Investigation of levels of some heavy metals (Cr, Cu, Zn, As, Se, Cd, and Pb) present in cattle fodder, water, milk, hair and manure in Malta

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To my late parents' souls

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Abstract

The objective of this study was to assess the levels of seven heavy metals, four essential elements, Cr, Cu, Zn and Se, and three toxic elements, As, Cd and Pb, through the life cycle of cattle from the dairy cattle farm in Malta, to determine the pollution levels, by using two different equipment. The first technique used was Atomic Absorption Spectrometry (AAS) in which 110 milk, 110 hair, 15 fodder, and 11 manure samples were collected in 11 weeks, started at the last week of September and finished in mid-December, and their heavy metal concentrations were determined for Cu only. The conventional method was used to prepare all these samples. Cu concentration in milk samples showed high levels in each animal separately during this study's period, which was found to be higher than the maximum level of 0.010mg/l, in accordance with FAO & WHO. The concentration was significantly high in Autumn, whereas it reached its lowest in Winter.

On the other hand, the hair samples results had much higher concentrations than those in milk. The Cu concentration was relatively affected by colour. The highest Cu concentration was noted in black haired cows, while the cows with brown hair colour presented similar results but less than the black-haired one. In addition, Cu concentration in fodder showed that there is a significant difference between several types of fodder. Moreover, in manure samples the highest Cu concentration was in week 5, while the lowest was noted in week 7. Manure, fodder and hair had no significant seasonal effect.

The second technique, Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES), was used on all seven heavy metals for a period of one year for all samples, except for hair which had a period of 8 months instead, to observe the seasonal effect. The microwave digestion preparation method was used. Cr resulted to have the highest seasonal effect, for all types of samples, during Winter, Spring and early Summer, with its highest concentrations being 0.880]] ppm in fodder, 0.720 ppm in hair, and 0.027 ppm in milk. Cu had the highest seasonal effect during the seasons of Winter and Spring for all samples and was also high in early Summer in hair. Its concentrations were 17.9 ppm in fodder, 10.9 ppm in hair, and 0.213 ppm in milk. Zn was found to be predominant in all samples during the season of Winter, along with milk and hair also having high levels during Summer, with concentrations of 63.1 ppm in fodder, 149 ppm in hair and 6.81 ppm in milk. As had the most seasonal effect during Summer or Winter, with the majority being in Summer. The concentration in fodder was 0.780 ppm, 1.22 ppm in hair, and 0.181 ppm in milk. Se reached seasonal effect during each season except for Winter, with most elements being mostly affected in Spring and Summer. Its highest fodder concentration was 0.740 ppm, hair was 0.130 ppm and milk 0.113 ppm. Cd concluded with seasonal effect being mostly present during Spring, except for Manure, which was highest during Winter, with concentrations of 0.080 ppm in fodder, 0.060 ppm in hair and 0.017 ppm in milk. The overall concentrations of Cd were lower than the permissible limit of 0.005 ppm. Lastly, Pb, resulted in being affected in each season, except for Autumn, with concentrations of 0.380 ppm in fodder, 0.130 ppm in hair and 0.007 ppm in milk. Pb was found to be higher than the permissible limit of 0.020 ppm. With regards to water, all samples were seasonally affected, however, their concentrations remained within the permissible limit. As for manure, it had the highest concentrations out of all the inputs and outputs, which is a positive result since manure does not directly affect the human food chain.

The analysis of all the seven heavy metals’ concentrations in milk were determined using two digestion methods and comparing them for the preparation of samples. It was concluded that good recovery was achieved for both methods, in which method 1 presented higher concentrations in all elements, except for As and Se. Zn presented no changes. Furthermore, a comparison between black and white hair was carried out, resulting in no noticeable significance, except for a mild difference in Cr.

This study concluded that the presence of some heavy metals’ concentrations are alarming indicators of high level of pollution in Malta.

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1. Introduction

1.1 Pollution

Natural environments can be impacted adversely by contaminants, which may provide various forms of pollution. This is not solely limited to chemical contaminants but may take the form of noise, heat, or light within particular situations. Pollutants themselves can either be naturally occurring or derived from anthropogenic sources. Chemical substances can be a form of pollution which may impact both the environment and human health depending on their composition. There is a need to understand how pollution occurs, its type and the exact medium it has an impact on, for both our environment and living creatures are to be protected. Indeed, some pollutants build up in our environment/food chains in unexpected ways and have long-term consequences. Microplastic debris from deep-sea sediments in the Atlantic Ocean, the Mediterranean Sea and the Indian Ocean have been found to be four times the concentration of surface waters (Woodall *et al.*, 2014). Certain heavy metals contaminating terrestrial ecosystems have impacts on soil organisms, plants, aquatic organisms (due to runoff) and can subsequently build up in food chains and food webs with consequences on human health (de Vries, Groenenberg, Lofts, Tipping, & Posch, 2013). Understanding these influences requires a basic knowledge of the various types of pollution being faced in today's world.

The importance of understanding these different types of pollution which include atmospheric pollution, water pollution, pollution of soil and eventually food is vital since most of these eventually impact human consumption and ultimately health. In the following sections these various forms of pollution will be examined in turn.

1.1.1 Air Pollution

Any contamination of the atmosphere, which upsets the natural composition and chemistry of the air is defined as air-pollution. This could be in the form of particulate matter that is dust or of a gaseous nature. When excessive quantities of particular gases build up in the atmosphere and are not removed through natural cycles there will be consequences the environment. Global warming, ozone depletion with an associated increase in solar UV radiation are all consequences of air pollution (Boubel, Vallero, Fox, Turner, & Stern, 2013).

Air pollution comes from a wide variety of sources such as the burning of fossil fuels for transport and enegry, forest fires, volcanic eruptions, dry soil erosion, building construction or demolition, industries and manufacturing activities, household, and farming chemicals. The effects of this pollution can vary, depending on the concentration of air pollutants and their toxicity. Chemical reactions involving air pollutants can also produce acidic compounds (acid rain) which can harm vegetation and wildlife. Higher levels of pollution can cause a decrease in crop yields due to insufficient oxygen levels particularly in urban areas. Such air pollution, as from traffic, can also induce higher rates of asthma and allergic reactions especially in children (Brauer *et al.*, 2002).

1.1.2 Water pollution

Water pollution involves any contaminated water, which could be chemical, particulate, or bacterial in nature that degrades the quality and purity of water. Water pollution can occur in oceans, rivers, lakes, and underground reservoirs. The interconnectivity between water bodies may cause the pollution to spread even further. Causes of water pollution include:

- Increased sediment from soil erosion;
- Improper waste disposal and littering;
- Leaching of soil pollution into water supplies; and
- Organic material decaying in water supplies.

The effects of water pollution include decreasing the quantity of drinkable water available, lowering water supplies for crop irrigation, and impacting fish and wildlife populations that require water of a certain purity for survival. Persistent Organic Pollutants (POPs) have been found in deep-sea areas suggesting that these may be acting like global sinks for such long-term pollutants (Froescheis, Looser, Cailliet, Jarman, & Ballschmiter, 2000).

1.1.3 Soil pollution

Soil pollution is contamination of the soil that impedes natural growth and balance in the land whether it is used for cultivation, habitation, or as a wildlife preserve. Some acts of soil pollution, such as the creation of landfills, is deliberate, while much more is accidental and can have widespread effects. Soil pollution sources include:

- Industrial activity.
- Agricultural activity.
- Waste disposal.
- Acid rain.
- Hazardous waste and sewage spills.
- Non-sustainable farming practices, such as the heavy use of inorganic pesticides; and
- Strip mining, deforestation, and other destructive practices.

Soil contamination can lead to poor growth and reduced crop yields, loss of wildlife habitat, water and visual pollution, soil erosion, and desertification. Crops grown on contaminated land can absorb much of the pollution and pass these on to consumers. Soils may become contaminated by heavy metals and metalloids through emissions from industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, application of fertilizers to the land, livestock manures, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and even atmospheric deposition. Heavy metals most commonly found to contaminate sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni). While organic contaminants can be oxidised to become carbon (IV) oxide by microbial action, heavy metals do not undergo similar degradation and hence their total concentration in soils can persist and indeed accumulate over time (Khan, Cao, Zheng, Huang, & Zhu, 2008).

1.1.4 Food contamination

Pollutants from industrial waste, much of which have been already mentioned above, enter into the vegetation and livestock production systems and then into the food chain. Figure 1.1 shows the cycle of pollution to the cows’ feed.

a

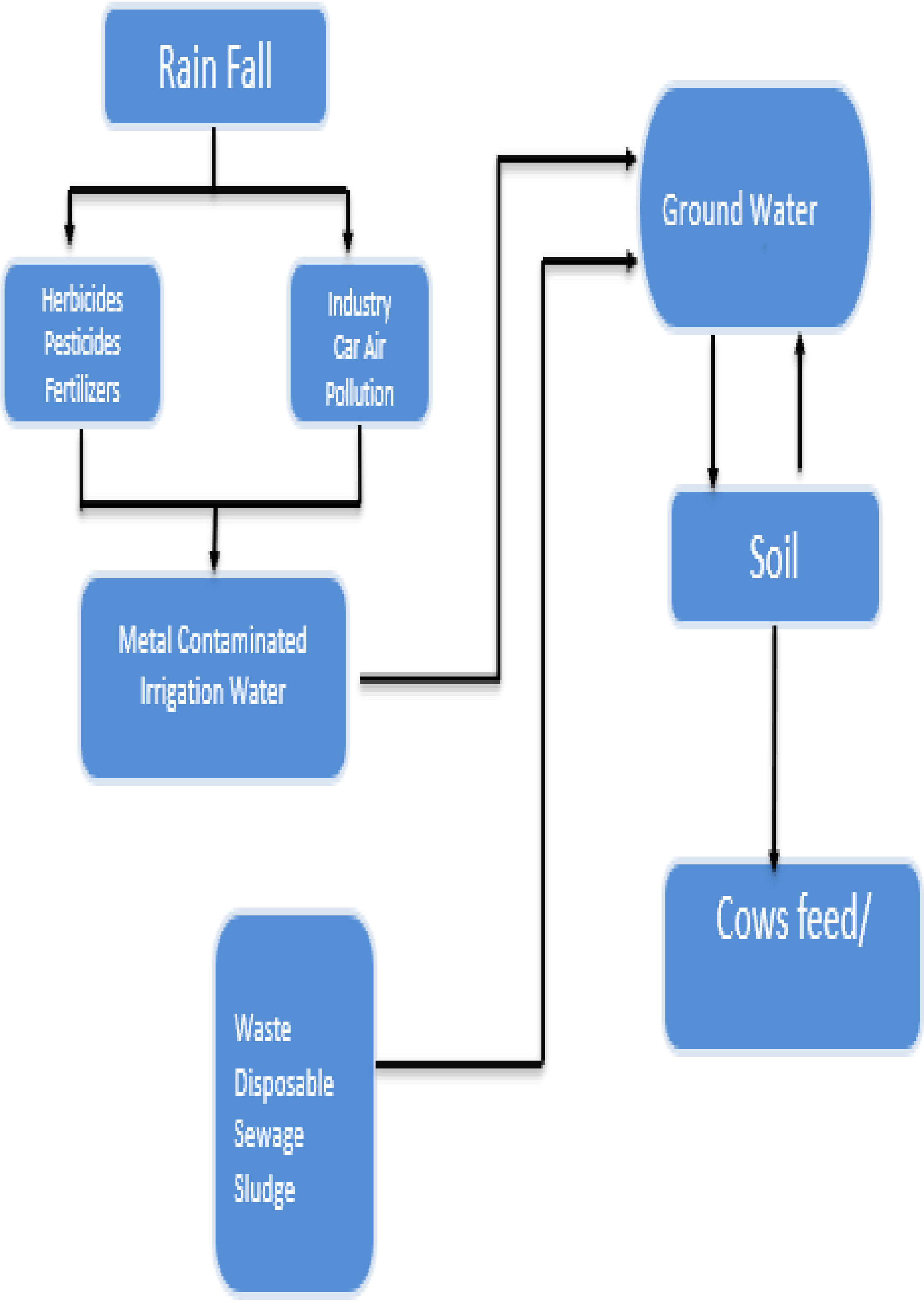

Figure 1.4 The cycle of pollutants in relation to a dairy farm

In most developing countries it is often caused by industrialization with improper waste disposal. In developed countries, the impact of pollution on domestic and wild animals can be due to chemical toxins. Pollution is a serious problem in the world threatening animal and human health. Heavy metal pollution is becoming a serious health concern in recent years because of its tendency to accumulate in the food chain. Heavy metals from industrial waste can contaminate drinking water, soil, fodder and food. These heavy metals have effects on the vascular plants, reducing development and growth of roots and shoots. Their presence also reduces the nutrient content of plants (Boubel *et al.*, 2013; Brauer *et al.*, 2002).

Aquatic organisms exhibit the effects of heavy metals via gill function, nervous systems as well as growth and reproductive rates. For humans, the presence of heavy metals in the food chain especially the toxic heavy metals like Cadmium and Mercury (Houston, 2007) and to a lesser degree Lead influence the human biological functions, altering the hormone system and growth. Many heavy metals accumulate in one or more of the body organs in food animals and are transmitted through food production and can cause serious public health hazards. These toxicants are accumulated in the vital organs including the liver and kidney exerting adverse effects on domestic animals and humans. Many surveys involving human populations in industrial, mining and urban areas have indicated toxicities due to effluents. Pesticides, heavy metals and other agro-chemicals are some of the major causes of environmental toxicity in farm animals. The impacts of pollution on animals can result in serious economic losses.

1.2 Pollutants in Complex Systems

When studying pollution in a particular setting, be it in the water, soil or air, it is very important to take the air-water- soil interactions into consideration to fully comprehend the influence of pollutants on our environment. Although these have been treated separately in the above section, for clarity of understanding, this interaction is very real. Also, similarly the presence of metals in the food chain influences many of the organs of the human body and its functioning. This too is a complex system that interacts with the human body in different ways depending on the metal element and its concentration and absorption.

The following two sections examine the heavy metal elements studied in this dissertation focusing on how the metal is used and also the health consequences of overexposure to humans.

1.2.1 Essential trace elements

A deficiency of certain elements leads to impairment of vital biological process but the same elements in excess become toxic. Copper is an essential trace element and a normal constituent of animal tissues and fluids. Its presence is necessary in haemoglobin synthesis and in the functioning of other enzymes (Muhib *et al.*, 2016; Tapiero & Tew, 2003). However, essential elements, which are needed for health, can be toxic when their concentration levels exceed 40 to 200 times their recommended threshold value (Rao, 2005). Toxic levels of Cu may lead to Wilson's disease (which causes excessive accumulation of Cu in liver, brain, kidney and cornea) and Menkes disease (Tapiero & Tew, 2003). However, micro elements such as Cu, Fe, Se, and Zn at appropriate concentrations are essential for human growth (Babu *et al.*, 2018).

Zinc metal is used in dry cell batteries and mixed with other metals to make alloys like brass, and bronze (e.g. a zinc and copper alloy is used for coinage in many countries). Zinc compounds are used to make paint, rubber, dyes, preserve wood and in ointments. Exposure to large amounts of zinc can be harmful. It can cause stomach cramps, anaemia, and changes in cholesterol levels (Agency for Toxic Substances and Disease Registry, 2014b).

Copper, like zinc, is found in electrical wires, plumbing pipes, and sheet metal. Copper is also combined with other metals to make brass and bronze pipes and faucets. Copper compounds are used to treat diseases in plants like mildew. It is incorporated in treating water and used as a preservative for wood, leather, and fabrics. Excessive levels of copper can produce harmful effects such as irritation of the nose, mouth and eyes, vomiting, diarrhoea, stomach cramps, nausea, and even death (Agency for Toxic Substances and Disease Registry, 2015b).

Selenium, is a vital essential element which impacts a range of body functions and plays a role in healthy cognitive functioning, helps maintain a good immune system and is instrumental in sustaining fertility in both sexes.

Selenium sulfides are used in some anti-dandruff shampoos. The burning of coal and other fossil fuels together with other industrial processes such as the production of rubber release selenium to the environment. Selenium is also used in the electronics industry, in the glass industry and as a nutritional supplement. It is a component of pigments in industry. Selenium is used as a nutritional feed additive for poultry and livestock and in pesticide formulations. Exposure to higher levels of Selenium can cause neurological effects and physical complaints like brittle hair and deformed nails. Workers inhaling selenium vapours may experience dizziness, fatigue, irritation of mucous membranes and respiratory effect (Agency for Toxic Substances and Disease Registry, 2014a). In addition to this, Hamza & Gharbi (2017) did a study in which they determined that selenium intake is reflected through the concentrations of blood and urine. Hair or nail selenium content can also be analysed in order to monitor intakes for longer periods of time, as well as the quantification of selenoproteins, for example glutathione peroxidase and selenoprotein P, as a practical measure for selenium status.

Chromium is an essential element for human beings, as it acts to maintain normal glucose tolerance (Babu *et al.*, 2018). Chromium is used in metal plating, electroplating, mining, dye production with around 90% of all leather tanned using chromium. However, the waste effluent is toxic, so alternatives are being investigated. Chromium compounds are used as industrial catalysts and pigments (in bright green, yellow, red and orange colours). However, there are several types of chromium that exist. Cr (III), found in many food and nutrient supplements, is an essential nutrient of very low toxicity, whereas Cr (VI) compounds have been shown to be potent occupational carcinogens. Stainless steel vessels appear to be the main source of chromium contamination of foodstuffs (Babu *et al.*, 2018).

The health effects of chromium exposure in high levels can be ulcers, skin irritation, liver and kidney damage (Kumar et al, 2006). There is evidence of damage to the sperm and male reproductive system when exposed to Cr (VI). The International Agency for Research on Cancer (IARC) has determined that Cr (VI) compounds are carcinogenic to humans (Agency for Toxic Substances and Disease Registry, 2015a).

1.2.2 Toxic heavy metals

Originally, inorganic arsenic compounds were used as pesticides, primarily on cotton fields and in orchards. Although, inorganic arsenic compounds are no longer used in agriculture, organic arsenic compounds are still used as pesticides, principally on cotton. Some organic arsenic compounds are used as additives in animal feed. Arsenic is added to other metals, in small quantities, forming metal mixtures or alloys with better properties. The largest use of arsenic in alloys is found in lead-acid batteries for automobiles. Arsenic compounds are also used in the production of semiconductors and light-emitting diodes. Arsenic in large quantities can cause death. In smaller quantities, it causes irritation of the stomach and is linked to cancers of the liver, bladder and lungs. It can reduce red and white blood cells. Inhaled inorganic arsenic can increase the risk of lung cancer. This is noticed in workers exposed to arsenic at smelters, mines, and chemical factories, and those who live near smelters and arsenical chemical factories (Agency for Toxic Substances and Disease Registry, 2015b).

Cadmium, even in low concentrations, has a toxicity level, with its biological half-life ranging from 10-33 years, causing most countries to prioritise it as one of its main monitored elements (Mohod & Dhote, 2013). Cadmium in high doses causes severe damage to the lungs and irritates the gastrointestinal tract causing vomiting and diarrhoea. Long-term exposure to lower levels builds up in the kidneys causing kidney disease, lung damage and fragile bones. However, the complexity of the interaction of cadmium with the human body is shown by the fact that smokers have four or five times the amount of cadmium than non-smokers. Alternatively, a high fiber diet reduces the amount of cadmium which is absorbed in the body whereas those with low iron show a higher percentage of resorption cadmium. Low intakes of vitamin D, calcium and a lack of trace elements like zinc and copper increase the uptake of cadmium (Godt *et al.*,2006). An increase in cadmium level may cause the human body to replace it with zinc in the arteries, making them inflexible and fragile (Afridi *et al.*, 2006). Some heavy metals, such as cadmium, are of particular risk in the environment as it is efficiently taken up by plants from the soil. The consumption of contaminated plants by herbivores leads to the transfer of this toxic heavy metal from the soil to animals and ultimately the human food chain. There are geographical regions, which have areas of naturally high levels of Cd in the soil, such as Ireland, and as a result of the transfer from soil to plant to animal the bovine kidneys, especially in older animals, exceed MRL (Canty *et al.*, 2014). This is a result of a longer lifetime exposure to this metal by consuming plants grown on these contaminated soils. Such situations can be further complicated on agricultural lands treated with phosphate fertilisers and pesticides which serve to increase the levels of heavy metals in the soil (Hashemi, 2018; Radha, Kumutha, & Marimuthu, 2014).

Moreover, lead is known for its devastating effects on brain development (Bryee-Smith, & Stephenes, 1982). For this reason, it is important to understand how lead finds its way into the food chain. One pathway is via lead consumed by chicken through contaminated feed ending up in their bones and soft tissue. Lead can also be found in drinking water; should the concentration of Pb be higher than the permissible limit, it can lead to metabolic poison and enzyme inhibition, (Gebrekidan *et al.*,2011). The main results of chronic exposure to Pb are the development of abnormalities, deficits in intelligence quotient and neurotoxicity effects in infants, incidents of constipation, colic and anaemia (Hariri *et al.*, 2015). On the other hand, it can substitute calcium at mineral sites for long term replacements in bones(Mohod & Dhote, 2013).

These elements are transferred through the food chain and do not serve any essential biological function (Liu, 2003). Children are more sensitive to Cd and Pb than adults and since effects are cumulative, these elements gradually accumulate in the tissues (Tripathi, Raghunath, Sastry, & Krishnamoorthy, 1999). Consequently, even regular absorption of small amounts of such elements, such as Pb, may have serious effects on growing children’s health. (Salma *et al.*, 2000). Since milk is a major source of nutrition for children and given that their susceptibility to heavy metal toxicity it is, therefore, vital to monitor the levels of such trace elements in cow’s milk.

It is vital to understand how heavy metals persist in the environment and causes serious environmental and health hazards, in order to avoid contamination of the food chain. This literature review will examine many elements impacting heavy metal content in agriculture including milk, hair, fodder and manure of cattle and also water. Initially, water and milk will be examined, to understand how heavy metals find their way into the respective food systems (i.e. cow and human, respectively).

Many studies have been carried out to examine heavy metal content in dairy products given their large-scale use in the human diet. Milk, in particular, is a focus of attention and heavy metal content has been studied and the results of these need to be analysed so that the present study of cow’s milk in Malta can be placed in context (Abdulkhaliq, Swaileh, Hussein, & Matani, 2012b).

Increased environmental pollution has accelerated the problems of milk contamination and caused uncertainties in milk quality (Farid & Baloch, 2012; Muhib *et al.*, 2016).

Given that milk plays an important role in today's diet and in particular that of children and infants any contamination of this important source of nutrition is of concern. Indeed, Malhatet *et al.* (2012) found that contamination in milk is considered to have become one of the main dangerous threats to health within the last few years (Malhat, Hagag, Saber, & Fayz, 2012).

Recently, the discovery of trace elements and heavy metals in dairy products and milk has alarmed many authorities in various countries and regions and is becoming a threatening phenomenon. So far around 38 micro and trace elements have been reported in raw milk from different regions around the world (Dobrzanski, Kolacz, Górecka, Chojnacka, & Bartkowiak, 2005; Muhib *et al.*, 2016).

Milk is an ideal food due to its combination of macronutrients (i.e. proteins, carbohydrates, and lipids) and micronutrients (i.e. vitamins and minerals) that are absolutely essential during the first months of a baby's life. Some micronutrients are stored by the baby within the uterus and are sufficient for the first 4-6 months of life, such as iron. Other essential elements such as selenium are required right from the moment of birth and need to be available in the milk as a food. For this reason, human and cow milk are quite low in Fe (0.200 to 0.800 mg/l) (Aleixo & Nobrega, 2003). Trace elements in cow milk are of particular interest as depending on their nature (essential or toxic) and their concentration levels, they may lead to health problems. For example, Cr and Mn are essential but can be toxic at higher levels, while Pb and Cd are toxic even at low levels and can be cumulative (Ataro, McCrindle, Botha, McCrindle, & Ndibewu, 2008). For this reason, Cd and Pb are some of the elements that have consequences in terms of adverse effects on human health.

The first time, some heavy metals was measured in milk from sheep, goats and cows in Malta and Gozo was in 2017, according to Spiteri & Attard (2017). The purpose of this study was to evaluate the mineral content of milk derived from cow, sheep and goat farms in Malta and Gozo and to identify any significant differences in milk between the islands. Since small ruminant farms are the most prevalent in the islands, goat and sheep milk were determined to compare with cow's milk.

In this study, 190 samples were collected from different farms in Malta and Gozo between October 2013 and March 2014, the samples were taken from the bulk tank or from the bucket, depending on the farm’s procedure of milking. Most of the samples were taken from the tank to find a general status of herds in Malta and Gozo. All samples prepared using conventional method and analysed using microwave plasma-atomic emission spectrophotometry. No significant differences were noted between localities for the metals in the milk. Mn, Cr and Cd were not detected in the three types of milk, and K was the highest in cow milk, while Ca was the highest in sheep milk. On the other hand, sheep milk presented the highest value for Cu, Fe, and Mg, while cow milk the exhibited highest concentration for Zn and Ba. For the micro minerals, the analysis of the main component indicated that cow and sheep milk samples were separated into two distinct groups, while goats' milk samples were dispersed across the two groups. This indicates that sheep's milk is distinguished from the other two types of milk (Spiteri & Attard 2017).

Studies of heavy metal content in cow milk and dairy products in Palestine have been carried out. The metals examined were copper, lead, cadmium and iron in commercially available cow milk using GFAAS. These indicated that highest concentrations of Pb (0.930µg/g), Cd (0.057µg/g) and Cu (12.9µg/g) were found in the powdered milk and the lowest levels (Pb was not detected, Cd 0.022µg/g, Cu 3.20 µg/g) were found in the liquid milk. Pb and Cd, in particular, exceeded the maximum allowed limits in the liquid milk of 0.020µg/g for Pb and 0.010µg/g for Cd (Ayar, 2009). This investigation identified the need to understand why these high levels of Cd and Pb were occurring in cow milk. These elevated levels could be due to contamination either during processing or as a result of heavy metal presence in the raw milk (Abdulkhaliq *et al.*, 2012b).

Heavy metals such as As, Cd, Hg and Pb have health consequences if they are consumed at more than maximum permissible levels. The maximum permissible level of Pb in milk which has been recommended by Codex Alimentarius Commission (2015) and the European Union Commission (EC) no. 1881 European Union (2006) is 0.020mg/ml (European Commission, 2006). Other researchers have indicated that the maximum allowed limit for Pb in milk is 0.020 and 0.100 mg/ml (the later value varies from that mentioned earlier in other studies) (Babu *et al.*, 2018; FSSAI, 2011).

Heavy metal content in raw cow milk fluctuates due to several factors i.e. the lactation period of cows, health conditions, seasonal variations, climatic conditions, annual feed composition and environmental contamination (Muhib et al., 2016; Yahaya, Ezeh, Musa, & Mohammad, 2010). In addition to this, particular conditions for processing of milk may also impact the contents and degree of minerals in the total composition of milk (Abd-El Aal, Salah Fathy Ahmed, 2012; Babu *et al.*, 2018).

Other studies have shown that levels of heavy metals in milk samples of different species are already above the maximum permissible range in some developing countries (with poor water treatment, improper waste disposal and widespread pollution). In Pakistan, a recent study was conducted to analyse the presence of heavy metals (Ni, Cu, Cr and Fe) in the milk samples of cattle and humans using Atomic Absorption Spectrometer. Results showed that most of the cattle and human milk samples were contaminated with heavy metals (copper, nickel and chromium) to levels well above the maximum permitted range. According to the Joint Expert Committee on Food and Agriculture and the World Health Organisation, the maximum level for Cu is 0.010mg/L (Ruqia Nazir *et al.*, 2015).

While there is concern about excessive levels of toxic heavy metals such as cadmium, in the case of some essential heavy metals like selenium (although toxic at high levels) the main concern relates to the levels of this heavy metal which has indeed beneficial effects on human health. Despite the need for selenium for physiological processes, its concentration in cow milk can vary from as much as 2-1270 μg/L depending on the food chain. The intake of Se, in Europe, has worryingly declined over the years, although its beneficial effects are well known. The G(F)AAS has been used to analyse the Fe and Se contents of milk directly without the need for a digestion procedure hence reducing the possibility of contamination. In such a study, the concentration of Fe varied from 0.610 to 1.17 mg/L and the Se concentrations were in the 17–122 mg/L range (Aleixo & Nobrega, 2003).

Selenium concentration in cow milk was determined by several workers as 10.9±0.300 μg/L in Belgium, 17.0±5.00 μg/L in Greece, 25.7 ±5.25 μg/L in India, 22.4 μg/L in United State, 5.40±11.7 μg/L in Hungary and 11±17 μg/kg in the Netherlands. Obviously, the selenium content in milk can vary substantially from region to region. Soil and water can have a natural selenium level in one area completely different to another influencing the associated levels found in cereals and plants etc. For most metals, there are clear guidelines for optimum concentration in milk but for selenium, there is no clear agreement. Aspila (1991) has suggested that the selenium concentration in milk for human consumption should be at least 20 µg/L (supplying more than 10% of our dietary requirement for this element) . The difficulty here is that when there is such a natural variation in selenium content in our food/water sources from one region to another dietary intake may not be the deciding factor. In fact, values reported for selenium in cow milk varies significantly due to the cow’s origin, the cow’s feed, the soil, and even the season. According to researchers (Ford *et al.*,1986), the concentrations of Fe, Cu, Na, K and Ca in raw cow milk remained relatively stable during the 12 months period samples were taken (Muñiz-Naveiro *et al.*, 2005).

On the other hand, Se, Mg and Zn concentrations varied considerably according to the season with the lowest mean concentrations occurring in autumn. This seems to indicate a seasonal variation in some heavy metals but not in others. This is not the only factor however to influence milk content. The mineral content of milk also varies widely due to lactation period, animal breed, the dietary composition of animal feed and soil contamination (Rodrıguez, Alaejos, & Romero, 2001).

Accumulation of heavy metals in agricultural soils may have a long-term negative impact on soil productivity by restricting soil microbial and fauna populations. This can become a risk to the health of animals, humans, and the entire ecosystem (Li, McCrory, Powell, Saam, & Jackson-Smith, 2005).

The quantity and quality of dairy milk depends on the good health condition of the cows and this entails meeting all their essential mineral needs. These include the inorganic mineral elements necessary for nutrition which are Ca, P, Fe, Cu, Mn, Mg, Na, K, I, Co and Zn according to Atherton and Castle (1982) and Watkins (1984). Of course, an excess of these quite beneficial elements can be toxic and even lethal to the animal. Therefore, the addition of minerals, particularly trace elements, should be carried out carefully. The nutrients are removed from the blood by mammary glands which affect the quality of milk. For healthy milk production, animals must be fed, either directly, or indirectly via body reserves of nutrients. Additional trace elements, however, found in environmentally polluted areas might reach the animal’s milk through foraging. Therefore, growing environmental pollution has increased concerns in relation to milk quality (Farid & Baloch, 2012).

Cadmium is highly toxic and has been involved in poisoning through food on certain occasions. A three times higher level of Cd in city areas was detected than in rural areas for cow and buffalo milk. The levels found were considerably lower than the cadmium concentration detected by Khattak and co-workers (2004) and Koen and co-workers (1982) but almost the same as that found by Lokeshwari and Chandrappa (2006). The latter study showed that the cadmium concentration was lower than the permissible level of 0.100 ppm (Lokeshwari & Chandrappa, 2006). Chromium in the animal milk collected from polluted areas had about 0.290 ppm as compared to the (0.100ppm) one obtained in the unpolluted area (Farid & Baloch, 2012).

In a similar study high levels of chromium were seen in the milk samples collected from the areas concerned. Lead concentration from polluted areas (0.110 ppm) was five times higher than those milk samples collected in unpolluted areas (0.020 ppm). The same high level of lead in milk was reported by Dwivedi and Swarup (1995) in their studies on urban areas of India. Values below the permissible values were found according to Hough and coworkers (2003) and Loeshwari and Chandrappa (2006) of 0.020 ppm in the milk. Environmental factors can play a critical role in the elevated level of forage which in turn influences the final Pb concentration in the milk. (Khattak *et al.*, 2004; Tripathi *et al.*, 1999; Ayyadurai et al., 1998) This is particularly the case where city effluents were used for raising forage (Farid & Baloch, 2012).

