Effects of chromium contamination on the soil microbiome and phytoremediation potential of crop plants

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Abstract

Soil pollution as a result of heavy metal contamination has become of major concern, with rising levels as a result of industrial effluents and waste. This is the case within Dindigul, Tamil Nadu, India, where tannery pollution has negatively affected soil health and caused land abandonment by farmers, reducing incomes and the availability of food for the local population. The extent of tannery pollution in Dindigul was determined by comparing soil samples from 11 sites within an area known as the Tannery Belt to 6 control sites. The level of contamination varied across the Tannery Belt, with several areas deemed moderately to highly polluted with Cr, as well as with Cd, Cu and Zn and salts. Salts and soil properties including pH, electrical conductivity, organic matter, and soil moisture all demonstrated correlations with heavy metals contributing to detrimental impact on the overall soil quality.

Soil metagenomics analysis showed that Cr pollution affected soil microorganism communities, causing changes in abundance of individual bacteria, fungi, nematode and protozoa in polluted soils compared to controls. Bacteria *Thermomicrobiales* and *Tistrellales,* fungi *Eurotiales* and *Capnodiales*, nematode *Rhabditida* and protozoa *Phytomyxea,* all showed significant resistance to the presence of pollution within the contaminated soil samples and demonstrated a large increase in frequency within the samples between control and contaminated. Bacteria *Tepidisphaerales,* fungi *Hypocreales,* nematode *Dorylaimia* and *Tylenchida* and protozoa *Gregarinasina* all showed sizable reduction in the relative frequency levels between the control and contaminated soil samples.

Six crop plants were identified as potential candidates for the cost-effective and sustainable remediation of Cr. From a pot experiment exposing the six crops to different chromium levels, tomato, sunflower, and sorghum demonstrated the ability to maintain biomass in high levels of Cr contamination. Cr uptake within edible structures did not exceed permissible limits for these edible crops, suggesting that the risk to human health is minimal. Sorghum was identified as the most appropriate crop for phytoremediation at highly polluted sites such as the Tannery Belt, with a chromium uptake potential of 38% when exposed to 700 mg/kg Cr, whereas tomato and sunflower could be planted in moderately contaminated sites.

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Declaration

The work in this thesis was developed by the author between September 2018 and March 2022. I declare that all the work contained within this thesis, apart from work whose authors are clearly acknowledged, is the result of my very own and original work.

1. Introduction

Tanneries and the leather industry

Leather is an extremely versatile material with many applications in other industrial sectors. It can be cut and made into shoes, clothing, leather goods, furniture, book binding and many other items of daily use around the world (Joseph and Nithya, 2009). The raw material (hides or skins) in the production of leathers is most commonly a by-product of the meat industry. Tanners recover and collect the hides and skins from slaughterhouses, where they would be discarded as a waste product, and convert them into a stable material which can then be used in the creation of a wide multitude of items and products to be sold. The leather industry on the whole is predicted to be worth $100 billion per year in trade (UNIDO, 2010) with the total worth of the industry being predicted at $394.12 billion in 2020 (GVR, 2021). The top producers of leather in the world include China, Brazil, Russia and India producing between 1,560 and 6,170 million sq.ft of leather annually (UN Comtrade, 2022).

Tanneries in India

Leather tanning is one of India's oldest industries (Nath, Saini and Sharma, 2005) and has been a part of Indian culture since 400 BC (Pavithra, 2019). Tanneries in their modern form established within India during the First World War (Thangaswamy et al., 2015). By 1913 25 large scale - tanneries had opened across India and by 1941 that number had increased to 114. The number of tanneries grew exponentially over the next few decades: by 1999, 1,083 tanneries were located in the Indian states of Uttar Pradesh, Tamil Nadu, Karnataka, Andra Pradesh and West Bengal alone, with 40 – 60% of the country's tanneries residing in Tamil Nadu (Thangaswamy et al., 2015). There is now thought to be in the region of 2,500 tanneries in India, with 80 % of these partaking in the process of chrome tanning (Shukla, Rai and Dubey, 2009; Chandra, Bharagava, Kapley and Purohit, 2011).

These leather tanneries grew out of a necessity to use the waste animal parts that were - and still are - being produced in large quantities in India (Kesarwani, Jahan and Kesarwani, 2015). According to statistics from the Food and Agriculture Organization of the United Nations, in 1980 66.3 million animals were slaughtered for food production and by 2004 that nearly doubled, to 126.3 million animals (Kesarwani, Jahan and Kesarwani, 2015). Without the leather industry using and converting the skins and hides of slaughtered animals, the burying of these waste parts in landfill or being burned could create major environmental pollution issues (Kesarwani, Jahan and Kesarwani, 2015).

Tannery processes

There are 5 main forms of tanning leather, as described below:

I. Vegetable tanning

This is the oldest form of leather tanning and is still in use today. The tanning of the hides is carried out using tannins (mixtures of phenolic compounds). These tannins are found within a number of natural sources such as vegetables and tree barks such as oak and chestnut (Bashar, 2012; Bhavya et al., 2019; Abid, Mughal, Saddiqe and Anwar, 2020). Leathers produced using vegetable tanning are resilient and are most commonly used for carving, stamping and book binding (Bashar, 2012) . however, there are a number of issues with leather tanned using this method: if the hides get wet, they are liable to discolouration, shrinkage and hardening (Bashar, 2012) due to their lack of waterproof properties (Baquero et al., 2021).

II. Chrome tanning

Often referred to as wet-blue tanning due to the pale blue colour that chromium salts initially dye the hides (Bashar, 2012), usually using chromium sulphate (Bhavya et al., 2019). Chrome tanning is known to be a highly used method of tanning globally (China et al., 2020) and is the most common method used in the tanning industry, especially in India (Bhavya et al., 2019), making up 85% of the worlds leather production (Maina, Ollengo and Nthiga, 2019). The leather produced is very pliable and can be used in all areas of leather work (Maina, Ollengo and Nthiga, 2019).

III. Aldehyde tanning

This involves the application of oxazolidine or glutaraldehyde to the hide which gives the leather white-cream colour (giving it the common name wet-white leather) (Bashar, 2012). Aldehyde tanning is the most common method used when chrome tanning is not desirable due to the product specifications, for example in the seats of cars or for infant's shoes (Bashar, 2012). Within this category, there are more than one tanning method commonly adopted. Brain-tanning is a highly labour-intensive process that requires emulsified oils that are often found in the brains of animals. Chamois tanning, like brain-tanning uses the oils from cod in order to produce the aldehydes required for the tanning process. Both these methods produce an exceptionally soft and absorbent leather (Bashar, 2012).

IV. Synthetic tanning

These hides are tanned using aromatic polymers such as novolac or neradol and were developed during times of vegetable tanning shortage, such as during the Second World War (Bashar, 2012). It also still required chrome to be used (Ramírez-Estrada et al., 2018). This produced leather that is white in colour, hence the name of the process being wet-white tanning (Wu et al., 2020).

V. Alum-tanned leather

This involves the use of aluminium salts (China et al., 2020). This can produce very light shades of leather; however, the leather material is not as supple as leathers produced through some of the other methods such as vegetable-tanned leather (Bashar, 2012). It has been used and developed as a chrome-free alternative tanning system to chrome tanning (Liu et al., 2020).

All leather processing can be split into three stages of production: preparation, tanning and post tanning/crusting (Hu and Deng, 2016). The preparatory stage is the first stage of all tanning process where the skin of the animals is prepared for the tanning process and is outlined in Table 1.1. After the preparatory stage is where the different methods of tanning will take place as mentioned previously. After tanning takes place post tanning/crusting will be carried out as per table 1.2 to finish of the product to specified standards for further manufacturing.

Process	Description
Wet-Salting curing	When the hides for tanning are stacked and covered in salt. These are then left to cure for at least a month to allow the salt to be absorbed into the skin of the hides. This version on the curing process is used less frequently due to being a time consuming and long process.
Brine-curing	More commonly used then the wet-salting due to being a faster and overall easier method. In this technique the hides are placed carefully into vats along with mixtures of salt and glycolipid surfactant. The hides are then kept within the vats for between 10 and 16 hours to allow for the hides to completely cure, at which point they are removed.
Soaking	The cured hides are soaked in water within a rotating drum to remove dirt, blood and excess salt not absorbed during the curing process.
Liming	The hides are now soaked in a mixture of lime and sodium sulphide for 24-36 hours. This is to remover hair, nails and soluble proteins such as mucin, it also dissolves some fats and grease. Swelling of the collagen within the hides also takes place and this stage.
Un-hairing and de-fleshing	At this stage, all tissues, flesh, fats and hairs not dissolved in liming, are removed. This is done by a roller mounted knife that is rolled over the surface.
Re-liming	Liming can take place again in order to remove more proteins that could have been missed
De-liming	Ammonium chloride is added to counteract the alkali of the liming process. The fibres now shrink back to original size.
Bating	Protease enzymes such as Palkobat or Palkocid are added to the leather to remove unwanted proteins that are still on the hides. This allows the hide to soften and become more pliable
Pickling	Sulphuric acid is added to the hides, as well as salt to prevent acid swelling and reduce the pH. The acidity of the hides after they have gone through the pickling process will typically stay between pH of 2.8-3.2. The hide can now be stored for a long period of time without degrading, ready for the tanning to begin.
De-pickling	The pH of the hides is raised out of acidic ranges in order to facilitate the penetration of certain tanning agents

Table 1.1. The preparatory stages of the tanning process (adapted from Bashar, 2012; Bhavya et al., 2019; Maina, Ollengo and Nthiga, 2019; Abid, Mughal, Saddiqe and Anwar, 2020).

Process	Description
Wetting back	Rehydration of the partially processed leather occurs.
Samming or sammying	The leathers is passed through rollers in order to remove water.
Splitting	The sections of leathers are split into grain and flesh splits.
Skiving	The sections of grain leather is sliced down to an even thickness and any irregularities are removed from the reverse side of the hide.
Neutralising	The leathers now need to be neutralised in order to remove any unwanted acids that could later go on to damage the piece during the drying process. This is done by the addition of a mild alkaline to the leather.
Re-tanning	More of the tanning agents used can be applied if required at this stage to impart desired properties.
Dyeing	Dyeing of the leather into many different colours happens at this stage. Some are surface dyed, and some are penetrated dyed. Many different dyes can be used and if penetration dyeing is required, a rotating drum is used.
Greasing of Fat liquouring	Reactive oils, fats or waxes are used to treat the surface of the leather. The oils interact with the fibrous structures of the leather and cause it to become softer by lubricating the surface.
Filling	Chemicals are added to the leather if a hardened end product is required.
Stuffing	Oils and waxes are added between the leather's fibres.
Stripping	Excess tannins are removed from the leather.
Whitening	If desired, the colour of the leather is lightened.
Fixation	All excess and unbound chemicals within the leather are chemically bonded or removed from the leather
Setting	The leather is flattened to produce a larger area and flatness of the grain, this also help to remove some of the last water content.
Drying	Drying is carried out by ether attaching the leather to a frame and placing it in an oven or laying the leather out on a hot plate with another plate on top pressing down. This sucks water out the leather by creating a vacuum giving the process its name, vacuum drying. The moisture level reached after this stage is between 14 – 25%
Conditioning	If required. The moisture content of the leather is raised to between 18 – 28%
Softening	Leather fibres are physically separated to improve the softness of the leather.
Finishing and Buffing	Leather is given a surface treatment which coats the outer layer of the leather which can protect it or give it a last colour change using pigments or dyes. This also removes scaring and damage defects from the surface,

Table 1.2. The post tanning and crusting stages of the tanning process (adapted from Bashar, 2012; Bhavya et al., 2019; Maina, Ollengo and Nthiga, 2019; Abid, Mughal, Saddiqe and Anwar, 2020)

Tanneries as a pollution source

In tanning, a large number of chemicals are used from several different tanning processes, including chromium sulphate, magnesium oxide, sodium formate, sodium sulphide, sodium chloride, sodium carbonate, ammonium chloride, acids, tannins and dyes (Thangaswamy et al., 2015). These tanneries produce a vast volume of wastewater due to the amount required in each step of the tanning process. For every 100 kg of hide that is processed, up to 4000 litres of wastewater is generated. The amount of effluent has increased gradually as production capacity of leather increases and this will continue (Gerek, Yılmaz, Koparal and Gerek, 2019). The effluent from these tanneries contains several heavy metals such as chromium (Cr), cadmium (Cd), copper (cu), lead (Pb), zinc (Zn), nickel (Ni), mercury (Hg) and iron (Fe) (Thangaswamy et al., 2015; Abdel-Shafy, Hegemann and Genschow, 1995). This makes tanneries a significant source of pollution for the areas that surround them, making them the source of the popular source-pathway-receptor model (Li et al., 2020) as shown in figure 1.1.

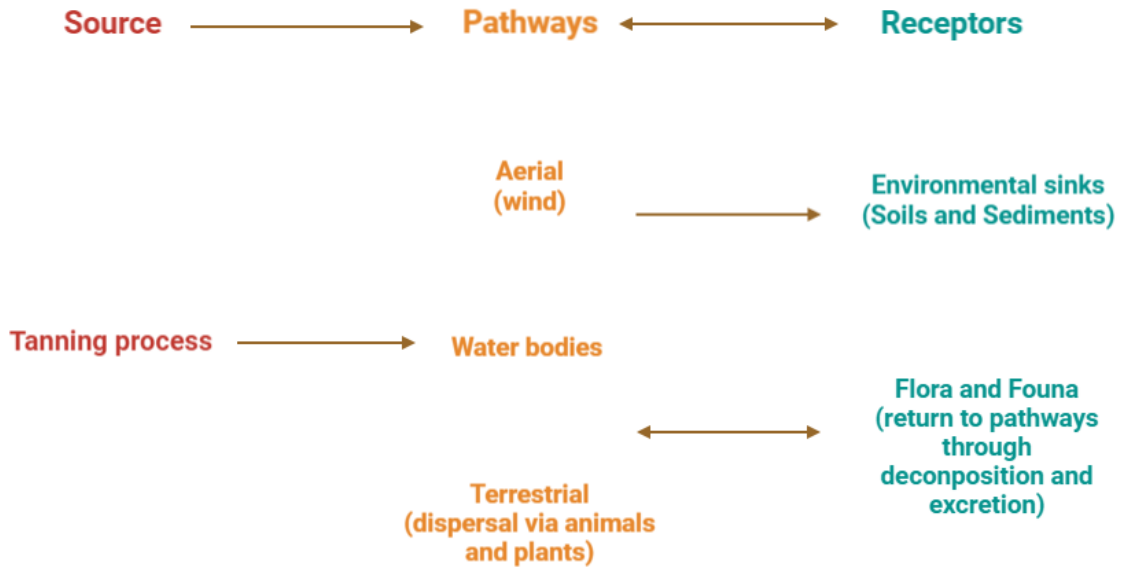

Figure 1.1 Source-Pathway-Receptor Model

Of the many potentially harmful constituents in tannery effluent, the pollutants posing the biggest ecological threat include sodium chloride (salt), pesticides, and chromium salts (Mwinyihija, 2010). Sodium chloride is the second-most important pollutant in this process. Chloride is introduced into tannery wastes as sodium chloride for use in the hide and skin preservation or the pickling stages of the tanning process. Being highly soluble and stable water, are unaffected by effluent treatment and natural remediation and can easily remain in the environment (UNIDO, 2000). Levels of salt can rapidly rise above the maximum level that is acceptable for drinking water. Increased salt content in groundwater, especially in areas of high industrial density, is now becoming a serious environmental hazard (UNIDO, 2000). However, chromium has the potential to be far more hazardous in the environment and will therefore be the focus of this project.

Leather industries are a major source of chromium pollution and play a big part in discharging chromium-related waste into the surrounding ecosystem (Oruko et al., 2020). Cr in the hexavalent phase (Cr-Vl) is more toxic than in trivalent state (Cr-III) (Nigam et al., 2018). Cr (VI) is recognized as a highly toxic, mutagenic and carcinogenic element for mammals including humans, however, Cr (III) is an essential trace element which is essential to humans but can also be carcinogenic at high intakes (DesMarias and Costa, 2019).

Tannery pollution pathways

Effluent discharged from the tanning process is generally stored on site in large, purpose-made lagoons (Thangaswamy et al., 2015). These lagoons provide a pathway, where insufficient safety measures are implemented to avoid a pollution pathway (i.e. pond linings), as the chromium salts dissolve in the effluent and percolate out into the surrounding soils and ground water (Thangaswamy et al., 2015). This process of contamination occurs frequently in sites that do not properly treat the effluent being produced before disposal (Karthikeyan, Lakshmanan and Vg, 2010). These heavy metals are deposited in the soils of the surrounding area and pass to agricultural land (Thangaswamy et al., 2015). Issues with incorrect disposeal are also a common occurrence with a large number of tanneries historically and recently, discharging waste directly into water systems (Whitehead et al., 2019).

Effects of heavy metals on plants

Plants can respond to external stimulus such as toxic or polluted conditions via a number of different mechanisms. These mechanisms include the sensing external stress, transmission of signals into the

cell, and the triggering of tolerance mechanisms to assist in combating negative effects of the heavy metals (Singh et al., 2016). This would be done by altering physiological, biochemical, and molecular processes within cell, binding to cell walls, reducing uptake of excess metal within the plasma membrane, chelation of metals within the cytosol via the use of various ligands including metal binding proteins, metallothionines and phytochelatins, repair of any proteins damaged by stress and the compartmentalisation of heavy metals within the vacuole using tonoplast-located transporters (Hall, 2002; Singh et al., 2016; Balzano et al., 2020).

Heavy metal accumulation and hyperaccumulation in plants relies greatly upon plant species and the efficiency of these plants in accumulating different heavy metals and is assessed by either plant bioaccumulation factor of contaminants or soil/plant transfer factors (Khan et al., 2008). A number of heavy metals (i.e. Cd and Pb) are not essential for a plant to grow healthily, as they do not perform any immediate physiological function within the plants. A number of other heavy metals (i.e. Cu, Co, Fe, Mn, Mo, Ni and Zn) are all essential growth, metabolism and general functioning of plants. However, these can easily cause toxic effects when concentrations reach high levels (Garrido et al., 2005; Rascio and Navari-Izzo, 2011). Once entered into the soils, heavy metals are then taken up by plants through the cortical tissues within the roots. This is due to the similarity that the heavy metals have with some other essential micronutrients and adopt symplastic and apoplastic pathways which allow the metals to reach xylem transport system (Salt and Rauser, 1995).

The root cells of plants tend to attempt to combat or lesson the uptake of heavy metals they are exposed to. This is done by a few methods including the sequestering of heavy metal ions to within the apoplast of cells, locking them down within the cell walls and cellular exudates or by preventing their translocation around the plant (Tangahu et al., 2011,). Hall (2002) states that with the root cell wall coming into direct contact with any metals while they are in the soil solution, the absorption of these metals must be limited onto the cell wall and as a result, this would limit the effects of metal activity upon the surface of the plasma membrane. Root exudates have also been shown to assist in decreasing the uptake of heavy metals taken into cell walls of the roots and counts as an avoidance mechanism (Ghori et al., 2019).

Upon contact with heavy metals within the soils, plants will attempt to stabilise them via absorption into the roots of the plant or by modification of the heavy metal ions (Dalvi and Bhalerao, 2013, Yan et al., 2020) Root exudates (also known as natural low molecular weight organic acids or NLMWOA) have been looked at in more detail for their involvement in metal tolerance thanks to their variety of roles they play for the plant including that of a metal chelator and also play a significant part in the mobility of heavy metals within soils (Nigam, Srivastava, Prakash and Srivastava, 2001) as well as

increasing the growth of the host plant roots (Tinker and Lanchli, 1998) and play an important role in the transformation of toxic heavy metals into organo-metal complexes that help to alleviate any toxic effects on plants and there uptake (Kim et al., 2009). Root exudates are also important in the attainment of several essential heavy metals that reside within the soils. These root exudates can vary massively between different plant species as well as depending on the composition of the soil they are grown in (Montiel-Rozas, Madejón and Madejón, 2016). For example, grass species are able to produce organic acids called siderophores form their roots which have been revealed to enhance bioavailability of iron in soils (Kanazawa et al., 1994) and also showed some affinity to making zinc bioavailable as well (Lasat, 1999; Gao, Zhang and Hoffland, 2009). Another important role of root exudes for increased heavy metal uptake is the use of root exudates that can help change the bioavailability of the heavy metals within the soil solution making them ether more or less available for uptake respectively (Hao et al., 2012). One way this can be done is the introduction of root exudates into the surrounding groundwater, causing the precipitation of heavy metals out, making them less available for uptake through the roots (Kumar et al., 2017). They can also change the state and fractions of which the heavy metals reside as within the soils, which can both increase and decrease their availability as some different states of metals are more easily taken up within plant roots and structures then others (Hao et al., 2012). More recently it has been found that complexes with chelators can form when heavy metals enter into the root cells which immobilise the ions both in the extracellular and intracellular spaces (Ali, Khan and Sajad, 2013). This can lead to its subsequent translocation out of the cells and up into the shoots and leaves of the plant (Yan et al., 2020).

Heavy metals can have a major effect on seeds. Depending on the plant, these effects can vary from abnormalities and decrease in germination, reduced growth and reduction in the dry weight (Ahmad and Ashraf, 2011, Ghosh and Sethy, 2013). All these different effects can contribute to levels of seed toxicity and as a result productivity loss during germination (Ghosh and Sethy, 2013).

Heavy metals and the soil environment

Soil that has been contaminated by heavy metals is one of the greatest issues that is being investigated around the world (Lin, Ye, Hu and Shi, 2019). The presence of heavy metals pollution can cause a number of negative effects on plant quality and those plants yields. Chen et al. (2010) proposed that the presence of heavy metals such as cadmium caused a reduction in richness of certain bacterial species and an increase in soil actinomycetes. They go on to suggest that this can cause a decrease in biomass and diversities of the communities of bacteria. The research of Karaca, Cetin, Turgay and

Kizilkaya (2010) went into more depth and reported that the activities of the enzymes are directly influenced in different ways by different heavy metals and their ions. This is due to the difference in chemical affinities of each enzyme in the soil.

Each different soil has a different level of sensitivity to heavy metals within the soil matrix. Karaca, Cetin, Turgay and Kizilkaya, (2010) go on to say that the order of inhibition of urease enzyme activity generally decreased because of heavy metal presence in order of the sequence Cr > Cd > Zn > Mn > Pb. Diversity within the soil microorganism comnunities are extremely important, with essential processes being recycling and cycling of plant nutrients, detoxification of contaminates and the control of pests (Jamir et al., 2019). As such, they are known to be important indicators orgnainsums for the quality of soil, also referred to as bioindicators (Schloter, Nannipieri, Sørensen and van Elsas, 2017). If the soil microbes in an area are being affected by heavy metal pollution, then these aspects of the soil will either be maintained to different levels or not be affected at all.

Effects of heavy metals on human health

The introduction of heavy metals in the food chain via there uptake and biomagnification are a major risk to human health and that of wildlife (Järup, 2003; Ali and Khan, 2018). Uptake of the heavy metals within root structures is how a large proportion of heavy metal contamination can enter in the food chain and bioaccumulate up (Jordão et al., 2006). The uptake of these heavy metals contaminating the soil can cause significant risk to human health when bioaccumulation within the food-chain is taken into account (Chary, Kamala and Raj, 2008). The use of crops that are contaminated from exposure to heavy metal contaminated soils is the main way humans can get exposure to high levels of heavy metals that in turn can lead to threat of life (Chary, Kamala and Raj, 2008).

It is the accumulation of heavy metals within the body due to them not being able to be metabolised that is what causes toxic effects to occur (Sobha et al., 2010). Many heavy metals will have negative implications to humas if continuously ingested over the course of many years (Khan et al., 2008). As a result of exposure, effects that can occur range from mild to severe with the symptoms ranging from nausea and muscular weakness to much more severe exposure which can even result in death (Duruibe and Ogwuegbu, 2007).

Chromium – what it is, its many uses as well as tanning industry and it is soil

Chromium is rarely found as metal and does not occur naturally in its elemental form; it is almost exclusively found in compounds within the earth's crust and can be released via weathering and erosion (de Sousa et al., 2016). It is usually found at concentrations between 15 to 100 µg g^{-1} depending on soil and groundwater characteristics (Dotaniya et al., 2014), and in higher quantities in ultramafic rocks (Saha, Nandi and Saha, 2011). Chromium is the seventh most abundant element in the earth's crust and the 21st within crustal rocks as a compound (McGrath, Smith and Alloway, 1990).

Chromium is frequently and is a preverlent metal contaminant. It found on the top 20 contaminants on the Superfund priority list of hazardous substances for 20 years (Chrysochoou, Johnston and Dahal, 2012). The chromium ore that is often used commercially is chromite (FeCr2O4). Other less utilised sources of chromium are crocoite (PbCrO4) and chrome ochre (Cr2O3) (Mukherjee, 1998).

Chromium is an elemental component of several chemicals used within many industrial processes. Most of the chromium found in the environment is a result of extensive use of compounds containing hexavalent chromium within industries (Fozia et al., 2008) such as tanning, electroplating, petroleum refining (Saha, Nandi and Saha, 2011), photography, galvanometric and electrical procedures, metal cleaning, plating and electroplating, leather, and mining (Unal, Isik and Sukatar, 2010), and from agricultural activities including the application of chemical fertilisers, composts and sewage and tannery sludges (Jaishankar et al., 2014), as shown in table 1.3. This has resulted in 2 million hectares of agricultural land across Europe is at ecological risk from high contamination levels of chromium (Tóth et al., 2016) and areas as large as 50,000 ha in areas of India such as Tamil Nadu (Rangasamy et al., 2015). Chromium is the most used heavy metal contaminant found in the tannery effluent, being required for 40% of the total industrial processes carried out in the leather making process. Chromium used by the tanning process to tan the hides is not used completely taken up and utilised by the leathering process and comparatively large amounts are discarded into the environment in the industries effluent (Dhal et al., 2013).

When not in a compound, chromium exists in different oxidation states. Pollution research focuses on two common oxidation states: trivalent chromium, Cr(III) and hexavalent chromium, Cr(VI). Cr (VI) has 100 times the toxicity of Cr (III) towards humans due to its high solubility and mobility within water (Saha, Nandi and Saha, 2011) and it is this which has the greater health and environmental implications compared to trivalent chromium. Both Cr(III) and Cr(VI) are highly stable within soils once deposited, with an estimated residence time of between 10^3–10^4 years (Alloway, 2013). As these forms of chromium will not degrade quickly over time, the pollution can persist even as land is converted for different uses, such as agriculture or residential development, resulting in an increased risk to human health (Saha, Nandi and Saha, 2011).

Uses	**Hexavalent chromium chemicals**	**References**
Pigments of paints, inks and plastics	Lead chromate, zinc chromate, barium chromate, potassium dichromate, sodium chromate	Das and Mishra, 2010; Morrison and Murphy, 2010; Saha, Nandi and Saha, 2011; Das, Das and Dash, 2017; Begum et al., 2019
Stained Glass	Chromium salts, Chromium Oxide	Bouchard, Smith and Carabatos-Nédelec, 2007; Morrison and Murphy, 2010
Insulation in metal industry	Chromite	Morrison and Murphy, 2010
Anti-corrosion coatings / Stainless steel	Chromic trioxide, zinc chromate, barium chromate, calcium chromate, sodium chromate, strontium chromate, chromic acid.	Baral and Engelken, 2002; Das and Mishra, 2010; Saha, Nandi and Saha, 2011
Textile dyes	Ammonium dichromate, potassium chromate, sodium chromate	Das and Mishra, 2010; Saha, Nandi and Saha, 2011
Wood preservatives	Chromium trioxide, chromated copper arsenate	Morrison and Murphy; 2010; Das, Das and Dash, 2017; Saha, Nandi and Saha, 2011
Leather tanning	Ammonium dichromate, chromium sulphate	Saha, Nandi and Saha, 2011; Bhavya et al., 2019
Agriculture – Fertiliser and pesticides	Tanning by-products	Kolomaznik, Adamek and Barinova, 2007; Ciavatta et al., 2012
Sewage	Water chromium-containing compounds from industry	Kolomaznik, Adamek and Barinova, 2007; Saha, Nandi and Saha, 2011

Table 1.3. Anthropogenic sources of chromium-containing compounds.

Cr (VI) can readily negatively affect soil environments (Khan, 2001). Soils and groundwater are easily affected by Cr pollution from as a result of year of unregulated disposal of Cr related waste from a number of industries including tanneries (Bhattacharya et al., 2019). Chromium does not remain in one state within the soil. Chromium is found in soils as Cr (III) and Cr (VI) most readily (Mwinyihija, 2010). Cr (VI) is a strong oxidizer as such is highly toxic within plants and humans. Cr (III) is a micronutrient and a non-hazardous species being of little risk (Garnier et al., 2006). Cr (VI), unlike Cr (III) has shown to the affects on the composition of soil microorganisms (Huang et al., 2009). Cr (III) and Cr (VI) are the states which are most at concern regards to biological organisms. Chromium is a necessary metal in small amounts to organisms however, chromium in its hexavalent form, however, is extremely toxic and carcinogenic to them (Rahmaty and Khara, 2011).

Chromium and human health

In its trivalent state, chromium is an essential element to animals and humans and is relatively safe to humans (Saha, Nandi and Saha, 2011), with contact dermatitis being one of the only symptoms of trivalent chromium exposure in humans when exposed to high amounts (Bini, Maleci and Romanin, 2008). The recommended maximum levels of trivalent chromium are a daily intake of 25µg for adults, and 0.1–1µg for children (Bini, Maleci and Romanin, 2008). However, chromium in its hexavalent state is highly toxic even in and is ranked 7th on the top 20 hazardous substances as put forward by the Agency for the Toxic Substances and Disease Registry (Oh et al., 2007). The detrimental effects of chromium to health are greatly dependent on several factors, including the chromium form, exposure time and concentration, and the age, sex and health of the person exposed (Sathwara et al., 2007). Hexavalent chromium is toxic to people due to the process of reduction it carries out within the body, where it is converted into other forms of chromium, including trivalent chromium (Kawanishi, Inoue and Sano, 1986). During this process, free radicals are released, interacting with the organs and proteins in the body and creating the toxic effect on the body and causing chronic illness (Kadiiska, Xiang and Mason, 1994).

A common route of exposure to chromium is via contaminated food consumed regularly in a person's diet (Wang et al., 2011). This is often caused by crops that are grown on areas of brownfield land that have been previously used for industry or landfill (Zupančič et al., 2009). In addition, crops that have been treated with fertilisers, sewage sludge or tannery effluent that contain high levels of chromium are also at risk, especially in less developed countries such as India and Pakistan, where many of the population are vegetarian and rely heavily on these crops in their diet (Khan, Malik and Muhammad,

2013). Chromium exposure does not only occur through ingestion of contaminated crops - chromium can also accumulate within aquatic life from contaminated groundwaters. This can mean that eating fish that has be exposed to high levels of chromium can cause toxic exposure (Sankhla et al., 2016). This same effect can be prevalent in livestock such as cows and pigs that ingest contaminated plants and then when are consumed for food, pass on their accumulated heavy metals (Ugulu et al., 2021). With heavy metals such as chromium being notoriously hard to or non-biodegradable, they are difficult to remove from biological tissues once they have accumulated since the body cannot break these down internally or excrete them (Tandy et al., 2004). Therefore, increasing concentrations of chromium within the environment are an ever-growing threat (Ojuederie and Babalola, 2017).

Industrial processes that expose workers to dust and contaminated soils have also been considered a hazard to health. There is evidence that there is a high carcinogenic risk via these exposure routes, and is thought to be a potential cause of lung cancer as a result (Smith and Steinmaus, 2009; Saha, Nandi and Saha, 2011). The effect of long-term exposure can directly cause kidney and liver damage as well as damage to the circulatory systems and nerve tissue (Wilbur et al., 2012).

Remediation of heavy metal contamination

Many different methods for the removal of contamination from within contaminated sources are implemented, with a large number of then being energy intensive and costly methods that can be highly invasive and damaging to the local ecosystems. These include extraction of soils, the washing or incineration of the soils or the addition of extra chemicals into the soil to stabilise the heavy metal pollution that is within to prevent it from being taken up by plants and entered into the food chain. These methods are displayed in table 1.4. Two methods are shown to be less invasive, electrokinetic treatment and phytoremediation. Phytoremediation, in more impoverished areas is the most suitable candidate, due to its low cost and lack of energy required for the process, as opposed to electrokinetic treatment.

Remediation method	Definition	Issues	Reference
Extraction	Excavation and disposal of contaminated soil at landfill	Relocates the contaminants from one area to another	Gomes, 2012, Vidonish et al., 2016; Bauddh, Singh and Korstad, 2016
Incineration	Soil in burnt to volatise contaminants	Costly and can greatly damage the soil biota	Gomes, 2012; Vidonish et al., 2016; Bauddh, Singh and Korstad, 2016
Soil - washing	Dissolution or suspension of contaminated soil in a wash solution which is then filtered out using additional adatives if required	Not cost-effective, and residues rich in contaminants require additional treatment.	Gomes, 2012; Bauddh, Singh and Korstad, 2016; Beiyuan et al., 2017; Fatdillah and Pauzi, 2018
Stabilization / solidification	Contaminants present in the soil are stabilized or solidified either by physical or chemical interactions between the contaminant and a stabilizing agent	The addition of additional chemicals can be unwanted and costly	Gomes, 2012; Bauddh, Singh and Korstad, 2016; Sahnoune and Moussaceb, 2019; Wang et al., 2019
Vitrification	Heat is used for melting and subsequently solidifying the contaminants in a solid material	Energy intensive and can require additional treatment units	Gomes, 2012; Bauddh, Singh and Korstad, 2017; Fatdillah and Pauzi, 2018
Electrokinetic treatment	Electrical potential passed through soil resulting in movement of contamination via the cathode or anodes	Energy intensive, only works with some contaminants	Gomes, 2012; Bauddh, Singh and Korstad, 2016; Fatdillah and Pauzi, 2018
Phytoremediation	Application of plants and soil communities to remediate contamination		Yan et al., 2020; Gavrilescu, 2022

Table 1.4. Remediation Methods and their associated issues

Phytoremediation

Utilising plants and in some cases soil community microorganisms, phytoremediation is a cost effective, environmentally friendly method of remediation which is still considered to be an emerging remediation technology compared to other methods of remediation (Gavrilescu, 2022). The success of phytoremediation as a pollution remediation management technique is based on the plant's capacity to both sequester heavy metal pollution into the structures of the plant, mainly its root, stem, leaves, flowers, fruit (Gavrilescu, 2022), and on the plants ability to do this as a hyperaccumulator without the levels of heavy metals having adverse effects on the plant's growth. A number of plant families have known and recognised hyperaccumulators including Brassicaceae*,* Fabaceae and *Lamiaceae*, which encompass over 500 hyperaccumulating plant species (Yan et al., 2020).

Bioaccumulation and biomagnification

Bioaccumulation refers to the process of pollutants building up within the organic tissues and cells of an organism that is able to cope with these excessive levels (Ernst, Verkleij and Schat, 1992; Aslam, Yasmin and Sohail, 2019), resulting in a higher concentration of contaminants such as heavy metals relative to the ambient levels (Blowes et al., 2003). This requires the uptake of the contaminant to be faster than it can be metabolised by the organism or excreted, and thus it bioconcentrates (Raskin, Kumar, Dushenkov and Salt, 1994). This process can happen in plants when they are exposed to high concentrations of a contaminant for a prolonged time (Levizou et al., 2020). If a large quantity of contaminated material is then ingested by animals or humans (Fantke, Arnot and Doucette, 2016), these contaminants then start to become concentrated is various tissues; this is referred to as biomagnification (Ali and Khan, 2018).

Study aims

This study had several aims in order to impact remediation of tannery-contaminated land in Dindigul, Tamil Nadu, India. First, the extent of pollution from tanneries was analysed and the effect this pollution had on the soil physical, chemical and biological properties was determined. Next, target crops were identified and tested for their phytoextraction capabilities for Cr since this is the key contaminant from tanneries. In order to be cost-effective and sustainable, the phytoremediator plant would have to demonstrate an ability to both remove heavy metal contamination, specifically Cr, but

also produce a crop that can be of economic benefit (i.e., as a food source for the local population). The five main aims of the study are as follow:

(i) Determine the extent of the contamination around the Tannery Belt

(ii) Understand the relationship between heavy metal contaminants and other soil physical and chemical properties

(iii) Investigate how tannery contamination altered the soil microbiome, focussing on the identification of pollution-sensitive organisms (potentially resulting in a loss of ecosystem functioning) and pollution-resistant organisms (potentially leading to future work on the use of these organisms to assist with phytoremediation)

(iv) Determine crop plants that do not bioaccumulate chromium in quantities considered dangerous to human health that can therefore be recommended as safe food crops

(v) Identify which safe crop plants also bioaccumulate over a minimum threshold to make this a timely, cost-effective and viable remediation method.

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2. Mapping Heavy Metal Pollution "Hot Spots" from Tanneries to determine risk to Health and Ecosystems

Abstract

Heavy metal pollution is an ever-growing concern globally, causing issues within ecosystems and the wider environment, with the potential in extreme cases to threaten public health. Tannery processes and effluent produced contain high levels of contaminants including heavy metals. These reach the soils of farms and public areas via leaching or dumping, posing a significant threat to the local populous. With India being a world-leading producer of leather and leather products, concern over risk to ecology via soil pollution and the local public through the ingestion of contaminated crops has led to significant changes in process and waste management. However, there is a lack of data concerning concentrations of pollutants in field conditions. Furthermore, studies rarely consider soil parameters affecting the mobility of these pollutants. In this study, 153 soil samples were collected from 13 field sites at distances from working and disused tanneries, ranging from 3 meters to 6 km. The samples were analysed using an ICP-OES determining concentrations of chromium and other heavy metals including copper, zinc, cadmium, and lead. Soil parameters including pH, electrical conductivity, water content, organic matter and nutrient content were also quantified. The data was processed in ArcMap using inverse distance weighting to identify hot spots of pollution and areas of specific ecological threat. These maps used data on pollution levels and soil parameters to identify influences on the mobility of heavy metals and subsequent effects on soil quality. Levels of high contamination within highly populated areas show a significant health risk and must be identified and managed accordingly.

Keywords – chromium, IDW, pollution, GIS, tanneries.

Introduction

Tamil Nadu

Historically, the tanning industry has been concentrated in a few districts within the State of Tamil Nadu. Tanneries are found in Chennai (Pallavaram, Chrompet, and Madhavaram), Ranipet and surrounding areas, Ambur and surrounding areas, Pernambut, Vaniyambadi, Erode, Tiruchi and Dindigul, mostly in clusters, however most tanneries are situated within the Vellore and Dindigul districts within Tamil Nadu (Princy, Sathish, Cibichakravarthy and Prabagaran, 2020). It is stated that the tanneries have been producing leather products in the state of Tamil Nadu for more than 200 years (Joseph and Nithya, 2009). In Tamil Nadu, the leather industry has a vital role and is extremely important to its economy and the wellbeing of the local population. Tamil Nadu, exports 55 per cent of total leather that is exported from India and tans 70% of the nation's hides (Nihila, 1993).

Dindigul

The areas of Dindigul made up of hard rock terrain and is situated in Tamil Nadu, South India. It is situated at 10.35° N latitude and 77.98° E longitude (Selvam, Venkatramanan, Chung and Singaraja, 2016). The area is displayed in Figure 2.1.

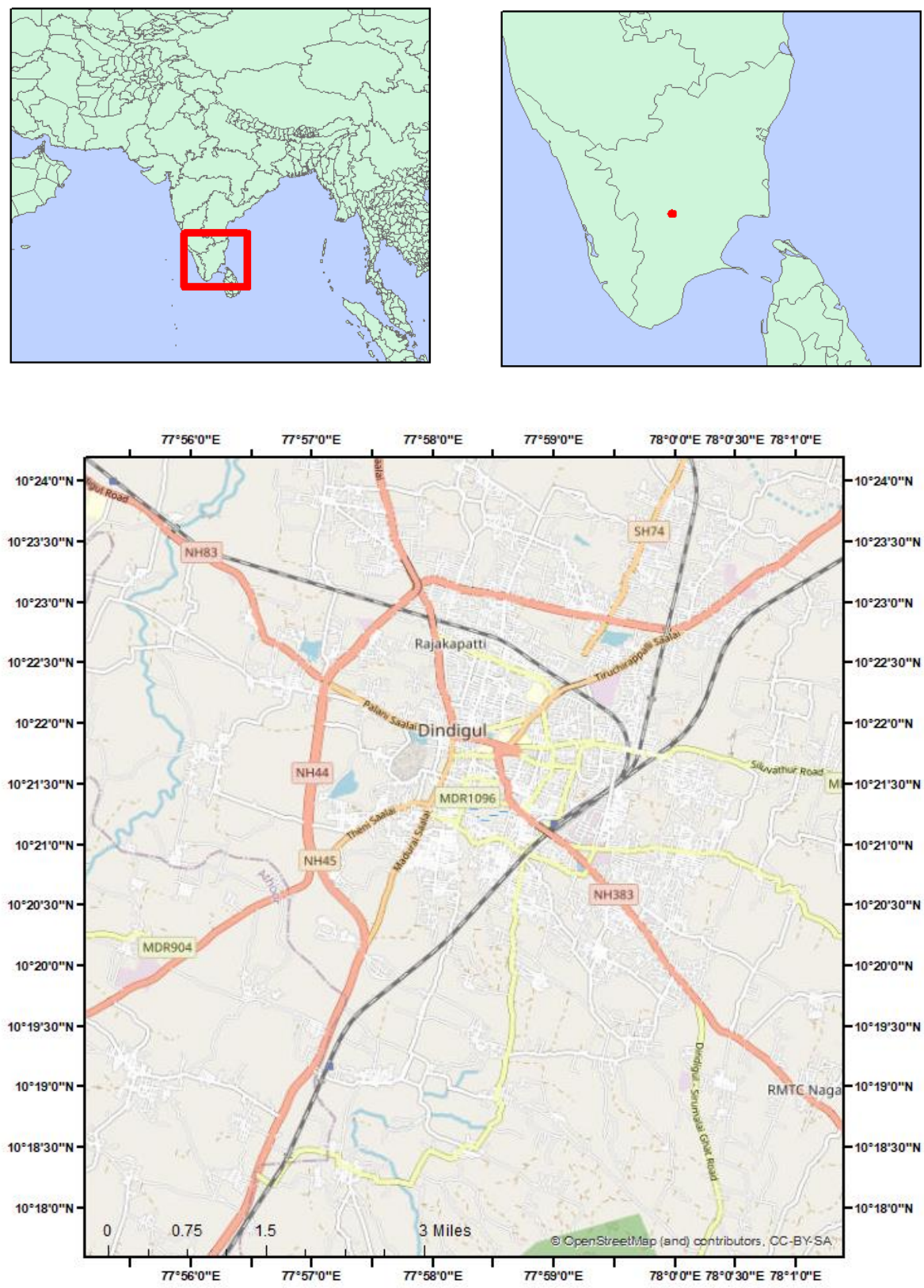


Figure 2.1 – Map showing the location of Dindigul

The District of Dindigul is a major area for the tanning industry in southern India (Selvakumar, Subramanian, Natarajan and Solai Ramatchandirane, 2021). Around 80 tanneries are situated in Dindigul city (Mondal, Saxena and Singh, 2005). These 80 tanneries are suggested to have the ability to process around 200Mt of hides and skins into various types of leather (Selvam, Venkatramanan, Chung and Singaraja, 2016). It is estimated that up to 85,600kg of leather is produced in Dindigul every day (Magesh, Chandrasekar and Soundranayagam, 2010). Most of the city's tanneries are situated along the roads of Madurai, Batla Gundu, and Ponmandurai. It is here that due to inadequate management systems being in place for the disposable of effluent produced by the tanneries (Vijayanand et al., 2008) large amounts of chromium containing effluent is dumped, being made to go into open land, streams and other water bodies (Vijayanand et al., 2008; Princy, Sathish, Cibichakravarthy and Prabagaran, 2020).

Soil properties and heavy metals

Soils are massively diverse and vary greatly from location to location, whether it is its soil texture (Wilson et al., 2016), the nutrients that are available within the soils, (e.g. Indian red soils are high in iron) (Titirmare et al., 2019) or the soil communities that inhabit there (Fierer, Wood and Bueno de Mesquita, 2021). Plants that grow in these soils have ways of manipulating the soil media around them in order to make it more habitable for themselves.

Soil that has been contaminated by heavy metals is one of the most pressing environmental issues that is being investigated throughout the world (Munir et al., 2021). The presence of heavy metal pollution can result in several negative effects relating to the quality of plants including crops and those plants expected yields. They can also affect the size and abundance of the microbial community (Speir, Kettles, Percival and Parshotam, 1999). Consequently, heavy metals pose a major threat as an important source of pollution. The effects of heavy metals on the biological and biochemical properties of soil are well known (Kelly, Häggblom and Tate, 2003). However, these soil properties can also affect on the extent the heavy metals within the soils effect the biological and biochemical properties (Kelly, Häggblom and Tate, 2003; Jiang et al., 2019).

Mobility and the availability of heavy metals are known to be influences by the processes of adsorption and desorption features of soils (Caporale and Violante, 2015). A number of soil properties, including pH, organic matter content, cation exchange capacity and oxidation-reduction have been found to effect the absorption and desorption of heavy metals (Antoniadis et al., 2008; Usman and Mohamed, 2009; Violante, Pigna, Cozzolino and Huang, 2012; Caporale and Violante, 2015). Soil pH has been

found to be an important aspect in determining metal speciation (Huang et al., 2017), and bioavailability of the heavy metals (Wang et al., 2018) such as Cr, Pb and Zn (Speir et al., 2003). A correlation between reduction in soil pH and the availability of heavy metals for uptake to plants has been found with decreases in soil pH increasing heavy metal desorption for Cd, Pb and Zn (Sukreeyapongse et al., 2002, Bang and Hesterberg, 2004). The bioavailability of a number of heavy metals has also been found to increase with decreased soil pH allowing for more uptake into the flora of the environment and pose more of a risk to human and ecological health (Riba, García-Luque, Blasco and DelValls, 2003; Ferguson, 2017).

Chromium pollution – a persistent issue

Heavy metals are elements that occur naturally in the environment in varying concentrations depending on location and soil type and that have a density and atomic weight at least 5 times greater than water (Tchounwou, Yedjou, Patlolla and Sutton, 2012). Heavy metals are normally found in trace levels (<1000 ppm) and occur through processes such as weathering (Wuana, Okieimen and Ogoh, 2012). These concentrations found in the environment are increasing at a steady rate due to the input of rapidly expanding industry and their activities, putting more of these pollutants into the environment that have a long lifetime within the soils (Clemens et al. 2013; Luo et al., 2014). In small amounts many heavy metals are essential to the growth of plants for growth and maintenance, however higher concentrations lead to their toxicity to plants. This can cause a reduction in species fitness or even mortality, leading to a reduction in plant community biodiversity. This can cause fluctuations in the structure and functionality of an established ecosystem (Mayor et al., 2013). Due to chromium contaminated areas being left unused and abandoned, in countries such as India, degradation of land caused by this pollution is threatening food security (Francis, Edinger and Becker, 2005).

Chromium and human health

In its trivalent state, chromium is an essential element to animals and humans and is relatively safe to humans (Saha, Nandi and Saha, 2011), with contact dermatitis being one of the only symptoms of trivalent chromium exposure in humans when exposed to high amounts (Bini, Maleci and Romanin, 2008). The recommended maximum levels of trivalent chromium are a daily intake of 25µg for adults, and 0.1–1µg for children (Bini, Maleci and Romanin, 2008). However, chromium in its hexavalent state

is highly toxic even in and is 7th on the top 20 hazardous substances as compiled by the Agency for the Toxic Substances and Disease Registry (Oh et al., 2007; Shahid et al., 2017). The detrimental effects of chromium to health are greatly dependent on several factors, including the chromium form, exposure time and concentration, and the age, sex and health of the person exposed (Sathwara et al., 2007). Hexavalent chromium is toxic to people due to the process of reduction it carries out within the body, where it is converted into other forms of chromium, including trivalent chromium (Kawanishi, Inoue and Sano, 1986). During this process, free radicals are released, interacting with the organs and proteins in the body and creating the toxic effect on the body and causing chronic illness (Kadiiska, Xiang and Mason, 1994).

Degradation and desertification of farmland

With a large proportion of the farmland around the city of Dindigul, much of the land has been left to go not managed due to the contamination risk of crops grown there. This leads to large areas of land that in normal circumstances would be covered in foliage in large cases barren, with very little cover. This lack of management paired with disturbance via anthropogenic sources (Jiang et al., 2019) such as industry and pollution, can lead to a processed called desertification, especially in arid or semiarid regions (Akbari, Shalamzari, Memarian and Gholami, 2020).

Much of the land surrounding the present and historical tannery sites south of Dindigul, Tamil Nadu has been left empty and barren due to the local population's perception of the contamination of the area. Large areas of land are left to degrade through lack of foliage. Leaving such areas of land open to wind and rain erosion can cause reductions in biodiversity on these areas as soil quality is reduced via the loss of nutrients and soil organic matter content. Due to the arid nature of the area with which the land resides, the term desertification could be used to describe the process due to the nature of the cause of the degradation of the land being attributed to the human activity of tanneries and non-sustained farming of the area (Mutti, Lúcio, Dubreuil and Bezerra, 2019; Xu, You and Xia, 2019). This process of desertification can also be exacerbated and increased due to the introduction of high salt levels (Singh, 2009), such as the amount that is used in the process of chrome tanning. However, whether the area is going through this process is undetermined, and the cause if so unsure as changes in climate and weathers such as rainfall and subsequent soil moisture content can also be an attributing factor.

Aims of the study

In this contribution to the study area, samples are taken from across the main rural tannery locations, to the south of Dindigul city which are known to be inversely impacted by the presence of contamination from the local industry. The study will evaluate the levels of contamination by heavy metals through statistics, geographical imaging systems and indices using geo-accumulation index and contamination factor and degree. Mutual relationships between Cr, heavy metals and soil properties will also be drawn to deduce which factors are having the largest effect on the availability of chromium in these agricultural areas. Heavy metals that were chosen for this study included Cr, Cd, Cu, Pb and Zn. This was due to their known use within the tanning industry and presence in tannery effluent (Mwinyihija, Strachan, Meharg and Killham, 2005; Abioye et al., 2018; Sawalha et al., 2020)

Hypothesis

- Levels of heavy metal contamination significantly higher than background levels will be present in levels across the site above permissible limits and a danger to human health/the ecosystem.

- Heavy metal contamination levels and soil properties will correlate significantly across the tannery belt site.

- Desertification will have significantly increased across the tannery belt sight and surrounding areas.

Methods

Site description

The main cluster of tanneries in rural Dindigul are situated around a triangle of roads to the south of the main city (Depicted in Figure 2.2). This area, forming a tannery industry triangle (Figure 2.3), sits within longitude $77^0$56'37'' – $77^0$58'01'' and latitude $10^0$21'21'' – $10^0$20'05''. These roads are the main route in and out of the city from the south. The bottom of this triangle of roads is a belt of tanneries that sit closest to the agricultural lands of the city, with the northern end being situated within the built-up limits of the city. This 'tannery belt' (Figure 2.4) consists of both current and abandoned tanneries, other industry, main body of water used by local villages as a supply and agricultural land that has been left abandoned.

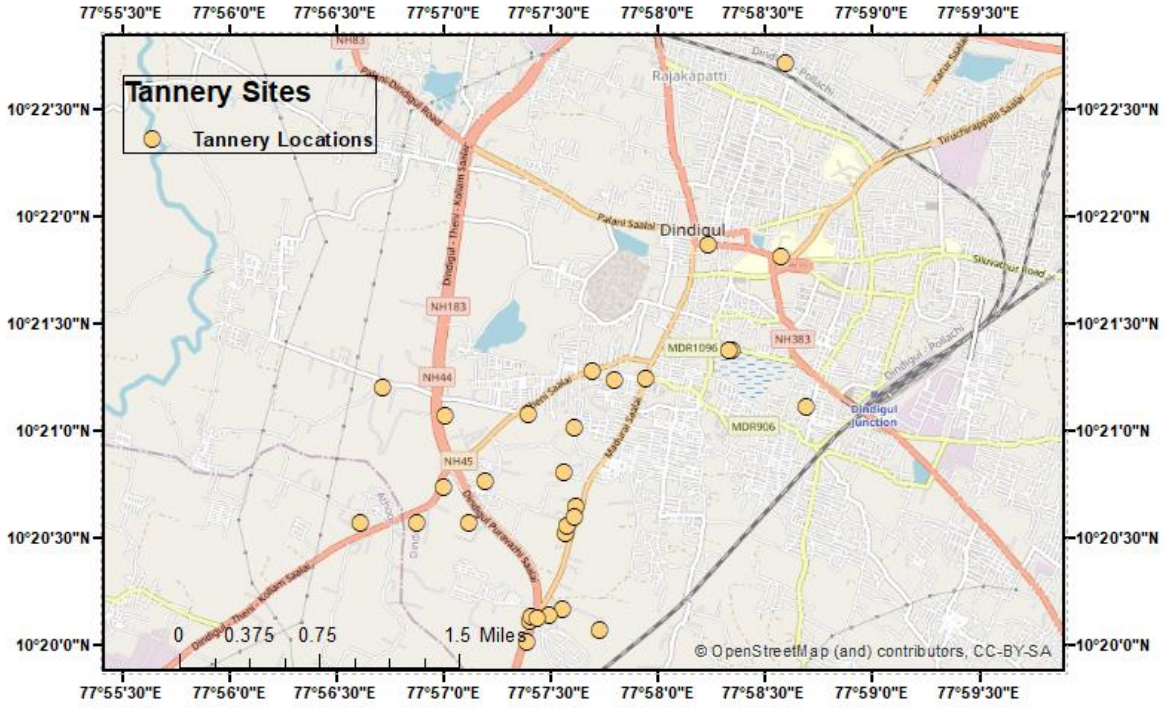


Figure 2.2. Locations of precent and historical tannery sites identified during study

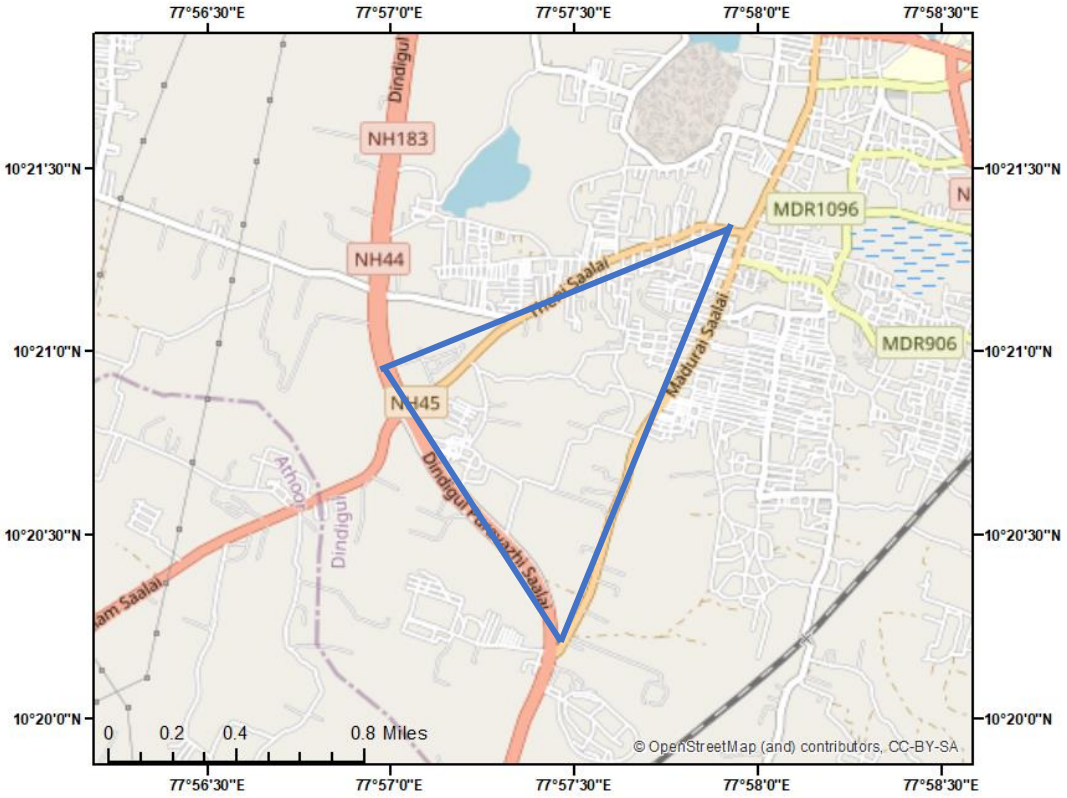


Figure 2.3. Map showing the 'Tannery Triangle' site

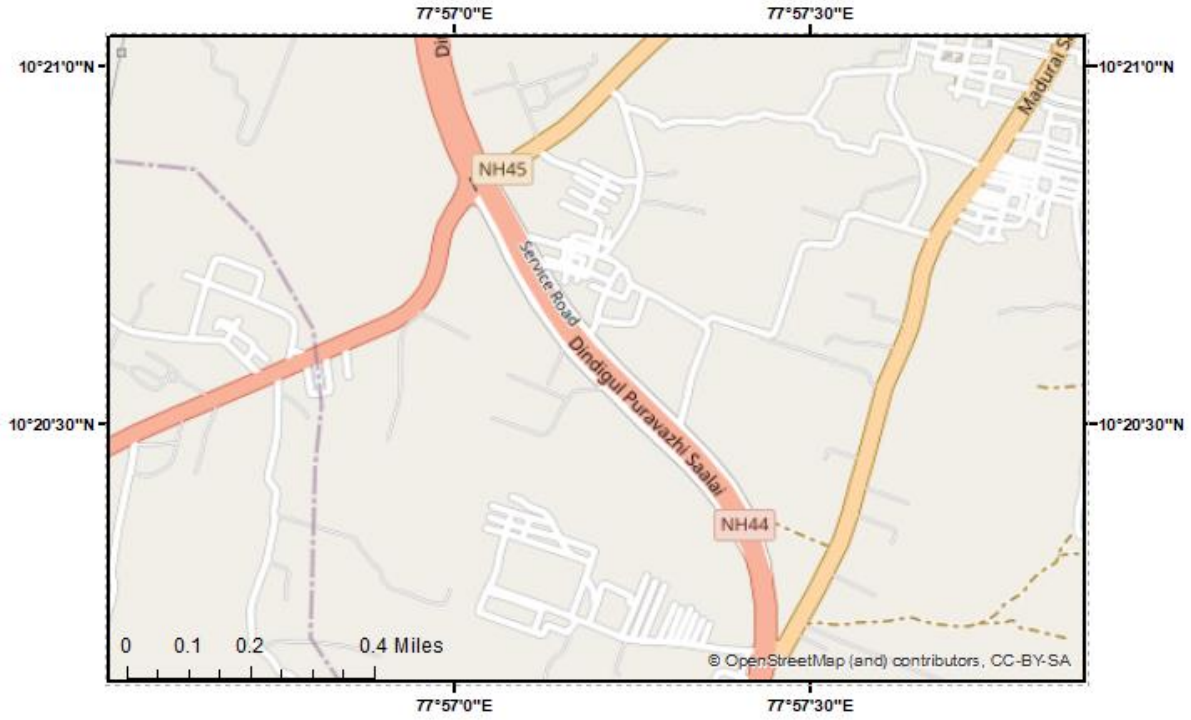


Figure 2.4. Map showing the 'Tannery Belt' site

Soil heavy metal analysis

A total of 153 soil samples taken from 17 sites ranging from 3 meters to 6 km from active/disused tannery sites were taken. This consisted of 11 tannery and tannery adjacent sites and 6 control and background reading sites (Figure 2.5) made up of a mix of monoculture, agroforestry and natural areas, these controls were taken to provide a varied background reading for comparison to the areas suspected of heavy tannery contamination. Sites not including background sites were all situated around the tannery belt rural/agricultural area (Figure 2.6). A handheld GPS devise was used to record each samples location as it was collected and were divided up into their respective site location (Figure 2.7). The samples were taken using a soil corer to the depth of up to 15 cm allowing for the representation of the topsoil. Each sample was stored individually and airdried for shipping in accordance with UK soil licensing law. Once returned to the laboratory, samples were oven dried at 50^0C for 5 days to slowly remove soil moisture.

Before digestion of samples occurred, microwave digester vessels were run on a digestion cleaning cycle using HN03 (68% / 16.23 M) and HCL (37% / 10.15 M) to remove residue metals and organics from the vessels. 0.5g of each Sample was digested using a 3ml of 68% $HN0_3$ and 7ml of 37% HCL mix using a microwave digester, with each sample being filtered using Whatman filter paper no. 42 and diluted up to 50 ml in a volumetric flask before being stored at 4^oC.

The samples were analysed using inductively coupled plasma optical emission spectrometry (The Optima™ 8000 ICP-OES, Perkin Elmer) to determine concentrations of chromium, copper, zinc, cadmium, and lead. Reference standards were placed periodically between samples as QC to ensure accuracy throughout each cycle. The operational conditions for the ICP-OES were RF power and frequency – 1.3kW and 40 MHz, nebulizer flow rate – 0.7 L/min, auxiliary flow rate – 0.2 L/min, plasma flow rate 10 L/min, and the Cr emission line – 267.716nm. The data was processed in ArcMap identifying "hot spots" of pollution and areas of specific ecological threat using inverse distance weighting.

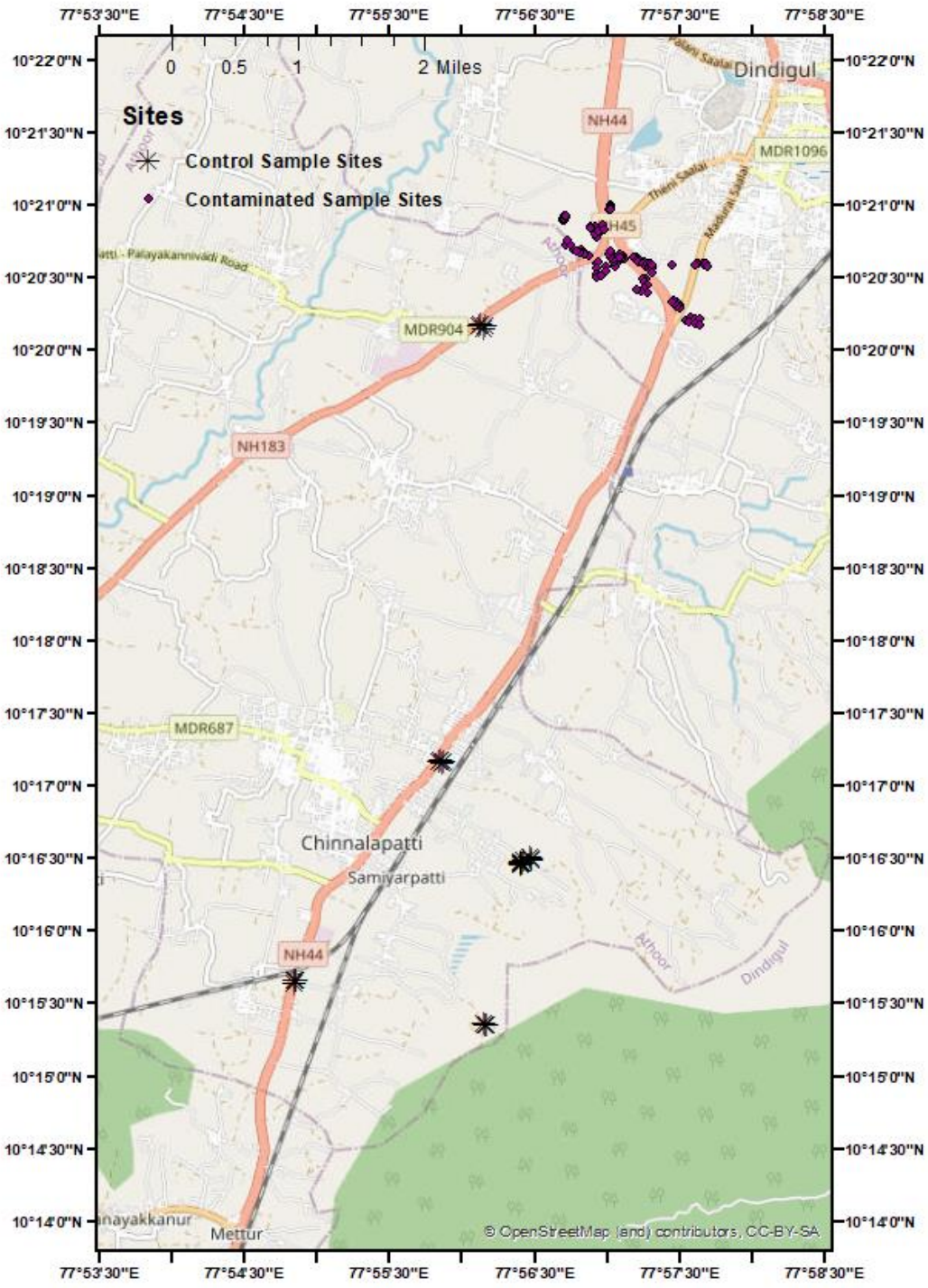


Figure 2.5. Map of all sampling locations

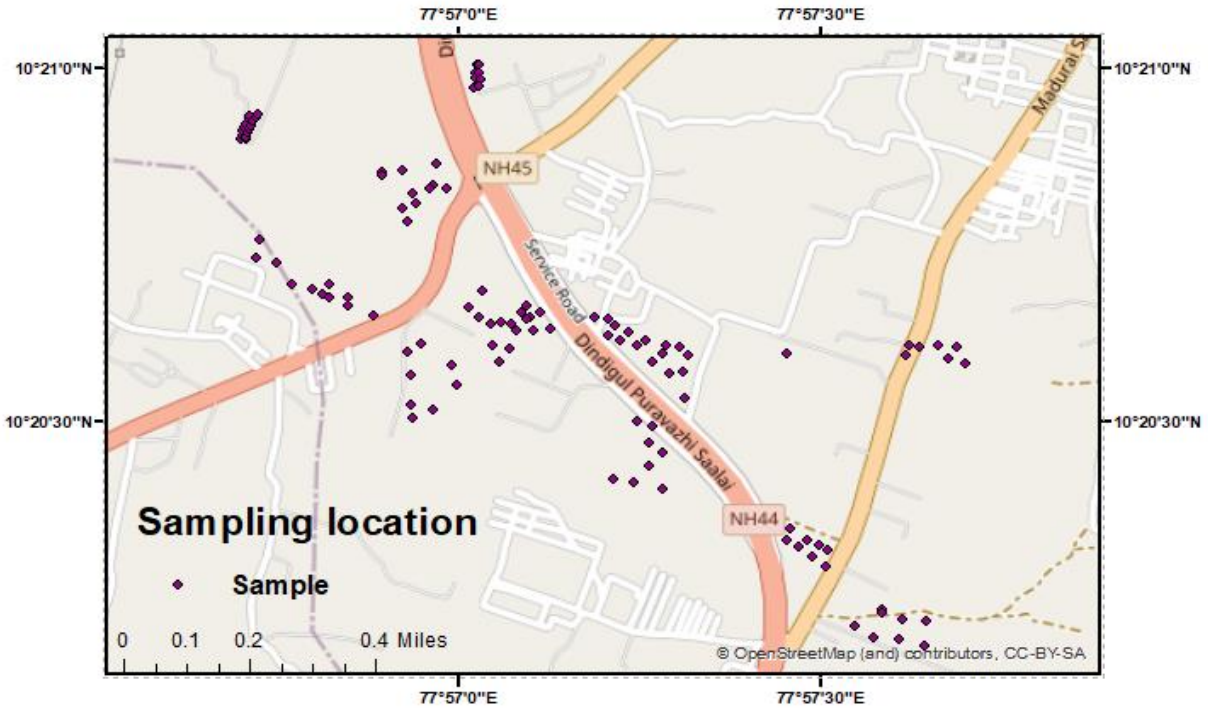


Figure 2.6. Contaminated sampling locations along the 'tannery belt'

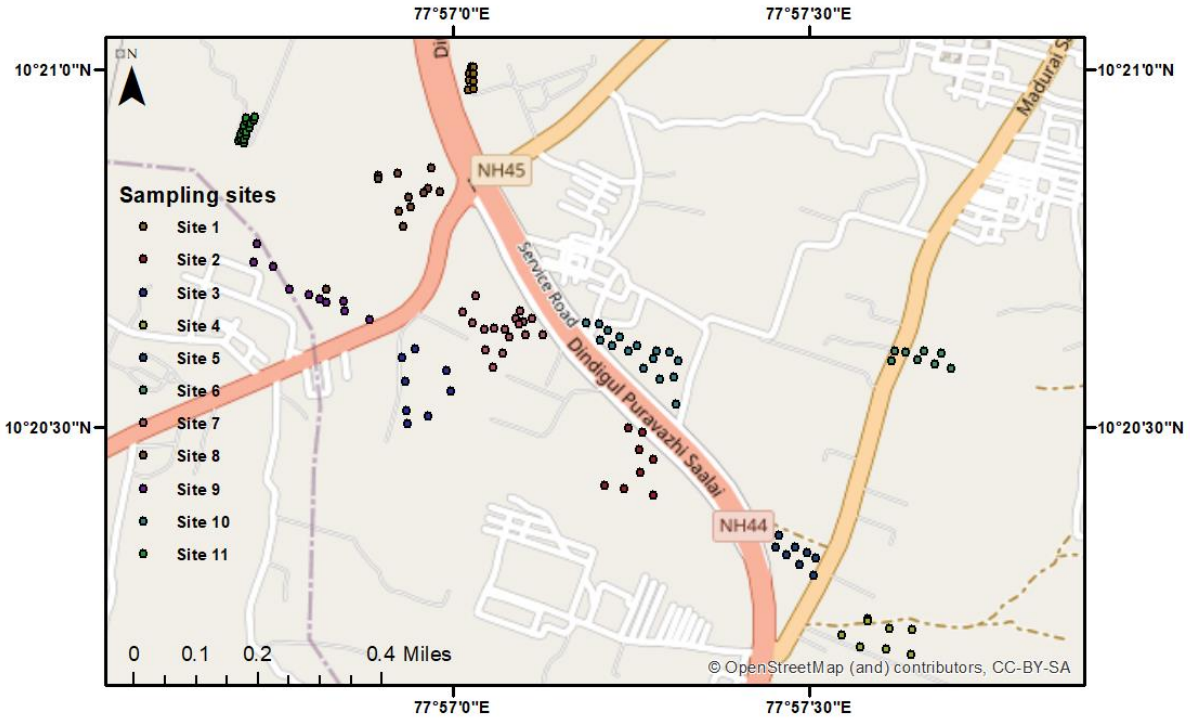


Figure 2.7. Sampling locations shown by designated sites.

pH and electrical conductivity

Samples were prepared by placing 3g of oven dried soils sample into a 25ml tube. The tubes were then filled with 15 ml of distilled water to produce a 1:5 dilution. Samples were then shaken in a Excella E25 heated orbital shaker for 1 hour at 25^{o}C at 120RPM. Samples were left to settle for 10 minutes, to allow partial separation of the water and soil. Readings for pH were taken using a Palintest Micro 500 pH probe and electrical conductivity readings were carried out with the use of a WPA CMD 200 electrical conductivity meter.

