samples in the vicinity of Narora Atomic Power Station (NAPS), Narora, India, content of cobalt was reported to be in the range of 0.75-32.6 mg/kg (Singh *et al*., 2014).

Manganese is another essential element required for the activation of several enzymes which are involved in various physiological functions. Also, it aids in splitting of water during photosynthesis. Manganese in excess, can cause toxic effects to plants leading to necrosis, chlorosis and browning of plant (Nagajyoti *et al*., 2010). In the present study, content of Manganese was found to range from 62.5-86.96 mg/kg. In all the sites, content was higher in summer samples as compared to those in winter samples. In an earlier study, to estimate physicochemical parameters in agricultural soil under rice cultivation manganese content varied from 282.5-422.1 mg/kg (Kaur *et al*., 2014a). In agricultural soil irrigated with city effluent in Faislabad, Pakistan, concentration of manganese ranged from 164.1-217.4 mg/kg (Cańigueral and Vanaclocha, 2010). Concentration of Manganese in soil around Narora Atomic Power Station (NAPS), Narora, India varied from 3.6-35.5 mg/kg (Singh *et al*., 2014).

5.1.4. CHNS/O analysis

Elemental analysis was carried using CHNS analyzer. The organic carbon and nitrogen content of soil is an important parameter for the determination of soil quality. In the present study, the percentage of nitrogen in soil samples from site 3 which is irrigated with wastewater was found to be much higher than other two sites. This can be attributed to domestic sewage and sludge which have contributed to the nitrogen content of soil. Also, the percentage of organic carbon is higher in samples from the same site. The continuous irrigation of soil with wastewater is known to increase the organic matter in soil (Solís *et al*., 2005; Xu *et al*., 2010; Ghosh *et al*., 2012). Results from present study also reveal the similar effect. The C/N ratio in soil samples from the present study is very high, this situation creates a nitrogen deficient environment in soil for plants as it leads to mineralization of nitrogen and thus making it less available for the plants (USDA-NRCS, 2011). It is to be noted that C/N ratio of samples from sites 1 and 2 was higher than that of sample from site 3.

Pearson's correlation matrices for various parameters of soil is given in Table. 36. pH of soil was found to be positively correlated with content of zinc and cadmium, while was negatively correlated with chromium content. The mobility of zinc and cadmium increases with decrease in pH (Kabata-Pendias and Pendias, 2001). Thus, the increase in pH would stabilize these metals in soil and thus are not taken up by plant or leached through soil solution, leading to their increased concentration in soil. Nitrates in soil were positively correlated with phosphates, magnesium, iron, carbon and nitrogen. Significant positive correlation between nitrates and phosphates has also been documented in another study with reference to Amritsar (Kaur *et al*., 2014a). This can be attributed to the biogeochemical fixation. Also, the nitrification process which leads to the formation of nitrates releases protons in soil solution, which thus release the magnesium or other metal ions from the exchange sites (Poss and Saragoni, 1992). This explains the positive correlation of nitrates with magnesium and iron. Positive correlation of phosphates was observed with magnesium, carbon and nitrogen. The phosphorus cycle in soil is correlated with mineral complexes; the phosphates are released in soil by desorption from the mineral complexes. This explains the correlation between phosphates and magnesium. Both nitrates and phosphates were found to be positively correlated with carbon and nitrogen, this could be because of increased fertility by these nutrients, thus increasing living organisms and their residues. Calcium correlated positively with potassium, as K^+ ions are released in soil solution from the minerals and thus possibly releasing the Ca^{2+} ions. Also, magnesium correlated positively with potassium, this could be because of competition for exchange sites between calcium and magnesium (Poss and Saragoni, 1992). Also, magnesium content in soil significantly correlated with iron, carbon and nitrogen. Content of zinc was positively correlated with content of copper, chromium and zinc. Also, both copper and zinc have affinity for organic matter (Reichman, 2002).