Although essential elements are important in our diets, as pointed out earlier, heavy metals can prove toxic if found in high levels in cow milk. A study investigating heavy metals Zn, Cu, Cr, As, Cd, and Pb in the fodder used for cows was carried out in dairy farms in the United States. Highest metal contents were found in purchased feeds, particularly mineral supplements and also lower levels occurred some in corn/soybean-based concentrates. Due to low levels of Zinc and copper in home-grown feeds, these metals are added as a mineral supplement for livestock (NRC, 1980; European Commission, 2003a, b). The deliberate addition of these metals results in half the feeds used being over the US recommended levels. The concentration of Cd was found close to the US maximum acceptable levels and indicate potential long-term risk if levels continue to rise.

Arsenic has been used as a food additive for disease control and improvement of weight gain in both swine and poultry in quite high concentrations for a considerable time. However, no evidence exists that arsenic is an essential nutrient for dairy cattle. Nevertheless, there is evidence that ruminating cattle do not show toxic effects to arsenic unless levels reach greater than 200 to 300 mg/kg of inorganic as (NRC, 1980).

Selenium can be found in a wide range of high protein foods such as meat, seafood, milk, eggs (Chaney *et al.,* 1979). Selenium levels in food are determined by the selenium content of the soil because minerals move from the soil to the plants growing on it. This is then passed up the food chain to the animals that eat plants (Inam & Somer, 2000).

Research done by Alonso 2003 has shown that dairy cattle may be more susceptible to the accumulation of heavy metals than beef cattle, so it is vital that sources of Cd in the dairy production system are identified and minimized to prevent problems associated with Cd accumulation in the dairy soil system in the long-term. The main sources of Zn, Cu, Cr, As, and Cd in fodder originated from the imported feed. The progressive addition of heavy metals in the dairy feed is likely to be linked to an accumulation of these metals in soils where manure is applied (Alonso *et al.,* 2003)

Understanding heavy metal accumulation in water, fodder and manure is an important feature of this present study. Having examined the impact of heavy metals on milk in both the fodder and supplements, now heavy metal presence in water, manure and hair will be dealt with.

When metals from industrial effluents enter the soil and water they will subsequently be found in plants and animals. As a result, there can be a build-up of heavy metals in crops, animal fodder and other living bodies so the bioaccumulation of such elements occurs (Rao & Praveen, 2014).

The soil can act as a long-term sink for heavy metals, with residence times ranging from hundreds to thousands of years depending on the specific metal and also the properties of the soil (Alloway, 1995). Given the potential long-term impacts of heavy metals on soil fertility also the legislation on permitted levels of cadmium and lead concentrations in the food chain, there is a need to measure/monitor metal inputs to agricultural soils and be able to determine which soils are particularly vulnerable to heavy metal pollution. If heavy metals flow within agricultural systems is understood, potential risks could be appropriately managed and reduced. Heavy metal inputs to agricultural soils include atmospheric deposition, sewage sludge, animal manures, agrochemicals and inorganic fertilisers (Nicholson *et al.*, 1998).

Arid and semi-arid parts of Pakistan have a mean rainfall of around 20 cm (Nazir, 1994). Due to economic impacts crops are frequently grown by the use of city effluent in order to sustain different crops including fodder in areas around large cities. Unfortunately, this effluent is contaminated with untreated industrial effluents (Ghafoor *et al*, 1994) (Farid & Baloch, 2012).

Such effluents may be contaminated with heavy metals like Cadmium, Chromium, Nickel and Zinc (Khan, *et al*., 1994). In some instances, these effluents may be an alternative to fertigation as these contain several nutrients and organic matter (Ahmad *et al.*, 1994). Consequently, farmers do not need to add fertilisers for the production of fodder. Farid and Baloch (2012) determined the extent of heavy metal contamination (Cd, Cr, Pb and Ni) in the city effluent, soils fodder and milk.

To this end, metals such as Cr, Pb and Cd have also been found within dairy feed due to phosphate-containing concentrates and supplements (Li *et al.* 2005; Wang et al. 2013; Ding *et al.* 2018). In an even more recent study, done by Jing Li *et al.* (2019), soil and aquatic environments absorb most of the heavy metals within feed due to faecal and urine contamination (Li, Xu, Wang, & Li, 2019).

In this present study, the conditions of fodder production were taken into account as environmental factors affect fodder cultivation, due to the potential presence of pollutants in the environment, originating for example from effluent. Such fodder which is imported to Malta (grown abroad) and fed to cattle here needs to be analysed for heavy metal content given that the air in which it is grown, the water that it is irrigated with and the fertiliser (effluent) which is used to encourage growth all may have contributed to heavy metal content. Farid and

Baloch (2012) reported cadmium and chromium levels which were four times and twenty-seven times respectively higher in fodder crops irrigated by city effluents than those irrigated with uncontaminated water. The average values of Pb in fodder were found more than twelve times in the case of polluted areas as compared to unpolluted areas.

Having looked at the heavy metal content as biomarkers in a number of matrices, another valuable source of information is the use of animal hair to give more insights into effects of such content. This has been used successfully in a range of animals and humans to provide more details of the interaction of heavy metals in mainly livestock and its impacts on health both of animals and humans.

There are many advantages of using hair as a biopsy specimen. It causes little trauma when collected and can be stored safely for long periods as it does not deteriorate easily. More importantly, it accumulates trace elements at concentrations ten times higher than those found in blood and urine (Maugh, 1978). In fact, hair is so effective as a biopsy marker is that it records elements being deposited in the hair matrix very quickly and then this information is stored in the hair shaft as it grows from the follicle. This time-dependent record of trace element concentration allows a longer examination of heavy metal content than more transitory serums such as blood and urine (Combs, Goodrich, & Meiske, 1982).

The main disadvantage of using hair is that mineral concentration in hair is impacted by many factors other than diet. These include season, breed, hair colour and body location. When hair analysis is carried out, precautions must be taken to compare values from test animals with those from animals of similar breed, sex, season, size and colour. Hair analysis, however, can be used to determine severe deficiencies of some essential minerals or exposure to some heavy metals (Combs *et al.,* 1982).

A study by Anke (1965) indicated that sampling hair should be done during certain set periods to avoid times when hair was being shed and new growth was taking place. The most optimal periods were from December to mid-February and from July to August.

O'Mary *et al.* (1969) examined hair from Hereford cattle and also found seasonal variations impacted results in measuring heavy metal content. In August there were higher concentrations of Na, Ca, Cu, Mg, Mn and K but lower Fe concentrations. In contrast, both Zn and P remained constant between seasons. Another study by Strain *et al.* (1966) showed conversely that for human hair Zn content was higher in summer than any other season. A seasonal variation in Zn was also noted by Miller *et al.* (1965) in Holstein cattle with Zn levels lower in winter than any other season. In general, it would seem that seasons do impact heavy metal content, but the manner of that influence is not yet completely understood.

Even the colour of hair can influence the results of heavy metal content. When O'Mary (1970) looked at white hair from Holstein cattle it contained higher levels of Na, Ca and K than from the black-haired Hereford cattle. Holstein black hair, in turn, had more Na, P, Mg and Ca than red hair from Herefords. Even the site of hair sampling can change results found. Miller *et al.* (1965) found elevated concentrations of Zn in white tail hair than in either black or white body hair. One study Miller *et al.* (1965) proposed that Zn content contributes to the stiffness of hair in rats and humans. Since switches from tails are much stiffer and of course this could be indicative of Zn content in this particular hair as compared to hair found on the rest of the body.

Hair sampling has been so closely linked to heavy metal content that on occasions, levels of metal content in hair have been used to monitor the health of cows. Indeed, Gardiner, (1966) and Hidiroglou *et al.* (1968) linked levels of Se in cow hair with white muscle disease in their calves due to selenium deficiency, in part. Cows with Se at levels between 0.060 and 0.230 ppm had calves with the disease whereas cows with Se at levels above 0.250ppm gave birth to calves with no disease.

Having established that season and hair colour impact heavy metal content it is also clear that the environmental conditions around the cows are also a determining factor. When Dorn *et al.* (1974) looked at hair from cattle on a farm close to Pb smelter and compared the results to a farm free of industrial impacts they found much higher levels of Cd in the hair of cattle within the first setup. Their respective Cd levels also varied with seasons. For the cattle close to the smelter highest levels of Cd were found in the spring but conversely Cd levels in the farm far from industry effects higher levels in hair were observed in winter. In both groups lowest levels of Cd were found in the summer.

Other studies (Miller *et al.*, 1968,1969: Neathery *et al.*, 1974; Doyle et al 1974) have found that in the case of cadmium, hair is better at detecting environmental exposure due to external effects than that produced via diet. In order to measure Cd content in the diet, they suggest that the kidney, liver and small intestine are much better indicators of dietary Cd intake in cattle than hair (Combs *et al.,* 1982). This may indicate valuable future areas of research in this study on Malta.

Hair is very easily used as a non-invasive method of finding heavy metals in the body. Unlike blood and urine analysis, hair provides an average concentration of the heavy metal exposure over time.

The fact that cattle hair and other different mammals, such as human hair and monkey hair pose similar effects by heavy metals, it would be of interest and useful to showcase the studies done by other researchers, since it can portray the levels of heavy metals in hair in a clearer light.

A study carried out in Pakistan examined scalp hair of hundreds of men living in urban environments and compared them in terms of heavy metal content (Cd, Cu, Fe, Ni, Cr, Pb and Fe) and found a link with hypertension and the presence of certain metals in the hair of those investigated. Certain metals were found in higher levels in those with hypertensive sensitivity and there was also a deficiency in some essential trace elements, they used a microwave digestion method which was substantially faster than the more commonly used acid digestion and gave reasonable results when checked with a certified reference material (CRM), Atomic Absorption Spectrometry was used to determine the concentration of heavy metals in samples taken (Afridi *et al.,* 2006).

Apart from diet, environmental pollution can contribute to heavy metal accumulation in humans. In Hyderabad City, the heavy traffic and high population density combined with many industrial units and frequent burning of plastics produce many toxic elements. High levels of cadmium and lead are found in the hair of humans living in this city. Very high levels of chromium and lead can lead to symptoms such as headaches and dizziness. Interactions between heavy metals in the human body can have dramatic results on health. Cadmium can replace Zn in arteries resulting in brittleness and a lack of flexibility. Cadmium can also accumulate in kidneys producing high blood pressure and kidney disease. In this study trace, essential and toxic elements were assessed in the hair of both hypertensive patients and normal healthy men of the same age group (Afridi *et al.,* 2006). The hypertensive group showed lower levels of iron across both age groups compared to the other subjects. As iron plays a major role in transporting oxygen from the lungs to the whole body, a lack of this element is one of the most prevalent nutritional disorders in the world with between 600-700 million suffering from iron deficiency anaemia. Whole-wheat flour in the diet (due to a high level of phytate) strongly inhibits iron absorption. It also lowers zinc levels and therefore increases levels of hypertension. Also, vegetarian diets usually have high phytate content and this can lead to a reduction in iron and zinc absorption. However, beef and meat contain much higher amounts of Cu and Cd compared to that found in a vegetarian diet. Cu, Cd and Ni levels were all found to be significantly higher in the group with hypertension compared to the normal subjects. Cadmium and lead have already been found to significantly increase blood pressure. Aside from diet, it was seen in this study that smoking hypertensive patients had much higher levels of toxic metals than non-smoking hypertensive patients. Diet would seem to influence heavy metal content in hair as does environmental pollution, including smoking with increased health consequences as a result (Afridi *et al.,* 2006).

Another study on hair and heavy metal content examined imported rhesus monkeys from southwest China and measured the heavy metal content in the hair of the monkeys. This included levels of cadmium, copper, iron, nickel, chromium, lead and zinc. On examining the monkey's hair from China researchers found concentrations of heavy metals in parts per million of As (0.654±0.331), Be (0.005±0.003), Cd (0.034±0.022), Cr (11.3±4.26), Fe (87.1±30.1), Pb (0.916±0.619), Hg (0.916±0.619), and Se (3.20±0.735). Monkeys share a lot of immunological and physiological characteristics with humans and most significantly how they respond to toxic exposure (Rice, 1992).

China has experienced rapid industrialisation and a corresponding rise in heavy metal contamination as a result (Jiang *et al.,* 2005; Wang, He, Wei, & Feng, 2006). This study examined rhesus monkeys reared in China to determine if their hair showed contamination through environmental pollution via soil, water, air and food. The results showed that the levels of Be, Cr and As between males and females was significant with females showing higher concentrations of these metals. Mercury content was higher than that seen in free-range monkeys in Nepal but no clinical signs of Hg damage to the immune systems/birth defects etc were seen in the examined monkeys from China. The results found for lead in this study were lower than that found in previous studies in Nepal and Singapore. It must be noted with 23 females and only 5 males, participating in this study, any comparisons between the sexes in terms of heavy metal content is difficult to ascertain (Lee *et al.*, 2012).

Having established the variations in heavy metal content in hair due to the season, place of sampling, colour and environmental pollution there has also been a study of human hair in adults from Vienna and Rome (Jiang *et al.*, 2005). This found that there is a variation due to sex and the dramatic contribution smoking makes to levels of heavy metal found in human bodies. Looking at levels of heavy metal content between Pakistan and Western Europe smoking is important as a source of heavy metals but environmental industrial pollution can contribute still further to bioaccumulation (Wolfsperger, Hauser, Gößler, & Schlagenhaufen, 1994).

Hair can be seen as a useful biomarker to determine heavy metal content. Smoking in humans is an important factor (including passive) but in examining levels of heavy metals in developing countries where industrialisation is combined with high levels of pollution the heavy metal content is considerably higher. This indicates that the use of monitoring hair in cattle, with appropriate season/age/colour/breed factors being included, is a worthwhile area of research. This present research of cattle in Malta is important as it is a heavily populated island of roughly 475.701 people per square mile (Eurostat).

The concentration of heavy metals within drinking water can be a measurer of its quality. High heavy metal concentrations can leave a marked effect on the aquatic flora & fauna which through biomagnification moves into the food chain, including water, and ultimately affects the human's health as well (Ram S Lokhande *et al.*,2011).Nevertheless, the assessment of heavy metal within water would be imperative in this study, since it can suggest ways on how to lower the risks of toxic heavy metals contamination of drinking water, keeping in mind its hazardous nature since it can lead to health threats. Natural weathering of the earth's surface results in almost all types of water containing heavy metals (Newcomb & Rimstidt, 2002). Adding to this problem is the use of wastewater to irrigate agricultural land. Effluent from city sewage and industrial wastewater further adversely affect water quality, which is why the water needs to be continuously assessed for the quality of ground and surface water sources (Muhammad Iqbal *et al.*, 2018). Heavy metals from human activities can migrate or infiltrate into aquifers and interact with groundwater (Dawson & Macklin, 1998, Charlesworth & Lees, 1999).

The presence of such heavy metals can cause a toxic reaction in plants and as a result its use must be limited. There is research that has led to recommended maximum allowable levels of heavy metals in irrigation water being indicated either for short term use or for longer term watering (Todd, 1980; FAO, 1985; Rowe and Abdel-Magid, 1995; Chapman, 1997). Rowe and Abdel-Magid (1995) have reported on the toxicity of several heavy metals impacting plants. One instance of this is when high concentration lead inhibits plant cell growth. Another example occurs when manganese, usually in acid soils, is toxic to a number of crops at concentrations ranging from a few-tenths to a few mg/L. In contrast, iron is not toxic to plants in aerated soils, but can cause soil acidification and result in the loss of essential phosphorus and molybdenum from the land. Copper has been found to be toxic to a number of plants at concentration levels ranging from 0.100 to 1.000 mg/L in nutrient solutions. Furthermore, zinc has been found toxic to many plants at a wide range of concentrations. Zinc toxicity is reduced in certain conditions (pH > 6.0) in fine textured or organic soils. Cadmium is found to be toxic to beans, beets and turnips at even concentrations as small as 0.100 mg/L in nutrient solutions. However, these heavy metals, at trace levels, including manganese, zinc and copper are vital to the physiological functions of living tissue and may even regulate many biochemical processes (Nwude, Okoye, & Babayemi, 2011).

The knowledge of green fodder production is especially essential in arid and semiarid regions. Hydroponics improves on average the quantity of crops in the same space, as traditional soil-created farming and can reduce water expending compared to traditional farming methods. Limited studies have been carried out on the use of hydroponic milk and fodder quality. A study to compare between traditional (Malta farm) and hydroponic fodder (Gozo farm) has been conducted in Malta by (Agius, Pastorelli, & Attard, 2019), 20 cows were chosen from two farms, and milk samples were collected once a week for a period of 1 month. The finding indicated differences in fat content and pH, showing higher values ($P<0.050$) in milk samples of cow’s fodder with the hydroponic fairly than the traditional fodder. Heavy metals such as Cu and Pb content was significantly higher ($P<0.001$) in the Gozo farm than in Malta, whilst Zn content presented higher values in Malta ($P<0.001$) than Gozo. Even though the proximate findings were similar for both farms, except for the higher fat content for the Gozo farm, principal component analysis revealed that milk quality for the Gozo was greater than the one of the Malta

Having considered milk, fodder, manure, hair and water, it is clear that heavy metal content is influenced by many factors. The present research will build on existing information gathered to understand the particular situation for dairy cattle, feed supplements (often containing heavy metals) and fodder living in a heavy populated island like Malta to see how heavy metal content is bioaccumulated in all the samples taken from a farm. Understanding environmental impacts in addition to diet, breed etc. will shed valuable insights in this field of research.

1.3 Preparation Samples

1.3.1 Analysis of preparation samples

Elemental analysis of most organic and inorganic compounds requires the partial or total dissolution of the sample before instrumental analysis takes place. Very few direct methods allow analysis of samples without any prior preparation, their main disadvantage being a lack of dependable calibration. Sample preparation not only can produce separation and /or pre-concentration of analytes but also permits several determination methods (Kingston & Jassie, 1988; Barnes, 1991; Jarvis *et al.,* 1992) to be used.

Sample preparation in this study involves digestion, extraction and preparation of analysts before analysis. This sample preparation is laborious and time consuming compared to rest of the procedure (requiring 61% of the total time) and introduces considerable error (30% of the total analysis error) (Oliveira, 2003).

For optimum results, the time taken should be reduced, contamination minimised, use of reagent kept low and production of large amounts of waste avoided. In order to achieve effective analysis, several steps of sample preparation should be taken; These should be kept simple and straightforward incorporating the use of high purity water and reagents in appropriate amounts with careful cleaning of any equipment used. Parallel to sample preparation there should also be blank preparation. Finally, validation of the methodology used is very important and ideally is backed up by certified reference materials (Bock,1979).

1.3.2 Sample decomposition technique

Sample decomposition changes the composition in which a particular element is found into a specific form removing contaminating substances from the mixture and leaving the element in a homogeneous and easily accessible matrix. Choosing the appropriate decomposition technique must take into account what element is to be analysed, the composition of the whole sample, the possible obstacles to clear results, the risk of losses and addition of unnecessary contaminants, the laboratory procedures to be followed and any safety hazards in the experiment (Harvey, 2000). Alternative decomposition methods include dry ashing, wet digestion and microwave digestion (Soylak, Colak, Tuzen, Turkoglu, & Elci, 2006).

Sample digestion processes prior to quantification of heavy metal content include closed or open digestion systems and the use of various combinations of acids, such as HNO_3, HCl, $HClO_4$ and others (MOGES, 2014), as well as oxidants such as H_2O_2. The recovery of heavy metals from organic residues will depend on their chemical composition, the degree of polymerization and the presence of molecules difficult to digest (De Abreu, Berton, & De Andrade, 1996).

The choice of digestion method will have to consider both the residue and recovery rate of the heavy metals investigated.

1.3.3 Digestion technique

Wet digestion in an open system, according to Azcue & Mudrock (1994), takes some time, can suffer contamination and loss of some of the sample contents by volatilisation. When using muffle furnace digestion, if the analyte measured is volatile (as is the case for As, Cr, Pb and Cd), some of the elements are lost to the atmosphere and can be also adsorbed onto the furnace walls because of the high combustion temperature, (typically 500-550 ^{o}C). There is also the possibility of cross-contamination of samples using this technique (Azcue & Mudroch, 1994).

The use of microwave digestion of wet samples has greatly accelerated the analysis of trace elements and lowered both contamination risks or amount lost due to volatile chemical elements. The advantage of open systems is that they allow the analysis of a larger number of samples, help in encouraging evaporation of acids used and drying of the digested samples which serves to minimise analytical problems in the stages after digestion (Sastre, Sahuquillo, Vidal, & Rauret, 2002).

Microwave digestion technique is a particularly efficient, fast, and reproducible sample preparation method. By using this method, reaction times are significantly reduced. In addition, it reduces the chances contamination as compared to open digestion techniques. All the closed microwave digestion vessels are armed with a safety system, which opens instantly to control any excessive pressure, by means of either a safety membrane or safety valve to release some of the gases. As the volatile elements are occasionally lost when gases are expelled from the closed vessels this may be responsible for the lower recovery levels in digestion. Fusion is another technique which can be used for difficult matrices. This technique is very labour intensive and can be very expensive; in addition, the particularly high salt load and problems with contamination as a result of the fluxing agent are the main disadvantages of this technique (Aras & Ataman, 2007; Gholami, Behkami, Zain, & Bakirdere, 2016).

Zheljazkov and Warman confirmed that HNO_3 with $HClO_4$ digestion produced a higher recovery of Cd and Pb in comparison to that achieved in a muffle furnace when examining organic matrices (i.e., dry ashing) (Zheljazkov & Warman, 2002). Hseu also obtained reasonable results in the recovery of Cd and other metals using HNO_3 digestion. The use of a combination of heat and mineral acids are called wet digestion methods. Acids typically used in this technique are H_2SO_4, HNO_3 and $HClO_4$, either combined or singly (Balcerzak, 2002).

To increase the reaction speed and ensure complete digestion Hydrogen peroxide is also used. The use of $HClO_4$ has reduced in laboratories due to the risk of explosion. Wet digestion can use open vessels, in tubes, on a hot plate or in an aluminium heating block. It can also be done in closed containers at elevated pressures (digestion bombs) and temperatures (including the use of microwaves). Methodol-assisted digestion has become an attractive and convenient method, particularly for small samples. For example, in all atomic spectrometric techniques, nitric acid is the most commonly used reagent. In spite of sometimes observing signal suppression due to the nitric acid presence (e.g.in ICP-OES), no severe analytical problems are encountered in all atomic spectrometric techniques at concentrations beneath 10% (or on occasion even greater concentrations) as long as its concentration is consistent in calibration and sample solutions. Hydrogen peroxide added in most mineralization procedures also rarely causes analytical problems (Arruda, 2007).

The presence of hydrochloric acid in ICP-OES analysis, does not create any problems. However, hydrochloric acid use is prohibited in GFAAS analysis because of the possible formation of volatile and difficult-to-dissociate analyses chlorides that could cause interference in the experimental results (Welz & Sperling, 2008).

1.4 Atomic Absorption Spectrometry (AAS)

Although alternative spectrochemical methods are available, AAS is one of the most extensively used laboratory techniques for identifying elements with both exactness and accuracy. The major advantages of AAS with atomisation, in a widespread air-acetylene flame, are its reduced operational costs and fairly good analytical performance (Salinas, Montero De Espinosa, Osorio, & Lozano, 1994).

Atomic absorption spectrophotometry (AAS) has been used routinely as a technique for analysis of elements in water samples. The extensive use of AAS around the world is because it is well established, its cost-effectiveness and its ease of use compared to other techniques including the use of inductively coupled plasma.

AAS is a sensitive technique, able to detect elements in up to ng/mL levels when a graphite furnace is used for atomization. In addition, AAS can have a range of irradiation sources chosen to provide good selectivity (Nwude *et al.,* 2011).

1.5 Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

The difference between the two techniques AAS and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) is that AAS relies upon an atomic absorption process while ICP-OES is an atomic/ionic emission spectroscopic technique. However, in this research AAS was used, followed by the use of the ICP-OES.

Nowadays, the most used multi-element technique for metal analysis in a wide range of matrices depend on the inductively coupled argon plasma, in combination with either optical emission (OES) or mass detectors (Montaser, 1998; Nölte, 2003; Payling & Nelis, 2007). This present research used Inductively coupled plasma optical emission spectroscopy (ICP-OES). ICP/OES is one of the most powerful and popular analytical tools in a myriad of sample types for the analysis of essential and trace elements due to its high sensitivity, its speed, and ability to detect the largest number of elements compared to other elemental analysis techniques. It also has the advantage that (ICP-OES) has a wide dynamic range which detects the ions themselves rather than the emitted photons. This provides isotopic information, and works with small sample volumes. It is has only limited spectral and isotopic interference which is another benefit (Karlsson, Sjöberg, & Ogar, 2015) . The determination of the concentration and types of various elements in plant fertilizer has been carried out using the AA (AOAC Official Method 965.09).

The features of ICP as an analytical atomic emission source are so striking that almost all other emission sources (flame, microwave-induced plasma (MIP), direct current plasma (DCP), laser-induced plasma (LIP), and electrical discharge) are only used in specific, highly defined situations. Indeed, areas of research that used to incorporate AAS using both the flame and graphite furnace atomic absorption spectrometry (GFAAS), are now utilizing the ICP. In comparison to these other techniques, ICP-OES runs at higher atomization Approximately 8000 °C, provides a more inert environment, and can provide identification simultaneously for up to 70 elements. This makes ICP less prone to matrix interferences and also allows for corrections for them if they do occur. In those cases where the sample volume is not limited, ICP-OES provides detection limits comparable or even better than its closest competitor, GFAAS, in all but a few elements. Even when these specific few elements are to be studied, the simplicity with which the ICP-OES instrument is operated often offsets the loss in sensitivity (Hou, Amais, Jones, & Donati, 2016).

1.6 Aim

The main objective of this study is to understand how the accumulation and seasonal pollution level of heavy metals, within a very complex pollution system occurs, in order to clarify heavy metal content in dairy cows in Malta and how it filters into a human's food chain. This is done using a small ecosystem.

What a very complex pollution system is, the interaction of pollutants on our food chain, this study will focus on a small ecosystem to try and clarify heavy metal content in dairy cows in Malta.

Fodder, milk, hair and manure of these cows were firstly studied throughout a period of 11 weeks using AAS. Again, this time using ICP-OES, samples of milk, manure, water and fodder were studied for a period of twelve months, as well as, hair which was studied for a period of eight months, to investigate the presence of some toxic heavy metals (Pb, Cd, As), and essential elements (Zn, Cu, Cr, Se) in the samples as it is intended that it will help our understanding of heavy metal content in dairy cows. By including what they digest (fodder and water), the milk they produce, the hair they grow (often used to determine heavy metal content), and the manure they produce, a clearer picture will emerge as to how heavy metals enter, are absorbed or expelled in this particular environment. This has implications for our environment, food chain and its subsequent impact on human consumers.

It is important to note that heavy metals are non-biodegradable in nature and accumulate in the food chains via three processes, biotransformation, bioaccumulation and biomagnification (Aslam, Javed, & Khan, 2011; Muhib *et al.*, 2016).

In light of this, recent increases in heavy metals being observed in milk production raise the possibility that the consumer population of the country could face significant health threats in the longer term from consuming contaminated milk and milk products. It is useful to understand these processes in a schematic manner that indicates this cycle(Muhib *et al.*, 2016).

The collected results help shed light on the heavy metal contamination coming from intensive dairy farms. To explain this pollution chain figure 1.2 below illustrates the cycle of pollutants to humans through cows.

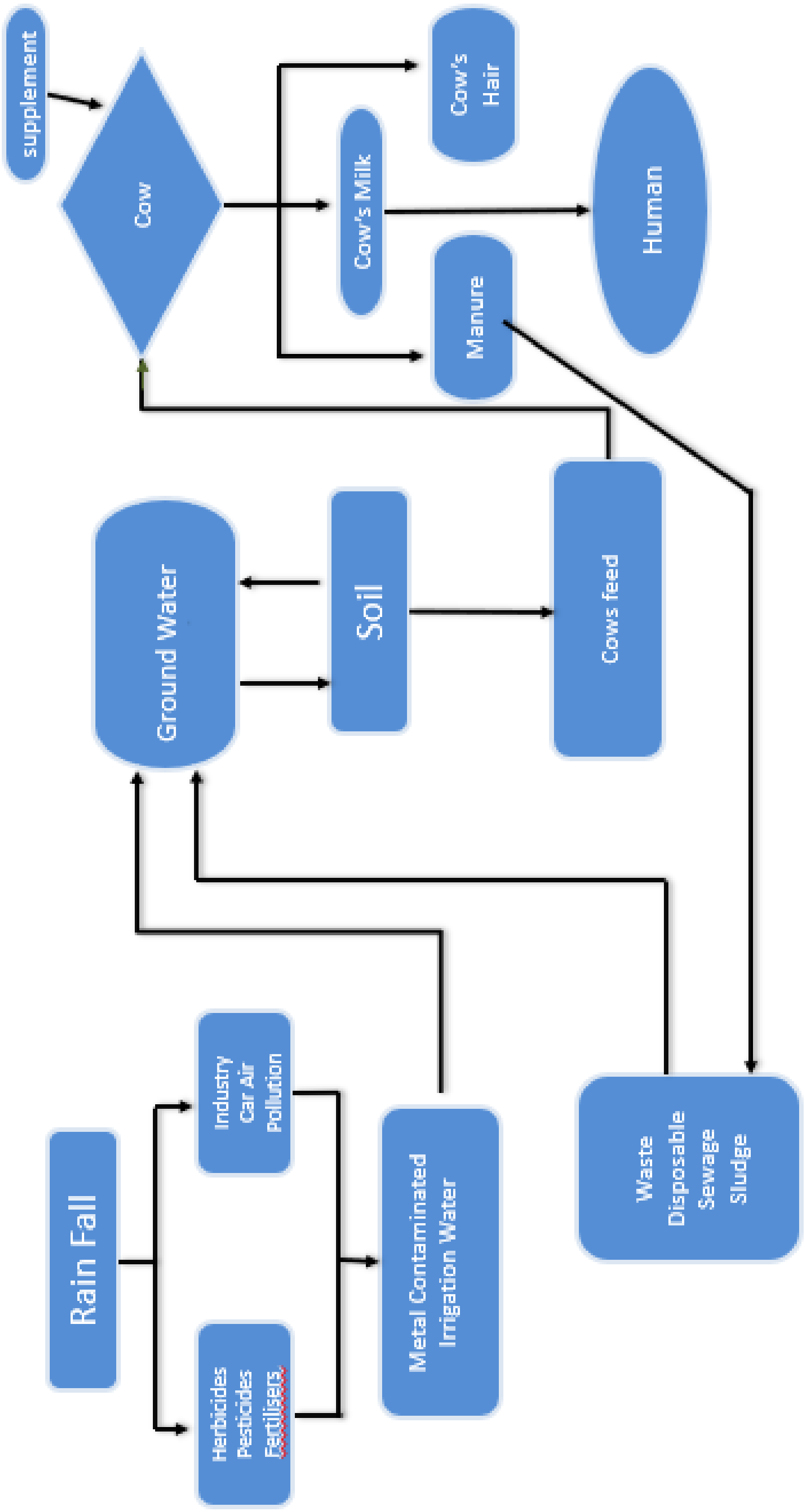

Figure 1.2 The cycle of pollutants to human through cows

1.7 Summary

In this current chapter, the various forms of pollution, air, water, soil and food that help describe the environmental contamination of heavy metals were discussed. The next section moved on to examine the importance of essential trace elements studied and the impact of toxic heavy metals. In order to understand exactly what environmental pollution was occurring, samples were taken of bovine fodder, water, manure, milk, and hair in a specific dairy farm in Malta.

This was followed by descriptions of how samples are prepared. This included details of digestion, extraction and preparation of analysts before analysis and how the samples are treated to remove contaminating substances from the mixture to be studied.

Then, the scientific techniques used, in general, to analyse these prepared samples were introduced including Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). These allow the concentration and type of heavy metal contaminate to be determined.

The last section focuses on the aim of this study which examines a small ecosystem to try and clarify heavy metal content in cows, on a particular dairy farm in Malta, by studying the fodder, milk, manure, and water over a period of eleven months and hair over eight months.