Organic matter (loss on ignition)

Organic matter of the soil samples was measured using the widely used method of loss on ignition (Hoogsteen, Lantinga, Bakker and Tittonell, 2018). A known weight of samples (approximately 1g) was added to a pre-weighed porcelain crucible. Samples were placed in a muffle furnace at 550^{o}C for 6 hours and then left to cool to room temperature. Once room temperature was achieved, the samples were re-weighed and the weight of the crucible removed from the total, giving a percentage of organic matter equal to the mass lost during heating.

Moisture content

The moisture content of the soil samples was calculated by the initial weighing of the whole sample before being placed within a cardboard tray. These were then all placed into an oven at 50^{o}C and not higher as to not affect the organic matter or volatile compounds within the sample. The samples were left in the oven for a period of 1 week to ensure complete drying out of the sample. The samples were then weighed separately from there vessels to determent loss of mass, which was used to indicate a loss of moisture allowing for the calculation of percentage moisture content of the given sample.

Soil nutrients

For the analysis of total organic nitrogen and phosphate content of the soil, 3g of oven dried soils sample was placed into a 25ml tube. The tubes were then filled with 15 ml of distilled water to produce a 1:5 dilution. Samples were then shaken in a Excella E25 heated orbital shaker for 1 hour at 25^{o}C at 120rpm. Each sample was filtered using Whatman filter paper no. 42 to remove the solids. A small

sample of the liquid was taken an analysed by use of a discrete analyser to determine levels of total organic nitrogen and phosphate. For the analysis of sodium and potassium, the same preparation was carried out as per previous (3g dry soil to 15 ml distilled water and filter). Samples were then tested by flame photometer against calibration of known concentrations in order to determine the levels of sodium and potassium.

Geostatistical spatial interpolation

Due to the high cost of using high-precision instruments when trying to achieve a high resolution in samples, especially using a system such as inductively coupled plasma optical emission spectrometry, a combination analysis of high accuracy inductively coupled plasma optical emission spectrometry and geostatistical special interpolation allows for analysis of a larger site (Kim and Choi, 2019). Spatial interpolation is a method in which readings from known sample locations are taken to draw predications of the unknown values between these sampling sites. The larger the initial data set that is used during the interpolation analysis, the better the predictions created by the model. The utilisation of spatial distribution analysis as a pollution assessment tool exists within the literature by several studies including by Ferguson (2017), Yang et al. (2017), Cui et al. (2018) and Emam and Soliman (2021). Spatial distributions that utilise geostatistical methods allow for unbiased estimates of heavy metal concentrations in locations within the experimental area that have not been directly sampled. This visual way of displaying and interpreting data offers a beneficial method of identifying areas of high heavy metal pollution and hotspots what otherwise would not be visible from the originally sampled locations (Liu et al., 2017).

Inverse distance weighting can be used to estimate concentrations of heavy metals between sample locations, using gradients between the known points in order to create a heat map of pollution (Fei et al., 2019). In this analysis, Inverse Distance Weighting (IDW) was used for the spatial interpolation via the use of the ArcGIS 10.3.1 desktop programme by ESRI. This method takes the inverse of the distance raised to the 2nd power and is the most commonly used method for IDW (Gong, Mattevada and O'Bryant, 2014). This was used to predict both heavy metal contamination levels across the tannery sites as well as the soil properties and ion levels.

Ordinary Kriging, another geostatistical special interpolation method that can be utilised to achieve similar results as IDW, could have also been implemented into this study. This method takes into account not only the distance between the measured sample locations and the predicted location but the spatial arrangement of all of the measured points and allows for the minimization of the

estimation error variances for predicted heavy metal contamination levels (Lv and Wang, 2018, Qiao et al., 2018, Fei et al., 2019). It has been found that ordinary kriging is more accurate than IDW in certain cases, especially when there is a more uniform contamination across a site (Milillo and Gardella, 2008; Gong, Mattevada and O'Bryant, 2014). However, a major issue with implementing kriging with the dataset produced during this research is that the estimates given during interpolation are highly vulnerable to extreme outliers (Ma et al., 2019) and can only really fit a really met requirement of uniform distribution (Zhou and Michalak, 2009), which due to the nature of taking all measured points into account can cause the entire produced 'heatmap' to be skewed by these extreme outliers and not predict accurately across the site.

Normalized difference vegetation index

Normalized difference vegetation index (NDVI) being recognised as one of the most important indicators of desertification (Jiang et al., 2019, Rajbanshi and Das, 2021). The NDVI allows for foliage cover to be estimated from the relative biomass of an area based of satellite imagery. A vegetation index (VI) is a method of transforming spectral data that has been retrieved from LANDSAT satellites to examine any relationships between the variability of the spectral bands (Gandhi, Parthiban, Thummalu and Christy, 2015, Ndungu et al., 2019). This Index utilises the contrast between the red band absorption of chlorophyll and the reflectivity of plant material regarding the near-infrared band. As such, the NDVI is calculated through the difference between near-infrared (NIR) and red (RED) spectral bands and shown in Equation 1 (Gessesse and Melesse, 2019).

$$\mathrm{NDVI} = \frac{\mathrm{NIR} - \mathrm{RED}}{\mathrm{NIR} + \mathrm{RED}}$$

Equation 1 – NDVI Calculation

This calculation gives a reading of the ratio and is a value for the photosynthetic activity, giving values of between -1 and 1. The lower NDVI values represent areas of barren soil or of vegetation that is experiencing moisture stress, while the higher values indicate increased densities of green foliage (Gessesse and Melesse, 2019).

Geo-accumulation index

The Geo-accumulation index (I_{geo}) is calculated using the formula displayed below (Equation 2), where C_n denotes the calculated level of heavy metal contamination from the sample and B_n denotes the background levels of heavy metals in the area (Nagarajan et al., 2019; Zafarzadeh et al., 2021).

$$I_{geo} = \log_2(C_n / 1.5B_n)$$

Equation2. Geo-accumulation index

I_{geo} can be split into seven different categories, each showing an increase in the severity of the pollution of the sample or site (Ahmadi Doabi, Karami and Afyuni, 2019) and were initially categorised by Muller (1969). These categories are as follows; unpolluted (Igeo≤0), relatively unpolluted (0< Igeo≤1), relatively polluted (1< Igeo≤2), relatively to highly polluted (2< Igeo≤3), highly polluted (3< Igeo≤4), highly to extremely polluted (4< Igeo≤5), and extremely polluted (Igeo>5) (Muller, 1969; Kim et al., 2018; Zafarzadeh et al., 2021).

Contamination factor and degree of contamination

The contamination factor (Cf) of a soil can be used to indicate the quantity of heavy metals added to it via introduction through anthropogenic activities (Ahmed et al., 2016; Bali and Sidhu, 2021) and is widely used in studies relating to heavy metal contamination (Alahabadi and Malvandi, 2018). Contamination factor can be seen to be similar to another index called pollution load. The difference being that pollution load can only be calculated using background soil levels that came pre-industrial levels (Kowalska, Mazurek, Gąsiorek and Zaleski, 2018), whereas contamination factor deals with levels precent in the earth's crust now (Masto, George, Rout and Ram, 2017). The values of Cf can be obtained by dividing the concentration of each heavy metal by the background concentration as shown by Hakanson (1980).

From the contamination factor, degree of contamination can be calculated as suggested by Hakanson (1980). This gives an overall assessment of the pollution by heavy metals of the site, considering all heavy metals at once. At least 5 metals are required to allow for this index to work and the result is compared to background levels of the site to decern total contamination level (Pejman et al., 2015).

$$C_{\mathrm{deg}} = \sum_{i=1}^{n} C_{\mathrm{f}}$$

Equation 3. Degree of contamination

Data analysis

The analysis of data was carried out via the use of Minitab statistical software (ver. 21). The Anderson-Darling test for normality was carried to determine normal distribution of the data. The data was deemed to be not normally distributed on all accounts and so non-parametric test were chosen for analysis. Mann-Whitney was carried out to determine significance in difference between the contaminated sample sites and the background control sites. Spearman's correlation matrix was also carried out to analyse correlations between all the heavy metals and soil properties and the p values determined to obtain if these correlations were of significance. Descriptive statistics were obtained for general analysis of the overall distribution of the heavy metals and soil properties.

Results and Discussion

Descriptive statistics and Mann-Whitney

For heavy metals in the experimental area, it is expected that significant difference from the background levels recorded and deviation from normal distribution of the data set would suggest influence from anthropogenic sources, be that agriculture, tanneries, or urban construction (Adimalla, 2019).

Descriptive statistics for the heavy metal concentrations from the sites are displayed in table 2.1 and a visual representation in the form of bar charts displaying the comparison between sampling sites and control sites (Figure 2.7). The table shows the mean values and other statistics from across all the test sites. The mean concentration of the heavy metals in the soil samples were in the following order: Zn > Cr > Cu > Cd > Pb. All heavy metals apart from Pb showed a maximum concentration that was higher than the permissible limits used from the EU (European Union, 2006) and as stated in guidelines for India by Singh, Sathya, Verma and Jayakumar (2018). The concentration of Zn and Cd in control

soils were found to be very high compared to that of the permissible limits with a mean concentration of 626.1 mg/kg for Zn compared to a permissible limit of 300mg/kg, and a mean of 17.18 mg/kg for Cd compared ot a permissible limit of 3mg/kg. From the p values attained from the Mann-Whitney statistical test, both Cr and Pb showed significant differences in tannery sites compared to the background concentrations in control sites ($p=0.025$ for Cr and $p=0.049$ for Pb). However, Cu, Cd and Zn showed no significant difference ($p>0.05$) in the concentration at tanneries compared to controls. However, Cd and Zn both showed mean background levels above the EU guidelines of 3 mg/kg and 300 mg/kg respectively, which suggests that contamination from the tanneries could have been more widespread than originally thought.

The plots in figure 2.8 demonstrate the difference in heavy metal concentration at the tannery sites compared to the control sites, with error bars representing the standard deviation between samples. For Cr and Pb, the standard deviations demonstrate that there is a higher variation within the contaminated samples to the control samples. This would be expected as any contamination caused by the tanning industries along the 'tannery belt' wouldn't be dispersed evenly out from the industry locations but would be irregular due to the nature of the dumping (Mandal, Vankayala and Kumar, 2011) and use of effluent on agricultural land (Juel, Hasan, Al-Mizan and Hashem, 2021), spreading the contamination out and in varying concentrations. This variation would be less expected in control sampling areas due to the lack of industry in these areas. Although Cd has a higher mean concentration in controls than tannery sites, and varies more in the controls then the, also this was not statistically significant ($p= 0.062$). These Cd levels, along with the elevated levels of Zn also in the control samples does support the notion that the contamination from the tanneries and potentially other industries in the areas isn't contained to the industrial and agricultural areas around the tanneries but could be becoming more widely spread due to the application of tannery waste in organic fertilisers (Barajas-Aceves, 2016).

Heavy Metal Content	Min	Max	Mean	Median	Standard Deviation	Background value
Cr (mg/kg)	35	7757	498	103	1341	87.69
Pb (mg/kg)	0	54.488	8.197	6.227	9.639	4.9
Cu (mg/kg)	12.17	244.26	32.71	26.35	28.66	28.09
Cd (mg/kg)	0	97.4	9.13	0.83	26.58	17.18
Zn (mg/kg)	0	4159.3	725.8	673.6	568.3	626.1

Table 2.1. Descriptive statistics for heavy metals at sample sites

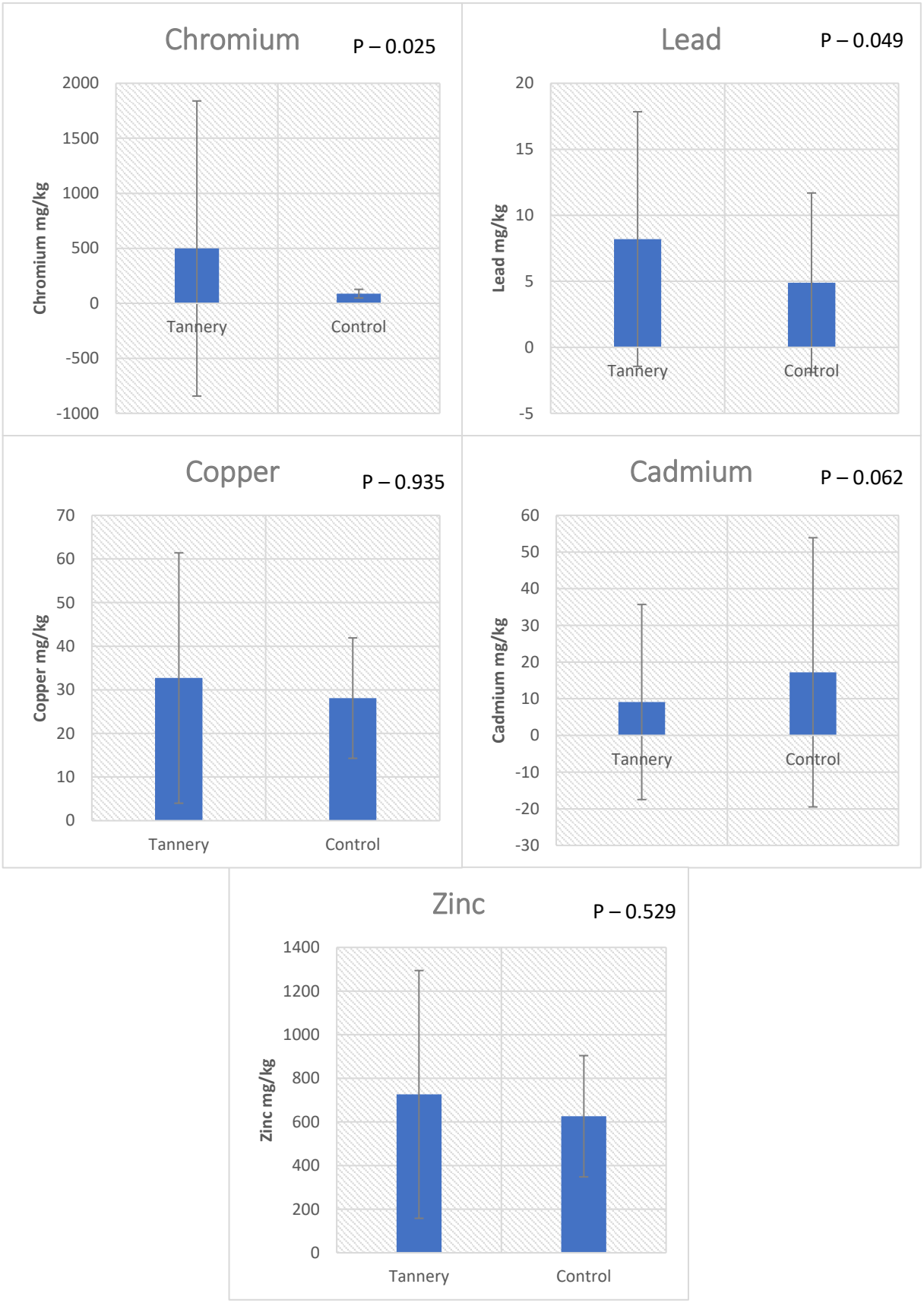


Figure 2.8. Plots of means for heavy metal contaminated sites (Tannery) against control sites (Control) for each heavy metal and standard deviations (including M-W p value of significance) Error bars represent standard deviation.

Additional soil analysis determined several other physical and chemical properties of the soil samples that can help to determine overall soil health. The descriptive statistics for these soil properties can be seen in table 2.2 and means and standard deviation are displayed in Figures 2.9a and 2.9b. All properties varied greatly across the 11 sample sites showing a large difference in the soil characteristics at each site. This would result in differing degrees of mobility and availability of heavy metals, meaning that each site could pose different levels of contamination risk to the local ecosystem and to the health of the local population.

With sodium and phosphorous being used in many forms during the tanning process and thus being prevalent in the effluent produced (Sawalha et al., 2020), it stands to reason those levels of salts would vary greatly as the effluent and solid wastes are disposed of through the dumping and/or landfilling (Sawalha et al., 2020). This variation compared to background levels can be seen in Figure 9b. Variations in the electrical conductivity as an indicator of salinity indicated by the large standard deviation could imply anthropogenic influence on the levels in the area (Yan et al., 2020), supported by the low levels in the rural, agricultural background levels. This is further supported by the p values obtained from the Mann-Whitney testing, showing that the electrical conductivity, sodium, phosphorous and potassium levels were all strongly significant from the background examined values, all having p values <0.01. All other soil properties showed no significant difference between contaminated sampling sites and the control background sites analysed.

Figure 9a and 9b shows visually the relationship between the contaminated sites and the background control sites for each soil property. Electrical conductivity, nitrogen, sodium, phosphate, and potassium all show much higher means in the contaminated sites as supported by their P- values <0.001. Organic matter as measured by loss on ignition showed a much larger variance in the control samples than in the contaminated samples, with the contaminated sites having constantly lower organic matter. However, no statistical significance between the two was found. The pH values of the soils varied greatly from 4.4 and 8.7. This shows that the soil samples varied from a very acidic to a fairly alkaline soil. Sodium, nitrate, phosphate and potassium were all found in varying quantities, ranging from 1 – 8930 mg/kg , 0.13 – 305.88 mg/kg , 0 – 2937.5 mg/kg and 0 – 516.73 mg/kg, respectively. Some of these properties would be expected to be higher near tanneries: for example, sodium is used heavily in the tanning process so would be expected to be found in large quantities at these sites, whereas low nutrient contents could be due to depletion through soil chemical degradation of the abandoned land.

Soil Properties	Min	Max	Mean	Median	Standard Deviation	Background value (mean at control)	M-W (p value)
Phosphate (mg/kg)	0	2937.5	210.9	49.9	478.6	18.87	0.000
Nitrate (mg/kg)	0.13	305.88	10	3.5	30.87	8.82	0.642
Sodium (mg/kg)	1	8930	710	43	1390	20.83	0.001
Potassium (mg/kg)	0	516.73	37.42	20.11	65.6	5.125	0.000
pH	4.4	8.7	6.5042	6.6	0.9476	6.539	0.939
Electrical Conductivity	36	25610	3038	452	4884	140.8	0.000
Organic Matter (%)	0.1	40.8	6.047	3.8	6.588	10.33	0.964
Moisture Content (%)	0.063	43.167	7.823	4.608	7.788	4.422	0.466

Table 2.2. Descriptive statistics for soil properties and characteristics at sample sites.

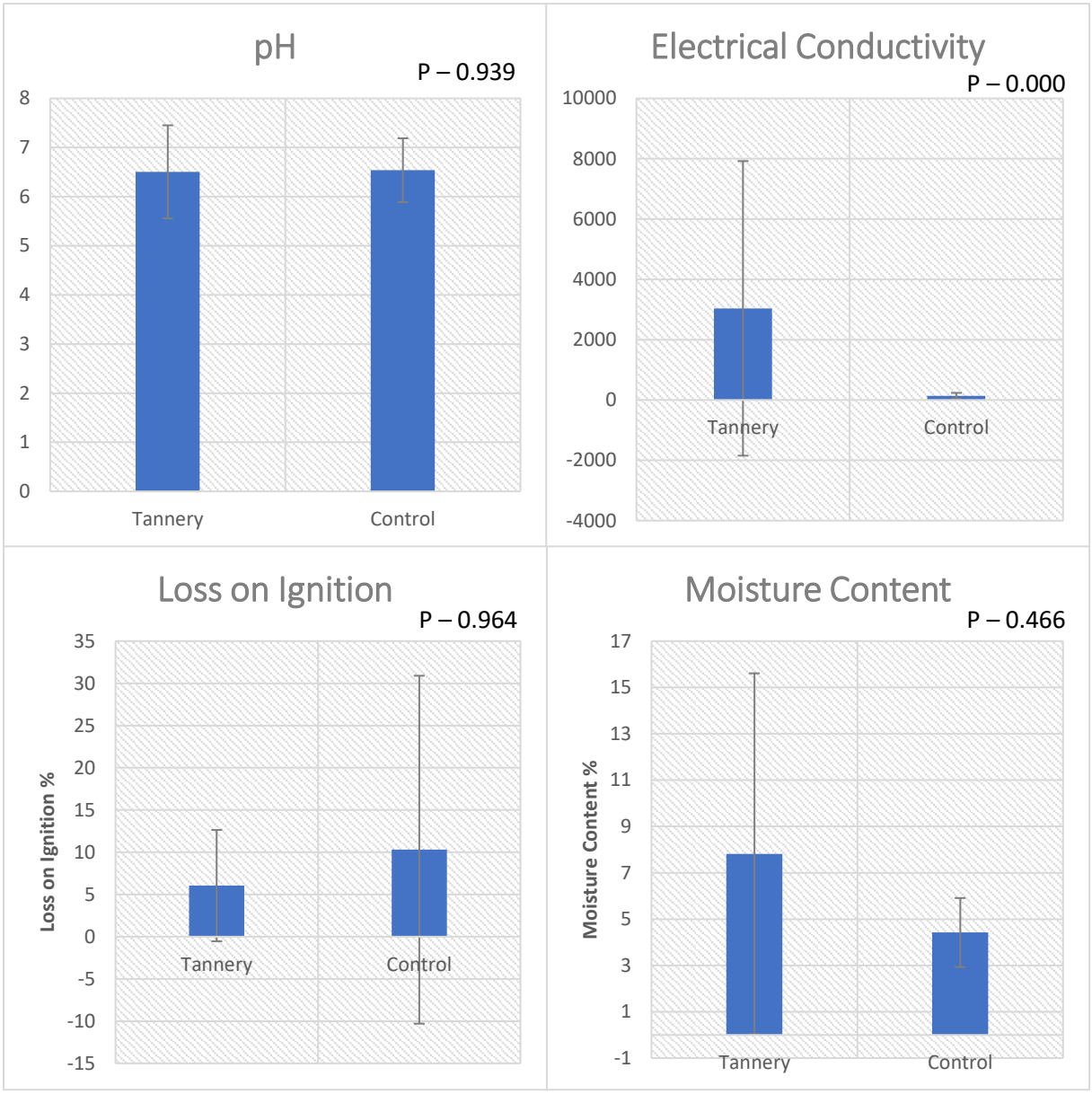


Figure 2.9a. Plots of means and standard deviations for heavy metal contaminated sites (Tannery) against control sites for pH, electrical conductivity, Loss on ignition (organic matter content) and moisture content (including M-W p value of significance) Error bars represent standard deviation.

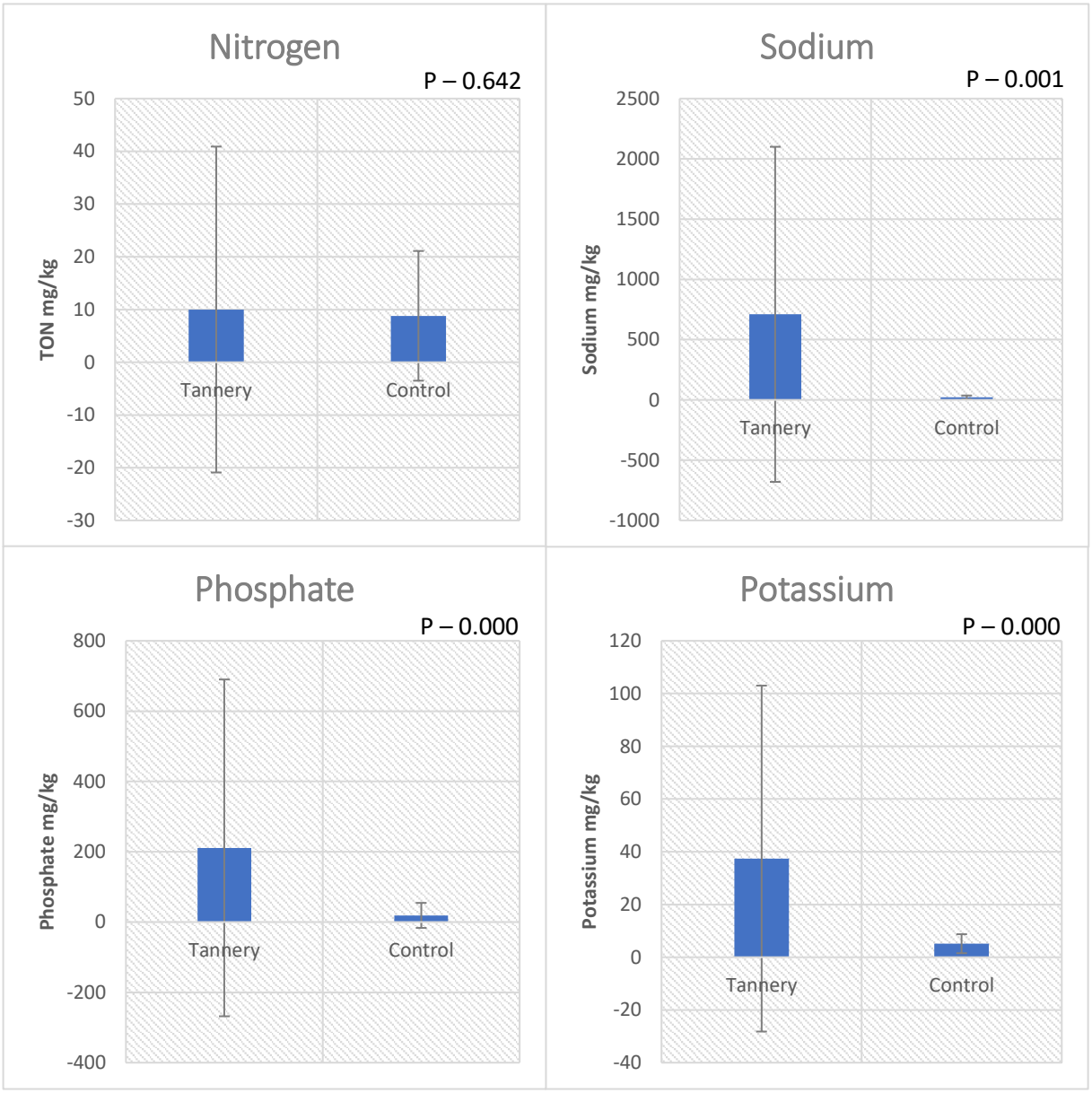


Figure 2.9b. Plots of means and standard deviations for heavy metal contaminated sites (Tannery) against control sites for nitrogen, sodium, phosphate, and potassium (including M-W p value of significance) Error bars represent standard deviation.

Spearman's correlation

A Spearman's Rank correlation matrix, as shown in Table 2.3, was utilised to determine correlations between any of the heavy metals and soil physical and chemical properties. Values close to zero

indicate no relationship between the two variables, whereas the strongest relationship possible would be a value of 1. Out of the five heavy metals analysed, all but Cu showed a significantly positive correlation with each other ($p<0.05$). Cu only showed a significantly positive correlation with Cr. Zn negatively correlated with all other heavy metals, with a correlation coefficient with Cr of -0.553. Zn showed the same negative correlation pattern with soil moisture content, potassium, and phosphate to a significant degree. Heavy metals that showed to have a high correlation value could indicate that these contaminants originated from the same source, such the tanning industry, as suggested by Yan et al., (2020). Varying degrees of relationships were found between the heavy metals Cr and Cd and all soil properties ($p<0.05$), except for nitrogen. The Spearman's Rank correlation indicated that many complex relationships between heavy metals and soil properties are occurring within the soil environment, with properties such as organic matter and pH known for their effects on influencing the mobility of heavy metals within the soils.

	Chromium	**Lead**	**Copper**	**Cadmium**	**Zinc**	**Total Phosphate**	**Total Nitrogen**	**Sodium**	**Potassium**	**pH**	**Electrical Conductivity**	**Soil Organic Matter**
Lead	**0.420**											
Copper	**0.347**	0.107										
Cadmium	**0.203**	**0.485**	0.025									
Zinc	**-0.553**	**-0.341**	-0.095	**-0.428**								
Total Phosphate	**0.283**	0.112	**0.156**	**0.223**	**-0.212**							
Total Nitrogen	0.023	0.055	-0.054	-0.063	-0.080	0.016						
Sodium	**0.210**	**0.204**	0.033	**0.226**	0.021	-0.055	-0.110					
Potassium	**0.316**	**0.232**	**0.153**	**0.366**	**-0.155**	**0.199**	0.036	**0.589**				
pH	**0.275**	**0.229**	**0.258**	**0.276**	-0.023	0.086	-0.168	**0.578**	**0.376**			
Electrical Conductivity	**0.211**	**0.212**	**0.188**	**0.563**	-0.177	0.142	**-0.229**	**0.690**	**0.659**	**0.559**		
Soil Organic Matter	**0.441**	0.143	**0.428**	**0.328**	-0.139	**0.280**	**-0.186**	**0.480**	**0.551**	**0.492**	**0.622**	
Soil Moisture Content	**0.309**	**0.184**	**0.352**	**0.454**	**-0.256**	0.146	**-0.293**	**0.415**	**0.497**	**0.493**	**0.720**	**0.617**

Table 2.3. Spearman's Rank correlation matrix of heavy metals and soil properties (p values <0.05 shown in bold).

Inverse distance weighting of heavy metals and soil properties

Spatial distribution is a very useful tool when analysing the extent of heavy metal contamination across a site, being essential in the documentation of hotspot zones across an area, allowing the identification of safe and unsafe locations where action must be taken as a top priority (Adimalla, 2019; Deng et al., 2019). Spatial distribution patterns of heavy metals and soil properties and the sampled contaminated sites around the 'tannery belt' are shown in figures 2.10 to 2.22. Spatial analysis via inverse distance weighting also allows for the prediction of concentrations of contaminants in untested locations based of the levels of the sampled sites in a "heat map" (Qiao et al., 2018). These can be useful to estimate ecological and/or human health risk for the whole area. It also allows for the identification of areas that exceed limits that have been deemed safe, such as the EU safe limits for heavy metals in soil used in this study. In order to accurately predict contaminations, good spatial resolution is required (Habib et al., 2018). If not then the predictions of the contamination could be misleading,

Cr levels mapped via inverse distance weighting can be seen in figure 2.10. The majority of the tested and simulated area can be seen to be above the permissible limits for Cr of 100 mg/kg. Sampling sites situated at the northern end of the experimental area, mostly including site 1 show levels ranging from 12 to 80 times the permissible limits of Cr in soils with the rest of the area that does exceed the limits, exceeding the limits by 1 to 12 times the permissible limits. The northern area of the site is situated next to an important body of water used by the local surrounding villages as a main water source. The IDW maps indicate high levels of contamination near this lake, therefore demonstrate a substantial risk to health from Cr leaching into water supplies. In fact, personal communication with locals suggested that this has already taken place and that there are negative health effects on populations using this as a water source, particularly for children. Areas similar to the background readings of 87.79 mg/kg or below are mostly towards the south-east of the site indicate less influence of the contamination towards that end of the 'tannery belt'

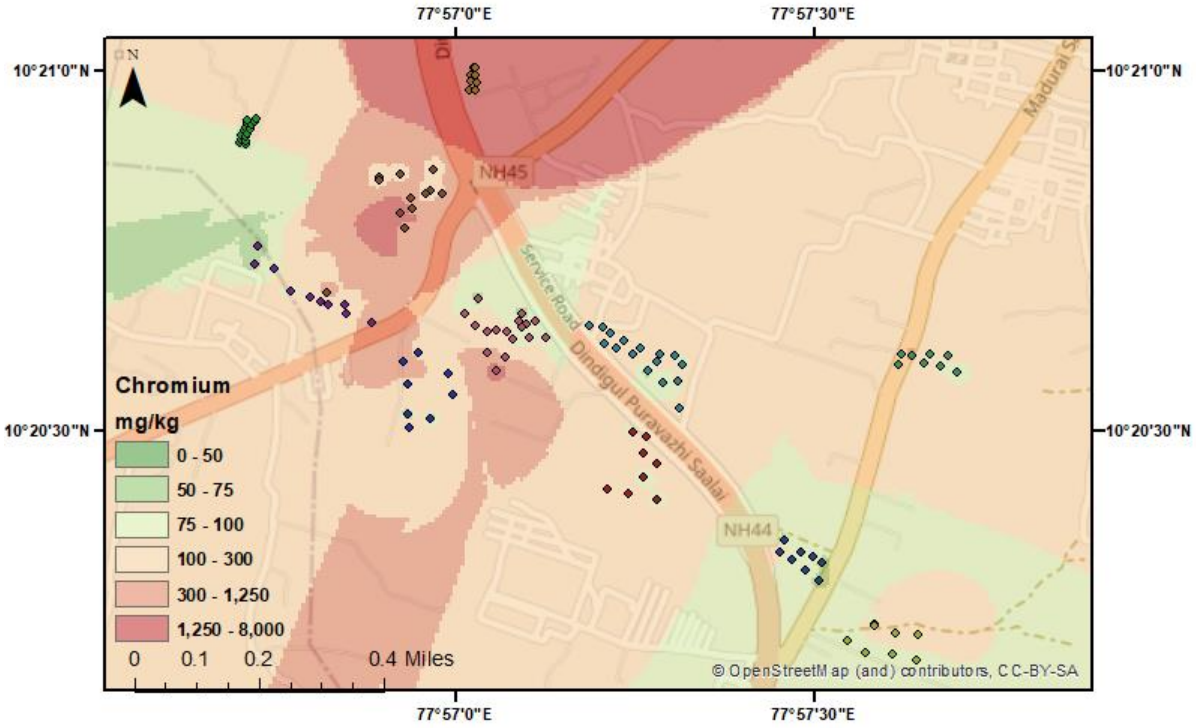


Figure 2.10. Spatial distribution by Inverse Distance Weighting of Cr (mg/kg)

Figure 2.11 depicts the spatial distribution by inverse distance weighting of Cd. Across much of the experimental site, Cd remains below the permissible limits of the EU of 3 mg/kg with most of the area showing levels of 2 mg/kg and below. Similar to Cr, the northern end of the 'tannery belt' and the north-west of the experimental site show increased levels of Cd in the industrial soils surrounding the vulnerable water body, with levels of Cd reaching levels of more than 30 times permissible limits. This again raises concern as to ecological and health effects due to the critical limit for ingestion via drinking water being 0.003 mg/l (de Vries, Römkens and Schütze, 2007). A less widespread distribution of Cd compared to Cr is to be expected as background levels across the world are below on average 0.2 mg/kg (WHO, 2000).

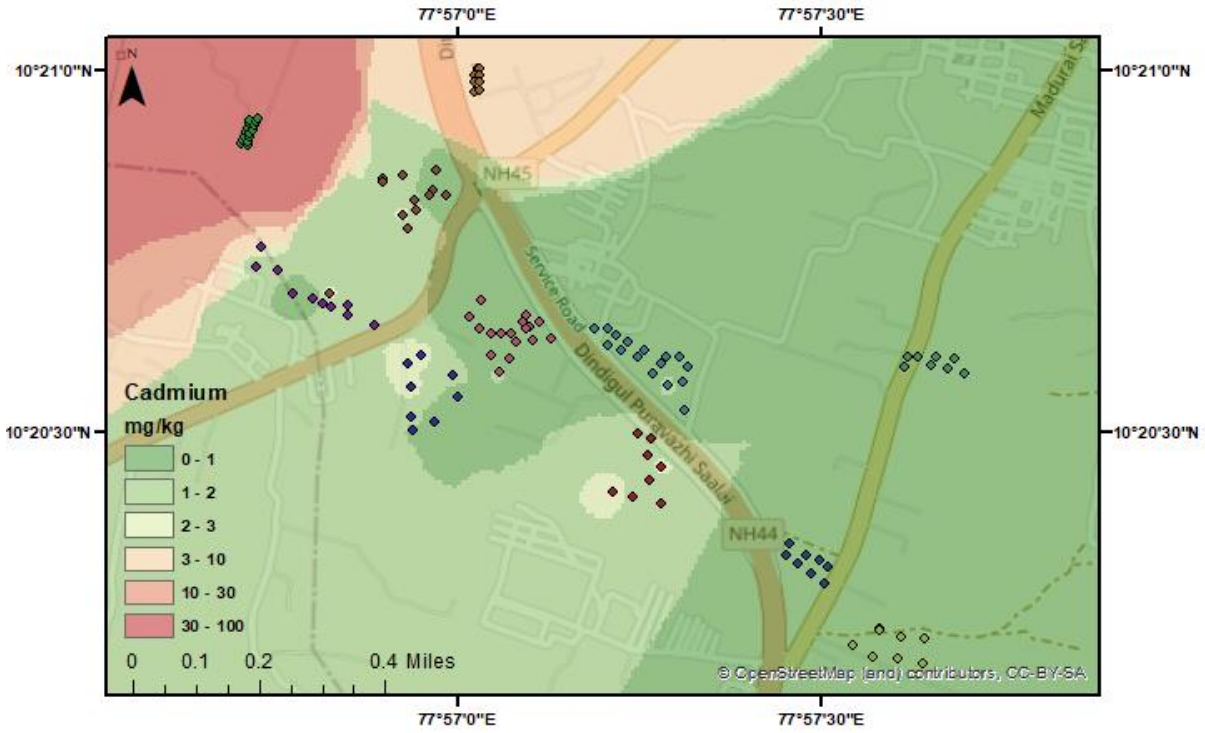


Figure 2.11 - Spatial distribution by Inverse Distance Weighting of Cd (mg/kg)

As can be seen in figure 2.12 and by the descriptive statistics, Pb did not exceed the permissible limit of 100 mg/kg across the entirety of the sampled site. The highest reading of Pb was measured at 54.5 mg/kg, situated just to the west of the 'tannery belt' site, therefore it does not appear that Pb is associated with tannery pollution. Similar findings can be seen in figure 2.13 for the inverse distance weighting of Cu. Three isolated samples at 3 of the sites examined showed a level of Cu that exceeded the permissible limit of 100 mg/kg. However, across the site, there was no significant difference from the background site to the south, with a M-W p value of 0.935. This similarity in lack of contamination from known chemicals used in the tanning industry, specifically in chrome tanning, the lack of correlation between Pb and Cu in the soils indicate that they have come from different sources from each other and likely are not significantly caused by pollution from the tanneries.

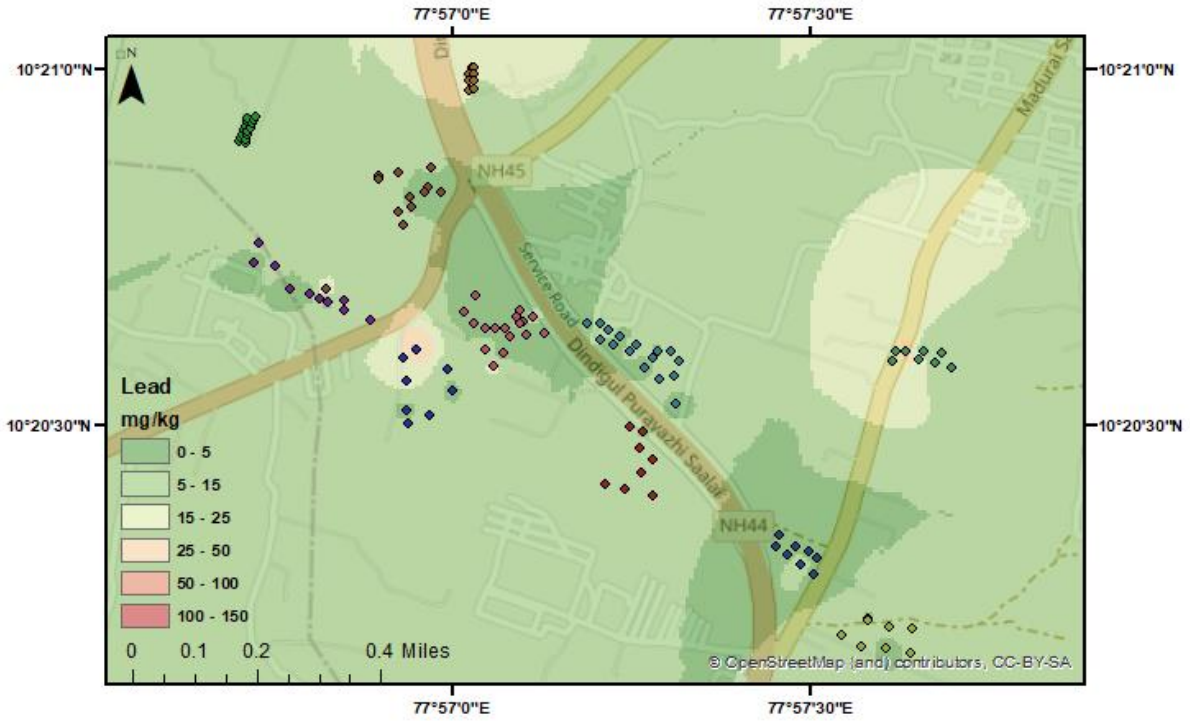


Figure 2.12 - Spatial distribution by Inverse Distance Weighting of Pb (mg/kg)

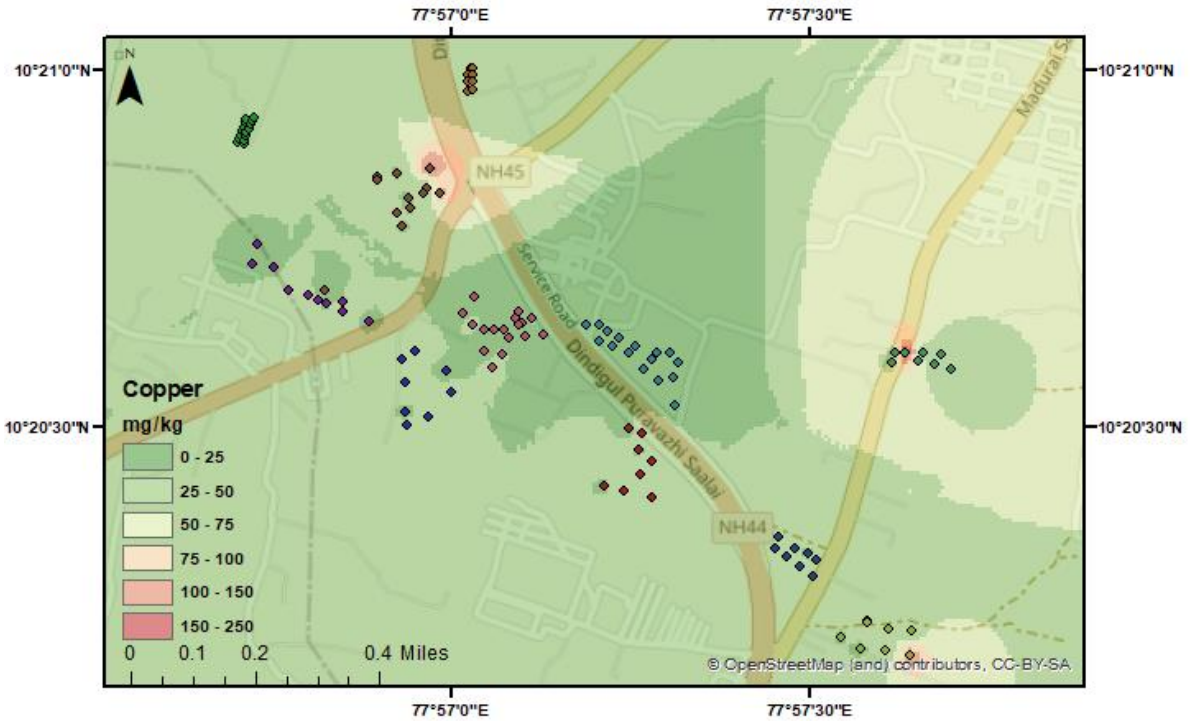


Figure 2.13 - Spatial distribution by Inverse Distance Weighting of Cu (mg/kg)

The distribution of Zn across the site, as shown by inverse distance weighting figure 2.14, visualises the reason for the negative correlations Zn was found to have with all the other heavy metals, with 3 of those relationships being statistically significant ($p<0.05$). The majority of the experimental area is shown to be above EU permissible limits of 300 mg/kg, including around the water body to the west of the 'tannery belt' and to the north-east towards the residential areas of the Dindigul. The higher levels of Zn, exceeding 1000 mg/kg and reaching towards 4,100 mg/kg, are towards the south of the experimental area, which is nearer the agricultural areas of Dindigul's outskirts. This can pose issues in regard to bioaccumulation of the Zn in the soil in to crop plants such as rice (Satpathy, Reddy and Dhal, 2014) and tomato (Gupta, Nayek, Saha and Satpati, 2007) which are both known to accumulate large amounts of Zn into their structures, including fruit, which could be a threat to health of both animals and humans with consumption and subsequent bioaccumulation through its high levels in the body or inducing of other deficiencies such as copper (Plum, Rink and Haase, 2010).

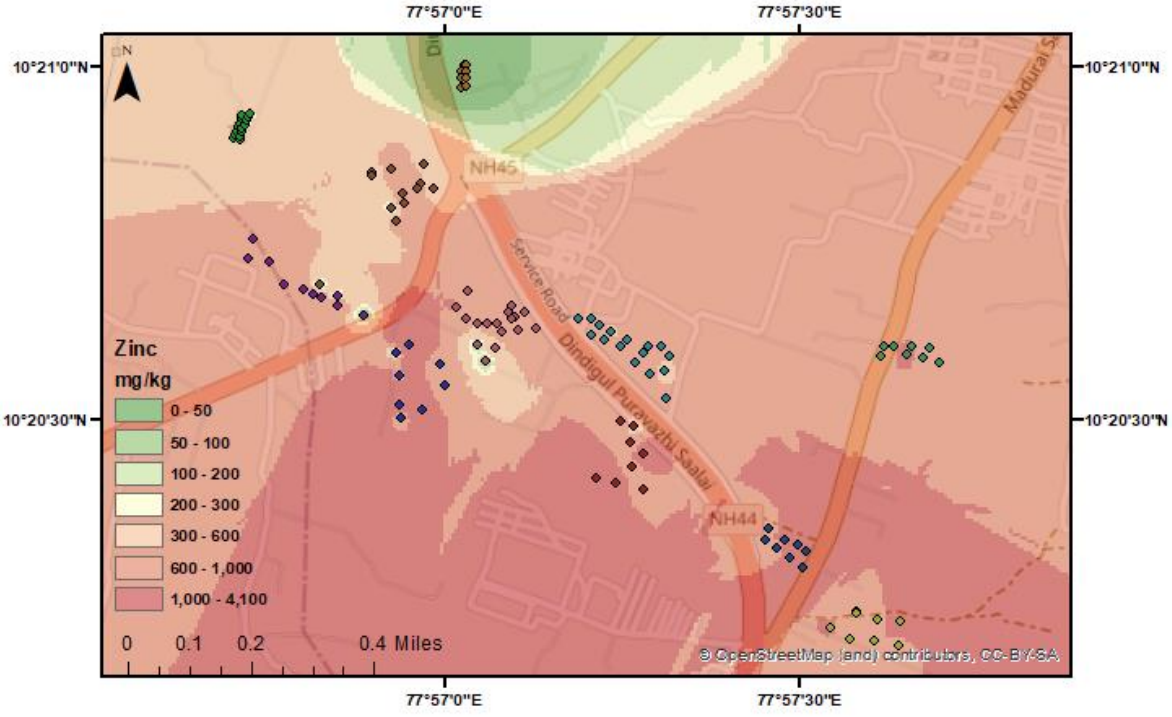


Figure 2.14 - Spatial distribution by Inverse Distance Weighting of Zn (mg/kg)

Soil properties were also analysed via the use of spatial distribution by Inverse Distance Weighting. Figure 2.15 shows pH and its distribution across the experimental site. Firstly, there is a notable similarity between the distribution of pH and the distribution of Cr across the site (Fig.10). The highest levels of Cr are situated in the same locations where the pH of the site is more neutral or moving towards the slightly alkali of the site. This is also supported by a statistically significant positive correlation between the two variables from the Spearman's Rank correlation ($p<0.05$). This corresponds to the higher levels of Cr being in areas where the Cr (VI) levels are likely to be higher due to higher pH levels (Unceta, Séby, Malherbe and Donard, 2010). However, in these higher pH areas, mobility and availability of the heavy metals is overall reduced, meaning a potential decrease in uptake into any crop plants in the area (Sukreeyapongse et al., 2002). To the same extent as Cr, pH was shown by Spearman's Rank correlation analysis to also have statistically significant correlations with Pb, Cu and Cd.

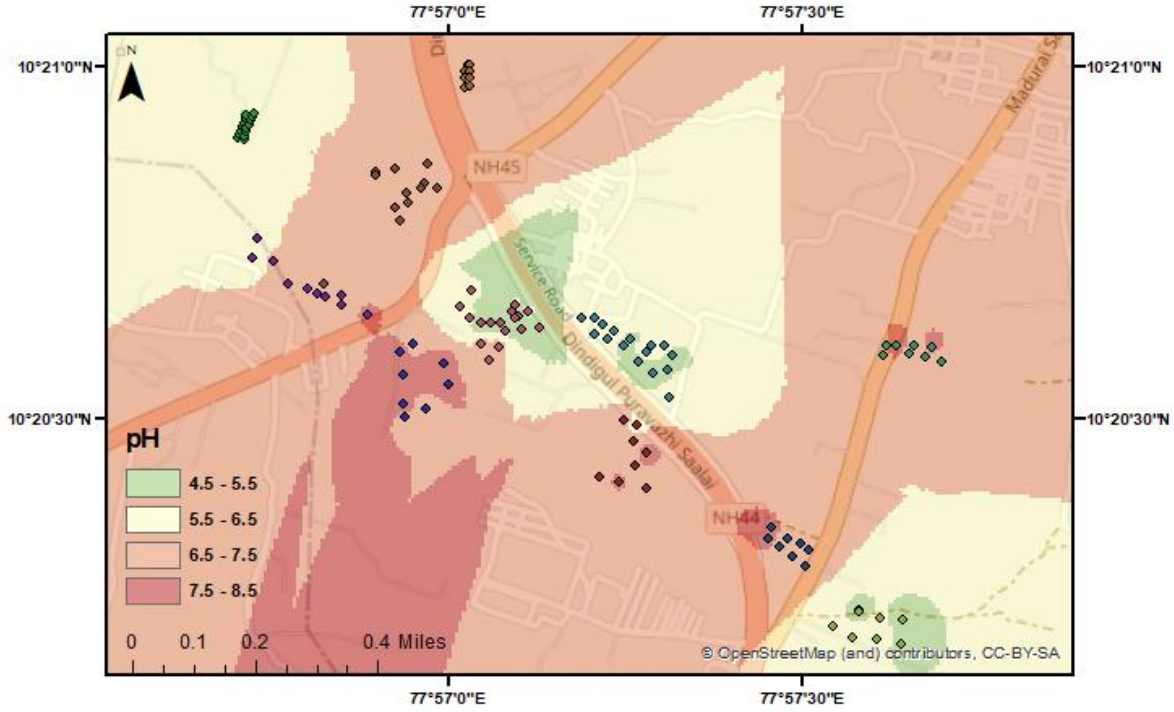


Figure 2.15 - Spatial distribution by Inverse Distance Weighting of pH.

Increases in salinity has been shown to cause an increase in the mobility of heavy metals from the presence of many different salts including those containing sodium (Acosta et al., 2011). With sodium

chloride and potassium chloride salts being used heavily within the curing process within tanneries (Wibowo, Anggriyani and Yuliatmo, 2018), high levels across the examination site were expected.

Very strong statistically significant correlations were found by Spearman's Rank correlation analysis between sodium, potassium and electrical conductivity, as the salts containing the sodium and potassium are discarded if in effluent and in hair waste, they cause the salinity of the soils to increase. This relationship can be seen in figures 2.16, 2.17 and 2.18 were the highest levels of electrical conductivity show the same distribution patters across the site, with figure 2.17 and 2.18 showing sodium and potassium showing an almost identical distribution pattern. These distribution patterns converge on the west of the experimental site. This is where the local body of water is situated which on inspection during the collection of the samples, was covered in a 'salt crust' that correlates with the increased levels of salts and salinity shown in figures 2.16, 2.17 and 2.18.

With electrical conductivity and pH effect mobility of heavy metals, but pH reduces this mobility as it increases whereas electrical conductivity increases this mobility as it increases. As such, it is hard to determine just how mobile heavy metals will be within the soil solution across the experimental site as the areas with the highest pH values also have increased electrical conductivity as displayed in figures 14 and 15.

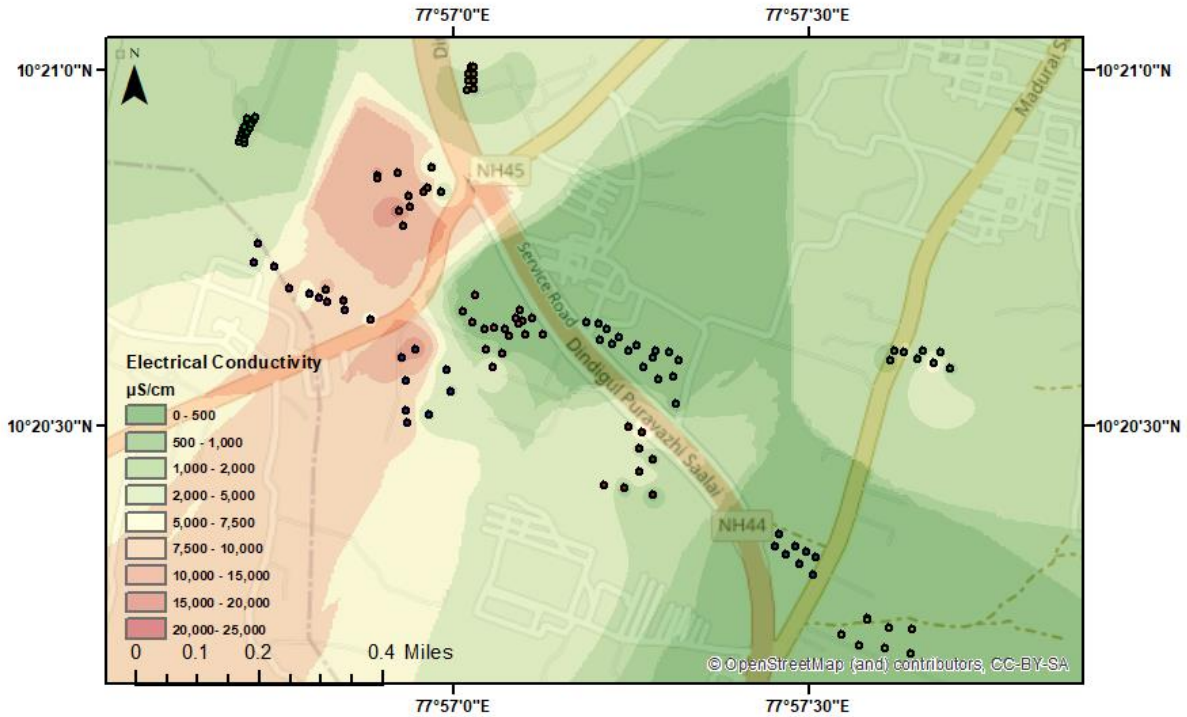


Figure 2.16 - Spatial distribution by Inverse Distance Weighting of electrical conductivity.

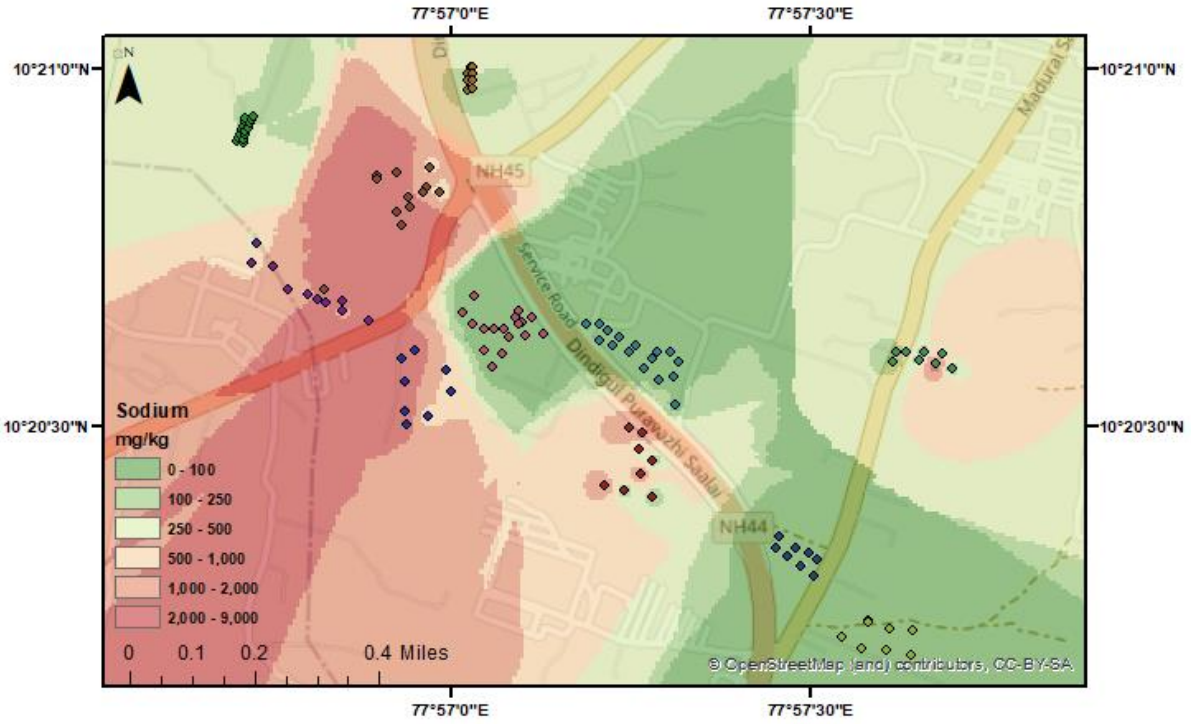


Figure 2.17 - Spatial distribution by Inverse Distance Weighting of sodium (mg/kg).

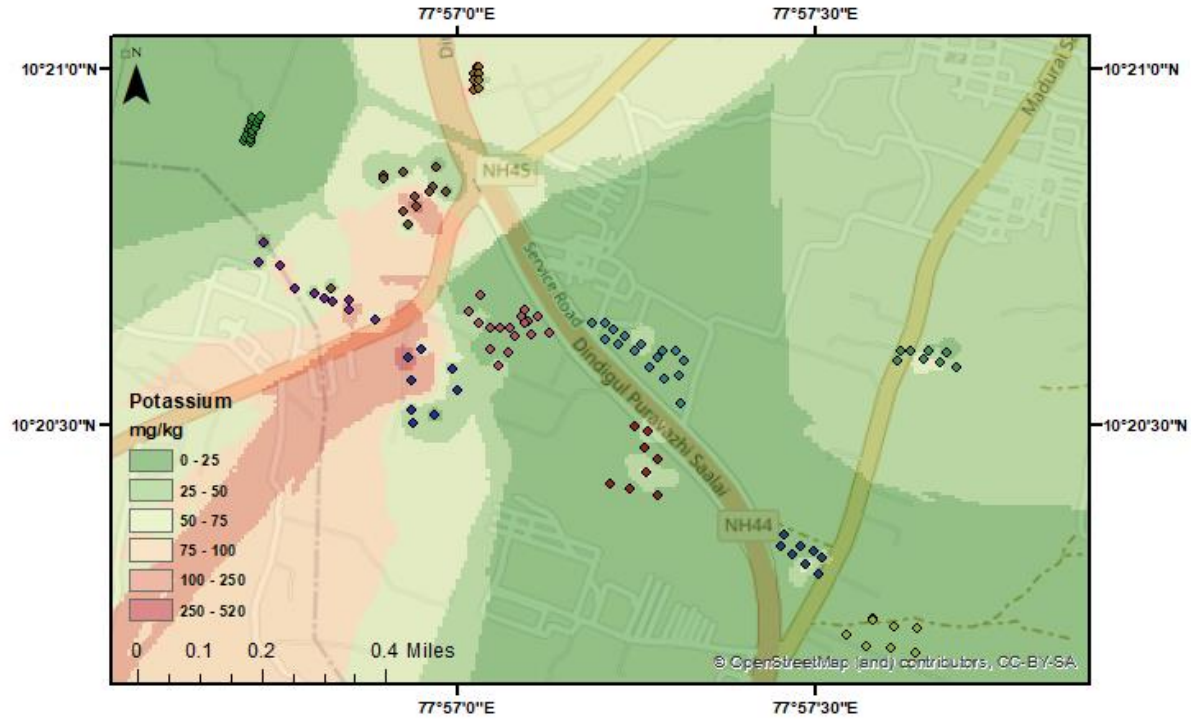


Figure 2.18 - Spatial distribution by Inverse Distance Weighting of potassium (mg/kg).

Figure 2.19 shows the inverse distance weighting of the soil organic matter. Organic matter content of soils is known to have significant effects on mobility, availability, and reduction of certain heavy metal ions and is heavily affected by microbial communities and decomposition or plant material in the area. Higher levels can indicate increased levels of both. Statistically significant positive correlations ($p<0.05$) were observed between organic matter and the metals Cr, Cd, and Cu which can be seen in the inverse distance weighting figures 2.19, 2.10, 2.11 and 2.13 respectively. This positive correlation between organic matter and Cr levels can have a number of effects including and increased rate of reduction from Cr (VI) to Cr (III), increased concentrations of Cr (III) and a reduction in the leaching of the Cr throughout the soil (Banks, Schwab and Henderson, 2006). With the highest levels of soil organic matter being from the site 1, the same as the highest reading for Cr on the site, the likelihood that a higher proportion of the contamination in Cr (III) is supported by the literature. This effect of increased organic matter reduction in mobility and availability has also been found relating to Cd, Pb and Zn (Kwiatkowska-Malina, 2018). This means that in the vast areas of the experimental site with low concentrations of organic matter, as seen in figure 2.19, the organic matter will not be there to form complexes with the heavy metals within the soils and their mobility and potential toxicity would be increased.