5.2. Monitoring of vegetable samples

5.2.1. Heavy metals in vegetables

Water scarcity, crunch of arable land and need to meet food demand of exponentially growing human population has led to an increase in malpractices in agriculture. Throughout the world, cultivation is being carried out in the contaminated areas or by using wastewater for irrigation. These practices have led to an increase in heavy metal content in the crops, especially vegetables since they are hyperaccumulators. In the present study, we collected vegetable samples from the three sites and analyzed heavy metal content in them and compared the vegetables from all the sites. The bioaccumulation/bioconcentration factor (BAF/BCF) was calculated for different metals in each vegetable from different sites (Table 37). The BCF is the ratio of metal concentration in root to metal concentration in soil (Yoon *et al.*, 2006). The BAF is the ratio of metal concentration in shoot to metal concentration in soil (Cui *et al.*, 2007). Depending on the edible part of vegetable, BAF or BCF was calculated. BAF/BAC value higher than one indicates that plant is a hyperaccumulator for a particular metal.

In the present study, the most abundant metal among all vegetables was iron followed by cobalt, copper and cadmium and least accumulated metal was lead. Content of iron in vegetables ranged from 16.8-740 mg/kg. Leafy vegetables like fenugreek, coriander, mint and spinach were amongst the major hyperaccumulators. Though iron is an essential element but higher concentration of iron is known to produce stress responses in humans and animals. It has been demonstrated that concentration of iron as high as 60 mg/kg a day causes serious stress responses to humans (Abhilash *et al.*, 2013). In a study which was carried out in Ponzan, Italy, to estimate heavy metal content in vegetables, the content of iron was found to vary from 12.9-228.5 mg/kg (Bosiacki and Tyksiñski, 2009). In another study in Sri Ganganagar, Rajasthan, India, the iron content in wastewater irrigated vegetables varied from 116-378 mg/kg. Also, spinach and mint accumulated maximum iron content (Arora *et al.*, 2008). In another study pertaining to estimation of heavy metals in wastewater irrigated vegetables, content of iron ranged from 62-348.75 mg/kg (Nayek *et al*., 2010). The content of iron in food crops under optimum conditions worldwide ranged from 25-130 mg/kg (Alloway, 2013). Iron content in many of the samples in the present study was found to be much higher than that reported in previous studies. Content of iron in vegetables from site 3 which was irrigated with wastewater was significantly higher than that of other two sites. The BAF/BAC of iron was below 1 for all samples.

Cobalt though is an essential element, but its excess is known to cause phytotoxic effects in plants, also, it interferes in uptake of other essential elements (Nagajyoti *et al*., 2010). Human intake of higher concentrations of cobalt are known to cause serious toxic effects which can be attributed to its affinity to sulfhydryl group (Simonsen *et al*., 2011). Concentration of cobalt in all samples ranged from 9.2-130.67 mg/kg. Accumulation of cobalt was highest in spinach samples from both site 1 and site 3 (92 mg/kg and 130.67 mg/kg, respectively), while being highest in coriander samples (69 mg/kg) from site 2. Among all the sites, least concentration of cobalt was found in bulb vegetables. In general, samples from site 3 showed significantly higher content of cobalt as compared to samples from other two sites. The typical range of cobalt in food crops grown in optimum conditions varied from 8-100 mg/kg (Alloway, 2013). 8 out of 12 vegetable samples from site 1 and all of them from site 2 and 3 showed BAF/BCF higher than 1 for cobalt.

Copper is also amongst essential elements required for plant growth but has been reported to be second most hazardous metal after lead due to its high uptake in food crops (Cherfi *et al*., 2014). It was also third most abundant metal in the vegetable samples of the present study. The content of copper in vegetable samples varied from 4.2-81.33 mg/kg. Copper, though an essential element but its acute exposure (200mg/kg) can cause toxicity and may even lead to death (FAO and WHO, 2011). Arora *et al.* (2008) reported the concentration of copper in spinach in the range of 15.9-17.4 mg/kg. In the present study, it was observed that concentration of copper in ground water irrigated site (in vicinity of wastewater drain) was many folds higher than as reported by Arora *et al.* (2008). The higher concentration of copper in groundwater irrigated samples indicates the percolation of contaminants from wastewater drain to soil and groundwater. In another study, to assess heavy metal content in wastewater irrigated vegetables, maximum content of copper *viz.*17.95 mg/kg was found in tomato (Singh *et al*., 2010b). Highest content of copper was observed in spinach (81.33 mg/kg) samples from site 1 in the present study. In wastewater irrigated area in Shah-Re-Iran maximum content of copper in spinach was found to be 22.74 mg/kg (Bigdeli and Seilsepour, 2008). In Titagarh, among different vegetables the maximum content of copper (35.41 mg/kg) was also reported in spinach (Gupta *et al*., 2008). 6 out of 12 vegetables from site 1, and 2 vegetables from site 3 had BAF/BAC value higher than 1 for copper.