This first chapter having provided an introduction and literature review of pollution, heavy metals, milk, fodder, hair, manure, water and preparation of samples with analytical techniques used, is followed by chapter 2 which illustrates the methodology undertaken in this study. Chapter 3 will include the statistical analysis, results, discussions and conclusion for fodder, manure, milk and hair within a period of twelve weeks with regards to Cu only using AAS.

Chapter 4 will investigate statistical analysis, results, discussions and conclusion for fodder, water, manure, and milk throughout twelve months, and hair for eight months, for all elements of interest using ICP-OES. The correlations between the intake and outtake concentrations of all the studied inputs and outputs samples will also be discussed.

Chapter 5 will deal with two different comparisons of heavy metal concentrations; starting with two methods of preparation of samples, followed by comparison between black and white hair.

The final chapter, 6, will pertain of the study’s closing remarks and recommendations for future work

2. Methodology

In this chapter, the determination of strategy concerned with the collection of samples will be discussed. Moreover, all chemical, glassware and apparatuses will be shown. This chapter will also focus on preparation samples, digestion methods and the instruments that were used as well.

2.1 Sampling and digestion

Milk, hair, fodder and manure samples were collected over two different periods from a dairy cattle farm located in Salina, Malta.

2.1.1 Weekly samples

Ten cows (ages 2 to 6 years) were chosen as representatives of this farm. These were observed over three months, starting with the first week of September 2016 until the first week of December 2016, which is the first period of this study.

Samples of milk and hair were taken from the same cows once a week. Overall, 110 milk samples and 110 hair samples were used in this research.

2.1.1.1 Milk samples

Samples were collected immediately after milking the ten cows, with 30 mL of each sample being put in polyethylene containers each time and frozen until they were ready to be prepared for analysis.

2.1.1.2 Hair samples

Samples were trimmed from the tails; each sample, which weighed approximately 1 g, was stored separately in polyethylene bags.

2.1.1.3 Fodder and Manure

All samples were collected over the same period once a week. Fifteen fodder samples were taken from different types of fodder that they use to feed the cows, those types being, pellets, hay, corn and mixed seeds powder. All samples were accumulated and stored individually in polyethylene bags.

A manure sample was taken per week from the manure clamp after mixing the tank well, which was then kept in polyethylene bags as well, for 11 weeks. The samples were then dried in an oven at 75 ± 5 ℃ (Afridi *et al.*, 2006); and stored in polyethylene bags upon drying.

2.1.2 Monthly samples

All the monthly samples were taken from cows at a random manner (unlike the weekly sampling which focused on the same ten cows), once a month for a period of approximately one year. In the case of milk, samples were collected each month from September until December 2016. The collection of milk and hair samples resumed for a period of eight months starting again in the month of January 2019 until the month of August 2019. In addition, samples of manure and water were collected once a month during one year in 2019, starting in January. However, fodder samples were collected in two periods. According to purchased fodder for the farm, the first period was in January 2019, and the second period was in May 2019. The same strategy was applied for the collection of samples and storage of the weekly samples. Water was collected from the drinking through of cows then kept in a clean and dry plastic container directly from sources of drinking water for animals at the farm.

2.2 Chemicals and glassware

Ultrapure water, 18 Ω, was prepared using the Elga Purelab Classic purification system. Nitric acid and hydrogen peroxide were ultra-trace analysis grade from Fisher chemical UK, acetone and Triton X-100 from BDH, England. Standard solutions of Cu were prepared by dilution of certified standard solutions (1000 ppm, Mono-element, Indian) for AAS. While certified reference material (1000 ppm, calibration standard (IV) 23, CPAchem. Bulgaria) for ICP was used to prepare the standard solutions of all the elements Cr, Cu, Zn, As, Se, Cd and Pb included in this research.

All glassware and polyethylene material used was previously treated for at least 24 hrs in 10% nitric acid and rinsed with distilled water and then with ultrapure water.

2.3 Instruments

Microwave digestion was used to prepare the samples, while Atomic Absorption Spectrometry and Inductively Coupled Plasma-Optical Emission Spectrometry were used to analyse the samples.

2.3.1 Microwave digestion

A microwave oven, ETHOS Up model MA182 with up to pressure 50 bar, with maximum temperature 270°C, was used from Milestone Srl, Sorisole (BG), Italy. This microwave system allows heating via pulsed and non-pulsed irradiations without contact for a range of materials, dependent on the specific material dipole present.

2.3.2 Atomic absorption spectrophotometer

The contrAA 700 was used to allow sequential analysis of metal and non-metal traces in liquid and dissolved samples, from Analytik Jena, Germany. The contrAA 700 provides a highresolution continuum source atomic absorption spectrometer for flame and graphite tube techniques used to determine the copper metal content in liquid and dissolved samples in this research.

The calibration curves were constructed using standard solutions of the metal ions by following the procedure given in the manual using appropriate detectors in the wavelength range suitable for the concentration range. The AAS parameters used are shown in table 2.1.

Table 2.1 Measurement conditions for AAS

Element	Line	Wave length(nm)	Oxidant (air) (Kg/cm^2)	Fuel (acetylene) (Kg/cm^2)
Cu	Cu324	324.754	1.6	0.2

2.3.2.1 Preparation of standard solutions for AAS

Calibration curve for Cu was prepared using five standard solutions. The usual procedure in quantitative analyses method is to prepare a series of standard solutions over a concentration range suitable for the sample being analysed, i.e., such that the expected sample concentrations are within the range established by the standard. These standards for Cu prepared by dilution from 2 ppm stock solution were as follows: 0.040 ppm, 0.100 ppm, 0.200 ppm, 0.300 ppm and 0.400 ppm. Calibration curves were drawn for Cu by plotting absorbance versus Cu ion concentration.

2.3.2.2 Sample preparation

In order to determine the concentration of minerals and trace elements, milk samples were taken out of the freezer and left long enough to melt. Subsequently, 3 ml of each sample were used in the digestion procedure described below.

Before the digestion of hair, each sample was cut into approximately 0.500 cm long pieces and mixed to allow a representative subsample of the cow’s hair. After cutting, each sample was washed with diluted Triton X-100. The samples were rinsed with distilled water and then with ultrapure water. Later, the sample was rinsed with acetone three times (Afridi et al., 2006), (Assarian & Oberleas, 1977), (Salmela, Vuori, & Kilpiö, 1981). The samples were then dried in an oven at 75 ± 5°C, and 0.2 g of hair were taken from the dry sample to continue the digestion procedure below.

Furthermore, fodder samples (Maltese and imported hay) were washed with ultra-pure water and dried at 105 °C in an oven. All the samples (Maltese hay, imported hay, pellets and mixed seeds) were grounded into powder, passed through a 0.020 mm sieve and mixed to homogenize the sample (Matthews-Amune & Kakulu, 2012. Subsamples of 0.200g from the dry product (fodder and manure) were weighed and then digested as in the procedure below.

2.3.2.3 Digestion Methods

In this section the conventional method was used to prepare all samples. A volume of 3 mL for each milk sample was transferred to a Pyrex beaker, then 5–10 mL conc. HNO_3 was added to the flask. These were covered using watch glasses and heated on an electric hot plate to decompose the organic matter in samples at 70–80 °C for 1–2 hours. The digest was then treated with additional 5 mL conc. HNO_3 and a few drops of H_2O_2 and heated on a hot plate at about 80 °C for 1–2 hours until the colour of the digestion solution became a clear transparent yellow. It was then cooled at room temperature and diluted to a volume of 25 mL in volumetric flasks with ultrapure water (Afridi *et al.*, 2006).

The same procedure as outlined above was performed for the solid samples (hair, fodder and manure). However, in these particular samples, 0.200 g was used for each one.

2.3.2.4 Samples Analysis

A solution of 1% KCl was added as an ionization buffer for the samples after the acid digestion (0.050 g KCl to 50 mL of each prepared sample), which were then placed on the carousel of AAS and were ran, after the calibration curve for Cu was determined. It was ensured that the calibration range was taken in the linear range. The method inputted into the AAS for Cu using acetylene/air fuel was as following: wash time between each sample was 20 s. The absorbance values only were given; the acceptable RSDs of repeated injections was 10%. After each 5 samples the QC blank was passed again to account for any drifting.

2.3.2.5 Statistical Methodology

The following details statistical methodology which will be used throughout the AAS chapter in this exploration:

1. **Descriptive statistics tables**: These tables will contain information about the mean, standard deviation and (where appropriate) 95% confidence intervals of the mean of various quantities along different categories. 95% confidence intervals interval estimates for which there is 95% confidence that are these contain the true mean.
2. **Error bars**: these are graphical tools which will display the information in descriptive statistics tables graphically, the centre of the error bar represents the mean, and the bar represents the standard deviation.
3. **Hypothesis testing for multiple repeated measures**: The main tests used were

    a. Repeated measures analysis of variance (ANOVA) (parametric test)
    b. Friedman test (a non-parametric test).

The difference between (a) and (b) is that (a) makes strong assumptions about the distribution and properties of the population from which the data is coming from, while for non-parametric tests some of these assumptions are relaxed. These tests are used to compare population differences in copper samples from milk and hair between weeks/periods. For both tests, the null and alternative hypotheses will be:

H0: The copper measurement for the cow population is not different between periods.
H1: The copper measurement for the cow population is different for some periods.

The level of significance we use is 0.05. This means that there is not enough evidence to reject the null hypothesis if the p-value exceeds the 0.05 level of significance, while if the p-value is below 0.05 has rejected the null hypothesis.

4. **Diagnostic tools**: The following are tools for checking parametric assumptions of repeated measures ANOVA. If these are not satisfied, we resort to the Friedman test.

a. Energy test: Repeated measures ANOVA requires multivariate normality of the multiple responses from each subject (in this case, the cows). This is available in R. See Szekely and Rizzo (2005) for test, and Szekely and Rizzo (2017) for description of package. In this null hypothesis, this test assumes multivariate normality, while the alternative hypothesis rejects it.

b. Mauchly’s test for sphericity: Repeated measures ANOVA in its standard form requires that the variances of the differences between all possible pairs of within-subject effects (in our case the different periods/weeks) are equal. To check for this, Mauchly’s test for sphericity was applied. In the null hypothesis, the test assumes that sphericity is present, while the alternative hypothesis rejects it. If sphericity is not present, it is not necessary to resort to the Friedman test, but one can instead consider one of the corrections for lack of sphericity (in the forms of e.g., Greenhouse-Geisser, Huynh-Feldt or lower-bound) in repeated measures ANOVA. This was not the case in our study.

5.**Post-hoc tests**: Repeated measures ANOVA and the Friedman test check whether there is a difference between the weeks but does not indicate where the differences lie. To check whether these different means actually differ significantly from each other, post- hoc tests must be used when the null hypothesis is rejected. For repeated measures ANOVA, all possible pairwise comparisons using the paired samples t-test were conducted, while for the Friedman test, on the other hand, all possible pairwise comparisons using the Wilcoxon test are conducted. The paired-samples t-test is a parametric test for twice-repeated measures, while the paired Dunn test is a non-parametric test for twice-repeated measures. The hypothesis for both is the same as repeated measures ANOVA and Friedman test but for two levels. For this current study, the Bonferroni p-value adjustment was used as the post-hoc tests. The Bonferroni p-value adjustment basically multiplies the p values of these tests by the number of possible pairwise comparisons. If this exceeds 1, then the given p-value will be 1. The Bonferroni approach ensures that under the assumption that the null hypothesis is true for all m pairwise comparisons, the probability of accepting the null hypothesis in all cases is at least 0.95. Similarly, the probability of wrongly rejecting the null hypothesis in at least one of the pairwise comparisons is at most 0.05. Other post-hoc tests which have tried gave similar results.

6. **Outlier detection**: For outlier detection, a commonly known method called Tukey's fences was used. If LQ and UQ represent the lower and upper quartiles respectively of a set of observations, any points less than LQ – 1.5(UQ – LQ) and greater than UQ + 1.5(UQ – LQ) are deemed to be outliers.

7. Sequence plot: A plot which displays the patterns of an ordered data set (particularly one ordered in time).

2.3.3 Inductively coupled plasma-optical emission spectrometry

ASpect PQ is the control and analysis software for the ICP-OES PlasmaQuant PQ 9000 Elite from Analytik Jena AG Germany. The method parameters for the measurement procedures can be optimized to the specific demands of the sample to be analysed.

Table 2.2 Measurement conditions for ICP-OES

ICP-OES RF power (W)	Plasma gas flow (L/min)	Auxiliary gas flow (L/min)	Nebulizer gas flow (L/min)	Replicates	Replicate time (s)	*Stabilization time (s)*	Emission lines (nm)
1000	12	0.50	0.50	2	10	30	Cr205.552 Cu327.396 Zn206.200 As188.979 Se196.028 Cd288.802 Pb220.353

2.3.3.1 Preparation of standard solutions for ICP

The calibration curve for all the elements was prepared using five standard solutions. The routine procedure in the quantitative analysis method is to prepare a series of standard solutions over a concentration range appropriate for the sample being analysed, i.e. ensuring that the expected sample concentrations are within the range given by the standard. The same standard procedure for ICP-OES was incorporated with minor changes, as used in ASS.

These standards, prepared by dilution from 1 ppm stock certified reference material solution content, all the elements were as follows: 0.100 ppb, 1 ppb and 100ppb, followed by 1000 ppb and 2000 ppb were prepared from 100ppb. Calibration curves were drawn for each element by plotting absorbance versus the ion concentration.

2.3.3.2 Sample preparation

The same procedure used in section 2.3.2.2 for AAS was carried out for ICP-OES samples.

2.3.3.3 Digestion Methods

A microwave oven (mentioned in section 2.3.1) was used to digest all the samples gathered for this research, including milk, hair, fodder and manure, and excluding water. An explanation for each one has been detailed below, starting with milk.

1. **Milk**

The concentration of all trace heavy metals in milk was determined by placing 3 mL of the cow’s milk in a high-pressure Teflon bomb and this was then digested with 10 mL of nitric acid (HNO_3) on the microwave workstation. The microwave digestion program was applied and included the following temperature stages: 25–140 °C for 10 minutes, 140–150 °C for 4 minutes, 150 °C for 7 minutes, 150–180 °C for 10 minutes, and 180 °C for 20 minutes, all of them at a power of 1000 W. The vessels were cooled at room temperature (20 minutes), and carefully opened. After the digestion process, the acid-digested sample solution was then diluted in a 25 mL volumetric flask with ultrapure water (Sola-Larrañaga & Navarro-Blasco, 2009).

2. **Hair**

The prepared hair samples were then cut into approximately 1cm pieces so that they could be inserted in the microwave digestion vessels. The digestion was carried out using the microwave digestion program, specifically for hair on the microwave equipment. A sample of 500 mg of the cattle's hair was placed in the digestion vessel with 10 mL of nitric acid (HNO_3). This was placed in the microwave workstation. The microwave digestion program was started at a temperature of 16℃ increasing up to 200℃ within 15 minutes. The temperature remained stable for another 15 minutes. The digested sample was then left to cool until it was at room temperature. Following this procedure, the prepared sample was added to a 25mL volumetric flask so that dilution with ultrapure water could be carried out.

3. Fodder

The dried and powdered fodder samples were digested using the microwave digestion program designed for fodder on the microwave workstation. A sample of 500 mg of each type of fodder, of powdered fodder (seven different categories), was placed in the microwave digestion vessels. With the addition of 8 mL of nitric acid (HNO_3) and 2mL of Hydrogen Peroxide (H_2O_2). This was left on the microwave workstation for half an hour. The program was started at a temperature of 16℃, which increased for 15 minutes until it reached 200℃, proceeding with it being stable for another 15 minutes. After cooling at room temperature, the digested sample solution was then diluted in a 25 mL volumetric flask with ultrapure water.

4. Manure

In order to determine the concentration of all trace heavy metals in manure by ICP, it was necessary to digest the dried sample. This was done by putting the prepared sample through the specific microwave digestion program designed for manure on the workstation. 200 mg of each prepared manure sample was positioned in the vessel of the microwave digestion workstation and then 8 mL of nitric acid (HNO_3) and 2mL of hydrogen peroxide (H_2O_2) were added. This was placed in the microwave workstation for half an hour, starting at a temperature of 16℃ which lasted for 15 minutes until it reached 200℃. The sample was left for some time to cool until it reached room temperature. Following this, a 25mL volumetric flask was used to dilute the sample with ultrapure water.

5. Water

No digestion is required for the water samples, however, in order to analyse these samples of water in the ICP equipment, it was necessary to ensure that they were at same acidic concentration of 2% like all samples, including standards. This was done by spiking 1.5mL of concentrated nitric acid to 50mL of the sample of water, in order for it to be ready for investigation.

After this procedure, all the samples of milk, hair, fodder, manure and water were ready to be inserted in the ICP-OES equipment for detection of the specific heavy metals studied in this present research.

2.3.3.4 Validation of Method

Taking into account all that has been discussed in the previous section (2.3.3.3), in order to test the method used for the digestion of samples, using ICP, the following validation method was carried out.

The first step was to prepare a mixture of 1.5 mL of milk with 1.5 mL of Ultrapure water. This was digested with 10 mL of nitric acid (HNO_3) using the same procedure as for the digestion of milk samples depicted in the previous section under milk. The second step was to prepare 1.5 mL of milk with 1.5 mL of quality control that was used during the analysis of the sample. This was digested in the same manner as milk and ultrapure water, with the same amounts of HNO_3. Finally, the last step was to prepare 1.5 mL of water with 1.5 mL of the same quality control used with milk, followed by the same amount of HNO_3, as in the previous mixtures.

These mixtures were analysed by means of ICP using the same conditions set for the equipment, to test the linearity for the standard solutions and the digestion method. The results obtained, showed that the method chosen for the digestion of samples is valid and appropriate, as explained in detail in the appendix 2.3.

2.3.3.5 Statistical Methodology

The following details statistical methodology which will be used throughout the ICP chapter in this exploration:

1. Descriptive statistics tables:
2. Error bars

With regards to the above two statistical tools, the same content applies as in section 2.3.2.5.

3. Hypothesis testing for paired measures: The test used was the paired Wilcoxon test, which does not assume normality in the differences of pairs of readings. This test is used to compare heavy metal concentrations between two periods, for different types of fodder. The null and alternative hypotheses will be:

> H0: There is no difference in heavy metal concentration between period 1 and period 2.
>
> H1: There is a difference in heavy metal concentration between period 1 and period 2. The level of significance used is 0.05.

4. Hypothesis testing for independent measures: The test used was the paired Kruskal Wallis test, which does not assume normality in the differences of pairs of readings. This test was used to compare heavy metal concentrations between two periods, for different types of fodder. The null and alternative hypotheses will be:

> H0: There is no difference in heavy metal concentration between different months.
>
> H1: There is a difference in heavy metal concentration between different months.

It is important to note that the parametric versions of Hypothesis testing for paired measures and for independent measures i.e., the paired t-test and the one-way Anova will not be considered due to the several combinations of tests required, hence the need to use a common tool. The level of significance used is 0.05.

5. Post-hoc tests: The Kruskal Wallis test checks whether there is a difference between months but does not indicate where the differences lie. To check whether these different means actually differ significantly from each other, post- hoc tests must be used when the null hypothesis is rejected. For the Kruskal Wallis test, all possible pairwise comparisons using the Dunn test are conducted. The Dunn test is a nonparametric test for independent measures. The hypotheses are the same as the Kruskal Wallis test but for two levels. For this current study, the Bonferroni p-value adjustment was used as the post-hoc tests. The Bonferroni p-value adjustment, in short, multiplies the p values of these tests by

the number of possible pairwise comparisons. If this exceeds 1, then the given p-value will be 1. The Bonferroni test used as explained in section 2.3.2.5

6. Correlation: Pearson correlation is a test statistic that measures the relationship between two quantitative variables. Values close to zero indicate that the two variables are independent, statistically significant positive values indicate that the relationship is a positive one (i.e., the two variables both tend to increase or decrease simultaneously), while statistically significant negative values indicate that the relationship is a negative (i.e., when one variable increases, the other variable tends to decrease, and vice versa). The null and alternative hypothesis in this case will be:

H0: The correlation between the two variables is not significant.
H1: The correlation between the two variables is significant.

While the standard level of significance used here is 0.05, other levels of significance (such as 0.01 or 0.1) shall be considered to highlight the significance of the correlation.

7. Moving average: A centred moving average is a series of averages applied symmetrically around any point to sequential/time series data which is used to infer the presence of any trend or seasonal behaviour. The size of the window around any point is referred to the span of the moving average. A simple moving average is used, as explained in detail by Chatfield (2013). The original series and the moving average will be presented as overlapping sequence plots.

2.3.3.6 Statistical Analysis for Comparison

The following details statistical methodology which will be used throughout the Comparison chapter in this exploration:

1. Descriptive statistics tables

2. Error bars

With regards to the above two statistical tools, the same content applies as in section 2.3.2.5.

3. Hypothesis testing for paired measures: The tests used were the paired samples t-test and the paired Wilcoxon test. These were used to compare concentration measurements between two methods for different heavy metals. The null and alternative hypotheses, for both tests, will be:

H0: There is no difference between the measurements of heavy metal concentration for the two methods.

H1: There is a difference between the measurements of heavy metal concentration for the two methods. The level of significance used is 0.05.

4. Hypothesis testing for two independent measures: The tests used were the independent samples t-test and the Mann Whitney test. These were used to compare heavy metal concentrations between black and white hair. The null and alternative hypotheses, for both tests, will be:

H0: There is no difference in heavy metal concentration between black and white hair.

H1: There is no difference in heavy metal concentration between black and white hair.

The level of significance used is 0.05.

5. Diagnostic tools: The following are tools for checking parametric assumptions of paired sample t-test in the case of (a) and independent sample t-test in the case of (a) and (b).

a. Tests for normality: For paired data the normality of the paired differences need to be tested, and for independent data, normality for each of the two groups needs to be tested. Two tests for normality are used: the Kolmogorov-Smirnov test and the Shapiro-Wilk. In both tests, the following hypotheses are present:

H0: The data comes from a normal distribution.

H1: The data does not come from a normal distribution.

b. Levene's test for the homogeneity of variance: When there are multiple independent groups, this test is used to check whether the variances between groups are homogenous (equal). In this test, the following null and alternative hypothesis are present:

H0: The groups have equal variances.

H1: Not all groups have equal variances.

If the parametric assumption (a) is not satisfied then the non-parametric equivalents of these tests are resorted to, the paired Wilcoxon test and the Mann Whitney test, respectively. If parametric assumption (b) is not satisfied but parametric assumption (a) is satisfied, then the independent t-test can still be used but the p-value needs to be considered, which considers the discrepancy between variances. For both (a) and (b), the level of significance used is 0.05.

2.3.4 Limit Of Detection (LOD) and Limit Of Quantification (LOQ)

For both AAS and ICP-OES, Limit of Detection (LOD) and Limit of Quantification (LOQ) are the lowest possible concentration of the measured that can be detected at a specified level of confidence. The LOD is most known as the concentration giving an absorbance signal equal to three times the standard deviation of the blank solution. On the other hand, the LOQ is most often defined as the concentration giving an absorbance signal equal to ten times the standard deviation of the blank solution. Concentrations that are above the LOQ can be easily quantified while the concentrations that are below the LOD are not detected. Having said that, concentrations between the LOD and LOQ are detected but not quantified (Butcher and Senddon, 1998) (Robenson J.W. *et al.*, 2005).

The instrumental LOD and LOQ were determined based on sample replicates measurement. Three replicates were secured at a blank solution (0.5 N HNO3) for each instrumental experiment. LOD and LOQ values were calculated using the formulas below:

$$\text{LOD} = 3 \times \text{SD} \quad \text{and} \quad \text{LOQ} = 10 \times \text{SD}$$

where SD is the standard deviation of the blank.

The linear calibration curve of Cu was established for AAS, while for ICP, the linear calibration curve was for Cr, Cu, Zn, As, Se, Cd and Pb. The determination of the concentrations' ranges occurred when the linear regression correlation coefficient was greater than 0.99.

2.3.4 Software used

For most of the analysis and outputs IBM SPSS version 26 has been used. However, R was used to extract results for the energy test for multivariate normality.

3. AAS results and discussion

3.1 Introduction

Heavy metals that are of interest in this study are Cr, Cu, Zn, As, Se, Cd and Pb. As a preliminary study, the AAS was used in this chapter to determine increasing or decreasing levels of Cu in the life cycle of cows, starting from the feed through the process of mechanical digestion within the body. The digestion process was examined in order to observe the remaining levels of Cu in milk and what is extracted through hair and manure. However, during the lab experiment work, the AAS was damaged and stopped working, hence the rest of the heavy metals were not analysed. Therefore, ICP was used to perform the ultimate analysis in the next chapter. Nevertheless, the results of the Cu from AAS have led the study to collect the samples on a monthly basis. Therefore, in this section, the findings of Cu concentration in the weekly samples (as mentioned in section 2.1.1) of fodder, manure, milk and hair are presented using the statistical tools, mentioned in section 2.3.2.5.

3.2 Fodder analysis

The fodder found in this farm was both of local and foreign origin. The feed was stored on the farm to be available for consumption, and when due, the fodder is bought according to what is available in the market. The fodder acquired by the farm is not based on seasonal requirements of a cow’s need for a particular feed.

Table 3.1shows the mean Cu concentration of various types of feed collected. It can be seen that there is a significant difference in the observed Cu concentration between different types of fodder. It can be clearly note that the dairy pellet has the highest average among the others, followed by the grounded maize which is close to the maize (small pellet). The Maltese hay and the large pellet both have slightly similar results. However, the mix seeds have the lowest mean concentration in the current study.

According to the results, Cu concentration is not affected by the seasons and no statistically significant differences can be noted. This is reported in Table A.3 (in the appendix), where Maltese Hay, which was collected in the first week of September and in the first week of November, show similar concentration of Cu 3.80 and 3.91 ppm, respectively.

Table 3.1 Mean Cu concentration in fodder (ppm)

Fodder type	**Mean ± SD (ppm)**
Imported hay	**4.93±0.751**
Maltese hay	**3.73±0.224**
Grounded maize (mixed corn)	**9.81±0.774**
Maize (small pellets)	**7.33±0.837**
Large pellets	**2.83±0.844**
Mixed seeds	**1.08±0.069**
Dairy pellets	**16.1±0.000**

According to Ghafoor *et al.* (1994) & Farid & Baloch (2012), growing crops are affected by economic consequences. This situation is combated by frequently using city effluent in order to maintain various crops, including fodder in the surrounding large cities. Unfortunately, these effluents are contaminated with untreated industrial effluents. However, these effluents are a good source of irrigation, nutrients, and organic matter but also rich in heavy metals (Ahmed et al, 1994) and contaminants.

In addition, one of the limitations in this study, with regards to fodder analysis, was information on the production and storage of the vegetal materials at the site of production and during its transit to the farm from local and foreign sources. However, since all types are imported from EU countries, they fall under the auspices of the European Union. Therefore, it can be assumed that each type was monitored in a strictly manner before being exported to Malta.

3.3 Manure analysis

Results of the contents of Cu for manure, are shown in Table 3.2 and Figure 3.1. The results, as illustrated in Figure 3.1, show that the highest Cu concentration was noted in week 5 while the lowest was in week 7. Similar to the fodder findings, there is no significant difference based on seasonal effect. However, there is a significant fluctuation of concentrations during the study period which has no relation to change in temperature.

Table 3.2: Cu concentration in manure (ppm)

Sample number	**Cu concentration (mg/Kg)**
1	**14.2**
2	**10.2**
3	**12.1**
4	**9.30**
5	**22.0**
6	**11.2**
7	**7.80**
8	**13.2**
9	**15.9**
10	**14.2**
11	**9.81**

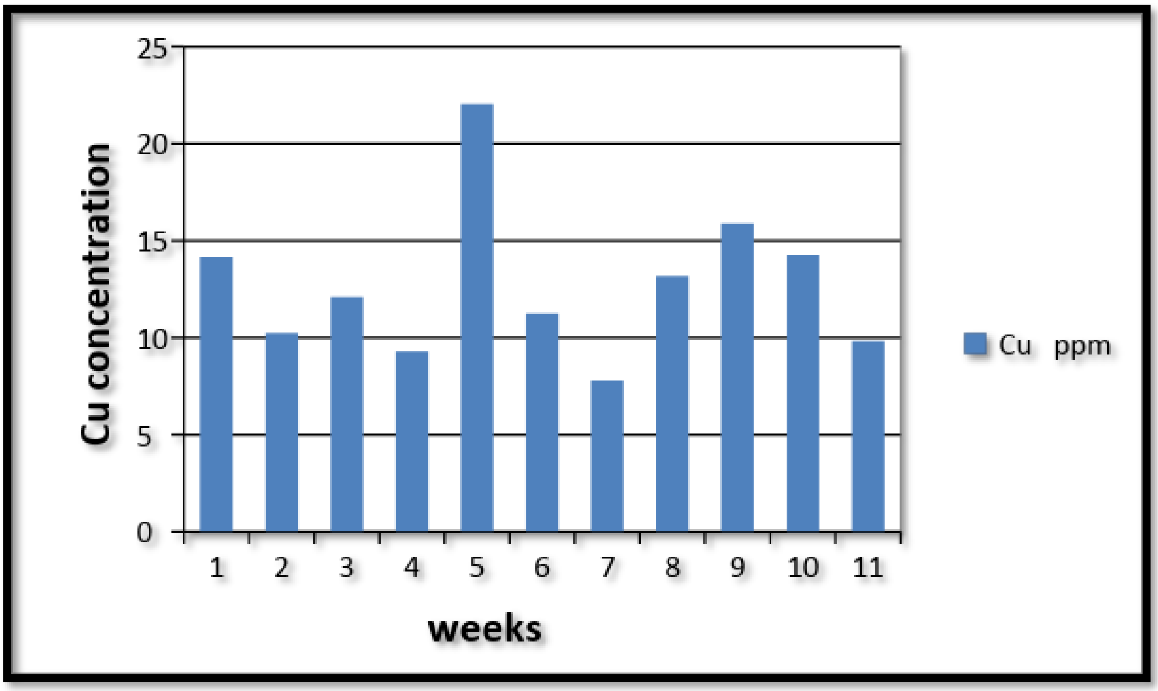

Figure 5.1 Copper concentration (mg/Kg) in manure with number of weeks

3.4 Milk and hair analysis

3.4.1 Introduction

In this section, Cu concentration in milk and hair were studied. The samples were collected from the same 10 cows over a period of 10 weeks, and the data analysed via some exploratory data analysis and statistical techniques. The aim is to assess whether there is a difference in the mean of Cu concentration between the weeks for both the cows' milk and hair samples. Therewithal, it is desirable to investigate whether there is a variation of the mean concentration between these cows. Following this, an overview of the methodology used, as previously mentioned, is discussed in the next section.

3.4.2 Milk and Hair Standards

Linearity for Cu

Figure 3.2 and Figure 3.3 represent typical calibration graphs for concentration of Cu in milk and hair, respectively. From both figures, the slope of milk and hair are $R^2 = 0.999$ and 0.998, respectively, which implies perfect linearity. Furthermore, most samples analysed were present within the calibration range and no appropriate dilution were performed.