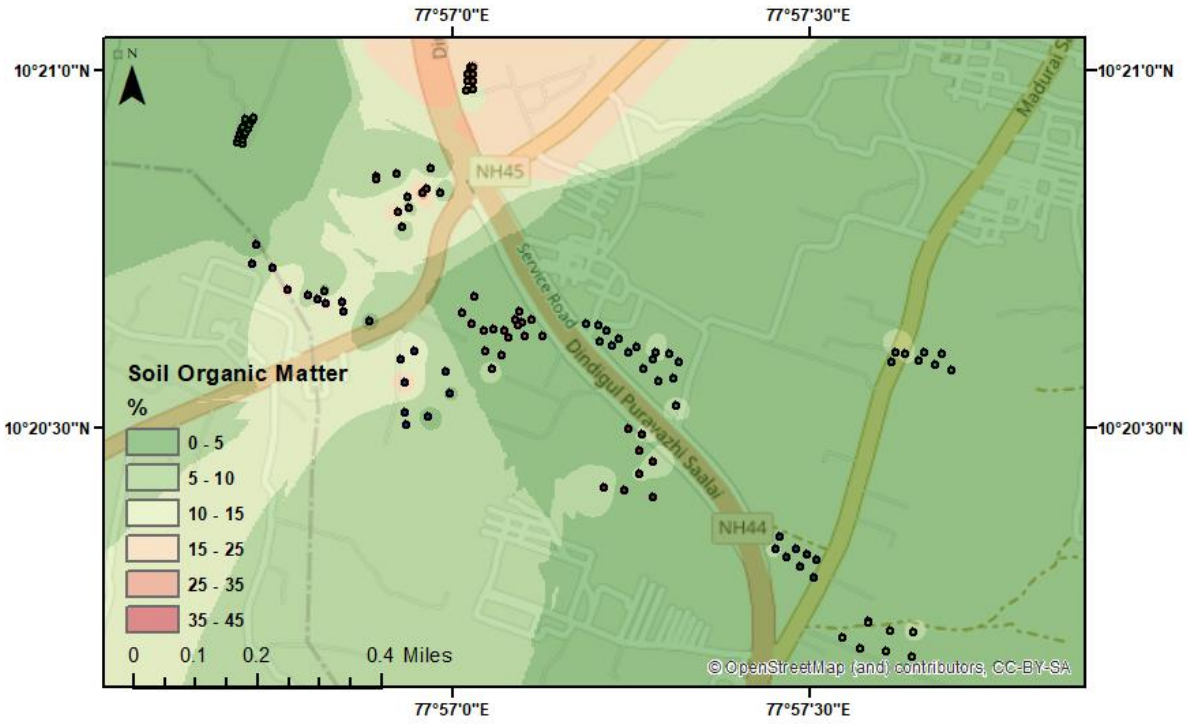


Figure 2.19 - Spatial distribution by Inverse Distance Weighting of Soil organic matter (%).

In the arid soils of around Dindigul, the increased levels that can be seen in figure 2.20 showing the inverse distance weighting for soil moisture content can be attributed to the contaminated waterbody situated in the west of the experimental site. Soil moisture content positively correlated significantly with Cr, Cd, Pb and Cu and negatively correlated with Zn, all statistically significantly (p value <0.05). This would indicate a link between the soil property and the heavy metal contamination, however from looking at the spatial distribution, it is not possible to conclude one.

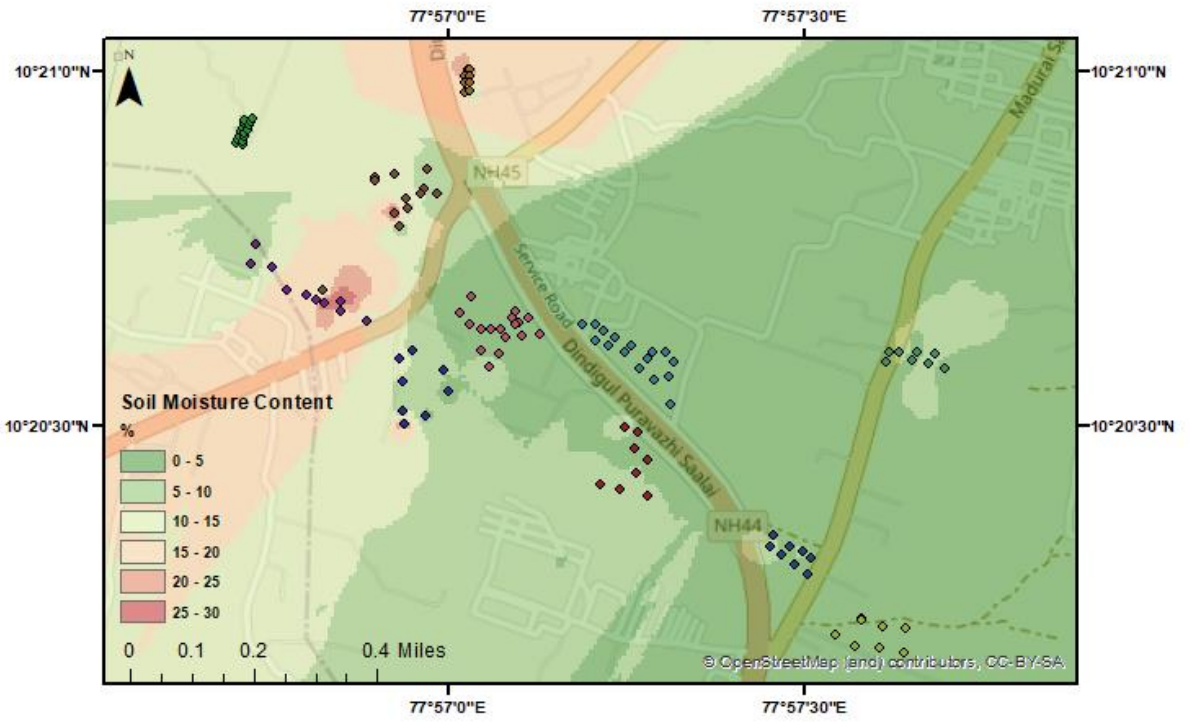


Figure 2.20 - Spatial distribution by Inverse Distance Weighting of Soil moisture content (%).

Phosphorous and nitrogen are extremely important the maintenance and increasing of plant diversity, being of extreme importance to the health of crop plants (Blumenthal et al., 2008; Thakur, Kaushal and Shyam, 2014). It has also been suggested that increased levels of both phosphorous and nitrogen, could provide an increased resistance to plants to heavy metals at contaminated sites (Huang et al., 2020). From figures 2.21 and 2.22, we can see that the experimental area has a high distribution of levels of phosphate, with very high concentrations focused on site 1, matching that of Cr. However, levels of nitrogen are seen to be very low across large areas of the experimental site (Fig.20). These reduced levels could be due to degradation of the land around the contaminated site, leading to loss

through wind or water erosion as well as many other way due to being a extremely susceptible macronutrient to loss in the environment (Mahmud, Panday, Mergoum and Missaoui, 2021).

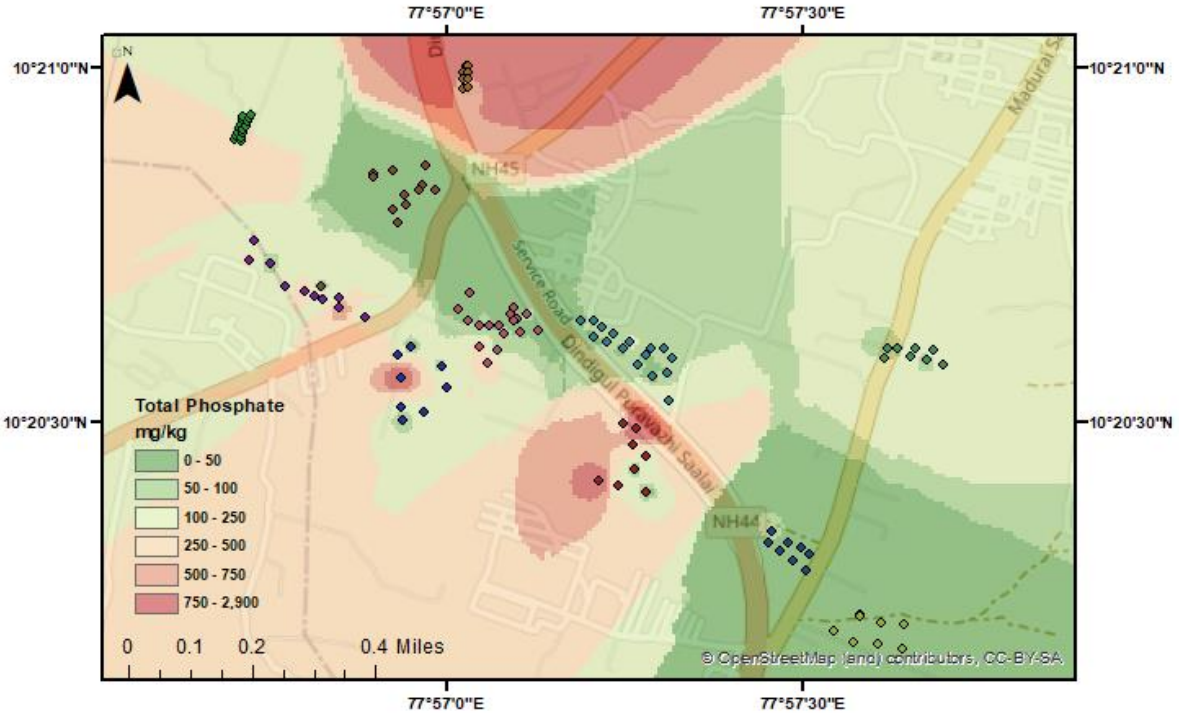


Figure 2.21 - Spatial distribution by Inverse Distance Weighting of Total phosphate (mg/kg)

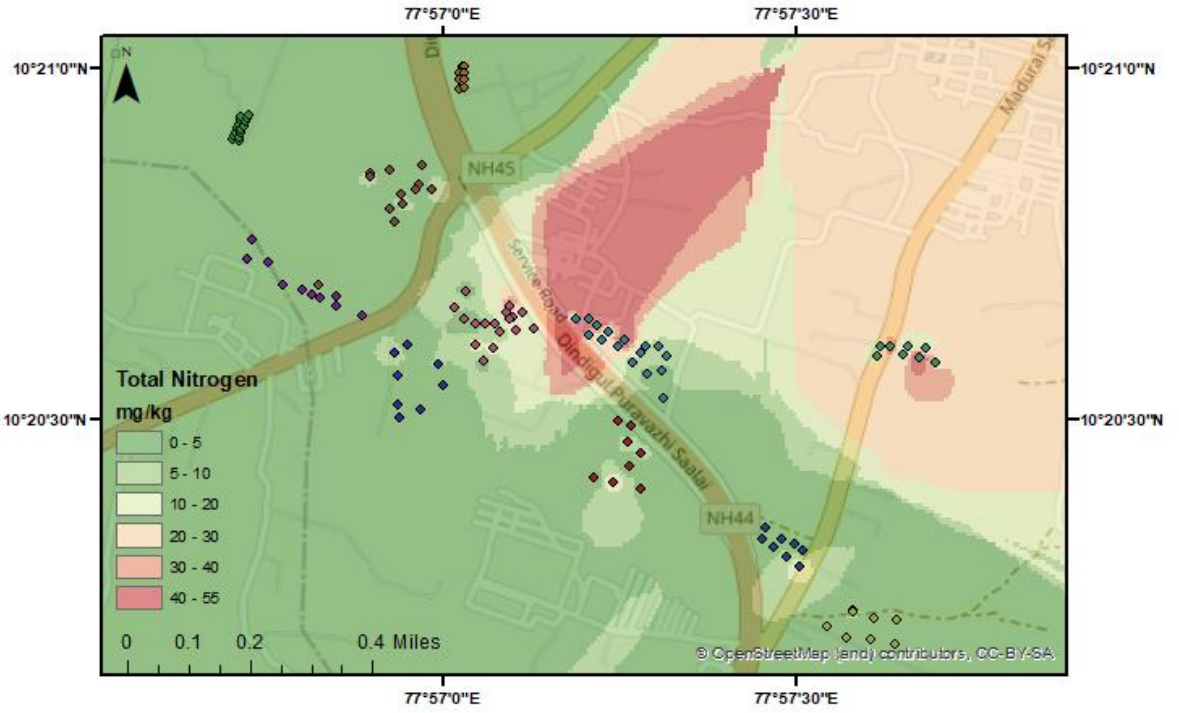


Figure 2.22 - Spatial distribution by Inverse Distance Weighting of Total Nitrogen (mg/kg)

Overall, from the spatial distribution by inverse distance weighting carried out on all the heavy metals and soil properties analysed, clear 'hot spot' Were identified. Areas situated around site 1 to the north, to the west of the tannery belt near the local drinking water supply and to the south, all show varying degrees of contamination and these 'hot spot' levels of contamination. The large variations in soil properties along with their associated statistics and correlations, lead to the suggestion that the areas has been greatly affected by local anthropogenic activity and tanneries, similarly to the research carried out by Paul, Choudhary, Gupta and Jose (2014).

Geo-accumulation Index of heavy metals

Geo-accumulation index was conducted and is displayed in table 2.5 using the classifications within table 2.4, originally put forward by Muller (1969) and built upon by Loska et al., (1997). Table 5 shows the classification values based of computed values for each site individually and varied to some extent across the experimental site. This calculation was done using bother the background levels that were obtained from the control sites situated to the south of the experimental site (as seen in figure 2.5) and against EU permissible limits (European Union, 2006). This was due to the different methods of which the calculation for B_n was being carried out across similar research stating the use of ether background or reference levels (Ahmed et al., 2019; Sulaiman, Salawu and Barambu, 2019; Rubasinghe, Gunatilake and Chandrajith, 2021). The subsequent contamination levels for the heavy metals were mapped though spatial distribution to visually depict both the extent of the contamination and how it compares between background and permissible limits.

0	Igeo < 0	Practically uncontaminated
1	0 < Igeo < 1	Uncontaminated to moderately contaminated
2	1 < Igeo < 2	Moderately contaminated
3	2 < Igeo < 3	Moderately to heavily contaminated
4	3 < Igeo < 4	Heavily contaminated
5	4 < Igeo < 5	Heavily to extremely contaminated
6	5 < Igeo	Extremely contaminated

Table 2.4. Description of each Igeo classification

	Control Background					EU Permissible Limits				
	Chromium	**Lead**	**Copper**	**Cadmium**	**Zinc**	**Chromium**	**Lead**	**Copper**	**Cadmium**	**Zinc**
Site 1	6	2	0	0	0	5	0	0	1	0
Site 2	0	1	0	0	1	0	0	0	0	2
Site 3	1	2	0	0	1	1	0	0	0	2
Site 4	0	0	1	0	1	0	0	0	0	2
Site 5	0	0	0	0	1	0	0	0	0	2
Site 6	1	2	1	0	0	1	0	0	0	1
Site 7	1	0	0	0	0	1	0	0	0	1
Site 8	3	1	1	0	0	3	0	0	0	1
Site 9	1	0	0	0	0	0	0	0	0	1
Site 10	0	0	0	0	0	0	0	0	0	1
Site 11	0	1	0	2	0	0	0	0	4	1

Table 2.5. Igeo classification per site for both measured background levels and EU permissible limits.

All heavy metals showed some extent of contamination across the experimental site when compared with background levels. Cr showed this to the greatest extent, with site 1 registering as extremely contaminated and site 8 as moderately to heavily contaminated. This was similar to the classifications given by the permissible limits, with a slight downgrading of site 1 to classification 5 from 6. Inputting the data into the spatial distribution, shown in figure 2.23, a large proportion of the experimental site under Igeo with background input shows between category 1 and 6 meaning some level of contamination of the site has occurred. This changes when analysed using permissible limits with much of the site becoming category 0 and such, classified as uncontaminated. This shows that the B_n used in the calculation can have a drastic effect on the information produced.

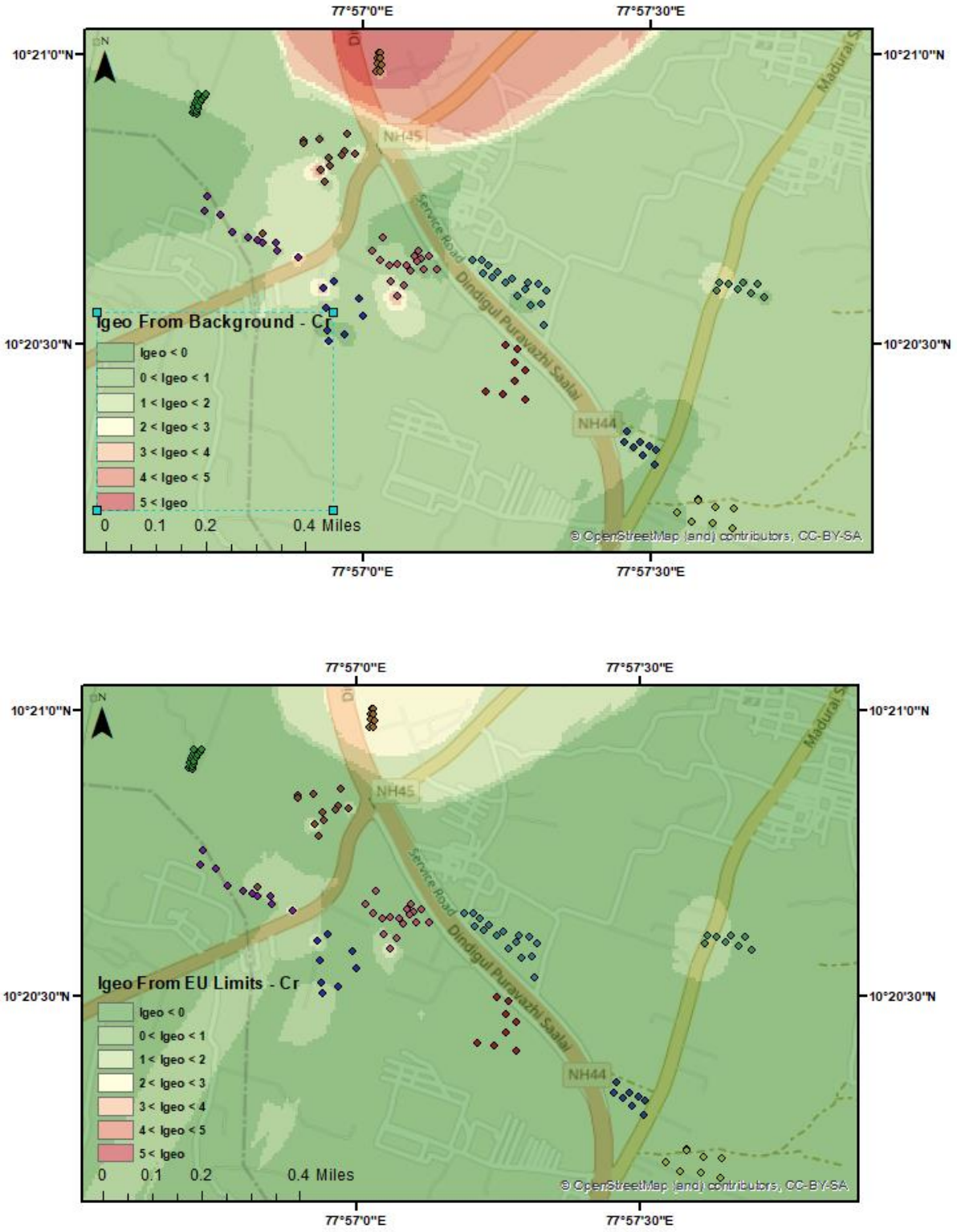


Figure 2.23. Spatial distribution of Igeo readings for Cr utilising background and permissible limit values.

Pb Igeo showed stark differences between background and permissible limits on categorising of the site. As visually displayed in figure 2.24, Site categories ranged from between 0 and 2 Igeo category, showing between uncontaminated to moderately contaminated levels across the wider site. Permissible limits however, showed no contamination at all by Pb, with all sites being categorised as 0 meaning uncontaminated. This shows that contamination is occurring regarding the natural levels of the area, as shown by the categorising and spatial distribution against controls, but when looking at levels compared to permissible limits, the conclusion that can be drawn is that there is no threat to health due to the levels of Pb in the area, as stated by limits by the European Union.

We see the same with Cu, slight contamination of the sites registers as category 1 when against background levels which is not seen as contaminated within the permissible limits with category 0. As can be seen in figure 2.25 however, 4 individual samples reach to higher Igeo category levels of 2 and 3 against background levels. This in the general analysis of the sites as wholes against each other is lost, meaning that a loss of resolution across the experimental area is lost that was only visible due to the implementation of the spatial distribution maps.

Cd and Zn both show the opposite issue to that of the other heavy metals. As visualised in figures 2.26 and 2.27, Cd and Zn as analysed by Igeo showed worse contamination levels when compared to the permissible limit levels as to the background levels of the area. Cd showed levels of category 4, meaning heavily contaminated at site 11, however as visible by spatial distribution, most of the site showed levels of category 3 with a high of 4. This means that site 11 raises contamination worry and risk to human and animal health. Site 11 is also situated near the local villages drinking supply, making this site an increased risk area. Zn, as compared to the relatively low to zero levels of perceived contamination shown by Igeo of background levels, showed light to moderate contamination across much of the site.

From the geo-accumulation index on the whole, contamination of the site compared to background levels was found to be of moderate levels or higher by Cr, Pb and Cd at different sites. Cu and Zn showed mild category 1 levels at a small number of sites. Cr, Cd and Zn showed contamination levels of moderate and higher (category 2 or more) when compared to permissible limits, showing these metals to be at levels across the site of concern towards contamination levels an there threat towards the local populations health and wellbeing. Pb and Cu showed no contamination at across the site when compared to permissible limits, suggesting less threat from these heavy metals towards the local population through bioaccumulation in crop plants on inhalation due to dust from desertification.

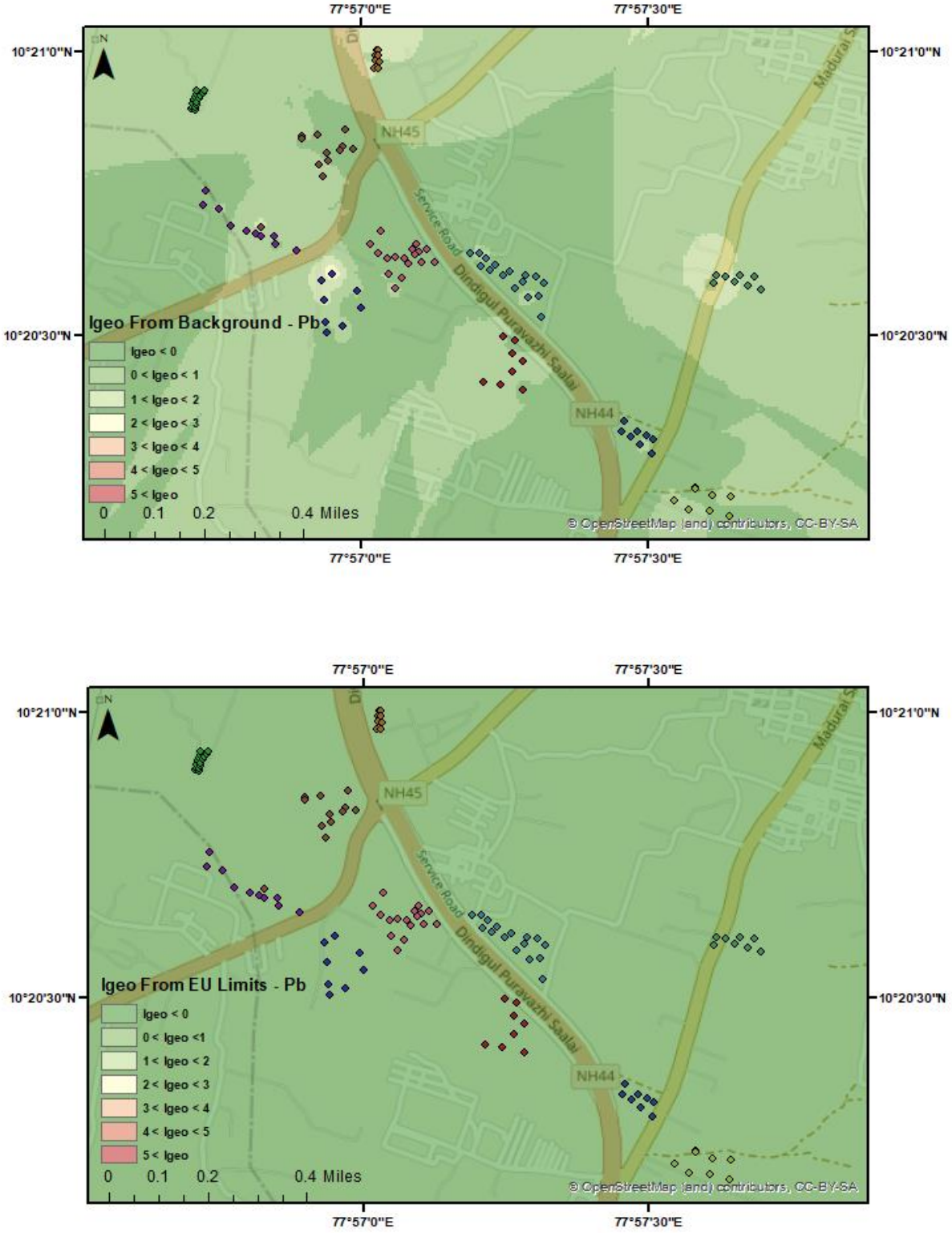


Figure 2.24. Spatial distribution of Igeo readings for Pb utilising background and permissible limit values.

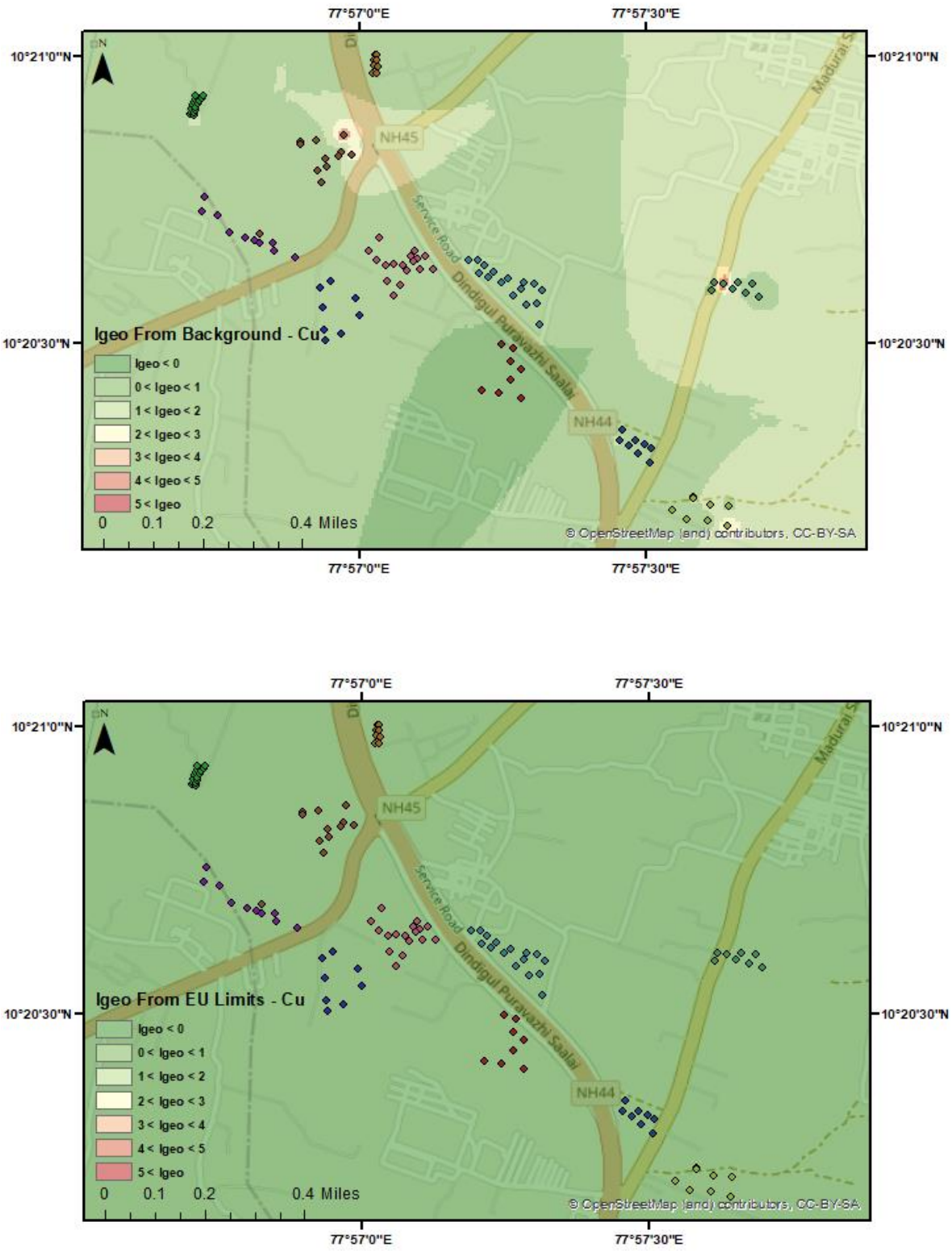


Figure 2.25. Spatial distribution of Igeo readings for Cu utilising background and permissible limit values.

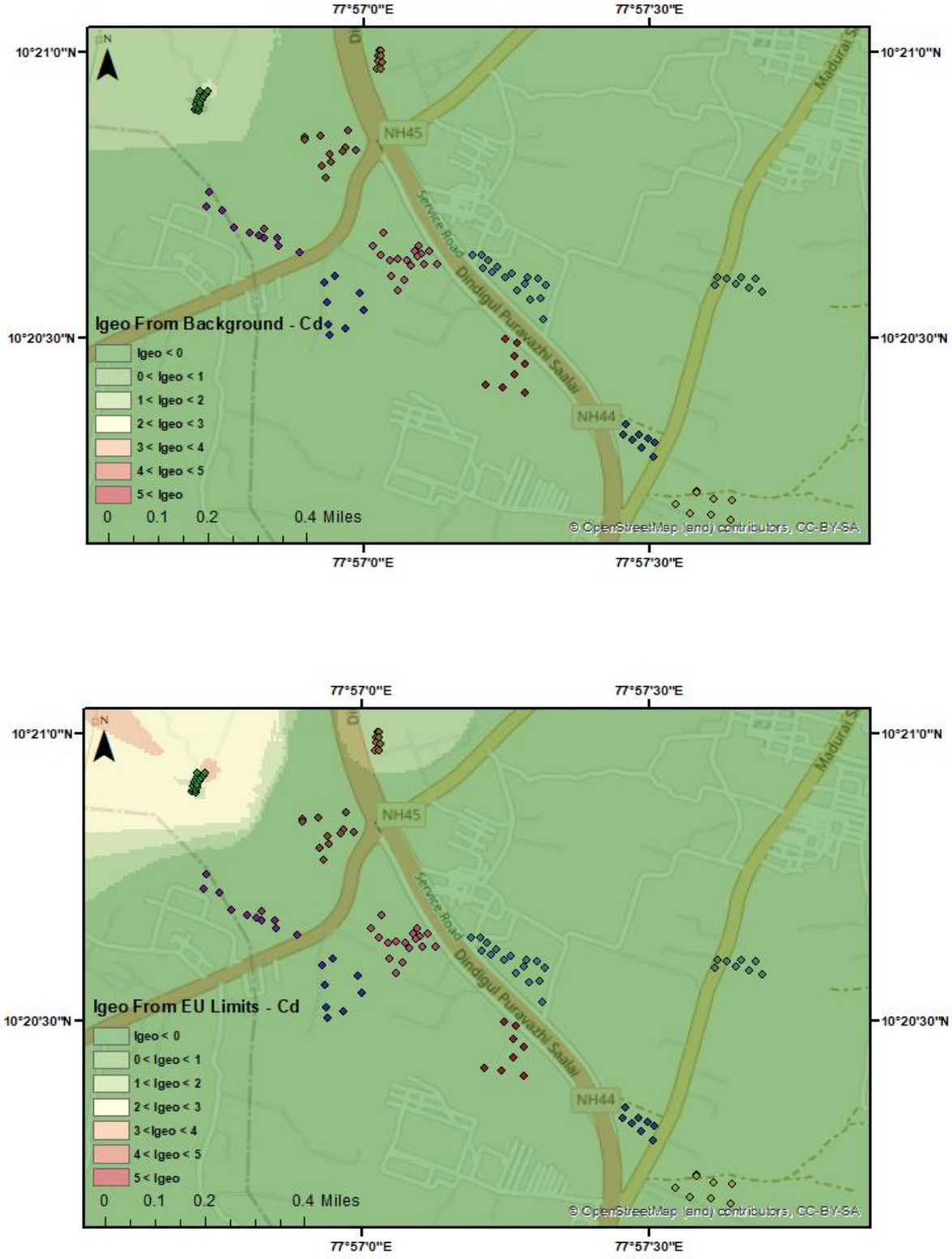


Figure 2.26. Spatial distribution of Igeo readings for Cd utilising background and permissible limit values.

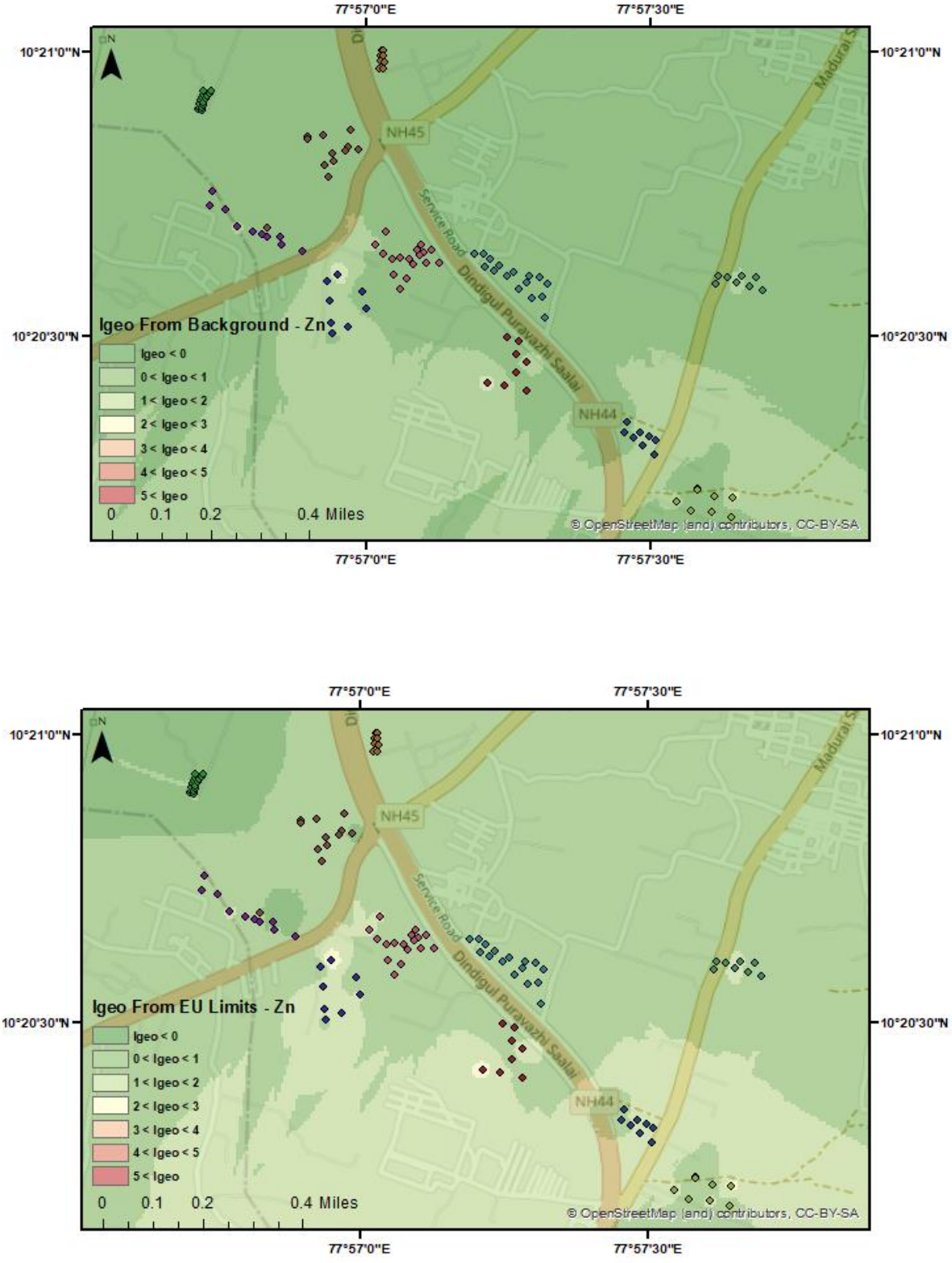


Figure 2.27. Spatial distribution of Igeo readings for Zn utilising background and permissible limit values.

Contamination factor index and contamination degree of heavy metals

The results for the contamination factor index (Cf) and degree of contamination index (Cdeg) are both shown in table 2.7. The classifications used to categorise the level of contamination are displayed in table 2.6 with readings across the whole experimental site displayed per heavy metal and in total for Cdeg in figures 2.28 – 2.33. Most sites showed at least a moderate contamination by each heavy metal except for Cd, only showing high levels of contamination at site 11 and none to low at all others. Cr was the only metal with contamination factor registering at very high, happening at two sites (1 and 8). These corresponded with Igeo levels of Cr for both those sites, but with Igeo showing site 8 to be moderately contaminated. Pb and Cu showed low level contamination factors at sites 7,8 and 9 with Cu showing moderate in the rest and Pb showing moderate in all but sites 1 and 6 where high contamination factors were found. Zn showed more contamination than displayed by Igeo, registering 8 sites as moderate contamination factor against 4 sites of 1 Igeo category on uncontaminated to moderate contamination.

In all both Igeo and Cf showed similar patterns in contamination prediction across the sites, however, contamination factor registered more moderate levels than Igeo. The majority of Cf categories of high to very high contamination factor was seen at sites to the north of the experimental area and situated around the left of the tannery belt, around the drinking water source as displayed in figures 2.28 – 2.33.

Cf < 1	Low contamination factor
1 < Cf < 3	Moderate contamination factor
3 < Cf < 6	High contamination factor
6 < Cf	Very High contamination factor

Cdeg < 8	Low contamination degree
8< Cdeg <16	Moderate contamination degree
16< Cdeg <32	High contamination degree
32< Cdeg	Very high contamination degree

Table 2.6. Contamination factor index (top) and degree of contamination index (bottom) classifications

Degree of contamination at each site is used to determine the extent of the contamination when all contaminants are taken into account at once. This would allow for sites that pose more of a threat to be highlighted. Sites 2, 4, 5, 7, 9, and 10 showed Cdeg of below 8. This means the sites showed a low contamination degree and as such are at a low priority for management for the area. Sites 4, 8, and 11 displayed moderate contamination degree levels meaning they are of concern to the local area. These three sites are all situated around the west side of the tannery belt. Site 1, as has been the case through all index analysis, showed contamination degree levels of above 32, meaning it is of a very high contamination degree. The actual value reached was 50, showing the real extent of the contamination at this location. This site is again situated to the north-west of the tannery belt and is, as the with sites 4,8 and 11, situated around the location of a local drinking water source (Figure 2.33).

	Contamination Factor - Cf					Contamination Degree - Cdeg
	Chromium	Lead	Copper	Cadmium	Zinc	
Site 1	6 < Cf	3 < Cf < 6	1 < Cf < 3	Cf < 1	Cf < 1	32< Cdeg = Very high
Site 2	1 < Cf < 3	1 < Cf < 3	1 < Cf < 3	Cf < 1	1 < Cf < 3	Cdeg < 8 = Low
Site 3	1 < Cf < 3	1 < Cf < 3	1 < Cf < 3	Cf < 1	1 < Cf < 3	8< Cdeg <16 = Moderate
Site 4	1 < Cf < 3	1 < Cf < 3	1 < Cf < 3	Cf < 1	1 < Cf < 3	Cdeg < 8 =Low
Site 5	1 < Cf < 3	Cf < 1	1 < Cf < 3	Cf < 1	1 < Cf < 3	Cdeg < 8 = Low
Site 6	1 < Cf < 3	3 < Cf < 6	1 < Cf < 3	Cf < 1	1 < Cf < 3	8< Cdeg <16 = Moderate
Site 7	1 < Cf < 3	Cf < 1	Cf < 1	Cf < 1	1 < Cf < 3	Cdeg < 8 =Low
Site 8	6 < Cf	1 < Cf < 3	1 < Cf < 3	Cf < 1	Cf < 1	8< Cdeg <16 = Moderate
Site 9	1 < Cf < 3	Cf < 1	Cf < 1	Cf < 1	1 < Cf < 3	Cdeg < 8 = Low
Site 10	1 < Cf < 3	Cf < 1	Cf < 1	Cf < 1	1 < Cf < 3	Cdeg < 8 = Low
Site 11	1 < Cf < 3	1 < Cf < 3	1 < Cf < 3	3 < Cf < 6	Cf < 1	8< Cdeg <16 = Moderate

Table 2.7. Contamination factor and degree of contamination for sites studied south of Dindigul

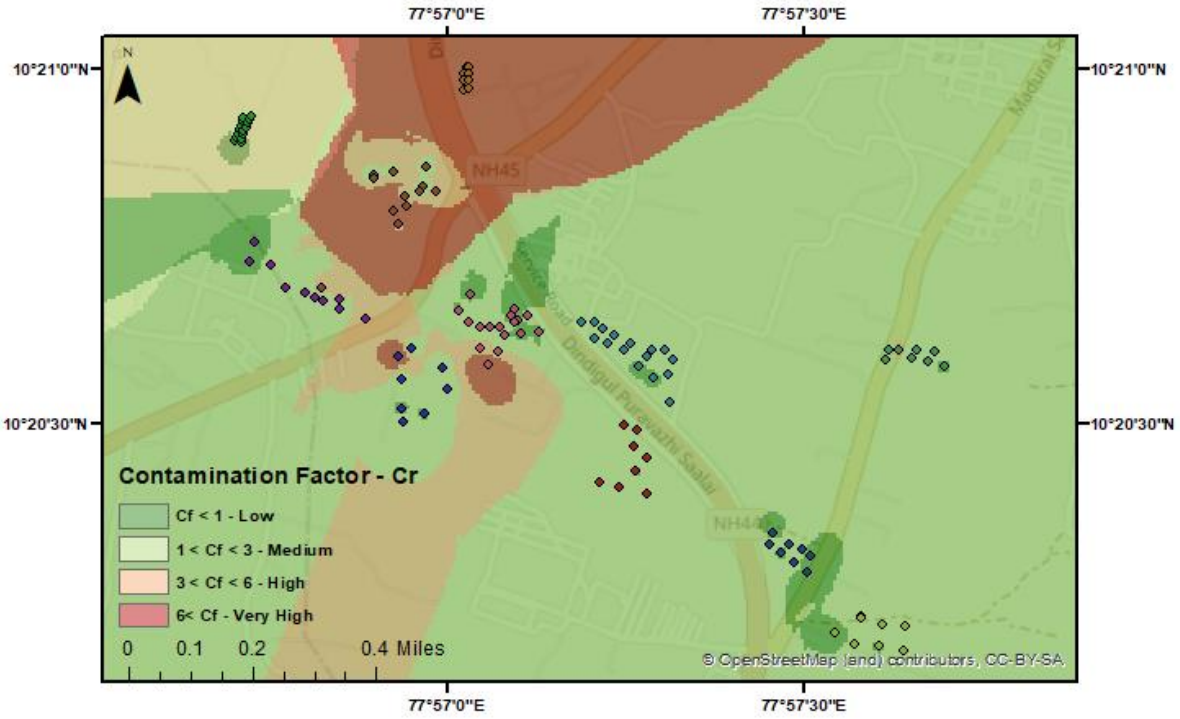


Figure 2.28. Spatial distribution of contamination factor - Cr

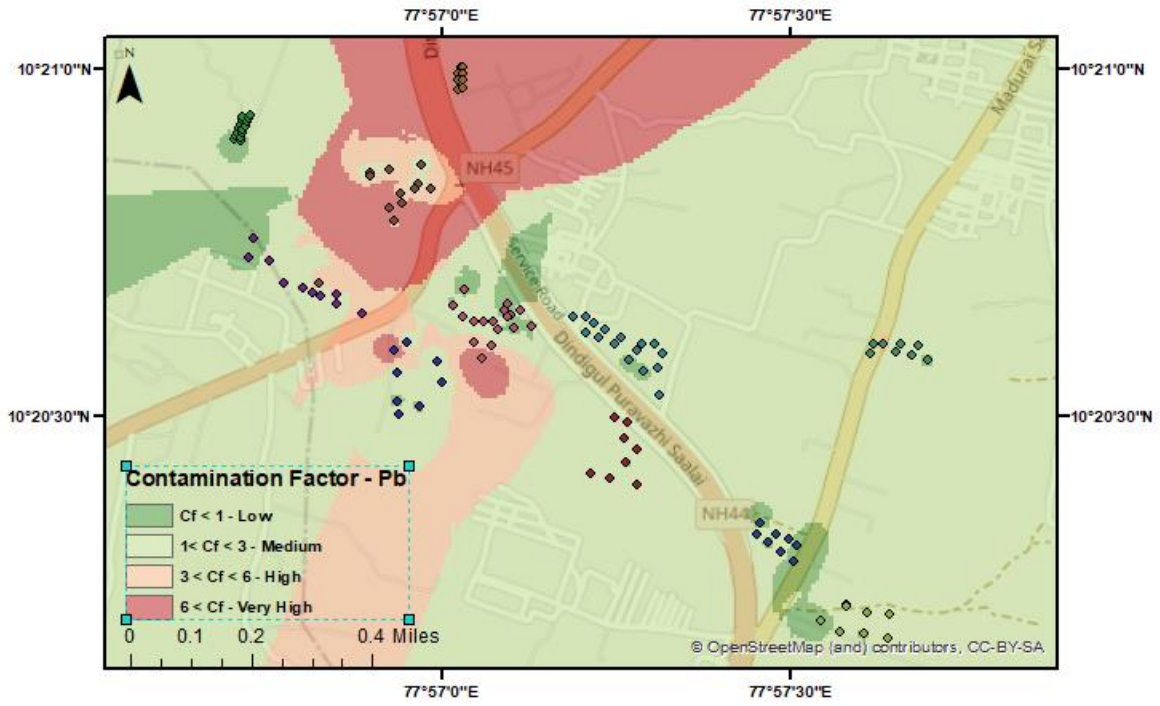


Figure 2.29. Spatial distribution of contamination factor - Pb

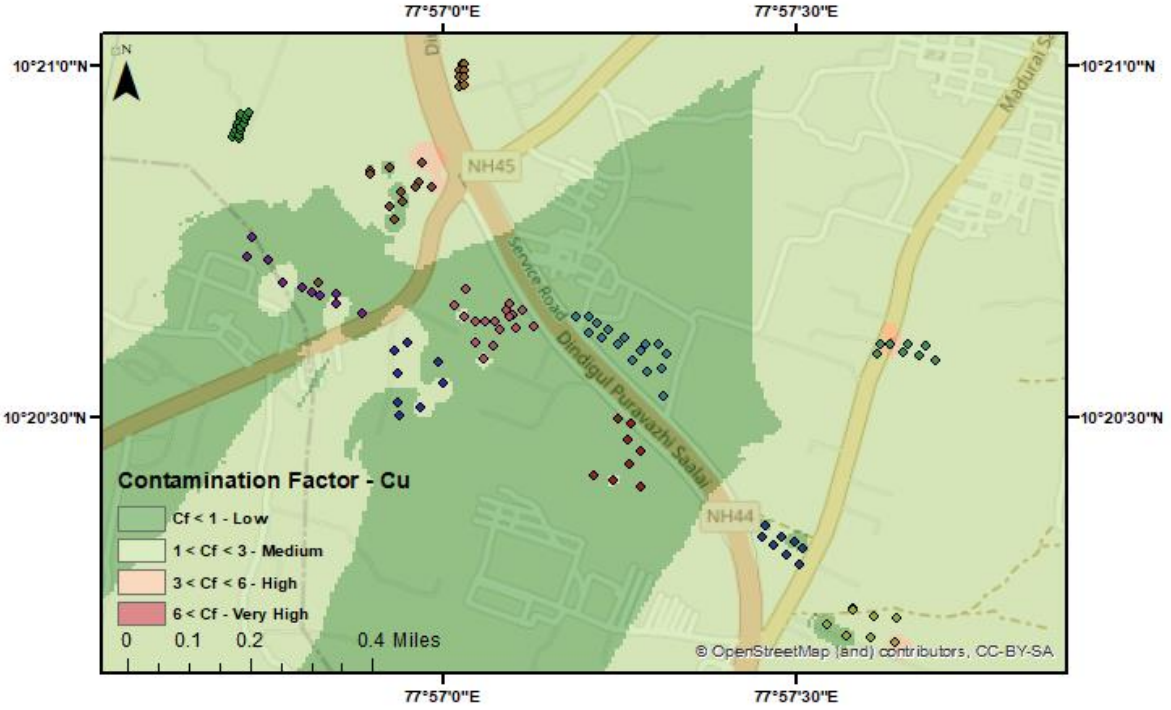


Figure 2.30. Spatial distribution of contamination factor - Cu

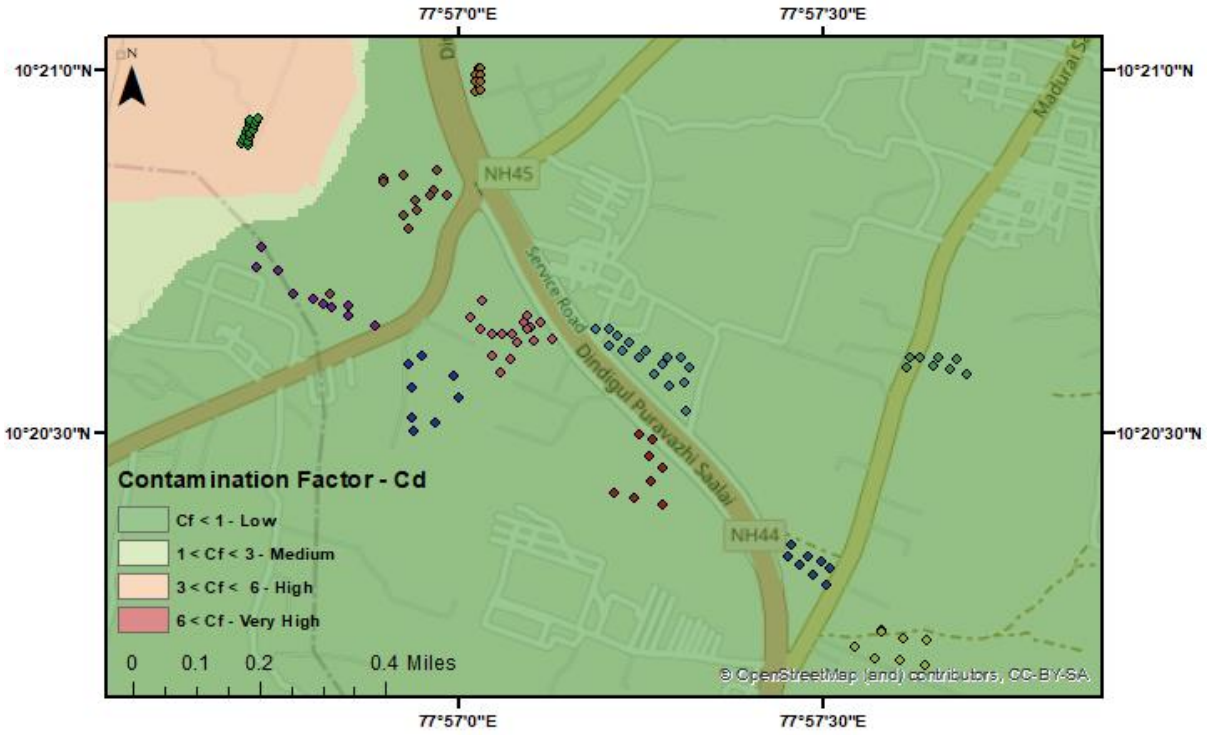


Figure 2.31. Spatial distribution of contamination factor - Cd

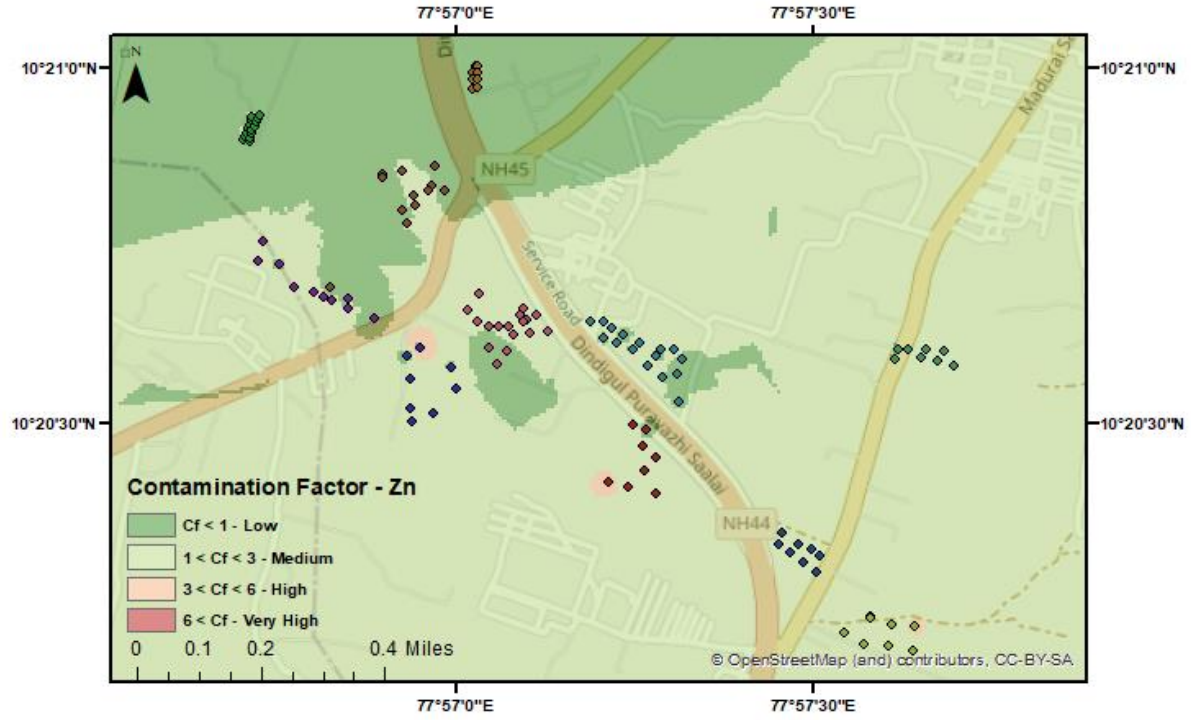


Figure 2.32. Spatial distribution of contamination factor - Zn

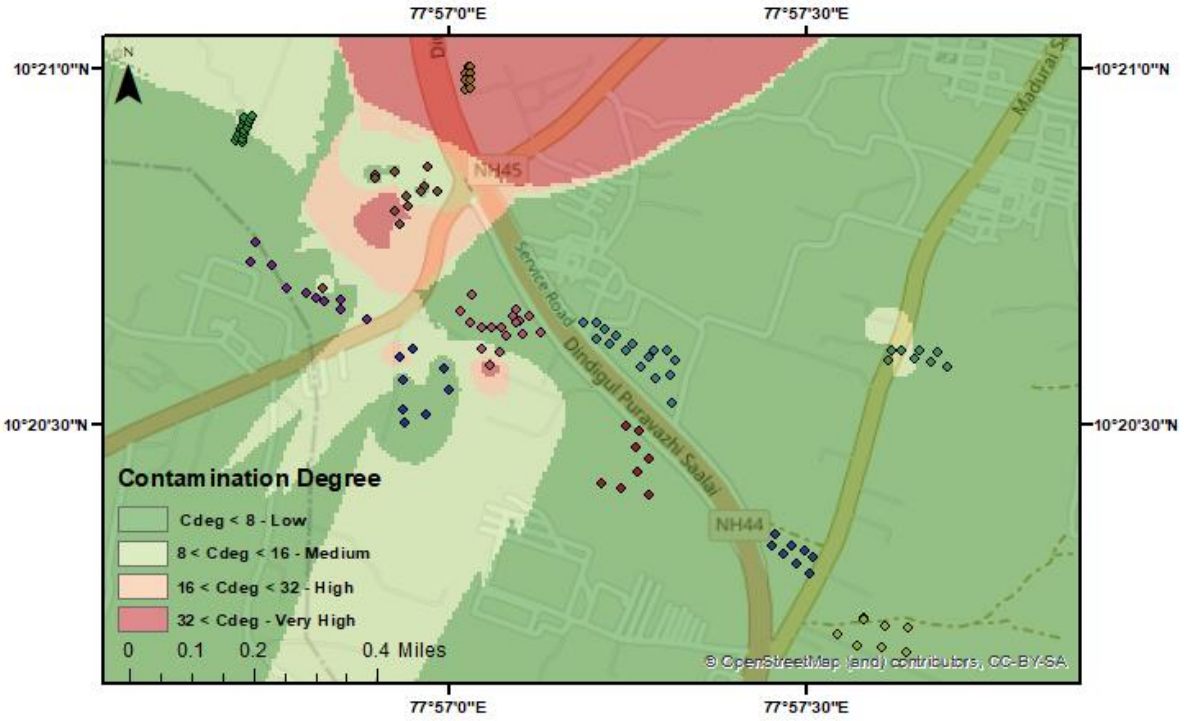
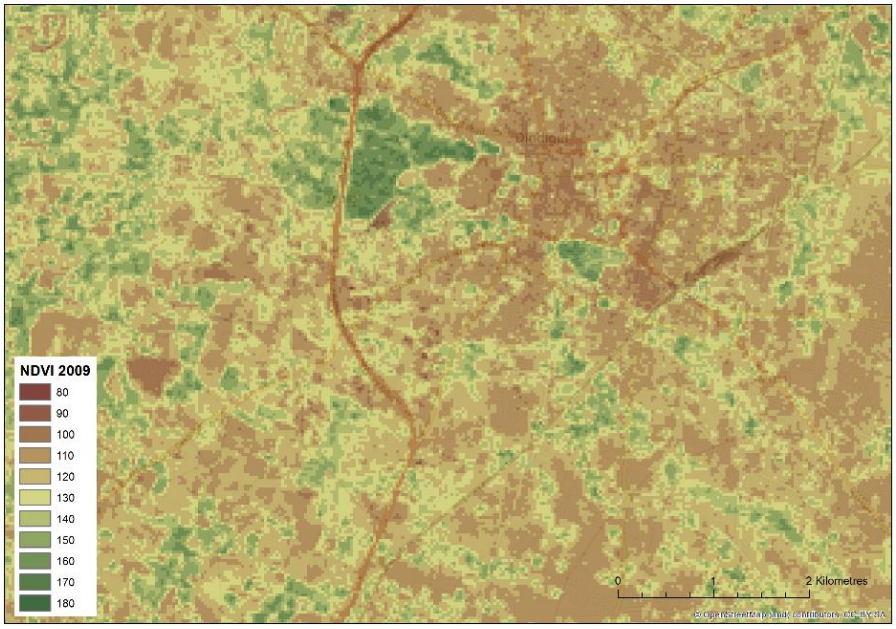


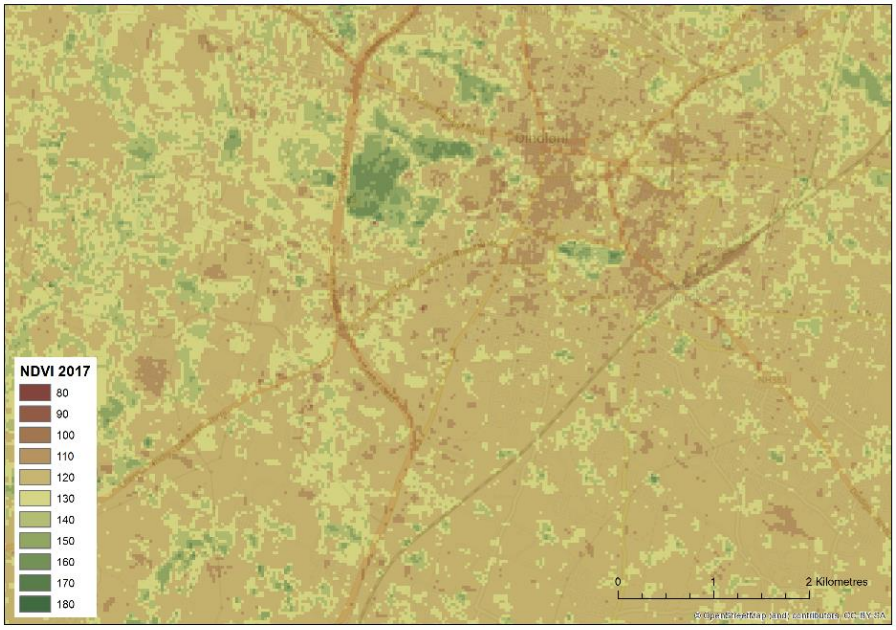
Figure 2.33. Spatial distribution of degree of contamination

NDVI values and soil moisture index values for the tannery area

Figure 2.34 shows the normalized difference vegetation index (NDVI) for the region surrounding the experimental sites, consisting of abandoned and degrading farm and scrubland. NDVI was calculated using Red Visible band 3 (0.63-0.69µm) and Near Infrared (0.77-0.90µm) band 4 (Ghosh and Porchelvan, 2018). Days within the month of March were selected for the years of 2009, 2017 and 2021, with care taken to avoid satellite images that had cloud cover obscuring the experimental area as this would adversely affect the NDVI images.

With green shades representing the increased foliage cover, there is a notable increase in the foliage of the whole area across the three years taken. With concerns over the desertification of the area due to a reduction in the use of the agricultural land surrounding the tannery belt district of Dindigul, an increase in the foliage cover of the area will help to reduce demineralisation and degradation of the soil environments more than they already have. This increase in foliage would offer protection from weather related erosion such as wind and water and potentially lead to an increase in the soil quality (Ziadat and Taimeh, 2013), even if the land remains unused for agricultural purposes. Rodrigo-Comino et al., (2018) supports the findings from this study with the increase of foliage. Rodrigo-Comino et al., (2018) states that although initially soil degradation can occur from the disuse of the one utilised land, such is the case with the sampling sites in this study, dense vegetation has been seen to eventually take over the land if not on steep slopes. This does mean that land degradation can occur before the colonisation of the land by the dense vegetation and could be the reason so the dispersal of contamination to further reached of the site where background soils levels were taken.





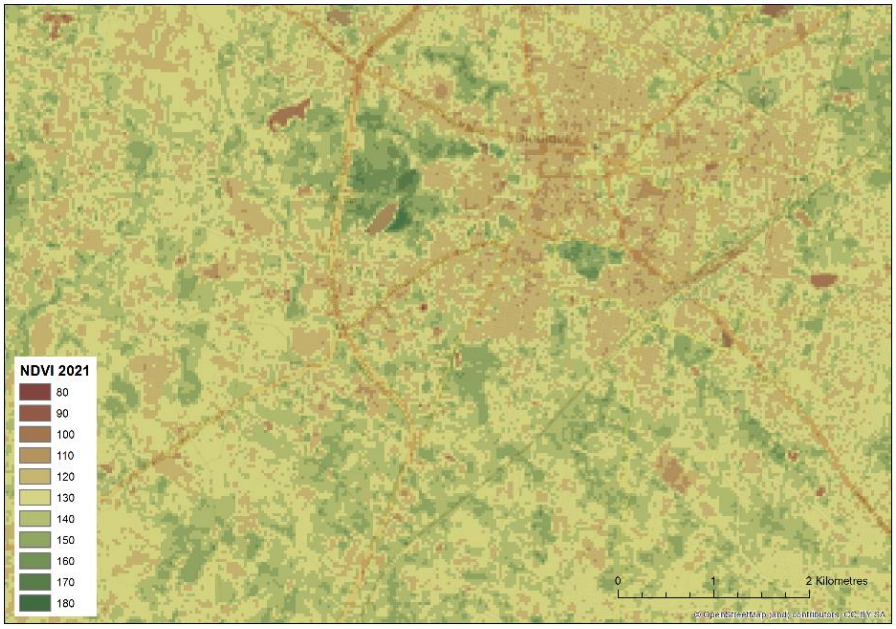


Figure 2.34. NVDI from 2009 to 2021 via LANDSAT satellite imagery showing changes in foliage cover for the areas surrounding the tannery sampling sites south of Dindigul.

Conclusion

Contamination from five heavy metals (Cr, Pb, Cu, Cd, Zn) known to be in contaminated effluent from tanneries was found to be a differing contamination levels across the site of the tannery belt, situated to the south of the city of Dindigul. All heavy metals analysed apart from Pb showed levels at the experimental site that exceeded permissible limits with limits also being exceeded within several background sites with Cr and Pb showing significant increases compared to background levels. This suggests that contamination from the tannery sites could be dispersing to a wider area then first thought via fertilisation using tannery waste and natural erosion. Cr showed levels 77 times the permissible limit of 100 mg/kg , Zn 13 times limit of 300 mg/kg, Cd 32 times limit of 3 mg/kg and Cu 2.5 times limit of 100 mg/kg. Statistically significant correlations were identified between several the heavy metals and the soil properties. Excessive levels of electrical conductivity, sodium and potassium were found, specifically around a local drinking water supply and relates to the large amount of salt used within the tannery process. These correlations between soil contamination and properties, will have greatly differing effects on the mobility and availability of the heavy metals precent for uptake into crop plants and the surrounding ecosystem and thus, going on to affect the health of the surrounding human population.

Contamination Indexes including geo-accumulation index, contamination factor and degree of contamination were carried out with varying results as the extent of contamination across the site. These were dependent on certain data being inputted into the equations, in this case, background reading or permissible limits. Igeo using background control levels and Cf showed similar contamination results for the heavy metal contamination across the sites analysed. Cdeg gave the concluding analysis into the extent of contamination, with several moderate and high contamination degrees being identified in the experimental site which warrant further investigation and subsequent remediation in the future. Lastly, suspected soil degradation was inspected using NDVI to analyse foliage cover. It was found that foliage cover from between 2009 and 2021 have been increasing across the surrounding area of the tannery triangle. As such threat of soil degradation will be reduced as threat from water and wind erosion are reduced due to increased foliage in the area.

The study concludes that the area surrounding the site of the tannery belt to the south of Dindigul is contaminated to differing degrees with Cr, Cd, Cu and Zn as well as with excessive amounts of salt related elements. All these contaminates can be linked back to the local tanning industry which is the areas main industrial focus. As such it is suggested that remediation methods are implemented as to improve the local agricultural land to a state safe for human health as a matter of priority due to the low social economic standing of the area.