Lead is a toxic metal and is responsible for causing most of the hazard associated with the consumption of heavy metal contaminated food (Cherfi *et al*., 2014). Permissible concentration of lead for fruit, tuberous and bulb vegetables is 0.1 mg/kg, while that for leafy vegetables is 0.3 mg/kg (FAO/WHO, 2014). In our samples, the concentration of lead varied from 0.07 mg/kg to 0.3 mg/kg. It was observed that mean concentration of lead in 7 out of 12 vegetable samples from site 3 was higher than the permissible limits. Content of lead in some vegetable samples from site 1 and 2 were also found to be higher than the permissible limits. Maximum concentration was found in coriander from site 3 (0.33 mg/kg). Concentration of lead in samples studied was lesser than the concentration reported across the world. Concentration of lead in coriander sample growing across Musi river, Hyderabad was found to be 3.4 mg/kg (Chary *et al*., 2008). In wastewater irrigated area in Pakistan, concentration of lead was 2.65 mg/kg (Farooq *et al*., 2008). Though the concentration of lead isn't as much as reported in other studies but was still higher than permissible limits in many of the samples studied. Toxicity associated with consumption of lead containing food is widely reported (Nagajyoti *et al*., 2010; Dorne *et al*., 2011). The BAF/BCF for lead couldn't be calculated as the content of lead in soil samples was BDL.

Cadmium is a possible carcinogen and dietary intake of cadmium affects kidneys and liver. Permissible limit for cadmium in bulb and fruits vegetables is 0.05 mg/kg and for leafy and tuberous vegetables is 0.1 mg/kg (FAO/WHO, 2014). Mean concentration of cadmium in all samples from all sites was higher than permissible limits. Highest concentration of cadmium (1.2 mg/kg) was reported in raddish samples from site irrigated with ground water (site 2). Maximum concentration of cadmium in the present study was reported in raddish samples from site in vicinity of wastewater drain but irrigated with ground water (1.2 mg/kg). In wastewater irrigated areas of Tehran, concentration of cadmium in radish samples was found to be 1.4 mg/kg (Harati *et al*., 2011). In wastewater irrigated fields in China, the content of cadmium in raddish samples was 0.88 mg/kg (Khan *et al*., 2008). Also, in Shah re Iran, the radish samples irrigated with wastewater showed maximum concentration of cadmium *viz.* 0.59 mg/kg (Bigdeli and Seilsepour, 2008). At Site 1 uptake of cadmium was found to be maximum in fenugreek (0.8 mg/kg) followed by spinach (0.6 mg/kg). This site is closest to industrial discharge points and even though is irrigated with ground water but in close proximity to wastewater drain. Site 3 which is irrigated with wastewater showed maximum uptake of cadmium in turnip (1.06 mg/kg) followed by fenugreek (1 mg/kg) and raddish (0.8 mg/kg). The level of cadmium in vegetable samples from wastewater irrigated site was significantly higher than that of ground water irrigated sites, but the concentration in all sites was higher than permissible levels. The typical range of cadmium in food crops grown under optimum conditions is 0.13-0.28 mg/kg (Alloway, 2013). It is to be noted that concentration of cadmium in vegetables from sites in the vicinity of wastewater drain was comparable to concentration of cadmium in vegetables irrigated with wastewater across the world. 3 vegetables each from site 2 and 3, and 1 vegetable from site 1 showed BAF/BAC value higher than 1 for cadmium.