LOD of Cu was obtained by multiplying the standard deviation of the reagent blank by 3, and the values was: $\text{LOD} = 3\times \text{SD}_{\text{blank}}$;

$$\text{LOD}= 3\times 0.000160=0.000480 \text{ mg/L}.$$

While LOQ of Cu was determined by multiplying the standard deviation of the reagent blank by 10, and the values was: $\text{LOQ} = 10\times \text{SD}_{\text{blank}}$;

$$\text{LOQ}= 10\times 0.000160=0.00160 \text{ mg/L}.$$

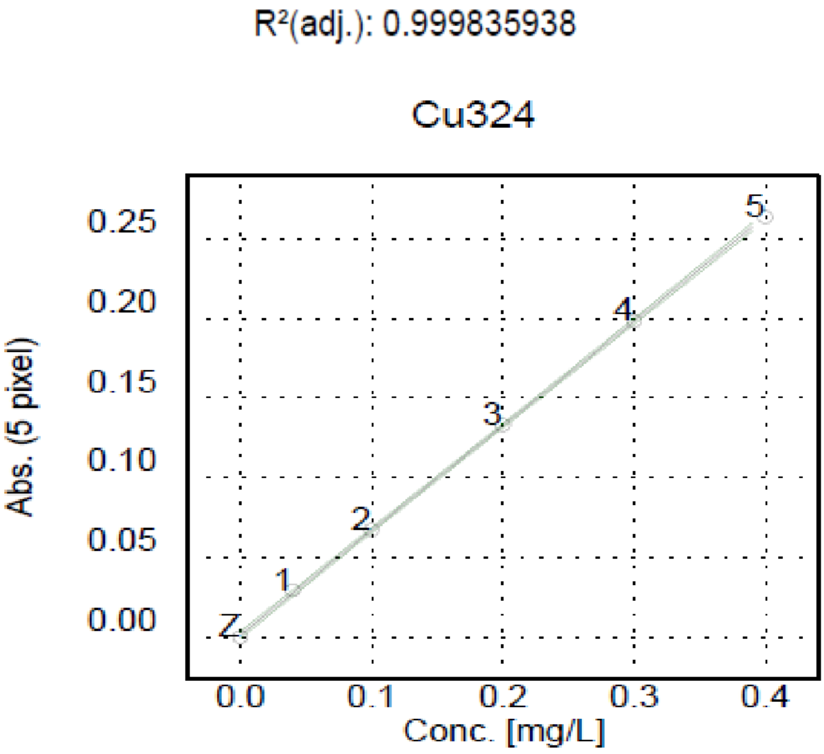

Figure 3.2 Calibration curve of Cu (milk)

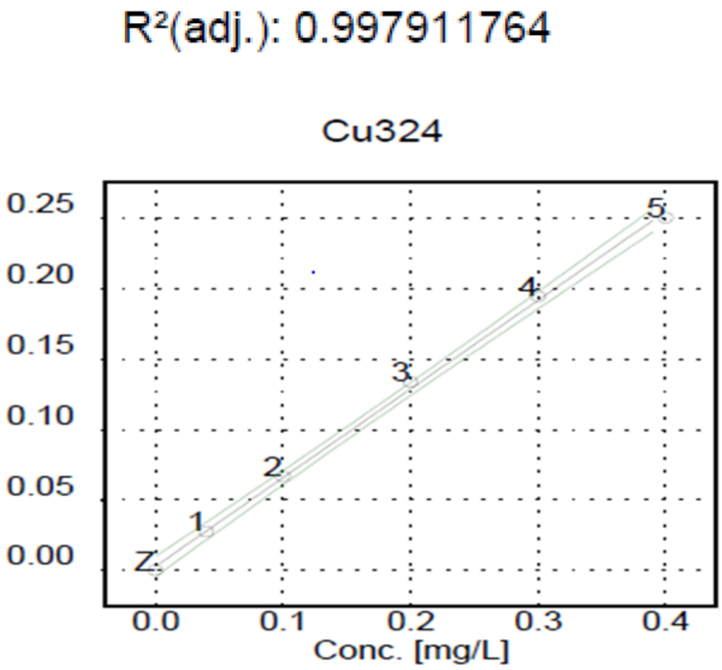

Figure 3.3 Calibration curve of Cu (hair)

3.3.3 Exploration of Data Analysis

The various descriptive statistics tables and error bar plots related to the data being studied are discussed and analysed here. All the statics and tools used to interpret cattle milk and hair analysis are described in Chapter 2 Section 2.3.2.6. The Cu concentrations from milk samples are investigated, and the same analytical process was conducted on the hair trials.

3.4.2.1 Milk samples

In Table 3.3, the descriptive statistics demonstrate the mean, standard deviation, lower and upper 95% confidence bounds for Cu samples in milk. It can be seen that the mean is consistently decreasing from week to week, with exceptions occurring on Week 4 and Week 9. It can also be spotted anomalously large standard deviations in Week 1 while the standard deviation appears to be decreasing in later weeks, and spikes again in Week 7.

Table 3.3 Mean and standard deviation of copper concentration (mg/Kg, milk) for each week

Week	Mean ± SD (mg/Kg)
1	.0767 ± .0948
2	.0689 ± .0302
3	.0586 ± .0375
4	.0600 ± .0399
5	.0419 ± .0230
6	.0318 ± .0176
7	.0310 ± .0397
8	.0159 ± .0163
9	.0186 ± .0068
10	.0176 ± .0099

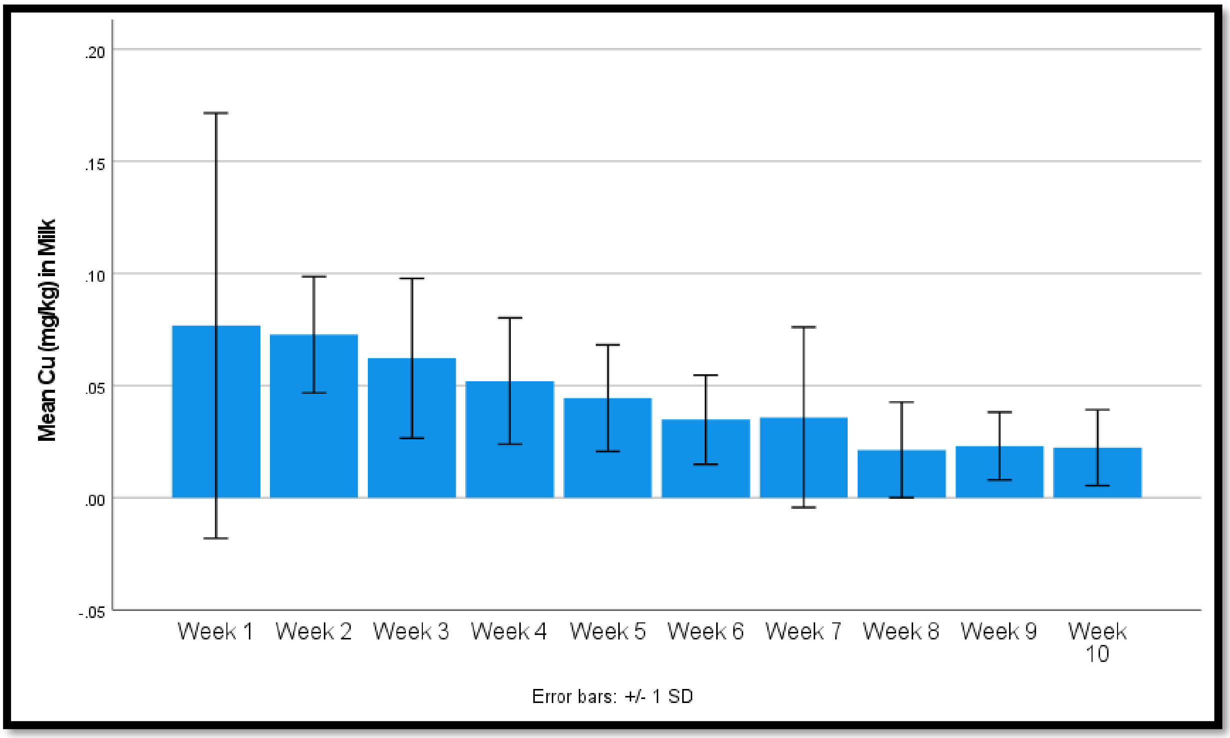

Figure 3.4 Error bar of copper concentration (milk, mg/Kg) for each week

The information depicted in Table 3.3 was also represented using the error bar graph shown in Figure 3.4, which gives the means for each week (dot) and the confidence intervals (line). Particularly, large confidence intervals for Week 1 and Week 7 confirm the large variance occurring during these weeks.

Similarly, the mean and standard deviation for each cow was considered. The confidence intervals in this case are not derived, due to the fact that the readings are repeated observations from the same subject. Therefore, the independence required to calculate these confidence intervals cannot be assumed. It can be observed in Table 3.4, that the readings from Cow 1 have a very large variance. To a lesser extent, the readings from Cow 7 too was found to have a significant variance.

Table 3.4 Mean and standard deviation of copper concentration (mg/Kg, milk) for each cow along weeks

Cow	Mean ± SD (mg/Kg)
1	.0856 ± .099
2	.0332± .0219
3	.0335 ±.0195
4	.0356 ±.0203
5	.0370 ±.0397
6	.0395 ±.0233
7	.0490 ±.055
8	.0412 ±.030
9	.0345 ±.024
10	.0320 ±.0230

Figure 3.5 shows a sequence plot of readings from each cow along the whole 10 weeks. One can observe that there are what appear to be outliers in a number of weeks, however the most remarkable outlier is Cow 1 on Week 1. In comparison to the rest, Cow 7 on Week 3 and Cow 1 again, on Week 7, appear to partially have a large reading which potentially make them outliers. Therefore, taking in consideration weeks in a coupled manner rather than every week individually can help to avoid this problem. In other words, one can adopt averaging two-week over five periods.

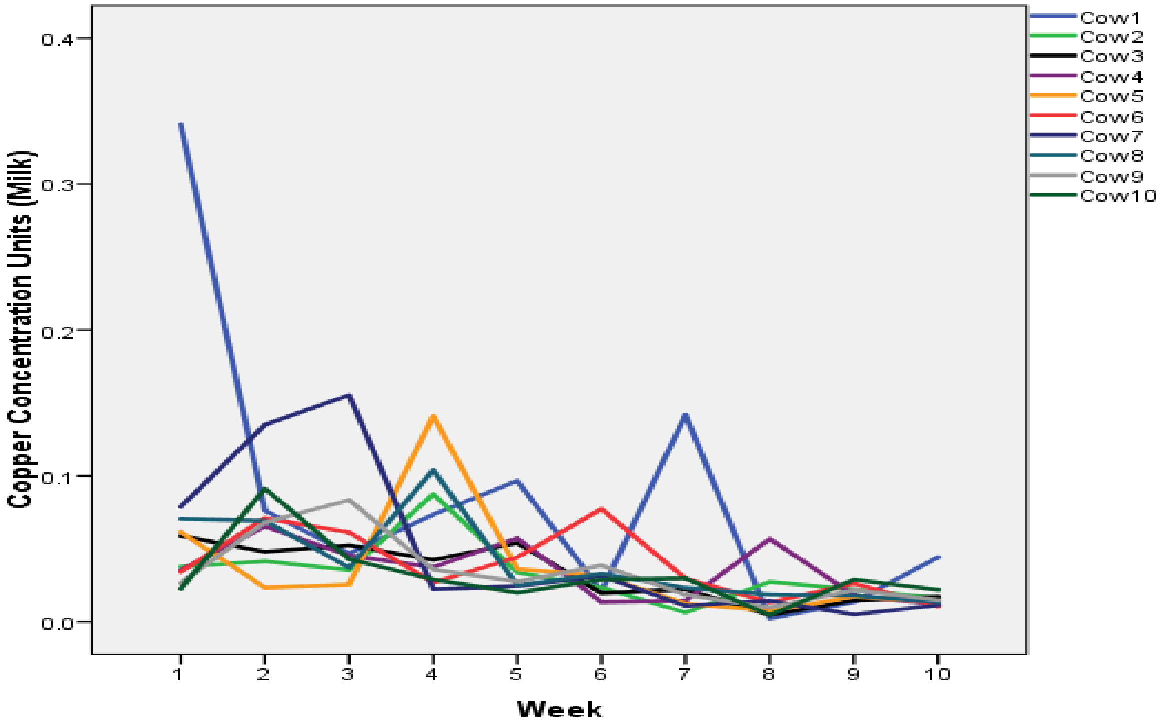

Figure 3.5 Sequence plot of copper concentration (mg/Kg) in milk for the various cows from Week 1 to Week 10

The same type of description and figures used for weekly readings will be used to show periods, which will also be repeated for hair samples.

Table 3.5: Mean, standard deviation and 95% confidence interval of copper concentration (mg/Kg, milk) for each period.

Period	Mean ± SD (mg/Kg)
1	.073 ± .052
2	.059 ± .017
3	.037 ± .013
4	.023 ± .019
5	.021 ± .006

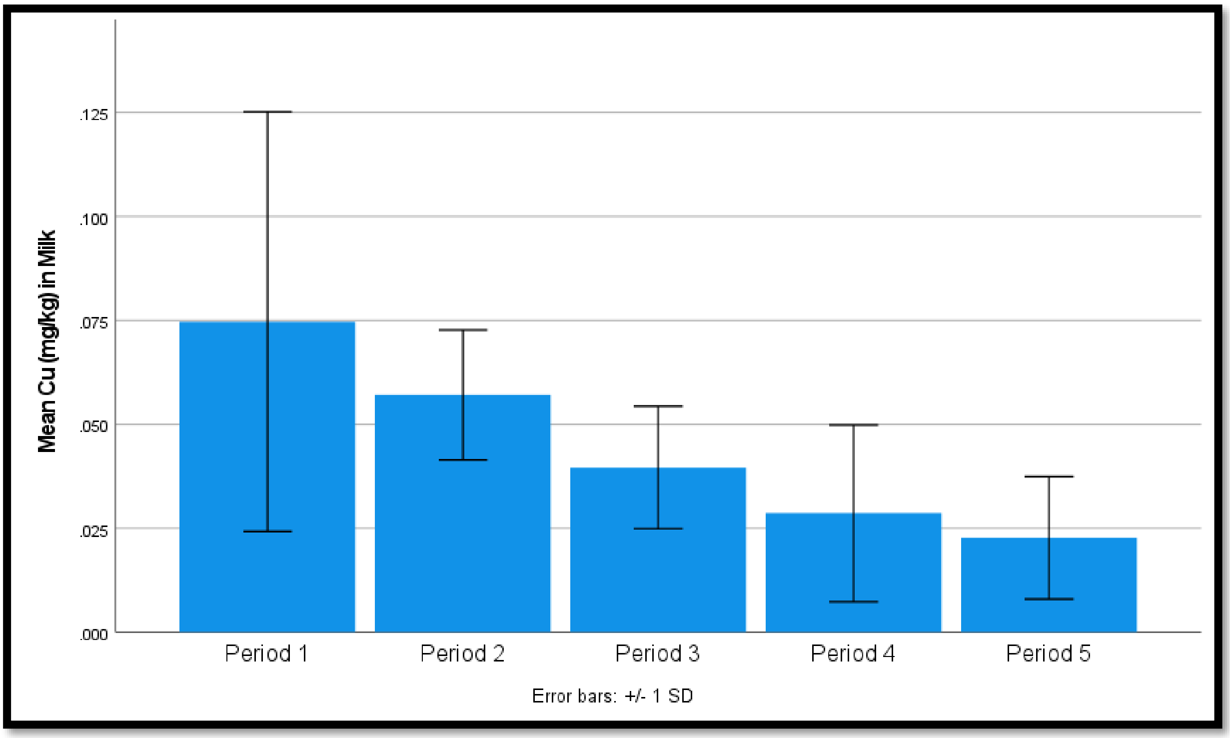

Figure 3.6 Error bar of copper concentration (mg/Kg, milk) for each period.

In Table 3.5, a decreasing mean trend according to period can still be seen. Despite the high standard deviation present in Week 7 in Table 3.3, the corresponding standard deviation in Week 4 is not remarkably different from that of other periods. However, just like Week 1, the standard deviation in Period 1 is still comparatively high. Table 3.5 also gives the 95% confidence intervals for each period. This trend is similar to Figure 3.4, however in this case, the mean and confidence interval are for periods. For milk, the Cu means, and standard deviations are listed in Table 3.6 and sequence plot for cows according to period are seen in Figure 3.7.

Table 3.6: Mean and standard deviation of copper concentration (mg/Kg, milk) for each cow along period

Cow	Mean ± SD (mg/Kg)
1	.086 ± .071
2	.034 ± .017
3	.034 ± .018
4	.038 ± .011
5	.036 ± .027
6	.038 ± .018
7	.050 ± .047
8	.042 ± .026
9	.034 ± .021
10	.034 ± .017

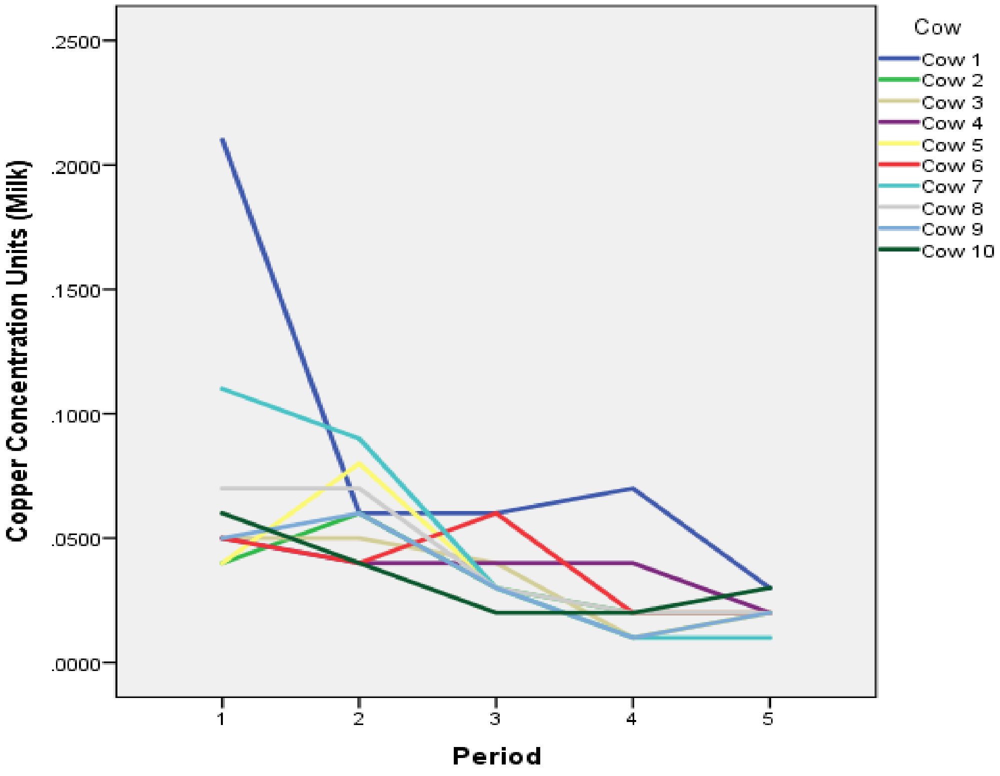

Figure 3.7 Sequence plot of copper concentration (mg/Kg, milk) for the various cows from Period 1 to Period 5

Once again, despite the averaging, Cow 1 still appears to partially have strong outlier in Period 1. Therefore, later in this chapter, an assumption will be concluded as to whether findings change when outliers are eliminated.

3.4.2.2 Hair samples

Moving on to the hair samples analysis, the Cu concentration of the same cows is given in Table 3.7, which shows the mean, standard deviation, lower and upper 95% confidence bounds of the Cu. Also, these results are depicted in Figure 3.8 including the error bar. In this case, the highest mean appears to occur in Week 3 and the lowest is in Week 1; however, from the findings of the table there does not seem to be an increasing or decreasing trend, as can be seen in Figure 3.8. Nevertheless, it does not mean that the variances between the different weeks will not be significant. In fact, there may still be week-to-week variation which is not due to the trend. Furthermore, it is noticeable that Week 3 and Week 5 have the highest standard deviations while the lowest standard deviation is in Week 1. Similarly, the widest 95% confidence intervals were demonstrated in Week 3 and Week 5 with the narrowest occurring in Week 1.

Table 3.7 Mean, standard deviation and 95% confidence interval of copper concentration (mg/Kg) in hair for each week

Week	Mean (mg/Kg)	Std. Deviation
1	1.97	1.06
2	4.10	1.79
3	8.09	9.21
4	5.66	1.41
5	6.45	5.28
6	5.21	2.26
7	4.48	1.12
8	4.11	1.31
9	3.42	2.12
10	4.55	3.13

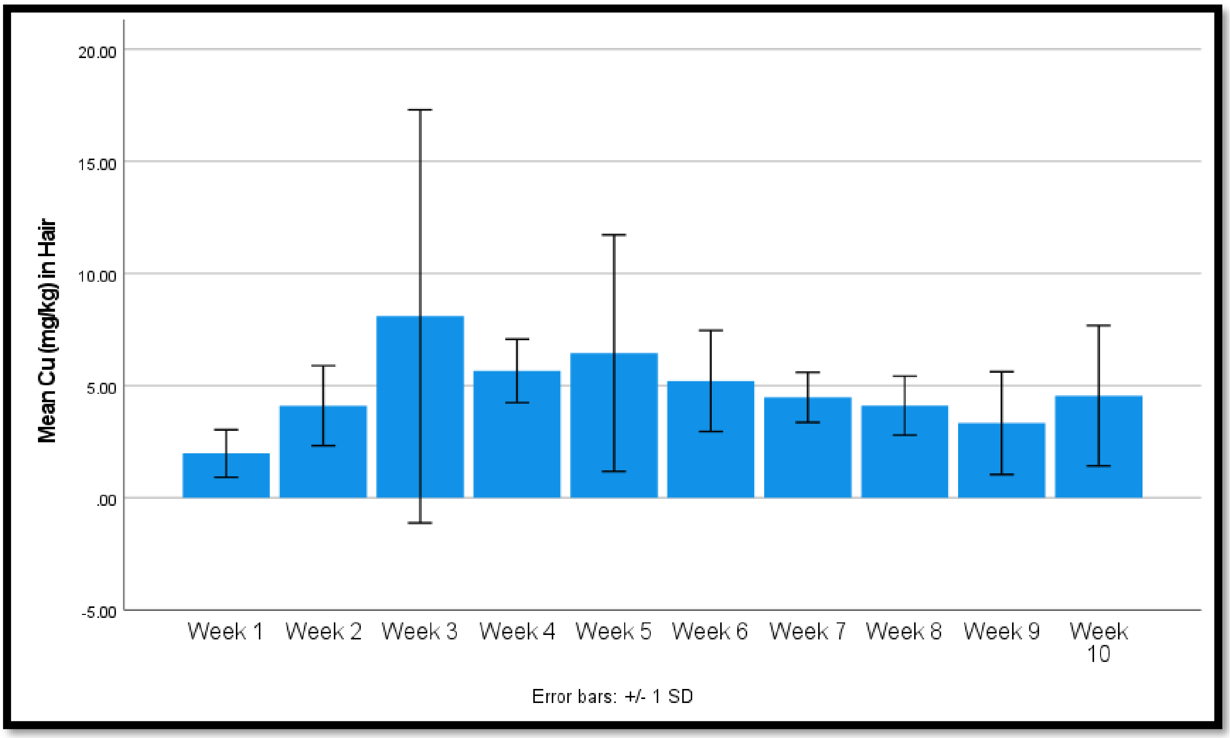

Figure 3.8 Error bar of copper concentration (mg/Kg, hair) for each week

Once again, from Table 3.8 and Figure 3.9, the presence of outliers is evident, particularly in Cow 1 and Cow 9. From Table 3.8 it can be seen that Cow 1 and Cow 9 have the largest standard deviations. Furthermore, this shows that Cow 1 appears to be an outlier in Week 5, and to a lesser extent in Week 10, and Cow 9 appears to be an outlier in Week 3.

Table 3.8 Mean and standard deviation of copper concentration (mg/Kg, hair) for each cow along weeks

Cow	Mean (mg/Kg)	Std. Deviation
1	6.36	5.98
2	4.18	1.83
3	4.76	1.27738
4	4.09	2.66
5	4.79	3.11
6	4.05	1.41
7	4.3418	1.34
8	5.00	2.41
9	6.79	9.50
10	3.68	1.40

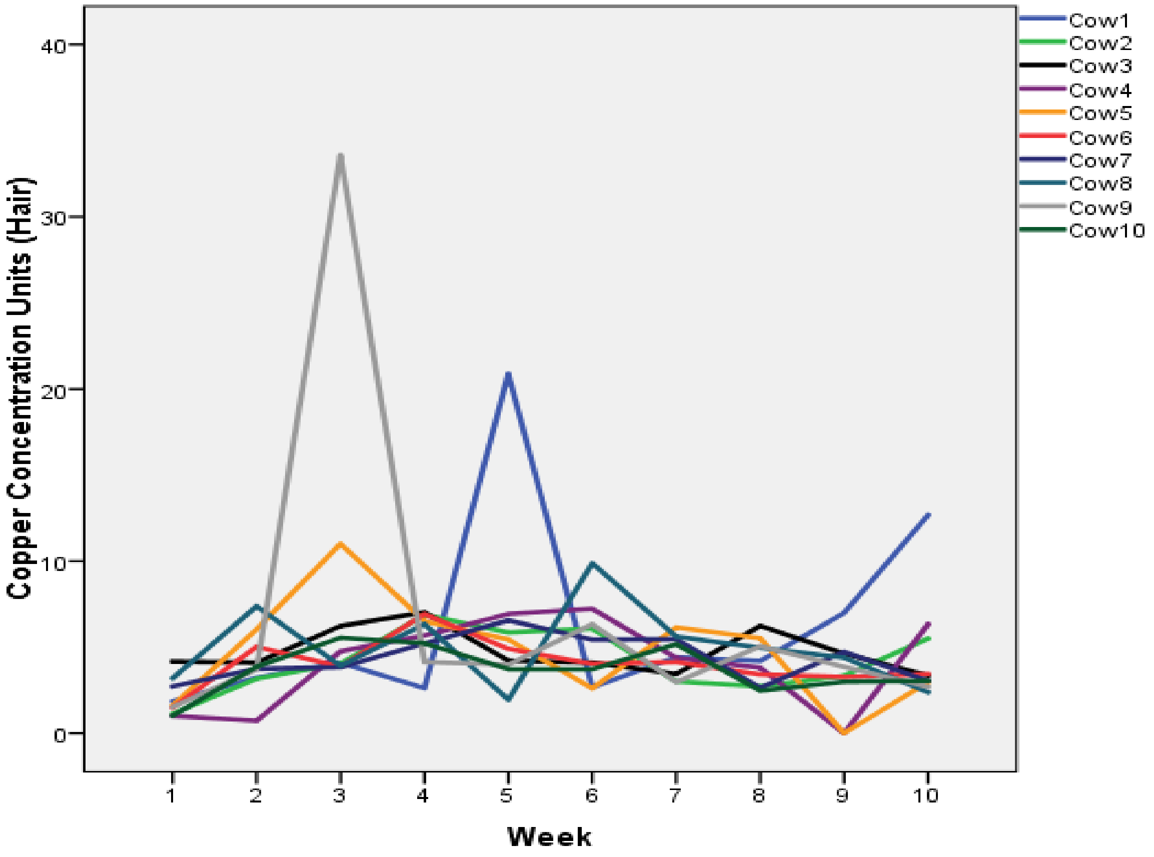

Figure 3.9 Sequence plot of copper concentration (mg/Kg, hair) for the various cows from Week 1 to week 10

For the duration of period consideration (the average of two weeks), the descriptive statistics table and the error given in Table 3.9 and Figure 3.10 constitute of five periods.

Table 3.9 Mean, standard deviation and 95% confidence interval of copper concentration (mg/Kg, hair) for each period

Period	Mean (mg/Kg)	Std. Deviation
1	3.04	1.22
2	6.88	4.44
3	5.83	2.35
4	4.29	.843
5	3.98	2.20

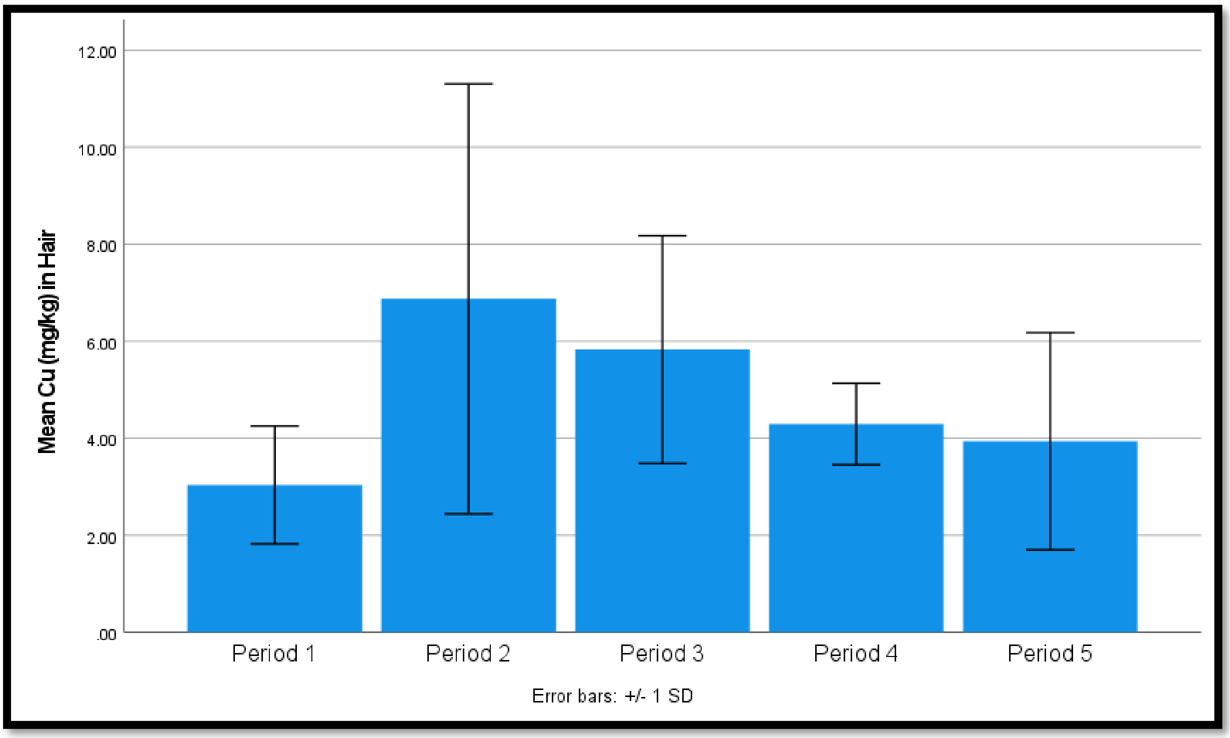

Figure 3.10 Error bar of copper concentration (mg/Kg, hair) to each period

Table 3.9 and Figure 3.10 illustrate that Period 2 has the highest mean and Period 1 has the lowest mean. From the first glance, it can be noted that period 2 also has a considerably higher standard deviation than the rest, and a considerably wider 95% confidence interval.

Table 3.10 Mean and standard deviation of copper concentration ((mg/Kg, hair) for each cow along periods

Cow

	N	Mean (mg/Kg)	Std. Deviation
1	5	6.36	4.16
2	5	4.18	1.64
3	5	4.75	1.10
4	5	4.09	2.32
5	5	4.79	2.71
6	5	4.05	.887
7	5	4.34	1.04
8	5	5.00	.949
9	5	6.79	6.81
10	5	3.69	1.11

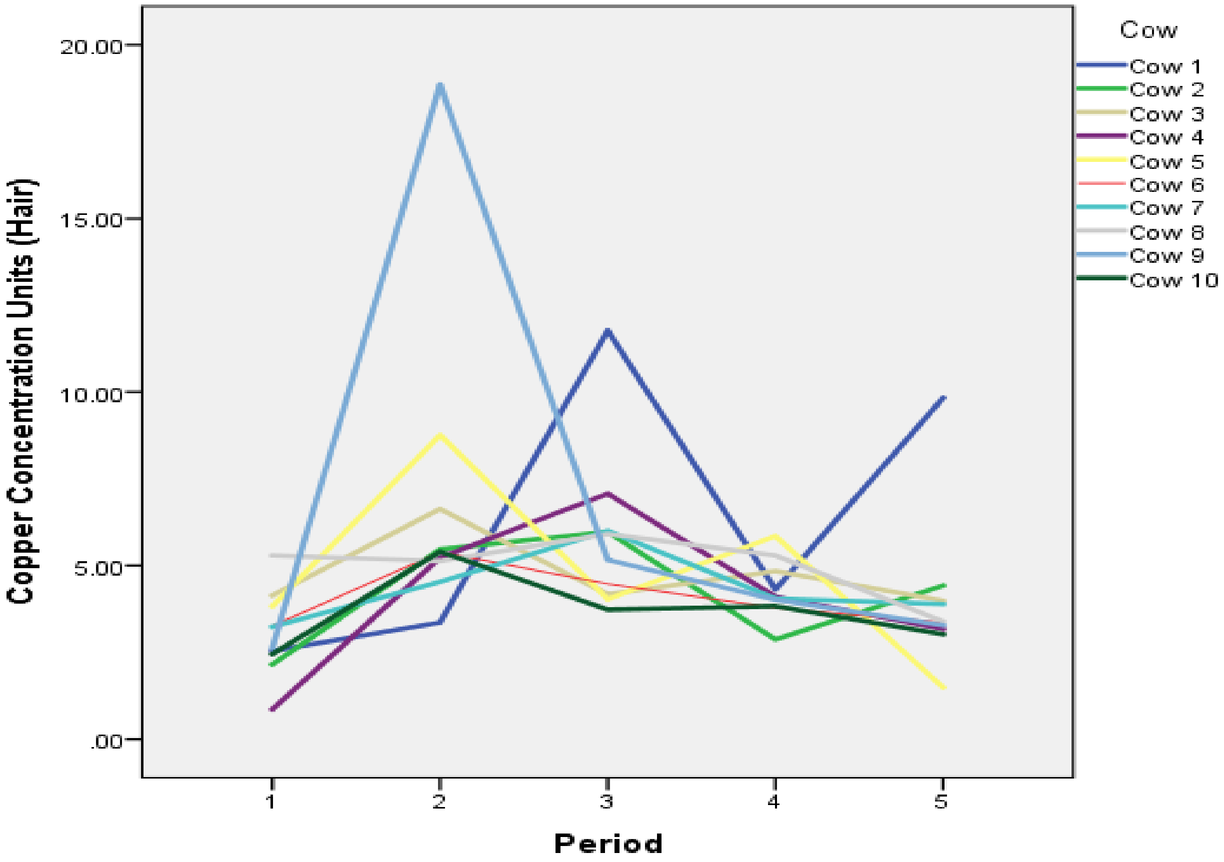

Figure 3.11 plot of copper concentration (mg/Kg, hair) for the various cows from Period 1 to Period 5

Subsequently, the Cu means, standard deviations and sequence plot for cows according to periods, are seen in Table 3.10 and Figure 3.11. The standard deviations for Cow 1 and Cow 9 are still partially high in Table 3.10, while in Figure 3.11 there is a considerably large outlier for Cow 9 in Period 2. Similarly, Cow 1 also produces a huge outlier in Period 3.