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3. Metagenomics of bacteria, fungi, nematode, and protozoa communities with tannery contaminated soils containing Cr and potential for assisted phytoremediation

Abstract

Samples previously tested for the chromium content after being collected from tannery contaminated industrial land situated near the tannery belt, Dindigul, Tamil Nadu, were analysed for their soil microbial communities. Bacterial, fungal, nematode, and protozoan communities within the soil microbiome were analysed via isolation via 16s, ITS and 18s specific primers and then subsequent sequencing via Illumina MiSeq platform and Nextera XT v2 primer indexing. Sequences were analysed via alpha and beta diversity index utilising the Qiime 2 package to determine if tannery contamination would have a significant effect on the abundance and diversity of the soil microbiome populations. Alpha diversity found significant differences in the ITS and 18s populations for their community structure, especially when abundance was involved, as shown by the Simpsons index. Beta diversity showed a significant dissimilarity between the control and contaminated samples in all analysed sequences via PERMANOVA. Orders of interest for each organism type were identified. Bacteria *Thermomicrobiales* and *Tistrellales,* fungi *Eurotiales* and *Capnodiales*, nematode *Rhabditida* and protozoa *Phytomyxea,* all showed significant resistance to the presence of pollution within the contaminated soil samples and demonstrated a large increase in frequency within the samples between control and contaminated. Bacteria *Tepidisphaerales,* fungi *Hypocreales,* nematode *Dorylaimia* and *Tylenchida* and protozoa *Gregarinasina* all showed sizable reduction in the relative frequency levels between the control and contaminated soil samples.

Introduction

The soil environment is known to be the most diverse and abundant microbiome that exists on earth (Bahram et al., 2018). The soil community is composed of a diverse array of soil biota, including bacteria, fungi, nematodes, protozoa, algae and earthworms to name a few (Fortuna, 2012; Thompson et al., 2017, van den Hoogen et al., 2019). These communities are not only diverse; there is an incredible abundance of organisms in the soil, especially of microscopic organisms. In a spoonful of

soil, the abundance of bacteria can be in the billions, fungi can have hundreds of meters of hyphae, protozoa can reach populations in the 100,000's and nematodes can reach hundreds of individuals. These populations differ depending on land use type, as shown in table 3.1 (Fortuna, 2012; Sollen-Norrlin, Ghaley and Rintoul, 2020).

	Land Type		
	Agricultural	Grassland	Forest
Bacteria	100 million - 1 billion	100 million - 1 billion	100 million - 1 billion
Fungi	Several Meters	10's - 100's of meters	1 - 60 kilometres
Protozoa	1000's	1000's	100,000's
Nematodes	10's	10's - 100's	100's

Table 3.1. Organisms per gram of soil (Adapted from Fortuna, 2012)

The diversity within soil communities matters greatly (as shown in figure 3.1, with the wider community of organisms the fixing of nitrogen (Chen, Zhu and Zhang, 2003) and also allowing for the increased health of plant and protection against pest and diseases (Francis, Jacquemyn, Delvigne and Lievens, 2020). It also includes the breaking down of dead organic matter and releasing the nutrients locked within back into the soil to be utilised again (Li et al., 2018) sequestering carbon into the soil media (van den Hoogen et al., 2019). Even with diversity being so important to so many aspects of the ecology of their ecosystems, global patterns of the microfauna and how they interact is vary comparatively under research (Bahram et al., 2018). providing many ecological functions. These include providing essential nutrients in mutualistic relationships with the resident flora (Ramakrishna, Yadav and Li, 2019; Hicks et al., 2021).

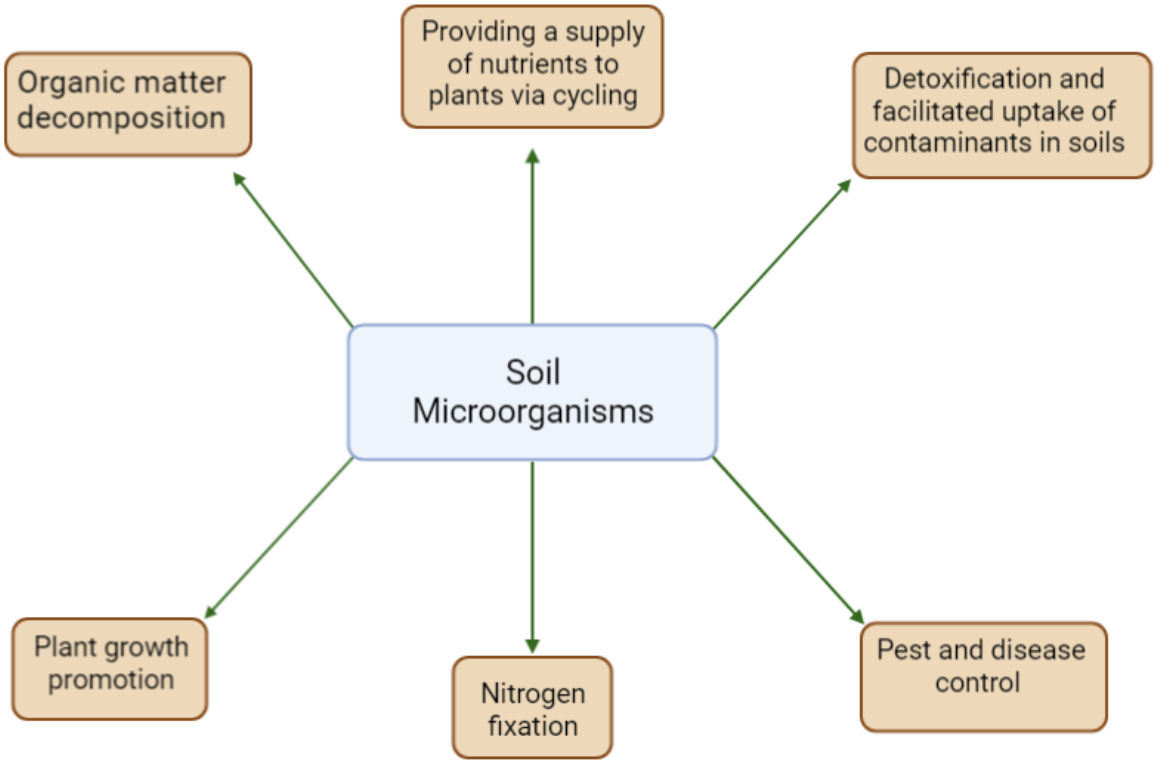


Figure 3.1 Key functions and roles of soil microorganisms

Although soil communities are thought of as essential in modern climate modelling and decision relating to the environment (van den Hoogen et al., 2019), most biogeographical modelling does not take the soil organisms into account on a global scale (Chu et al., 2020). This, however, is starting to change with broad range diversity and taxonomic data for all aspects of soil communities being researched within the Earth Microbiome Project being the start of study on these organisms on a grand scale (Thompson et al., 2017). Many studies focusing on the soil microorganisms relating to soil properties and substances within the soil media, look at a single taxonomic group. In order to gauge the full effect that a diverse and abundant soil community has on the soil properties, there effect on uptake of nutrients and pollutants, and on the other soil organisms present it is important that studies covering all biotic relations and interaction biogeographically are explored which in recent years is only beginning to be the case (Bahram et al., 2018).

Soil-biota relations

The soil biodiversity of a given ecosystem is one of the most important factors affecting many different aspects of the properties of the soil. Soil organisms have been demonstrated to impact the hydrology (Colica et al., 2014), pH (Msimbira and Smith, 2020; Shen et al., 2019), organic matter (Chen et al., 2015), mobility of nutrients (Jacoby et al., 2017) and the uptake and mobility of contaminants (Mishra, Singh and Arora, 2017). In turn, the composition of these soil communities has been proven to be greatly affected by these same soil properties, as well as other factors such as day length and geology of the area (Thompson et al., 2017). A high diversity of the microbiome of the soil and the resources available within can produce the environment for a highly diverse above ground ecosystem, largely through the increase in nutrient availability and the breakdown of detritus (Hooper et al., 2000). As such, microorganisms are significant in the development and biodiversity of the aboveground ecosystem (Bardgett and van der Putten, 2014), having a particularly strong influence on the health and diversity of plant communities (Bahram et al., 2018). Since the aboveground community interacts with a highly diverse community of soil organisms present in variable population sizes, it is necessary to understand which of these soil organisms are the key drivers of plant health, abundance, and diversity aboveground.

Hydrology

Hydrology of the soils, including its ability to retain and capture water, has been seen to be affected by the microorganism's present (Colica et al., 2014). A number of ways this has been seen to occur include the secretion of exopolysaccharides allowing for the retaining of water within certain soils (Colica et al., 2014; Sneha et al., 2021), and the improving of soil structure allowing for a structure that allows water to infiltrate into it and is able to contain more water within (Swaby, 1949; Coban, De Deyn and van der Ploeg, 2022; Jafarpoor, Sadeghi, Zarei Darki and Homaee, 2022). Changes in hydrology also effect the soil microorganisms, having effects of the community structure (Peralta, Ludmer and Kent, 2013) and diversity as a result in changes in soil moister content (Griffin et al., 2019).

pH

Some soil microbes have the ability to alter the pH of their surrounding soil by acidifying it as a way of competing with other microbes (Msimbira and Smith, 2020). However, pH has a large effect on the

soil microorganism communities and their structures (Zhalnina et al., 2014), with different microorganisms requiring different pH levels for their internal functions (Msimbira and Smith, 2020). As such, some microorganisms thrive at the optimal pH for plant growth of mildly acidic 5.5 – 6.5 (Zifcakova, 2020) and some such as *Acidobacteria*, *Alphaproteobacteria* (Shen et al., 2019) and some fungi prefer higher acidities (Msimbira and Smith, 2020).

Organic matter

Although fundamental in the creation of organic matter that is in a form that can be utilised by plants and other organisms, soil communities can be greatly affected by organic matter quality and content in soil (Chen et al., 2015). Common anthropogenic sources of organic matter include livestock (Li et al., 2018; He, Zhang, Zeng and Zhang, 2016) and mulching (Liu et al., 2014). This can alter the biomass of the microorganisms or the structure of the soil community itself (Philippot, Raaijmakers, Lemanceau and van der Putten, 2013, Chen et al., 2015, Scotti et al., 2015). These additions of organic carbon allow for and increased microbial abundance and diversity (Staddon, Duchesne and Trevors, 1997, Li et al., 2018).

Nutrients

Nitrogen and phosphorus are not readily available for plants to take up from the soils due to being trapped within organic compounds (Jacoby et al., 2017). Microorganism's however are able to mineralize the organic forms of these nutrients including important species of them such as ammonium, nitrate, phosphate (van der Heijden, Bardgett and van Straalen, 2008) which are subsequently released into the soil media through death and cell lysis (Richardson, Barea, McNeill and Prigent-Combaret, 2009; Jacoby et al., 2017).

Heavy metals

The increased mobility of the heavy metal ions as the result of soil microorganisms can affect accumulation of contaminants, with the process being referred to as rhizoremediation (Mishra, Singh and Arora, 2017), as depicted in figure 3.2. Bioaccumulation and biosorption refer to processes involving the uptake of heavy metals into the organism (Olaniran, Balgobind and Pillay, 2013), making it unavailable to the resident flora by reducing the concentration in the soil (Ayangbenro and Babalola,

2017). Both of these mechanisms are beneficial to the microorganism but to what degree depends greatly on the properties of the species present including chemical and physical attributes (Pratush, Kumar and Hu, 2018). Biotransformation and biodegradation involve the detoxification of heavy metal ions via a number of ion channels, changing the oxidation state of the metals and thus making them less harmful to the local ecology (Mishra, Singh and Arora, 2017, Kumar and Bharadvaja, 2020). Some of these microorganisms can indirectly affect the heavy metals within the soil by inducing their uptake into plants via assisting in plant growth (Yan et al., 2020) and making the heavy metals more mobile (Mishra, Singh and Arora, 2017) or prevent uptake by inferred resistance (Rizvi and Khan, 2018).

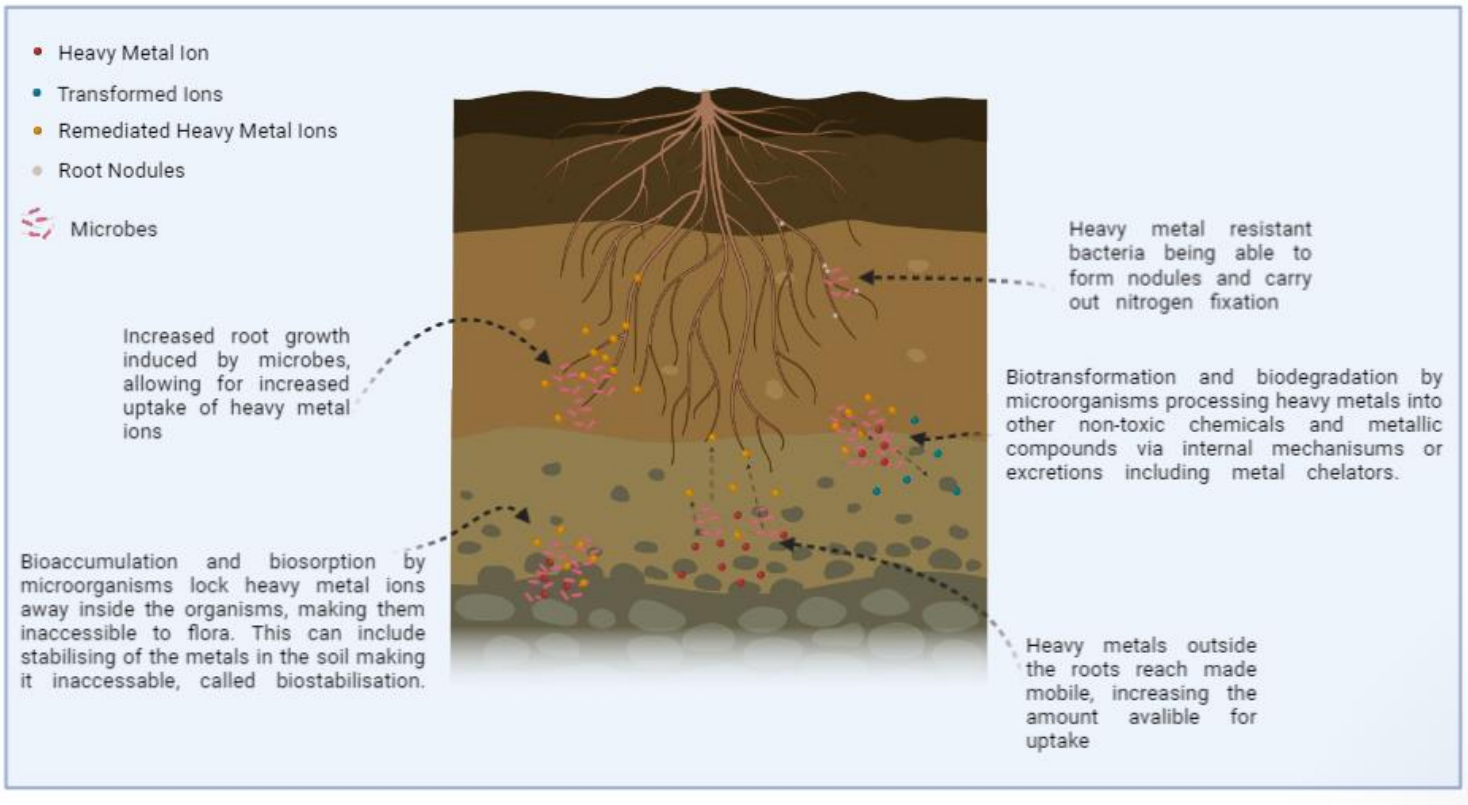


Figure 3.2. Microbial effects on heavy metals in the rhizosphere

Pollution can have a devastating effect on microbial communities in the rhizosphere. Heavy metals also affect enzymatic activities within the soils by affecting the microbial communities which synthesizes the enzymes (Zhao, Huang, Lu and Sun, 2019). Heavy metals can cause toxicity towards microorganisms within the soil by influencing processes and as a result kill of microbes and the activity

of the soils microorganism communities (Zhao, Huang, Lu and Sun, 2019). The presence of heavy metal pollution can cause issues such as fragmentation of the DNA (Mishra, Singh and Arora, 2017) leading to cell death of the microbial organism (Fernández et al., 2008), reductions of enzyme activity and respirations rates (Chu, 2018). Equally, long-term effects of heavy metals can lead to increases in the tolerance of bacteria as well as in arbuscular mycorrhizal (AM) fungi (Mora et al., 2005). These effects the metals have, play a significant role in the remediation of polluted ecosystems. Remediation technologies focused on microbial remediation are thought to be extremely effective for heavy metal removal due to their natural affinity and being environmentally friendly (Tiwari and Lata, 2018).

Plant – soil microbe Interactions

Root-microbe communication is a significant process that takes place within the rhizosphere and the surrounding area. Some plant root exudates have shown an important part in these interactions (Liu et al., 2017). These exudates can act as signalling and transfer of nutrients between the plant and beneficial soil organisms within the soils, or more directly to symbiotic organisms as nutrients or flavonoids (Haichar, Santaella, Heulin and Achouak, 2014). They can also act as signalling molecules for plant growth promoting rhizobacteria (PGPR) causing chemotactic movement towards the plant (Chaturvedi and Singh, 2016), however there is little known about these molecules (Haichar, Santaella, Heulin and Achouak, 2014). Plants also produce amino acids, carbohydrates and carboxylates and release them as exudates to promote an increased biomass and activity of the microbial community (Rohrbacher and St-Arnaud, 2016). They can also act as a protective barrier against pathogenic organisms (Haichar, Santaella, Heulin and Achouak, 2014). As a result, root exudates can be used to control the levels of both beneficial and harmful microorganisms using positive and negative interactions (Philippot, Raaijmakers, Lemanceau and van der Putten, 2013). These are all depicted in figure 3.3.

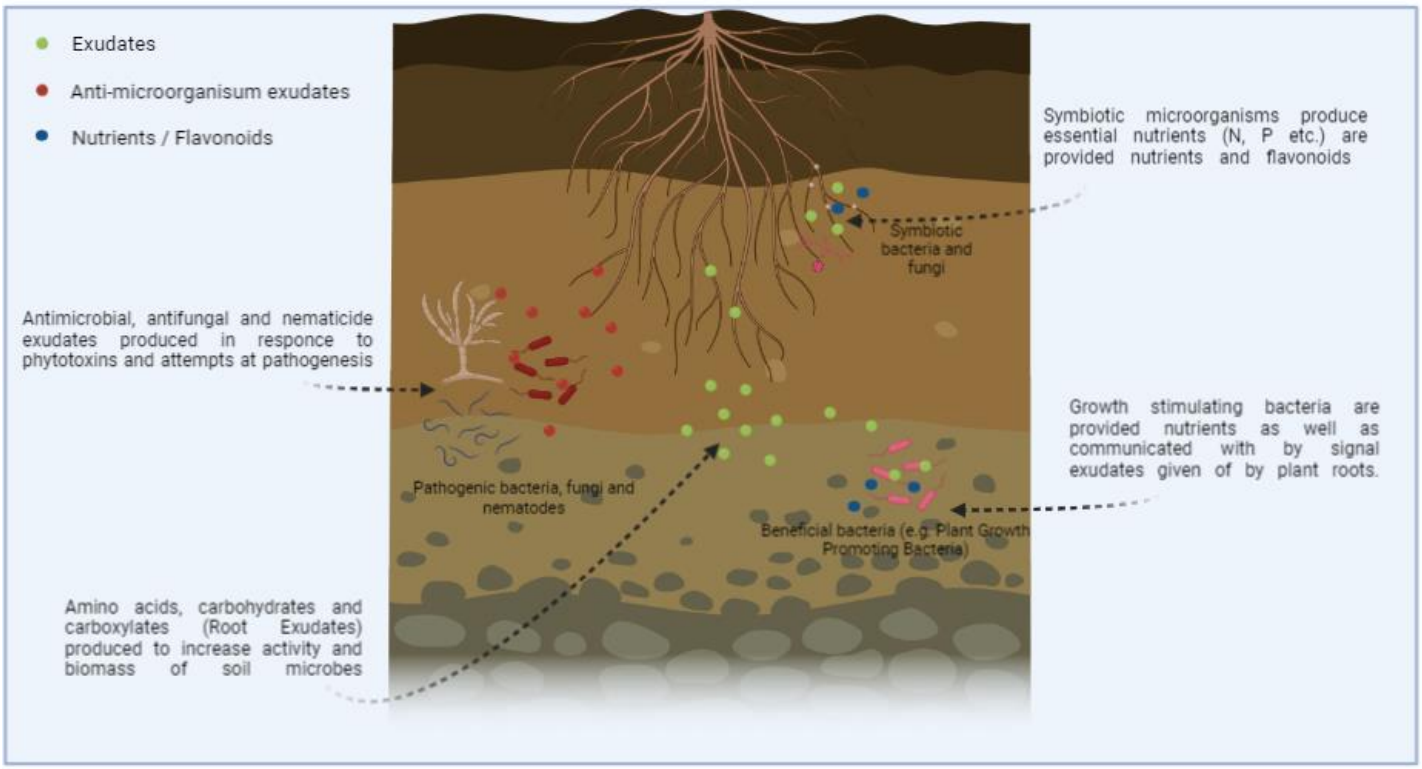


Figure 3.3 – Plant–soil microbe Interactions

With these interactions taking place below the surface, making them more difficult to observe and analyse, they have been mostly overlooked due to the most accurate way of analysis, DNA sequencing being expensive and expertise intensive to run. Many functions have been found to be related to root exudation, including the protection of roots, stabilization of minerals in the soil, and the selective storage of ions (Rougier, 1981; Hawes et al., 2000). The different root exudates also produce a chemotactic response within the microbial community which helps lead to the colonisation of the root structures (Kumar and Bharadvaja, 2020). A number of papers (Chaboud and Rougier, 1984; McCully and Boyer, 1997; Oburger and Schmidt, 2016) have also shown the hydraulic component of root exudation in the maintenance of stabilization and promotion of root growth within the rhizosphere, with Young (2006) showing the rhizosphere to be wetter than that of the bulk soil surrounding it leading to suggestions that the exudates also help in the withholding of moisture within this area. As such these studies show that root exudation maintaining interconnections between the soil and roots of plants in the rhizosphere contributing to the enhanced growth of root networks and the survival of resident plants.

Soil communities

Soil bacteria

Soil bacterial communities are known to be important components of ecosystems, particularly in agricultural systems as they greatly effecting the health and yields of crop plants (Hayat et al., 2010). This is unsurprising when it is estimated that 10,000 species and 1 billion individual bacterial cells can live in 1 gram of soil (Roesch et al., 2007). With such a high abundance of organisms and species diversity within this group, bacteria can cover a wide variety of different functions and roles and have been separated into a number of different categories by the European Commission (Turbé et al., 2011, Wagg et al, 2014). These include chemical engineers, biological regulators and ecosystem engineers (Saccá, Barra Caracciolo, Di Lenola and Grenni, 2017).

Chemical engineers include detritovorous bacteria that are involved in the breakdown of organic matter such as that allow the recycling of nutrients and make them available again for uptake by the plant (Wagg et al., 2014). They are key in the degradation of chemicals through their metabolic pathways, making them important in bioremediation (Saccá, Barra Caracciolo, Di Lenola and Grenni, 2017). Biological regulators include parasitic and predatory bacteria, regulating the populations of organisms both in the soil and the plant life above (Wagg et al, 2014, Saccá, Barra Caracciolo, Di Lenola and Grenni, 2017). Lastly, ecosystem engineers modify the surrounding soils and as a result can change the availability of certain chemicals and nutrients for other organisms through modification of pore networks in soils creating hotspots (Saccá, Barra Caracciolo, Di Lenola and Grenni, 2017).

All the aforementioned types of bacteria are referred to as rhizobacteria due to their interactions mostly with the roots of plants within the rhizosphere (Reinhold-Hurek et al., 2015). Further categorisation is given depending on their interaction with the plants themselves; for example, plant growth-promoting rhizobacteria (PGPR) or plant-beneficiary bacteria (PBR) are free-living soil bacteria that promote plant growth and yield in the colonised plant (Wu, Cheung, Luo and Wong, 2006; Zhuang et al., 2007; Hayat et al., 2010; Mendoza-Hernández José et al., 2016; Sharma et al., 2020) by stimulation or by protecting the plant from the toxin (Abou-Shanab et al., 2009; Abou-Shanab, El-Sheekh and Sadowsky, 2019; Halim, Rahman, Megharaj and Naidu, 2020). They are also known for being beneficial for agricultural plants by assisting with the uptake of nutrients, resulting in an increased nutrient availability in soil and improved quality of agronomically important crop plants (Choudhary et al., 2018).

PGPR achieve their plant growth mechanism through a number of different means, including chemical excretions and improved survival relating to stress conditions by their detoxifying nature (Mustapha and Halimoon, 2015). This can be accomplished via the production of phytohormones (Mendoza-Hernández José et al., 2016; Keswani et al., 2020), including auxins and cytokinins, that directly benefit plant growth through the modulation of different processes of the plant (Boivin, Fonouni-Farde and Frugier, 2016; Akhtar, Mekureyaw, Pandey and Roitsch, 2020). This can allow for improved responses for plants that are under direct heavy metal stress (Manoj et al., 2020; Zeng et al., 2020). Cytokinin and auxin interactions within the soils and important, especially relating to the development of the plant's roots (Vacheron et al., 2013). These can lead to improved root meristem formation and maintenance, internal structure formation of the roots and the formation of branching and lateral roots (Schaller, Bishopp and Kieber, 2015).

Auxins are a group of small molecules which are all known for their ability to influence growth within plants (Ma, Grones and Robert, 2017). They were the first group of phytohormones to be discovered with indole3-acetic acid (IAA), in 1885 (Teale, Paponov and Palme, 2006; Enders and Strader, 2015; Keswani et al., 2020). Auxins are important in the stimulation of a plants root systems by improving cell division and vascular bundle and root nodule formation (Chen et al., 2017; Manoj et al., 2020). The application of IAA-producing bacteria has been shown to directly enhance root length and area (Ali, Charles and Glick, 2017). IAA has also been shown to assist in reducing toxic effects of heavy metals such as lead through increased cell division and formation of vascular tissues (Israr and Sahi, 2008).

Cytokinins are hormones that are able to be synthesised by a number of bacteria (Arkhipova et al., 2007) and are known to be of major importance to the growth of plants (Santoyo, Moreno-Hagelsieb, del Carmen Orozco-Mosqueda and Glick, 2016). These hormones help to promote cell division within various structures of the plant including roots and shoots and assist in differentiation processes within tissues and stomatal operation (Arkhipova et al., 2007; Cassán, Vanderleyden and Spaepen, 2013; Ali, Charles and Glick, 2017). Endophytic bacteria - bacteria that live within internal plant tissues without ADL causing damage or any negative effects (Chaturvedi and Singh, 2016) - can form a number of beneficial mutualistic or symbiotic relationships with the plants they inhabit (Ma, Rajkumar, Zhang and Freitas, 2016; Abou-Shanab, El-Sheekh and Sadowsky, 2019). Most endophytic bacteria achieve this relationship be creating small aggregates within the tissues of the plant (Perez, Perez and Chamorro, 2013), allowing the exchange of nutrients to occur i.e. via nitrogen fixation (Franco-Franklin, Moreno-Riascos and Ghneim-Herrera, 2021). These relationships between the bacteria and it's host will usually entail the bacteria providing nutrients to the plant in exchange for improved health and overall growth (Ryan et al., 2008; Franco-Franklin, Moreno-Riascos and Ghneim-Herrera,

2021). To this end, a number of endophytic bacteria are also classed as PGPR - one of the only major differences is that they reside within the plant, unlike rhizobacteria (Ali, Charles and Glick, 2017).

The bioavailability of the heavy metals within the rhizosphere is thought to be extremely important in the eventual effectiveness of both the translocation of the heavy metal and the stabilisation in the soils through phytostabilisation (Abbaszadeh-Dahaji, Omidvari and Ghorbanpour, 2016). Thus, rhizobacteria that are able to increase the availability of heavy metals to the plant can be greatly beneficial for phytoremediation (Manoj et al., 2020; Abou-Shanab, El-Sheekh and Sadowsky, 2019). In addition, they are effective in both biotic and abiotic conditions (Ma, Rajkumar, Zhang and Freitas, 2016; Kumar et al., 2021).

With such a variety of effects on the soil environment, bacteria have been identified as highly important in phytoremediation of soils contaminated with heavy metals (Zhuang et al., 2007). Many bacteria have been found to be a viable option in the remediation of heavy metal contamination via a number of routes, including accumulation into the organism, causing precipitation and oxidation-reduction (Tiwari and Lata, 2018; Dhaliwal, Singh, Taneja and Mandal, 2019). As such, these bacteria do not degrade the heavy metals, but instead change their chemical and physical properties (Dhaliwal, Singh, Taneja and Mandal, 2019). Examples of this include research carried out by Singh, Verma and Gaur (2013) showing that *Bacillus cereus* can detoxify hexavalent chromium in tannery effluent, Ashokkumar, Loashini and Bhavya (2017) demonstrating biosorption for Cu, Pb and Cr by *Sphaerotilus natans* and by Abioye et al (2018) stating the effectiveness of *Bacillus subtilis* and *Bacillus megaterium* for biosorption of Pb, Cr and Cd. *Bacillus subtilis* has also been used to promote nickel accumulation in *Brassica Juncea* (Zaidi, Usmani, Singh and Musarrat, 2006) and *Azotobacter chroococcum stimulated plant growth assisted with Pb and Zn uptake* (Wu, Cheung, Luo and Wong, 2006). With chromium, research has shown that in many cases Cr becomes immobilised when inoculated with bacteria via absorption and reduction rather than increasing uptake within the plant itself (Tirry et al., 2018).

Plant bacteria interactions

Soil microbial communities, especially in the rhizosphere, are important in the removal of pollutants within the soil media, such as heavy metals (Barra Caracciolo and Terenzi, 2021). This removal occurs by detoxification processes using chelation (Cobbett, 2000). Phytochelatins have been identified as a group of highly important metal chelators (molecules that bind to heavy metal ions to detoxify or remove from solution) (Chaudhary, Agarwal and Khan, 2018). As such, these phytochelatins reduce the binding capacity of heavy metals to a plant's cell walls while also detoxifying the cells (Chaudhary,

Agarwal and Khan, 2018). Originally found in plants (hence the name phytochelatins), these have now been found to be produced - or have the potential to be produced - by many different microorganisms, including bacteria (Sharma et al., 2016).

Metallothioneins are a type of phytochelatin which contain a group of cysteine amino acids which allow for the binding of heavy metals within their structures. This results in the detoxification of heavy metals (Sharma et al., 2016; Ojuederie and Babalola, 2017; Chaudhary, Agarwal and Khan, 2018) and protecting the bacteria and plants from metal toxicity (Benhalima, Amri, Bensouilah and Ouzrout, 2020) alleviating plant toxicity stress (Sharma et al., 2016) and potentially helping with phytoremediation via plants (Rono et al., 2021). Finding these metallothioneins within bacteria and other microorganisms is a relatively new area of research, however this new discovery suggests that metallothionein-producing bacteria may be more common than originally suspected (Robinson, 2008).

Siderophores are a type of metallophore (Ortúzar, Trujillo, Román-Ponce and Carro, 2020) - a metal chelator which removes metals from the soil solution. These usually benefit the plant to obtain functionally important metals that are scarce and/or hard for plant roots to reach such as Fe (Ahmed and Holmström, 2014; Barra Caracciolo and Terenzi, 2021). An example of the is iron, which is made available by the siderophores of microbes to enhance plant growth in Fe-deficient soils (Ojuederie and Babalola, 2017). Bacteria that are already known to have beneficial functions for plants - such as the nitrogen fixing bacteria *Frankia* spp - produce an array of different metallophores when exposed to toxic metals in the rhizosphere (Deicke et al., 2019).

Heavy metal stress and tolerance in bacteria

Bacteria that are able to assist a plant in its growth are only part of the puzzle when looking for successful phytoremediation mechanisms. The bacteria will also have to be resistant themselves to the toxifying effects of the heavy metals (Tirry et al., 2018). Tirry et al. (2018) found that a strain of *Cellulosimicrobium* spp. not only showed a resistance to heavy metals and the ability to reduce Cr(VI), but also produced plant growth promoting products including IAA, improving plant growth by up to 28% in alfalfa. Increases in Cr, Zn and Cu were also observed within the plant's roots. Although stated before that Cr pollution can become immobilised when inoculated with bacteria rather then taken up by plants, it has been reported that inoculation with Cr-resistant bacteria strains can greatly increase uptake within plants compared to those that are not inoculated. Examples include bacterial inoculation of two plant species already identified as phytoremediators: *Pseudomonas lurida* in

sunflowers (Bahadur et al., 2016; Bahadur et al., 2017; Kumar et al., 2021) and *Bacillus cereus* in vetiver grass (Nayak, Panda, Basu and Dhal, 2018). However, this area of research is very new, and more research is required to fully understand the interworking of the bacterial communities in the soils and their benefits towards the uptake of heavy metals within many hyperaccumulating plants (Kumar et al., 2021).

Mycorrhizal fungi

Mycorrhizal fungi form a mutualistic relationship with certain higher plants. These associations are common in nature, with 80- 90% of vascular plant species having an association with mycorrhizal fungi (Bucking, Hans and Heyser, 2007; Bonfante and Genre, 2010). This occurs when mycorrhizal fungi colonise the roots of the plant. Mycorrhizal fungi produce hyphae - branching threadlike filaments (Dodd, 2000) (figure 3.4) - that proliferate into areas of the soil previously inaccessible to the larger plant roots or that are out of reach of root tips (Saint, 2021) (Shown in figure 3.5), and therefore allow contact with extra resources such as water and nutrients (Gong and Tian, 2019). This allows for plants to grow in areas that they would normally struggle due to common issues such as an inability to fix nitrogen or to mobilise certain essential nutrients (Bonfante and Genre, 2010). Mycorrhiza is derived from the Greek words mýkēs ("fungus"), and rhiza ("root"), and is descriptive of its development of the widespread hyphal networks connecting plant communities together, often referred to as the fungal mycelium (Bonfante and Genre, 2010) or the “wood wide web” (Helgason et al., 1998).

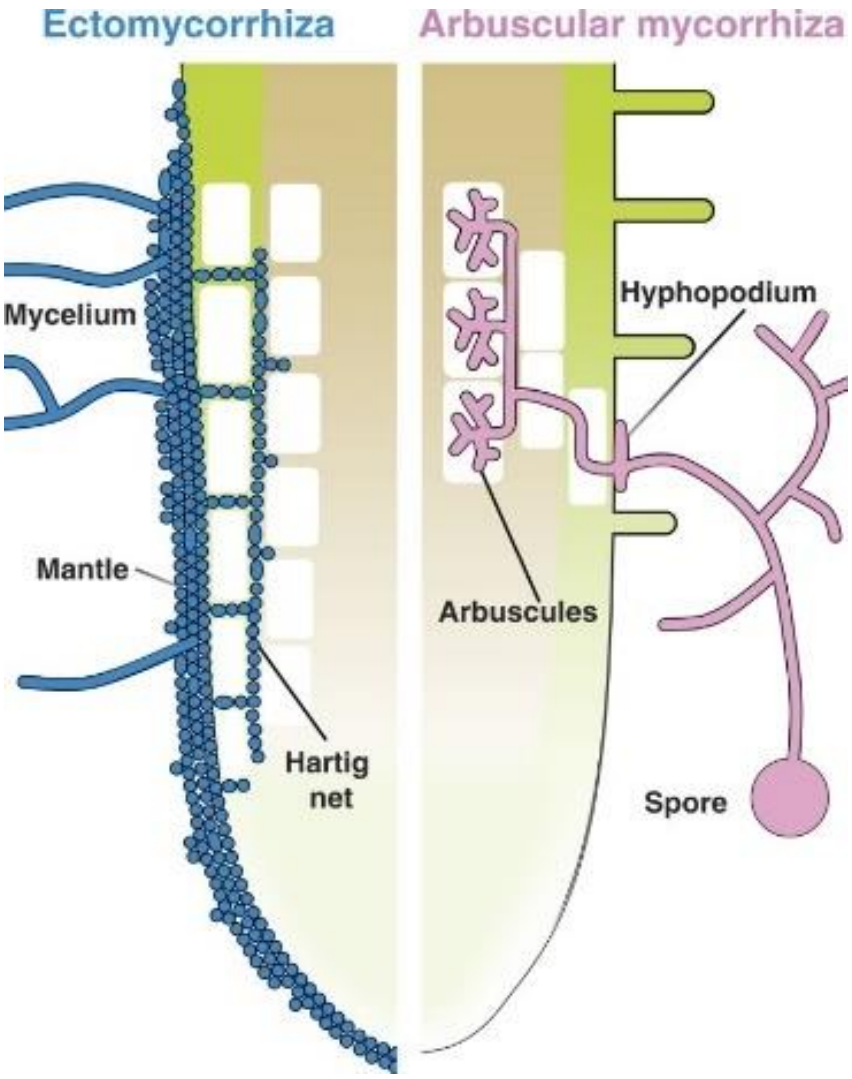


Figure 3.4. Root colonisation by ectomycorrhizal and arbuscular mycorrhizal fungi (taken from Bonfante and Genre, 2010)

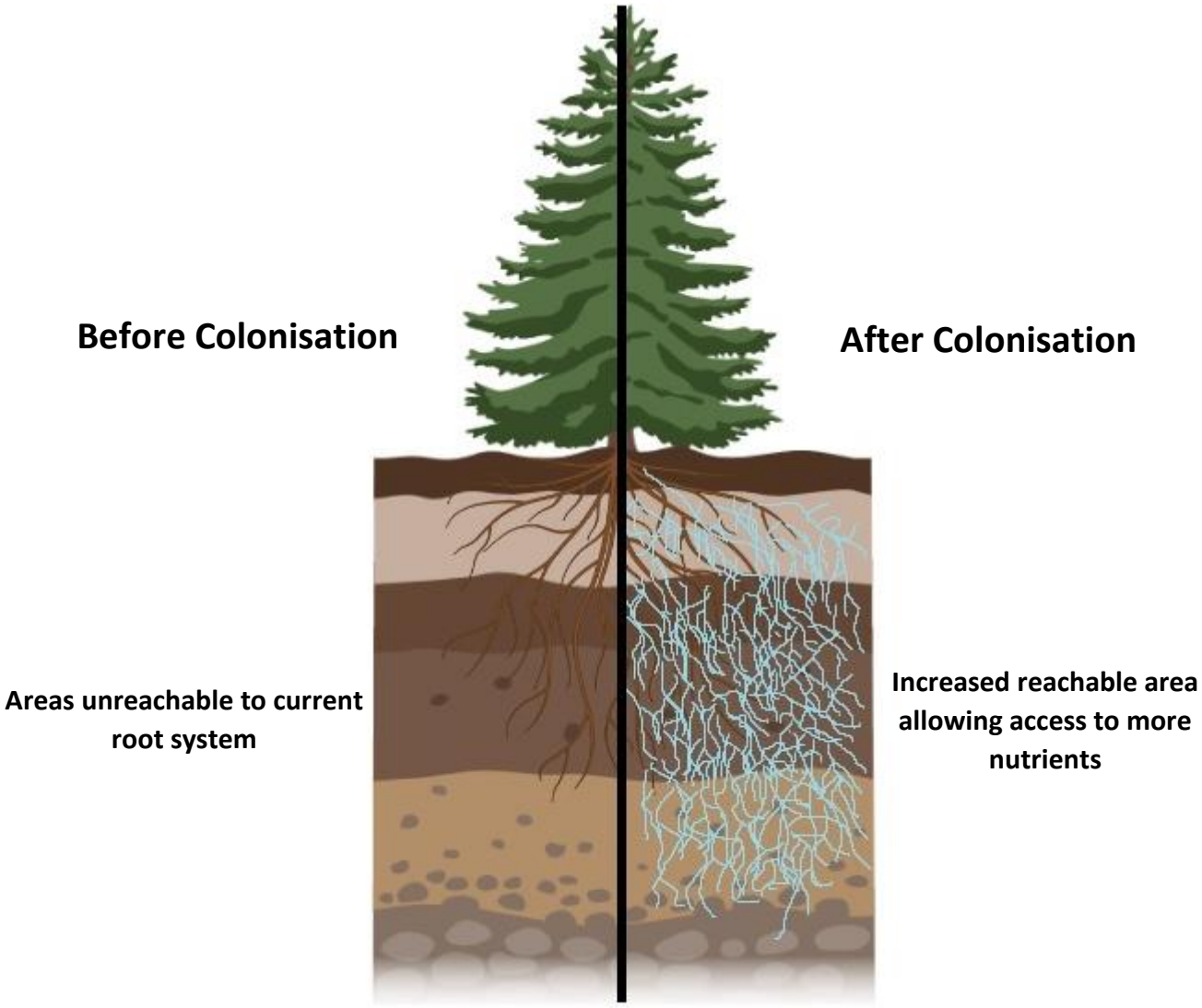


Figure 3.5. Mycorrhizal colonisation of tree root system depicting increased reachable area due to fungi colonisation.

Ectomycorrhizal fungi

Ectomycorrhiza are one type of mycorrhiza that has been said to have been integral in the shaping of the world's forests as we know them, with families of tree including *Pinaceae*, *Fagaceae*, *Dipterocarpaceae* and *Caesalpinoidaceae* all being shown to interact with hundreds of different species of ectomycorrhiza (Smith and Read, 2008; Bonfante and Genre, 2010). Hyphae of

ectomycorrhiza penetrate into the plant root cortex and form a hyphal sheath around the root surface. This acts as a barrier, preventing heavy metals from getting into the host plants (Lindahl et al., 2007; Yu et al., 2020). This restricts heavy metal movement into the host roots allowing for toxicity resistance. These mechanisms include the absorption of toxic metal into the hyphal sheath of the mycorrhiza, a reduction in access to the apoplast as a result of hydrophobic properties of the hyphal sheath, chelation as stated before and the absorption of the toxic metals and transport into the mycorrhizas extended external mycelium network (Jentschke and Godbold, 2000). However, these are likely to vary greatly depending on the heavy metals present, the speciation of the metal ion and the species of plant that are involved in the relationship (Jentschke and Godbold, 2000; Urban, Puschenreiter, Strauss and Gorfer, 2008).

Ectomycorrhizal fungi are known for their ability to modulate cellular, physiological and molecular processes within the host plant, and as a result the plant will have an altered response to their environment (Luo et al., 2014). This is conducted through a relationship with the root tips of the host species they have developed a mutualistic relationship with (Smith and Read, 2008). Almost all host plants that ectomycorrhizal fungi interact with are woody perennials (Reddy and Saravanan, 2013) and in rarer cases herbaceous perennials (Hoeksema et al., 2018). Interactions between ectomycorrhizal fungi and plants include three structures; a sheath of fungal tissue that encloses the root tip, an inward growth and interconnecting hyphae network, known as a hartig net, that the connects the epidermal and cortical cells but never enters into the lumen of the plants root (Reddy and Saravanan, 2013; Hurst, 2021). This allows for the transport of nutrients and water between both organisms, giving the host plant access to nutrients that the plant roots would be unable to access (Smith and Read, 2008; Bonfante and Genre, 2010; Nehls, Göhringer, Wittulsky and Dietz, 2010; Luo et al., 2014). However, there are exceptions to this, where different developments of the Hartig net, mantle (a sheath around the root) (Li et al., 2021) and external mycelium can occur. As a result, the ectomycorrhizal-plant relationship allows for the obtaining of water and essential nutrients from the surrounding (otherwise unreachable) soils, while the fungi itself gains energy from stored carbohydrates from the host plant in a mutualistic beneficial relationship (Pena et al., 2010; Luo et al., 2014).

Endomycorrhizal fungi

Arbuscular mycorrhiza fungi are extremely important endophytic fungi that are associated with over 80% of land plants (Smith and Read, 2008; Mnasri et al., 2017). The symbiosis between fungi and plant

offers several benefits that the host plant can profit from, mainly the acquisition of phosphorus and other nutrients from the soils that the plant unable to acquire itself at required levels (Karimi et al. 2011). As well as nutrient aquisition, the presence of arbuscular mycorrhiza fungi can improve plant resistance to both biotic and abiotic stresses such as certain soil microbials (Pineda et al., 2015), salination of soil (Jayne and Quigley, 2014) and the presence of contamination (Harrier and Sawczak, 2000; Whitfield, Richards and Rimmer, 2004). It has been shown that the presence of arbuscular fungi increases uptake of nutrients, increases plant biomass and results in a heightened resistance to stress and pathogens (Smith and Read, 2008). They also create a large hyphal network that provides an enhanced area for root absorption of essential nutrients and water (Smith and Read, 2008). This is also beneficial in the use of these organisms in the increased effectiveness of a plant as a hyperaccumulator of heavy metal pollution (Lasat, 2002) allowing for the sequestering of heavy metals into plant root and preventing the translocation into shoot (Giasson, Jaouich, Gagné and Moutoglis, 2005). In fact, the hyphal network of the arbuscular mycorrhiza have been shown to have the capability to absorb and translocate several different toxic heavy metals via both the compartmentalisation mycelium and fungal sheath (Jentschke and Godbold, 2000) and the inferred change the mutualistic relationship has on the overall uptake by the host plant, especially for metals such as Cu and Zn (Smith and Read, 2008).

Studies relating to arbuscular mycorrhizae and its effectiveness for heavy metal tolerance have increased in recent years, with studies demonstrating that the presence of arbuscular mycorrhizal fungi alleviates heavy metal stress in host plants (Heydarian, Moghadam, Donath and Sohrabi, 2018; Sushma et al., 2021). Since increased plant growth and root biomass is thought to give plants a higher tolerance to heavy metals (Yan et al., 2020), the addition of arbuscular mycorrhiza hyphae benefits the plant by extending the plant root network, and therefore increasing root biomass, thus increasing resistance to heavy metal toxicity (Khade and Adholeya, 2007; Wei et al., 2012; Gong and Tian, 2019). This is supported by studies demonstrating that the presence of mycorrhizal host plants increases in heavy metal contaminated mining sites, suggesting conference of tolerance and other beneficial attributes (Karimi et al., 2011). However, research does not show a clear consensus on whether mycorrhizal fungi associations always result in an increase in metal absorption into plant root and shoot structures (Gong and Tian, 2019). For example, Weissenhorn, Leyval, Belgy and Berthelin (1995) observed a range of effects, from reduced to increased metal absorption from contaminated soils into plant root and shoot structures. More recently, Lasat (2002) demonstrated that the effect of mycorrhizal fungi on plant heavy metal uptake is heavily dependent on the metals and plants involved, with different combinations having varied results. Gong and Tian (2019) support this and go further by stating that no consistent result is caused by either the stimulation of uptake in the plants or the

sequestering of the heavy metals within their own structures and that it can also depend on the environmental conditions. This inconsistency was also identified by Smith and Read (2008). Thus, to understand how to improve conditions for phytoremediation, experiments mimicking environmental conditions at the location being researched are required, rather than relying on previous data that may have been obtained from experiments run under very different conditions.

It is known that arbuscular mycorrhizae metabolise heavy metals, reducing toxicity, as well as producing metallothionines to also assist in detoxification (Bano and Ashfaq, 2013). Although experiments show an increase uptake and accumulation of metals such as Cu, Pb, Zn and Ni, many have shown the opposite or have shown that although the total accumulated levels of the toxic heavy metals has increased compared to levels with no arbuscular mycorrhiza fungi, actual concentrations of the metals and the toxic effects of the metals as a result have reduced (Weissenhorn, Leyval, Belgy and Berthelin, 1995; Joner and Leyval, 2001; Smith and Read, 2008).

Heavy metal stress and tolerance in mycorrhizal fungi

Research has shown previously that arbuscular mycorrhizal fungi are able to survive in - and tolerate - high levels of heavy metal contamination. They accomplish this through a combination of molecular processes that can bind metals to the walls and other structures of the fungi (Riaz et al., 2021) and via chelators such as metallothioneins that work alongside metal transporter molecules, actively lowering metal levels in the cytosol structure and allowing the fungi to survive (González-Guerrero, Benabdellah, Ferrol and Azcón-Aguilar, 2008). This resistance has been well documented for arbuscular mycorrhiza fungi and it's associated host plant. Fungi-assisted stress tolerance and phytoremediation has been documented in many host crop plants (Riaz et al., 2021) including Cd, Cr, Ni, Pb for *Zea Mays* (Malcová, Vosátka and Gryndler, 2003; Singh, Pankaj, Chand and Verma, 2019); Cd, Cr, Mn, Ni, Cu, Zn, Al, Pb, Co, Mo, Fe and Si within *Helianthus annuus* (de Andrade, da Silveira, Jorge and de Abreu, 2008; Sayın, Ali Khalvati and Erdinçler, 2019) and Cu in *Sorghum bicolor* (Toler, Morton and Cumming, 2005), with the majority of the fungal species within the *Glomerales* order of fungi (Riaz et al., 2021).

The resistance and improvement of heavy metal uptake within the host plant is caused by symbiosis through the hyphae (Cui, Ai, Chen and Wang, 2019), allowing for improved nutrient availability and uptake via the host plants such as for phosophorus (Smith, Smith and Jakobsen, 2004) and causing changes in the plant's morphology and enzyme activation (Riaz et al., 2021). Due to this very important symbiosis between arbuscular mycorrhizal fungi and host plants within heavy metal contaminated

sites, and evidence of improved phytoremediation of these contaminants, arbuscular mycorrhizal fungi are very useful organisms to study in phytoremediation experiments.

Mycorrhizae are effective in reducing the effects of heavy metal toxicity on affected host plants (Jentschke and Godbold, 2000; Lasat, 2002; Marschner, 2011), however being able to identify the mechanisms that are involved in this interplay between the two organisms has proven difficult. This is due to the mechanisms being specific depending on the species of mycorrhiza or plant involved. This is evident in the literature, where experimental results vary widely depending on the species of fungi and/or plant and the heavy metals present (Hall, 2002).

Protists

Protists are a group of single-celled microscopic organisums that includes amoebas, flagellates, ciliates, sporozoans, and many other forms (Adl et al., 2012; Geisen, Bonkowski, Zhang and De Jonckheere, 2015; Geisen et al., 2018). Being able to survive in all earth biomes to the most extremes in temperature, salinity and pH, they are the most abundant eukaryotes on the planet apart from plants (Geisen, Bonkowski, Zhang and De Jonckheere, 2015; Geisen et al., 2018).

Protists that reside in soil environments can provide a wide range of different functions that greatly shape the soil community and their interactions with the resident flora (Geisen et al., 2018). These microscopic organisms are already used widely in municipal waste treatment (Rehman and Shakoori, 2001; Rehman, Shakoori and Shakoori, 2006), breaking down waste into less harmful and useful nutrients via mineralisation and the predation of bacteria (Madoni, Davoli, Gorbi and Vescovi, 1996). They make available minerals such as nitrogen that are often not readily available to plants and thus the main constituent of many fertilizers. In particular, ciliate protozoa have been found to greatly improve effluent quality of activated sludge from wastewater treatment (Martín-González et al., 2006). This nutrient cycling is also known to be important in the sequestering of carbon in habitats such as in peatland (Jassey et al., 2015). Another well-known process that protists are involved in is silicon cycling, with many studies findings that protists are associated with the uptake of Si to build structures, with the protists' involvement being equal to that of trees (Aoki, Hoshino and Matsubara, 2007; Sommer et al., 2013; Puppe, Kaczorek, Wanner and Sommer, 2014; Geisen et al., 2018).

Cycling of nutrients isn't their only function, with many heterotrophic protists contributing to the release of nutrients into the soil environment through bacterivore (Geisen et al., 2018) - the consumption of bacteria (Ducklow, 2001). Protists don't only target bacteria, with some known to

consume fungi, algae and nematodes as well (Geisen, 2016; Seppey et al., 2017; Xiong et al., 2017). Nitrogen and other nutrients are made available through this consumption and excretion allowing for its uptake by plants and other microorganisms (Geisen et al., 2018).

The presence of the vast number of protists in soils has a direct impact of plant growth: increasing growth and yield from the releasing of nutrients through consumption and then their subsequent uptake is known as the 'microbial loop' (Bonkowski, 2004; Xiong et al., 2017; Geisen et al., 2018). They have also been found to stimulate PGPR (Jousset, 2011) and to maintain soil fertility through their constant predatory control over the soil community (Xiong et al., 2017) by the selecting of specific targets such as pseudomonads (Flues, Bass and Bonkowski, 2017). This releases much required nitrogen and carbon for the plants to then utilise (Bonkowski and Clarholm, 2012; Koller et al., 2013). Plant parasitic protists are also important in the promotion of plant growth by providing nutrients in conditions of nutrient stress (Barthlott, Porembski, Fischer and Gemmel, 1998).

Heavy metal stress and tolerance in Protozoa

A number of protozoans found within heavy metal polluted media have shown a remarkable ability to resist and tolerate this toxicity (Rehman, Shakoori and Shakoori, 2006). For example, the ciliate protozoa *Vorticella microstoma* showed a high level of resistance to Cr(VI) as well as a heightened ability to take up the heavy metal ions (48% in 192 hours in cultured medium), whilst also enabling plants to increase nutrient and heavy metal uptake (Shakoori, Rehman and Riaz-ul-Haq, 2004). Although known for resistance, Madoni et al. (1996) reported a mortality of 55% of total protozoan community at chromium concentrations of 150 µg ml^{-1} and 90% mortality at 293 mg ml^{-1}. Rehman, Shakoori and Shakoori, (2008) found that *Euplotes mutabilis took up 90-93% of chromium in solution, showing a great affinity for heavy metal uptake.* Like bacteria, protozoa can produce phytochelatins, specifically metallothioneins, to assist in this removal of the heavy metals (Martín-González et al., 2006).

Nematodes

Nematodes are microscopic, round-bodied, wormlike organisms that live in water films and water-filled pore spaces within terrestrial soils, marine environments, and freshwater ecosystems (Bala and Vyas, 2015). There are thought to be over 100,000 species of nematode, with some estimating species richness to be over 1 million (Nielsen et al., 2014), making them the most diverse group of multicellular

animals (Lü, Chen, Xue and Zhang, 2020). In addition, they are found in nearly all terrestrial habitats (Rodríguez Martín et al., 2014; Sávoly and Záray, 2014; Šalamún, Hanzelová and Miklisová, 2018). A detritovore organism, they recycle minerals and other nutrients from bacteria, fungi, and other substrates and returns them to the soil (Nielsen et al., 2014; Gutiérrez et al., 2016). This is where the nutrients can be taken up by plants which otherwise would have been unable to reach these essential nutrients.

Interestingly, nematodes can be indicators of the presence of other soil microorganisms. With populations of bacterivorous and frugivorous species correlating with the populations of bacterial and fungal populations respectively (Ferris and Bongers, 2006; Nielsen et al., 2014). As well as this, soil properties, precipitation and temperature were shown to affect nematode abundance and community composition (Nielsen et al., 2014). As a result, nematodes have been identified for use as bioindicators for many years, especially when relating to anthropogenic disturbance such as cropping and tillage (Sánchez-Moreno, Minoshima, Ferris and Jackson, 2006) or the presence of heavy metal pollution (Chauvin et al., 2020). Although many nematodes are known for their negative effects on many organisms by predating them including other nematodes, larger invertebrates and invertebrates (Dodds and Whiles, 2010) as well as bacteria, fungi (Thakur and Geisen, 2019) and protozoa (Shaw et al., 2018), some are beneficial to the soil ecosystem and in turn (Abd-Elgawad, Askary and Coupland, 2017). This is done by the the release of nutrients into the soil environment via the consumption of bacteria and fungi and relinquishing nutrients back into the soil (Lambert and Bekal, 2002).

Heavy metal stress and tolerance in nematodes

Heavy metal pollution can greatly affect nematode communities within soils, and this has been widely studied (Lü, Chen, Xue and Zhang, 2020). Certain groups of nematodes are highly susceptible to levels of heavy metal (Chauvin et al., 2020) including chromium contamination (Nagy, 2003). In areas with elevated levels of heavy metals there has been a reduction in the species richness of nematode communities (Rodríguez Martín et al., 2014). This effect that heavy metals have could be used as an indicator for evaluating disturbance levels of soil ecosystems (Gutiérrez et al., 2016). However, Gutiérrez et al., (2016) also found that the presence of pollution within the soil did not significantly change the number of soil nematodes within the test, but the diversity and overall structure of the nematode communities was greatly affected (Rodríguez Martín et al., 2014). It was also found that pharmaceutical pollutants had a greater effect on nematodes than heavy metals, theorising that historical adaptation of the nematode communities could be the reason (Gutiérrez et al., 2016).

However, the sensitivity of nematodes towards pollution such as heavy metals vary greatly between species (Martinez, Torres, dos Santos and Moens, 2018) with a number showing high tolerance (Monteiro et al., 2018).

Phytochelatin are a peptide that some organisms such as plants, molluscs and nematodes can produce that can detoxify heavy metals such as chromium through chelation or binding. This has been found to be the case in one of the most used model organism *Caenorhabditis elegans* (Singh and Tripathi, 2007; Essig, Webb and Stürzenbaum, 2016). This however is a rather new discovery with very few pieces of research having been carried out apart from the discovery of the Phytochelatin gene (Vatamaniuk, Bucher, Ward and Rea, 2002; Srivastava, 2016).

Some of the main ways that nematodes can assist in the uptake and/or remediation of heavy metals from the soil is by assisting in the mitigation of stresses on the plant (Haldar, Kumari, Ghosh and Ghosh, 2021). However, research into how nematodes can assist in the remediation of heavy metals and not hinder the plants that host them is in its infancy, with a area of significant interest being that of the excretion of metal metallothionein ether induced within the host plant (Li et al., 2021) or more recently discovered to potentially come from the nematodes themselves (Monteiro et al., 2018; Chatterjee et al., 2020).

Aims of the study

In this chapter, the effect of chromium contamination within the contaminated land along the tannery belt, south of Dindigul, Tamil Nadu on the soil microbiome is studied. The relationship between the soil community and Cr contamination will also be examined to decern to what extent soil communities are driven by Cr or whether other soil properties also have an effect. The effects these organisms might have on the uptake and hyperaccumulation potential of crop plants will be discussed. Four groups of organisms found in the soil microbiome were chosen for this study: bacteria, fungi, protozoa and nematodes. These were selected following a review of previous research that has been carried out indicating their potential resistance to heavy metal contamination and/or ability to enhance attributes of plants potentially leading to hyperaccumulation or toxicity resistance by crop plants. Bacteria, fungi, nematodes, and protozoa were all analysed in order to evaluate their presence and abundances in the soils

Hypothesis

- Samples taken from control sits and contaminated sited will have a significant change in the soil microorganism community, abundance, and similarity.

- Soil microorganism communities with significantly change because of Cr contamination within the soil sampling areas

- A number of organisms from bacteria, fungi, nematode and protozoa will be identified that show levels of resistance to Cr contamination that have not been previously identified.

Methods

Site description, soil sampling and chromium analysis

Details on the sampling site, sampling locations, sampling methods and chromium analysis methods are detailed in chapter one of this thesis. From these soil samples, between 4 - 8 samples were randomly selected from each site depending on the original number of samples from the site. The samples had been stored at -20^{o}C between Chromium sampling and DNA analysis. These were the samples used for the determination of soil microorganisms

DNA extraction from soil samples

In order to prevent cross contamination of the samples, all DNA extraction processes were carried out using DNA free equipment which also went through UV sterilisation prior to extraction. DNA extraction was carried out with the use of the DNeasy® PowerSoil® Pro Kit (Qiagen). 250 mg of soil was collected from each sample. Soil samples were homogenised before the DNA extraction sample was obtained to ensure a complete mix a genetic material throughout. The DNeasy® PowerSoil® Pro Kit contained all required reagents and consumables required for the extraction process. As such, samples were processed in accordance with the protocol supplied by the manufacturer. 250mg of each soil samples was loaded into a Power Bead Pro Tube with 800 µL of solution CD1 and horizontally vortexed for 20 minutes to allows for the lysis of microorganisms cells within the soil sample. This was centrifuged at

15,000 x g for 1 minute, and the supernatant transferred into a 2 ml microcentrifuge tube. 200 µL of CD2 was then added to each sample to cause non-DNA materials to precipitate out and increase the purity of the DNA. Samples were centrifuged at 15,000 x g for 1 minute and supernatant transferred to a new microcentrifuge tube. Solution CD3 is added to increase the salt concentration of the sample and allow for the DNA to bind to the subsequent column. The lysate is then loaded into an MB Spin Column and centrifuged at 15,000 x g for 1 minute. At this point, the DNA from the sample will be bound to the silica of the spin column. This was repeated until all of the lysate for the sample had passed through the column. The column is then removed and placed in a new 2ml collection tube and the lysate discarded. 500 µL of Solution EA was added to each column and centrifuged at 15,000 x g for 1 min to remove residual proteins and contaminants from the column. The lysate was discarded and 500 µL of Solution C5 was added to the column to further clean the DNA of salts and other contaminates and centrifuged at 15,000 x g for 1 min. The lysate was discarded, and the columns centrifuged at 16,000 x g for 2 min to remove and residual C5 and the column placed into a new elution tube. 100 µL of Solution C6 was added to the membrane of the column to bind and remove the DNA back into solution. The column is then Centrifuged at 15,000 x g for 1 min and the resulting lysate collected. This is the sample containing the DNA extraction.

The final elution of each DNA sample was 100 µL. DNA concentrations were subsequently measured by a Qubit 3.0 fluorometer (Invitrogen, Life technologies) using a Qubit dsDNA HS (high sensitivity) kit. DNA samples were stored at − 20 °C until the next stage of sequencing.

PCR amplification and sequencing

Methods for DNA PCR and sequencing were constructed and adapted from methods used by FERA (UK) for commercial and research-based soil DNA sequencing. Similar protocols have been used in resent research on soil micro-organisms (Wasimuddin et al., 2020; Iturbe-Espinoza et al., 2021).

Each extracted DNA sample was distributed into a 96 well plate for PCR amplification of the 16s region for bacteria (Ruan et al., 2017; Mukhopadhyay, Mitra, Choudhury and Ganguli, 2021; Illumina, n.d.), ITS region for fungus (Ruan et al., 2017; Vujanovic, Islam and Daida, 2019l; Giampaoli et al., 2020) and 18s for nematode and protozoan populations (Ahmed et al., 2019; Kenmotsu, Uchida, Hirose and Eki, 2020; Kenmotsu et al., 2021) as shown in table 3.2. PCR was carried out using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific), sample DNA template and associated primers for 16s, ITS and 18s. Once PCR was completed, gel electrophoresis of the PCR product was conducted as a quality control stage to make sure amplification had taken place. After successful PCR

amplification was confirmed, purification was carried out via the application of Agencourt AMPure XP magnetic beads. Samples containing individual PCR amplifications (16s, ITS and 18s) were pooled at this stage into one 96 well plate. Indexing PCR was carried out to allow for sequencing of samples via PCR amplification and the Nextera XT v2 Indexes kit group A (Illumina USA). A second run of purification using Agencourt AMPure XP magnetic beads was carried out on the Index PCR products to further remove and debris. Quantification was again carried out via Qubit 3.0 fluorometer (Invitrogen, Life technologies) using a Qubit dsDNA HS (high sensitivity) kit. The results of the quantification allowed for a library to be pooled to a total concentration of 20 Mm which is then once again measured for concentration using Qubit 3.0 fluorometer (Invitrogen, Life technologies). The libraries were sent to FERA (UK) for sequencing of the amplified and Indexed DNA where it was carried out using Illumina next generation sequencing via a targeted sequencing method for 16s, ITS and 18s.

	Primer	Nucleotide sequence (5'-to-3')
Bacteria - 16s	Amplicon PCR Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
	Amplicon PCR Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
Fungi - ITS	ITS1-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTTAGAGGAAGTAA
	ITS2-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC
Nematodes + Protozoa - 18S	NF1_MiseqF	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTGGTGCATGGCCGTTCTTAGTT
	18Sr2b	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTACAAAGGGCAGGGACGTAAT

Table 3.2. Primers used to sequence organisms from soil samples

Data analysis

Reads were received from the sequencing centre with barcodes and adapters attached. All analysis was conducted using the Qiime 2 environment. Sequence files were received with multiple samples that where indexed and pooled together in a multiplex and required demultiplexing. Demultiplexing of the samples utilised for the study was carried out initially to create single sample data, (Tian and Imanian, 2022) followed by the removal of adapters. For data pre-processing, the DADA2 pipeline was followed (Callahan et al., 2016) utilising the built-in automation and specification as described by Qiime 2. Denoising via truncation was carried out based on quality profiles generated during trimming to allow for the reads of only high quality to be used for the analysis (Tian and Imanian, 2022). Feature tables as well as alpha (the evenness of abundance and richness of the species withing the samples) (Willis, 2019) and beta diversity (the amount of similarity in the composition of the species communities compared between different sites) (Zhou, Wang and Luo, 2020). Alpha indexes carried out were; the Shannon's index, used to obtain the diversity of the community within the sample (Thukral, 2017), Simpson's index, a measure of diversity, utilising evenness and richness, and Principle coordinates analysis (PCoA), looking at dissimilarity, taking into account abundance via Bray -Cutis and Jaccard plots (Almela, Justel and Quesada, 2021) . These were all carried out in order to determine differences in abundance and similarity between the communities within the control and contaminated sampling locations. Lastly, classifiers trained against region specific databases, after which, the Operational Taxonomic Units (OTU) were assigned taxonomy down to species and taxa bar plots were produced. The significances of the differences among communities were analysed using permutational multivariate analysis of variance (PERMANOVA) to determine if Cr polluted samples had a significant effect on the variation between the control and contaminated samples.

Results and Discussion

Comparison of microorganism community structure and composition from Cr contaminated soils

Alpha diversity

Soil microbiome communities for both control and contaminated sites were analysed together to allow for the investigation of differences in the community compositions and structures for each organism analysed. The Shannon-Weiner (Shannon's) Index, an alpha diversity measurement, was used to determine species richness and evenness in the control and contaminated communities with the higher the value, the large the diversity. Simpson's diversity was also conducted to determine diversity focused on dominance, with the high ether number, the lower the diversity. Significant differences were determined via Kruskal-Wallis.

The alpha diversity for bacterial communities is shown in figure 3.6. For bacterial communities, in both the Shannon and the Simpson indexes, the alpha diversity of soil microbiome relating to 16s OTUs indicated no significant difference between that from the control sites and the readings from the contaminated sites, indicating that anthropogenic effects on the soil caused by the tanning industry had no significant effect on the diversity of the bacterial soil community. Similar was found by Miranda et al, (2018), who found that the addition of tannery sludge to soils didn't affect the overall diversity of the bacterial communities in the short term. However, larger amounts of sludge that signifies long term addition of high levels of tannery related contamination could affect the diversity of bacterial communities significantly (Miranda et al., 2018). It can also be seen that the low H value for Shannon (0.066) but high Simpson's index indicate that the bacterial communities are largely dominated by a small number of species and/or a very low evenness across the samples. This disparity in the evenness across the samples can be visualised in figure 3.7, with several of the samples showing a very low number of reads (between 1-8) and others showing dozens. This shows how the differences in the two alpha indexes can have a great effect on the apparent diversity of a site or sites with Shannon showing low diversity due to low evenness but Simpsons indicating a much higher diversity with control samples near 1 and all samples above 0.7 out of 1.