The present study reveals that concentrations of all metals were significantly higher in wastewater irrigated site as compared to groundwater irrigated sites. But, sites 1 and 2 though were irrigated with groundwater also, had high concentration of various metals in vegetables and this can be attributed to adjoining wastewater drain. The heavy metals/other contaminants percolated from the drain can cause serious health issues pertaining to the buildup of heavy metals in vegetables.

5.2.2. Metal Pollution Index (MPI)

Metal pollution index is considered as a precise and authentic tool to measure metal pollution associated with wastewater irrigation (Usero *et al*., 1997). In 10 out of

12 vegetables, MPI was higher in samples from wastewater irrigated site than groundwater irrigated site. All the leafy vegetables had higher MPI than the tuberous and fruit vegetables. The results were in accordance with previous studies, where leafy vegetables tend to exhibit higher MPI (Singh *et al*., 2010b). MPI of spinach from site 1 was comparable to that with site 3 thus exhibiting the fact that irrigation with wastewater and percolation of wastewater from adjoining drain can have similar effect. The MPI in both the tuberous vegetables was comparable at all sites. In a study it was found that MPI decreased by the intermittent use of clean water during the wastewater irrigation (Singh *et al*., 2009). Lesser MPI of certain vegetables like, garlic, onion and green chilli can be attributed to less accumulation of certain metals into these vegetables. Also, garlic is a post monsoon crop and thus lesser MPI is a result of dilution of metals due to rainfall. MPI of onion and garlic at site 3 is high as this is irrigated with wastewater and post monsoon dilution didn't have any effect as a result of continuous irrigation with wastewater.

5.2.3. Hazard Quotient (HQ)

Health risk associated with the consumption of heavy metals through vegetables can be assessed by estimating hazard quotient. Although, it may not provide the quantitative estimation of chances of serious health effects that can be caused due to heavy metals but it is considered as a reliable tool to estimate the risk associated with exposure through heavy metals in food (Chary *et al*., 2008; Guerra *et al*., 2012; Hu *et al*., 2014). In the present study, hazard quotient was calculated for children and male and female adults using the formula given by USEPA (1989). The reference oral dose to calculate the hazard quotient for a metal is given by USEPA. As per USEPA guidelines there is no reference oral dose value available for cobalt. Hence, to calculate HQ associated with cobalt, the reference oral dose given by Food and Nutrition Board (2004) was used. Hazard quotient turned out to be maximum for the cobalt. Among other metals the most hazardous metal on the basis of hazard quotient was copper. In a food survey of heavy metals in vegetables across the world it was identified that after lead, copper contributes maximum to the total hazard quotient (Cherfi *et al*., 2014). Consumption of copper beyond permissible limits can cause serious health hazards. In our study, lead was present in relatively lesser concentration and thus the hazard quotient associated with lead was within the safe limits. After copper maximum hazard quotient was exhibited by cadmium followed by iron. Hazard quotient provides an approximate idea about risk associated with heavy metals. Cadmium is identified as a carcinogen by USEPA and the concentration beyond a permissible concentration can be genotoxic and carcinogenic. The hazard quotient and metal concentration in food crops in sites irrigated with wastewater was in accordance with previous studies (Gupta *et al*., 2010; Pedrero *et al*., 2010; Singh and Agrawal, 2010; Masona *et al*., 2011; Guerra *et al*., 2012; Orisakwe *et al*., 2012). In the present study, we observed that plants growing in vicinity of wastewater drain also pose a significant threat to human health. Various studies have reported increase in number of cancer cases, DNA damage and high frequency of micronuclei in buccal mucosa in people from village Mahal along the wastewater drain (Gandhi, 2004 and Sambyal *et al*., 2004). This can be attributed to consumption of these vegetables. Apart from various other toxicity affects, USEPA (2015) identifies cadmium to target kidneys, cobalt targets endocrine gland and copper and iron to target gastrointestinal tract, thus these metals can cause serious health effects.