3.4.2.3 Hypothesis testing and post-hoc tests

Following on, the effect of time duration on the Cu levels in milk and hair was considered. Furthermore, in order to investigate discrepancy related to this effect, post-hoc tests were applied, further details on this type of analysis can be found in chapter 2 section 2.3.2.6. The following subsection demonstrates the results for milk samples. The outcomes of hair samples were also examined.

1. Milk Samples

With respect to Cu, and its different concentrations in milk, data from weeks 2, 4, 6, 8 and 10 were analysed. The means for these weeks can be seen in Table 3.3 and Figure 3.4. There appears to be a consistent decrease from Week 2 to Week8, followed by a slight increase in Week 10. The first step is to start by testing multivariate normality of the 5-week copper sample of 10 cows using the energy test, and also perform Mauchly’s test for sphericity. The energy test conducted in R does not reject the multivariate normality hypothesis (E=1.10, p=0.255). In addition to this, Mauchly’s test for sphericity does not reject the sphericity hypothesis either (W=0.136, p=0.104), meaning that when the hypothesis of the variances of the differences between all possible pairs are equal is rejected. It is therefore possible to resort to the repeated measures ANOVA test. The output for this test can be found in Table 3.11.

Table 3.11: Repeated measures ANOVA for copper (mg/Kg, milk) over weeks 2,4,6,8 and 10

Source		Type III Sum of Squares	Df	Mean Square	F	Sig.
Time	Sphericity Assumed	.024	4	.006	7.96	.000
	Greenhouse-Geisser	.024	1.87	.013	7.96	.004
	Huynh-Feldt	.024	2.37	.010	7.96	.002
	Lower-bound	.024	1.00	.024	7.96	.020
Error(time)	Sphericity Assumed	.027	36.00	.001		
	Greenhouse-Geisser	.027	17.00	.002		
	Huynh-Feldt	.027	21.3	.001		
	Lower-bound	.027	9.00	.003		

Since the sphericity assumption was satisfied, in Table 3.11 Mean, standard deviation and 95% confidence interval of Cu concentration (milk) for each week were considered the ‘Sphericity Assumed’ p-value. One can conclude that the hypothesis of no difference in mean between weeks is rejected (F=7.96, df=4, p=0.00).

As mentioned, post-hoc tests for the Friedman test are multiple paired t-tests. If post-hoc tests are applied for repeated measures ANOVA with no p-value adjustment, which means assessing each pairwise comparison individually using a paired samples t=test, the following significant differences at the 0.05 level are obtained:

- Week 2 and Week 6 (p = 0.007)
- Week 2 and Week 8 (p = 0.001)
- Week 2 and Week 10 (p = 0.001)
- Week 4 and Week 10 (p = 0.009)
- Week 4 and Week 8 (p = 0.012)

On the other hand, for the Bonferroni correction the following have been found to be significant at the 0.05 level:

- Week 2 and Week 8 (p = 0.010)
- Week 2 and Week 10 (p = 0.006)

Similarly, the same analysis between periods were performed. The descriptive statistics table in Table 3.5 and the error graph in Figure 3.6 demonstrate a decreasing trend from Period 1 to Period 5, with Period 1 having a considerably higher standard deviation than the rest. The energy test of multivariate normality, the multivariate normality assumption is rejected at 0.05 level of significance (E = 1.11, p = 0.025). Furthermore, the sphericity assumption is rejected (W=0.015, df=9, p=0.000), meaning that the hypothesis of the variances of the differences between all possible pairs are equal is rejected. Since the multivariate normality assumption was rejected, the Friedman test was performed. The output for this is found in Table 3.12.

The Friedman test concludes that there is a significant difference in medians between periods (χ^2 =28.4, p=0.000), as expected.

Table 3.12 Friedman test for copper (mg/Kg, milk) over Periods 1, 2, 3, 4 and 5

N	10
Chi-Square	28.4
df	4
Asymp. Sig.	.000

Post-hoc tests for the Friedman test are multiple pairwise Wilcoxon tests. When no p-value adjustment is applied, the mean differences between the following groups are found to be significant:

- Period 1 and Period 3 (W = 1.6, p = 0.024)
- Period 1 and Period 4 (W = 2.7, p = 0.000)
- Period 1 and Period 5 (W = 2.9, p = 0.000)
- Period 2 and Period 4 (W = 2.4, p = 0.001)
- Period 2 and Period 5 (W = 2.6, p=0.000)

With the Bonferroni p-value correction applied, it can be seen that the mean differences between the following groups are found to be significant:

- Period 1 and Period 4 (W = 2.70, p = 0.001)
- Period 1 and Period 5 (W = 2.90, p = 0.000)
- Period 2 and Period 4 (W = 2.40, p = 0.007)
- Period 2 and Period 5 (W = 2.60, p=0.002)

2. Hair Samples

The descriptive statistics table in Table 3.7 and the error bar in Figure 3.8 show that there is no correlation between Cu concentration in cow's hair and weeks 2, 4, 6 and 8. However, it is important to verify potential discrepancy between weeks. Initially, the energy test of multivariate normality was performed. The multivariate normality assumption was rejected at 0.05 level of significance (E = 1.17, p = 0.04). On the other hand, the sphericity assumption is not rejected (W = 0.191, df = 9, p = 0.208). While the sphericity assumption is not rejected, the Friedman test was still applied due to the fact that multivariate normality is rejected.

Table 3.13 Friedman test for copper (mg/Kg, hair) over Weeks 2, 4, 6, 8 and 10

N	10
Chi-Square	8.64
Df	4
Asymp. Sig.	.071

In table 3.13, the null hypothesis of no difference between medians is not rejected at 0.05 level of significance, but it is rejected if 0.1 level of significance (χ^2 =8.64, p=0.071) had to be taken. For this reason, the post-hoc analysis for the Friedman test was still performed. If post hoc tests are applied for the Friedman test with no p-value adjustment, meaning assessing each pairwise comparison individually, then it can be seen in table 14 that there is a significant difference between the following at 0.05 level of significance:

- Week 4 and Week 8 (W = 1.60, p = 0.024)
- Week 4 and Week 10 (W = 1.60, p = 0.024)

There is also a significant difference at a 0.1 level between:

- Week 6 and Week 8 (W = 1.30, p = 0.066)
- Week 6 and Week 10 (W = 1.3, p = 0.066)

However, after applying the p-value adjustment from the Bonferroni method, none of these differences remain significant at either 0.05 or 0.1 level of significance.

For periods, the descriptive statistics table in Table 3.9 and the error bar in Figure 3.10, also show that there is no relationship between Cu concentration in cow’s hair and periods 1, 2, 3, 4 and 5. However, as mentioned earlier, it could also be the case that there are differences not related to trend but to exogenous circumstances. It may also be the case that trends, and seasonality need a longer period of time to be seen, and a longer time series would be required. Consequently, the energy test of multivariate normality was performed, where it can be seen that the multivariate normality assumption is rejected at 0.05 level of significance (E = 1.18, p = 0.025). Furthermore, the sphericity assumption pattern was also rejected at the 0.05 level (W = 0.007, df = 9, p = 0.000). Since neither normality nor the sphericity assumption are satisfied, the Friedman test to check whether there is any difference between periods was applied. The Friedman test concludes that there is a significant difference between periods (χ^2 =28.4, p=0.000), as expected. This means that, even though no trend is evident over the periods considered, a difference between period medians has been registered across the whole cow sample used in the experiment.

Table 3.14 Friedman test for copper (mg/Kg, hair) over Periods 1, 2, 3, 4 and 5

N	10
Chi-Square	25.200
Df	4
Asymp. Sig.	.000

If post-hoc tests for the Friedman test with no p-value adjustments are applied, the mean differences between the following groups are found to be significant:

- Period 1 and Period 2 (W = -2.70, p = 0.000)
- Period 1 and Period 3 (W = -2.80, p = 0.000)
- Period 1 and Period 4 (W = -1.90, p =0.007)
- Period 2 and Period 5 (W = 2.10, p = 0.003)

- Period 3 and Period 5 (W = 2.20, p = 0.002)

With the Bonferroni p-value correction applied, it can be seen that the mean differences between the following groups are found to be significant:

- Period 1 and Period 2 (W = -2.70, p = 0.001)
- Period 1 and Period 3 (W = -2.80, p = 0.001)
- Period 2 and Period 5 (W = 2.10, p = 0.030)
- Period 3 and Period 5 (W = 2.20, p = 0.019)

It is interesting to note that when dealing with average over two-week periods, a significant difference was detected. This, on the other hand, was not detected when dealing with bi-weekly readings, possibly because readings were missing from the 'odd' weeks which were responsible for these differences. This may indicate that averaging over two-week periods rather than even weeks was better, as it led to not neglect important information.

To conclude, data processing procedure, so far, has not allowed the complete elimination of the presence of outliers. In the next section, the identification and treatment of outliers was carried out, whereby these tests were performed again to see whether any differences in the conclusions are reached.

3.4.2.4 Treating Outliers

It was evident that some of the observations were outliers. A possible way of dealing with outliers is to delete them from the dataset, but when dealing with repeated observations this is not that simple, as to get rid of one outlier, one has to eliminate all the readings from that subject (in this case that particular cow). When the sample is based on a small sample of 10 cows, the power of the test is already compromised, so removing further observations will further compromise it. Furthermore, outliers for a particular subject (cow) did not happen every single week, but only on particular weeks, meaning that eliminating all the readings for that subject is tantamount to eliminating a number of potentially valid readings. The following approach was therefore taken:

Initially, identification of the major outliers in each week was carried out – these are points that are severely anomalous in comparison to other readings. This was done by the box-and-whisker plot. The box in the box-and-whisker plot contains a line in the middle representing the median, while the lower and upper edges of the boxes represent the lower and upper quartiles. The lower whisker represents either the minimum reading, or the smallest data point within one and a half times the interquartile range of the lower quartile. Similarly, the upper whisker represents either the maximum reading, or the highest data point within one and a half times the interquartile range of the upper quartile. If any other data lie outside this range, these are considered as major outliers and represented as points on the box plot.

When these outliers were identified, these points were treated as though they were missing observations and imputation was carried out. Imputation involved replacing missing observations (or in this case outliers) with a representative value. There are various imputation methods to deal with this, as observed by Little and Rubin (2002). One of the simplest methods of imputation used here is the mean imputation. This involved replacing the outlier by the sample mean obtained from the other subjects in that particular week.

After identifying and treating outliers, a repletion of all the procedure on both Week 2, 4, 6 and 8, and two-week averages for Period 1, Period 2, Period 3, Period 4 and Period 5 for both milk and hair was carried out. Firstly, the analysis of the copper concentration in milk was carried out, followed by the analysis of Cu concentration in hair.

1. Milk samples

Cu concentration in milk samples data post treating outliers was reprocessed where Figure 3.12 shows box-and-whisker plots for each week.

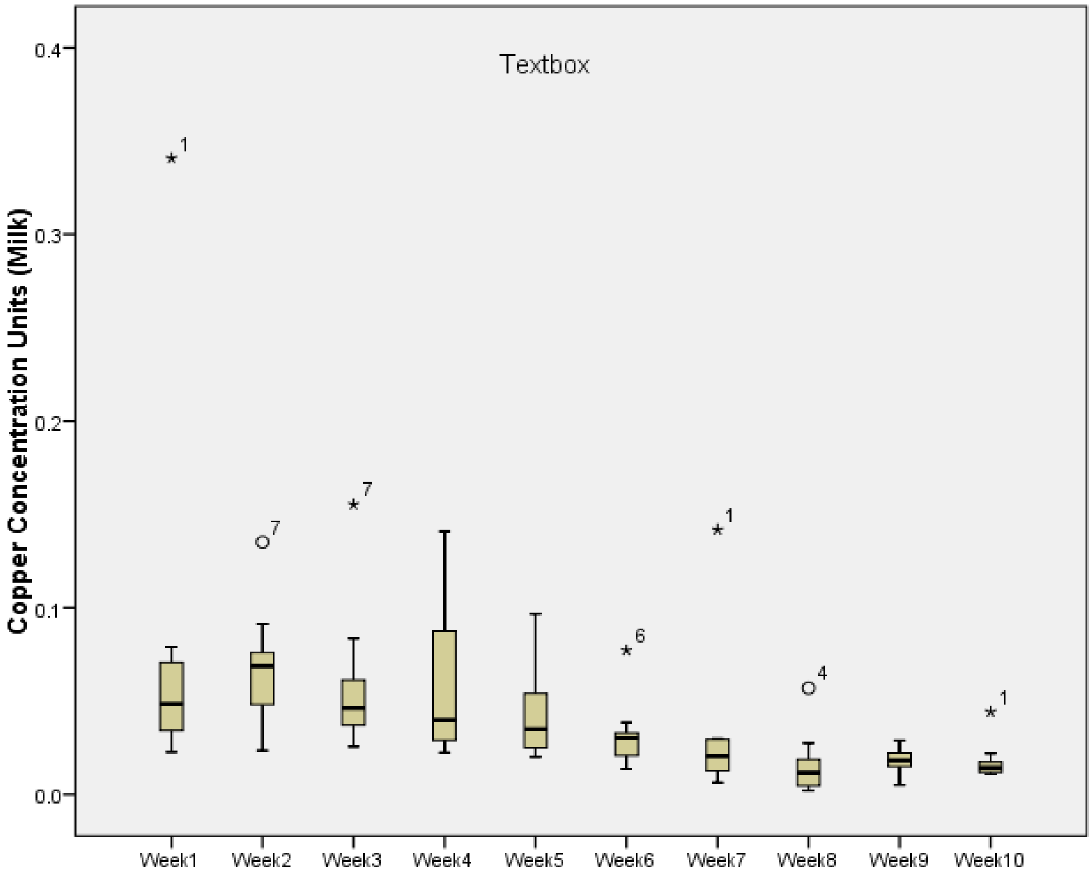

Figure 3.12 Box plot for copper concentration (mg/Kg, milk) in cows for Weeks 1 up to Week 10

The outliers identified are the following:

- Observation for Cow 1 in Weeks 1, 7 and 10
- Observation for Cow 7 in Weeks 2 and 3
- Observation for Cow 6 in Week 6
- Observation for Cow 4 in Week 8

On the other hand, no outliers were detected in Week 4, Week 5 and Week 9.

The data with respect to Weeks 2, 4, 6, 8 and 10 were analysed first. The multivariate normality hypothesis in the energy test is once again not rejected in this case (E=1.08, p=0.280). However, this time round, the Mauchly test for sphericity rejects the sphericity hypothesis (W=0.007, df=9, p=0.000). This means one cannot assume sphericity in the repeated measures ANOVA test (Table 3.15).

Table 3.15 Repeated measures ANOVA for copper (mg/Kg, milk) over weeks 2,4,6,8 and 10 after outliers have been treated

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Week	Sphericity Assumed	.024	4.00	.006	12.9	.000
	Greenhouse-Geisser	.024	1.28	.019	12.9	.003
	Huynh-Feldt	.024	1.40	.017	12.9	.002
	Lower-bound	.024	1.00	.024	12.9	.006
Error(Week)	Sphericity Assumed	.016	36.0	.000		
	Greenhouse-Geisser	.016	11.5	.001		
	Huynh-Feldt	.016	12.6	.001		
	Lower-bound	.016	9.00	.002		

Since the assumption sphericity cannot be used, it is important to look at the p-values related to the Greenhouse-Geisser, Huynh-Feldt and lower-bound corrections. In all cases, the outcome is the same, though with minor differences in the p-value:

- Greenhouse-Geisser (F=12,9, df=1.28, p=0.003).
- Huynh-Feldt (F=12.9, df=1.40, p=0.002).
- lower-bound (F=12.9, df=1, p=0.006).

Therefore, the null hypothesis of no difference between the week means at 0.05 level of significance was rejected again. Once again, all possible pairwise t-tests were performed. When no p-value correction was applied, the following differences are significant at a 0.05 level:

- Week 2 and Week 6 (p=0.000)
- Week 2 and Week 8 (p=0.000)
- Week 2 and Week 10 (p=0.000)
- Week 4 and Week 6 (p=0.005)
- Week 4 and Week 8 (p=0.021)
- Week 4 and Week 10 (p=0.017)
- Week 6 and Week 8 (p=0.001)
- Week 6 and Week 10 (p=0.001)

On the other hand, when applying the Bonferroni correction, the following differences are significant at the 0.05 level:

- Week 2 and Week 6 (p=0.004)

- Week 2 and Week 8 (p=0.001)
- Week 2 and Week 10 (p=0.000)
- Week 4 and Week 8 (p=0.035)
- Week 6 and Week 8 (p=0.009)
- Week 6 and Week 10 (p=0.010)

It can be seen that removing outliers has increased the number of significant pairwise differences. Prior to treating outliers, only 5 pairwise differences were significant, as opposed to 8, when to no p-value adjustments are made. Furthermore, when using the Bonferroni correction, now that outliers are treated, there are 6 significant pairwise differences whereas previously it had only two.

On the other hand, when analysing periods, the multivariate normality hypothesis in the energy test is once again rejected as in the case where the outliers were not treated (E=1.17, p=0.015). Mauchly's test for sphericity rejects the sphericity hypothesis (W=0.079, df=9, p=0.030). This means that sphericity cannot be assumed in repeated measures ANOVA. Therefore, the Friedman test was used. Table 3.16 shows that the null hypothesis of no difference between medians along the periods is rejected.

Table 3.16 Friedman test for copper (mg/Kg, milk) over Periods 1, 2, 3, 4 and 5 after outliers have been treated

N	10
Chi-Square	35.4
Df	4
Asymp. Sig.	.000

Once again, all possible pairwise Wilcoxon tests were performed. When no p-value correction is applied, the following differences are significant at 0.05 level:

- Period 1 and Period 3 (W = 1.70, p = 0.016)
- Period 1 and Period 4 (W = 3.30, p = 0.000)
- Period 1 and Period 5 (W = 2.80, p=0.000)
- Period 2 and Period 3 (W = 1.50, p = 0.034)
- Period 2 and Period 4 (W = 3.10, p = 0.000)
- Period 2 and Period 5 (W=2.60, p=0.000)

- Period 3 and Period 4 (*W* = 1.60, *p* = 0.024)

With the Bonferroni p-value correction applied, it can be observed that the mean differences between the following groups are found to be significant:

- Period 1 and Period 4 (*W* = 3.30, p= 0.000)
- Period 1 and Period 5 (*W* = 2.80, p=0.001)
- Period 2 and Period 4 (*W* = 3.10, p = 0.000)
- Period 2 and Period 5 (W=2.60, p=0.002)

It is evident that removing outliers has not considerably affected the number of significant pairwise differences. Prior to treating outliers, 5 pairwise differences are significant, as opposed to 6, when to no p-value adjustments are made. Furthermore, Period 2 and 3, and Period 3 and 4, are now significantly different when before they were not, while Period 1 and Period 3 cease to have any significant difference. In addition, when using the Bonferroni correction, the same four significant differences are detected. Since the Friedman test deals with medians, rather than means, the likely reason for this occurrence is that means are robust to outliers. Hence, eliminating outliers has not greatly impacted the outcome of the results.

2. Hair samples

Similarly, hair sample analysis was reprocessed post treating outliers for Cu concentration levels and Figure 3.13 demonstrates the box-and-whisker plot for each week.

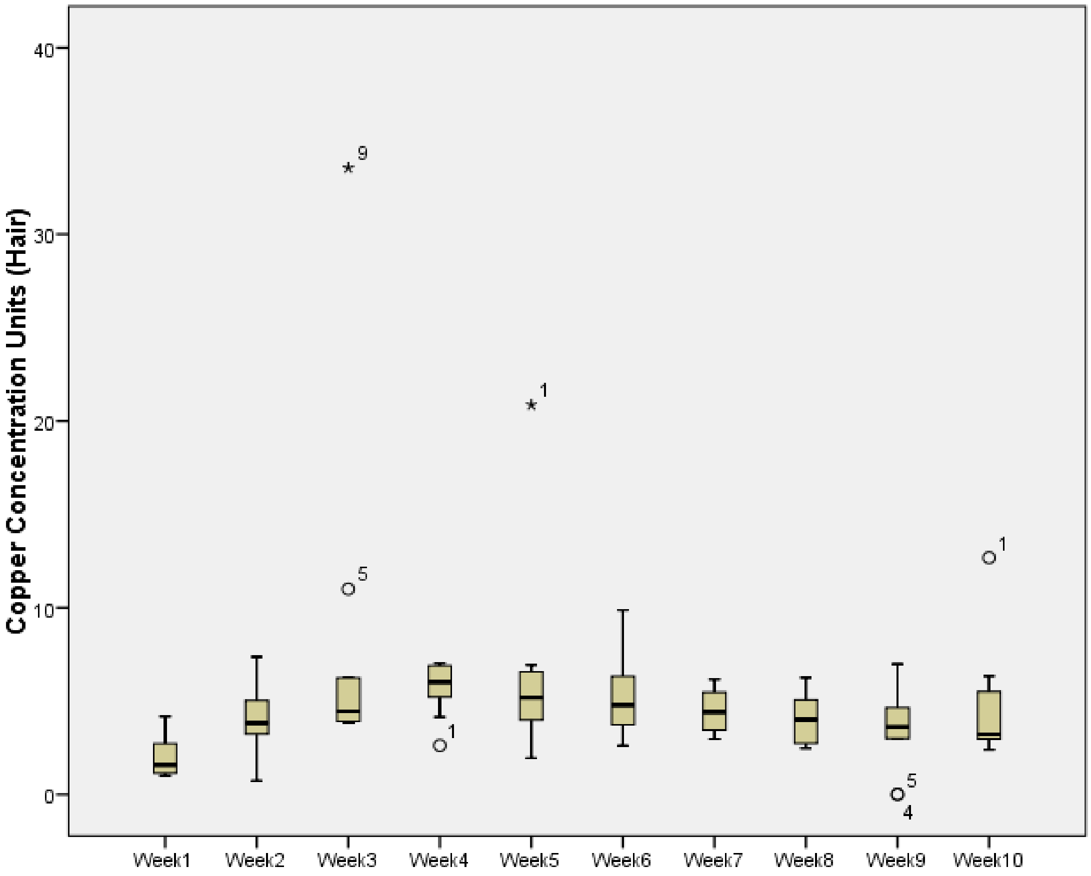

Figure 3.13 Box plot for copper concentration (mg/Kg, hair) in cows for Weeks 1 up to Week 10

The outliers identified are the following:

- Observation for Cow 1 in Weeks 4, 5 and 10
- Observation for Cow 4 in Week 9
- Observation for Cow 5 in Weeks 3 and 9
- Observation for Cow 9 in Week 3
- Observation for Cow 4 in Week 8

On the other hand, no outliers were detected in Weeks 1, 2, 6, 7 and 8. The analysis of the data with respect to Weeks 2, 4, 6, 8 and 10 was carried out. The multivariate normality hypothesis in the energy test is not rejected in this case (E=1.08, p=0.205). On the other hand, the Mauchly test for sphericity rejects the sphericity hypothesis (W=0.094, df=9, p=0.046) at 0.05 level of significance (albeit marginally). This means that sphericity in repeated measures ANOVA cannot be assumed.

Table 3.17 Repeated measures ANOVA for copper (mg/Kg, hair) over weeks 2,4,6,8 and 10 after outliers have been treated

Measure: MEASURE_1

Source		Type III Sum of Squares	Df	Mean Square	F	Sig.
Week	Sphericity Assumed	37.3	4.00	9.31	3.72	.012
	Greenhouse-Geisser	37.3	2.57	14.5	3.72	.031
	Huynh-Feldt	37.3	3.68	10.1	3.72	.015
	Lower-bound	37.3	1.00	37.3	3.72	.086
Error (Week)	Sphericity Assumed	90.1	36.0	2.50		
	Greenhouse-Geisser	90.1	23.1	3.90		
	Huynh-Feldt	90.1	33.1	2.72		
	Lower-bound	90.1	9.00	10.0		

Since sphericity cannot be assumed, one needs to consider the p-values related to the Greenhouse-Geisser, Huynh-Feldt and lower-bound corrections. In two of the three cases, the null hypothesis of no difference between means is rejected (the only exception is for the lower-bound case). Therefore, the most reliable results are as follows:

- (F=3.72, df=3.68, p=0.015).
- lower-bound (F=3.72, df=1, p=0.086).

Under the assumption of sphericity (which has only been marginally rejected), the conclusion is also that of rejecting the null hypothesis (F=3.72, df=4, p=0.012). Though inconclusive, there was an inclination to reject the null hypothesis of no difference between the week means at 0.05 level of significance. This contrasts with the previous conclusions when outliers were not treated, where the Friedman test did not reject the hypothesis of no difference for medians. Once again, all possible pairwise t-tests were performed. When no p-value correction was applied, the following differences were found to be significant at 0.05 level:

- Week 2 and Week 4 (p=0.008)
- Week 4 and Week 8 (p=0.003)
- Week 4 and Week 10 (p=0.001)

Furthermore, Week 6 and Week 10 are significantly different at the 0.1 level (p=0.074). On the other hand, when applying the Bonferroni correction, the difference between Week 2 and Week 4 is not rejected at the 0.05 level, but at the 0.1 level (p=0.077), as following:

- Week 4 and Week 8 (p=0.027)
- Week 4 and Week 10 (p=0.005)

It is clear that removing outliers has, in this case, also made the test more sensitive to differences. Prior to treating outliers, Week 4 and 8, and Week 4 and 10 were only significant prior to any p-value correction.

Finally, the data was analysed with respect to Periods 1, 2, 3, 4 and 5. The multivariate normality hypothesis in the energy test is not rejected in this case either (E=1.07, p=0.41). On the other hand, the Mauchly test for sphericity rejects the sphericity hypothesis at a 0.05 level of significance (W=0.168, df=9, p=0.162) This means that repeated measures ANOVA and assume sphericity shall be applied.

Table3.18: Repeated measures ANOVA for copper (mg/Kg, hair) over period 1,2,3,4 and 5 after outliers have been treated

Measure: MEASURE_1

Source		Type III Sum of Squares	Df	Mean Square	F	Sig.
Week	Sphericity Assumed	31.7	4	7.94	8.41	.000
	Greenhouse-Geisser	31.7	2.21	14.4	8.41	.002
	Huynh-Feldt	31.7	2.95	10.6	8.41	.000
	Lower-bound	31.7	1.00	31.7	8.41	.018
Error(Week)	Sphericity Assumed	34.0	36	.944		
	Greenhouse-Geisser	34.0	19.8	1.71		
	Huynh-Feldt	34.0	26.6	1.28		
	Lower-bound	34.0	9.00	3.78		

Since sphericity was assumed, the corresponding p-value in Table 3.18 was considered. It could be concluded that the null hypothesis of no difference between period means is rejected at 0.05 level of significance (F=8.41, df=4, p=0.000). Once again, all possible pairwise t-tests were performed. When no p-value correction was applied, the following differences were found to be significant at 0.05 level:

- Period 1 and Period 2 (p=0.000)
- Period 1 and Period 3 (p=0.009)
- Period 1 and Period 4 (p=0.002)

- Period 2 and Period 4 (p=0.009)
- Period 2 and Period 5 (p=0.002)
- Period 3 and Period 5 (p=0.020)

When applying the Bonferroni correction, on the other hand, the following significant differences at the 0.05 level were obtained:

- Period 1 and Period 2 (p=0.003)
- Period 1 and Period 4 (p=0.020)
- Period 2 and Period 5 (p=0.025)

Furthermore, for the remaining, the significant can be found at 0.1 level:

- Period 1 and Period 3 (p=0.092)
- Period 2 and Period 4 (p=0.089)

3.4.2.5 Discussion (Milk Samples)

The concentration of trace elements affects milk quality because the nutrients are removed from the blood by mammary glands, converted into milk and secreted in the udder. For nutritious milk production, animals must be fed either directly or indirectly via body reserves of nutrients, which come directly from the animals' feed. Heavy elements produced by various environmental pollution sources might reach the animal’s milk through fodder. Therefore, growing environmental pollution has increased concerns about milk quality (Cullison, 1975; Sabir *et al.,* 2003; Oskarsson *et al*., 1995). In addition to fodder intake, this study provides an analysis of environmental occurrence on a cows’ life cycle tracing Cu concentration in milk to characterise its levels and toxicity.

In this study, despite the fact that the cows studied had almost identical results during the study period, except from the first cow (possibly due to the initial reading being significantly higher than the other analyses), the results showed high levels of concentrated Cu in each animal separately during this period.

On the other hand, when studying the relation between the Cu concentration and the weeks, Cu concentration in the milk samples was higher as compared to the maximum level of 0.01mg/L according to FAO & WHO (Ruqia Nazir *et al.*, 2015) in weeks 1 to 7. It then decreased almost nearby reaching the maximum level in the last three weeks.

According to previous researchers (Sola-Larrañaga & Navarro-Blasco, 2009), referring to the effect of seasons in heavy metals, it was noted that the highest concentration found in this current study observed in the month of September, was still under the effect of the Summer season. Furthermore, the weekly Cu concentrations showed a significant effect along the season of Summer, which then proceeded to decrease until it reached the lowest values at the end of the study period. In addition, this fact can be explained by two different factors: firstly, feeding and metabolic adaptation during the climatic season, and secondly, it could be related to modification in food habits between climatic seasons with incorporation of fodder into the diet. Moreover, this kind of fodder (diet that used to feed the cattle in this research) is a natural source of Cu and Mn, which influences dietary intake of these heavy metals (Mata, Sánchez, & Calvo 1996).

Furthermore, the usual practice of the intake of alfalfa, fodder and cereals causes a small increase in fat content of cow milk, and a reduced need to drink water by cattle. Hence, cows meet their salt requirement not by a salt lick but rather by means of the diet, intrinsically associated with a lower intake of drinking water. This is one of the most important reasons for the increase in the concentration of Cu during the Summer season, and this is confirmed in this research. The present chapter showed that the milk had high concentration of Cu in comparison to the permissible level.

3.4.2.6 Discussion (Hair Samples)

It is well known that hair tissue analysis is widely used to monitor their health status. Hair plays an important role to demonstrate environmental contamination caused by pollution effect on soil, water, air and food. Hence, it can be a useful biomarker to determine heavy metal content. The main disadvantage of using hair is that mineral concentration levels in hair is not only impacted by feed but also by seasons of the year, hair colour and its position on the cattle. Hair analysis, however, can be used to determine severe deficiencies of some essential minerals or exposure to some heavy metals (Combs *et al.*, 1982).