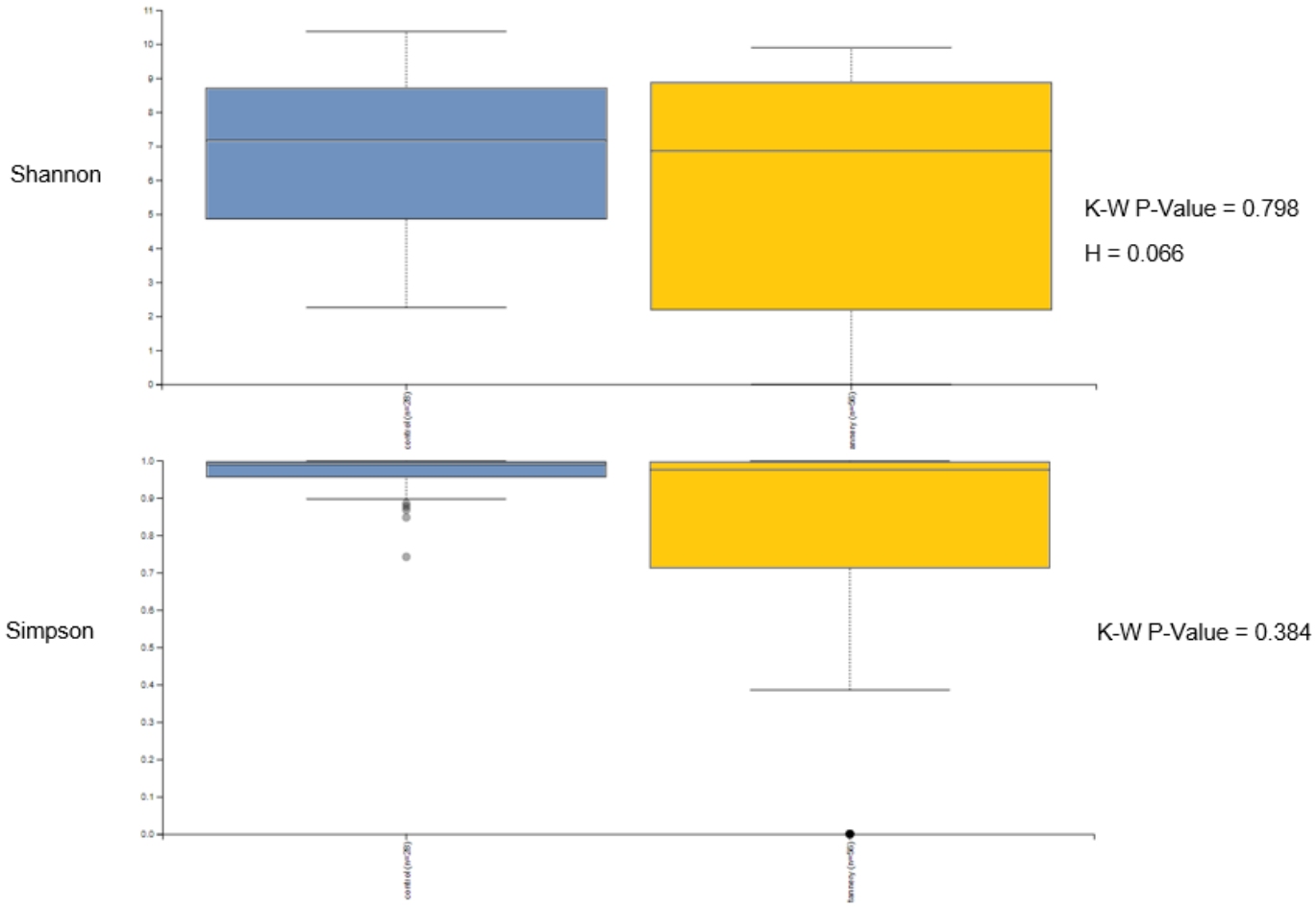


Figure 3.6. Analysis of alpha diversity of 16s communities within control and contaminated soil samples via Shannon's and Simpson's Indexes visualised using Boxplot

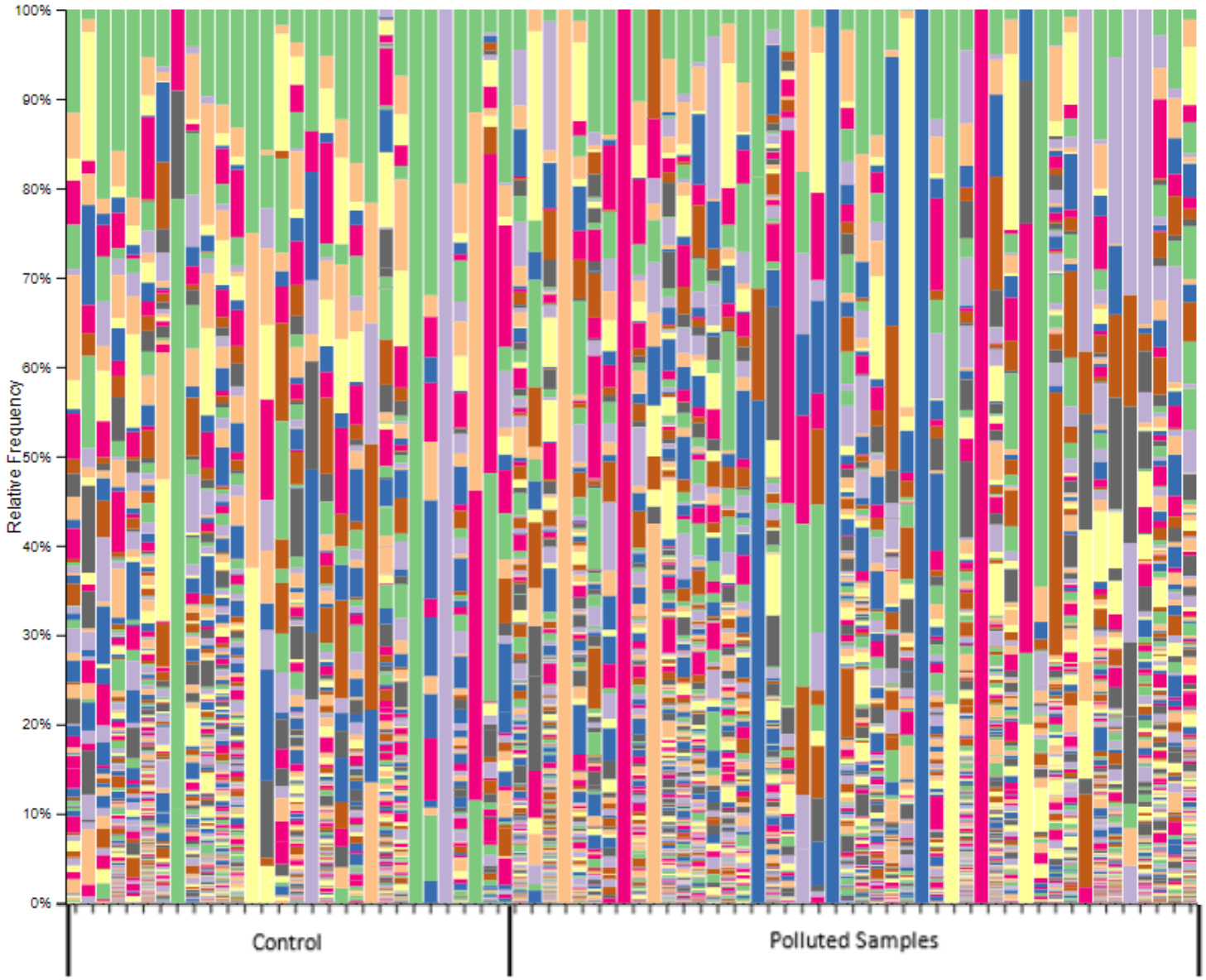


Figure 3.7. Relative frequencies of the species of bacteria via 18s sequencing

Alpha diversity relating to the fungal species determined through ITS sequencing are shown in figure 3.8. As opposed to that of 16s OTUs, ITS representing fungal communities, showed significant differences in the diversity (Shannon p value = 0.013) and the dominance (Simpson p value = 0.030) of the fungal communities of the control and contaminated samples. The Shannon Index showed an H value of 6.151 overall, showing a high average diversity of all the samples for ITS. But the average Shannon index for contaminated was near 5 but control samples showed a higher diversity of 6. With reading for the Simpson index for both control and contaminated samples being close to 1, this indicates a high diversity within both groups. Although lower, contaminated samples still showed high diversity scores for both tests, which is opposite to the results found by Kumar and Adholeya (2018). Kumar and Adholeya (2018) found that the diversity of fungal species dropped significantly to 21 within a controlled trap experiment treated with tannery sludge. This significant reduction in diversity was also witness with the addition of non-metallic tanning agents (Li et al., 2021). The relative high abundance still found within the contaminated soil samples could be attributed to the natural nature of the site as compared to these referenced studies, which were both conducted under greenhouse conditions as well as the potential for different concentrations of the many different chemicals that are utilised in the tanning industry.

The alpha diversity for 18s are shown in figure 3.9. Although showing the diversities of both protozoa and nematodes, the 18s OTUs contained many other organisms including rotifers, invertebrates, and some fungi. The results for the alpha diversity of the 18s were like that of ITS, with high Simpson's diversity scores of near 1. Diversity as determined by Shannon showed a relatively diverse community with no significant difference in the diversity of the controls and the contaminated samples. Simpson's diversity however did show a significant difference in the diversities of the two groups, indicating that the diversity of the contaminated group is negatively affected by the presence of the tanning industry similarly to fungi. This reduction in diversity and a change in dominance within protozoan species has been observed before with the addition of chromium containing tannery sludge (Samaras et al., 2009). Samaras et al., (2009) reported that dominant species of protozoa changed with the addition, shifting from sessile to carnivorous species with the addition of high levels of Cr. In regard to nematodes, it has been found that abundance can increase of certain nematodes in very high contamination environments (Chauvin et al., 2020) whereas some will decrease, but will only decrease in new pollution and in areas with historical contamination, it has little effect on the diversity as the nematodes have adapted (Gutiérrez et al., 2016).

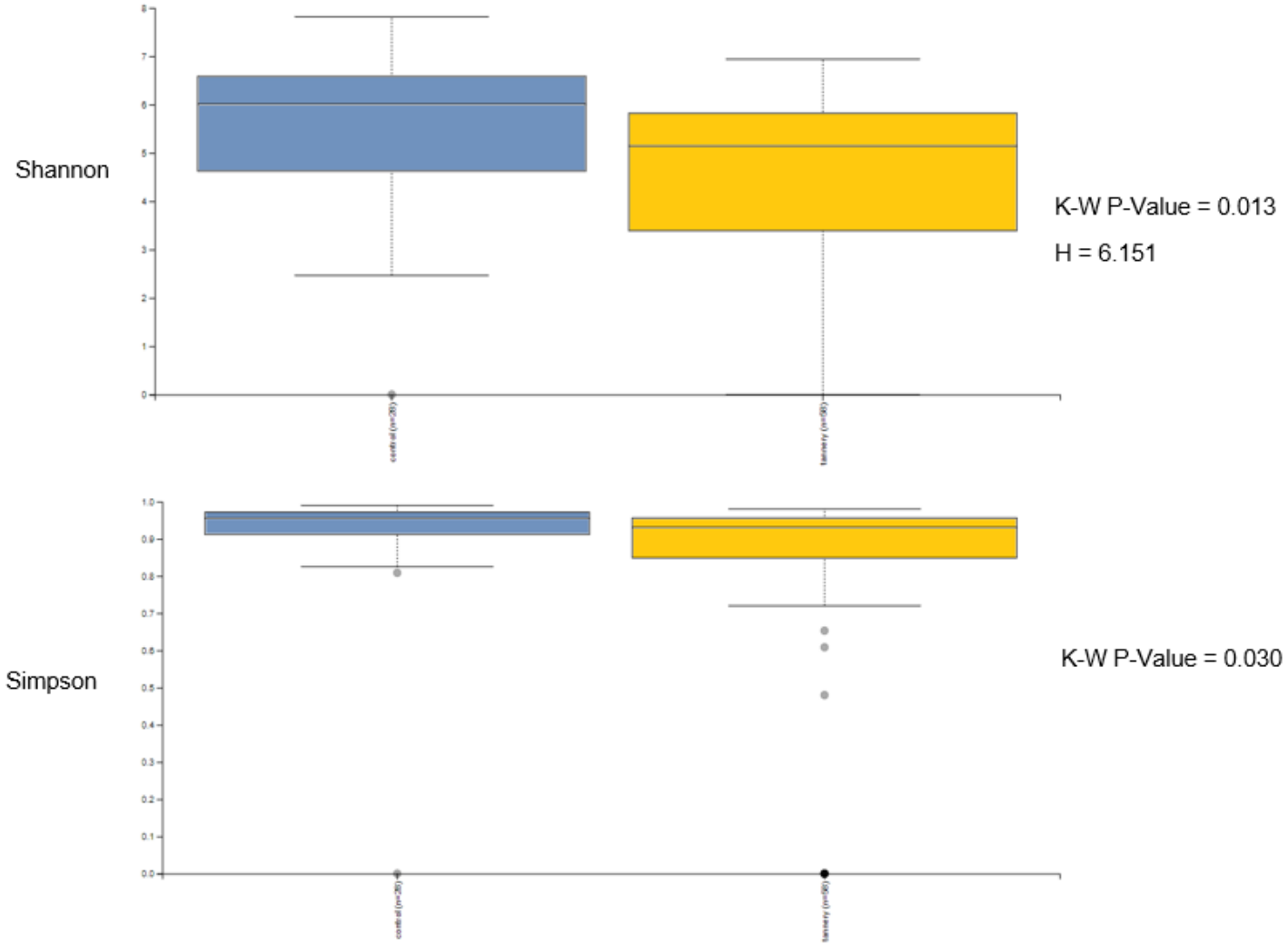


Figure 3.8. Analysis of alpha diversity of ITS communities within control and contaminated soil samples via Shannon's and Simpson's Indexes visualised using Boxplot

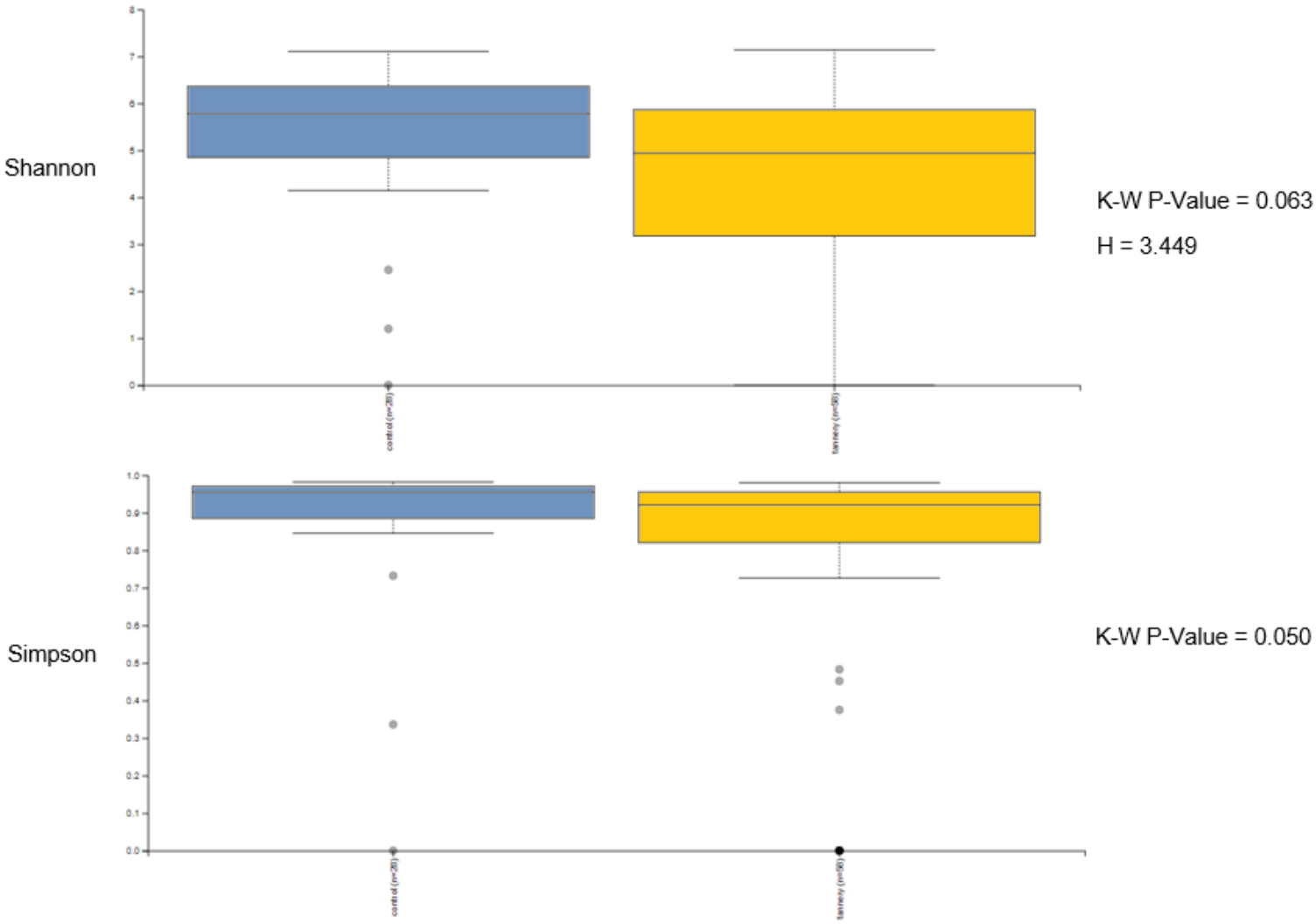


Figure 3.9. Analysis of alpha diversity of 18s communities within control and contaminated soil samples via Shannon's and Simpson's Indexes visualised using Boxplot

Beta diversity

Principle coordination analysis (PCoA) was carried out in order to determine the effect that tannery related pollution had on the microbial communities, specially relating to chromium. Two methods were used to show the similarity in the communities of organisms within the control and contaminated samples. Jaccard Emperor distance metric and Bray Curtis metric were used to look at

shared features within the samples from the sites and to analysis feature similarity with abundance considered respectively. While both sets of analysis look at the similarity between the control and contaminated microorganism data sets, however Bray Curtis similarity also take abundance of the microorganisms within the dataset into account. Beta diversity can also show how the main pollutant focus of this study, Cr, is represented within the similarity of the samples across the metrics. Significance in the dissimilarity of the communities were assessed via permutational multivariate analysis of variance (PERMANOVA).

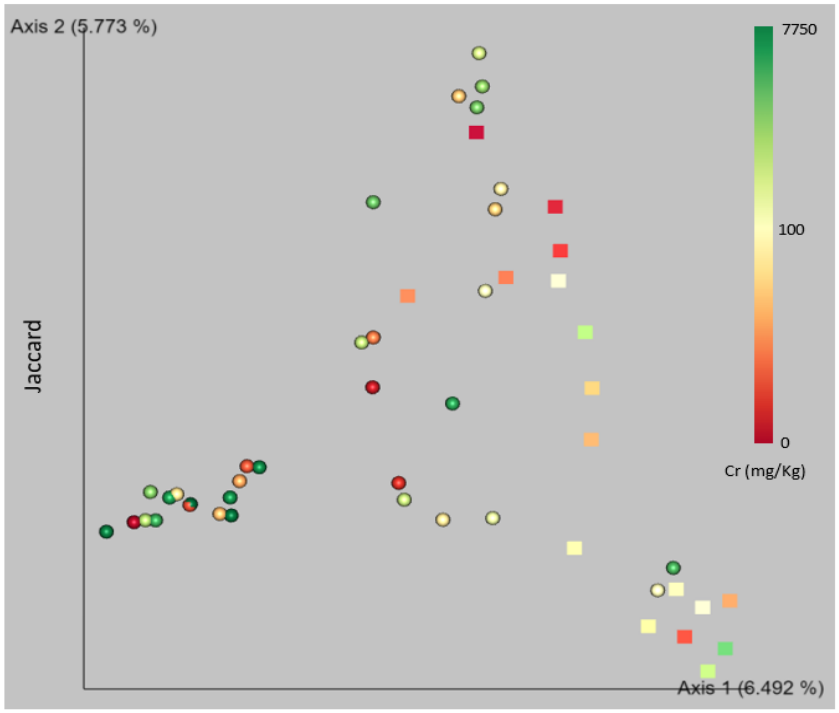
Beta diversity for 16s OTUs, as shown in figure 3.10, shows that abundance has little effect on the overall similarity of the samples for both the control and contaminated sites with little distinguishable change occurring between the Jaccard and Bray Curtis metrics, but it does increase the overall dissimilarity when abundance is included, as can be observed by the increase in the axis % explanation for dissimilarity of samples. PERMANOVA indicated that the control and contaminated samples differed significantly (p value = 0.009) in their composition of bacterial communities through there similarity. This has been observed in previous studies where the dominant species of bacteria shifts with the presence of Cr (Desai et al., 2009) as well as other parameters that tannery effluent effects, such as soil carbon (Miranda et al., 2018) and other heavy metal contaminants (Hemmat-Jou et al., 2018) However, in the results obtained in this study a link between Cr and bacteria community similarity appears to be visible. Samples containing higher levels of Cr contamination are found to have a high similarity to each other and high dissimilarity from controls. This could be the result of not just Cr by soil properties within the soils, as has been discussed by Kamal, Prasad and Varma (2010) who stated that reductions in diversity can occur from shifts in soil properties such as pH as well as the presence of toxic element. This fits with the reduction in diversity in tannery soils seen from the alpha diversity indexes: it can be hypothesised that elevated chromium causes the populations to be similar, where a few Cr-resistant species dominate the soil community in tannery soils. These might be of interest if they also show to have relations with plant growth promoting and the uptake of Cr into plant structures. This subsequent screening of Cr resistant bacteria had been seen in research by Hemambika, Balasubramanian, Kannan and James, (2013) *Pseudomonas sp.* VRK3 was identified as Cr-resistant and was found to assist in plant growth via bioremediation into there own structures as well as mobilising the Cr in the soil, making it more available to take up within plants.

As shown in figure 3.11, fungal OTUs as determined by ITS also showed through beta diversity there was a significant dissimilarity between the communities with control and contaminated soil samples as shown by the p value achieved from PERMANOVA (p=0.001) showing a change in the community structure as a result of tannery pollution. However, with a change in the distribution and % explanation of similarity on the axis of the Bray Curtis as opposed to the Jaccard dissimilarity metric, it suggests

that abundance of the OTUs for fungi found have a large effect on the similarity of the soil samples fungi community structures. This is also seen by Li et al. (2021) with changes in the community structure occurring with the addition of tanning agents to the soil media. This reduction in the abundance of fungi could be as a result of reduced spore counts as discussed by Nakatani et al., (2011). With the addition of higher concentrations of tannery sludge, the arbuscular mycorrhizal fungi spore density may be reduced, and the community may have changed within the soil environment, causing

the sporulation of certain species to become more prevalent and changing the community structure of the soil microbiome (Nakatani et al., 2011).

A



PERMANOVA – p value = 0.009

B

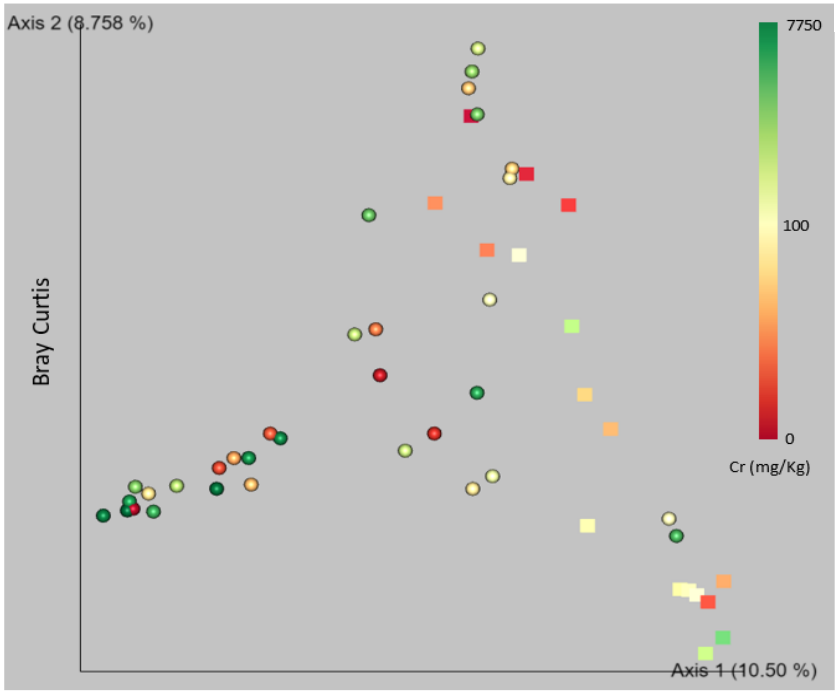
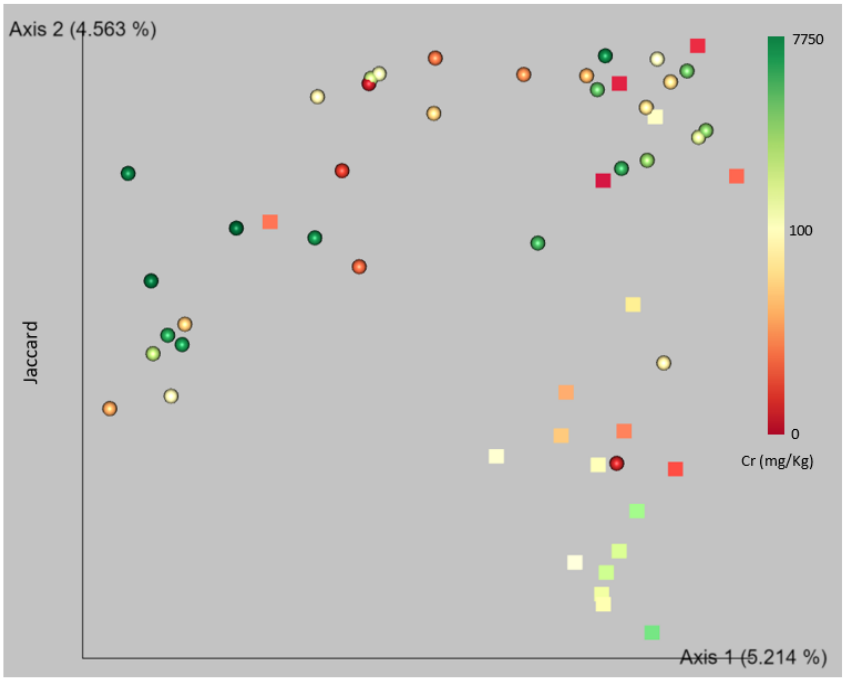


Figure 3.10. Principal Coordinate Analysis (PCoA) based of (A) Jaccard Emperor dissimilarity metrics and (B) Bray Curtis dissimilarity metrics for the inclusion of abundance, showing the distances in the bacterial communities in control and contaminated site samples. PERMANOVA was ran to discern significant differences in the control and contaminated sites

A



PERMANOVA – p value = 0.001

B

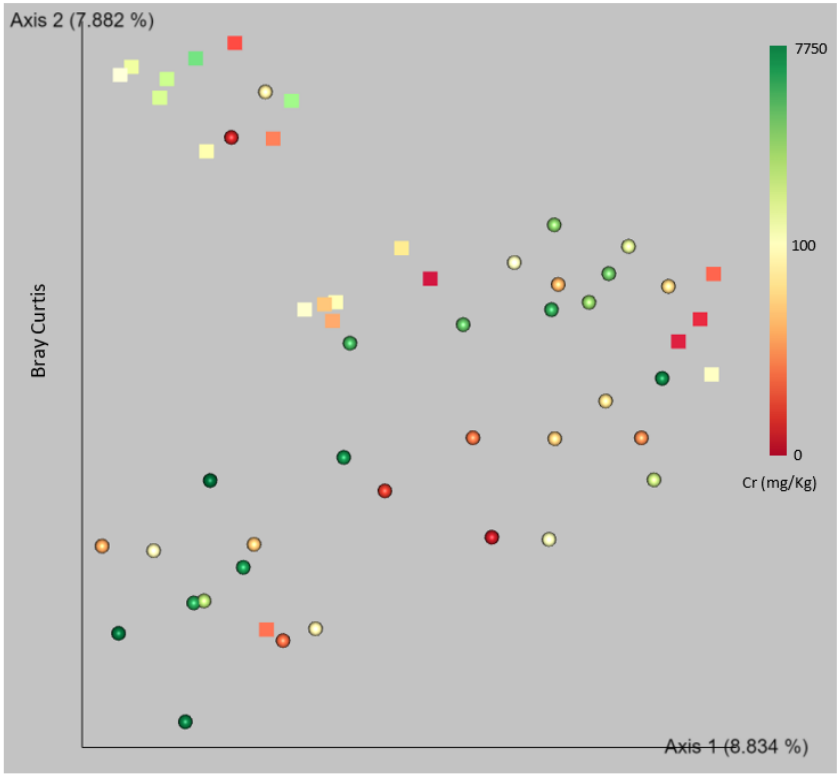
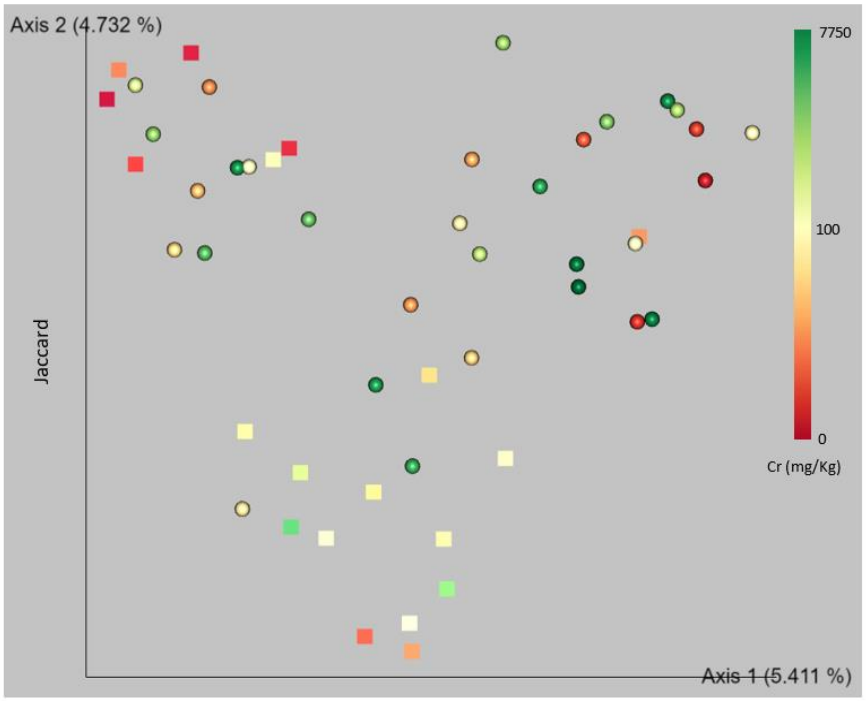


Figure 3.11. Principal Coordinate Analysis (PCoA) based of (A) Jaccard Emperor dissimilarity metrics and (B) Bray Curtis dissimilarity metrics for the inclusion of abundance, showing the distances in the fungal communities in control and contaminated site samples. PERMANOVA was ran to discern significant differences in the control and contaminated sites.

18s beta diversity analysis (Figure 3.12) showed a significant difference in the similarity of the control and contaminated communities with a PERMANOVA p value of 0.014. This demonstrates that a significant effect of the tannery contamination on the communities within the 18s analysis. Similarly, to the fungal Bray Curtis, the Bray Curtis similarity metric showed there to be an effect of abundance on the similarities of the samples. Research relating to protozoa and nematode within soils contaminated via heavy metals or tannery industrial processes are few, but research has shown that contaminants such as polyaromatic hydrocarbons (PAH) can have a significant effect on nematode communities to the extent that they can be used as a bioindicator organisms for PAH (Parihar et al., 2021) or even heavy metals as seen if fish guts (Azmat et al., 2008). What has been found is that heavy metals within soils can of toxic to certain groups of protists including ciliates (Wu, Chao, Shu and Qiu, 2022), which if removed from the soil via contamination, would cause a large divergence in the populations protist populations in the soils and as a result the similarity to normal soils.

A



PERMANOVA – p value = 0.014

B

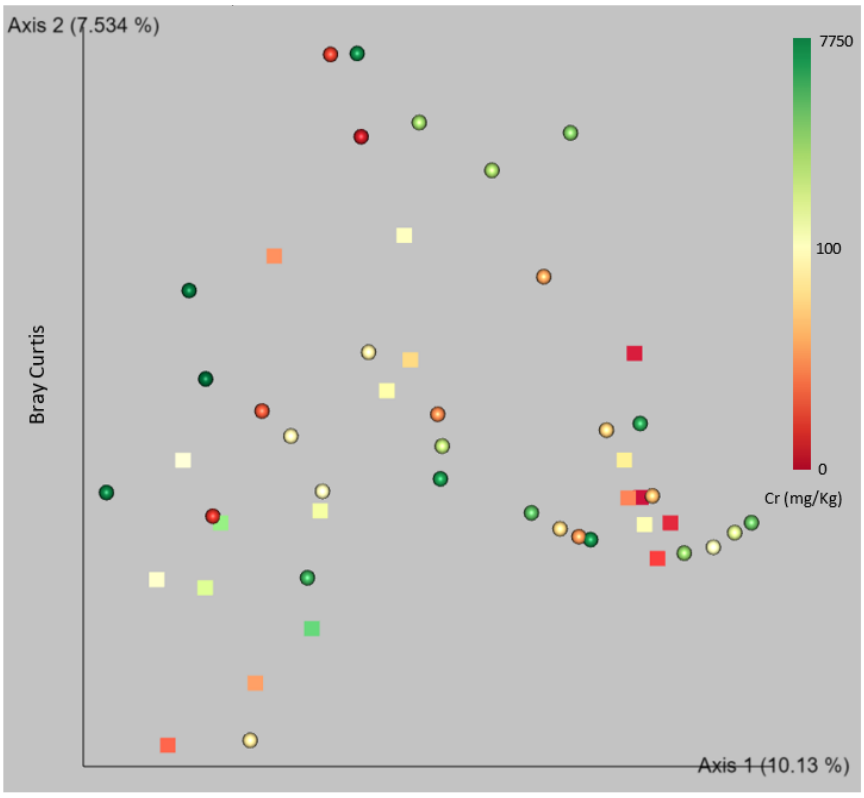


Figure 3.12. Principal Coordinate Analysis (PCoA) based of (A) Jaccard Emperor dissimilarity metrics and (B) Bray Curtis dissimilarity metrics for the inclusion of abundance, showing the distances in the 18s (protozoa and *Nematoda* included) communities in control and contaminated site samples. PERMANOVA was ran to discern significant differences in the control and contaminated sites.

Phyla in the metagenomes

For 16s, ITS and 18s, all 100% of the OTUs were able to be classified against a hit, however down to order and species level for fungi showed unidentified OTU levels of between 0.1% to 44.1%.

Out of the 16s PCR products run using the Illumina MiSeq platform by FERA (UK), *archaea* were only found in 12 samples, all of which were contaminated. The proportion of archaea were observed between 1.2% and 33.4% relative frequency of the sample's readings for 16s OTUs. The most dominant bacterial phyla found in the soil samples (table 3.3 and figure 3.13) were *Chloroflexi* (36.83% control and 46.06% contaminated), *Actinobacteria* (32.92% controls and 20.69% contaminated), *Planctomycetota* (10.60% control and 11.73% contaminated), *Proteobacteria* (3.97% control and 7.48% contaminated), *Firmicutes* (2.10% control and 1.98% contaminated), *Acidobateria* (4.32% control and 2.13% contaminated) and *Gemmatimonadota* (5.06 % control and 2.59% contaminated). The total relative frequencies of the bacterial top phyla frequencies across samples ranged between 55% and 100 % in control samples and 45% to 100% in the tannery polluted samples gathered from the industrial zone (Figure 3.6). All seven of the most abundant bacterial phyla are widespread in soil distributions (Hemmat-Jou, Safari-Sinegani, Mirzaie-Asl and Tahmourespour, 2018). As can be seen from table 3.3, *Chloroflexi* and *Planctomycetota* showed increased frequency within contaminated samples compared to polluted samples suggesting an increased tolerance to the contaminations of the site. *Actinobacteria, Acidobateria* and *Gemmatimonadota* all show a noticeable reduction in frequencies within contaminated soils with the largest being by *Actinobacteria* showing a sensitivity to the tannery contamination.

For fungi (table 3.3 and Figure 3.14), the highest fungal phyla dominance within the soil samples was found to be *Ascomycota* (74.52% control and 77.53% in contaminated), *Basidiomycota* (7.65% control and 8.52% contaminated), *Chytridiomycota* (1.40% control and 1.36% % contaminated), *Glomeromycota* (9.67% control and 5.20% contaminated), *Aphelidiomycota* (0.05% control and 0.80% in contaminated), *Mortierellomycota* (2.96% control and 3.18% contaminated) and *Rozellomycota* (0.38% control and 2.81% contaminated). As well as these phyla, unidentified proportions of the sequenced reads down to phyla can be seen in 66 of the samples, ranging between 0.5% to 34% relative frequency. Relative frequencies of the top 7 phyla discounting unidentified readings, made up between 40% to 100% of control sample and 50% to 100% if contaminated sample relative frequencies (Figure 3.14). *Ascomycota, Basidiomycota, Aphelidiomycota, Mortierellomycota* and *Rozellomycota* all showed increased relative frequencies within contaminated samples to that of the controls

inferring higher levels of resistance to contamination then *Chytridiomycota* and *Glomeromycota*. However, *Chytridiomycota* only decreased in frequency by 0.04% between control and contaminated and as such stays relatively stable.

In the taxonomy bar plots produced showing the relative frequencies when using the 18s selective primers (table 3.3 and Figure 3.15), a large array of different *Eukaryota* were identified as well as a number of protists and *Nematoda*. The top protozoa phyla produced via 18s sequencing were shown to be *Ciliophora* (6.53% control and 10.24% contaminated)*, Apicomplexa* (16.33% control and 4.03% contaminated)*, Cercozoa* (6.91% control and 16.01% contaminated)*, Chlorophyta* (6.97% control and 7.62% contaminated)*, Amoebozoa* (1.00% control and 0.80% contaminated)*, Protalveolata* (0.23% control and 0.48% contaminated)*, Holozoa* (0.11% control and 0.30% contaminated) *and Discoba* (0.08% control and 0.12% contaminated). These top phyla for protozoa made up between 0.2% to 90 % of the relative frequency within the samples (Not including 2 samples where no protozoa in the top 7 were found). *Nematoda* ranged between 0% to 25.5% relative frequency within these same samples. As can be seen in table 3.3, *Ciliophora* and *Cercozoa* both showed large increase in relative frequency from between control and contaminated samples. This implies resistance mechanisms within these phyla allowing for them to be able to increases their proportion of the protozoan community.

	Phyla		Relative frequency %	
			Control	Contaminated
Bacteria	*Chloroflexi*	Mean ± SD	36.83 ± 18.39	46.06 ± 22.31
	Actinobacteria		32.92 ± 23.51	20.69 ± 19.71
	Planctomycetota		10.60 ± 8.70	11.73 ± 9.37
	Proteobacteria		3.97 ± 7.00	7.48 ± 11.15
	Firmicutes		2.10 ± 7.22	1.98 ± 3.06
	Acidobateria		4.32 ± 7.27	2.13 ± 2.08
	Gemmatimonadota		5.06 ± 6.57	2.59 ± 3.11
Fungi	*Ascomycota*	Mean ± SD	74.52 ± 17.88	77.53 ± 23.07
	Basidiomycota		7.65 ± 7.05	8.52 ± 12.87
	Chytridiomycota		1.40 ± 2.12	1.36 ± 2.10
	Glomeromycota		9.76 ± 9.86	5.20 ± 7.57
	Aphelidiomycota		0.05 ± 0.21	0.80 ± 1.90
	Mortierellomycota		2.96 ± 9.87	3.18 ± 14.89
	Rozellomycota		0.38 ± 1.78	2.81 ± 14.38
Protozoa	*Ciliophora*	Mean ± SD	6.53 ± 5.11	10.24 ± 10.42
	Apicomplexa		16.33 ± 24.06	4.03 ± 8.22
	Cercozoa		6.91 ± 4.52	16.01 ± 19.41
	Chlorophyta		6.97 ± 7.98	7.62 ± 13.73
	Amoebozoa		1.00 ± 0.80	0.80 ± 0.78
	Protalveolata		0.23 ± 0.39	0.48 ± 1.55
	Holozoa		0.11 ± 0.14	0.30 ± 0.60
	Discoba		0.08 ± 0.13	0.12 ± 0.18

Table 3.3. Mean relative frequency % of top Phyla within control and contaminated samples (± standard deviation)

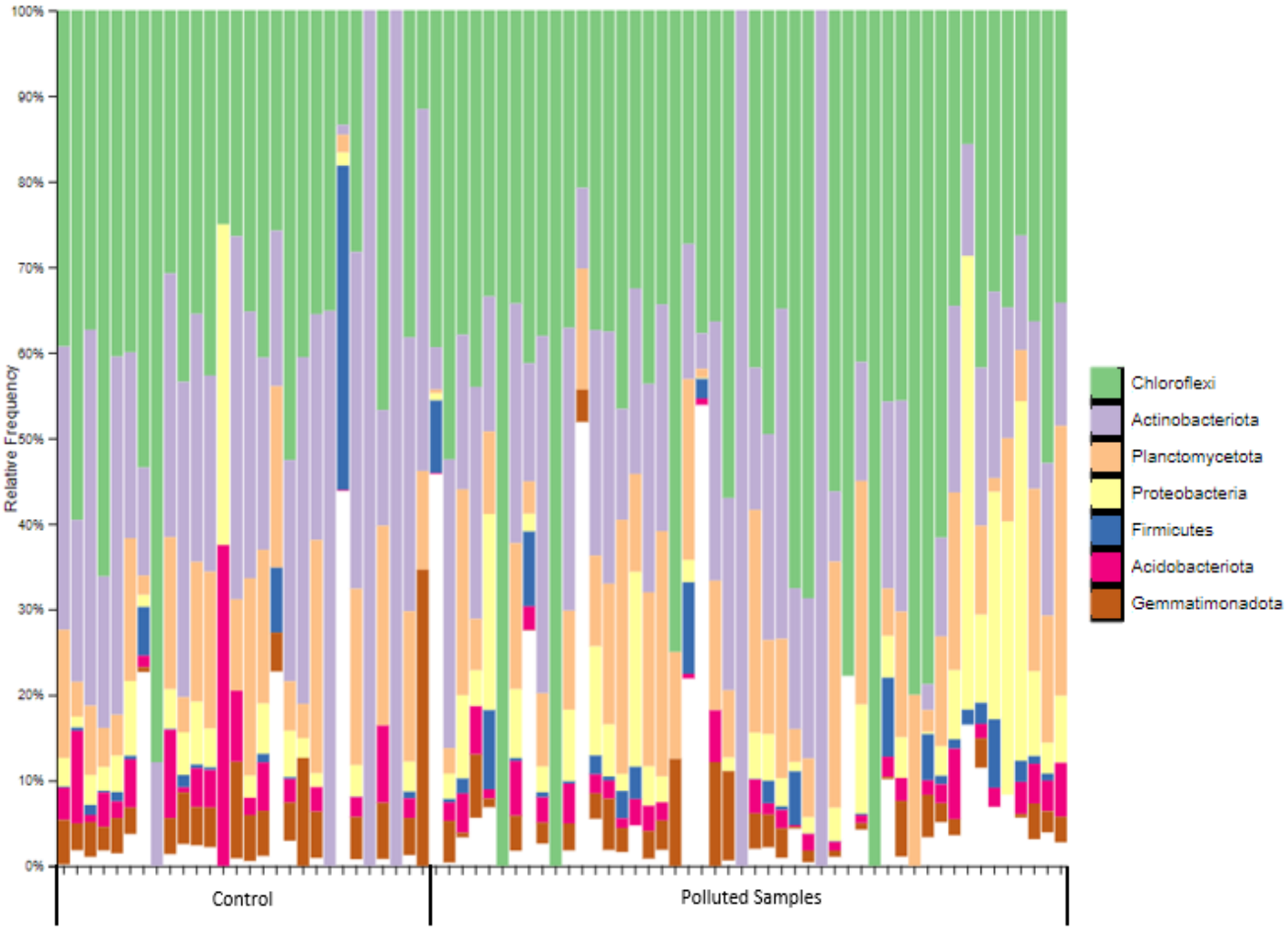


Figure 3.13. Relative frequencies of the top 7 phyla of bacteria though 16s sequencing (White indicates relative frequency relating to all other phyla discovered within the samples that are not in the top 7 abundant).

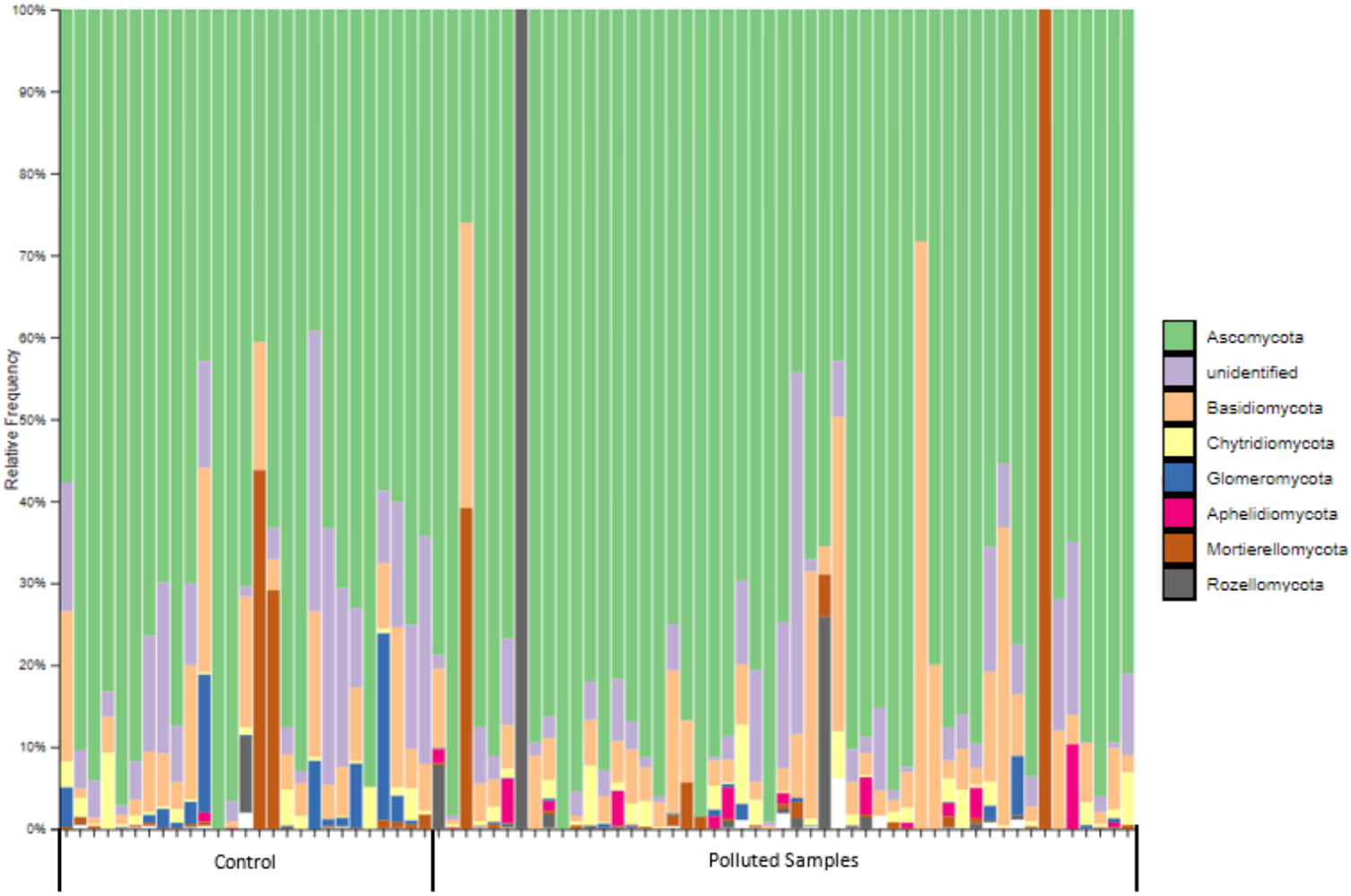


Figure 3.14. Relative frequencies of the top 7 phyla of Fungi and unidentified phyla through ITS sequencing (White indicates relative frequency relating to all other phyla discovered within the samples that are not in the top 7 abundant)

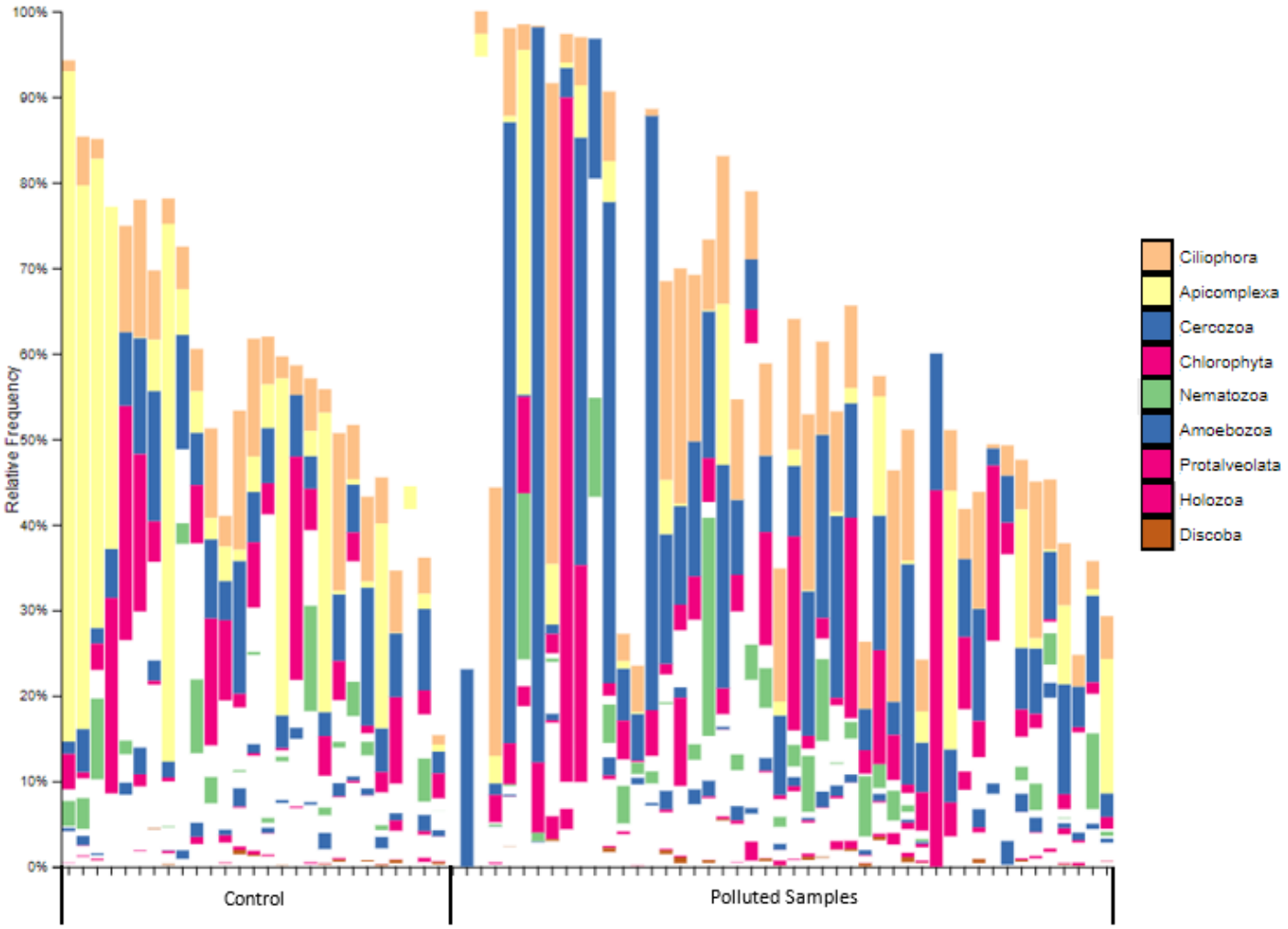


Figure 3.15. Relative frequencies of the top 8 phyla of protozoa and *Nematoda* determined through 18s sequencing (White indicates relative frequency relating to all other phyla discovered within the samples that are not in the top 8 abundant or not relating to protozoa)

Nematoda class and order in the metagenomes

In analysing the relative frequencies of nematode class and order via taxonomic bar plots, OTUs relating to the two classes *Chromadorea* and *Enoplea* were found with the relative frequencies varying greatly across the samples both for control (*Chromadorea* 66.12% , *Enoplea* 33.88%) and within the chromium polluted samples (*Chromadorea* 72.49% , *Enoplea* 27.51%) as shown in Figure 3.16. This shows a shift in the nematode communities towards more abundance of *Chromadorea* within the tannery polluted samples. This indicates more resistance to the contamination levels within the samples by *Chromadorea* as opposed to individuals from the *Enoplea* Class.

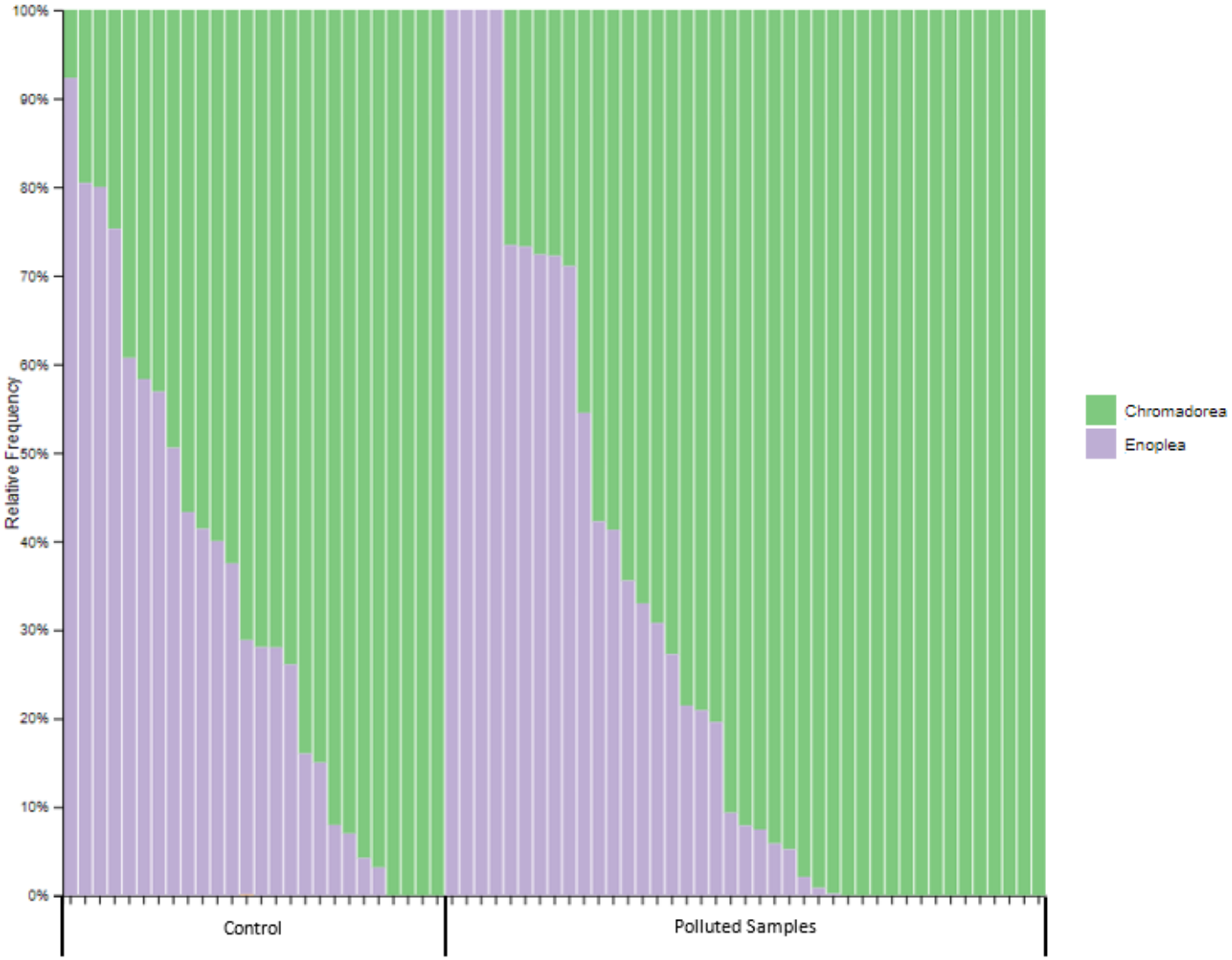


Figure 3.16. Relative frequencies of the Class of Nematode via 18s sequencing

A total of nine taxonomic orders for nematode were identified. Six orders under the class *Chromadorea* and three under the class *Enoplea.* Seven of these orders showed in more than one contaminated sample. These orders were *Rhabditida* (25.97% control and 42.66 % contaminated), *Dorylaimia* (24.28% control and 12.93% contaminated), *Tylenchida* (27.28% control and 13.28% contaminated) , *Araeolaimida* (8.90% control and 9.27% contaminated) , *Triplonchida* (6.84% control and 5.79% contaminated), *Enoplida* (2.75% control and 8.78% contaminated) and *Monhysterida* (2.02% control and 6.80% contaminated) (table 3.4 and Figure 3.17). These orders contribute between 80% to 100% for control and 90% to 100 % of the sample nematode relative frequencies with the rest of the % being made up of OTUs that could not be assigned to an order with a large proportion (18 samples of 41 samples) having over 50% of its relative frequency dominated by ***Rhabditida***. As per table 3.4, it can be seen that ***Rhabditida,*** *Araeolaimida, Enoplida* and *Monhysterida* all demonstrated increased frequency within the contaminated soil samples than the control, indicating heightened resistance. *Dorylaimia* and *Tylenchida* both showed halving if their control frequencies within the contaminated site suggesting they do not possess the same resistance mechanisms as the more resistant nematode orders.

	Order		Relative frequency %	
			Control	Contaminated
Nematode	*Rhabditida*	Mean ± SD	25.97 ± 29.93	42.66 ± 36.32
	Dorylaimia		24.28 ± 27.00	12.93 ± 22.39
	Tylenchida		27.28 ± 30.56	13.28 ± 24.40
	Araeolaimida		8.90 ± 16.40	9.27 ± 25.40
	Triplonchida		6.84 ± 12.84	5.79 ± 16.76
	Enoplida		2.75 ± 6.50	8.78 ± 24.62
	Monhysterida		2.02 ± 7.87	6.80 ± 22.24

Table 3.4. Mean Relative frequency % of top Nematode Order within control and contaminated samples (± standard deviation)

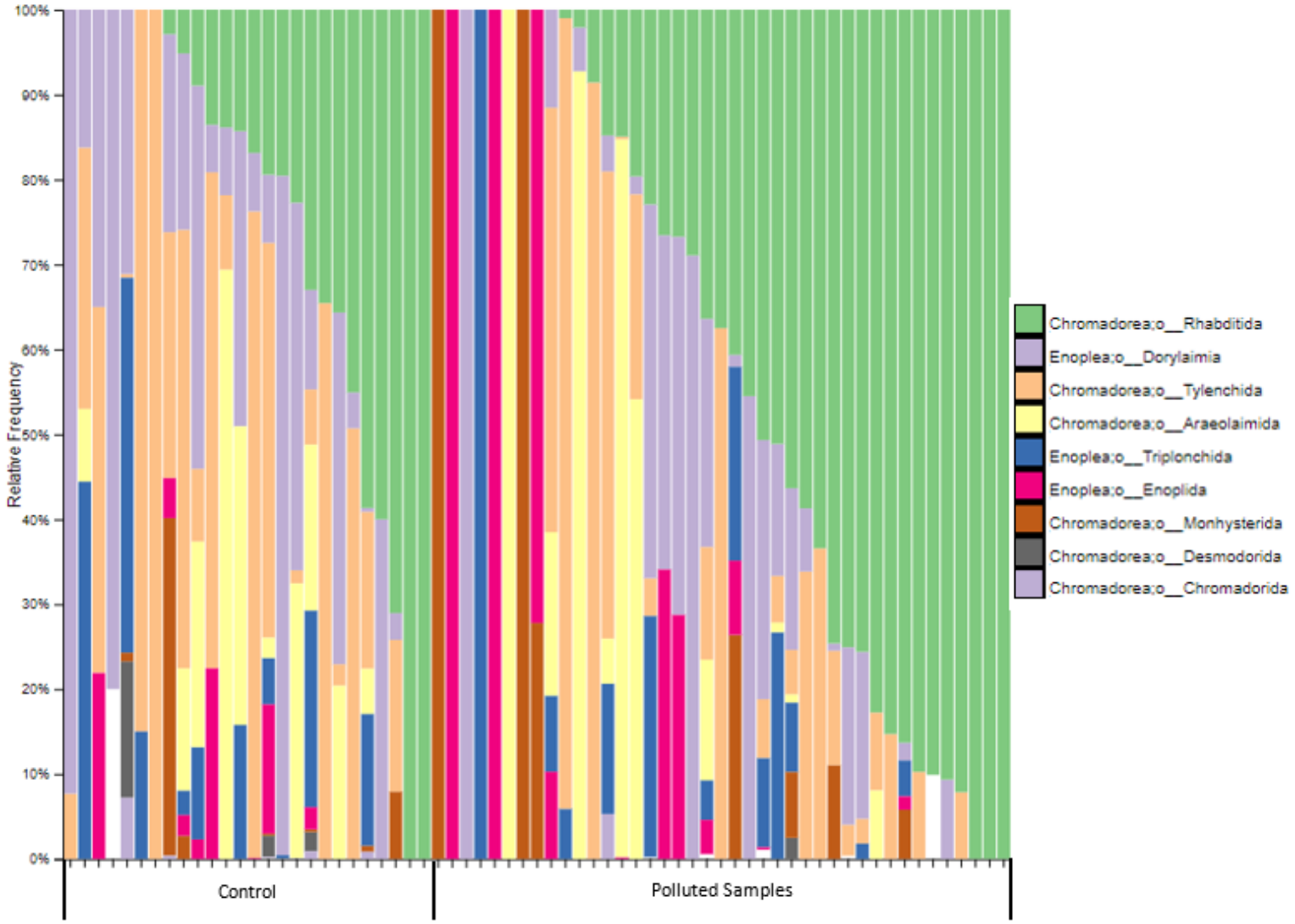


Figure 3.17. Relative frequencies of the orders of Nematode via 18s sequencing

Orders of promise identified during metagenome analysis

The top Orders for bacteria, fungi, nematodes, and protozoa were all collated from the data of the taxonomic relative frequencies. Each will be discussed in turn to determine the effect pollution had on its frequencies within samples and its potential suitability for application to remediation projects. Mean relative frequencies and their standard deviation are shown in table 3.5.

Bacteria

Several taxonomic orders of bacteria showed a resilience or even an increase in abundance to the increased levels of Cr. However, there was a large number of orders that showed low relative frequencies and as such will not be assessed in this research. *Thermomicrobiales, Kallotenuales* and KD4-96 of the phyla *Chloroflexi, Tistrellales* of the phyla *Proteobacteria, Peptostreptococcales-Tissierellales* from the phyla *Firmicutes* and *Tepidisphaerales* form the phyla *Planctomycetota* were all identified as bacteria with the potential of Cr resistance mechanisms due to there levels found within elevated Cr contamination levels.

Thermomicrobiales has been seen to be enriched by the presence of contamination previously in hexafluoropropylene oxide-dimer acid contaminated soil (Liu et al., 2022) and heavy metal contaminated soils (Zeng et al., 2022 In levels exceeding 100 mg/kg Cr, *Thermomicrobiales* was found in relative abundances of 17.67% in control to 20.21% of the bacterial OTUs in contaminated samples. This showed *Thermomicrobiales* to increases in abundance within contaminated samples. This was still the case to levels above 1000 mg/kg Cr were the relative abundance ranged between 15.7% to 27% of the bacterial OTUs. The results for *Thermomicrobiales* at increased levels of Cr along with its identification in research relating to assisting the uptake of other associated heavy metals utilised in tannery processes, make *Thermomicrobiales* an interesting target organism for future research. .In the research conducted by Zeng et al., (2022), *Thermomicrobiales* was seen to be enriched within the rhizosphere of *Arundo donax* L while it was being utilised as a pioneer plant in intercropping for the uptake of heavy metals, assisting in the uptake of 72.16% of Cu contamination by the *Arundo donax* L. However, there has been no research into the ability of *Thermomicrobiales* to resist tannery contamination or elevated Cr levels.

Kallotenuales have been identified in soils in historical areas of the sampling region of Tamil Nadu (Ennis, Dharumaduri, Bryce and Tisa, 2020), as well as other post-industrial sites including mining sites, although at relatively small levels (Gabay, Rotem, Gillor and Ziv, 2022). Communities of *Kallotenuales*

were present throughout all concentrations of chromium. They were found at 5.30% in control soils and 6.04% in contaminated soils. These levels of relative frequency dropped above these levels with the high contaminated levels 5924 mg/kg Cr and 7757 mg/kg Cr only consisting of a relative frequency of 0.48% and 0.38% respectively. Whether these levels infer a promising of chromium resistance of as a phytoremediation assisting microbe in unclear as there is no prior research that has been carried out in this area. These relative frequencies suggest a slight resistance to the presence of pollution at low levels of Cr, which is subsequently lost at higher levels. Zhang et al., (2022) found the *Kallotenuales* frequencies were negatively correlated with pH levels of soil, with increases causing drops in the community. This supports the findings from this study as high Cr levels were identified as being within high pH soils. The suggests that the drop in *Kallotenuales*at high Cr levels could also be attributed to the increased pH.