5.3. Mitigation strategies

On the basis of monitoring studies, we selected copper for the remediation/mitigation studies considering the hazardous nature of copper and its content observed in soil and vegetables. Maximum hazard quotient/content of copper was observed in the spinach and thus mitigation studies were carried out using spinach. Though, treatment of wastewater is the prior step while considering the mitigation of heavy metals and reducing their transfer in food chain, but for the soil which is already contaminated and to further reduce the mobility of metals in soil, many amendments can be used. These amendments can be inorganic or organic in nature and can reduce the mobility by forming complex with metal ions, adsorption or by increasing the pH of soil (Castaldi *et al*., 2005; Khan *et al*., 2013b; Mahar *et al*., 2015). Biochars are also used as amendments to stabilize heavy metals in soil. These biochars are derived from food byproducts or other organic wastes (Beesley *et al*., 2010; Clemente *et al*., 2015; Smebye *et al*., 2015). Studies are being carried out to use wheat straw ash, bagasse and other food products as soil amendments (Ping *et al*., 2008; Bian *et al*., 2014; Mohee *et al*., 2015). In the present study, we attempted to measure the stabilization potential of food by products which are easily available and are generally free of cost.

For preliminary studies, we used seven different easily available amendments i.e., compost, onion peels, mausami peels, groundnut peels, bagasse, used tea leaves and limestone. The growth of spinach in used tea leaves and limestone amended soil was negligible, thus further experiment was carried out with the five amendments using different concentration of copper to analyze the saturation capacity of each amendment. In the present study, amendments were not digested to form charcoal before the usage rather they were sun dried and powdered before use. This aids in the reduction of industrial cost involved for the production of biochar.

The spinach growing in each type of amendment was estimated for the uptake of copper and other metals, biochemical and growth parameters and CHNS content. The results were statistically analyzed. Pearson's correlation coefficient was calculated and correlation matrix for each amendment was prepared to compare various parameters. This is the first study in which different amendments are directly added to soil to reduce the metal uptake.

5.3.1. Growth parameters

Growth of spinach in different treatments was measured in terms of germination percentage and root-shoot length of plant. Germination percentage in most of the amendments decreased with increase in copper concentration. At highest copper concentration maximum germination percentage in lab conditions was observed in soil amended with compost followed by bagasse. While in field experiment, at maximum copper concentration highest germination percentage was observed in soil amended with groundnut peels followed by bagasse and onion peels. In soil with no amendment there is significant decrease in germination percentage with increase in copper concentration in soil. It has been reported that at higher concentration of copper seed germination of mung bean plant was inhibited (Verma *et al*., 2011). Other studies have also revealed that higher concentration of copper hinder the seed germination by inhibiting the activity of alpha-amylase or enolase (Sethy and Ghosh, 2013). Also, in soil amended with mausami peels, the germination percentage was very low at maximum copper concentration. At higher concentrations of copper, the use of amendments (except mausami peels) have led to an increase in germination rate as compared to non-amended soil implying that the use of amendments helped in the growth of plant. No significant difference in root-shoot length was observed with increase in copper concentration but the maximum shoot length was observed for Cu_1000 in soil amended with mausami peels also the uptake of copper was very high in this treatment. Overall, maximum total plant length was observed for Cu_500 in soil amended with groundnut peels. Our results are in conformity with other studies, where the use of amendments have significantly helped in growth of plants (Fang and Wong, 1999; Angelova *et al*., 2010; Bauddh and Singh, 2015). Use of lime and diatomite has resulted in increased growth in Cd-stressed *Zea* mays (Guo *et al*., 2011). It has been reported that the use of chicken manure and coconut tree saw dust increased yield in case of copper stressed spinach (Kamari *et al*., 2014). Also, use of clay and saw dust increased the growth of *Jatropha curcas* in heavy metal stressed condition (Majid *et al*., 2012). Also, in copper stressed *Brassica juncea* the use of greenwaste biochar increased the biomass as compared to control (Park *et al*., 2011a). Like other composted or charred amendments, use of dried food products in the present study has also helped in the growth of plant.