The results of the present study do not provide a clear view of the effect of seasons on Cu concentration as observed in Figure 8. It depicts an increase in the first three weeks and then a decrease in concentrations in weeks 6, 7, 8 and 9. Moreover, an increase is depicted again in week 10. The reason behind this behaviour is interpreted in relation to the seasonal impact (as mentioned earlier), which will be taken in quest in the next Chapter, which will be discussing the seasonal effect in more detail.

Since the colour of hair can influence results of heavy metal content, hair samples of different colours were considered in this study, as in Table 3.19. However, the number of cows involved were not enough to realise such effect. The high concentration of Cu was noted in black hair, cows 1 and 9 (6.07, 6.43 ppm) respectively, while the cows who do not have the brown colour such as cow 2,4,6 and 10 presented similar results (4.07, 3.95, 3.92, 3.59 ppm) respectively. Nevertheless, cow 5 who has mixed colour, brown and black, had findings close to the brown cows (4.49 ppm). This supports the observations of previous studies in relation to the effect of hair colour on the concentration levels of heavy metals. In fact, it was found that the concentration of Cu is relatively affected by colour.

Table 3.19 Colour of cow`s hair

Cow	**Colour of hair**
1	Black
2	Brown
3	Blonde
4	Brown
5	Brown and black
6	Brown
7	White
8	Blonde and black
9	Black
10	Brown

3.5 Conclusions

Evaluation of levels of heavy metals in livestock for measuring the potential effects of pollutants on non-grazing cattle is very important, and for quantifying pollutant intakes by humans. Essential elements, such as Cu is toxic when consumed in excess. The results obtained show that the Cu concentration testifies to a considerable accumulation discrepancy from week-to-week than from period-to-period. This is more obvious, in most cases, after treating outliers, the post-hoc tests demonstrated extensive divergence between week-to-week and period-to-period duration. Furthermore, the multivariate normality that was being rejected earlier was accepted in two more instances after outliers were treated, showing that the multivariate normality hypothesis was being rejected due to these outliers.

Throughout this study, the outlier behaviour was distinguished in relation to readings from a particular cow (or cows). Indeed, one of the cows in the milk and hair sample (referred to as Cow 1) was recognized as an outlier six times. This incidence is unlikely to be coincidental and can be interpreted as being due to subject's habits, health conditions or the environment playing a major contribution. In the next chapter, it may therefore be worth considering repeating the readings on different cows, so as not to perpetuate any anomalies arising from the subject frequently in the study.

To conclude, Figure 3.14 gives an overall perspective on the Cu contribution within feed, manure, milk and hair, acting as input and output parameters with regards to the cow's system (Udo, 1978) (McDowell, Lee, McMullan, Fohrman, & Swett, 1954) . It can be observed that the Cu input coming from feed does not tally to the Cu output through manure, milk and hair. Manure was found to have a high concentration of Cu, in comparison to the other input and output parameters. The reasoning behind this may be due to Cu entering the system through other input parameters, Cu accumulation and outputs release. This allows for the basis of further studies whilst taking into consideration more inputs, for example atmospheric pollution and drinking water, as well as urine in liquid manure as outputs.

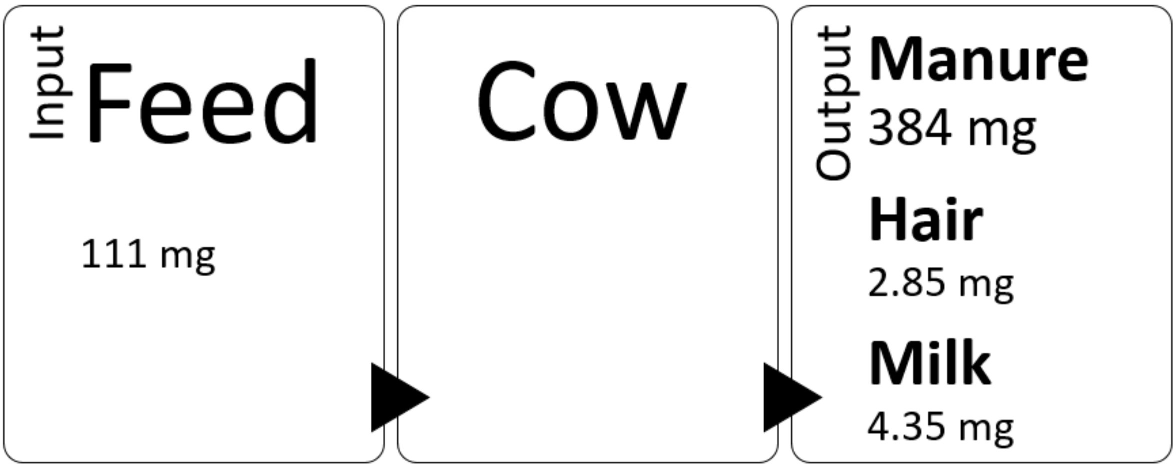

Figure 3.14 The input and output parameters considered in this study, and the copper contribution of each parameter on a daily basis.

Summing up, further research done on a long-term basis, need to be carried out to identify whether any trends of seasonality are present with regards to the presence of Cu in cows, as well as other possible pollutants. In addition to this, environmental and climatic factors need to be analysed in order to assess for their influence to the presence of Cu and other pollutants. An example of this may be the reflection of relevant climatic and environmental factors during the time that these readings were taken, as these may lead to the detection of not only drifts, but also seasonality and other differences due to time, as well as its underlying cause.

4. ICP Results and Discussion

4.1 Introduction

In an earlier chapter, the presence of copper in samples was examined by AAS to understand how the digestion of copper in feed impacts the resultant heavy metal contamination of milk, hair and manure of cattle. As the AAS equipment was subsequently unavailable to complete the measurement of the other heavy metals (Cr, Zn, As, Se, Cd and Pb) content ICP was used instead to examine their presence in fodder, water, manure, milk and hair. In this section, the concentration levels of the heavy metals focused on this study (Cu, Cr, Zn, As, Se, Cd and Pb) were determined using ICP for the various types of samples. This will provide a more comprehensive overview of the degree to which a wide range of heavy metals are ingested, digested and subsequently expelled from the cattle. Understanding exactly how much of the heavy metals enter the animal, via fodder or water is important. The degree to which these heavy metals are subsequently digested and appear either in the milk, hair or manure allows for a clear indication of how heavy metals can enter the food chain. Given the high toxicity of some heavy metals and dairy milk's present human consumption levels, it is essential to investigate the heavy metal life cycle in cattle. In this chapter, first fodder will be investigated using ICP for heavy metals, followed by water, manure, milk and hair respectively.
Before results are presented and discussed, earlier sections 1.2.1 and 1.2.2 introduced the beneficial effects of heavy metal and the toxic impact that high concentrations can have on health. In addition to this, details regarding the impact the metals in fodder and its effect on humans through the animals are included below.

Certain heavy metals are potentially toxic compounds, including heavy metals such as Cr, As, Cd and Pb, which can negatively impact the quality of an animal fodder. Such contaminated animal fodder can have consequences to not only the animal's health and welfare but can also increase human exposure to these toxic heavy metals and organic contaminates. It is therefore vital to understand clearly how such pollutants find their way into the food chain.

Table 4.1 below gives some examples of various heavy metals and their impact on the human body in more detail (Briffa, Sinagra, & Blundell, 2020).

Table 4.1: Some Metals and their daily severe exposure and chronic exposure.

Metal	Severe exposure of a day or less	Chronic exposure usually months or years
Cr	• Gastrointestinal hemorrhage (bleeding) •Hemolysis (red blood cell destruction) • Acute renal failure	• Pulmonary fibrosis (lung scarring) • Lung cancer
Cu	• Irritation of the nose • Mouth and eyes • Vomiting • Diarrhoea • Stomach cramps • Nausea	• Wilson's disease (which causes excessive accumulation of Cu in liver, brain, kidney and cornea) and Menkes disease (Tapiero & Tew, 2003) and even death
Zn	• Stomach cramps • Anaemia • changes in cholesterol levels • Nausea and vomiting,	• Decrease in high-density lipoprotein (HDL) cholesterol, • Impaired immune function,
As	• Nausea • Vomiting • Diarrhea • Encephalopathy • Multi-organ effects • Arrhythmia • Painful neuropathy	• Diabetes • Hypopigmentation/Hyperkeratosis • Cancer
Se	• Neurological effects • Brittle hair	• Cancer • Cardiovascular disease

	• Deformed nails.	• Cognitive decline • Thyroid disease
Cd	• Pneumonitis (lung inflammation)	• Lung cancer • Osteomalacia (softening of bones) • Proteinuria (excess protein in urine; possible kidney damage)
Pb	• Nausea • Vomiting • Encephalopathy (brain dysfunction)	• Anemia • Encephalopathy • Foot drop/wrist drop (palsy) • Nephropathy (kidney disease)

Linearity for the heavy metals

The linearity for the heavy metals used in this study (Cr, Cu, Zn, As, Se, Cd and Pb) was tested by analysing the certified reference material standard solutions having four concentrations ranging from 0.1 to 2000 µg/L. The Figures A.1 to A.7 in the appendix 2.1-ICP show a plot for concentration of each element, all the correlation coefficients were higher than 0.988 presenting that the response for all the elements are linear at the range of concentration from 0.1 to 2000µg/L. Furthermore, most samples analysed were present within the calibration range and no appropriate dilution were performed.

The LOD and LOQ of the ICO-OES method for each element are listed in Table 4.2. In the present work, the LOD and LOQ are defined as 3 and 10 times the standard deviation of three repeated measurements of the blank solution, respectively. Moreover, the linear correlation coefficient is bigger than 0.999 for all the elements.

Table 4.2 illustrates the correlations, also the limits of detection and limits of quantitation of all the metals under this study.

Table 4.2 The correlation coefficient (R2), limits of detection (LOD) and limits of (LOQ) of each element.

Element	R^2	LOD µg/L	LOQ µg/L
Cr	0.999	0.381	1.27
Cu	0.999	0.285	0.745
Zn	0.998	0.781	2.60
As	0.999	9.04	30.1
Se	0.999	0.526	1.75
Cd	0.999	0.138	0.460
Pb	0.999	0.054	0.178

4.2 Fodder analysis

The study on fodder was carried out over a duration of 12 months sequenced in two periods, according to purchased fodder to the farm, the first period was in January 2019, and the second period was in May 2019, (as depicted in section 2.1.2).

From the samples collected during the two periods of this section, the results of the mean concentration and the standard deviation for all the elements in this study, are shown in table 4.3 below.

Table 4.3 The mean concentration and the standard deviation for all the elements µg/kg

Period	Element	Mean (µg/kg)	Std. Deviation	N
Period 1	As	.578	.133	5
	Cd	.0407	.028	5
	Cr	.6058	.389	5
	Cu	8.71	5.80	5
	Pb	.202	.133	5
	Se	.497	.181	5
	Zn	35.6	26.2	5
	Total	6.60	15.4	35
Period 2	As	.572	.113	5
	Cd	.041	.022	5
	Cr	.498	.300	5
	Cu	7.69	6.62	5
	Pb	.177	.091	5
	Se	.517	.119	5
	Zn	30.0	19.4	5
	Total	5.64	12.6	35

In addition to the above table, figure 4.1 illustrates the mean of Cu concentration and the mean of Zn concentration, as well as the standards deviation across various fodders between Period 1(January 2019) and Period 2 (May 2019). It can be seen that there is no significant deference between the mean of Cu concentration in the two periods of the study, whereas the mean of Zn concentration seems to be slightly higher in period 1 than in period 2.

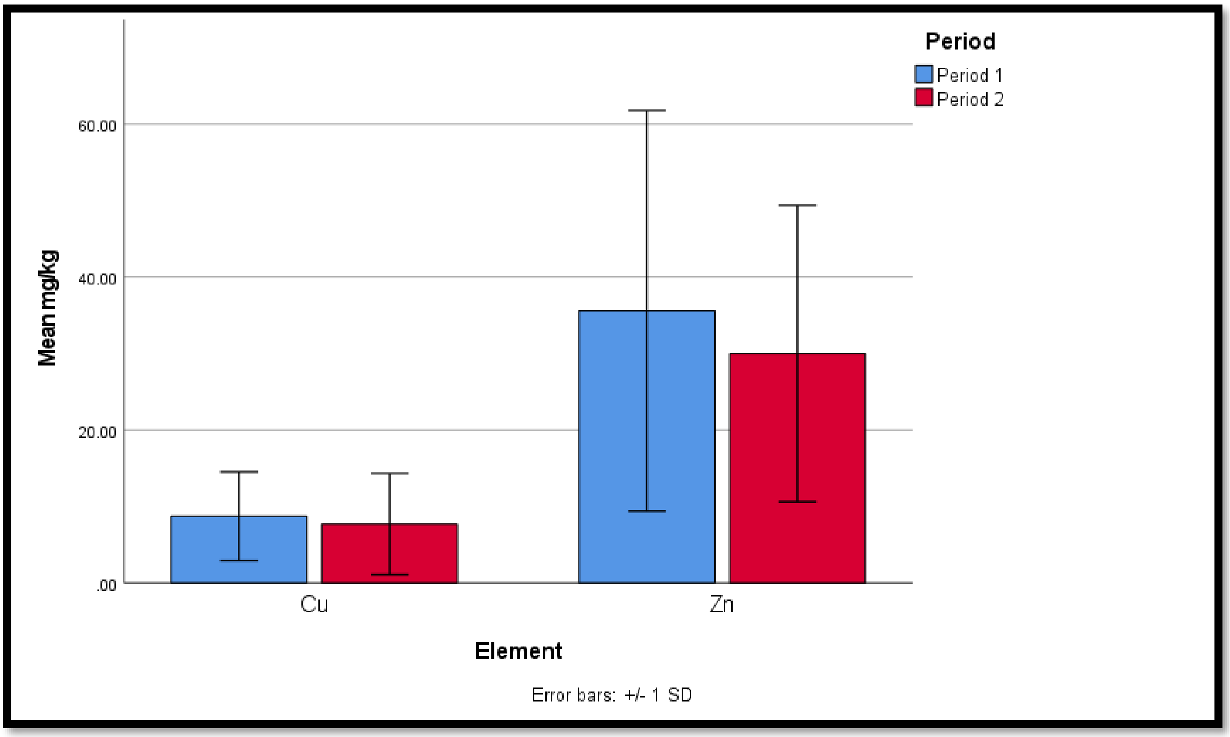

Figure 4.1 Mean Bar Graphs with Error Bar (SD) across fodders between Period 1 and 2 for Cu and Zn.

Moving onto figure 4.2, showing the means of As, Cd, Cr, Pb and Se concentrations and standards deviation across various fodders between Period 1 and Period 2, it is noticeable that there is no significant deferent between the means concentrations of As, Cd, Pb and Se in Period 1 and in Period 2, however, one can observe that the mean of Cr concentration is higher in Period 1 than in Period 2. The levels of all the elements in this study were detected in all various types of Fodders.

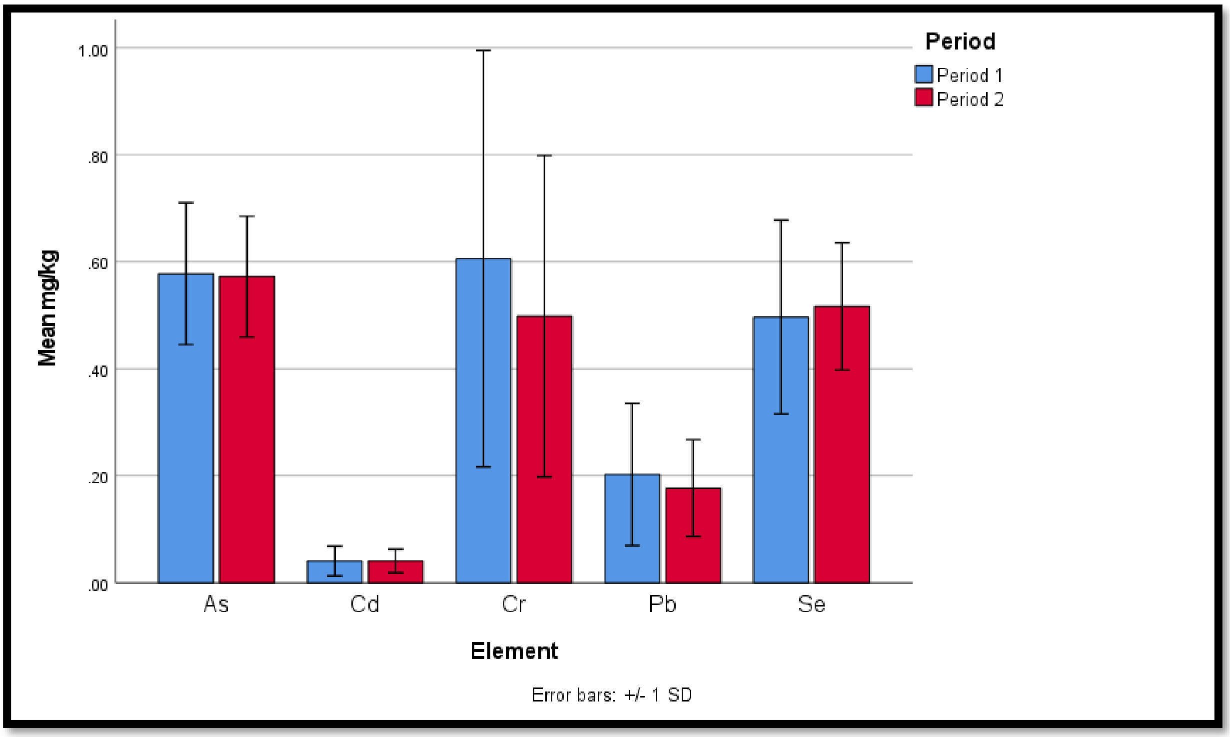

Figure 4.2 Mean Bar Graphs with Error Bar (SD) across fodders between Period 1 and 2 for As, Cd, Cr, Pb and Se.

Figures 4.1 and 4.2 depict the mean concentration for each element of interest within this study, in which were compared between the two periods of the study. Presently, the mean concentrations with standard deviation of all the essential and toxic elements of different types of fodder collected in this part of study, are presented in table 4.4 below. As Cu has already been studied using AAS in Chapter 3, it makes sense to begin the ICP investigation of this chapter on the same metal, Cu, followed by essential minerals, Zn, As and Cr, before moving on to the other toxic heavy metals.

Table 4.4 Mean ± SD for different combinations of fodder and element (mg/Kg)

	Cu	**Zn**	**As**	**Cr**	**Se**	**Cd**	**Pb**
Maize small pellet	17.9±0.960	63.1±10.5	0.49±0.01	0.43±0.02	0.74±0.06	0.04±0.00	0.160±0.020
ground maize	1.80±0.960	11.8±0.410	0.500±0.050	0.100±0.010	0.440±0.070	0.020±0.000	0.090±0.030
mixed seeds	9.28±2.75	50.6±7.70	0.540±0.060	0.430±0.080	0.570±0.010	0.080±0.010	0.170±0.020
Maltese hay	4.46±0.350	15.5±0.210	0.560±0.030	0.910±0.070	0.410±0.040	0.030±0.000	0.150±0.010
imported hay	7.53±0.520	22.9±1.34	0.780±0.030	0.880±0.220	0.370±0.030	0.030±0.000	0.380±0.070

It is noticeable that the highest mean concentration of Cu was found in Maize small pellet (17.9±0.960 ppm) while the lowest mean level of Cu was observed in ground maize

(1.80±0.960 ppm). This difference is surprising given that both are maize and ground maize. Despite this, significant differences were found between both of these maize's in terms of copper content. This difference could be influenced by several factors such as the pollution in the country of origin, including air, soil and water pollution. For example, polluted rainfall on the crops or contaminated irrigation water could cause substantial difference in levels of heavy metals in the maize. Farid and Baloch 2012 found that untreated city effluent was used for irrigation in growing crops which were later used as fodder in cattle's farms. They also found that the city effluent considerably increased the heavy metals concentration in both soil and fodder(Farid & Baloch, 2012).

It is clear that the largest standard deviation is found in mixed seeds (±2.75 ppm). However, the standard deviation appears to be less in the other types. Evidently mixed seeds has a wider range of copper content in the same type of feed possibly due to the mixed nature of seed content or samples of heterogeneous mixes.

Similarly, the highest mean concentration of Zn was shown in maize, (63.1±10.5 ppm) while the lowest was noticed in ground maize, (11.8±0.410 ppm). The highest standard deviation for Zn was found in maize (± 10.5 ppm) and mixed seed was second (± 7.70 ppm). The standard deviation was much smaller in the other types of fodder. Reasons for such variation in metal content of feeds can be due to the influence by season, as well as those factors already mentioned above (pollution by rain or irrigation water, season or mixed feed).

Moving to Se, the highest mean concentration of Se was seen in maize (0.740±0.060 ppm) while the lowest was noticed in imported hay (0.370±0.030 ppm). No significant difference was observed in standard deviation between different fodder.

Researchers (Nawaz et al. 2016) found that the impact of water scarcity, as a result of climate change, is not limited only to the productivity of food crops but also severely effects livestock feed production systems. Se is essential for animal health and reduces abiotic stresses in plants, however, understanding exactly how Se regulates the mechanisms to improve the nutritional status of fodder crops remains unclear. A study highlight the effects of external selenium on physiological and biochemical processes that could influence green fodder yield and the quality of maize particularly when there is a scarcity of water (Nawaz et al., 2016). The impacts of adding Se to address water scarcity might be one of the major reasons to increase the levels of

Se in the fodder, especially maize. However, not enough information on the presence of Se in maize grown in their country of origin is generally known.

In the case of Cr, the mean concentration of Cr was noted the highest in Maltese hay (0.910±0.070 ppm), although the lowest was in ground maize (0.100±0.010 ppm). Once again, no significant difference was observed in standard deviation for Cr between all types of fodder in this research.

In animals, Cr is thought to alleviate stress-associated effects. Cr supplements have been shown to enhance growth and lactating yield in ruminants exposed to thermal stress. Leaves generally have a higher Cr content than any other plant parts which might be the reason for the higher Cr content in some fodders (Mor, 2005a). This could explain why the highest concentration of Cr was observed in the imported hay.

Next, the toxic heavy metals As, Cd and Pb were examined. Starting with As it has the highest mean concentration in imported hay (0.780±0.030ppm), while the lowest mean was shown in maize (0.490±0.010 ppm). There was no significant deference in standard deviation between the various types of fodder.

Researchers Li et al. (2007) and Zhang et al. (2012a) have shown that it is usual to add essential minerals such as Cu, Zn and As to animal feeds as health supplements because of their antimicrobial and growth-stimulating effects. This explains the larger variation in these essential minerals in fodder.

In the case of Cd, it was found that it has the highest mean concentration in mixed seeds (0.080±0.000ppm), while the lowest mean was shown in ground maize (0.020±0.010 ppm). No significant difference was observed in the mean concentration of Cd for the different types of fodder used in this research. Even though Cd is not an essential element for growing animals(LI, Y. *et al.*, 2007), in this survey, all the fodder samples were noticed to contain this potentially toxic metal. The high level of Cd found could be an outcome of geological features, such as the use of phosphate fertilizer within the lands where the fodder is imported from(Saheed, Azeez, Jimoh, Obaro, & Adepoju, 2020).

The last element investigated in this chapter is Pb. It can be seen that the highest mean concentration of Pb was found in imported hay (0.380±0.070 ppm), while the lowest mean level of Pb was observed in ground maize (0.090±0.030 ppm). Similar to As, Se and Cr, in Pb there was not much significant difference seen in the standard deviation between fodders in this research.

Other studies have shown that Pb concentrations in fodder plants grown in a range of different locations, agricultural areas, industrial areas, and busy highways, have indicated that Pb contamination was considerably higher in industrial and highway sites as compared to rural locations(Mor, 2005a; Nawab *et al.*, 2015). This demonstrates the importance of knowing where fodder is grown in terms of Pb content. There is at present insufficient information on the conditions where fodder is grown in the country of origin, as pollution in that country could be the main reason for the large significant difference between all types of feed.

As shown in table 4.4 above, the concentrations of all selected heavy metals (Cu, Zn, As, Se, Cd, Cr and Pb) were distributed over a wide range, using feed samples. The results obtained were similar to the ones of Zhang *et al.* (2012), in which heavy metal content in animal feeds were assessed. Farms containing different sizes of cow herds were used, resulting in average values ranging from 0.380 mg/Kg (Cd) to 156 mg/Kg (Zn). Average heavy metal concentrations also varied widely depending on the type of feed and intensive livestock farms, as reported by Cang (2004).

Further analysis was carried out on the metals.

Table 4.5: Paired Wilcoxon test outcomes for each element

	Wilcoxon Z	Sig.
As	-0.135	0.893
Cd	-0.405	0.686
Cr	-1.753	0.080†
Cu	-0.944	0.345
Pb	-0.944	0.345
Se	-0.674	0.500
Zn	-1.753	0.080†

*Periods statistically different from each other at $p<0.001$***, $p<0.01$**, $p<0.05$*, $p<0.1^{\dagger}$*

Table 4.5 shows the paired Wilcoxon test applied across the two periods (January 2019 and May 2019), taking the type of fodder as the subject. According to the statistically significant parameter, there are no major differences across the fodders for most elements except for Cr and Zn. Both elements exhibit statistically significant differences at the 0.1 level. This indicates that Cr and Zn levels over all fodders were lower for Period 2 than Period 1 and this could be a result of seasonal variation. For further clarity, all elements show Wilcoxon Z negative values and, if this is statistically significant, it indicates that the median percentage change between Period 1 and Period 2 is significantly negative. Cr and Zn had the lowest Z scores (-1.75), both with a p-value of 0.080, indicating a negative change which is significant at the 0.1 level between Period 1 and Period 2 across all fodders for both elements. Furthermore, Figure 4.10, below, illustrates the error bars (mean ± SD) for percentage change across fodders between Period 1 and 2 for the seven elements. One can observe that Cu has the largest negative mean percentage change (in absolute terms) but also a considerably large standard deviation. Cr, Zn and Pb also have a negative mean percentage change, but the standard deviation is relatively small for Cr and Zn, indicating why these were the two elements which turned out to have statistically significant negative changes between Period 1 and Period 2. Furthermore, As, Se and Cd have zero or positive mean percentage change between Period 1 and Period 2 however, the Wilcoxon test detected no significant change in these cases.

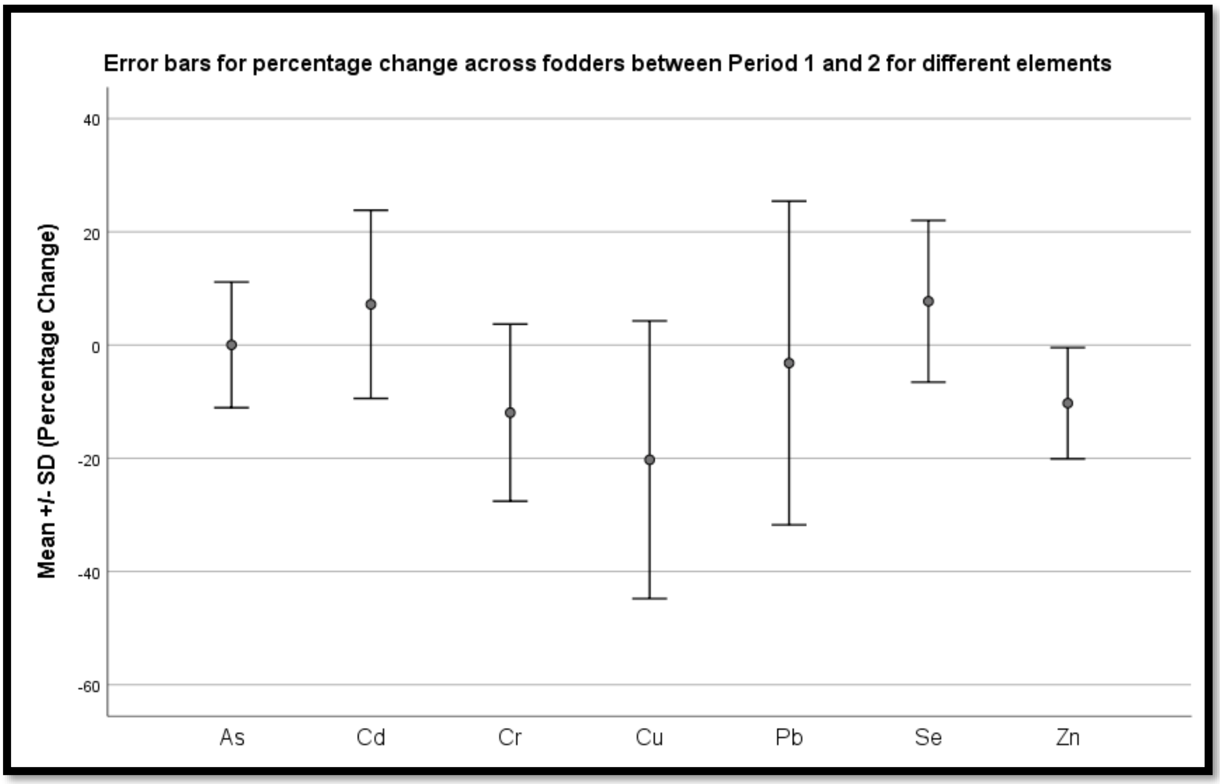

Figure 4.3: Error bars (mean ± SD) for percentage change across fodders between Period 1 and 2 for different elements

In a study carried out in China, the animal feeds were analysed for the content of heavy metals (Cu, Zn, As and Cd). Samples from different locations in China were collected, and their heavy metal concentration were determined by graphite furnace atomic absorption spectrometry. The concentrations of the mentioned elements in all cattle feed were in the range of 2.73-115mg/Kg, 11.1-346 mg/Kg, 0.010-6.12 mg/Kg and nd- 23.3 mg/Kg, respectively. (Zhang, Li, Yang, & Li, 2012b). Other researchers in China collected, from four different intensive dairy farms, samples of dairy feed in order to analyse the chosen heavy metal concentrations of Cu, Zn, Cr, Ni, Pb, and Cd.

This research resulted in the proof of heavy metal presence within all feed samples due to the wide use of additives within the intensive dairy farms, with results including concentrations of 6.10 for Cr and 17.1 for Pb, which are far greater than the average and recommended guidelines in China.(Li, J., Xu, Wang, & Li, 2019).

According to the Chinese Standard for Feeds (GB 13078-2001), Cd, Cr and Pb concentrations in animal feed are only allowed at a maximum rate of 0.5, 5, and 10 mg/ Kg, respectively. Thus, was not the case found in the studies carried out by researchers in China in which highlighted the wide use of additive concentrations within dairy farms. However, there are no Chinese guidelines at present regarding the maximum use of Cu and Ni levels in animal feed. Due to this, the researchers had to use the Russian standards and noticed that most of the feed samples obtained exceeded the allowed Cu (8 mg/ Kg) and Zn (100 mg/ Kg) values (Cang 2004).

Furthermore, another study was carried out in England about its livestock feeds (Nicholson, Chambers, Williams, & Unwin, 1999) in order to determine their contents of some heavy metal including (Zn, Cu, Pb, Cd, As, and Cr). The highest concentrations were for Zn and Cu in cattle fodder, with typical concentrations in cake/nuts of 0.190 mg Zn/Kg and 0.350 mg Cu/Kg. Grass silage, hay and straw fed contained 0.300 mg Zn/Kg and 0.600 mg Cu/Kg. Concentrations of the further elements (Pb, Cd, As and Cr) in all types of fodder were <5 mg/Kg.

In connection to the study about fodder, the mean concentrations of the elements investigated in this research, are 8.20mg Cu/Kg, 32.8mg Zn/Kg, 0.510µg Se/Kg, 0.580 µg As/Kg, 0.040µg Cd/Kg, 0.550µg Cr/Kg and 0.190µgPb/Kg. The mean concentrations of Cu, Zn, As and Cd are very close to those found in China by Zhang *et al.* (2012). However, these findings are greater than the ones determined by Nicholson in England. These feed samples results contained heavy metals, and as mentioned earlier, the Chinese Standard for Feeds (GB 13078-2001) allowed a permissible limit of 5, 20, 0.5 and 10 mg/kg for Cr, As, Cd, and Pb respectively.