KD4-96 showed to be consistently within the bacterial communities throughout all levels of chromium contamination, if at low relative frequencies (ranging between 0.25% and 12.26% in samples containing them). KD4-96 was identified in relative frequency of 2.80% in control and 2.16% in contaminated soils. KD4-96 affiliated OTUs have been previously known to be detected within contaminated soil areas (Wegner and Liesack, 2017) such as mining (Kujala et al., 2018). In relation to heavy metals, KD4-96 has been found to be present in the bacterial community of contaminated site in several papers (Gołębiewski et al., 2014; Yin et al., 2015; Girardot et al., 2020; Liu et al., 2020) and specifically in research of Cr containing contaminated soils, being in high levels of abundance as a core of the bacterial community (Wu et al., 2022; Zhang et al., 2022). Having been identified also as present within research surrounding phytoremediation and beneficial bacterial communities for resistance and mutualistic relationships, KD4-96 is a strong candidate for further research into plant-microbe relationships relating to Cr and heavy metal uptake into hyperaccumulators.

Tistrellales showed little to no relative abundance within the majority of samples across all concentrations with frequencies of 0.18% in control soils and 4.68% in contaminated soils, suggesting some resistance to the contamination of the site. Levels from 1000 mg/kg to 7760 mg/kg of Cr showed a relative abundance of between 10.57% and 53.04%. Apart from being identified as being able to aid in water purification (Wang et al., 2021), very little to no research has been carried out regarding *Tistrellales* around heavy metal tolerance, how its effected by industrial process, or any mutualistic relationships it may have for heavy metal uptake into plants. *Tistrellales*, even with a lack of prior research, show promise for resistance mechanisms for chromium and other heavy metals and such might be of interest for further research to see if this ability of two orders to resist Cr might aid in uptake of Cr into plants.

Peptostreptococcales-Tissierellales similarly to *Tistrellales* was found at its highest relative frequencies at higher levels of chromium contamination. Mean frequencies found were 1.18% in control and 1.08% in contaminated soils suggesting no change in the population as a result of the contamination. Also, similarly to *Tistrellales,* no research relating to heavy metals, industry and phytoremediation has been carried out in conjunction with *Peptostreptococcales-Tissierellales*. *Peptostreptococcales-Tissierellales* has been identified within human microbiomes including the brain and gut (Bedarf et al., 2021; Gubert et al., 2022)

Tepidisphaerales showed a consistent presence and relative abundancy across all samples of the study, with relative frequencies of 6.19% in controls and 4.92% in contaminated samples. *Tepidisphaerales* have been identified as a highly abundant order of bacteria within pollution studies (Campos et al., 2020) with the precent flora having a significant impact of the abundance of *Tepidisphaerales* within contaminated urban soils (Rosier et al., 2021). Once again, research relating to Chromium and phytoremediation has not yet been conducted.

Out of the six orders of bacteria, *Thermomicrobiales* and *Tistrellales* showed the most resistance to the presence of contamination within the soil locations. *Tepidisphaerales* showed a vulnerability to the contamination within the sample and was the most effected order analysed in this study.

Fungi

Within the OTUs that were identified to order level for fungi, five have been identified as of interest for Cr resistance mechanisms due to exhibiting consistent or increased relative frequencies at increased Cr concentrations, with an extra two being identified specifically in extreme Cr contamination levels. All five orders that exhibited the most relative frequencies at increased Cr levels came from the phylum *Ascomycota* which has already been identified as one of the most resistant and abundant phylum in heavy metal contaminated areas (Văcar et al., 2021). However, the two found in the most contaminated sample (7760 mg/kg) were both from the phylum *Rozellomycota.* The orders to be discussed are *Pleosporales, Hypocreales, Sordariales, Eurotiales, Capnodiales* of the phylum *Ascomycota* and

Pleosporales showed a consistent presence across all samples at all levels of Cr content with relative frequencies of 33.30% in controls and 32.44% in contaminated samples, showing that the precense of contamination has no detrimental effect on the frequency of *Pleosporales* to a significant level. At levels over 2000 mg/kg Cr to 6560 mg/kg, a continued consistently high relative frequency of *Pleosporales* at frequencies of 17.3% to 44% were identified. *Pleosporale* has been shown to have

heavy metal resistant qualities including against Hg, Pb (Văcar et al., 2021), Cd (Jin et al., 2017), low Cr levels (Zhang et al., 2021), as well as being identified as a dark septate endophyte (Jin et al., 2017), known for their ability to colonize plant roots and facilitate the growth of the plant that's hosting it (He, Wang and Hou, 2019) though the forming of melanized septate hyphae and microsclerotia in roots especially in areas such as polluted areas (Hou, Yu, Zhao and He, 2020). Due to being mainly saprotrophs (Moretti and Sarrocco, 2016), which are species that feed of dead organic matter such as wood and plant litter (Asiegbu and Kovalchuk, 2021), *Pleosporales* can promote plant growth though making available nutrients locked in these organic matter sources (Xia et al., 2019) as well as providing resistance to plants such as tobacco to heavy metal contamination (Jin et al., 2017). *Pleosporales* have been identified in many pieces of research relating to assistance in uptake of contamination within plants (Iffis, St-Arnaud and Hijri, 2017; Gómez-Sagasti et al., 2021) but specific research relating to *Pleosporales* is lacking.

Hypocreales was identified in many of the samples tested in both control and contaminated areas. Genus under the order *Hypocreales* such as *Trichoderma* have already been found to reside in heavy metal contaminated soils with resistant qualities (Ayad et al., 2018) and can act as a bioaccumulation method for Cr(VI) (Hlihor, Rodríguez, Gutiérrez and Moctezuma, 2004; Ayad et al., 2018). Seven species of *Trichoderma* were identified during this study constantly across all concentrations of Cr, but at low relative frequencies (3% and below). These levels do not support the reports of resistance of the *Hypocreales* order to the presence of contamination (Ayad et al., 2018; Văcar et al., 2021). Assisted phytoremediation has been identified with the enhancement on Ni uptake by *Brassica juncea* (Domka, Rozpaądek and Turnau, 2019) while also helping provide increased tolerance to the toxicity of the heavy metals (Cao et al., 2008) as such, another variable might be at play within the contamination at the tannery sites causing the reported resistance not to occur for *Hypocreales* in these samples.

Sordariales was found in higher relative frequencies between 90 mg/kg and 200 mg/kg (up to levels of 63%) representing a mild tolerance to Cr within the study. This is supported by a consistency of the relative frequencies found within this study of 9.58% in control and 10.11% in contaminated soils. *Sordariales* have been proven to be resistant to other heavy metals including Cu, Cd and Zn at high levels (Albert et al., 2019; Song et al., 2021), as well as potentially being involved in the reduction and stabilisation of Cr(VI) and Cr(III) respectively (Fu et al., 2021). *Sordariales* have also been found to help with plant growth and nutrition availability but have been found to reduce the amount of heavy metals that have been taken up into the plant that they form mutualistic relationships with (Barberis, Michalet, Piola and Binet, 2021).

Eurotiales displayed a high Cr tolerance and subsequent resistance, being present in samples containing Cr contamination levels from low to 6560 mg/kg. Relative frequencies of 2.77% were found in control samples and at 7.93% in contaminated samples, indicating improved abundance and potential resistance mechanisums. Species of *Eurotiales* have been identified to be able to grow in a large array of different environmental stress conditions (Urquhart, Chong, Yang and Idnurm, 2022) while also being thought to be able to resist heavy metal including Cr (Lotlikar et al., 2018; Urquhart, Chong, Yang and Idnurm, 2022). As well as resistance, high levels of heavy metals have been found to allow for abundances of *Eurotiales* to increase (Zeng, Li, Leng and Kang, 2020).

Analysis of the *Capnodiales* order of fungi showed that the relative frequencies of the OTUs were higher at high levels of Cr contamination. *Capnodiales* was identified within samples up to Cr levels of 6560 mg/kg with the relative frequency range being between 1.4% and 46.6% of the OTUs sequenced. This infers not just a resistance to the presence of tannery contamination and Cr but that the increase of Cr levels can allow for the abundance levels of *Capnodiales* to increase due to a decrease in other fungi populations. *Capnodiales* have been identified in microbial communities that are more resistant to contamination have heavy metals (Ye et al., 2020; Văcar et al., 2021), however specifics on *Capnodiales* seem to be lacking. This lack of research is also around its link to tannery microbial communities with one paper mentioning it in that context (Spennati et al., 2021).

Glomerales were not identified in high levels of Cr contamination with the use of ITS primers but instead were found by the 18s analysis to be at high relative frequency in medium level Cr contamination. *Glomerales* has been include in this discussion as although being identified in medium levels of Cr contamination, research has shown that this arbuscular mycorrhizal fungus is of high importance for its resistance to heavy metals and symbiotic relationships with plants (Riaz et al., 2021), providing assistance to plant growth and phytoremediation through rhizosphere modification (Riaz et al., 2021) and chelation of metallothioneins (González-Guerrero, Benabdellah, Ferrol and Azcón-Aguilar, 2008), and reduction of bioavailable heavy metals (Liu et al., 2019; Xiao, Zhao, Chen and Li, 2020). As such it is important to note its presence within the soil samples of the soils contaminated by the tannery industry as a native order.

Out of the five orders identified for fungi, *Eurotiales* and *Capnodiales* showed the most affinity to resistance of the contamination from the site, suggesting mechanisms at play to assist in survival. *Hypocreales* showed a intolerance to the presence of contamination, with a noticeable drop in the populations of *Hypocreales* within the contaminated samples.

Nematodes

Of the mentioned orders of *Nematoda* from previously in this study, four show the most promise for Cr resistance mechanisms due to exhibiting consistent or increased relative frequencies at increased Cr concentrations. The four orders that exhibited the most relative frequencies at increased Cr levels were *Rhabditida, Dorylaimia, Tylenchida* and *Araeolaimida.*

Rhabditida showed the highest consistent relative abundance across all of the samples analysed appearing in 53 of the 67 samples where *Nematoda* OTUs were identified. These relative frequencies showed as 25.97% in control and 42.66% in contaminated. The OTUs identified also at at extreme Cr contamination concentrations of between 2750 mg/kg and 6560 mg/kg. This suggests that *Rhabditida* make up a large and important proportion of the nematode communities in both contaminated and non-contaminated soils and that a resistance or mitigation by the *Rhabditida* organisms must be taking place. *Caenorhabditis elegans* is a model organism used widely in scientific research which belongs to the order *Rhabditida.* As such, they have been used to identify toxic levels of contamination and heavy metals for decades mostly though observation the inhibition of their reproduction (Díaz-Morales et al., 2021). Due to this research mechanisms for resistance to metals have been readily researched and found within *Caenorhabditis elegans* and more widely *Rhabditida* (Pastuhov, Shimizu and Hisamoto, 2017) including for Cr (Šalamún et al., 2012). Interestingly, despite the large amount of data and research on *Rhabditida,* none could be found relating to tannery and related contamination. Assisted phytoremediation has been found to occur in soils containing *Rhabditida* within the soil community (Wang et al., 2021) but research is minimal. This makes *Rhabditida* and *Caenorhabditis elegans* as a model organism of interest for further investigation in later studies. However, its worth noting that *Caenorhabditis elegans* was not identified within any of the samples collected form the Dindigul sites.

Dorylaimia OTUs were relatively abundant, being found at Cr contamination levels up to 440 mg/kg. relative frequencies of the OTUs for *Dorylaimia* were found to be at levels of 24.28% in controls and 12.93% of the nematode population in contaminated soils. This does not display a resistance mechanism and shows the contamination to have a detrimental effect on *Dorylaimia* populations. A lack of research relating specifically to *Dorylaimia* on resistances to heavy metals, Cr and assisted phytoremediation existed meaning that *Dorylaimia* is a prime Order of Nematodes for future research, with untapped potential for its medium resistance/tolerance to Cr and other associated tannery pollutants.

Tylenchida along with *Rhabditida* made up 100 % of the relative frequency for nematode OTUs for 10 of the 67 samples with populations of nematodes identified. *Tylenchida* relative frequencies showed at 27.28% in controls and 13.28% in contaminated samples, with the levels varying across the Cr concentrations. Although levels of *Tylenchida* were found at levels above 100 mg/kg up to 6560 mg/kg, the reduction in the population of *Tylenchida* indicates a lack of resistance. The levels of resistance found in this study support what has been identified in previous studies with Cu and Cr being shown to extremely reduce the levels of *Tylenchida* within the contaminated soils (Park, Lee, Ro and Kim, 2011). In other associated research *Tylenchida* is only mentioned for its involvement in the community effected by heavy metal (Rodríguez Martín et al., 2014) and not specifically focused on *Tylenchida* again around resistance, and assistance to phytoremediation.

Araeolaimida was only identified in 20 samples across the site, however relative frequencies of *Araeolaimida* showed to be 8.90% in control soils and 9.27% in contaminated soils. Again, *Araeolaimida* like *Tylenchida* has been shown to drop in abundance in high levels of Cu and Cr (Park, Lee, Ro and Kim, 2011). Also, the majority of research focuses on *Araeolaimida* as well as other nematode populations within aquatic environments, which are not applicable as such to soil polluted environments (Tahseen, 2012; Sufyan and Asif, 2022). Again, no research on chromium direct effect on *Araeolaimida* and assistance to phytoremediation has been conducted.

Out of the four nematode order, *Rhabditida* demonstrated a large tolerance to the presence within the contaminated soils, increasing its proportion of the nematode population from 25.97% to 42.66%. Both *Dorylaimia* and *Tylenchida* showed reductions in their populations as a result of the contamination levels within the polluted samples and as such demonstrated an intolerance towards the contamination.

Protozoa

Within the 18s OTUs that were sequenced within this study, four protozoan order OTUs showed a resistance to the levels of Cr at increasing concentrations. *Gregarinasina* demonstrated large intolerance to the presence of contamination within the soils with controls showing 15.98% relative frequencies but dropping to 2.46% within the contaminated soil samples. *Conthreep* was found to have a small increase in relative frequency from 6.40% in control to 7.95% in contaminated samples. This could suggest some sort of tolerance of *Conthreep* against the precense of contamination. *Phytomyxea* demonstrated the most resistance out of the protozoa analysed, with controls showing levels of 0.48% which increased to levels of 7.10% within the polluted soils. *Gregarinasina, Conthreep* and *Phytomyxea* although mentioned in research, have no relative research relating to these

resistance capabilities against heavy metals or Cr and as such are an idea target for future research in this area with relative frequencies reaching levels of 78.3%, 22.8% and 82.7% respectively and being found in Cr levels up to 7760 mg/kg.

Glissomonadida showed consistent if low abundance and relative frequency through concentrations of Cr, with increasing concentrations seeming to have no effect on its relative frequency showing levels of 2.36% in control samples and 2.65% in contaminated samples. . Unlike the other orders of *protozoa* identified as of interest in this study, *Glissomonadida* has been shown to be resistant to contamination from industry in past studies (Richardson et al., 2019; Giongo et al., 2020; Jia, Liang, Guo and Chai, 2021). However as with a number of the nematode orders, research relating to chromium resistance, tannery links and assisting in phytoremediation of benefits to plant growth has not been carried out.

	Order		Relative frequency %	
			Control	Contaminated
Bacteria	*Thermomicrobiales*	Mean ± SD	17.67 ± 11.56	20.21 ± 20.92
	Kallotenuales		5.30 ± 6.04	6.04 ± 14.75
	KD4-96		2.80 ± 3.19	2.16 ± 2.77
	Tistrellales		0.18 ± 0.37	4.68 ± 11.27
	Peptostreptococcales-Tissierellales		1.18 ± 5.64	1.08 ± 2.36
	Tepidisphaerales		6.19 ± 5.28	4.92 ± 5.26
Fungi	*Pleosporales*	Mean ± SD	33.30 ± 21.82	32.44 ± 21.83
	Hypocreales		15.40 ± 16.07	9.09 ± 11.47
	Sordariales		9.58 ± 12.87	10.11 ± 13.29
	Eurotiales		2.77 ± 3.01	7.93 ± 9.89
	Capnodiales		2.49 ± 4.30	5.30 ± 9.85
Nematode	*Rhabditida*	Mean ± SD	25.97 ± 29.93	42.66 ± 36.32
	Dorylaimia		24.28 ± 27.00	12.93 ± 22.39
	Tylenchida		27.28 ± 30.56	13.28 ± 24.40
	Araeolaimida		8.90 ± 16.40	9.27 ± 25.40
Protozoa	*Gregarinasina*	Mean ± SD	15.98 ± 24.24	2.46 ± 5.64
	Conthreep		6.40 ± 5.00	7.95 ± 6.86
	Phytomyxea		0.48 ± 1.09	7.10 ± 18.52
	Glissomonadida		2.36 ± 1.81	2.65 ± 2.25

Table 3.5. Mean Relative frequency % of top Order within control and contaminated samples (± standard deviation)

Conclusion

Soil metagenomics analysis identified populations of bacteria, fungi, nematodes and protozoa within the soils at tanneries and control sites. Alpha diversity analysis showed that ITS and 18S OTUs were had significantly different diversity and abundance in tannery-contaminated soils compared to the control samples. It was also found that 16s, ITS and 18s OTUs were significantly dissimilar when comparing control and contaminated samples using PERMANOVA. 16s and ITS showed a significant difference in community structure at higher level Cr contamination levels compared to lower Cr levels as determined via beta diversity and PERMANOVA. Through taxonomic bar analysis, species of bacteria, fungi, nematodes and protozoa were identified as candidates for research into relationships fore assisted phytoremediation. Bacteria *Thermomicrobiales* and *Tistrellales,* fungi *Eurotiales* and *Capnodiales*, nematode *Rhabditida* and protozoa *Phytomyxea,* all showed significant resistance to the presence of pollution within the contaminated soil samples and demonstrated a large increase in frequency within the samples between control and contaminated. Bacteria *Tepidisphaerales,* fungi *Hypocreales,* nematode *Dorylaimia* and *Tylenchida* and protozoa *Gregarinasina* all showed sizable reduction in the relative frequency levels between the control and contaminated soil samples. The suggests that these orders are negatively affected by ether the levels of Cr at the site or by one of the other contaminations or soil properties that are as a result of the tannery pollution. As such, further research on the orders that demonstrated resistance to the contamination of the site, would help to determine the best native communities of soil organisms to culture and enrich in order to assist in a remediation operation of the tannery contaminated farmland that surround the tannery belt of Dindigul, Tamil Nadu.

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4. Chromium phytoremediation within crop plants and their suitability for the remediation of the Tannery Belt, Dindigul.

Abstract

Chromium is a stable heavy metal pollutant that has become a growing concern due to its use in industry and damaging effects on ecosystems and human health. With large areas globally being contaminated with chromium in both its soils and water bodies (2 million ha across Europe and 50,000 ha in India) and an increasing demand for land for food and fuel, land remediation is required. Although several remediation technologies are available, phytoremediation offers a sustainable and cost-effective solution. Crop plants that can both remediate the pollutant from the agricultural soils in non-edible plant parts whilst still providing a source of income as a food crop are of increasing interest. An extensive literature review examines studies that have investigated chromium hyperaccumulation within important global crop plants and effects on plant biomass. This highlights which crops have been adequately studied, how research methodologies may affect the outcomes of these studies, and where there are gaps in the current research that may result in barriers to implementing successful phytoremediation projects in chromium-contaminated soils. Six crops were identified as crops of promise for both remediation of Cr contaminated land and of economic benefit and a food source for the local population. Tomato, sunflower, and sorghum showed the most promise after Cr uptake analysis via pot experiment. All maintained biomass through low, medium, and high exposure to Cr with sorghum showing BF of 0.381 at the highest Cr levels.

Introduction

Phytoremediation and hyperaccumulation of chromium

Remediation of heavy metals involves the removal of a pollutant in order to reverse potential environmental damage (Wuana and Okieimen, 2011). Phytoremediation is the process of utilising plants – often hyperaccumulators – to extract pollutants from the environment to clean contaminated land and has been seen to be a renewable alternative to other techniques (Lasat, 2002; Padmavathiamma and Li, 2007; Wan, Lei and Chen, 2016; Amin et al., 2019). In order for successful remediation to take place, hyperaccumulator plants must first be identified. Hyperaccumulators are defined as plants that can uptake an element in concentrations ranging from 100 to 1000 times that of a non-hyperaccumulating plant without it having a detrimental effect on the growth of the plant itself (Ojuederie and Babalola, 2017). A number of plants can naturally accumulate heavy metals within their structures but many of these plants have a low biomass and as a result are unsuitable for phytoremediation management due to the time-consuming nature of their use (Karimi et al., 2011).

The increased uptake of heavy metals by hyperaccumulators can be caused by a range of traits. Examples include a stronger metal sequestration mechanism within cells, a greater requirement by the plant for specific metals for growth and internal working, an ability to mobilise metals from within a less soluble soil faction compared to other plants or better mechanisms for storing potentially harmful compounds in plant tissues (McGrath, Shen and Zhao, 1997; Shen, Zhao and McGrath, 1997). These abilities are often why hyperaccumulating plants can be found in areas where there is a naturally high level of mineralisation, such as mines, spoil heaps or landfill sites (Pulford, 2003). Phytoremediation represents a more cost-effective, environmentally friendly and less invasive way than most other remediation techniques, such as soil extraction or chemical additives (Padmavathiamma and Li, 2007; Wan, Lei and Chen, 2016), this can be seen by the comparison of remediation methods in table 4.1.

Technology	Description	Applicability	Costs ($US/ton)
Containment			
Physical	Prevent movement by preventing fluid flow	Landfill covers and slurry walls	10–90
Solidification	Creation of an inert waste	Injection of solidifying chemicals	60–290
Vitrification	Application of electrical energy to vitrify contaminant	Shallow metal-contaminated soil, low volatility metals	400–870
Ex situ treatment			
Soil washing	Addition of surfactants and other additives to solubilize	For water soluble contaminants	25–300
Pyrometallurgical	Elevated temperature extraction and processing for metal removal	Highly-contaminated soils (5–20%)	200–1000
In situ			
Soil flushing	Water flushing to leach contaminants	For soluble contaminants	100–200
Electrokinetic	Application of electrical current	Applicable for saturated soils with low groundwater flow	Little info
Phytoremediation	Use of plants for metal extraction	Shallow soils and water	(50,000–200,000/acre)

Table 4.1. Remediation technologies and their associated costs (Ferguson, 2017)

Different phytoremediation mechanisms can take place within a hyperaccumulating plant and its surrounding environment (see Figure 4.1). Phytofiltration refers to the removal of contaminants via absorption and concentration/precipitation from an aqueous environment through submerged sections of the plant or its root system where its known as rhizofiltration (He and Chi, 2015; Favas et al., 2016). This is effective when plants have high root biomasses as well as high below water surface absorption and has been shown to remove heavy metals, radioactive elements and polycyclic aromatic hydrocarbons (PAHs) (Ali, Khan and Sajad, 2013; He and Chi, 2015; Favas et al., 2016). If it is seedlings being used for this process, then the process is referred to as blastofiltration (Ali, Khan and Sajad, 2013).

Phytostabilisation and phytoimmobilisation are the process of the inclusion of contaminants into humus or the plants root cell walls and preventing their mobilisation and diffusion within the soil, stabilising them (Singh, 2012; Favas et al., 2016). An example is the use of root exudates to precipitated metals into an insoluble form and trapping them in the soil matrix, however this method does not fully remove the contaminant and so is a management strategy not a permanent solution for areas with high contamination levels (Vangronsveld et al., 2009; Ali, Khan and Sajad, 2013; Seshadri, Bolan and Naidu, 2015; Favas et al., 2016).

Phytodegradation and rhizodegradation refer to the degrading the mineralising (convert into a mineral or inorganic structure) of organic pollutants such as herbicides, pesticides, petroleum and solvents (Newman and Reynolds, 2004; Ali, Khan and Sajad, 2013; Favas et al., 2016). This process is carried out by a variety of enzymes within the plants, transforming harmful contaminants into less toxic forms stored within the plant's tissues (Ali, Khan and Sajad, 2013; dos Santos et al., 2018). The process of phytovolatilisation allows for the uptake of organic pollutants and several heavy metals such as Se, Hg and As, and conversion into volatile and non-toxic forms that can then be released into the atmosphere. (Ali, Khan and Sajad, 2013; Favas et al., 2016; Limmer and Burken, 2016). Direct phytovolatilisation refers to the volatising (conversation to vapour) of compounds from the stem and leaves of the plant; when root activity results in volatilisation it is referred to as indirect phytovolatilisation (Limmer and Burken, 2016).

Phytoextraction, also referred to as phytoaccumulation, phytoabsorption or phytosequestration (Ali, Khan and Sajad, 2013; Favas et al., 2016), occurs when contaminants are absorbed through the roots of a plant and subsequently stored within the roots and aerial structures (Garbisu and Alkorta, 2001; Ali, Khan and Sajad, 2013; Favas et al., 2016; Napoli et al., 2019). This method is particularly important as it allows for the removal of a contaminant from tissues of hyperaccumulators as theytake up large

amounts of these pollutants (McGrath and Zhao, 2003; Favas et al., 2016; Suman, Uhlik, Viktorova and Macek, 2018; Napoli et al., 2019).

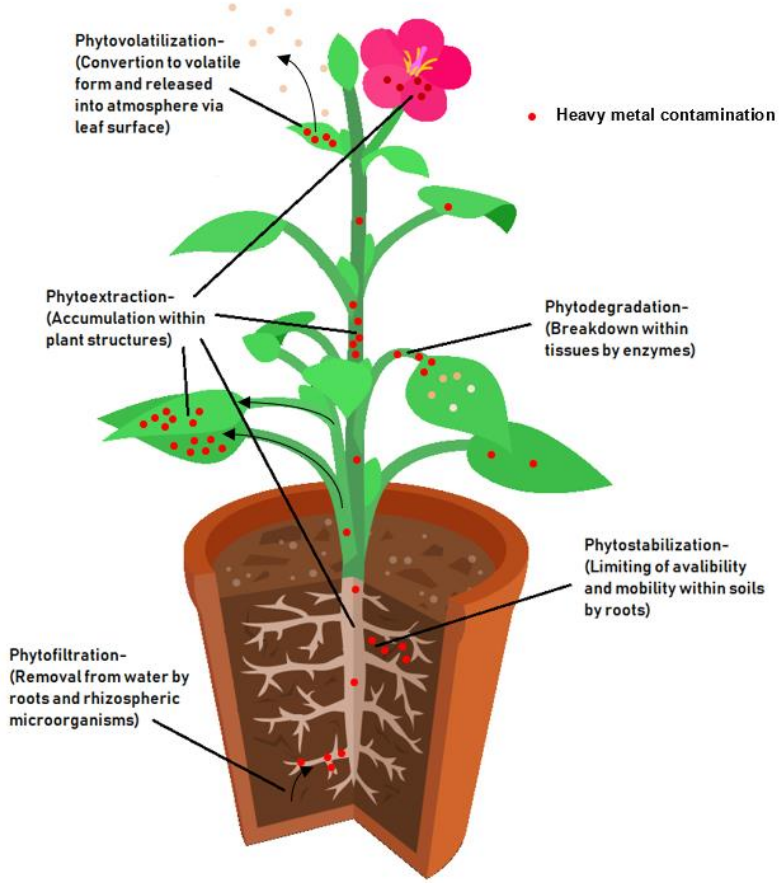

Figure 4.1. Phytoremediation mechanisms in plants

Different phytoremediation mechanisms are utilised by the plant within different mediums (figure 4.2). These processes are implemented in order to deal with different types of contamination, both organic and inorganic. Organic pollutants need to be managed differently by the plant in order to neutralise them via degradation, whereas inorganic compounds which can be stored readily by the plants that are taking them up (Batty and Dolan, 2013; He and Chi, 2015; Favas et al., 2016). Organic mechanisms appear not to store the pollutant within the plant structures; they instead either change its state and volatise or prevent it from being removed from the soil medium. This is due to the plant requiring the assistance of rhizospheric microbial communities and their mutualistic relationships with

the plant to be successful (Pilon-Smits, 2005; Batty and Dolan, 2013). Figure 4.2 also shows that although it is a suitable method of toxicity stress prevention, phytostabilisation does not fully remediate the contaminant as it will still be present in the soils (Ali, Khan and Sajad, 2013). Ali, Khan and Sajad (2013) also note that although phytovolatilization is counted as remediation, it only transfers the contaminant from one medium to another (soil to atmosphere) leaving it susceptible to being redeposited within the soils. Since plants have access to this large number of mechanisms by which they can remove or store different contaminants, they are well suited for remediation applications in a range of circumstances.

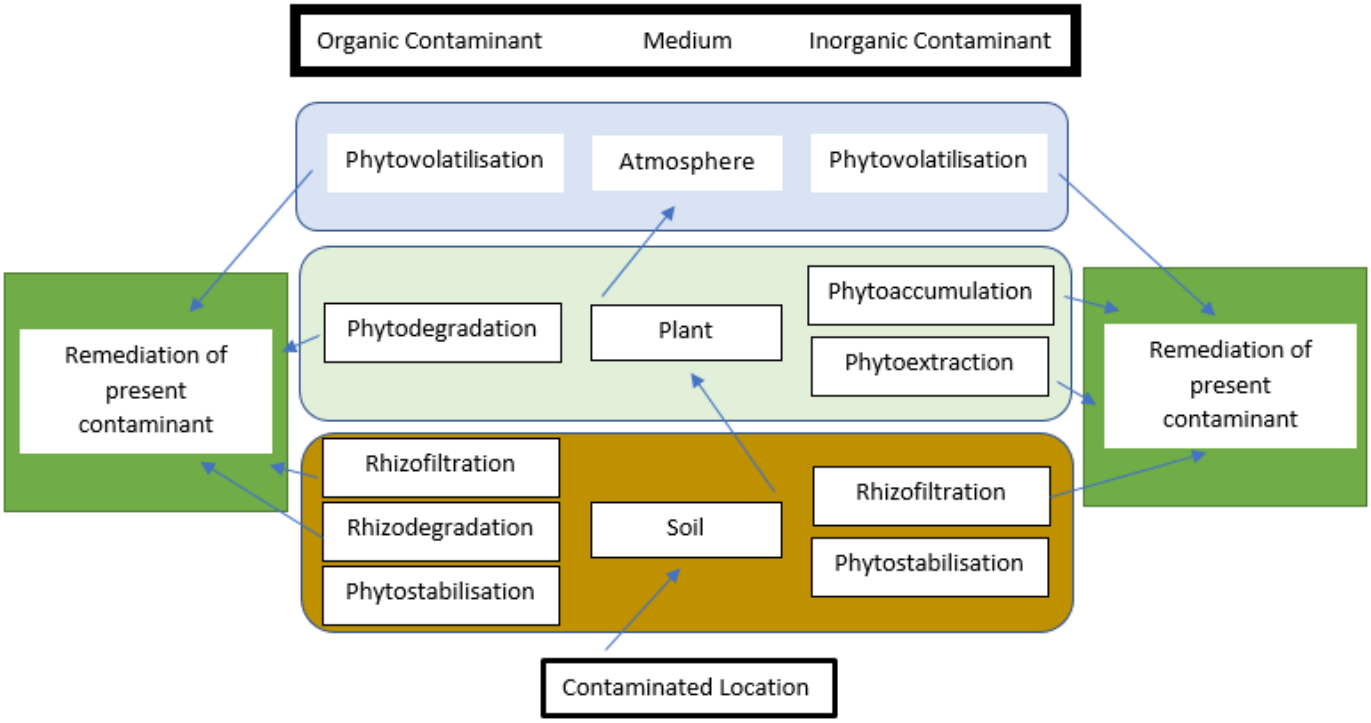

Figure 4.2 – The processes involved in phytoremediation

Despite extensive research on phytoremediation, few plants have been identified as good phytoremediators of chromium. Many pollutants that require remediation are also harmful to plants at high concentrations, such as those found at sites experiencing significant anthropogenic contamination (Nagajyoti, Lee and Sreekanth, 2010). For a plant to be able to accumulate toxic chromium, they must be able to convert hexavalent chromium into the much less toxic trivalent

chromium form or store it without being negatively affected (Cervantes, 2001). Removing chromium can protect the health of the environment and the local communities if it removes this contaminant from the food chain (Bini, Maleci and Romanin, 2008). However, phytoremediation must also be cost-effective: it is often carried out by landowners due to the potential for economic gain via increases in crop yield and quality. Landowners would also be able to build on disused brownfield sites previously contaminated allowing for economic gain through residential, commercial building or the selling off of land. Expansion would also allow an area to develop further, increasing economic gain and potentially the quality of life in the area. Although this is not the only driver, the socio-economic benefits of phytoremediation must be considered if phytoremediation is to be carried out on a large scale (Lepp, 1981). With the need to make the remediation of land that is unusable due to contamination for economically viable pursuits while also helping to feed a growing population (Francis, Edinger and Becker, 2005), researchers are looking for the 'holy grail' of the plant kingdom: a species that can (i) grow well in extreme chromium toxicity, (ii) remove chromium in significant quantities from the soil and (iii) bioaccumulate in non-edible parts to ensure the crop has an economic benefit to the land owner.

Phytoremediation potential of crops

Data from a total of 64 studies investigating uptake of chromium in crop plants are summarised in table 4.2. Studies were reviewed in order to determine both the extent of research carried out around potential crop plants suitable for farming near Dindigul, as well as to analyse inconsistencies within similar research across the scientific community to allow the construction of a comparable study.

From the table, a number of the species were identified as being of importance for one (or more) of the following reasons: (i) they have been used in several different studies and showed the potential of hyperaccumulation within their structures, (ii) they are major food crops globally and/or are common in less economically developed countries, or (iii) they could be used in energy production once they have hyperaccumulated land. Species that fit within one or more of these three categories include willow, sunflower, maize, wheat, oat, tomato and sorghum. Their importance, phytoremediation potential and suitability to be a crop grown within our contaminated area are discussed below.

Species	Common name	Root uptake (mg/kg)	Shoot/stem uptake (mg/kg)	Leaf uptake (mg/kg)	Fruit/flower/seed uptake (mg/kg)	Soil level (mg/kg)	Liquid media level (mg/l)	Plant uptake (mg/kg)	Uptake %	Number of papers	Reference
Sorghum bicolor	Sorghum	0.86 - 432.2	0.15 - 101	1.8 - 5.7		2 - 500 ppm	50 - 100 um	1.01 - 441.9	11 - 283	6	Shahandeh and Hossner, 2000; Shanker and Pathmanabhan, 2004; López-Luna et al., 2009; Revathi, Haribabu and Sudha, 2011; Dheeba and Sampathkumar; 2012; Karimi, 2013
Glycine max	Soybeans	15 - 700	5 - 27	1.25 - 21	0.6	25 ppm	1 Mm	21.85 - 748	17	2	Mei, Puryear and Newton, 2002; Amin et al., 2019
Solanum tuberosum	Potatoes				0.063 - 0.284	5 ppm	0 - 0.25 ppm	0.063 - 0.284	4 - 1370	3	Zayed and Terry, 2003; Kirkillis et al., 2012; Stasinos and Zabetakis, 2013
Triticum aestivum	Wheat	3.36 - 931.3	0.92 - 601.6	9.8 - 333.7	0.12 - 35	25 - 500 ppm	0.1 - 1 mM	2.61 - 1188.8	7 - 240	6	Sharma, Chatterjee and Sharma, 1995; Shahandeh and Hossner, 2000; Liu et al., 2008; Subrahmanyam, 2008; Chandra et al., 2009; López-Luna et al., 2009
Zea mays	Maize	3.12 - 4160	0.61 - 525	1.08 - 25.9	0.5 - 550	5 - 141 ppm	0.5 - 25 ppm	0.37 - 4182.8	4 - 46476	7	Mishra et al., 1995; Shahandeh and Hossner, 2000,;Sharma, Sharma and Tripathi, 2003; Zayed and Terry, 2003; Mallick et al., 2010; Dheeba and Sampathkumar; 2012; Kacálková, Tlustoš and Száková, 2014
Chrysopogon zizanioides	Vetiver Grass	319.8 - 1750	18 - 29.6			100 - 600		349.4 - 1768	293 - 349	2	Shahandeh and Hossner, 2000; Danh et al., 2009
Brassica napus	Rapeseed	4.9 - 298	1.9 - 372.2			66.3 - 1997.8		6.8 - 626.4	15 - 320	3	Shahandeh and Hossner, 2000; Marchiol et al., 2004; Brunetti et al., 2011
Helianthus annuus	Sunflower	0.56 - 1800	0.0002 - 33.7	0.004 - 10	0.57 - 5.1	20 - 5464	0 - 10 ppm	1.85 - 1806	2 - 1851	11	Shahandeh and Hossner, 2000; Davies et al., 2002; Mei, Puryear and Newton, 2002; Fozia et al., 2008; Santiago and Shenbagavalli, 2010; Dheeba and Sampathkumar; 2012; Aslam et al., 2013; Ranieri et al., 2013; Kacálková, Tlustoš and Száková, 2014; Lotfy and Mostafa, 2014; Stoikou et al., 2017
Brasicca juncea L.	Brown Mustard	60.29 - 1800	20 - 4100	15.76 - 650	6.26	100 -5177	1 - 5 ppm	7.78 - 4100	0 - 2050	8	Shahandeh and Hossner, 2000; Han et al., 2004; Marchiol et al., 2004; Bluskov et al., 2005; Diwan, Ahmad and Iqbal, 2007; Chandra et al., 2009; Diwan et al., 2010; Yuan et al., 2016; Annamalai, Sinha and Arunachalam, 2018
Brassica chinensis	Bok Choy					247 - 16,492		10.4 - 300	2 -4	2	Chen et al., 2014; Yuan et al., 2016
Gossypium hirsutum	Upland Cotton	0.06 - 1318.6	13.6 - 16.8	0.03 - 1.97		0 - 100		0.13 - 1320.57	23 - 6470	2	Daud et al., 2014; Lotfy and Mostafa, 2014
Cucurbita pepo	Field Pumpkin	16.7 - 23.5	9.6 - 12.7			148 - 213		26.3 - 36.2	17 - 18	1	Lotfy and Mostafa, 2014
Cymbopogon martinii	Palmarosa	1.269	8.9			3.52		10.17	289	1	Pandey et al., 2015
Brassica campestris L.	Field Mustard	60.26	35.38	15.76		313	9.38 ppm	11.4	36	1	Chandra et al., 2009

Solanum lycopersicum	Tomato	12.92	0.97	1.02	0.24 - 1.7	19.5 - 127.56		0.24 - 16.02	1.2 - 12.6	2	Olayinka and Ipeaiyeda, 2010; Murtic et al., 2018
Hordeum vulgare	Barley	1.84 - 225	3.73 - 11.2			0.3 - 100		5.67 - 233.4	42.3 - 6681	2	Shahandeh and Hossner, 2000; Ali, Ater, Sunahara and Robidoux, 2004
Avena sativa	Oat	1.43 - 214.8	0.53 -15.18	4 - 12.6		25 -500		0.53 -227	0.4 - 227	4	Shahandeh and Hossner, 2000; Radulescu et al., 2007: López-Luna et al., 2009; Wyszkowski and Radziemska, 2013; Amin et al., 2019
Salix aps	Willow	0.42 - 849.33	0 - 262.67	0 - 9.11		1 -117	1.6 -3159 ppm	1.29 - 1117.3	1 - 10526	10	Yu and Gu, 2007; Yu, Gu and Huang, 2007; Yu and Gu, 2008; Yu, Gu and Xing, 2008; Yu, Peng and Xing, 2009; Laidlaw et al., 2012; Kacálková, Tlustoš and Száková, 2014; Ranieri and Gikas, 2014, Terebova et al., 2017; Pilipović et al., 2019
Allium cepa	Onion	130	153 - 340	217 - 657	10	1 - 1345	0 - 0.25 ppm	0.19 - 894	1 - 336	5	Stalikas, Mantalovas and Pilidis, 1997; Zayed and Terry, 2003; Kirkillis et al., 2012; Stasinos and Zabetakis, 2013; Chen et al., 2014
Brassica oleracea	Cabbage	5 - 775	4.8 - 7.2	3.9 - 10.6	150	360	0 - 50mM	13.7 - 792.8	42	2	Stalikas, Mantalovas and Pilidis, 1997; Chatterjee and Chatterjee, 2000
Daucus carota subsp. sativus	Carrot				0.08 - 0.62	0.36 -5	0 - 0.25 ppm	0.08 - 0.62	2 - 2710	4	Stalikas, Mantalovas and Pilidis, 1997; Zayed and Terry, 2003; Kirkillis et al., 2012; Stasinos and Zabetakis, 2013
Apium graveolens	Celery	0.36 - 46.59	0.081 - 3.24	0.3 - 8.93		0.14 - 1345	0 - 1mM	0.441 - 58.71	2 -844	3	Stalikas, Mantalovas and Pilidis, 1997; Scoccianti et al., 2006; Chen et al., 2014
Pennisetum purpureum	Napier grass	26.3 - 33.8	12.6 - 16.8			148 - 213		38.9 - 50.6	24 - 26	1	Lotfy and Mostafa, 2014
Capsicum Frutescens	Red pepper				0.35 - 1.7	19.5 - 57.3		0.35 - 1.7	1.8 - 3.9	1	Olayinka and Ipeaiyeda, 2010
Vigna radiata	Mung Bean	0.08 - 301	0.061 - 259		0.32 - 1.47	50	200uM	0.32 - 560	0.3	3	Diwan et al., 2010; Singh and Agrawal, 2010; Dheeba and Sampathkumar; 2012
Oryza sativa	Rice Paddy	2.96 - 2781.7	0.11 - 120.46	1.02 - 75.35	0.107 - 15.39	0 - 500	0.5 - 25 ppm	3.65 - 2941.91	46 - 6622	6	Mishra et al., 1997; Yap et al., 2009; Zeng et al., 2010; Ahmad et al., 2011; Carolyn and Aqilah, 2014; Payus and Talip, 2014
Allium sativum	Garlic					247 - 1345		9.3 - 15.3	1 - 4	1	Chen et al., 2014
Raphanus sativus	Radish					247 - 1345		13.9 - 14.8	1 - 6	1	Chen et al., 2014
Spinacia oleracea	Spinach	2.98 - 66.85	1.09 - 14.98	130		0 - 320		4.35 - 130	7 -898	3	Stalikas, Mantalovas and Pilidis, 1997; Sharma, Brar and Malhi, 2005; Singh, Brar and Malhi, 2007
Arachis hypogaea	Peanut	0.8				50		0.8	1.6	1	Dheeba and Sampathkumar, 2012
Medicago sativa	Alfalfa	0.98 - 6.08	0.18 - 3.22			2 - 10		1.16 - 9.3	58 - 93	1	Karimi, 2013
Mangifera indica	Mango					17.01		0.52 - 0.66	3 - 4	1	Ahg and Ng, 2000
Psidium guajava	Guava					17.01		0.39 - 0.58	2 - 3	1	Ahg and Ng, 2000
Carica papaya	Papaya					17.01		0.58	3	1	Ahg and Ng, 2000

Table 4.2 – Data collected from 64 papers focused on the topics of chromium contamination and uptake within crop plants.

Willow

The use of certain crops for bioenergy has increased due to the move towards a potential carbon-neutral and eco-friendly sources of renewable energy, while also being able to be produced in such a quantity that it can meet the energy needs of the world today (Pandey, Bajpai and Singh, 2016). Of all energy consumed annually, about 17% in the USA (as of 2018) (CCES, 2020) is currently produced with the use of renewable fuel sources. This is just above the world average of 22.5%, with a slight increase of the use of biofuel energy worldwide from below 3% before 2000 (Ritchie and Roser, 2020). In all its many forms, biomass constitutes a large proportion of these renewable sources. This makes biomass fuels one of the most used renewable energy source after hydroelectric energy (Heller, Keoleian and Volk, 2003).

Globally about 450 different willow species are present with *Salix* being ideal candidates for use as phytoremediators able to colonise extreme soil conditions such as heavy metals (Paray et al., 2017; Punshon and Dickinson, 1999). Their ability to grow very large relatively rapidly allows for a significant harvestable biomass (Laidlaw et al., 2012; Jha, Misra and Sharma, 2017). Willows also have an effective nutrient uptake and a high evapotranspiration rate allowing for an increased potential for heavy metal uptake in the plant structures (Pulford, 2003, Zalesny Jr et al., 2019), making them prime candidates for heavy metal remediation due to an increased uptake over time potential of the plant.

Various willow species have been growing in popularity as crop plants as the need for renewable sources of energy grows, particularly in countries such as Sweden (Nordborg et al., 2018) by the burning of the wood to generate energy. Woody crops that require only a short rotation, are very fast to propagate and can achieve high biomasses annually are an ideal source of biomass for such energy production through the burning of biofuel (Volk et al., 2004; Meers et al., 2007). Thus, these trees are ideal for remediation while still being of economic value (Janssen et al., 2015).

This demonstrates a viable option for a hyperaccumulator which can be used to phytoremediate areas without the worry of being a danger to human health through their consumption as willow is not an edible crop. This does mean that how these crops are dealt with afterwards is important as to not reintroduce the contaminant into the environment via dispersal in the atmosphere by burning due to a large number of tree crops grown in a coppice fashion being grown specifically to be burnt.

Due to the timescale of this study and the aim to study fully mature crop plants for there uptake and phytoextraction potential, Willow species have been ruled out as a target plant. Although

demonstrating promise, the time it would take to get meaningful data would not sit inside the remit of this study.

Sunflower

Sunflowers are grown commercially for their seeds which are used to make oil for both cooking and biofuel industries (Myers, 2017) as well as being important as a feed crop to cattle (Stoikou et al., 2017). It has also been used to a slightly lesser extent as a fertiliser, pesticide, biological control and health supplementation (Arribas, 2014). Globally, 47.9 million tonnes of sunflower seeds were grown over 26.5 million ha annually in 2017. A large proportion of this production came from Europe (34.4 million tonnes), China (2.6 million tonnes) and India (211,000 tonnes). These figures are still on the rise (FAO, 2019). Vegetable oils, such as that from sunflowers, have gained popularity in recent years due to their environmental benefit as a renewable fuel source to replace fossil fuels in cars (Iriarte and Villalobos, 2013), with its energy content being similar to that of diesel (Baltacioglu et al., 2016).

Sunflowers have shown to be high metal accumulating plants but when exposed to chromium have a low tolerance to high levels as compared to other crop plants (Shahandeh and Hossner, 2000). In research by Stoikou et al (2017), levels of chromium found in sunflower oil taken from plants grown within increasing levels of chromium contaminated water did not exceed 0.028 ppm, well below permissible healthy limits to be ingested. This would as such be ideal for further research along with the low levels within the oils, to determine if sunflowers could be a viable crop for farmers to cultivate on land that is contaminated by chromium while not endangering the locals through ingestion of contamination.

Maize

Along with rice and wheat, maize provides at least 30% of calories to over 4.5 billion people in 94 developing countries (Shiferaw et al., 2011). Around 67% of maize production in the developing world originates in low and lower-middle-income countries; as a result, maize plays a crucial part in the livelihoods of millions of deprived farmers (Mulungu and N. Ng'ombe, 2020), including 900 million people for use maize as there main staple (Shiferaw et al., 2011). Maize is grown on 197.1 million ha

of land in 70 countries (Dowswell, Paliwal and Cantrell, 2019) all producing 1.1 billion tonnes of maize crop annually it, Maize is one of the most grown crops on the planet (FAO, 2019). Maize is also used as a livestock feed, with 66 % of global maize harvested being fed to livestock, and a further 8 % used in industrial processing (Dowswell, Paliwal and Cantrell, 2019). More recently, maize is also an ideal candidate to produce bioethanol in the move to mix ethanol and petrol in developing countries (Shiferaw et al., 2011). Even with the large quantity of maize already being grown, the high demand for the crop would potentially lead to the requirement of more maize to be grown in areas where land is already scarce. Thus, growing as a phytoremediating crop would allow for more a food crop to be grown while cleaning the contaminated areas of land.

Maize shows a great affinity for the uptake of chromium. Kacálková et al., (2014) identified bioaccumulation of 50% of contamination levels and Mallick et al., (2010) finding highs of 4160 mg/kg uptake within the maize structure. In these experiments, measurements within the edible seeds remained below permissible limits in the majority of studies. However, some studies did show higher concentrations of chromium in the seeds than most. For example, Mishra et al. (1995) reported chromium contamination up to 550 ppm within the seeds. This could mean that higher concentrations of contamination will cause an increase in the uptake of chromium into the plant (Mishra et al. 1995). As a result, accumulation of chromium in maize seeds crop may render the seeds inedible.

Wheat

Wheat is one of the most widely grown and cultivated crops on the planet and has been grown and milled for up to 10,000 years (Shiferaw et al., 2013). In 2017, 771.7 million tonnes of wheat were grown across 218.5 million ha globally (FAO, 2019). Early modified varieties spread quickly within areas with a high production output, which lead to its adoption in Latin America, West Asia and North Africa (Shiferaw et al., 2013). Now wheat is the primary crop production of over 100 countries and consumed worldwide (Shewry, 2009).

The adoption of wheat into diets and caused development of growing patterns leading to a boosted average yield. First, the yields increased by 3.6 % per year in developing countries. This level of yield growth was not sustainable; and the yield globally has continued to drop (Dixon, 2009). Wheat in diets provides around 500 kcal of energy per capita per day in China and India, as well as over 1400 kcal per capita per day in Iran and Turkey, making it extremely important to their daily diets (Dixon, Braun and Crouch, 2009). Without the Green Revolution, it is generally acknowledged that there would be large food deficits today (Shiferaw et al., 2013). Wheat is also viewed as an attractive option for use as

biofuel. Its high starch content is easily converted into sugars which can be turned into ethanol and used as an alternative renewable fuel source (Shewry, 2009)

A reduction in wheat seed germination by up to 60 % in 125 ppm of chromium, dependent on variety (Datta et al. 2011). Increasing levels of both Cr(III) and Cr(VI)caused large reductions in wheat seed germination (López-Luna et al., 2009). Low levels of chromium however have been shown not to affect seed germination rate of wheat (López-Luna et al., 2009). This is unusual for a crop plant (Bishnoi, Dua, Gupta and Sawhney, 1993; Peralta et al., 2001; Joshi, Menon and Joshi, 2019). Expression of chlorophyll a and chlorophyll b was also reduced in higher chromium concentrations (Nayak et al., 2015), although Chandra et al., (2009) found that this drop in chlorophyll levels was only observed after 90 days with an increase in levels for the first 60 days.

Wheat shows promise as a remediator of chromium. Several studies have found that increasing of the levels of chromium caused the uptake into the wheat plant to increase, and the roots immobilised more chromium than other structures and had poor translocation into aerial structures of the plant (Nayak, Rath and Patra, 2004; Chandra et al., 2009; Mohanty and Patra, 2012, Nayak et al., 2015).

Sorghum

Sorghum is the fifth most important cereal crop in the world's agriculturally (Revathi, Haribabu and Sudha, 2011; Proietti, Frazzoli and Mantovani, 2015). Being a major traditional staple food in many of the world's developing countries, sorghum is the main calorie intake and source of proteins for around a billion people in regions of the tropics (Rakshit and Wang, 2016). Sorghum is not produced as much as other cereal crops: 40.6 million ha of sorghum are planted globally every year, producing 57.6 million tonnes of cereal crop annually (FAO, 2019). As well as a food crop, it is still widely used as a renewable building material and for oils within important industrial chemicals (Dahlberg, 2019). Sorghum is also well known for being used as a staple in alcohol production, largely for human consumption in distilled spirits (Lopes, Genisheva, Nunes and Duarte, 2019). In countries such as the USA, states have started to use renewable fuels made from sorghum as a cost-effective substitute to maize (Dahlberg, 2019) with the implementation of sorghum for the production of bioethanol (Arif et al., 2019) given its characteristics making it ideal for intensive cultivation (Sytar, Brestic, Taran and Zivcak, 2016).

Sorghum displays an affinity to survive in high heavy metal contaminated stress conditions as compared to other crops such as mustard and millet (Padmapriya et al., 2015). There is evidence for the phytoremediation potential of sorghum in the current literature. Some studies have found that

young seedlings of sorghum have high mortality rates at levels of 100 ppm (Shahandeh and Hossner, 2000). Most of the chromium uptake is into the root structures, as has been observed in many other cereal crops (Revathi, Haribabu and Sudha, 2011). With crop plants use as a remediator of heavy metals hinging on its ability not to store contamination within its fruiting structures, the full potential of sorghum as a hyperaccumulator and remediator that could be implemented in the field is still yet to be covered, even with some studies stating its relevance as a potentially useful hyperaccumulator (Ravathi, Harbabu and Sudha 2011; Sytar, Brestic, Taran and Zivcak, 2016)

Tomato

Tomato is one of the most cultivated crops globally (Piscitelli et al., 2020) being grown within more than 170 countries commercially and with a production of 177 million tonnes (Kaur, Yadav, Sharma and Singh, 2020). It has become increasingly popular around the world for its use in sauces (Islam and Kabir, 2019). Tomato is mostly known for being grown in Mediterranean countries including Spain and Italy (Piscitelli et al., 2020) but India Is the 4th largest grower of tomatoes, being known to produce 7.4 million t and with a harvesting area of 520,000 ha (Costa and Heuvelink, 2005). India is also a major exporter of tomatoes to surrounding countries such as Bangladesh, Pakistan and Nepal (Kaur et al., 2013). Tomatoes form a large proportion of the Indian diet with it forming an integral part of many Indian dishes (Kaur et al., 2013), providing the main source of lycopene and phenols in the Indian diet owing to their availability all year round (George, Kaur, Khurdiya and Kapoor, 2004).

Tomato doesn't show the same affinity as other crop plants to resist against Cr contamination. Research has found that exposure to Cr stress can cause a number of issues relating to plant growth and nutrient uptake within the stems and leaves of the plant (Shanker, Cervantes, Lozatavera and Avudainayagam, 2005). Research on the phytoremediation potential of tomatoes is scares with some suggestion that tomato lacks the ability to phytoremediator Cr (Saleh et al., 2017). However, with the importance of the crop to the India diet and the lack of extended knowledge around its ability as a phytoremediator, analysis of tomato within this study will go ahead.

Oats

Oats are the sixth most grown cereal crop in the world (Zwer, 2004). Having first been domesticated in the Neolithic period, oats have been an integral part of diets for centuries (Marshall et al., 1992). Land growing oats cover a global area of 10.1 million ha and producing nearly 26 million tonnes of output yearly (FAO, 2019). The production of oats is recently in a decline as more effort is being put into the cultivating of crops that provide a greater source of protein (Marshall et al., 1992). They are used for a variety of different purposes including human consumption, animal feeds, pharmaceuticals for heart disease, cosmetics and industrial uses varying from cardboard and board material to furan compounds used for solvents and adhesives (Zwer, 2004; Butt et al., 2008). Like many cereal crops, oats can be used for biofuel production similar to that of maize and wheat (Ballesteros and Manzanares, 2019).

Oat species are adapted well to a cool and moist climates and areas that have acidic soils, unlike most other cereal crops. They are however sensitive to water deficit and high heat during seed formation and the plant's maturity (Marshall et al., 1992). Mahmood-ul-Hassan, Suthar, Ahmad and Yousra, (2017) also demonstrated that the uptake into the plant is greatly dependent on the type of soil the crop is being grown in. Amin et al., (2019) also reported that the phytoremediation potential of the oat plant, although always of a level to make it worthwhile as a hyperaccumulator, diminished as the levels of chromium in the soils increased.

A lack of literature focused on the uptake of chromium in aerial seeding structures of the oat plant means that no conclusions to possible health implications or benefits oat could bring as a crop for remediation. This issue is also prevalent due to the lack of focus on chromium within oat plants. A large amount of research has been carried out with regards to other heavy metals and the potential of oat as a hyperaccumulator, but the number focused on chromium are fewer. Interestingly, the addition of oat biomass to a chromium contaminated site was found to be an effective bio sorbent for the chromium removal (Gardea-Torresdey et al., 2000)

Analysis of experimental setup

Many studies will show a difference in results relating to Cr uptake in crop plants. These differences are not due to the variable they are testing however they could be instead due to differences in the experimental set up that has been implemented.

It is important to have an overview of these studies in order to fully understand where these differences are occurring and what impact they are having of the data being produced and to what extent variations in results are to do with the plants and contamination or to what extent is it in relation to confounding variables.

Consistency is important when drawing conclusions that allow for a method to be implemented in the real world and not just a lab. If conclusions are to be trusted and used as a basis for remediation of large-scale projects, all studies used need to be of the same level of depth and accuracy as one another. As well as this, the idea of collating data into big data analysis is highly important when it comes to real world application of research. If it isn't possible to collate and interpret data from multiple source due to the slight variations in study methods, causing a lack of consistency, then the usefulness of the variety of research occurring lessens.

Several charts (Figure 4.3 and 4.4) were constructed out of data collected from all 64 papers focusing on different aspects of the research carried out and their experimental design. In order to determine the methodological differences between experiments, several key aspects of the study were recorded and the proportion of studies following similar methods are shown

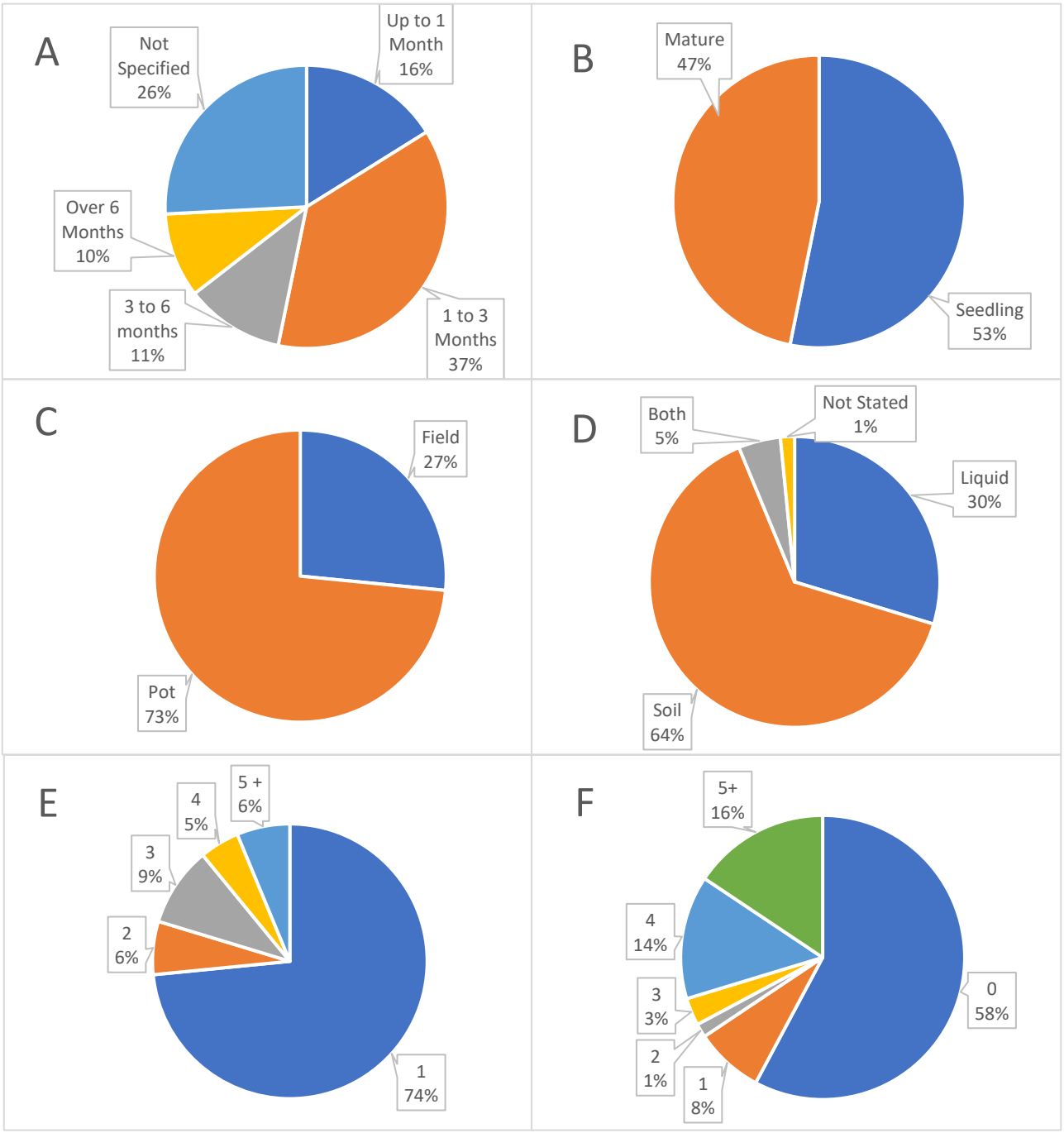

Figure 4.3 – A – Duration of Study. B – Plant age of grass and forbs. C – Type of study. D – Method of chromium application to the study. E – Number of species focused on in study. F – Number of additional heavy metals studied.

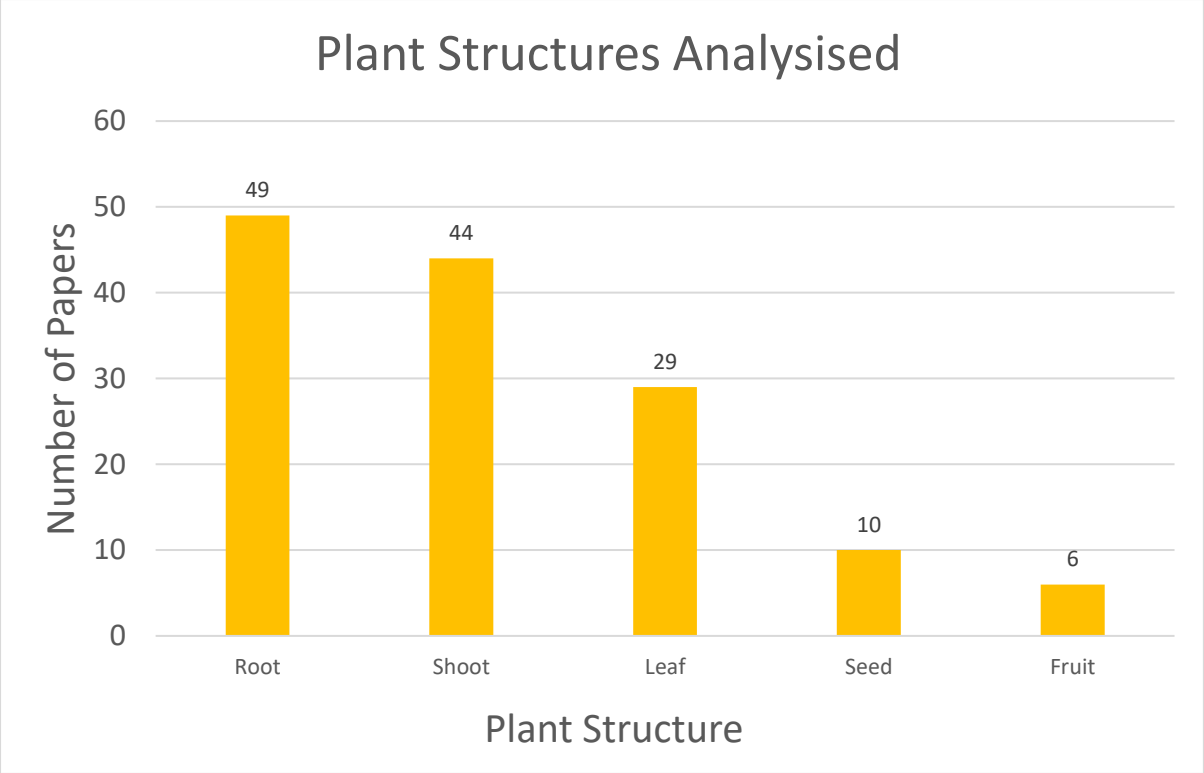

Figure 4.4. Different plant structures analysed within studies in literature review

Duration and Age

When looking at phytoremediation, the duration of the experiment is important in seeing a plant's full potential as a phytoremediator and hyperaccumulator since plants may be more susceptible to chromium toxicity at different stages of the life cycle and since phytoremediation potential is directly related to the time a plant is exposed for. Over 25% of studies investigating grasses and forbs were grown for under a month, with some of them being grown for as little as 14 days. For example, 66% of studies only grew their plants to seedling age (figure 4.3B), so the only parts of the plant that can be analysed for remediation potential are the seeds. Although important in determining how chromium and other metals affect seedling growth, without being grown to an extent where the stem is more established and other plant structures have formed, the full hyperaccumulation potential of the plant cannot be determined.

Even though approximately three quarters of studies had a duration for up to 3 months, many of these studies ran several seedling studies or shorted length studies within this time, rather than allowing time for plants to grow to maturity. This impacts the validity of conclusions based on

hyperaccumulation of a fully grown crop plant as the life cycle has not been completed within the experiment. For example, time to maturity for sorghum is 90-120 days (Carter et al., 2020), for oat is 70 days (Dan et al., 2008), for maize is 75-130 days depending on variety (Moriri, Owoeye and Mariga, 2010), sunflower is 90-125 days (Berglund, 2007) and wheat is 140 and 170 days depending on variety (Acevedo, Silva and Silva, 2002).

Pot or field

Over two thirds of experiments included in the literature review were pot experiments rather than field studies. The validity of any conclusion drawn from pot studies when they are extrapolated to real-life conditions could be brought into question when field trials haven't also been conducted (de Vries, 1980). This is because there are a large number of complex interactions within soil in field conditions, containing not just chromium but often several different metals (Zhao, Joo and Kim, 2021), as well as a large, diverse community of microorganisms (Vangronsveld et al., 2009) and different physico-chemical properties that affect heavy metal uptake (Zeng et al., 2011; Chirakkara and Reddy 2014; Shahid et al., 2017). This degree of complexity and heterogeneity can be difficult (or impossible) to reproduce in a pot experiment.

As well as extrapolation issues, Vangronsveld et al. (2009) state that field trials are important in getting the local public interested the issue at hand. Field trials can create a growing commercial awareness of what is taking place, which will help with the acceptance of change within a population is required as well as the creation of new legislation to prevent the contamination from occurring again. Other key differences between the use of pot vs field tests include the lack of microclimate effects that come with the growing of crops outside, having only one constant climate for the plants to exist in the greenhouse. This means the plant isn't truly demonstrating uptake within the outside environment.

Application method

The method of chromium application can significantly affect the outcome of experiments. Generally, chromium is applied either directly to the soils as a solid or via watering as a liquid medium (either once or repeatedly). Studies that use a liquid medium for the addition of chromium often show very high uptake percentages into the plants, even when exposed to relatively low chromium concentrations in the soil. These high readings could be due to pooling of chromium as it is added in liquid form near the surface of the soil, resulting in the contaminant being more readily available to

the roots to take up into the plant than form an approach that ensures homogenisation of the contaminant(s) within the soil. This produces an unrealistic impression of the phytoremediation potential, bringing the legitimacy of the results from these pot experiments into question.