5.3.2. Copper content in spinach

Copper is an essential element required for the growth of plants and is the main element required for photosystem 1 of plants as it can easily gain or lose electrons (Nagajyoti *et al*., 2010). But, higher concentration of copper is toxic not only to plant but if transferred through food chain it can cause serious health issues to animals and humans. Amendments to reduce the uptake of copper focused on the adsorption of metal ions on the lignocellulose rich materials. In laboratory conditions, upto 65.96% reduction in uptake was observed using groundnut peels as amendment. All the amendments resulted in significant reduction in uptake of copper at least till 500 mg/kg concentration. At higher concentrations, the amendments like, mausami peels (Cu_1000), groundnut peels and bagasse (Cu_2000) led to an increase in uptake of copper as compared to control. This is attributed to increase in shoot length in these treatments as compared to control and lack of ability of amendment to adsorb high concentration of metal ions. Though, maximum percentage reduction in both lab and field experiments was observed in soil amended with groundnut peels but at higher concentration groundnut peels did not seem to provide significant reduction. After groundnut peels, maximum reduction was observed in onion peels and it is to be noted that onion peels were able to reduce uptake of copper even at highest concentration. All the amendments used have high C/N ratio, onion peels had highest C/N ratio followed by mausami peels and groundnut peels. The high C/N ratio leads to slower decomposition and thus, managing the excess of nitrates. Since, vegetables are low C/N ratio plants, the slower decomposition may not create a nitrogen deficit situation (USDA-NRCS, 2011). Also, the increase in organic matter of soil buffers the pH and thus reduces the mobility of heavy metals (Leung and Kimaro, 1997; Morera Luzan, 1998; Kumpiene *et al*., 2008). Studies have revealed that Cu^{2+} ions have highest affinity towards the organic matter as compared to other metal ions thus increase in organic matter in soil stabilizes the copper in soil (Takamatsu *et al*., 1983; Adriano, 200; Warwick *et al*., 2005). Also, there are reports that reveal that increase in calcium in soil can reduce the availability of copper to the plant (Faba *et al*., 2010; Fan *et al*., 2011, 2012). And the elemental analysis of the amendments used revealed that maximum concentration of calcium was found in onion peels followed by bagasse and groundnut peels. Also, onion peels are rich in flavonoid content and the flavonoids are identified as copper chelators and thus bind with copper ions to form stable complexes (Lewis and Watts, 1958). Also, a recent study has revealed that increase in sodium in root zone also reduces the uptake of copper by plants (Matijevic *et al*., 2014). Among the amendments used in present study, maximum concentration of sodium was found in onion peels. Thus, the efficiency of onion peels to reduce copper uptake at even higher concentrations can be attributed to all the aspects i.e., highest C/N ratio, maximum calcium content, high sodium content and high flavonoid content. When compared to the use of inorganic amendments, Ping *et al.* (2008) reported that the use of limestone as an amendment reduced the uptake of copper by rice by 50.4%, much less than the maximum percentage reduction observed in the present study (Ping *et al*., 2008). Other amendments are also effective because of high C/N ratio and high calcium content. In a study, use of green waste biochar and chicken manure led to reduction in uptake in copper by *Brassica juncea* (Park *et al*., 2011b). In another study the use of clay and saw dust sludge increased copper uptake in *Jatropha curcas* (Majid *et al*., 2012). It is reported that use of farmyard manure reduced the copper uptake in *Beta vulgaris* (Singh *et al*., 2010a). In a three year study carried out on *Microchloa altera,* use of compost and limestone significantly reduced copper uptake (Shutcha *et al*., 2015). A study was carried out to study the effect of concarpus biochar on copper uptake in *Zea mays* and it was found that the use of amendment decreased the copper uptake in shoots of maize plants (Al-Wabel *et al*., 2014). Also, the use of chicken manure and coconut tree sawdust increased the yield of spinach and reduced the copper uptake (Kamari *et al*., 2014).