In comparison to these guidelines, the results obtained from the fodder research were smaller, however still in the same permissible limit as the Chinese standards. However, a report, titled 'Hygienic Standard for Feeds' (GB13078-2017), in China, found a new update of permissible limit for some heavy metals' concentrations on animals feed, those being, Cr ≤5 mg/Kg, As ≤4 mg /Kg, Cd ≤2 mg /Kg, Pb ≤30 mg /Kg. Only the chromium concentration did not change. Nevertheless, the findings of this survey were still under the allowed limit.

In addition to this, another study by Farid and Baloch (2012), untreated city waste was used as source of irrigation in Pakistan, for raising various crops including fodder which is utilized for feeding the animals in the cattle's farms. Heavy metals were determined in typical effluents, four soils and fodder crops. It was discovered that the city effluent increased heavy metal levels in the soil and eventually in fodder. Clearly, the contamination to the fodder from the irrigation vastly effects the heavy metals concentrations.

Similarly, heavy metals, such as Zn, Cu and As were used within agricultural activities in order to enhance commercial feeds by using the heavy metals as growth promoters and antimicrobials. (Li *et al.,* 2005; Moller *et al.*, 2007; Wang *et al.,* 2013). Nevertheless, not enough information was given on how the fodder samples were produced in this study, however, the effect of the irrigation into the feed should not be neglected.

Moreover, it is not known whether the fodder used in this study's farm came from polluted, industrial or agricultural areas, which might have played a role in the quality of the fodder. In Malta, which is where this study was conducted, feed is either imported or grown locally. The local fodder is then either harvested and silage or else dried in situ and harvested using bales. However, cows do not receive any necessary nutrients from fresh pasture. In a study carried out in Malta by (Agius *et al.,* 2019), the hydroponic fodder system was suggested as a solution for places with limited land capacity for growing their own fodder, since it does not take as much space, and can provide farmers with fresh and nutritious fodder on a daily basis, making it more efficient and less expensive than other systems of acquiring fodder for cows. This would be ideal since Malta has a lack of fresh and nutritious feed.

In connection to the research mentioned above, the hydroponic system suggested by Agius et al (2019), would have been an ideal solution for the farm used for this study in order to ensure that the fodder was of good quality. This is ideal because of the lack of knowledge on where the fodder used within this study was produced, as mentioned previously; whether it came from polluted, or industrialized or agricultural areas, as well as the effects the irrigation system might have had on the fodder.

4.2.1 Conclusion

Due to the increase in anthropogenic activities, heavy metals have the ability to accumulate significantly, which can be a serious detriment onto the environment and overall health of living organisms, (Khan *et al.,* 2008; Chen *et al.,* 2015; Jin *et al.,* 2019). As mentioned previously, heavy metals (Cu, Zn, and As) have been excessively found within growth promoters and antimicrobials in the commercial feeds used in industrial livestock farming, (Li *et al.,* 2005; Moller *et al.* 2007; Wang *et al.,* 2001). This in turn can leave an effect not only on the overall environment, but also on the quality of a human's health, through the consumption of cows and their produce.

Therefore, this section focused on determining the concentration levels of heavy metals within fodder using ICP. This gave clearer results as to what different types of feed contain. The results found for this study were those of seven heavy metals (Cr, Cu, Zn, As, Se, Cd and Pb), in which Cu and Zn were found to be the highest concentrations during the two periods of this study. These results are expected since these two elements are essential and non-toxic for a human's body, as long as they are under the permissible limit. Therefore, it is natural for the fodder to contain higher concentrations of Cu and Zn in comparison to the rest of the elements studied for this research.

Following Cu and Zn, the next three highest concentrations found within the fodder were other two essential elements, Cr and Se, and the toxic element, As. These were then followed by the two most toxic elements Cd and Pb, which both had lower levels of concentrations. As, Cd, and Pb concentrations too were within the permissible limit. As for the rest of the elements the permissible limit was not identified due to the shortcoming of studies and guidelines.

Furthermore, the study was carried out during two periods with different seasons. In reference to this, it was noted that the only two elements that differed between one season and another were Cr and Zn in which they had higher concentrations in Winter than in the end of Spring, beginning of Summer. However, it is hard to accommodate measurements for all the elements since these seven elements (Cr, Cu, Zn, As, Se, Cd and Pb) present in the samples, contain different concentrations depending on the particular feed.

All in all, the results from this study proved the presence of heavy metals inside fodder. This is normal for farms and cattle feed and is not harmful, as long as the concentration levels are within limits. However, the results might have been more accurate had there been resources about EU guidelines and the permissible limits for EU heavy metal concentrations in fodder, since the fodder used for this study was obtained from different countries within the EU.

4.3 Water analysis

Samples of water were collected once a month over a period of one year, starting from January till December 2019, as depicted in Section 2.1.2. In the following graphs different heavy metals can be seen with actual observations marked in blue, and their moving average in red. The purpose of moving average is for smoothing month-to-month fluctuations. A 3-point centred simple moving average is used for all elements in order to smooth and discern overall behavioural changes in the mean. However, as observed below, both methods had similar results.

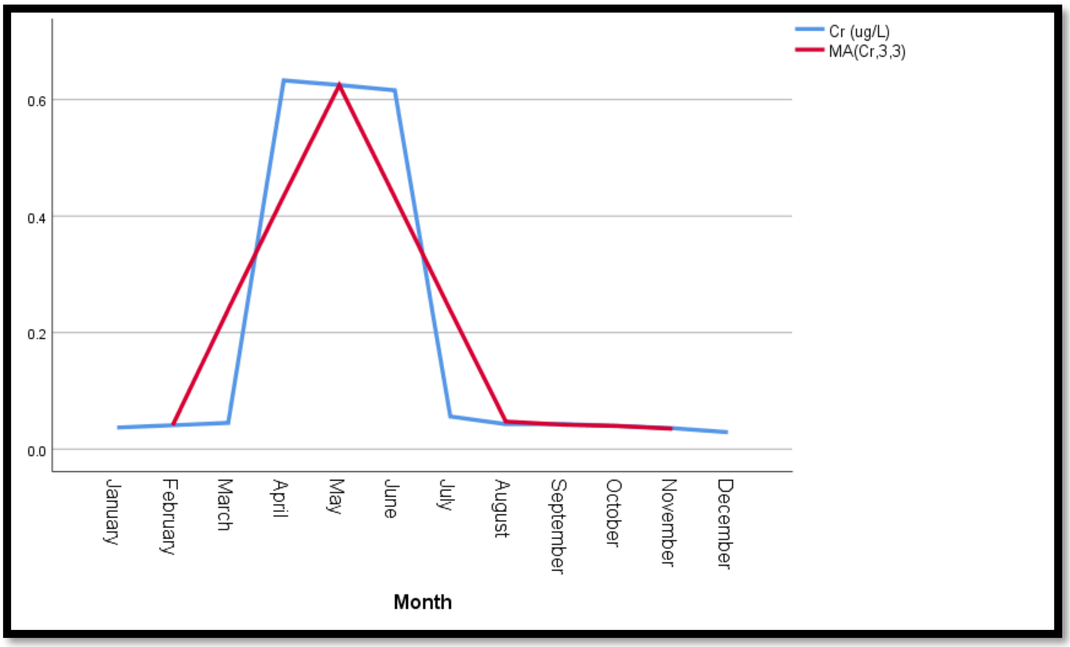

Figure 4.4: Sequence plots for actual observations (blue) and 4-point moving average (red) for Cr.

In figure 4.4, there are both the actual Cr observation and the 3-point centred simple moving average as mentioned previously. It can be seen that Cr had low levels of concentration, below 0.100µg/L, for most of the year, but increased to above 0.6 µg/L between April and June.

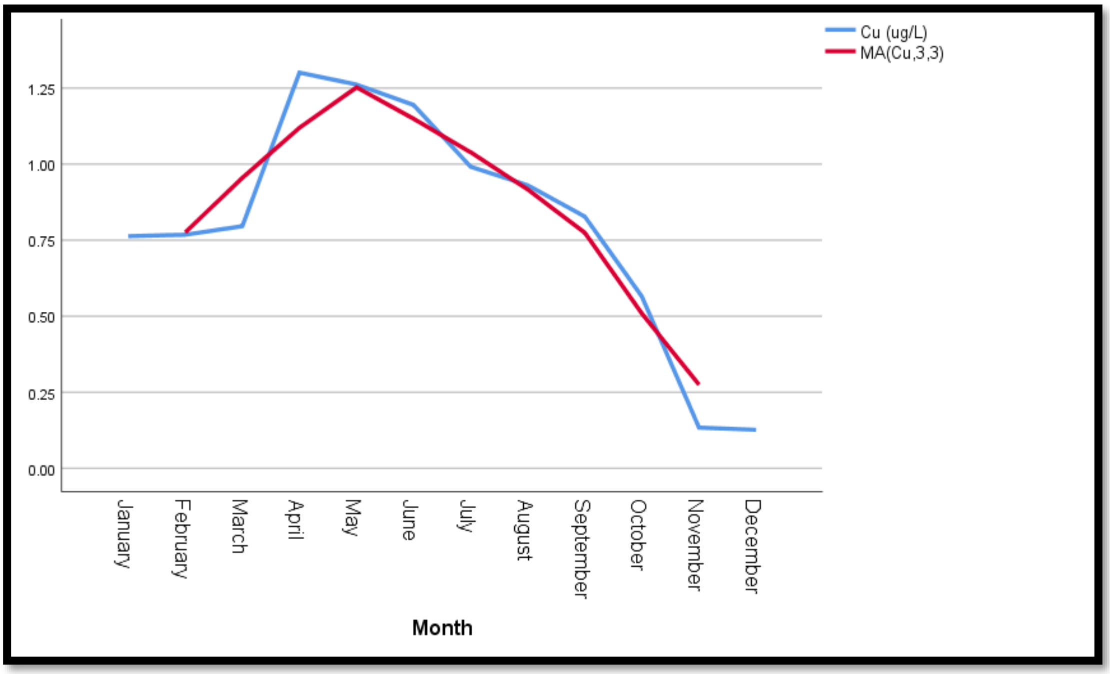

Figure 4.5: Sequence plots for actual observations (blue) and 4-point moving average (red) for Cu.

Figure 4.5, the above plot, Cu was just above 0.750 µg/L in the first three months of the year, then increased to above 1.25 µg/L in April. It then proceeded to gradually decline all the way down to November/December where it reached a lower level of just above 0.100 µg/L.

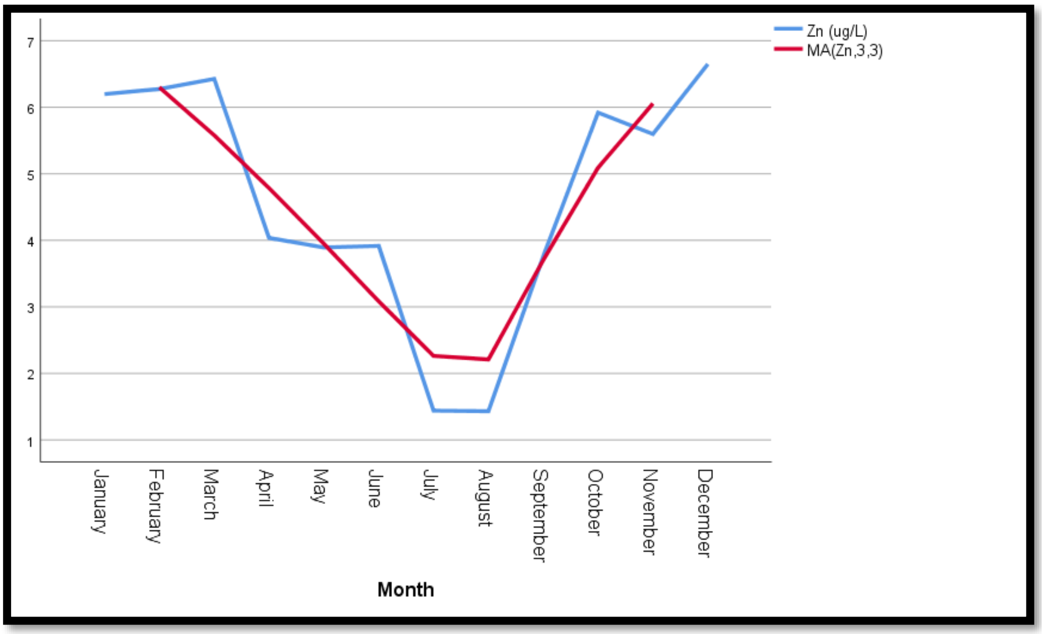

Figure 4.6: Sequence plots for actual observations (blue) and 4-point moving average (red) for Zn.

Using the actual Zn observation and the 3-point centred simple moving average in Figure 4.6, it can be seen that Zn was higher for the Autumn/Winter months (January – March, October - December) and lower for the Spring/Summer months (April – September). The peak is between 6 and 7 µg/L, and at its lowest levels it is between 1 and 2 µg/L.

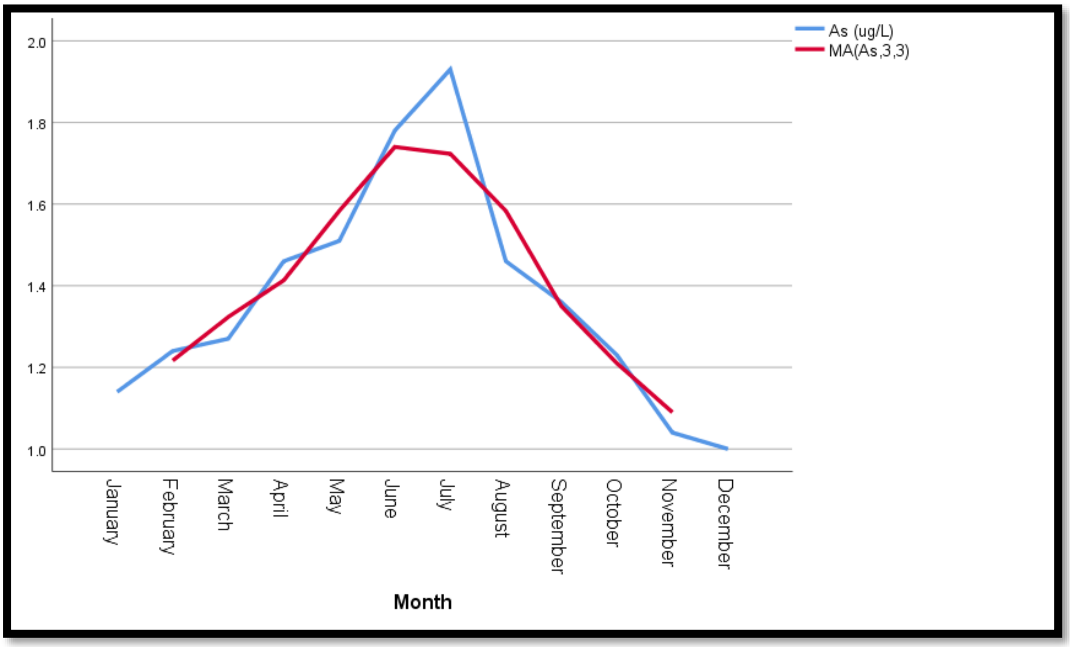

Figure 4.7: Sequence plots for actual observations (blue) and 4-point moving average (red) for As.

In Figure 4.7 it can be observed that As was lower in the Autumn/Winter months and higher in the Spring/Summer months. As peaks at around 1.90 µg/L but is close to 1 µg/L at its lower levels.

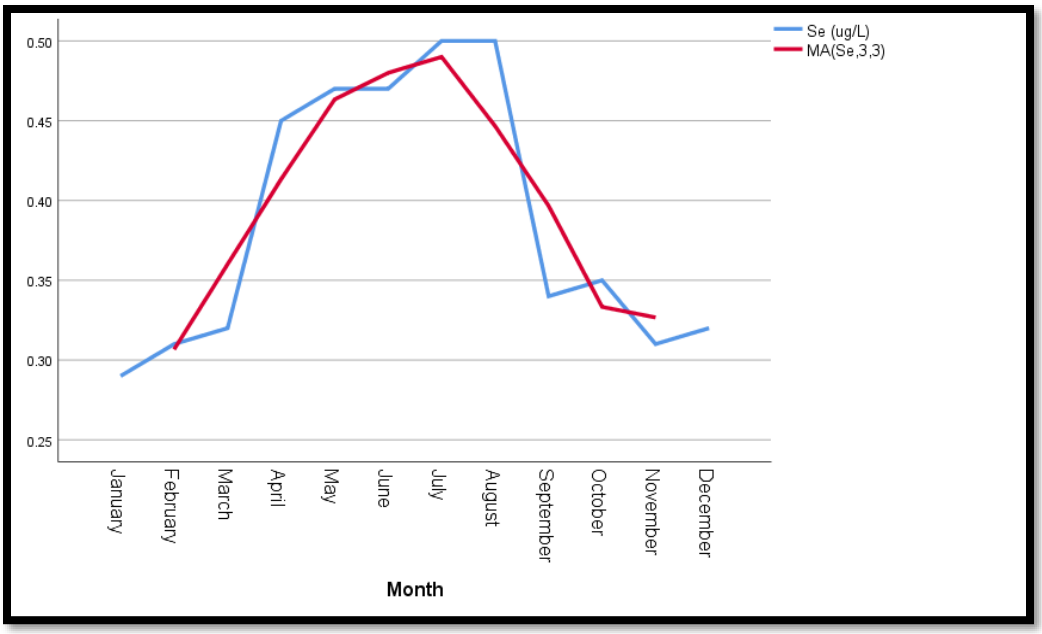

Figure 4.8: Sequence plots for actual observations (blue) and 4-point moving average (red) for Se.

It can be witnessed in Figure 4.8 that Se was higher between April and August than it was for the other months of the year. Se peaks at around 0.500 µg/L and is around 0.300µg/L at its lower levels.

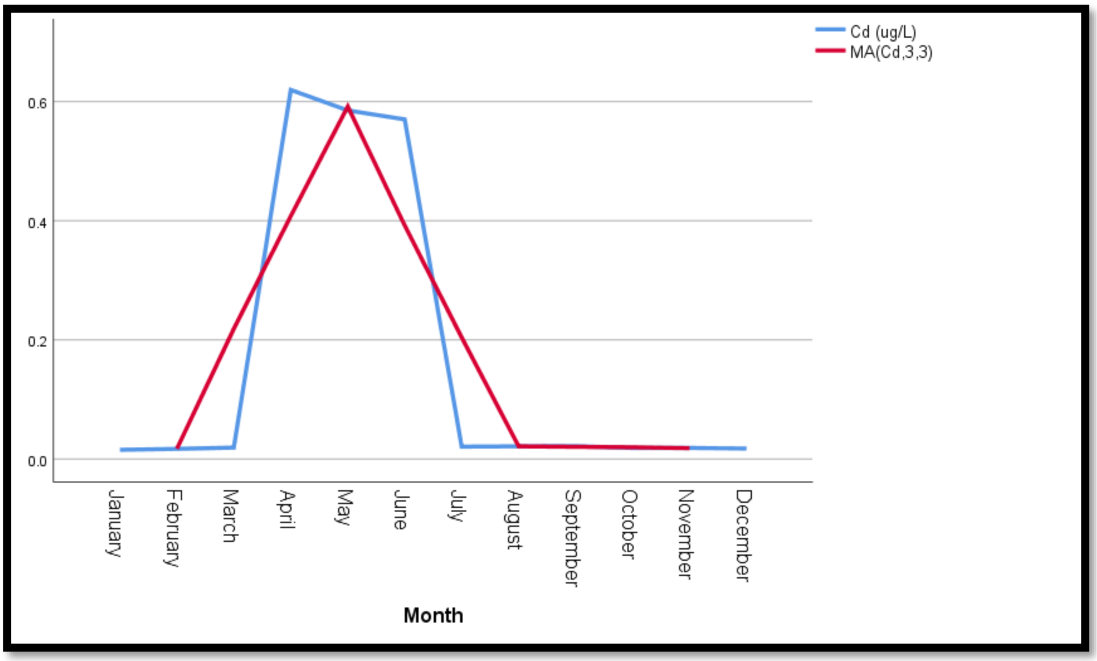

Fig4.9: Sequence plots for actual observations (blue) and 4-point moving average (red) for Cd.

In the above plot, Figure 4.9, Cd had low levels of below 0.100 µg/L for most of the year but increased to around 0.600 µg/L between April and June.

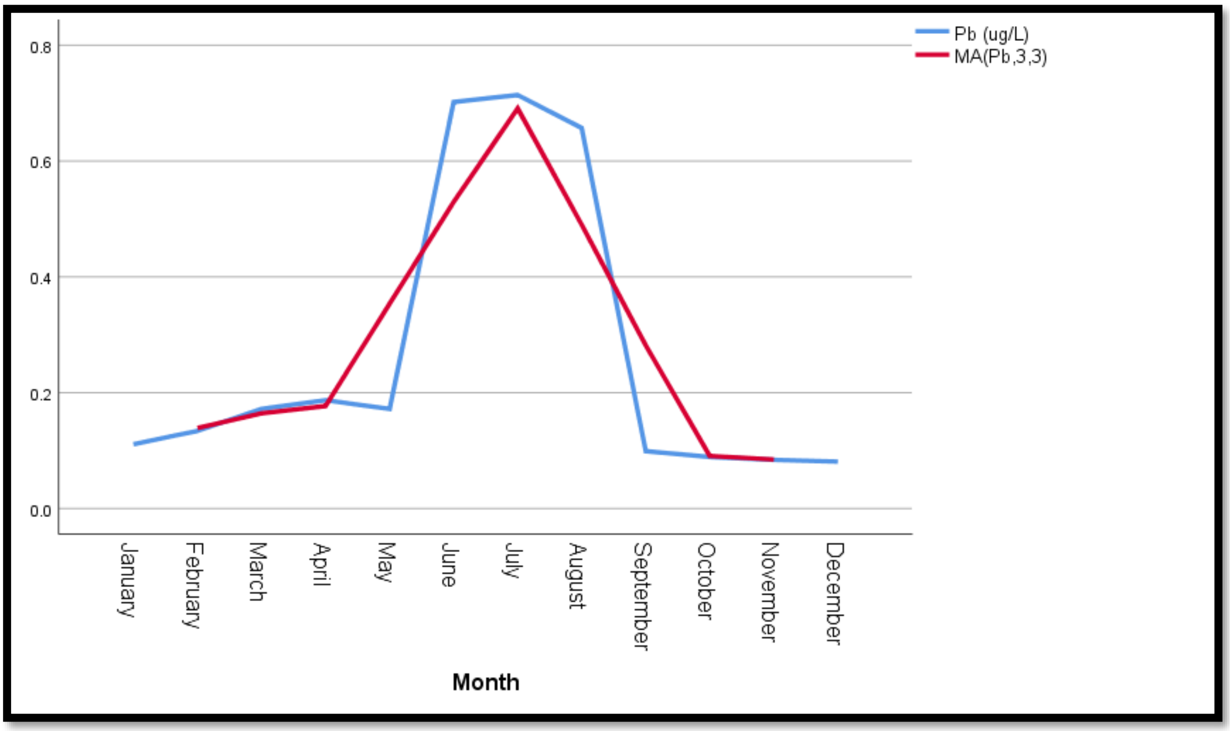

Figure 4.10: Sequence plots for actual observations (blue) and 4-point moving average (red) for Pb.

Finally, the above plot, shows that Pb had low levels (below 0.200 µg/L) for most of the year, however increased to between 0.600 and 0.800 µg/L between June and August.

Table 4.6: The highest concentration of Heavy Metals in Water (µg/L and mg/L) and WHO maximum permissible limit (mg/L) 2008

Metals	**µg/L**	**mg/L**	**WHO maximum permissible (mg/L) limit 2008**
Cr	0.600	0.0006	0.050
Cu	1.25	0.0013	0.050
Zn	7.00	0.0070	5
As	1.80	0.0018	--
Se	0.500	0.0005	--
Cd	0.600	0.0006	0.005
Pb	0.700	0.0007	0.050

Viewing the table above, it can be observed that all the metals resulted in having lower levels of concentration than the permissible limit established by WHO in 2008 for levels in drinking water for human health. The farm chosen for this study used tap water as drinking water for cattle, which in Malta's case is not harmful since the heavy metal concentration levels are low. Although there are no guidelines set by WHO (2008) for Arsenic and Selenium levels in drinking water, the results obtained from this study show that they are under control. Thus, the mean concentrations of all investigated metals in the waters tested were well below the maximum contaminant levels established by the WHO for levels in drinking water.

The present results seem to fluctuate depending on the season, therefore seasonal effect may be present within the study. The only element that had higher concentrations during the Winter season was Zn, resulting the highest in concentration in the first and last three months of the year. On the contrary to Zn, the rest of the elements (Cr, Cu, As, Se, Cd and Pb) had higher concentrations during the warmer seasons of the year, Spring and Summer. In comparison to Zn which had its highest concentrations during the first and last three months of the year, Se had its highest concentrations during Spring and Summer, from April to September. In addition to this Cr and Cd had their highest concentrations during Spring, from April to June, whilst As and Pb their highest concentrations were noted to be during Summer between June and August. Finally, with regards to Cu, as opposed to the other elements that had sudden upwards or downwards movements within their respective graphs, Cu had the highest concentrations in April and then started to gradually decrease until it hit its lowest concentration during the months of November and December. Overall, most of the seasonal pollution of heavy metals in water was during the months of Spring and Summer, with the exception of Zn which was during Winter.

In a study done in China by Rajeshkumar *et al.*, (2018), the water samples collected had resulted that the heavy metal concentrations of Pb, Cd, Cr and Cu had higher seasonal pollution in water during the Winter and Summer seasons than in Spring and Autumn. In comparison to the previously discussed results, similar conclusions were reached, with the majority of the same elements, as this study, having a higher concentration during the months of Summer than the rest of the Seasons. According to

Wong *et al*., (2001), one of the reasons that could explain the high concentrations of heavy metals during the seasons of Winter and Summer, might be attributed to an increase in evaporation and a decrease in rainfall. On the other hand, the lower levels of concentration during Spring and Autumn can be a result of rainfall which can increase the lixiviation process with regards to the dilution of heavy metals during the wet season, (Tekin-Ozan and Kir, 2008; Duman and Kar, 2012).

4.3.1 Conclusion

The most significant outcome of this study indicated that despite the difference in concentrations depending on the season, none of the elements analysed had higher concentrations within water than the permissible limit set by the WHO guidelines. Therefore, one can conclude that since water had low concentrations of heavy metals, it would not have an adverse effect on the drinking water quality of the cattle, and in turn would not have any effect on a human's health either. Had the concentration of heavy metals been higher than the permissible limit, the contamination of heavy metals within water would have had a negative effect on the quality of drinking water for the cattle, which eventually would have seeped through into a human's food chain. It would have been interesting to analyse the relation between fodder and water, however, since the fodder used for this study was mostly imported, it did not make much sense to compare the two together.

Furthermore, the drinking water used in the farms was tap water, which again resulted to be much lower than the permissible limit. From the point of view of heavy metals, tap water is not harmful, however, Maltese residents do not drink from it as it is more common to drink filtered water since tap water usually has a higher concentration than filtered water.

4.4 Manure analysis

In this section manure samples were collected for this survey over a duration of one year, starting in January 2019 until December 2019. The concentrations of seven heavy metals (Cr, Cu, Zn, As, Se, Cd and Pb) in manures are shown in figures 4.11-4.17.

In the following graphs one can observe the different heavy metals with actual observations marked in blue, and their moving average in red. The purpose of the moving average is for smoothing month-to-month fluctuations. A 4-point moving average was used for As and Se, and a 3-point moving for the rest. The reason for selecting a 4-point moving average for As and Se was because smoothing with this window size was more appropriate for discerning the overall behavioural changes in the mean.

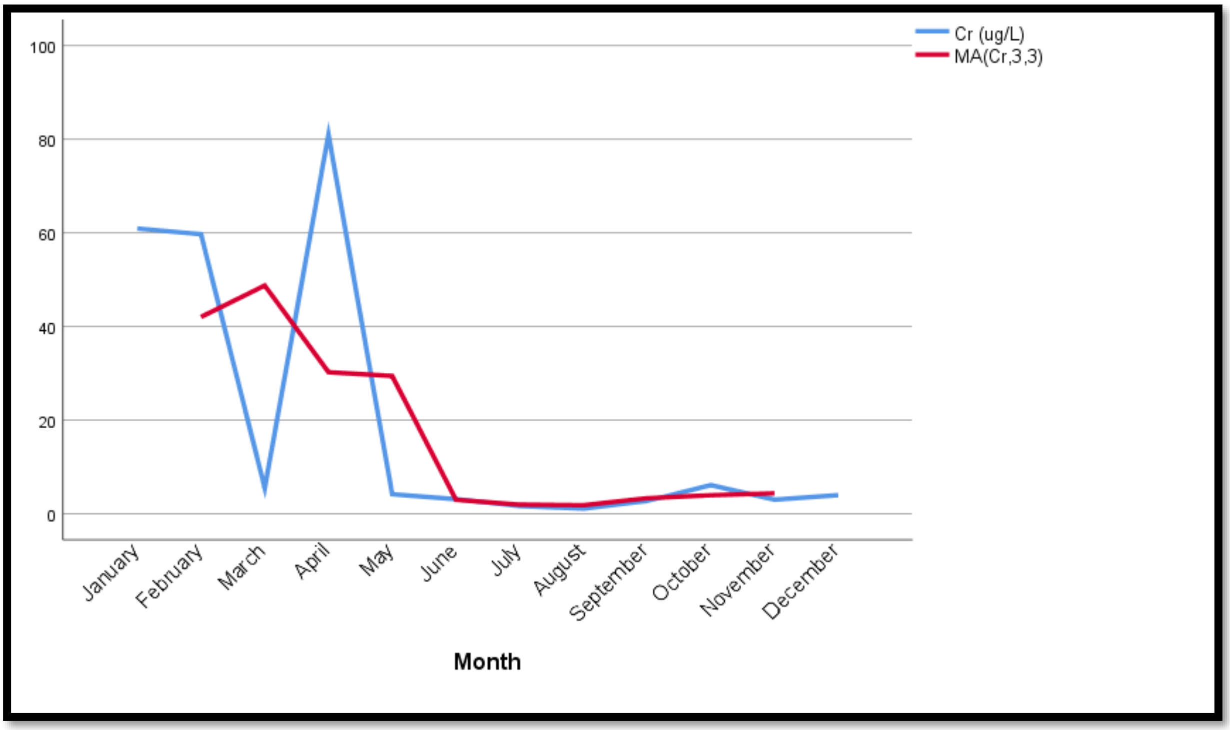

Figure 4.11: Sequence plots for actual observations (blue) and 3-point moving average (red) for Cr.

In, figure 4.11, the moving average sequence plot indicates the presence of higher levels of Cr towards the first 4-5 months of the year. Nonetheless, in the actual observations sequence plot, low levels of Cr can also be noticed during Spring season in the months of March and May.

Levels of Cr drop down abruptly to lower levels in the remaining months, during Summer, Autumn and Winter seasons.