Number of species

74% of studies looked at just one crop type within their research. Where this was the case, a larger number of samples on average were taken and more soil properties were considered in the analysis. This can make for a more in-depth overview for the phytoremediation potential of a given crop and as a result. On the other hand, since experimental setup varies widely between researchers, this does not allow for direct comparison of phytoremediation potential for multiple species.

Number of metals

Chromium, like any compound, can react with other contaminants within the soils. The majority of contaminated sites contain multiple contaminants. For example, tanneries produce multiple contaminants in addition to chromium such as lead, cadmium, copper and nickel (Cloquet et al., 2006; Taylor, Mould, Kristensen and Rouillon, 2014; Khan, Khan, Khan and Alam, 2017; Peng et al., 2019; Wyszkowski, 2019) and electroplating as well as chromium produces copper, nickel, zinc, iron and manganese (Özbaş et al., 2013; Wu et al., 2020). Contaminants all react differently in a soil environment, creating complexes within the soils and with each other and mean the bioavailability for uptake within a plant structure can be greatly affected (Ogundiran and Osibanjo, 2009). The presence of other contaminants can have effects ranging from stabilisating of chromium to rendering it more available for uptake by plants (Fendorf, 1995; Shahid et al., 2017). For example, Wyszkowska, Boros and Kucharski (2008) showed that that the application of additional heavy metals resulted in a maintained or even increased yield, but an increase in availability of chromium to the plants. similarly, Markiewicz-Patkowska, Hursthouse and Przybyla-Kij, (2005) stated that the adsorption of the heavy metals was reduced by the presence of multiple heavy metals, allowing the metals to be more mobile in the soil and potentially leading to more widespread contamination. Despite this, 60% of the studies reviewed only investigated chromium and thus could not consider the effects of other heavy metal pollutants.

Structures

The degree of uptake in different plant structures is important when looking for a good phytoremediator plant that can also be sold as a food, fuel or fibre product. For example, a good phytoremediator is not suitable as a food crop if uptake is mainly in edible parts of the plant. However, studies varied widely in their approach to examining different plant structures. Of the plant structures studied, these can broadly be split into five categories: roots, shoot, leaves, seeds and fruit.

The proportions of the research varied greatly on plant structures analysed, with a number of the studies some not looking at plant structures but at total uptake by the entire plant. The larger proportions analysing root, shoots and in some cases leaves, are an indication of the studies that didn't grow the plants to maturity, not looking at fruit or seed and choosing to go for shorter studies. The large variety of combinations of different plant structures analysed raises issues when it comes to comparing research on the same topic from different researchers. Having such a broad variety and an inconstant analytical method means that two papers can draw the same conclusion with greatly varying amounts of depth to their analysis. This is due to each having a fragmented analysis that cannot be brought together to build a case for a crop plant to be deemed a hyperaccumulator. This is the same issue when it comes to studies that have only looked at total uptake or focused on the solely roots of the crops. These studies don't show how the accumulation within a harvestable part of the crop is performing and so stating that a crop is a potential hyperaccumulator and could be used as a phytoremediation tool but not seeing if this contamination could then be passed on to edible structures, a large possibility for the use of the plant is being missed. It couldn't also be suggested to suggest whether the edible structures of each of the crops would be of a safe level for sale as a food stuff as such, info on these studies are not helpful when you what to find ether a secondary use for the crop or are trying to grow crops for the dual reason of remediation and food.

Aims of this study

In this study, pot experiments were run in order to deduce as to what extent levels of Cr contamination has on the overall growth and phytoremediation potential of each crop plant identified through the literature review as target crops for remediation of the Tannery Belt, Dindigul, Tamil Nadu. The study will determine the levels of phytoextraction via crop plants at four Cr contamination levels (0, 10, 150 300) based of average ranges of contamination discerned from soil analysis for Cr pollution levels in Chapter 1 of this thesis. Plants will be analysed for their resistance to Cr via biomass analysis, and Bioaccumulation factors (BF) / Translocation factors (TF) to determine the crops bioaccumulation

potential and how they store the pollutant. Lastly, the levels of Cr within pollinator and edible structures of the plant and the TF into these structures will be looked at to better understand the risk the crop plants could pose to human health and the wider ecosystem.

Hypothesis

- Phytoremediator potential species of crop identified from the literature review will significantly take up Cr into their structures.

- Cr contamination within edible structures of the crop plants will be of levels below permissible limits for edible plants.

- Biomass of all structures are reduced to some degree by the presence of Cr contamination.

- Suitable crop plants will be identified for the proposal of a phytoremediation method at the tannery belt site.

Method

Pot experiment set up

The experiment was conducted in a temperature controlled, plant growth laboratory with a constant temperature and growth lights. A series of identical pots with a holding capacity of 250g were used. 6 crop plants (tomato, sorghum, oat, sunflower, wheat and maize) were each set up with 4 treatments of soil as follows: 1) Control soil; 2) 10 mg/kg Cr; 3) 150 mg/kg Cr; and 4) 300 mg/kg Cr. Each treatment was replicated 12 times for each crop, totalling 288 plants (as shown in table 4.3). Potassium Dichromate was used for the addition of Cr into the soil mix and was added as a liquid mix to each pot and mixed to allow for complete homogenization of the Cr throughout the soil.

Crops were harvested and soils samples collected at the end of the 90-days experiment. The plant biomass was harvested, and each individual structure was separated, weighed and dried at 50^0C for 1 week. Roots were separated from soils and washed to remove attached soil. Soils were dried at 50^0C for 1 week. Each plant and soil sample were collected and stored separately to act as replicates. Plant samples were weighed for dry biomass, measured out for digestion, and ground down using a ball mill (Retsch Mixer Mill MM 400, Germany).

Crop plant grown in compost of known volume (250 g)	Initial concentration of Cr (0, 10, 150 and 300 mg/kg)	Number of replicate pots of 250g (pots with Cr addition and crop plant)	Duration of investigation
Tomato	0 mg/kg	12	90 Days
	10 mg/kg	12	
	150 mg/kg	12	
	300 mg/kg	12	
Maize	0 mg/kg	12	90 Days
	10 mg/kg	12	
	150 mg/kg	12	
	300 mg/kg	12	
Sorghum	0 mg/kg	12	90 Days
	10 mg/kg	12	
	150 mg/kg	12	
	300 mg/kg	12	
Oat	0 mg/kg	12	90 Days
	10 mg/kg	12	
	150 mg/kg	12	
	300 mg/kg	12	
Wheat	0 mg/kg	12	90 Days
	10 mg/kg	12	
	150 mg/kg	12	
	300 mg/kg	12	
Sunflower	0 mg/kg	12	90 Days
	10 mg/kg	12	
	150 mg/kg	12	
	300 mg/kg	12	
Total 6 species of crop plant		Total 12*24 = 288 pots	

Table 4.3. Set up of Cr pot experiment

Heavy metal analysis

Digestion of plant and soil samples was carried out using a Multicube 48 hot block digestion system. For soil, 0.5g of each Sample was digested using a reverse Aqua regia mix (9ml 70% $HN0_3$ and 3ml of 37% HCL) heated in stages to 120^0C for 3 hours and left to cool. Plant was digested using a 12ml 70% $HN0_3$ and 4ml 30% H_2O_2 mix heated in stages to 120^0C for 2.5 hours and left to cool. Each sample was then diluted up to 50ml in the Multicube 48 PP 50ml Class A specification Vial and then filtered using Whatman filter paper no. 42. All samples were stored at 4^oC until further analysis.

Cr analysis was performed by inductively coupled plasma – optical emission spectrometry (ICP–OES) (The Optima™ 8000 ICP-OES , Perkin Elmer). Reference standards were placed periodically between samples as QC to ensure accuracy throughout each cycle. The operational conditions for the ICP-OES were RF power and frequency – 1.3kW and 40 MHz, nebulizer flow rate – 0.7 L/min, auxiliary flow rate – 0.2 L/min, plasma flow rate 10 L/min, and the Cr emission line – 267.716nm.

Bioaccumulation factor

The bioaccumulation factor (BF) of Cr into the crop plants was determined as the proportion of the concentration of Cr within the crop plant to the concentration within the soil. The given value shows a plants ability to accumulate heavy metals from its soil (Tesfahun et al., 2021). The equation is:

$$BF = C_{PT} / C_{SS}$$

Where C_{PT} = concentration of Cr within plant tissues (mg/kg dry weight) and C_{SS} = concentration of metal in soil sample (mg/kg dry weight) (Ibrahim, Young, Deqiang and Mughal, 2021)

Translocation factor

The translocation factor of Cr between the structures of the crop plant were investigated showing a plants ability to store Cr within the different structures (Du, Yang, Liu and Wang, 2018). The equations are as follows:

$$TF_{i,Shoot/Root} = C_{i,Shoot}/C_{i,Root}$$

$$TF_{i,Leaf/Shoot} = C_{i,Leaf}/C_{i,Shoot}$$

$$TF_{i,Flower/Shoot} = C_{i,Flower}/C_{i,Shoot}$$

$$TF_{i,Fruit/Shoot} = C_{i,Fruit}/C_{i,Shoot}$$

$$TF_{i,Seed/Shoot} = C_{i,Seed}/C_{i,Shoot}$$

$$TF_{i,Tassel/Shoot} = C_{i,Tassel}/C_{i,Shoot}$$

Where C_ix is the respective plant structure. TF_i shoot/root, TF_i leaf/shoot, TF_i flower/shoot, TF_i fruit/shoot, TF_i seed/shoot and TF_i tassel/shoot are the translocation factors for Cr (i) from root to shoot, shoot to leaf, shoot to flower, shoot to fruit, shoot to seed and shoot to tassel respectively.

Data Analysis

The analysis of data was carried out via the use of Minitab statistical software (ver. 21). The Anderson-Darling test for normality was carried to determine normal distribution of the data. Data relating to the biomass of the crops was found to be normally distributed, all other data was found to not be normally distributed. For parametric data, one way ANOVA and Tukey tests were conducted to determine significant differences between treatments. For non-parametric data, Kruskal-Wallis and Mann-Whitney tests were conducted to discern significant differences between treatments. Descriptive statistics were obtained for general analysis of uptake and biomass changes.

Results and discussion

Theoretical vs Actual spiking levels

During the analysis it was found that levels of Cr within the pot experiments were higher than originally thought during dosage. Although correctly calculated, the amount of mg/kg produced from potassium dichromate to attempt to achieve a uniform and accurate level of Cr in the pot experiment, levels that were achieved were higher. This issue has been discussed in research surrounding theoretical verses actual spiking levels when using pot experiments and achieving higher levels through spiking than

targeted (Najafi and Jalali, 2016; Chen et al., 2020; Lee et al., 2021). A reason suggested in that the soil mix is not homogonous enough, causing samples to spike in concentration (Chen et al., 2020; Lee et al., 2021), which can be caused by using the addition of a liquid form of the contaminant and not a fine powder as suggested by Chen et al., (2020).

It is important to test all samples against the actual soil contamination levels and not the theoretical levels in order to achieve accurate data on the effects the Cr contamination if having on the crop plants and the actual BF levels that are being achieved by the crop plants.

This is shown within the relative figures within the discussion. As such each concentration with be referred to as treatment. Issues

Cr effect on biomass

Total biomass

Before the effectiveness of the selected crops at accumulating Cr contamination can be assessed, the effect on the growth and survivability of the crops must be assessed to allow for potential phytoremediators to be suggested.

To allow for an ideal candidate for the remediation of the Tannery Belt while also being of economic benefit to farmers and provide for the local population, the first aspect that needs to be analysed is the crop's ability to survive in the increase Cr soil environment and still produce food. Figure 4.5 shows the total Dry weight of Biomass for the 6 crop plants grown in different concentrations. Tomato was the only crop to reduce in biomass significantly negatively because of the increase of Cr from 40g to 34g. Oat also showed a decrease in biomass as a result of Cr; however, this was not to a significant degree when statistically analysed by Kruskal-Wallis test (p value – 0.502).

Sorghum showed little change in total biomass, contradictory to research suggesting reductions in the biomass of sorghum was observed with increasing levels of chromium with a loss of over 50% at 150 ppm according to Revathi, Haribabu and Sudha (2011) with levels above 200 mg/kg causing death of the plant. Results reported by Dheeba and Sampathkumar (2012) support this observation but state that low levels of 10 to 20 ppm of chromium don't have much effect on the biomass and growth of the sorghum.

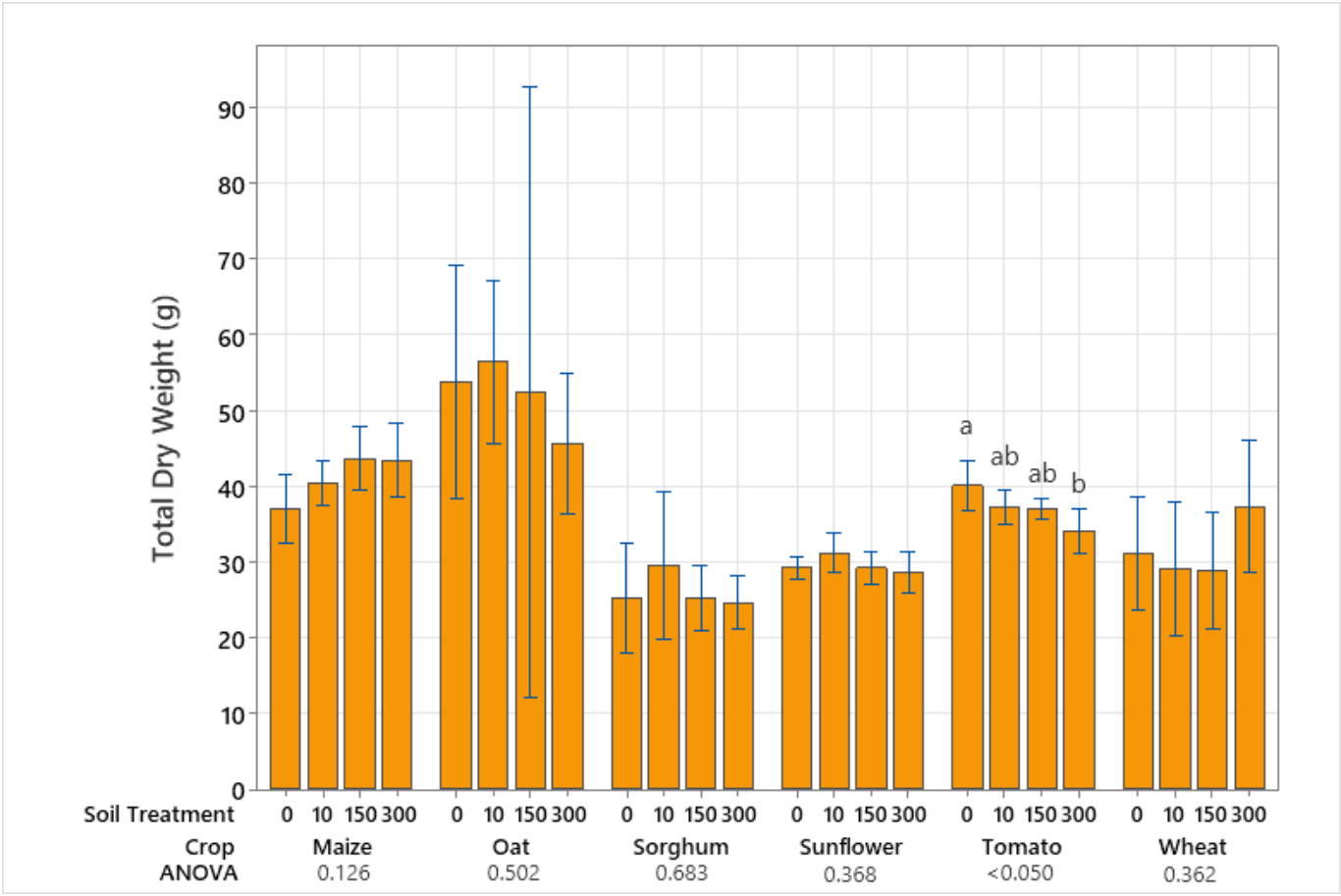

Figure 4.5. Total dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed. Significant results had subsequent Tukey test carried out. Bars of the same crop not sharing letters indicate significant difference

Wheat did not grow past the shoot phase of its growth cycle and so will be discounted at this stage due to a lack of data to support or deny its ability to resist levels of Cr treatment.

Root and shoot biomass

A reduction in biomass was also recorded within the roots and shoots of the oat and significantly for tomato and sorghum for root and (Figures 4.6 and 4.7 respectively). For tomato, Root growth has also been observed to be negatively affected with a reduction of the root growth of up to 90% when exposed to Cr (di Toppi et al., 2002) which supports the findings of a decrease in root and shoot biomass in this study.

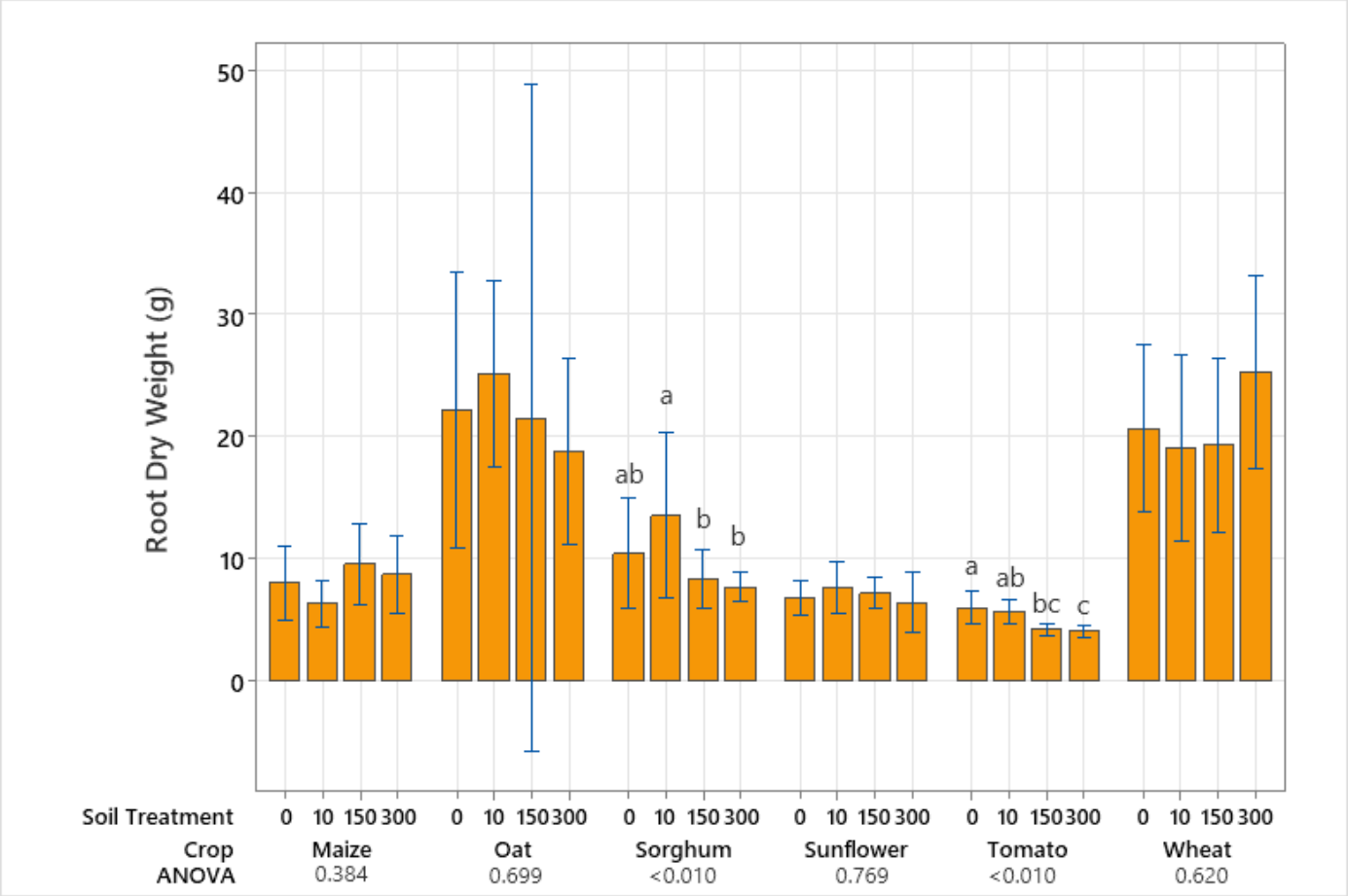

Figure 4.6. Root dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed. Significant results had subsequent Tukey test carried out. Bars of the same crop not sharing letters indicate significant difference

Joshi, Menon and Joshi (2019) found that sorghum had a lower reduction in root and stem lengths compared to another crop. Similarly, Dheeba and Sampathkumar (2012) reported decreases in root length of only 13% at up to 50ppm chromium. Sorghum was also exhibited an increase in the diameter of the root and number of root hairs produced (Shanker et al., 2005). Reductions of shoot length were observed to levels near 50% (Dheeba and Sampathkumar, 2012), with larger decreases in shoot length as concentration increased (López-Luna et al., 2009). With levels within this study increasing past the 300 mg/kg level, it is found that a decrease in roots of more than in the literature of 30% but no significant reduction in the shoots.

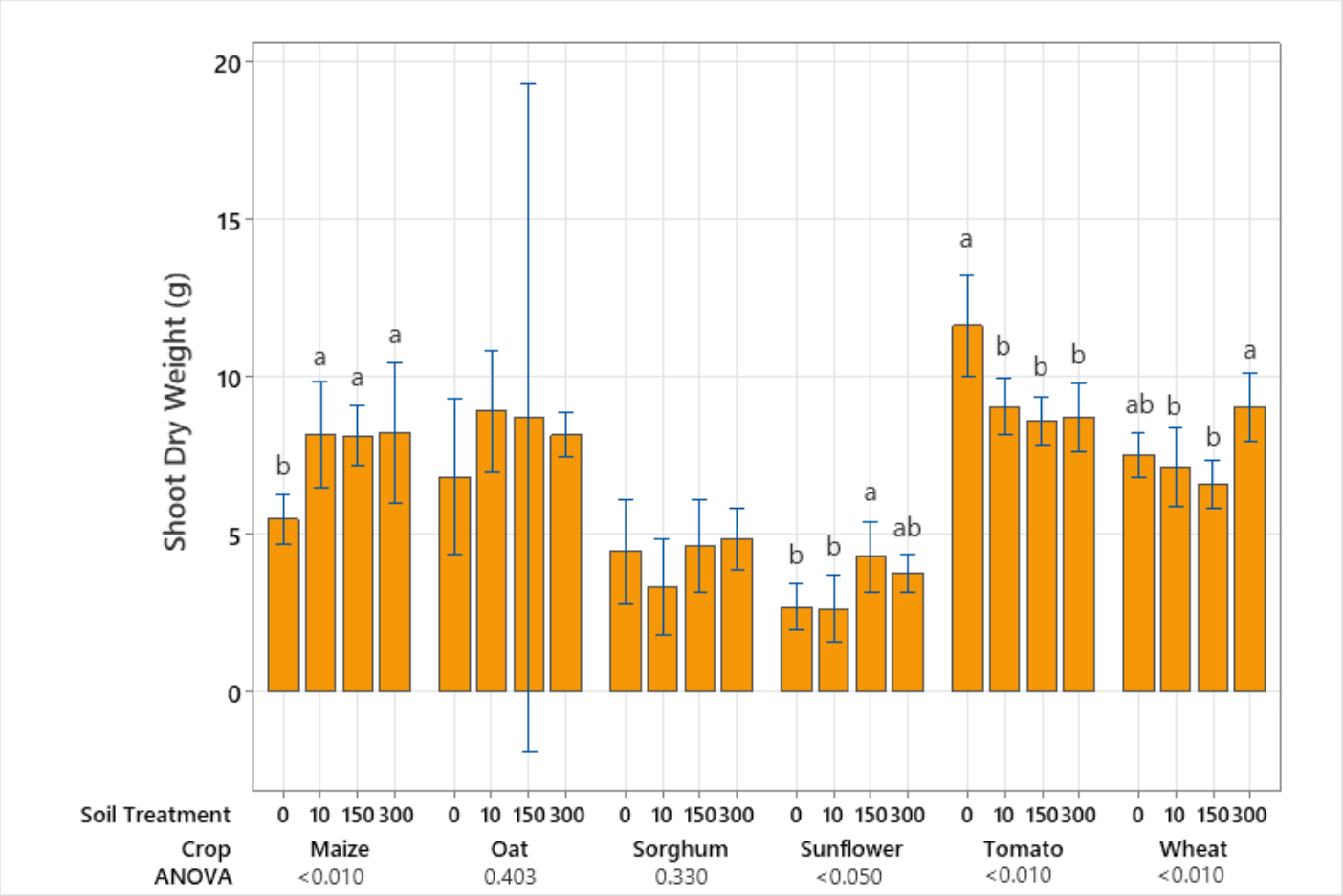

Figure 4.7. Shoot dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed. Significant results had subsequent Tukey test carried out. Bars of the same crop not sharing letters indicate significant difference

For oat, a reduction in the shoot length and overall plant height once the plant has reached maturity has been observed as the levels of chromium increase (Shanker et al., 2005). Another study recorded a reduction in root growth of nearly 50 % at 75 ppm, with no root of shoot growth once 100 ppm was reached (Amin et al., 2019). A reduction in root length was also observed by López-Luna et al., (2009) who found a root reduction of 50% and that shoot growth of the oat plant was more sensitive to the presence of chromium then its roots, especially at levels of 500 mg kg^{-1}. Chirakkara and Reddy (2014) also found a reduction in the biomasses of both the roots and the shoot when exposed to heavy metal contamination, as well as the overall biomass close to 80%. This suggest that although not found to

be significant in this study, oat root and shoot biomass is susceptible to elevated Cr concentration and as such would affect its efficiency as a phytoremediator for the purposes of this study.

Maize and wheat, although showing no significant difference in root biomass levels at increasing Cr treatments, showed a significant increase in shoot biomass within Cr contamination, with a consistent increase in shoot biomass for maize at 10, 150 and 300 mg/kg compared to the control and wheat showing significantly higher biomasses in 300 mg/kg compared to 10 and 150 mg/kg. This goes against previous research conducted where for maize it was found that the length of the primary root, as well as the number of lateral off-shoots, can be reduced by up to 65%, with the susceptibility of the plant increasing as the concentration increases (di Toppi et al., 2002). For wheat, a reduction in the growth of both stems and roots of the plant were observed by Datta et al. (2011) and López-Luna et al. (2009), causing large reductions in plant height (Sharma and Sharma, 1993). Similar results were published by Nayak et al. (2015), where elevated chromium resulted in a reduction in root and shoot length and dry biomass of the plant structures. Chromium was observed to affect roots more than shoots, with roots growth being inhibited before that of shoot even with shoots continuing to grow (Wong and Bradshaw, 1982; López-Luna et al., 2009). Due to the lack of growth of wheat past initial shoot stage, the wheat plants would not have started to put resources into the growth of extra structures and as such could be the reasoning for the lack of reduction that was observed in this study,

Sunflower showed no significant difference in root and shoot biomass, but a constant low level of root biomass produced and a significant increase in shoot length at 105 mg/kg Cr. This resistance to effects on the growth of the crop is contradictory to past research. Reductions in root length, shoot length, height, overall dry biomass, and overall nutritional content on the plant were observed have been observed (Fozia et al., 2008) with Davies et al., (2001) stating that they found that moderate levels of chromium negatively affected all growth aspects of the sunflower.

Leaf biomass

Leaf biomass was seen to be significantly affected by the presence of increasing Cr levels (figure 4.8). Significant reductions in leaf biomass were observed within tomato, oat, and sunflower. With the leaves being important for the survival of the crop plants, these loses in leaf biomass could signify a tolerance mechanism that could harm the effectiveness of these crops as a usable food crop on the contaminated sites.

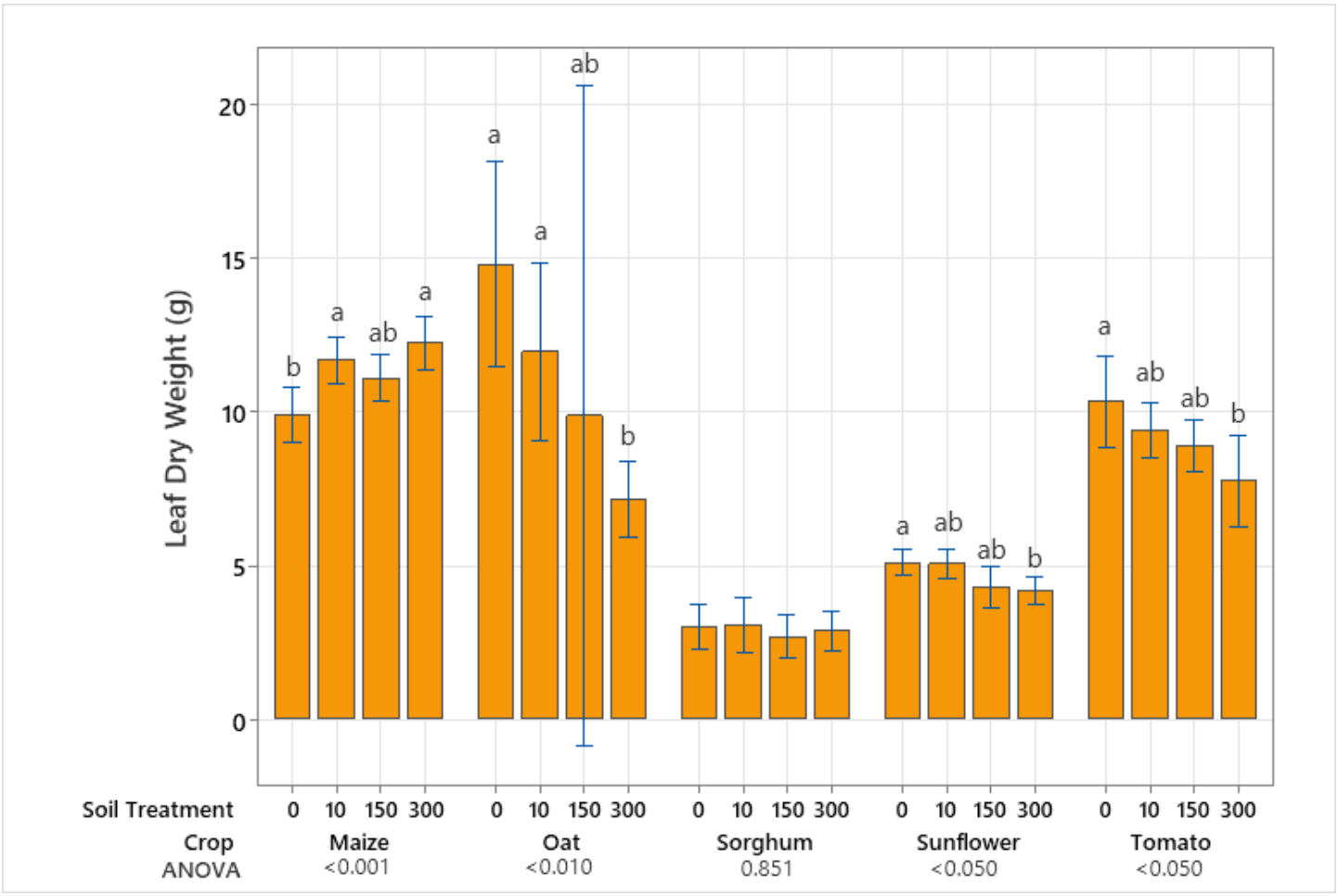

Figure 4.8. Leaf dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed. Significant results had subsequent Tukey test carried out. Bars of the same crop not sharing letters indicate significant difference

Reductions in tomato leaf growth biomass have been previously observed (Henriques, 2010) with induced oxidative stress being the cause at levels over 10 mg/L (Christou et al., 2021) and as a result of this reduced leaf growth and area, a reduction in photosynthesis has been observed, leading to a reduction in overall plant growth (Singh and Prasad, 2019). This Supports the significant decrease in total biomass observed in this study.

Gopal and Khurana (2011) found that chromium had major negative effects on the health and growth of the sunflower plants: yellowing of the plant, complete stopping of growth, reduction of height of 79% and a 90% reduction in total leaf area and many of the plants did not survive longer than 10 days. These negative effects were correlated with and increased levels of the chromium within the plant

tissues. Similar results were also found in research by Fozia, Muhammid, Muhammid and Zafar (2008) who found Cr to also have a detrimental effect on the growth and morphology of the sunflower in increased levels (Fozia, Muhammid, Muhammid and Zafar, 2008). A significantly negative correlation was also found within this study, but with lower reduction to that of the research found, with a reduction of the means of the biomass of the treatments only decreasing by 10%.

Oat has been shown to decreased considerably in chlorophyll a and b levels with a complete loss after 50 ppm (Amin et al., 2019) suggesting also detrimental effects on the leaves of the oat crops. With the leaves being important for the survival of the crop plants, these loses in leaf biomass could signify a tolerance mechanism that could harm the effectiveness of these crops as a usable food crop on the contaminated sites.

Maize again showed a significant increase in its biomass, this time within the leaves with an increase of nearly 25%. A 50% tissue growth inhibition was observed at levels as small as 5.2 ppm (Chang, Granato and Page, 1992), causing reductions in the number and sizes of the leaves (up to 10%) (Anjum, 2016) which goes against these findings of stimulation of growth in high Cr levels. Sharma, Sharma and Tripathi (2003), Dheeba, Sampathkumar and Kannan (2014) and Anjum (2016) also noted that reductions in the expression of chlorophyll a and b and multiple enzyme activities where all observed with the introduction of chromium up to 61%. This all taken into account, studies such as that by Kacálková et al.,(2014) state that maize plants have exhibited the most resistance to an increasing of heavy metal levels within soils when compared to other crop plants such as sunflowers and wheat, which supports the ability to thrive in Cr contaminated conditions such as the ones used within this study..

Sorghum, again shown no significance in leaf biomass weight because of Cr concentration. Studies have shown previously a reduction in the leaf biomass as a result of increased Cr (Revathi, Haribabu and Sudha, 2011) and also levels of both chlorophyll a and b both drop with levels of chromium rising, chromium a being inhibited to a larger extent than b (Dheeba and Sampathkumar, 2012). However, sorghum within this study is demonstrating a level of tolerance to the Cr contamination and as such a candidate for the contaminated areas of Dindigul.

Flower and tassel biomass

Flowering and the tassel structures of maize are important to analysis due to the requirement for pollination of the crops to provide edible structures. Flowers for both sunflower and tomato were analysed (figure 4.9) as well as the tassels of maize (figure 4.10).

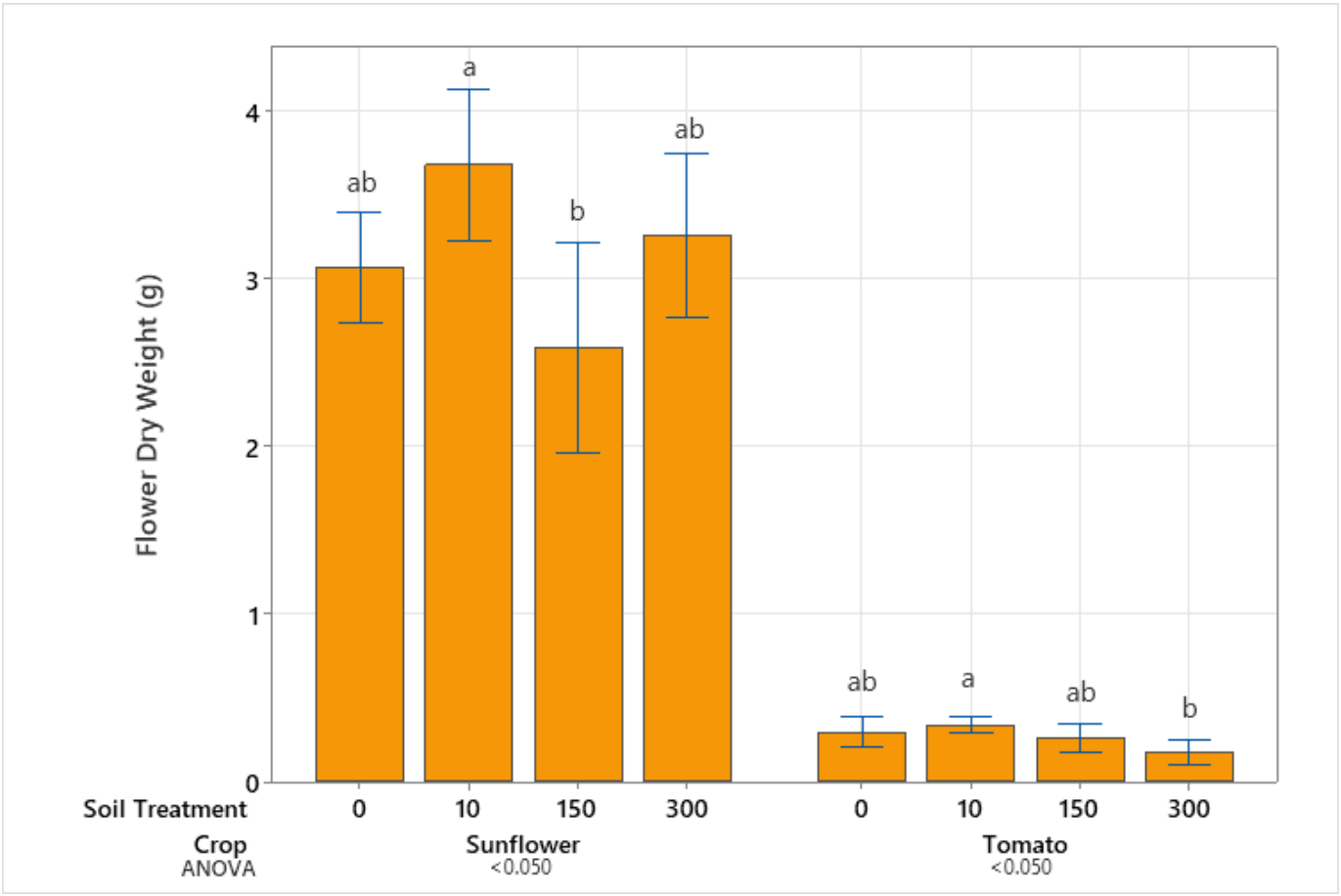

Figure 4.9. Flower dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed. Significant results had subsequent Tukey test carried out. Bars of the same crop not sharing letters indicate significant difference

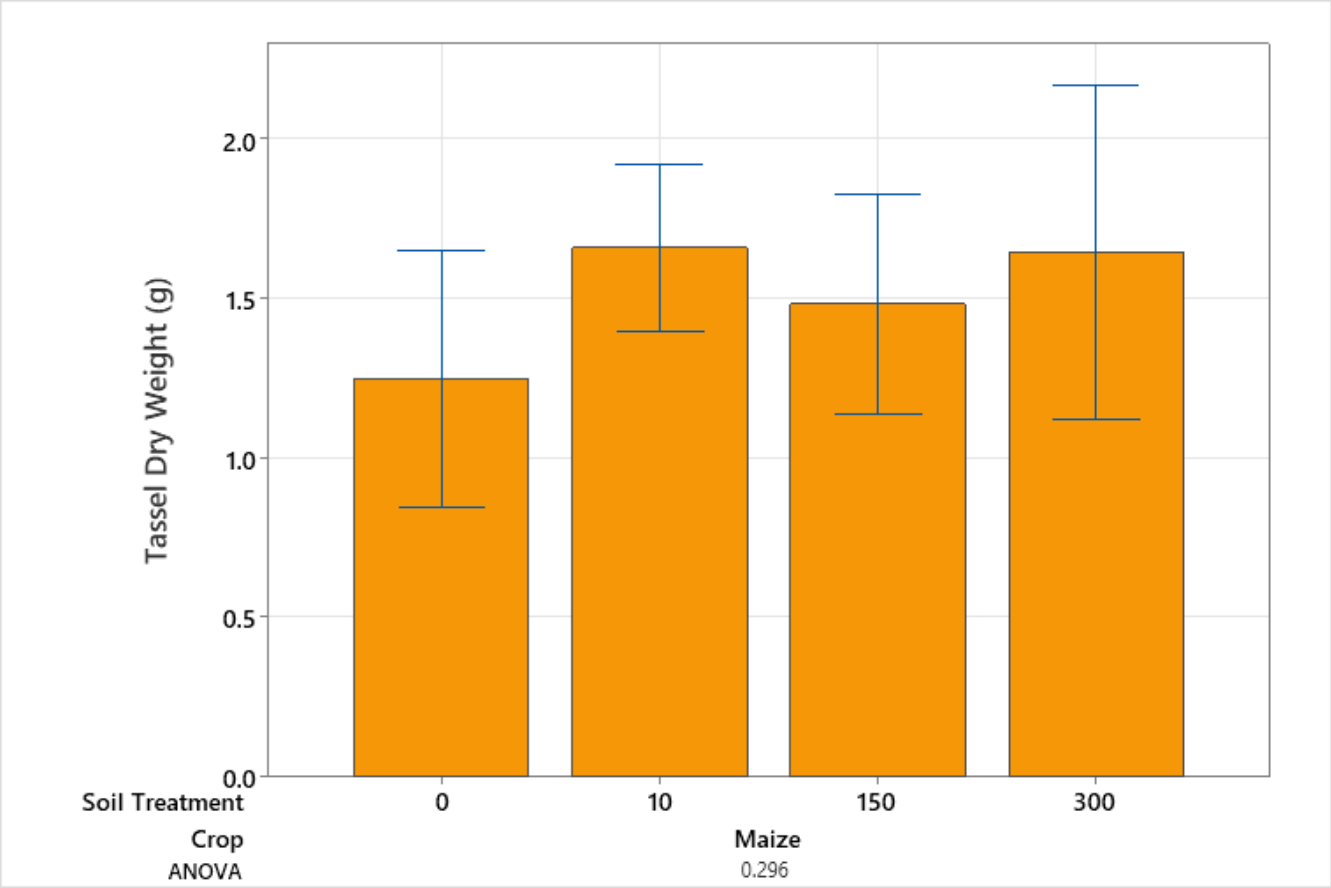

Figure 4.10. tassel dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed.

Sunflower and tomato showed an increased in flower biomass at 10 mg/kg. They also both showed no significant difference between controls and the highest concentrations of Cr. This is important to analyse as reductions in flowers at higher levels would result in a reduction in the yield of the crop plant when harvested. Reductions in the biomass of the flowering structure of sunflower has been reported by Davies et al., (2001), with increase concentration completely removing the flowering structure. This however was not seen within this study. Christou et al., (2021) found that no detrimental effect on flowering of tomato was identified which supports the findings here.

Tassels of the maize crop showed a trend increasing in biomass but not to a significant level. This indicates that this structure isn't affected by the increase in Cr concentration and would continue to allow for pollination to occur. Although decreases in biomass have not been reported, noticeable

decreases in the formations of tassels and by extension cobs at 0.05 mM chromium and no cobs forming at 1mM (Sharma, Sharma and Tripathi, 2003; Anjum, 2016) have been reported.

Fruit and seed biomass

Arguably the most important aspect of a crop plant being implemented, the biomass of the fruit and seeds produced by the crops in this study have been analysed in order to see any effects of Cr level on the yield of the crops (Figures 4.11 and 4.12 respectively).

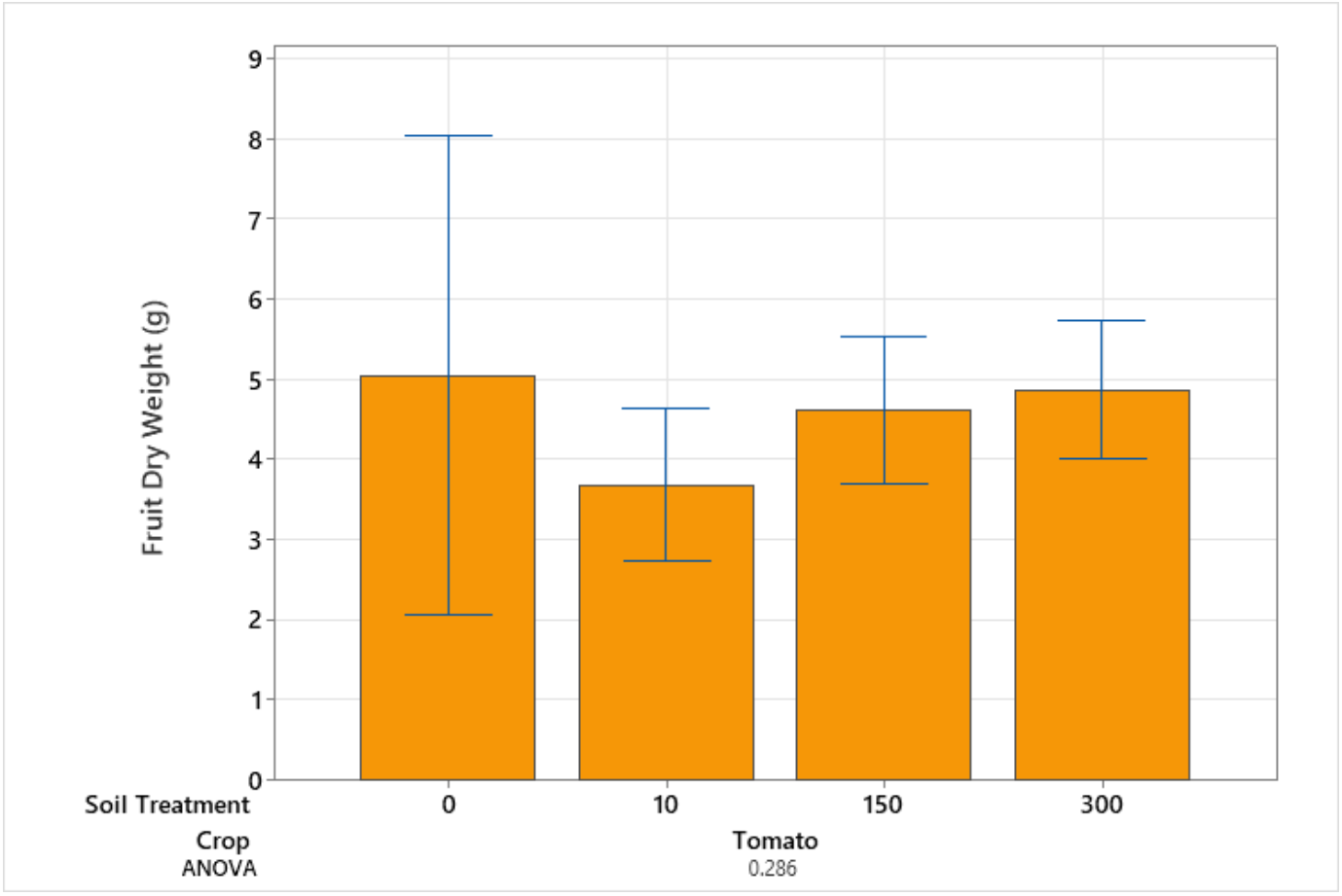

Figure 4.11. Fruit dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed.

Tomato fruits showed a drop of 1.4g with the initial exposure of 10 mg/kg Cr, with increasing levels causing dry weight of fruit increase back up to 4.9g. This is promising for tomato as a crop to be grown on the contaminated sites at Dindigul as it would theoretically be able to maintain yields across the site. Regarding the yield of the tomato crops, no significant effect on the tomato yield was observed by Christou et al., (2021) which is supported by previous research conducted by Moral, Pedreno, Gomez and Mataix (1995).

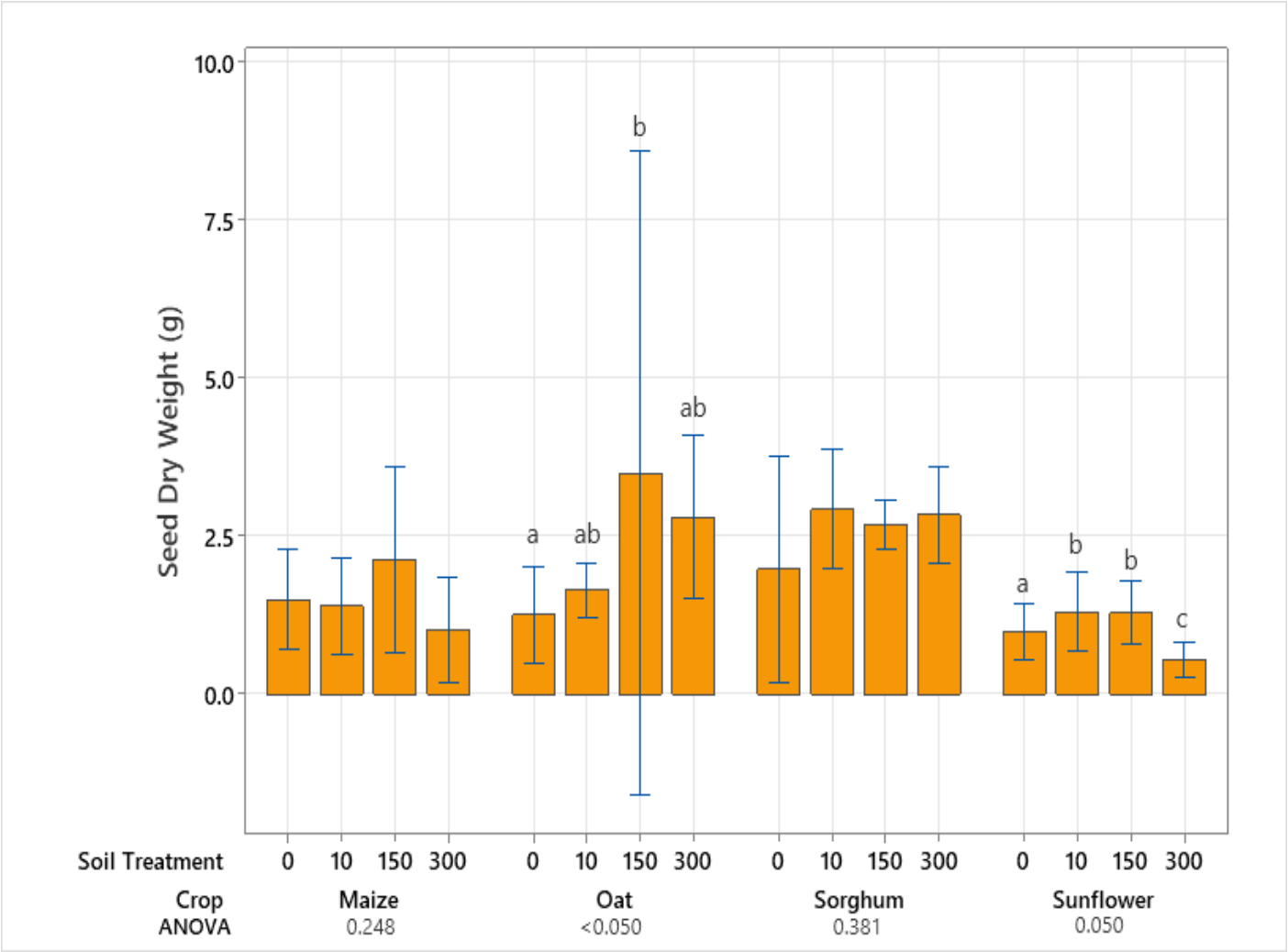

Figure 4.12. Fruit dry weight of crop plants grown at differing Cr concentrations. One-way ANOVA for significance is displayed. Significant results had subsequent Tukey test carried out. Bars of the same crop not sharing letters indicate significant difference

Sunflower showed a significant increase in the biomass of seeds produced at 10 and 150 mg/kg treatments but a significant decrease what exposed to 300 mg/kg treatments indicating that at the higher levels of contamination, the sunflower is unable to maintain seed production and the yield drops. This reduction at high levels of Cr is supported by Saleem, Asghar, Khan and Zahir, (2015) who

observed increasing Cr stress caused reductions in yield. Oat, with its decreases in biomass of other plant structures, showed a significant increase in the biomass of seed in all treatments opposed to the control, with 150 mg/kg treatment causing the highest yield. Wyszkowski and Radziemska (2013a) found the mass and yield of the plant was reported to drop 95% in soils contaminated with 80 mg kg^{-1} which contradicts what has been found in this study.

Both sorghum and maize show no significant difference in seed dry weight, with a slight decrease in seed from maize and an increase from sorghum. The yield of maize in research has shown to be affected with the number of grains and the weight of them reducing with increased levels of chromium (Shanker et al., 2005; Hayat et al., 2011). This supports the findings of this study. However, Kumar (2020) found in sorghum a reduction in yield exposed to Cr treatment with as little as 4 ppm causing a reduction. This is contradictory to the findings in this study with a maintained weight of over 2.5 g achieved at the 300 mg/kg treatment.

Although many of the crops show some level of detrimental effect cause by the presence of increased Cr, all crops tested maintained biomass levels that would indicate there use within the project of a utilisable crop plant for phytoremediation of the Cr contamination (not including wheat that failed to grow to maturity). This is due to research showing that the maintenance of biomass production by a plant growing within contaminated soils is vital for the efficiency of a phytoremediation method (Cui et al., 2021). Having passed the first stage crops need to be analysed to determine if they take up enough Cr to be deemed as a phytoremediator.

Cr bioaccumulation and translocation factor

The uptake, bioaccumulation factors and translocation factors for each crop and the constituent structures were measured. Overall uptake for each crop is displayed in figure 4.13. All crops showed a significant increase in the uptake overall, with sorghum and wheat showing the largest increases at the treatment of 300 mg/kg to 237 mg/kg and 166 mg/kg respectively. Maize also showed significance at each increase of treatment to a lesser extent as sorghum and wheat, reaching levels of 71.9 mg/kg. Sunflower and tomato showed significant increases up to treatment 150 mg/kg and then no significant difference between treatment 150 and 300 mg/kg. All of these high levels can be attributed to levels taken up within the roots of the plant structures (Figure 4.14) which is shown to be the case in the majority of Cr uptake studies as can be seen in table 4.2.

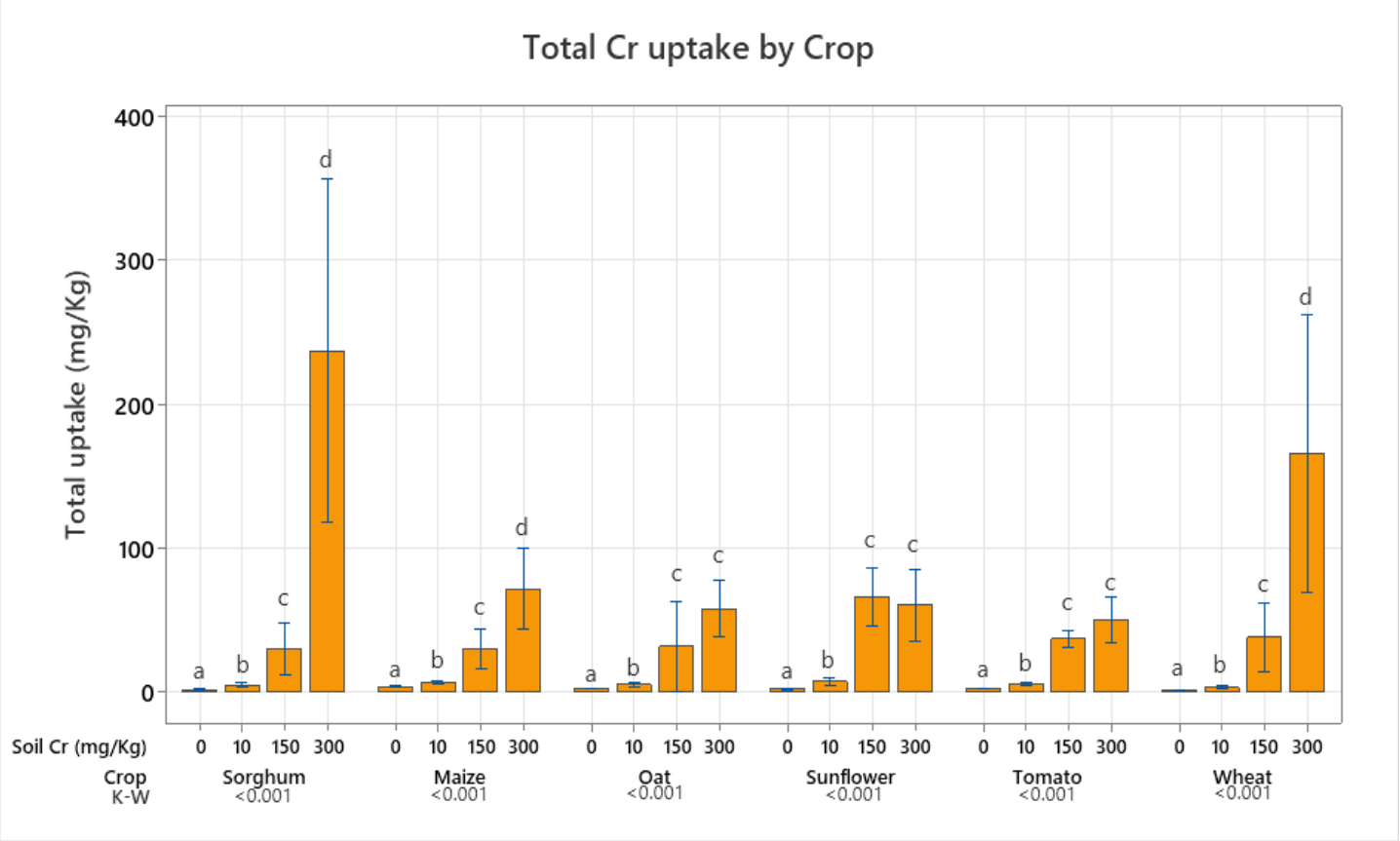

Figure 4.13. Total Cr uptake (mg/kg) at differing Cr concentrations. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Bars of the same crop not sharing letters indicate significant difference

Although remediation into the rooting structures of the crop plants is more difficult to remove from the remediation site and means the total removal of the plant, it does mean a larger proportion of the Cr can be removed at once and make a crop plant more viable if the methods are put in place to allow for the total removal of plant material.

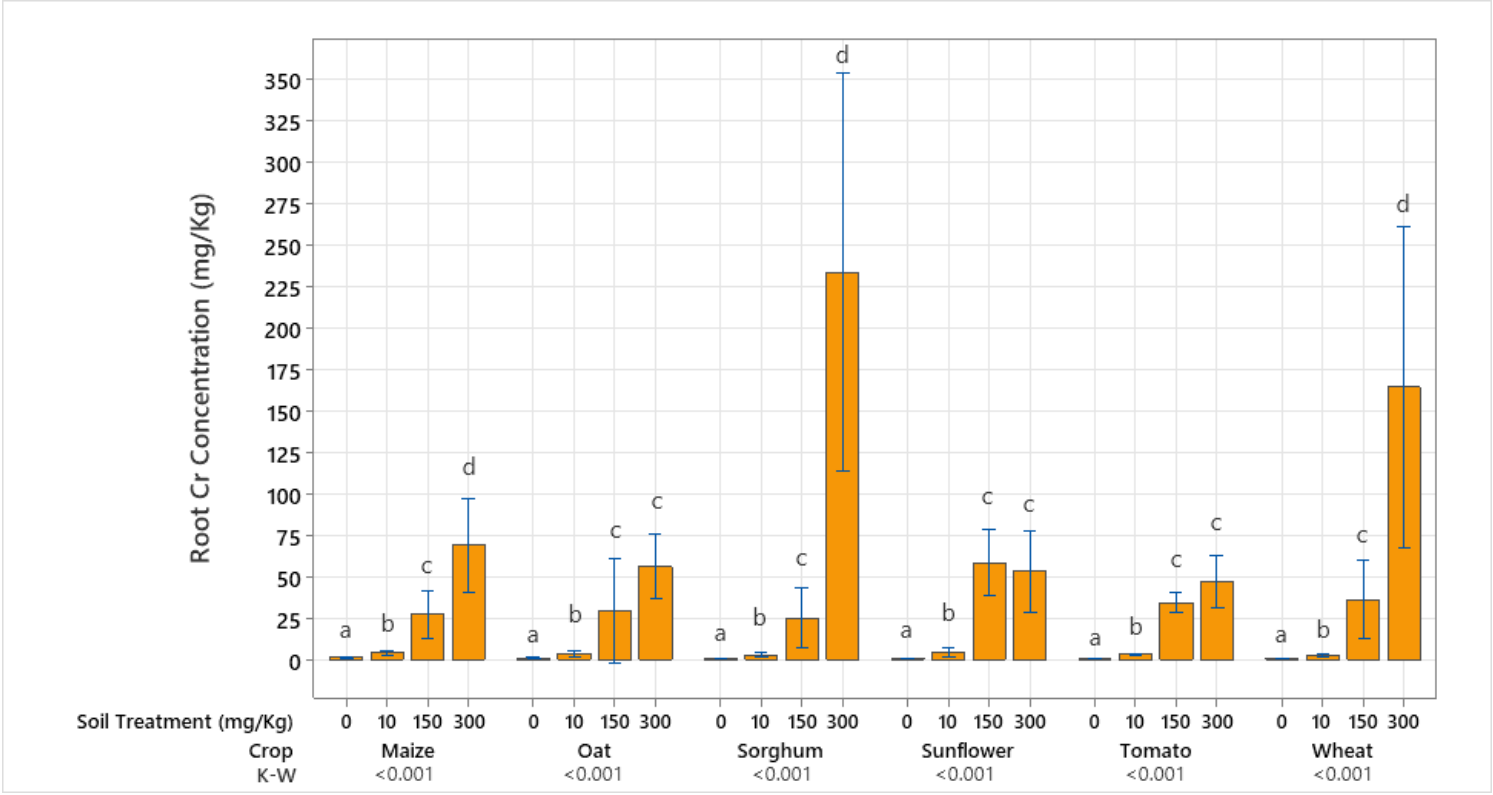

Figure 4.14. Root Cr uptake (mg/kg) at differing Cr concentrations. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Bars of the same crop not sharing letters indicate significant difference

To determine a crops suitability as a phytoremediator, the bioaccumulation factor (BF) of the crop and the translocation factor (TF) between structures must be identified together with Cr uptake.

Tomato demonstrated significant increases within root and shoot structures and slight but not significant increases in leaf and flower concentration (table 4.4). Fruit showed a significant decrease in the levels of Cr within the structure with an increase of Cr concentration. This is ideal for a crop that is to be grown on high concentrations of Cr and still be edible. BF (table 4.5) shows a significant drop as concentrations increase with BF within the total plant reaching 0.092 representing an uptake of around 9% comparable to the concentration within the soils. This drops to 0.0065 BF for just arial structures. Around 0.1 BF within this study will be deemed as a successful candidate for the remediation of areas of the contaminated sites in Dindigul. This is due to the outcome not only being for the remediation of the Cr contamination of the site, but also to produce an economically viable and edible crop for the local population. This means that a trade of for less remediation to allow an

increase of food for an area the requires and increase is acceptable. TF shows that sequestering of Cr within the shoots from the root and fruit from the shoots both decrease significantly as the Cr concentration increases (table 4.6). This is ideal for the fruit meaning less Cr is translocated into these structures however a lower TF shoot/root isn't ideal for a phytoremediator.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		Total Cr uptake (mg/Kg)	Root Cr Concentration (mg/Kg)	Shoot Cr Concentration (mg/Kg)	Leaf Cr Concentration (mg/Kg)	Flower Cr Concentration (mg/Kg)	Fruit Cr Concentration (mg/Kg)
Tomato	0 (n=12) **1.22**	Mean ± SD	2.566 ± 0.544	0.41382 ± 0.03341	0.8399 ± 0.1465	0.4563 ± 0.1971	0.3351 ± 0.0366	1.0423 ± 0.0807
		Min - Max	1.824 - 3.359	0.35818 - 0.46997	0.4639 - 1.0431	0.2295 - 0.9621	0.2872 - 0.3939	0.7718 - 1.2842
		CV / M-W	21.21% / a	8.07% / a	17.44% / a	43.20%	10.92%	18.95% / a
	10 (n=11) **23.77**	Mean ± SD	5.49 ± 1.21	2.915 ± 1.234	0.6222 ± 0.2486	0.3916 ± 0.1797	0.391 ± 0.421	1.1668 ± 0.2332
		Min - Max	4.00 - 8.34	1.704 - 6.251	0.1080 - 0.9128	0.1535 - 0.6967	0.011 - 1.594	0.9554 - 1.7525
		CV / M-W	22.00% / b	42.33% / b	39.95% / b	45.90%	107.53%	19.98% / a
	150 (n=10) **283.64**	Mean ± SD	36.93 ± 8.80	34.26 ± 8.79	0.8943 ± 0.2713	0.725 ± 0.796	0.3959 ± 0.1008	0.6488 ± 0.2483
		Min - Max	24.14 - 50.53	21.83 - 48.44	0.5448 - 1.2788	0.320 -2.968	0.2881 - 0.5917	0.3827 - 1.2816
		CV / M-W	23.82% / c	25.65% / c	30.34% / ab	109.75%	25.45%	38.27% / b
	300 (n=11) **602.76**	Mean ± SD	50.34 ± 23.44	48.53 ± 24.08	1.813 ± 0.472	0.770 ± 0.532	0.534 ± 0.345	0.3906 ± 0.1198
		Min - Max	25.47 - 105.14	22.72 - 102.12	0.814 - 2.238	0.386 - 2.091	0.272 - 1.270	0.2683 - 0.6200
		CV / M-W	46.57% / c	49.62% / c	26.01% / c	69.06%	64.58%	30.68% / c
		K-W	<0.001	<0.001	<0.001	0.091	0.502	<0.001

Table 4.4. Uptake of Cr within the structure of tomato at different treatments of Cr. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		BF Plant (Predicted)	BF Plant (Actual)	BF Arial Structures (Predicted)	BF Arial Structures (Actual)
Tomato	0 (n=12) **1.22**	Mean ± SD	NA	2.269 ± 0.841	NA	1.908 ± 0.770
		Min - Max	NA	1.125 - 3.622	NA	0.895 - 3.151
		CV / M-W	NA	37.09% / a	NA	40.36% / a
	10 (n=11) **23.77**	Mean ± SD	0.5487 ± 0.1207	0.2378 ± 0.0729	0.2572 ± 0.0603	0.11117 ± 0.03278
		Min - Max	0.3995 - 0. 8349	0.1673 - 0.3753	0.1844 - 0.2553	0.07891 - 0.16513
		CV / M-W	22.00% / a	30.67% / b	23.43% / a	29.49% / b
	150 (n=10) **283.64**	Mean ± SD	0.2462 ± 0.0586	0.1406 ± 0.0576	0.0177 ± 0.0054	0.01026 ± 0.00439
		Min - Max	16.09 - 33.68	0.0924 - 0.2649	1.25 - 1.57	0.00508 - 0.01882
		CV / M-W	23.82% / b	40.96% / c	30.31% / b	42.80% / c
	300 (n=11) **602.76**	Mean ± SD	0.1678 ± 0.0236	0.0924 ± 0.0489	0.0111 ± 0.0024	0.00653 ± 0.003294
		Min - Max	0.0849 - 0.3505	0.0392 - 0.2168	0.0081 - 0.0106	0.002347 - 0.01463
		CV / M-W	46.57% / c	52.93% / d	22.08% / c	50.44% / d
		K-W	<0.001	<0.001	<0.001	<0.001

Table 4.5. BF of Cr within total plant and arial structures of tomato. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		TF Shoot/Root	TF Leaf/Shoot	TF Flower/Shoot	TF Fruit/Shoot
Tomato	0 (n=12) **1.22**	Mean ± SD	0.3610 ± 0.1028	05649 ± 0.0815	0.4090 ± 0.0740	1.477 ± 0.679
		Min - Max	0.2303 - 0.5655	0.2483 - 1.3319	0.3180 - 0.6191	0.851 - 2.768
		CV / M-W	28.49% / a	50.00%	18.10%	45.94% / a
	10 (n=11) **23.77**	Mean ± SD	0.1266 ± 0.0626	0.999 ± 1.257	0.817 ± 0.890	2.630 ± 2.434
		Min - Max	0.0826 - 0.2810	0.196 - 4.597	0.017 - 3.096	1.113 - 9.619
		CV / M-W	49.43% / b	125.83%	108.97%	92.55% / a
	150 (n=10) **283.64**	Mean ± SD	0.1303 ± 0.0549	0.932 ± 1.141	0.4699 ± 0.2450	0.809 ± 0.467
		Min - Max	0.0835 - 0.0.2461	0.326 - 4.093	0.2607 - 0.9101	0.325 - 1.971
		CV / M-W	42.14% / b	122.43%	49.35%	57.68% / b
	300 (n=11) **602.76**	Mean ± SD	0.0859 ± 0.0464	0.4429 ± 0.2857	0.346 ± 0.318	0.2718 ± 0.1665
		Min - Max	0.0349 - 0.2022	0.1937 - 1.0052	0.131 - 1.183	0.1316 - 0.6484
		CV / M-W	53.99% / c	64.49%	91.93%	61.24% / c
		K-W	<0.001	0.216	0.080	<0.001

Table 4.6. TF between tomato structures. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference.