5.3.3. Elements other than copper

In the present study, we also estimated the effect of amendments on the uptake of other metals, including the trace metals. Of all the metals copper has highest affinity towards the humic substances and hence would be the first metal to be adsorbed by the humic substances present in soil (Alloway, 2013). In all the amendments, increase in concentration of copper has led to decrease in uptake of chromium except in soil amended with groundnut peels and soil amended with bagasse. In lower concentration of copper i.e., till Cu_500, use of amendments in soil has significantly reduced the uptake of chromium by plants. Maximum reduction was observed in soil amended with bagasse. Interestingly, onion peels have led to reduction in uptake of chromium at even higher concentrations of copper. At higher concentration of copper, the efficiency of all amendments (except onion peels) to reduce the uptake of chromium has decreased. This could be because of availability of more copper ions. Thus, amendments used are efficient in reduction of uptake of chromium too. Our results are in conformity with other studies, where use of amendments has affected the uptake of chromium. In a study on *Zea mays*, poultry manure reduced the uptake of both copper and chromium (Okieimen and Ikhuoria, 2011). Also, the use of farm yard manure reduced the uptake of copper and chromium in *Beta vulgaris* (Singh *et al*., 2010a). In an another study, the use of farmyard manure significantly reduced the uptake of copper, chromium, iron and zinc in wheat plant (Khan *et al*., 2013b).

No clear effect of amendments on the uptake of cobalt was observed but in soil amended with onion peels and mausami peels, the uptake of cobalt decreased significantly with increase in copper concentration in soil. It is to be noted that Pearson's correlation matrices have revealed significant correlation between chromium and cobalt in plants in case of soil amended with both onion and mausami peels. In a study on *Berkheya coddii*, it has been reported that there was no effect of chelating agent used on the uptake of cobalt (Robinson, 1999).

Soil amended with onion peels, bagasse and groundnut peels showed a significant reduction in uptake of iron at all concentrations of copper. Affinity of iron to organic matter is also high (Takamatsu *et al*., 1983; Warwick *et al*., 2005; Adriano, 2001). Also, iron has higher affinity towards the fulvic acid (Alloway, 2013). Accumulation of iron has reduced by the use of amendments in all treatments. Increase in iron uptake in soil amended with mausami peels could be because of low pH of mausami peels which increase the mobility of iron. In a study, where clay and saw-dust sludge were used as the amendments, the uptake of iron was increased in *Jatropha curcas* (Majid *et al*., 2012). A study to compare the organic and inorganic amendments revealed that the use of Triple super phosphate and farmyard manure reduced uptake of both copper and iron in wheat (Khan *et al*., 2013b). Also, use of farmyard manure, compost and mixture of both reduced uptake of iron and copper in wheat (Abd Elrahman *et al*., 2012). On the contrary, use of concarpus biochar had no effect on uptake of copper by *Zea mays* (Al-Wabel *et al*., 2014).

In lower concentrations of copper, all the amendments resulted in decrease in uptake of manganese but at high concentration of copper only soil amended with onion peels showed significant reduction in uptake of manganese. The decrease in uptake of manganese in spinach grown on soil with various amendments can be attributed to increase in organic matter (Reichman, 2002). Interestingly, in all amendments (expect mausami peels) uptake of manganese increased with increase in concentration of copper. It is because manganese ions are unable to compete effectively with Cu^{2+} ions to form complexes with organic matter (Reichman, 2002). Also, the affinity of copper towards fulvic and humic acids is much higher than that of manganese (Alloway, 2013). Thus presence of high amount of copper ions in the soil solution decreases the availability of bonding sites for manganese and makes it more bioavailable.

No specific pattern of zinc uptake was observed with increase in copper except in soil amended with onion peels and control soil. Soil amended with mausami peels has led to significant increase in uptake of zinc. At lower concentrations of copper, soil amended with onion peels led to decrease in uptake of zinc. After copper, zinc is the metal which has affinity to bind with the humic substances (Alloway, 2013).

At lower copper concentrations, C/N ratio has increased in amended soil and maximum ratio was observed in soil amended with groundnut peels followed by bagasse and onion peels. Effect of copper on nitrogen metabolism in certain plants have been reported (Azmat and Khan, 2011). Increase in plant defence mechanism and protease activity result in increase in nitrogen content under stress conditions. It is interesting to note that there is no significant correlation between increase in copper content in soil and nitrogen content in plants in case of soil amended with onion peels. In most of the treatments, in the present study, nitrogen content in spinach has increased with the use of amendments. Also, in another study it has been reported that the use of biochar has resulted in the increase in nitrogen content in cadmium and nickel stressed spinach (Younis *et al*., 2015a, 2015b). But the carbon and hydrogen content has significantly increased in the soil amended with onion peels. Sulphur is an important macronutrient required for various metabolic functions of plants. Sulphur content has significantly reduced with increase in copper content in soil. Assimilation of sulphur to form cysteine has been found to get influenced by heavy metal stress. Under stress condition the sulphur metabolism of plant gets hindered (Schützendübel and Polle, 2002; Gill and Tuteja, 2014). It is to be noted that organic sulphur content has decreased linearly with increase in copper concentration in case on non-amended soil and soil amended with compost while of all the amendments content of organic sulphur was maximum in soil amended with onion peels, thus indicating the assimilation of sulphur in the spinach.

5.3.4. Biochemical parameters

No certain pattern in content of chlorophyll was observed with reference to concentration of copper or amendments. In general, all the amendments have led to an increase in chlorophyll content in spinach with reference to control. This is in accordance with previous studies where use of amendments like Farmyard manure has significantly increased the chlorophyll concentration in radish plants (Singh and Agrawal, 2013). Reduction in chlorophyll content in vegetables irrigated with wastewater due to effect of heavy metals has been reported (Gupta *et al*., 2010). No correlation of chlorophyll with any other parameter was observed in the present study. In a study, extracts of *Picea abies* bark, *Castanea sativa* chestnuts shell and *Asclepias syriaca* plant were used to evaluate their effect on the cadmium accumulation in Oat plants and also the effect of amendments on biochemical parameters was observed and the mentioned amendments were found to increase the chlorophyll content in plant with respect to control (Stingu *et al*., 2012). No significant difference in carotenoid content was observed in carotenoid content with respect to all treatments. Also, the use of 3% lime has led to increase in chlorophyll content in *Vigna unguiculata* grown on soil treated with mine tailings (Kshirsagar and Aery, 2007). Also, use of biochar has resulted in significant increase in chlorophyll and carotenoid content in spinach grown in excess of nickel (Younis *et al*., 2015a). It is documented that photosynthetic apparatus of spinach is tolerant to high concentration of copper and is thus not harmed with increase in copper concentration (Tukendorf *et al*., 1984).

No significant difference in the protein content of spinach was observed in comparison to various amendments. But concentration of proteins increased significantly in soil amended with mausami peels and bagasse. In soil amended with mausami peels, there has also been reduction in iron and zinc with increase in copper concentration. A closer view of literature reveals that there is no effect on copper on the metalloproteins of spinach (Tukendorf *et al*., 1984). Though, in case of nickel stressed spinach, use of biochar has significantly increased the protein content in plant (Younis *et al*., 2015a).

Ascorbic acid is a stress indicator, and holds significant position in the plant physiology to understand the stress effects (Khan *et al*., 2011). In previous studies it has been documented that heavy metal stress increased the ascorbic acid content in spinach (Pandey *et al*., 2009; Younis *et al*., 2015b). The content of ascorbic acid has decreased in spinach grown on soil amended with various amendments but significant reduction was observed at higher concentration of copper in soil. With increase in concentration of copper, ascorbic acid increased in control and soil amended with compost while in soil amended with onion peels and mausami peels there was significant reduction in ascorbic acid with increase in concentration of copper in soil. In non-amended soil, the ascorbic acid showed positive correlation with iron and zinc content in plants. In soil amended with onion peels the ascorbic acid content has also showed strong positive correlation with chromium and cobalt but negative correlation with copper and manganese in plants. In soil amended with mausami peels, ascorbic acid exhibited strong positive correlation with chromium, cobalt, iron and zinc in plant. In the amended soils (except compost), no positive correlation was observed in ascorbic acid and copper content in soil.

We can summarize that all the amendments have helped in the growth of plant and also aided in the reduction of metal uptake.