Wheat demonstrated a significant increase of Cr within both the root and shoot structure (table 4.7) in increasing concentrations of Cr up to mean levels of 166 mg/kg total Cr uptake and max of 548 mg/kg in the 300 mg/kg treatments (795 mg/kg actual). The crop failed to develop to maturity and produce seeds meaning its usefulness as a crop phytoremediator cannot properly be analysed within this study. Within other studies, uptake of chromium reached 1188.8 high ppm, however up to 931.3 ppm of that is found within the roots, with Cr uptake in structures in the order leaves > shoot > seeds (Chandra et al., 2009). Stem and leaf uptake showed uptake levels of up to 601.6 and 333.7 ppm, respectfully while levels in the seeds didn't exceed 35 ppm (Chandra et al., 2009). It is also apparent that the higher levels of contamination of the soil, the more the wheat plants will uptake (López-Luna et al., 2009).

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		Total Cr uptake (mg/Kg)	Root Cr Concentration (mg/Kg)	Shoot Cr Concentration (mg/Kg)
Wheat	0 (n=12) **1.71**	Mean ± SD	1.275 ± 0.490	0.529 ± 0.501	0.7458 ± 0.0508
		Min - Max	0.735 - 2.272	0.047 - 1.536	0.6698 - 0.8382
		CV / M-W	38.41% / a	94.64% / a	6.81% / a
	10 (n=12) **28.61**	Mean ± SD	3.17 ± 1.46	2.048 ± 1.349	1.124 ± 0.749
		Min - Max	1.13 - 5.75	0.164 - 4.975	0.679 - 3.455
		CV / M-W	45.88% / b	65.88% / b	66.61% / b
	150 (n=12) **415.24**	Mean ± SD	38.10 ± 37.00	36.2 ± 37.1	1.909 ± 0.603
		Min - Max	8.10 - 141.40	5.8 - 140.2	0.905 - 2.786
		CV / M-W	97.07% / c	102.45% / c	31.57% / c
	300 (n=12) **795.16**	Mean ± SD	166.10 ± 152.00	164.1 ± 152.1	2.008 ± 0.537
		Min - Max	24.30 - 548.10	22.6 - 546.5	1.305 - 3.039
		CV / M-W	91.49% / d	92.68% / d	26.76% / c
		K-W	<0.001	<0.001	<0.001

Table 4.7. Uptake of Cr within the structure of wheat at different treatments of Cr. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

BF of the wheat plant did increase between the 10 mg/kg and 300 mg/kg treatment (28 – 795 mg/kg actual) (table 4.8) to 0.28 suggesting a 28% uptake of the Cr within the structures compared to the soils. TF shoot/root showed the same trend of an increase as the concentration increases after an initial drop from the control (table 4.9). This does suggest good indications of a utilisable crop for the purposes of this study however as a potential phytoremediator with studies showing wheat to have increased uptake of Cr to a significant level at increased Cr concentrations (Nayak et al., 2015) although González, del Mar Gil-Diaz and del Carmen Lobo, (2016) showed wheat to have a low potential for phytoremediation due to the adverse effects the Cr have on the health of the plant.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		BF Plant (Predicted)	BF Plant (Actual)	BF Arial Structures (Predicted)	BF Arial Structures (Actual)
Wheat	0 (n=12) **1.71**	Mean ± SD	NA	9.73 ± 20.42	NA	8.52 ± 18.69
		Min - Max	NA	0.17 - 58.59	NA	0.12 - 53.38
		CV / M-W	NA	209.89% / a	NA	219.36% / a
	10 (n=12) **28.61**	Mean ± SD	0.3172 ± 0.1455	0.1598 ± 0.1557	0.1124 ± 0.0749	0.0593 ± 0.0689
		Min - Max	0.1133 - 0.5752	0.0330 - 0.6024	0.0679 - 0.3455	0.0145 - 0.2556
		CV / M-W	45.88%	97.41% / b	66.61% / a	116.12% / b
	150 (n=12) **415.24**	Mean ± SD	0.2543 ± 0.2468	0.1466 ± 0.2792	0.0127 ± 0.0040	0.004936 ± 0.001845
		Min - Max	0.0537 - 0.9427	0.0145 - 1.0247	0.0060 - 0.0186	0.003211 - 0.008728
		CV / M-W	97.07%	190.50% / b	31.57% / b	37.38% / c
	300 (n=12) **795.16**	Mean ± SD	0.5540 ± 0.5070	0.2786 ± 0.3210	0.0067 ± 0.0018	0.002950 ± 0.001265
		Min - Max	0.0810 - 1.8270	0.0287 - 1.1532	0.0044 - 0.0101	0.000805 - 0.005781
		CV / M-W	91.49%	115.23% / b	26.76% / c	42.89% / d
		K-W	0.180	<0.001	<0.001	<0.001

Table 4.8. BF of Cr within total plant and arial structures of wheat. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		TF Shoot/Root
Wheat	0 (n=12) **1.71**	Mean ± SD	1.209 ± 1.776
		Min - Max	0.034 - 5.209
		CV / M-W	146.97%
	10 (n=12) **28.61**	Mean ± SD	0.1005 ± 0.0970
		Min - Max	0.0048 - 0.3469
		CV / M-W	96.60%
	150 (n=12) **415.24**	Mean ± SD	0.1416 ± 0.2780
		Min - Max	0.0105 - 1.0160
		CV / M-W	196.25%
	300 (n=12) **795.16**	Mean ± SD	0.2756 ± 0.3205
		Min - Max	0.0262 - 1.1497
		CV / M-W	116.30%
		K-W	0.073

Table 4.9. TF between wheat structures. Kruskal-Wallis for significance is displayed.

Sorghum, an already important crop plant within India (Sridhara et al., 2020), showed a consistency within its biomass and yield when exposed to increasing Cr levels and showed the highest levels of uptake out of all crop plants tested (Table 4.10). Levels of Cr significantly increased with the increase of Cr concentration with a max total uptake of 464 .8 mg/kg and a mean of 237 mg/kg at the highest levels of Cr. The same significant increase was seen in the root structures of the crop with most of the uptake found to be within these root structures supported by findings by Karimi, (2013) who also found increased levels of Cr within the root. Significant changes were also observed within the shoot, leaf, and seed concentrations, with the highest increase for all three structures being at the 150 mg/kg treatment (307.56 mg/kg actual).

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		Total Cr uptake (mg/Kg)	Root Cr Concentration (mg/Kg)	Shoot Cr Concentration (mg/Kg)	Leaf Cr Concentration (mg/Kg)	Seed Cr Concentration (mg/Kg)
Sorghum	0 (n=9) **1.88**	Mean ± SD	1.65 ± 0.405	0.542 ± 0.537	0.3495 ± 0.2147	0.6440 ± 0.2620	0.25695 ± 0.01572
		Min - Max	1.039 - 2.508	0.064 - 1.844	0.0115 - 0.6918	0.1503 - 0.6607	0.23482 - 0.27154
		CV / M-W	24.53 / a	99.11% / a	61.43 % / a	40.68% / a	6.12% / a
	10 (n=9) **25.70**	Mean ± SD	4.95 ± 2.03	2.680 ± 1.915	1.344 ± 0.972	0.6702 ± 0.2407	0.2888 ± 0.0726
		Min - Max	2.51 - 8.58	0.016 - 6.925	0.383 - 3.719	0.2703 - 1.0585	0.2301 - 0.4569
		CV / M-W	40.97% / b	71.46% / b	72.32% / b	35.91% / a	25.14% / a
	150 (n=11) **307.56**	Mean ± SD	30.24 ± 27.02	25.04 ± 27.04	3.49 ± 5.23	1.304 ± 0.362	0.451 ± 0.332
		Min - Max	9.37 - 108.01	6.26 - 104.68	1.29 - 19.21	0.719 - 1.961	0.039 - 1.306
		CV / M-W	89.35% / c	108.00% / c	149.78% / c	27.73% / b	73.57% / b
	300 (n=10) **745.40**	Mean ± SD	237.00 ± 166.90	233.5 ± 167.1	2.394 ± 0.702	0.885 ± 0.375	0.3307 ± 0.0721
		Min - Max	15.20 - 464.80	11.3 - 463.0	0.565 - 3.000	0.465 - 1.706	0.2110 - 0.4463
		CV / M-W	70.40% / d	71.57% / d	29.31% / c	42.39% / a	21.81% / ab
		K-W	<0.001	<0.001	<0.001	<0.010	<0.050

Table 4.10. Uptake of Cr within the structure of sorghum at different treatments of Cr. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

An increase in the concentration within the seeds is not what is wanted within this study due to the requirement of the crops as a food source for the local populous. However, if the levels remain below threshold values for eating then the crop could still be of utilised by farmers as the levels would not cause risk to human health via ingestion at such levels. BF of Cr for sorghum (table 4.11) showed a significant decrease in BF compared to the control but showed relatively high BF for this study across the Cr treatments. A significant decrease in the BF into arial structures across the Cr treatments shows that lest of the Cr will be in the easily removable structures of the plant, but also less to be taken up within the seed structures. This reduced uptake into above ground structures is noted as a consequence of crop plants and Vamerali, Bandiera and Mosca, (2009) states in their review of crop phytoremediation that crop plant remediators can compensate for the low uptake of heavy metals via a greater biomass yield, however more field studies need to be carried out to see this in action.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		BF Plant (Predicted)	BF Plant (Actual)	BF Arial Structures (Predicted)	BF Arial Structures (Actual)
Sorghum	0 (n=9) **1.88**	Mean ± SD	NA	1.746 ± 1.635	NA	1.234 ± 1.219
		Min - Max	NA	0.447 - 5.024	NA	0.118 - 3.817
		CV / M-W	NA	93.63% / a	NA	98.84% / a
	10 (n=9) **25.70**	Mean ± SD	0.4951 ± 0.2028	0.2499 ± 0.2082	0.2271 ± 0.0959	0.1189 ± 0.1050
		Min - Max	0.2506 - 0.8581	0.0830 - 0.7396	0.1175 - 0.4446	0.0429 - 0.3510
		CV / M-W	40.97% / a	83.29% / b	42.25% / a	88.28% / b
	150 (n=11) **307.56**	Mean ± SD	0.2016 ± 0.1802	0.1388 ± 0.1418	0.0347 ± 0.0351	0.02194 ± 0.02007
		Min - Max	0.0624 - 0.7201	0.0245 - 0.4520	0.0189 - 0.0240	0.00711 - 0.06391
		CV / M-W	89.34% / b	102.16% / b	101.12% / b	91.47% / c
	300 (n=10) **745.40**	Mean ± SD	0.7900 ± 0.5560	0.381 ± 0.359	0.0118 ± 0.0025	0.00581 ± 0.00424
		Min - Max	0.0510 - 1.5490	0.016 - 1.245	0.0060 - 0.0012	0.00174 - 0.01720
		CV / M-W	70.40% / ab	94.18% / b	21.13% / c	72.89% / d
		K-W	<0.050	<0.001	<0.001	<0.001

Table 4.11. BF of Cr within total plant and arial structures of sorghum. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

TF for sorghum as displayed in table 4.12, shows and initial decrease in TF from roots to shoots but then a gradual increase again up to the 300 mg/kg treatment. Significant decreases within the TF from shoots to both leaves and seeds shows a tolerance mechanism preventing the Cr from being stored within these structures at higher levels. In other research, sorghum has been found to be a poor translocation of the Cr to arial plant structures (Jadia and Fulekar, 2009; Dheeba and Sampathkumar, 2012; Karimi, 2013). López-Luna et al. (2009) state that translocation only started to occur into shoots at 1000 mg Cr kg^{-1} soil, however they also reported that less mobile Cr (III) translocated into the aerial structures of the sorghum more readily.

This would support that much of the chromium being moved within the arial structures of the sorghum will be the less harmful Cr (III), however more testing to deem this to be the case will need to be conducted. Sorghum remains a promising candidate for the continuation of this study, showing a good BF on average of 0.38 with a reduction of the translocation of the Cr into the fruiting structures at higher levels.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		TF Shoot/Root	TF Leaf/Shoot	TF Seed/Shoot
Sorghum	0 (n=9) **1.88**	Mean ± SD	0.512 ± 0.732	8.54 ± 18.22	0.6237 ± 0.1829
		Min - Max	0.076 - 0.259	0.44 - 6.14	0.3736 - 0.7777
		CV / M-W	142.88% / a	213.47% / a	29.33% / a
	10 (n=9) **25.70**	Mean ± SD	0.1310 ± 0.1174	0.757 ± 0.543	0.3039 ± 0.1951
		Min - Max	0.0007 - 0.3887	0.073 - 1.851	0.1229 - 0.6745
		CV / M-W	89.64% / b	71.73% / b	64.21% / b
	150 (n=11) **307.56**	Mean ± SD	0.1169 ± 0.1304	0.669 ± 0.381	0.2417 ± 0.2844
		Min - Max	0.0163 - 0.3880	0.068 - 1.520	0.0177 - 1.0120
		CV / M-W	111.56% / b	56.9% / b	117.67% / b
	300 (n=10) **745.40**	Mean ± SD	0.375 ± 0.355	0.473 ± 0.433	0.1840 ± 0.1602
		Min - Max	0.012 - 1.228	0.161 - 1.621	0.0703 - 0.5698
		CV / M-W	94.74% / ab	91.56% / b	87.07% / b
		K-W	<0.050	<0.010	<0.05

Table 4.12. TF between sorghum structures. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Sunflower showed significant increases of Cr uptake across all structures (table 4.13), with only shoots of the sunflower decreasing again at 300 mg/kg treatment after peaking at a mean of 4.119 mg/kg at the 150 mg/kg treatment. This increase signals a promising phytoremediator with arial structures holding more Cr than that of the other crops analysed. The phytoremediation potential of this crop plant is evident and well known (Arribas, 2014) with levels of up to 1806 ppm total uptake within the plant structures with a large proportion of this being stored within the easily harvestable stem, as also discussed by Francis (2017). Seed contamination levels also remained low ranging between 0.57 – 5.1 ppm (Francis, 2017).

BF for both the total plant and the arial structures significantly decreased (table 4.14) but showed a high mean BF of 0.54 at 150 mg/kg treatment (254.4 mg/kg actual) and even higher at the 10 mg/kg (23.28 mg/kg actual) treatment at 0.79. This shows sunflower to be a promising candidate for phytoremediation of low to mid-levels of Cr contamination with applications promising at higher levels also. TF (table 4.15) showed a reduction although not significant, in the TF of root to shoot translocation of Cr in increasing concentrations. Results for TF into shoots was found to be lower than has been stated in research by Lotfy and Mostafa (2014), who found that at levels between 148 and 213 mg/kg, translocation of Cr was 0.58 and 0.49 respectively. This is more than the averages for

sunflower across all levels of Cr carried out in this study, apart from background uptake from the control. Significant reductions in TF were found between shoot and flower as well as shoot and seed.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		Total Cr uptake (mg/Kg)	Root Cr Concentration (mg/Kg)	Shoot Cr Concentration (mg/Kg)	Leaf Cr Concentration (mg/Kg)	Flower Cr Concentration (mg/Kg)	Seed Cr Concentration (mg/Kg)
Sunflower	0 (n=12) **2.03**	Mean ± SD	2.159 ± 0.722	0.363 ± 0.366	0.529 ± 0.481	0.2090 ± 0.0942	0.797 ± 0.352	0.2848 ± 0.0957
		Min - Max	1.166 - 3.691	0.013 - 0.924	0.238 - 1.673	0.0569 - 0.3898	0.385 - 1.468	0.1414 - 0.4005
		CV / M-W	33.47% / a	100.81% / a	90.93% / a	45.10% / a	44.17% / a	33.59% / a
	10 (n=10) **23.38**	Mean ± SD	7.36 ± 3.92	4.30 ± 4.11	0.639 ± 0.0.920	0.358 ± 0.466	1.746 ± 0.639	0.3102 ± 0.0488
		Min - Max	3.17 - 16.63	0.33 - 14.60	0.023 - 3.230	0.023 - 1.612	0.711 - 2.731	0.2547 - 0.4088
		CV / M-W	53.25% / b	95.62% / b	143.91% / a	130.14% / a	36.63% / b	15.75% / a
	150 (n=12) **254.40**	Mean ± SD	66.13 ± 31.15	58.34 ± 30.95	4.119 ± 3.111	0.652 ± 0.533	2.796 ± 1.196	0.3392 ± 0.0529
		Min - Max	18.33 - 114.01	11.99 - 107.83	0.986 - 10.963	0.028 - 1.720	1.730 - 6.252	0.2374 - 0.4008
		CV / M-W	47.11% / c	53.05% / c	75.51% / b	81.76% / b	42.77% / b	15.60% / ab
	300 (n=12) 6**97.43**	Mean ± SD	60.30 ± 38.90	53.3 ± 38.7	2.328 ± 1.079	1.392 ± 1.054	2.978 ± 0.434	0.3969 ± 0.1056
		Min - Max	15.10 - 135.10	9.7 - 128.9	0.524 - 3.982	0.172 - 3.402	2.528 - 3.796	0.2866 - 0.6826
		CV / M-W	64.53% / c	72.59% / c	46.33% / b	75.75%/ b	14.59% / b	26.61% / b
		K-W	<0.001	<0.001	<0.001	<0.001	<0.001	<0.050

Table 4.13. Uptake of Cr within the structure of Sunflower at different treatments of Cr. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		BF Plant (Predicted)	BF Plant (Actual)	BF Arial Structures (Predicted)	BF Arial Structures (Actual)
Sunflower	0 (n=12) **2.03**	Mean ± SD	NA	3.37 ± 5.12	NA	2.81 ± 4.16
		Min - Max	NA	0.25 - 18.93	NA	0.19 - 15.21
		CV / M-W	NA	152.10% / a	NA	147.90% / a
	10 (n=10) **23.38**	Mean ± SD	0.7360 ± 0.3920	0.793 ± 0.844	0.3053 ± 0.1811	0.359 ± 0.419
		Min - Max	0.3170 - 1.6630	0.075 - 2.741	0.1552 - 0.7982	0.029 - 1.390
		CV / M-W	53.25% / a	83.29% / b	59.31% / a	116.90% / b
	150 (n=12) **254.40**	Mean ± SD	0.4409 ± 0.2077	0.540 ± 0.671	0.0520 ± 0.0197	0.0572 ± 0.0661
		Min - Max	0.1222 - 0.7601	0.047 - 2.393	0.0308 -0.0947	0.0137 - 0.2292
		CV / M-W	47.11% / a	124.24% / b	37.90% / b	115.55% / c
	300 (n=12) 6**97.43**	Mean ± SD	0.2011 ± 0.1298	0.0986 ± 0.0747	0.0234 ± 0.0038	0.01200 ± 0.00736
		Min - Max	0.0503 - 0.4504	0.0244 - 0.2690	0.0181 - 0.0299	0.00668 - 0.03406
		CV / M-W	64.53% / b	75.79% / c	16.31% / c	61.31% / d
		K-W	<0.001	<0.001	<0.001	<0.001

Table 4.14. BF of Cr within total plant and arial structures of sunflower. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		TF Shoot/Root	TF Leaf/Shoot	TF Flower/Shoot	TF Seed/Shoot
Sunflower	0 (n=12) **2.03**	Mean ± SD	0.558 ± 1.049	0.649 ± 0.439	2.365 ± 1.679	0.815 ± 0.478
		Min - Max	0.007 - 3.722	0.069 - 1.525	0.415 - 5.941	0.085 - 1.620
		CV / M-W	188.00%	67.61%	70.97% / a	58.66% / a
	10 (n=10) **23.38**	Mean ± SD	0.435 ± 0.464	0.669 ± 0.575	4.808 ± 2.550	0.856 ± 0.374
		Min - Max	0.027 - 1.351	0.074 - 1.797	0.845 - 9.368	0.127 - 1.436
		CV / M-W	106.79%	85.83%	53.03% / b	43.72% /a
	150 (n=12) **254.40**	Mean ± SD	0.483 ± 0.608	0.343 ± 0.533	1.262 ± 1.661	0.1565 ± 0.1236
		Min - Max	0.031 - 2.164	0.010 - 1.744	0.248 - 6.342	0.0367 - 0.4066
		CV / M-W	126.08%	155.68%	131.53% / a	78.96% / b
	300 (n=12) 6**97.43**	Mean ± SD	0.0866 ± 0.0716	1.045 ± 1.598	1.839 ± 1.611	0.2483 ± 0.1854
		Min - Max	0.0166 - 0.2566	0.056 - 5.828	0.697 - 5.702	0.0961 - 0.6739
		CV / M-W	82.72%	152.93%	87.60% /a	74.65% / b
		K-W	0.133	0.070	<0.010	<0.001

Table 4.15. TF between sunflower structures. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Oat displayed the lowest uptake of Cr out of all the crops analysed only achieving higher that tomato at 300 mg/kg treatment as seen in figure 11. In wider research, oat also showed less phytoremediation potential then other cereal crops analysed, taking up low levels in the stem and a varying uptake in roots (López-Luna et al., 2009). Amin et al., (2019) stated that the levels taken up by the oat crop increased with increased chromium content in the soils within all structures. However, levels within the roots are consistently higher than the shoots (Mahmood-ul-Hassan, Suthar, Ahmad and Yousra, 2017). The same is not represented by the data collected (table 4.16) as both the shoot and seed structures showed no significant change as the levels of Cr increased with a slight decrease in Cr content in both structured between the 150 and 300 mg/kg treatments. This low level of uptake makes oat a poor candidate for the remediation of the contaminated site. BF of Cr into oat (table 4.17) confirms oat is not suited for phytoremediation with a large significant decrease in the BF especially in arial structures with a factor of 0.0028, showing very little is taken up within the arial structures, which although good for the crop as a food source, as a remediator makes the removal of the contamination difficult. Amin et al (2019) found result that different from those found in this research, with oat displaying one of the higher Cr accumulations of the crop plants tested, However, levels tested only increased to 75 mg/kg within the tests, as opposed to 10 times those levels within this study.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		Total Cr uptake (mg/Kg)	Root Cr Concentration (mg/Kg)	Shoot Cr Concentration (mg/Kg)	Leaf Cr Concentration (mg/Kg)	Seed Cr Concentration (mg/Kg)
Oat	0 (n=7) **1.72**	Mean ± SD	2.315 ± 0.359	0.896 ± 0.449	0.3674 ± 0.0723	0.5469 ± 0.0531	0.505 ± 0.270
		Min - Max	1.803 - 4.420	0.347 - 1.500	0.2884 - 0.5018	0.4710 - 0.5945	0.314 - 1.024
		CV / M-W	15.51%	50.11% / a	19.68%	9.72% / a	53.37%
	10 (n=7) **30.25**	Mean ± SD	5.26 ± 2.02	3.607 ± 2.064	0.4080 ± 0.2096	0.7930 ± 0.2633	0.4482 ± 0.1056
		Min - Max	3.03 - 8.67	1.735 - 7.138	0.1010 - 0.6745	0.5075 - 1.2554	0.3757 - 0.6725
		CV / M-W	38.35%	57.22% / b	51.37%	33.21% / ab	23.57%
	150 (n=3) **414.45**	Mean ± SD	31.64 ± 12.75	29.70 ± 12.66	0.4732 ± 0.0408	0.9542 ± 0.1517	0.5087 ± 0.1392
		Min - Max	16.99 - 40.29	15.17 - 38.35	0.4384 - 0.5181	0.8210 - 1.1194	0.3820 - 0.6578
		CV / M-W	40.31%	42.63% / c	8.62%	15.90% /b	27.37%
	300 (n=7) **787.72**	Mean ± SD	57.89 ± 21.15	56.07 ± 21.10	0.3934 ± 0.0605	0.9674 ± 0.2060	0.4608 ± 0.0831
		Min - Max	17.67 - 79.44	16.00 - 77.94	0.3240 - 0.4913	0.6369 - 1.3240	0.4300 - 0.5584
		CV / M-W	36.53%	37.63% / c	15.37%	21.29% / b	18.03%
		K-W	<0.001	<0.001	0.271	<0.010	0.787

Table 4.16. Uptake of Cr within the structure of oat at different treatments of Cr. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		BF Plant (Predicted)	BF Plant (Actual)	BF Arial Structures (Predicted)	BF Arial Structures (Actual)
Oat	0 (n=7) **1.72**	Mean ± SD	NA	1.488 ± 0.557	NA	0.909 ± 0.313
		Min - Max	NA	0.717 - 2.385	NA	0.444 - 1.218
		CV / M-W	NA	37.43% / a	NA	34.41% / a
	10 (n=7) **30.25**	Mean ± SD	0.5257 ± 0.2016	0.1836 ± 0.0763	0.1649 ± 0.0337	0.05845 ± 0.02328
		Min - Max	0.3025 - 0.8685	0.0991 - 0.2896	0.1175 - 0.2306	0.03358 - 0.10660
		CV / M-W	38.35% / a	41.57% / b	23.48% / a	39.83% / b
	150 (n=3) **414.45**	Mean ± SD	0.2109 ± 0.0850	0.07421 ± 0.01204	0.0129 ± 0.0007	0.004937 ± 0.001405
		Min - Max	0.1033 - 0.2686	0.06105 - 0.08465	0.0122 - 0.0136	0.004076 - 0.006558
		CV / M-W	40.31% / b	16.22% / c	5.63% / b	28.45% / c
	300 (n=7) **787.72**	Mean ± SD	0.1930 ± 0.0705	0.0881 ± 0.0671	0.0061 ± 0.0009	0.002815 ± 0.001655
		Min - Max	0.0589 - 0.2648	0.0346 - 0.2336	0.0050 - 0.0078	0.001469 - 0.006330
		CV / M-W	36.53% / b	76.25% / c	14.45% / c	58.79% / c
		K-W	<0.010	<0.001	<0.010	<0.001

Table 4.17. BF of Cr within total plant and arial structures of Oat. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

This lack of uptake within the arial structures is supported by the results of TF root to shoot, showing a mean TF of 0.085 into the shoots from within the root structures (Table 4.18). The majority of this seems to be subsequently translocated into the leaves and seeds of the plants with TF of 2.04 – 2.5 and 1.077 – 1.59 respectively.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		TF Shoot/Root	TF Leaf/Shoot	TF Seed/Shoot
Oat	0 (n=7) **1.72**	Mean ± SD	0.579 ± 0.368	1.5162 ± 0.1858	1.440 ± 0.895
		Min - Max	0.201 - 1.248	1.1643 - 1.7187	0.704 - 3.307
		CV / M-W	63.62% / a	12.26%	62.19%
	10 (n=7) **30.25**	Mean ± SD	0.1251 ± 0.0691	2.657 ± 1.945	1.587 ± 1.240
		Min - Max	0.0458 - 0.2386	0.770 - 6.587	0.557 - 4.048
		CV / M-W	55.19% / b	73.19%	78.12%
	150 (n=3) **414.45**	Mean ± SD	0.06927 ± 0.01339	2.043 ± 0.487	1.077 ± 0.299
		Min - Max	0.05449 - 0.08057	1.585 - 2.554	0.871 - 1.420
		CV / M-W	19.33% / b	23.81%	27.79%
	300 (n=7) **787.72**	Mean ± SD	0.0852 ± 0.0249	2.501 ± 0.563	1.201 ± 0.323
		Min - Max	0.0313 - 0.2273	1.406 - 3.037	0.890 - 1.723
		CV / M-W	77.14% / b	22.51%	26.91%
		K-W	<0.010	0.071	0.899

Table 4.18. TF between oat structures. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Maize showed significant increases in the Cr uptake levels with an increase of Cr treatment in root structures (table 4.19) Kacálková et al., (2014) found that the chromium taken up by the maize plant was accumulated within the root structures, with 50% of total accumulated chromium found in roots compared to 27% uptake within the leaves. It is apparent that the chromium uptake is highest within the root structures of the maize plant (Dheeba, Sampathkumar and Kannan, 2014), which is a similar picture to many crop plants that have been assessed for their phytoremediation potential. Zayed and Terry (2003) and Dheeba, Sampathkumar and Kannan (2014) also have stated that the root network of the maize plant act as a barrier to the translocation of chromium from the root structures giving a

form of control to the uptake of chromium even with no specific plant mechanism system in place for the transport of chromium within plant structures. No significant increase was observed within the shoots of maize, achieving a high of 3.766 mg/kg and the 300 mg/kg treatment (723.65 mg/kg actual). This contradicts findings be Mallick et al., (2010) who found concentrations in the stem of the plant exceeded 500 ppm, reaching highs of 4160 ppm. Seed Cr concentrations significantly increased with the introduction of the lowest Cr treatment but no significant difference between the increasing treatments.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		Total Cr uptake (mg/Kg)	Root Cr Concentration (mg/Kg)	Shoot Cr Concentration (mg/Kg)	Leaf Cr Concentration (mg/Kg)	Seed Cr Concentration (mg/Kg)	Tassel Cr Concentration (mg/Kg)
Maize	0 (n=11) **0.54**	Mean ± SD	3.524 ± 0.801	1.1983 ± 0.3290	0.5615 ± 0.0814	0.858 ± 0.660	0.4722 ± 0.1623	0.5198 ± 0.1319
		Min - Max	2.401 - 5.591	0.8141 - 1.8665	0.4401 - 0.6942	0.472 - 2.824	0.2749 - 0.7248	0.3376 - 0.6763
		CV / M-W	22.74% / a	27.46% / a	14.50%	76.99%	34.37% / a	25.38%
	10 (n=11) **28.61**	Mean ± SD	6.60 ± 2.22	4.063 ± 2.249	0.5685 ± 0.1203	0.7873 ± 0.1297	0.7602 ± 0.0834	0.4596 ± 0.1152
		Min - Max	3.89 - 10.05	1.304 - 7.806	0.4241 - 0.7896	0.5128 - 0.9791	0.6296 - 0.8986	0.3495 - 0.7450
		CV / M-W	33.64% / b	55.35% /b	21.17%	16.48%	10.98% / b	25.06%
	150 (n=10) **296.14**	Mean ± SD	29.95 ± 19.64	27.16 ± 19.68	0.652 ± 0.351	0.965 ± 0.362	0.8139 ± 0.1174	0.4438 ± 0.0761
		Min - Max	7.72 - 74.89	5.62 - 72.25	0.285 - 1.382	0.613 - 1.758	0.6720 - 1.0148	0.2970 - 0.5763
		CV / M-W	65.56% / c	72.47% / c	53.90%	37.51%	14.43% / b	17.15%
	300 (n=9) **723.65**	Mean ± SD	71.90 ± 36.90	69.0 ± 36.8	0.803 ± 1.144	0.7887 ± 0.1880	0.7987 ± 0.1519	0.4850 ± 0.1035
		Min - Max	19.10 - 112.50	16.6 - 109.1	0.241 - 3.766	0.5675 - 1.1514	0.4822 - 0.9732	0.3763 - 0.6981
		CV / M-W	51.37% / a	53.33% / d	142.41%	23.84%	19.02% / b	21.33%
		K-W	<0.001	<0.001	0.202	0.218	<0.010	0.611

Table 4.19. Uptake of Cr within the structure of maize at different treatments of Cr. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

BF of Cr into the structure of the maize plant showed significant decrease in BF into total plant until 150 mg/kg treatment (296.14 mg/kg actual) and no significant difference with a subsequent increase (Table 4.20). BF of arial structures showed a significantly negative difference, decreasing with each increase in Cr concentration. This is supported by findings in review by Aladesanmi, Oroboade, Osisiogu and Osewole, (2022) who found that roots of the maize crop immobilized Cr within them, causing increased levels as compared to aerial structures of the plant. TF shoot/root (table 4.21) showed a significant decrease in higher concentrations of Cr whereas a significant increase was seen in TF seed/shoot compared to control with a high mean of 1.97 at the highest concentrations. This

significant increase in translocation into from shoot seeds is supported by results found by Kacálková, Tlustoš and Száková, (2014) who found that higher concentrations of Cr within seed compared to shoots with levels of up to 2.32 mg/kg Cr.

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		BF Plant (Predicted)	BF Plant (Actual)	BF Arial Structures (Predicted)	BF Arial Structures (Actual)
Maize	0 (n=11) **0.54**	Mean ± SD	NA	7.134 ± 2.931	NA	4.655 ± 2.209
		Min - Max	NA	3.082 - 13.537	NA	2.287 - 9.611
		CV / M-W	NA	41.08% / a	NA	47.48% / a
	10 (n=11) **28.61**	Mean ± SD	0.6597 ± 0.2219	0.4015 ± 0.1700	0.2534 ± 0.0163	0.1644 ± 0.0780
		Min - Max	0.3893 - 1.0045	0.1889 - 0.7180	0.2239 - 0.2776	0.0745 - 0.3110
		CV / M-W	33.64% / a	42.34% / b	6.43% / a	47.45% / b
	150 (n=10) **296.14**	Mean ± SD	0.1997 ± 0.1309	0.1001 ± 0.0559	0.0186 ± 0.0041	0.009985 ± 0.002846
		Min - Max	0.0515 -0.4993	0.0366 - 0.2071	0.0137 - 0.0274	0.004816 - 0.012507
		CV / M-W	65.56% / b	55.81% / c	22.08% / b	28.50% / c
	300 (n=9) **723.65**	Mean ± SD	0.2397 ± 0.1232	0.1040 ± 0.0544	0.0096 ± 0.0038	0.004506 ± 0.002994
		Min - Max	0.0635 - 0.3749	0.0207 - 0.1957	0.0059 - 0.0189	0.002391 - 0.011951
		CV / M-W	51.37% / b	52.34% / c	39.43% / c	66.45% / d
		K-W	<0.001	<0.001	<0.001	<0.001

Table 4.20. BF of Cr within total plant and arial structures of maize. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Crop	Concentration (mg/Kg) **Bold = Measured concentration**		TF Shoot/Root	TF Leaf/Shoot	TF Seed/Shoot	TF Tassel/Shoot
Maize	0 (n=11) **0.54**	Mean ± SD	2.479 ± 1.239	1.541 ± 1.160	0.869 ± 0.345	0.9497 ± 0.2994
		Min - Max	0.795 - 5.550	0.897 - 4.949	0.415 - 1.375	0.5564 - 1.4252
		CV / M-W	50.00% / a	75.30%	39.66% / a	31.53%
	10 (n=11) **28.61**	Mean ± SD	0.2371 ± 0.1390	1.431 ± 0.342	1.3880 ± 0.3228	0.872 ± 0.342
		Min - Max	0.0751 - 0.5579	0.864 - 1.907	0.9284 - 1.9893	0.451 - 1.589
		CV / M-W	58.62% / b	23.89%	23.26% / b	39.20%
	150 (n=10) **296.14**	Mean ± SD	0.0901 ± 0.0563	1.774 ± 0.961	1.507 ± 0.752	0.864 ± 0.419
		Min - Max	0.0262 - 0.1998	0.825 - 3.667	0.486 - 2.713	0.215 - 1.431
		CV / M-W	62.52% / c	54.18%	49.91% / ab	48.44%
	300 (n=9) **723.65**	Mean ± SD	0.0995 ± 0.0537	2.065 ± 1.044	1.965 ± 1.002	1.216 ± 0.583
		Min - Max	0.0183 - 0.1916	0.167 - 2.990	0.239 - 3.722	0.813 - 1.974
		CV / M-W	54.00% / c	50.53%	51.00% / b	47.91%
		K-W	<0.001	0.247	<0.050	0.320

Table 4.21. TF between maize structures. Kruskal-Wallis for significance is displayed. Significant results had subsequent Mann-Whitney test carried out. Results not sharing letters indicate significant difference

Ideal candidate species for remediation of the Tannery Belt Site

Ruling out wheat due to insufficient data collected during this study, the 5 remaining crops all displayed different affinities for resistance and uptake of the Cr contamination they were exposed to. Sorghum demonstrated the most promising attributes with the highest Cr uptake out of all crops tested and maintained biomass in the majority of its structures. The World Health organisation states a permissible limit of Cr within edible plants to be 1.3 mg/kg (Ogundele, Adio and Oludele, 2015). All seed and fruit structures showed levels below this level however maize, and oat showed the highest levels of uptake into these structures. The limit on ingestion of Cr changes depending of the proportion of Cr (III) and Cr (VI) with limits being between 1.5 and 0.003 mg/kg a day respectively (Agency for Toxic Substances and Disease Registry, 2012). As such, further analysis into what form the Cr is taken up into the structures would better inform further research for this study.

Tomato, sorghum and sunflower showed good uptake into arial structures at higher Cr contamination levels than that of oat and maize. Tomato, sorghum and sunflower also showed BF >0.1 at levels up to the 150 mg/kg treatment (between 250 – 310 mg/kg) representing an uptake in these areas of 10% of the total and of around 9% in the highest contaminated areas for tomato and sunflower and 0.381 or just below 40 % for sorghum. Due to the nature of this remediation, the target BF for a target remediator does not need to be >1 as is thought of to be the case for metal accumulators (Parihar, Parihar, Pakade and Katnoria, 2020). Instead, with the target of this study to identify crops that can be grown on the contaminated site while providing economically and as a food supply, a lower and longer remediation process would still make a significant difference to the local population who leave large areas of land empty due to worry with the contamination of the area.

As such the suggested crops to be utilised for further research into their suitability for the remediation process of the Tannery Belt in Dindigul would be the implementation of tomato and sunflower crops at low and medium levels of Cr contamination owing to their ability to take up medium levels of Cr contamination without accumulation within edible structures at risk levels at these Cr levels. The areas that reach into the high Cr contamination levels to be cover cropped with sorghum to allow for sustained remediation of the pollution with the higher BF of sorghum identified at these levels allowing for a quicker potential remediation of the site.

Conclusion

The literature review revealed that phytoremediation crops are a target for research within chromium contaminated areas, however many of the crops that were examined didn't have extensive research by many different researchers. For research in this area to be utilised applications such as phytoremediation of chromium using crop plants can not only be utilised as a management tool for contaminated sites but also deemed to be safe for the populations making a living off and eating the crops, more extensive and diverse research is needed to be carried out.

Looking at the data that has been summarised in this study many gaps can be identified. Several species examined in the literature had very little research on chromium uptake, although some of the published work does show promising results. As these are major crops and could be ideal phytoremediators, more research is needed. In addition, the experimental methods could be improved. Many of the studies focus on the uptake in juvenile plants and only quantify uptake within roots and shoots, but research needs to determine accumulation within other plant structures particularly in edible tissues. Studies are often very short (less than the plant life cycle) and researchers rely heavily on results from pot experiments. Most experiments only investigated chromium uptake despite real-life scenarios generally involving a number of contaminants at the same site. Moreover, studies used different chromium compounds and varied in how they applied chromium (e.g. as a solid or as an aqueous solution).

Out of the six crop plants selected from the review of Cr uptake in crop plants, wheat, maize and oat were discounted due to a combination of insufficient evidence, lack of sustained growth at higher levels of Cr contamination and highest levels of sequestration of Cr into the edible structures of the plant. This made them unsuitable for the intended task. Tomato and sunflower showed promise, with sustained biomass at increased Cr levels and increased levels of Cr uptake at higher levels without increases into edible structures. Sorghum showed the most promise, displaying maintained biomass across all levels of Cr, the highest BF at all levels of Cr in the plant including the highest treatment, and levels within the edible structures that fall below permissible limits for edible plants.

Further research into the forms of Cr being taken up by these crop plants while being grown on Cr contaminated soil to fully indicate the potential health risks that could be linked with the ingestion of the edibles.

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5. Conclusion

Overview of findings

This study was conducted in order to investigate effects of soil contamination from tannery effluent on the agricultural land of the Tannery Belt, Dindigul, Tamil Nadu. The study aimed to (i) determine the extent of the contamination around the Tannery Belt and (ii) understand the relationship between heavy metal contaminants and other soil physical and chemical properties and to what extent have these caused desertification of the site to occur. It also aimed to (iii) investigate how tannery contamination altered the soil microbiome, focussing on the identification of pollution-sensitive organisms (potentially resulting in a loss of ecosystem functioning) and pollution-resistant organisms (potentially leading to future work on the use of these organisms to assist with phytoremediation). In addition, the study aimed to investigate land management approaches by investigating uptake of chromium in pot experiments to (iv) determine crop plants that do not bioaccumulate chromium in quantities considered dangerous to human health that can therefore be recommended as safe food crops. Finally, using the crop plants found to be safe for consumption, the final aim is to (v) identify which safe crop plants also bioaccumulate over a minimum threshold to make this a timely, cost-effective and viable remediation method.

This study produced the following key findings based on these five aims described above:

(i) Determine the extent of the contamination around the Tannery Belt

Heavy metal contamination of Cr, Pb, Cu, Cd, Zn were all identified in the vicinity of the Tannery Belt, Dindigul, Tamil Dadu, in levels that exceeded regulatory levels as determined by the European Union and thus are likely to be a risk to human health and the soil microbiome. Contamination Indexes indicate moderate to high contamination reading for all metals (Cr, Pb, Cu, Cd, Zn) at sampling sites with contamination degree showing 5 field sites with medium to very high contamination of the land. In addition, salts, including sodium and phosphate, were also found in high concentrations that would be considered detrimental to soil health.

(ii) Understand the relationship between heavy metal contaminants and other soil physical and chemical properties

Significant correlations between heavy metals and several soil properties were found including pH, electrical conductivity, organic matter, and soil moisture. This is evidence that tannery contamination doent only affect heavy metal levels but can have indirect effects on soil physical and chemical properties. This will lead to degradation of the soil and reductions ion soil health as soil properties have major effects on plant health and soil organism communities. NDVI shows increasing of foliage cover in the local area, indicating a reduction in desertification risk. Although it was expected that this change in soil quality would lead to desertification the finding shows this not to be the case suggesting soils can be rejuvenated and have not reach strong or extreme desertification where land is not able to be restored or reclaimed as shown by Wild (2003).

(iii) Investigate how tannery contamination altered the soil microbiome, focussing on the identification of pollution-sensitive organisms (potentially resulting in a loss of ecosystem functioning) and pollution-resistant organisms (potentially leading to future work on the use of these organisms to assist with phytoremediation)

Chromium-rich tannery contamination significantly effects the soil microorganism population diversity, abundance and similarity for organisms identified via 16s, ITS and 18s sequencing.

Bacteria *Thermomicrobiales* and *Tistrellales,* fungi *Eurotiales* and *Capnodiales*, nematode *Rhabditida* and protozoa *Phytomyxea* showed evidence of resistance to increased levels of Cr contamination.These show potential for future research into microorganisum assisted phytoremediation. Bacteria *Tepidisphaerales,* fungi *Hypocreales,* nematode *Dorylaimia* and *Tylenchida* and protozoa *Gregarinasina* all showed evidence of pollution sensitivity, reducing their abundance and impacting potentially important roles within the soil microbiome such as nutrient cycling and the availability of nutrients to plants.

(iv) Determine crop plants that do not bioaccumulate chromium in quantities considered dangerous to human health that can therefore be recommended as safe food crops

Tomato, sunflower, sorghum, maize and oat demonstrated uptake of Cr into edible structures that fall below permissible limits for edible crop plants at all levels of Cr contamination. Wheat did not grow to seeding stage and so could not be assessed. Permissible limits for edible plants of Cr are 1.3 mg/kg (Ogundele, Adio and Oludele, 2015). Tomato, sunflower, sorghum and maize showed seed/fruit levels of below 0.4 mg/kg within the highest levels of Cr whereas oat showed levels of 0.46 mg/kg. These levels are below permissible limits and show all identified crops to be a safe food crop limits.

(v) Identify which safe crop plants also bioaccumulate over a minimum threshold to make this a timely, cost-effective and viable remediation method.

Tomato, sunflower and sorghum all showed promise as candidates for the remediation of the tannery belt site, Dindigul. Sorghum showed the highest levels of uptake out of the three identified crops. All crops showed highest levels of Cr uptake to within root structures with all showing low levels of uptake within aerial structures. This is not ideal owing to difficulties with the total removal of root structures during harvesting. Tomato and sunflower showed highest bioaccumulation factor of Cr at low and medium levels of Cr contamination (between 0.09 and 0.3), indicating these levels are best for their implementation as remediators. Sorghum showed the highest bioaccumulation factor (0.36) at high levels (700 mg/kg) of Cr and as such would be suited to be grown in these environments as a remediator. Although the bioaccumulation factors for these species are not as high a studies may aim for phytoremediators (>1), within this study the identified BF are ok due to the need for the crops to have a second purpose of food crop and economic benefit.

The conclusion chapter will discuss the implications of the key findings of this study in a broader context to demonstrate their importance for potential management and remediation of tannery-contaminated land, including the field site in this study: the Tannery Belt, Dindigul, Tamil Nadu. This will be discussed in the context of the five aims described above.

Extent of tannery related contamination across the tannery belt tannery site (aim (i))

India has a lack of sufficient regulations relating to permissible limits of several heavy metals (Ganeshkumar, Arun, Vinothkumar and Rajaram, 2019) compared to other regulatory bodies. As a

result, studies on contamination in India are forced to use guidelines based on legislation from other countries: for example, Narsimha, Qian and Wang (2019), Dogra et al. (2019) and Tamele and Vázquez Loureiro (2020) quote regulations from Canada, Sweden and The European Union, respectively. The European Union set out clear permissible limits for concentration of all the heavy metals that were examined within this study (European Union, 2006) and were therefore used to determine human health risk in this study. At tannery sites, all heavy metals studied (Cr, Zn, Cd and Cu) were found at levels that exceeded the EU permissible limits for soils at locations across the site however only Cr and Pb showed significant differences from the background controls. Cr showed concentrations 77 times the permissible limit of 100 mg/kg at its peak, Zn was 13 times the permissible limit of 300 mg/kg and was consistently above permissible limits across the site, Cd was 32 times permissible limit of 3 mg/kg at a few locations across the site and Cu was 2.5 times the permissible limit of 100 mg/kg at 3 locations. Levels of heavy metals across the site including background were elevated, this could be the result of the use of the tannery effluent in the surrounding area (Barajas-Aceves, 2016) or through leaching of the pollution.

With the utilisation of pollution indices, levels of contamination across the tannery belt site were deemed to be moderately to very highly contaminated. Using the contamination factor index, all heavy metals displayed a contamination factor of moderate or higher across the tannery belt, however Cr was the most concerning contaminant out of the four heavy metals analysed, where all sites showing a contamination factor of moderate of very high. Results of the geo-accumulation index differed depending on whether the background levels or permissible limits were used. Contamination of the site was indicated as higher with the use of background readings for the area compared to that of the permissible limits, with Cd being the only exception. Thus, it is difficult to compare these results with other studies that calculated a geo-accumulation index since the way that previous researchers calculated the geo-accumulation index differs widely between studies (Ahmed et al., 2019; Sulaiman et al., 2019; Rubasinghe et al., 2021). However, there seems to be a larger consensus on the use of background levels rather than permissible limits, with recent research utilising geo-accumulation index using the geochemical background values as the source for their background values, selecting to use average levels for the area (Rubasinghe, Gunatilake and Chandrajith, 2021; Tamrakar, Nair and Chatterjee, 2021; Zhao, Liao, Zhou and Zhou, 2022).

Pollution indications from contamination factor and geo-accumulation indices indicated that the most heavily contaminated areas of the site were situated near an important water source for local villages, with Cr, Zn, Cd all identified as being present at levels above EU permissible limits (European Union, 2006) and Na and K being also consistently high across the site. Mishra et al., (2018) has indicated that the accumulation of heavy metals into water can be attributed directly to the discharge of waste from

industrial processes, and Karthika et al. (2020) stated that contamination of water sources such as wells and surface water as a result of tannery pollution is an issue within the area of Dindigul. From the results determining the extent of pollution of tannery related contaminants situated around the tannery belt, areas to the North-West of the site showed the highest levels of combined contamination across the site. Thus, this area - and the lake mentioned previously - should be the main target for phytoremediation efforts in order to reduce the high levels of heavy metal and salt contamination that could be contaminating water sources and causing an increased risk to the health of the local population and ecosystem.

High levels of salts (Na and K) were found within the contaminated sites; however, K was found to be below permissible limits of 80 mg/kg (Raman and Narayanan, 2008) but Na was abover levels ddemed as high within research by 5 times (Krishnasamy, Bell and Ma, 2014). Both Na and K were correlated with the heavy metal contaminant, demonstrating that due to the mix of chemicals used within the tanning process that include heavy metal containing salt compounds, the areas surround the tanneries are impacted by contamination of both heavy metals and increased salinity. There were also significant correlations between Cr contamination levels with all tested contaminants and soil properties, excluding total nitrogen, with pH, electrical conductivity, organic matter and soil moisture all exhibiting positive correlations with Cr.

Effects of tannery pollution on the environment (aims (ii) and (iii))

Heavy metal pollution was found to have a significant effect on the quality of the soils in the tannery belt sites. Heavy metals are known to reduce soil quality, both in terms of physical and chemical soil properties as well as the soil biology in the form of the soil microbial communities (Burges, Epelde and Garbisu, 2015; Lwin et al., 2018; Mamehpour, Rezapour and Ghaemian, 2021). The findings of this study support this, where the majority of soil properties had a significant correlation with the heavy metal pollutants within the soil medium. The pH, electrical conductivity, and soil moisture content of the soil were all higher in soils with high heavy metal concentrations, including for chromium. Unceta et al. (2010) found that soils with a high pH also had higher speciation of Cr into Cr (VI). Although the chromium salts that are used in the tanning process are mostly in the form Cr(III) (Esmaeili, nia and Vazirineja, 2005), it has been noted that Cr within the tanning wastes produced is often converted to the more hazardous form, Cr(VI) (Erdem, 2006). This indicates that in the sites of high Cr and high pH, there may be an even greater risk to the local ecosystem and the health of the local population due to the form of chromium found in the soil. Samples with high electrical conductivity also tended to

have high levels of Na and K within the soil samples, which is to be expected since electrical conductivity is often used as a proxy for salinity. With Cr salts being heavily used in the chrome tanning process of leather, especially chromium (III) sulphate (Prokein, Renner, Weidner and Heinen, 2017), these relationships are a strong indication of tannery pollution.

Based on field observations at the site and findings other studies in the local area indicating the desertification and drought in the are at or near the field sites (Prawin and Masilamani, 2020; Thilagaraj et al., 2021), it was hypothesized that tannery pollution would have caused desertification. However, NDVI findings showed that within the tannery belt site and to the south of Dindigul where the majority of farmland in situated, the vegetation cover/density has actually increased over a 12 year period. This is widely used as an indicator for desertification and shows that the area studied is not currently going through desertification but is instead repopulating the disused areas of farmland and scrubland with new foliage. This is promising when looking at phytoremediation studies as it suggests the sites are able to be rejuvenated and are not destroyed indefinably.

The presence of tannery contamination was found to influence the soil community structure and the similarity of soil communities in tanneries compared to controlsAlpha and and beta diversity indexes significantly showed that in the presence of tannery pollution, there was a significant difference in the fungi and 18s communities. However, there was no significant change in the bacterial community diversity between control and contaminated samples. This in contrary to findings that increases in Cr pollution, such as that in the polluted samples has had a negative correlation with bacteria communities but not that of fungi (Zhang et al., 2021). It was found however that similarity between all three communities tested was shown to have a been significantly affected by the presence of tannery pollution contamination. It is also worth mentioning that samples that displayed high levels of Cr showed a closer similarity to each other in all 16s and ITS and 18s communities. . The relative frequencies of phyla identified through soil DNA sequencing and analysis showed that although many of the bacteria, fungi, nematodes and protozoa where species were found in both the tannery and control samples, the frequencies differed. Bacteria *Thermomicrobiales* and *Tistrellales,* fungi *Eurotiales* and *Capnodiales*, nematode *Rhabditida* and protozoa *Phytomyxea*, showed evidence of resistance of increased levels of Cr-rich tannery contamination. Bacteria *Tepidisphaerales,* fungi *Hypocreales,* nematode *Dorylaimia* and *Tylenchida* and protozoa *Gregarinasina* all showed evidence of pollution sensitivity, reducing their abundance and impacting potentially important roles within the soil microbiome The effects of pollution on the pH and electrical conductivity can also affect the activity of soil microorganisms with Kamal, Prasad and Varma (2010) stating that increases of pH

within soil environments can have a detrimental effect of certain groups of soil organisms, This would cause issues for future plans for remediation on the site, with the activity of soil microorganisms being heavily correlated with these soil properties.

Management of tannery-polluted sites (aims (iv) and (v))

The overall aim for the study was to identify suitable remediation methods for the area contaminated by tanneries in Dindigul that has been abandoned by the local agricultural community due to the contamination levels and perceived health risks from it, as well as for other similar sites. A combination of contamination levels, soil quality, effects on the soil microorganism and suitability of local and scientifically tested crop plants against the highest-level contaminant on site Cr has allowed for the suggestions to inform future research and land management practice at the Tannery Belt and for other chromium-contaminated sites. From the three main studies conducted in this project, several conclusions as to suggestions for remediation of the site and further research into suitability of organisms for the task have been drawn.

Tomato, sunflower and sorghum were identified as having a high potential for remediation of Cr. Contaminant accumulation by these crops was not at levels normally required to class as an appropriate a hyperaccumulator plant, where a BF or TF >1 is desirable (Nouri et al., 2010). However, the purpose of these crops is twofold: to not only remediate Cr and other heavy metals, but also to provide a secondary use as a food crop for the local population and thus a source of income.

Of the edible plant parts, the tomato fruit and sorghum seeds did not show significant bioaccumulation of chromium, but sunflower seeds had significantly higher chromium in their seeds in the elevated chromium treatments. Having said that, both tomato, sorghum and sunflower demonstrated levels of Cr contamination within the seed and fruit structures of less than the permissible limits of Cr within edible plants of 1.3 mg/kg (Ogundele, Adio and Oludele, 2015) and so can all be used as a potential phytoremediator. With all this data considered, suggestions as to suitable planting can be made for the contaminated site if research into proportion of Cr (III) and Cr (VI) is carried out and identified. The planting of tomato and sunflower crops for remediation within the areas of the tannery belt would be best suited for sites that fall within the low to medium pollution range. This would include areas along the main connective road, as well as to the south, southwest and east sides of the sampled areas. This would allow for the most effective remediation of the area with native crop plants, with the potential of providing for the local area as well. Sorghum, with its increased uptake of Cr at the higher levels of contamination compared to all other crops tested, would

be of benefit for cropping in the area due south of the tannery belt, as well as the highest contamination areas to the northwest of the site where Cr and other heavy metal contamination is at its highest levels.

If successful, crops can be subsequently rotated after annual testing of soil contamination levels to determine the best crop planting areas at the time. With the selected crops in place, low and medium levels sites for Cr could be remediated in a minimum of 2 years by sunflower or tomato, and highest levels in a minimum of 3 years with the planting of sorghum. However, these predictions are based on BF and it should be noted that the levels of uptake will differ year-on-year and will most likely decrease as the Cr concentration decreases over time. In addition, since bioaccumulation is high in the roots of the crops studied, remediation projects in the field would need to consider the possibility of harvesting the crop in it's entirety, including the root system, in order to remove as much contaminant as possible during each harvest.

Several different soil microorganisms from bacteria, fungi, nematode, and protozoa were identified as potentially resistant organisms to the varying pollution within the tannery polluted soils, with a number showing increasing abundance within the contaminated soils as compared to the background control soils. The implementation of such microorganisms within the remediation of the tannery belt site could allow for increased effectiveness by the crop plants if any inferred resistances of benefits for the plants can be deduced. Saranraj and Sujitha, (2013) discuss how the presence of microorganisms within tannery effluent, have been shown to be able to reduce the levels of Cr, with the higher the pH, the more reduction of the Cr be bacterial species. Within the tannery polluted soils, 7 orders of bacteria, 5 orders of fungi, 4 orders of Nematode and 4 protozoan orders showed promise to resistance of the levels of Cr contamination. These levels of apparent resistance varied between the orders identified. The bacteria *Thermomicrobiales*, the fungi *Pleosporales*, *Hypocreales* and *Glomerales* and the nematode *Rhabditida*, have all demonstrated assisted phytoremediation in another research. The bacteria *Kallotenuales* and KD4-96, the fungi *Eurotiales* and *Capnodiales,* and the protozoa *Glissomonadida*, although not previously demonstrating assisted phytoremediation, have all been previously identified as resistant to industrially contaminated soil environments.

The Orders of microorganisms that have been seen to assist in phytoremediation previously are obvious candidates for potential assistance to the tomato, sunflower and sorghum crops proposed for the tannery belt remediation project. *Thermomicrobiales, Pleosporales*, *Hypocreales* and *Rhabditida,* all showed above 25% relative frequencies at the higher extremes of Cr contamination at the tannery sites. This leads them to be suitable for further research into and assisted phytoremediation partnerships between these organisms and sorghum to ether increase the crops efficiently of uptake

of the contamination at the site or increase the overall health of the crop through inferred resistances, with the intended outcome to be an improved yield of the crop. *Glomerales* showed relative its highest relative frequencies at the 100 mg/kg levels of contaminated soil. This would make these fungi more of a target of assisted phytoremediation at low level contamination sites with ether tomato or sunflower crop. Tests to further determine the compatibility and potential of inferred assistance should be carried out of the stated organism initially.

Out of the Orders of microorganisms that have previously shown resistance abilities to polluted soils in previous research, KD4-96, *Capnodiales* and *Glissomonadida* show affinities to be present in in higher levels of Cr contamination within the tannery belt site where, *Kallotenuales* and *Eurotiales* showed affinities for levels around the 200 mg/kg level of Cr contamination. Analysis as to determine and inferred assistance to resident crop plants should be carried out to determine if these organisms could assist in the project going forward. The remaining identified Orders including bacteria *Tistrellales, Peptostreptococcales-Tissierellales* and *Tepidisphaerales*, the fungi order *Sordariales* (known to assist in nutrient uptake and plant growth (Barberis, Michalet, Piola and Binet, 2021), the nematode order *Dorylaimia, Tylenchida* and *Araeolaimida,* and the protozoan order *Gregarinasina, Conthreep,* and *Phytomyxea,* all showed affinities for Cr resistance but majority have not had research carried out upon them with regards to the abilities to resist contaminants or to assist with phytoremediation or beneficial effects on plants. All demonstrated an ability within this study to resist elevated levels of Cr up to the highest levels of contamination of Cr at the site. This indicates the potential identification of microbiome Order of bacteria, fungi, nematode and protozoa with potential resistance mechanisms to tannery pollution including Cr that have previously not been identified within scientific research previously.

Study limitations and suggestions for future work

Although the study provides a comprehensive insight into the extent of tannery contamination and provides suggestions for the remediation of the contaminated site, there are a number of limitations to the study and suggestions for future work, as described below:

1. Experimental setup

The initially intended sample dosage within the phytoremediation pot experiment was intended to be 0 mg/kg, 10 mg/kg, 150 mg/kg and 300 mg/kg based of levels selected from the chapter 2 extent of contamination study. However, the Cr concentrations achieved from the readings after the conclusion of the pot experiment displayed levels of more than double the intended value within all the crop plant soils. However, the experiment was still able to investigate the effects of low, medium, and high concentrations of chromium compared to controls and the concentrations achieved in the experiment were still comparable to the chromium levels found across the tannery belt site. It brings into question the accuracy of the reagents and equipment that where available for use in the project as this could have had an effect on the final concentrations of the solutions added. However, this appears to be a common problem encountered by researchers spiking soils with heavy metals. It has been suggested by other researchers that the addition of contaminants to a soil via a liquid-based addition can cause issues with homogenisation of the contaminant, even when thorough mixing is carried out. The addition instead of a finely ground powder version of the potassium dichromate and the mixing of the substrate using a more intensive mixer could help with this issue. A greater concern is that the majority of bioaccumulation studies do not appear to analyse the soil to ensure the desired concentration si the same as the actual concentration, which brings the validity of these studies into question.

As well as examining the effects of different concentrations of chromium, future research should also consider the form of Cr being used; for example, Cr(III) is likely to be less harmful than the same concentration of Cr(VI). In fact, the recommended daily allowance (RDA) for the intake of Cr is dependent on its form: Cr(III) RDA is 1.5mg/kg whereas Cr(VI) RDA is 0.003 mg/kg ; thus, the RDA for Cr(III) is 500 times higher than for Cr(VI) (Agency for Toxic Substances and Disease Registry, 2012). Inconsistencies in Cr uptake studies regarding form of Cr and Cr compounds used within pot experiments makes the comparability of result between studies difficult. Consistency is required amongst research in this area so that the full extent of contamination can be assessed. BF for tomato and sunflower were at their highest levels at the low treatments of Cr but remained high at medium treatments of Cr. Sorghum demonstrated the best ability to phytoextract Cr from within the soil treatments maintaining a BF of 0.381 at the highest levels of Cr contamination which decreased at lower levels of Cr. Sorghum also showed

the lowest TF of all the crop types at the highest levels of Cr from shoot to seeds and displayed levels in the seed below permissible limits.

It is worth noting that initial plans before the COVID-19 global pandemic this project aimed to plant phytoremediation field trial and community garden, carried out in subsequent years after the first year of initial sample collection. This would have consisted of a contaminated site and control site being planted up with crops to determine the uptake of Cr in soils that were identical to the soils that would require remediation as a target for this study. This would have been used to test the extend of crop health and uptake of pollutants within the soils that would need remediating and would then have been opened to the public once analysis had taken place. However, with a small selection of crop plants now selected through the research carried out in this study, the most obvious next step is to conduct field trials within the contaminated areas of the Tannery Belt (and/or other tannery-contaminated sites) to assess the extent of Cr phytoremediation in field conditions in order to determine their true potential for phytoremediation. However due to complications cause by the outbreak of the Covid-19 pandemic globally, the study had to be changed to allow for the carrying out of the study under stringent and regulated Covid-19 safety precautions. As such a pot experiment was derived to determine the effect of Cr on the indicated crop plants but model soil and seeds from Dindigul were also unobtainable due to restrictions in shipping put in place by the Indian government.
The collection of samples during the growing seasons of the proposed crop plants analysed within this study would have provided useful data on the availability of contamination including Cr, as well as show any seasonal change in the soil microbiome populations, so that these can be accounted for during the interpterion of the results during analysis.

It would be useful for the sampling and analysis of the contaminated soil at different times throughout the year as well as yearly to determine the effects of the seasons on the contamination levels and to see if levels are remaining constant or shifting across the site on an annual basis. This would allow for a more accurate interpretation of suitable crops to be grown as if availability of contamination changes over the course of a year, crops more suited for growth during the indicated season would be of more value to the project.

Due to the cost of DNA sequencing being relatively high, sample libraries had to be combined for the different organism target primers (16s, 18s and ITS). A protocol with Fera Science Ltd was devised in order for the primers to be subsequently separated out from each other prior to the demultiplexing and subsequent analysis of the OTUs. However, the number of unassigned readings compared to the running of libraries separately was higher, meaning a slight loss in the number of hits for each of the primers of about 3%. This is simply an issue with the amount of funding available and the study could be made better by the running of the libraries separately and getting a slightly more complete view of the bacteria, fungi, nematodes, and protozoa present. Since the loss was relatively small, this protocol does demonstrate a mor cost effective analysis of multiple OTU's within the same sample set. It would have been ideal to have had the additional resources to run addition primer sets to analysis more of the soil microbiome as what was run only symbolises a fraction of the diversity of organisms within the soil.

Since this research focusses on agricultural land and potential crops that could be grown in polluted areas, there are several additional variables that could be recorded to get a more comprehensive understanding of the effects of chromium on agricultural crops. The addition of growth measurements, flowering times and fruiting times for each crop would produce valuable information for local farmers.

The implementation of a field study to determine the extent of phytoremediation that the selected crop plants had within the target contaminated soils would allow for a clearer analysis as to suitability of the crops for the project. This would allow for the intended soil type as well as the presence of the complex mix of contamination precent in the tannery contaminated soils to be present and would allow for a crop plant suitable for the desired environment to be selected.

To determine the effects of the selected microorganisms of interest and their extent as potentials for assisted phytoremediation, a suggestion of experiments relating to extent of resistance of each microorganism to determine its tolerance to chromium as well as other pollutants including heavy metals and salts. This would allow for a more in-depth analysis on their suitability to the target soils. There testing with the target crop plant to analyse if there is an effect on the uptake of contaminants as a result of the addition of the microorganisms would also be of interest to the future of this study. Future research

could also identify target species from the identified orders of microorganisms that would be of use for the intended project.

2. Constraints with data collection

Resolution of the sampling sites across the tannery belt study area meant that several large areas across the site were left unsampled due to access issues and short length of time situated at the site. This could impact the resolution of the spread of the contamination across the sampled site as well as cause peaks of contamination to become diffused within modelling due to more consistent lower levels of contamination. However, this is a common issue with many studies and the spatial resolution was greater than in many GIS-based studies that implement heat mapping

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