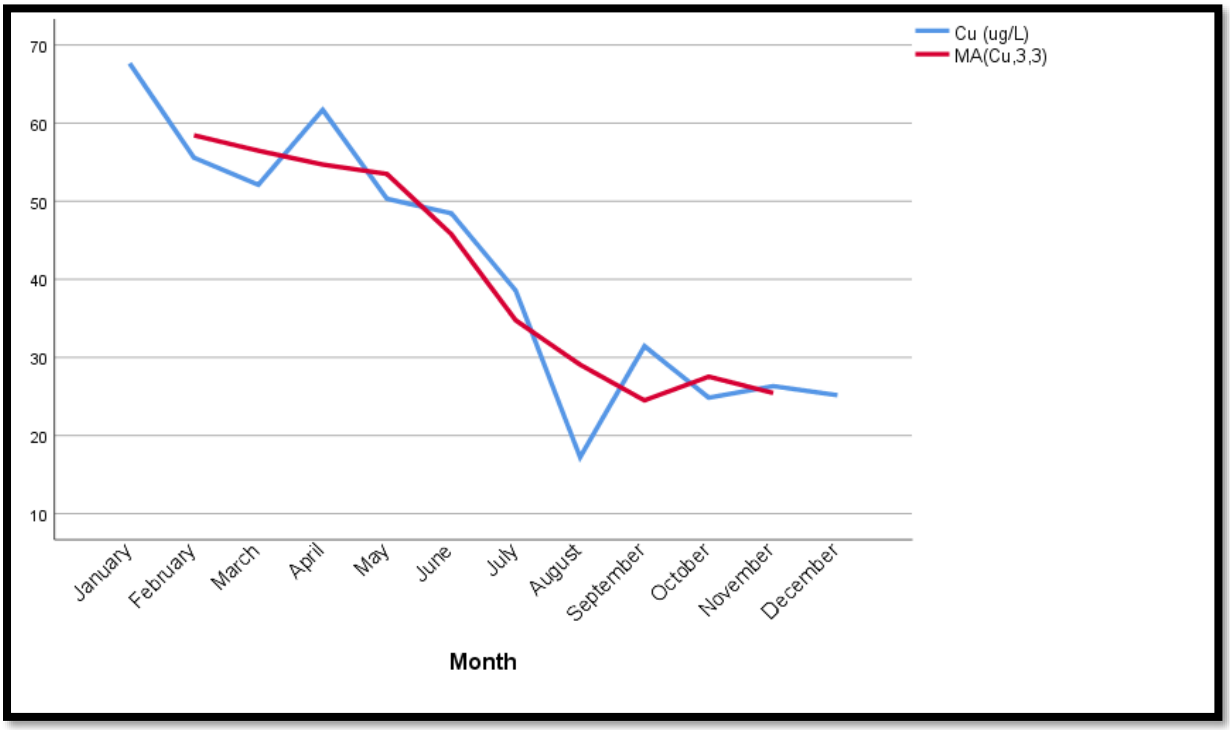

Figure 4.12: Sequence plots for actual observations (blue) and 3-point moving average (red) for Cu.

In figure 4.12, the moving average sequence plot indicates an overall decreasing trend in Cu throughout the year. From the actual observations sequence plot, the levels of Cu seem to settle at around 20-30 µg/g in the latter months of Winter (October-December).

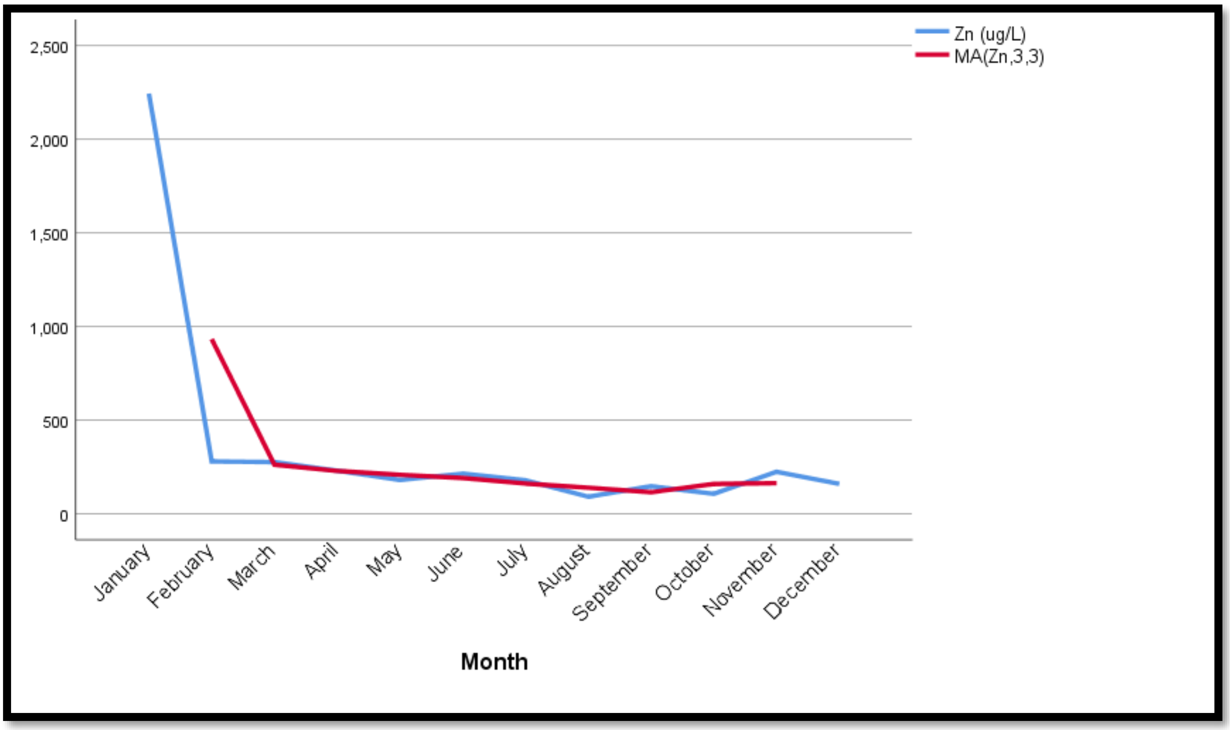

Figure 4.13: Sequence plots for actual observations (blue) and 3-point moving average (red) for Zn.

Figure 4.13 illustrates that only January has high levels of Zn from the actual observations sequence plot. The moving average indicates no trends between March and November.

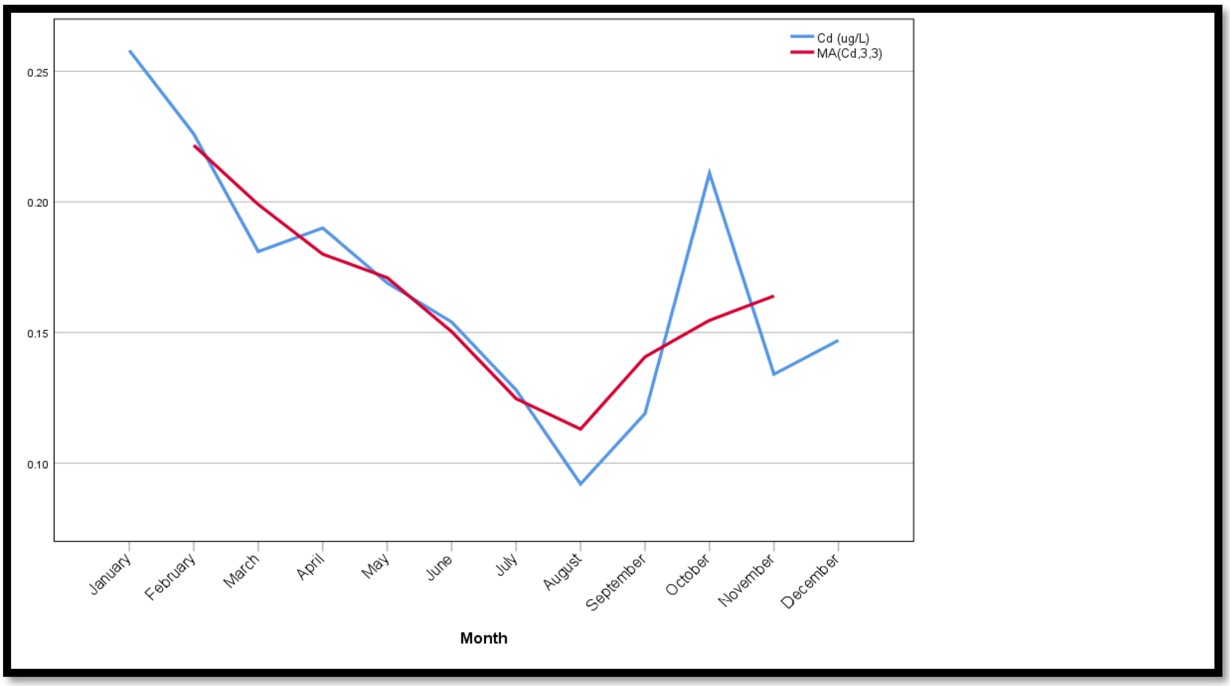

Figure 4.14: Sequence plots for actual observations (blue) and 3-point moving average (red) for Cd.

In figure 4.14, it can be seen that the highest levels of Cd are present towards the beginning of the year. This is evidenced both in the actual observations sequence plot and the moving average. The levels of Cd reach their low in August, then start increasing again. In October, a spike in Cd is observed in the actual sequence plot (compared to the moving average plot). Since the data is for one year only, there is not enough evidence to conclude that there is any seasonality in Cd, however this could be a possibility which could be studied further, due to higher levels of Cd in winter months.

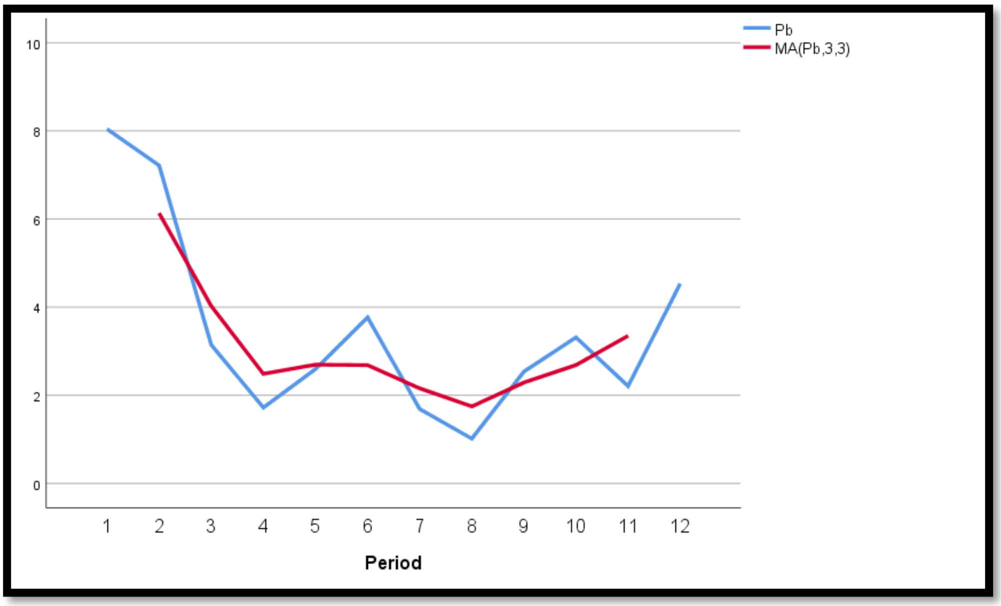

Figure 4.15: Sequence plots for actual observations (blue) and 3-point moving average (red) for Pb.

In the above graph (figure 4.15), both the actual observations sequence plot and the moving average indicate higher levels of Pb in the initial months of the year, which levels itself out towards the middle of the year. There seems, again, to be a slight increase towards the end of the year, therefore should the research be carried out for a longer period, the study of seasonal effect on Pb could be observed further.

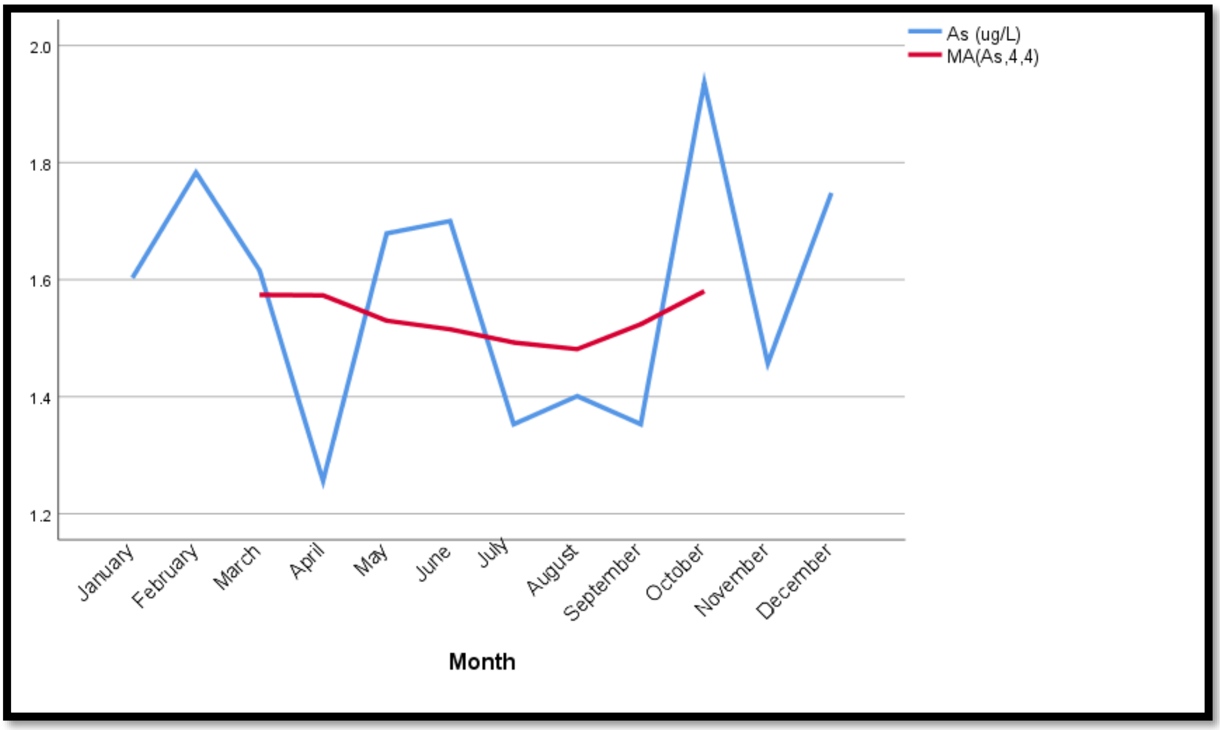

Figure 4.16: Sequence plots for actual observations (blue) and 4-point moving average (red) for As.

In figure 4.16, from the actual observations sequence plot, one can observe As fluctuated a lot around a mean of 1.50-1.60 µg/g. However, the moving average is relatively flat which seems to indicate that there is no trend or seasonality in As levels.

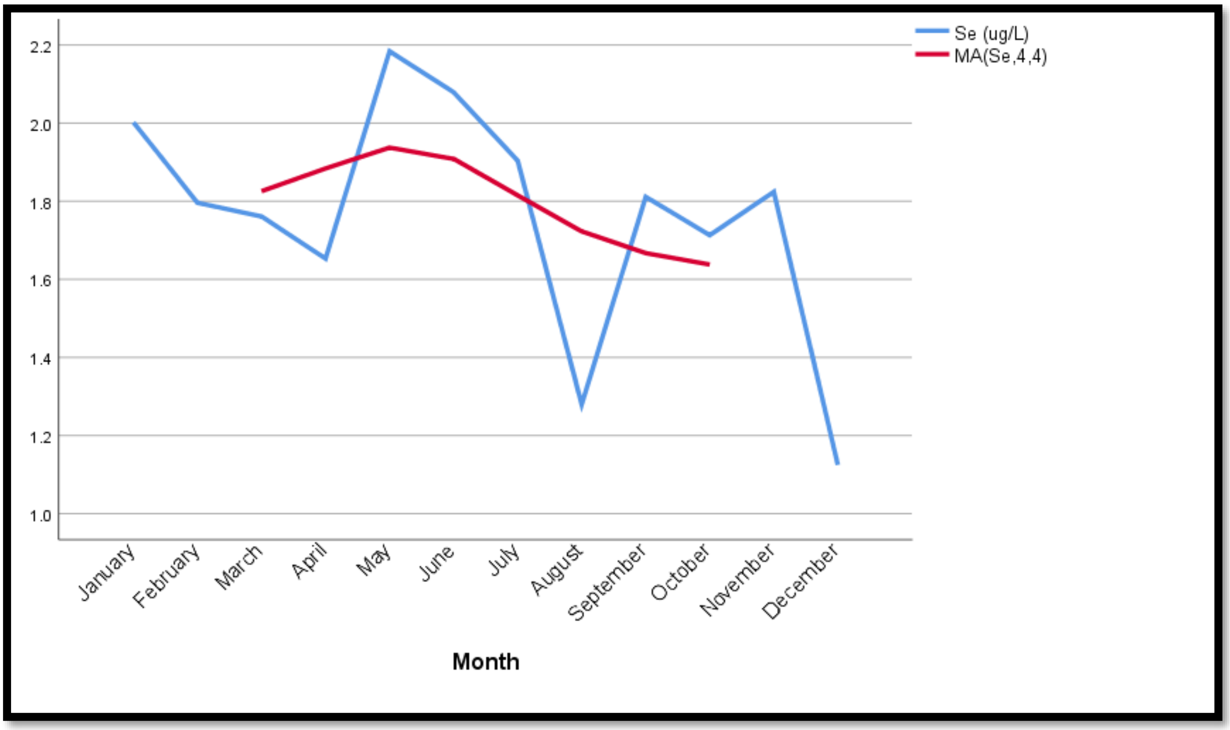

Figure 4.17: Sequence plots for actual observations (blue) and 4-point moving average (red) for Se.

In figure 4.17, from the actual observations sequence plot it is noticeable that Se fluctuates significantly from month to month, while from the moving average plot, there appears to be a decreasing trend in the latter months of the year.

As observed above, in the case of Cr, the low concentration was present during Summer, Autumn and early Winter, followed by a spike in concentration during January and the 4 months that followed. Similar to Cr, the concentration of Cu too started at a high-level during Winter, Spring and early Summer and slowly decreased as the year went by. Moving on, Zn presented high level of concentration during the month of January only, and then remained stable at a much lower level from the month of March till the end of the year. Following Zn, Cd had high levels at the very beginning of the year, however started to decline until it reached its lowest level in August, then started increasing again. This was noted as well for Pb. As for As, its concentration levels seem to be stable throughout the year with a slight, lower curve in August. Se too was stable throughout the year, however, opposite of As, it had a slightly higher curve in May.

A comparison of the fodder’s heavy metals concentration levels as intake and the manure’s heavy metals concentration levels as output, with regards to the cattle, can be discussed. Starting with the heavy metals that had a higher concentration in manure than in the fodder, Cr had concentration levels of 0.550µg/kg within fodder as opposed to manure which was 50 µg/kg. Following Cr, As too had higher concentration levels within manure with its concentration being that of 1.58µg/kg while fodder had a concentration of 0.580 µg/kg. Se had a concentration level of 0.510µg/kg in fodder and a concentration of 1.98µg/kg in manure. Cd had a concentration level of 0.040µg/kg in fodder and a concentration level of 0.220µg/kg in manure. Finally, Pb had a concentration level of 0.190µg/kg in fodder and a 6.20 µg/kg concentration level in manure. This means that the output was much higher than the intake which can lead to other sources such as water and supplements to be affected. This result could also be an effect from medical treatment; however, one cannot know for sure since the samples used for this study were given and collected in a random manner.

As opposed to the previous elements, only Cu and Zn resulted with lower concentration levels in manure than in fodder. Beginning with Cu, the intake within fodder was found to be 8.20mg/kg, whilst in manure 0.060 mg/kg. Similar to Cu, Zn too had lower results in manure than in fodder with fodder concentration level being that of 32.8mg/kg, while the manure concentration being 0.900 mg/kg. Since these two essential elements are necessary to the animal's health system, it is expected that the majority of intake is digested and absorbed by the animal's body.

In addition, a comparison can also be made between the water as an intake and the output of manure. For this comparison, the actual observation results were used for both water and manure. To begin with, in water, Cr had its highest concentration during the months between April and June. Due to this, manure had a high concentration in April too. Following Cr, Cu had a high concentration in water during April whilst in manure, it was during February. In the case of Zn, it reached its highest concentration during January, and had low concentrations during the months of Summer, both in water and in manure. The difference between water and manure is that in water, the concentration increased again during the last two months of the year. Furthermore, As in manure had a high concentration in October while its lowest was in April. In the case of As in water, its highest was between July and August. Whilst As had opposing concentrations, Se had high concentrations in both water and manure during May and low concentrations in December, winter season. The last two elements studied for this research, Cd and Pb were noted to have the highest concentrations in February and the lowest in August within manure samples. Then, with regards to the water samples, Cd had the highest concentration between April to June while Pb had the highest between June and August, opposite to the manure samples.

The concentrations within manure can be affected by any type of intake, including water, fodder, supplements and medical treatments. Even if the water had low concentrations during a particular month or season, other intakes, such as fodder can still result with high concentrations of manure, and vice versa. This applies to any type of intake, including supplements and medical treatments.

4.4.1 Conclusion

The concentration of heavy metals within manure can be seen as a reflection of the quality of the animal, as well as the feed and other sources of intake being digested (Sager, 2007). Manure is applied for agricultural activities in order to make it more organic and improve the fertility of soil. However, this in turn can result in dangerous environmental problems since heavy metals can accumulate on the surface of soils should the agricultural activity last for a long period, resulting in further negative effects on product quality of the cattle (Zhang, Li, Yang, & Li, 2012c).

With regards to this study, all heavy metals, except for Cu and Zn, had higher concentrations within manure than in fodder. This is beneficial to the cattle since most of the heavy metals that had higher concentrations in manure are toxic, resulting in the cattle being of better quality for human intake. In addition to this, seasonal effect was clearly present during the months of Summer, due to low concentrations of most of the elements. While it is advantageous for cattle to digest most of the heavy metal concentrations, it is also important for manure to have low concentrations of heavy metals, since high concentrations of heavy metals within manure could lead to negative effects onto the environment. This is due to the fact that manure is used as a fertilizer for crops leading to there being toxic heavy metal levels within agricultural activities (Nicholson *et al.*, 2003; Brock *et al.,* 2006). This can in turn also affect not only the animal's health and well-being, but a human's too.

For future reference, had the study had a longer research period, the seasonal effect on heavy metals might have been clearer and better analysed, in order to be able to note more accurate results.

4.5 Milk Analysis

For this section, samples were collected once a month from ten different cows for a period of twelve months. The first batch of samples were collected between the months of September and December in 2016, whereas the second batch were collected over a period of eight months, from January till August 2019.

Table 4.7 Kruskal-Wallis test outcomes for each element

	Kruskal-Wallis H	Df	Sig.
As	66.9	10	0.000
Cd	66.5	10	0.000
Cr	60.2	10	0.000
Cu	43.2	10	0.000
Pb	94.2	10	0.000
Se	69.7	10	0.000
Zn	57.4	10	0.000

The Kruskal-Wallis test is applied for all elements, with Bonferroni post-hoc tests. In Table 4.7, it can be seen that the p-value is 0.000 for all elements for the Kruskal Wallis test. This indicates significant month-to-month variation for each element. The results achieved from these samples were then statistically worked out to create the below table which presents the mean ± sd of all the elements for each month. For each element, the statistical significance with which each month is different from the other months when using Bonferroni post-hoc tests is also denoted with the suggested symbols in the superscript.

Table 4.8: Contents for Cr, Cu, Zn, As, Se, Cd and Pb along the months January 2019 – December 2019 (mg/L).

	Cr	Cu	Zn	As	Se	Cd	Pb
January	0.008±0.002*	0.022±0.007***	3.28±0.512***	0.003±0.001***	0.007±0.001***	0.012±0.002***	0.006±0.001***
February	0.011±0.006	0.027±0.010**	3.59±0.614**	0.003±0.000***	0.008±0.001***	0.014±0.003***	0.007±0.001***
March	0.008±0.002*	0.022±0.007***	3.28±0.512***	0.109±0.026*	0.106±0.015**	0.005±0.001***	0.024±0.005*
April	0.022±0.007***	0.213±0.090***	4.74±1.53	0.112±0.011*	0.075±0.015†	0.017±0.016**	0.183±0.153***
May	0.027±0.038	0.053±0.043	3.70±0.779**	0.130±0.011***	0.111±0.009***	0.010±0.010	0.051±0.029***
June	0.024±0.038*	0.029±0.021***	3.59±0.667**	0.137±0.020***	0.107±0.010**	0.006±0.001†	0.030±0.005***
July	0.008±0.002*	0.027±0.007**	6.81±0.636***	0.112±0.012*	0.097±0.007	0.005±0.000***	0.011±0.002***
August	**0.01**	**0.007**	**6.28**	**0.181**	**0.11**	**0.007**	**0.04**
September	0.006±0.001***	0.034±0.020*	3.06±1.25***	0.096±0.017†	0.097±0.016	0.005±0.001***	0.024±0.005*
October	0.006±0.000***	0.035±0.017†	4.12±0.864	0.127±0.025***	0.113±0.013***	0.006±0.001*	0.027±0.003**
November	0.030±0.065	0.049±0.028	4.65±1.50	0.111±0.036*	0.104±0.025***	0.011±0.017*	0.034±0.029**
December	0.006±0.001**	0.024±0.016***	7.70±1.39***	0.112±0.010*	0.112±0.006***	0.005±0.000***	0.012±0.001***

*statistically different from other monthly levels at $p<0.001$***, $p<0.01$**, $p<0.05$*, $p<0.1^{\dagger}$*

For further clarification the below figures have been displayed below, starting with the essential elements (Cr, Cu, Zn and Se), followed by the toxic elements (As, Cd and Pb). It is important to note that all elements had the lowest concentration during the month of August, however, this is also imperative to be knowledgeable of the fact that during the analysis of the August sample, the equipment used (ICP- OES), was damaged and repaired. For this reason, the findings for the August samples were imputed and are therefore closer to the months of July and September. Due to this the August results are presented in black and have no standard deviation. However, the actual finding for August were presented in the appendix 2.1.ICP figures A.8-table A.14.

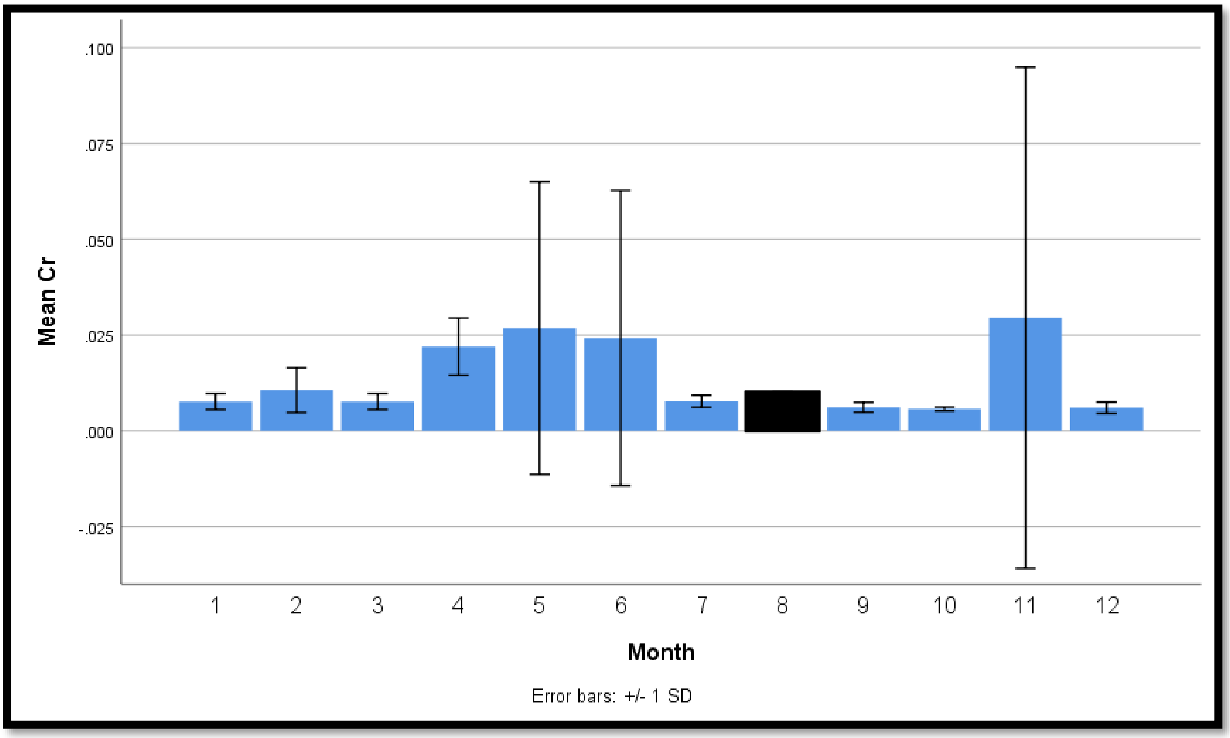

Figure 4.18: Error bar for Cr (mg/L) by Month

In figure 4.18, it can be observed that Cr had its highest mean concentration during the month of November, whereas its lowest mean concentration was during September, October and December. With regards to the month of November, it also had the highest standard deviation in comparison to the rest of the months. Looking at the figure, the mean concentrations in the first few months, January to May, were constantly increasing, with the exception of March. On the other hand, it decreased drastically until it reached its lowest level during September, October and December, with the exception of November which presented the highest mean concentration. Therefore, there seems to be a trend of continuous increasing and decreasing as time passes by. With regards to seasonality effect, the mean concentration reached the highest during Spring and beginning of Summer, with the exception of November which had the highest concentration during the end of Autumn. Opposite to this, most of the low concentrations are present during the seasons of Autumn and Winter.

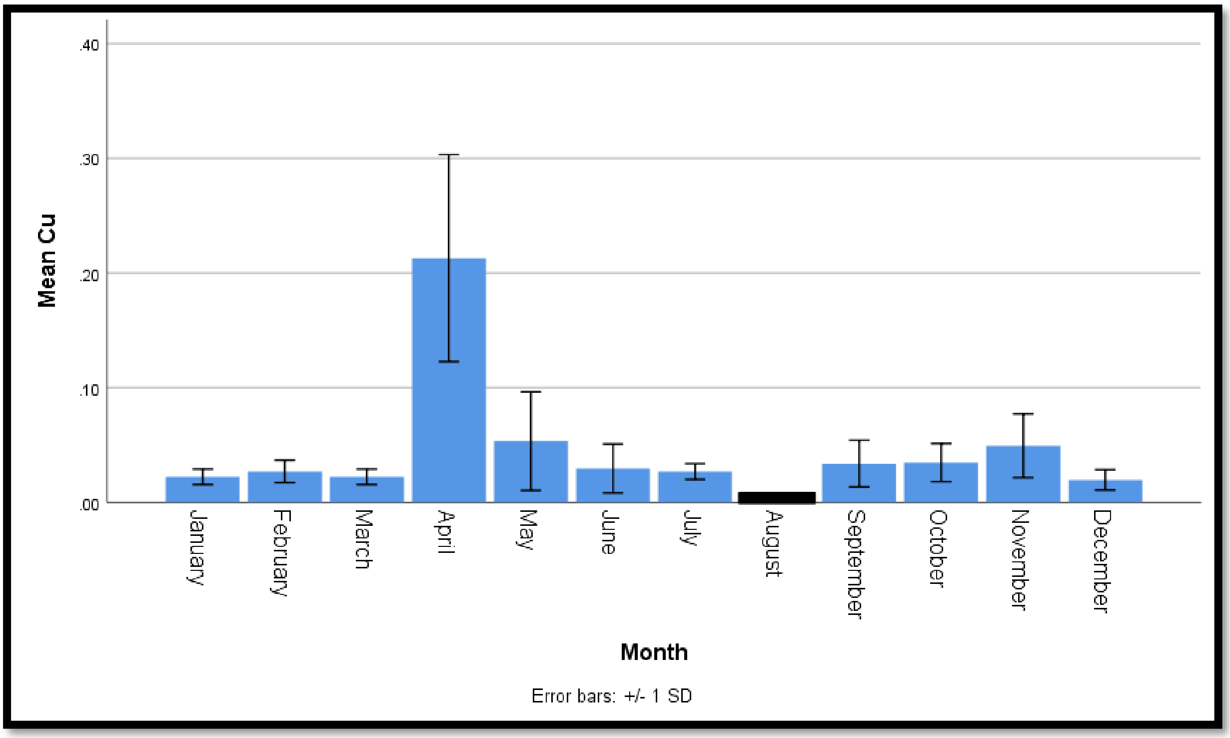

Figure 4.19: Error bar for Cu (mg/L) by Month

In figure 4.19, at first glance, the highest mean concentration of Cu is during the month of April, a much higher mean concentration than the rest of the months in which the lowest mean concentration was during the month of August. The concentrations had mild up and down fluctuations throughout the 12 months of the year, with the exception of April. It seems that seasonality is not clear since the readings were all close to one another, except for April which had a large gap from the rest. The large gap could be a result of the disruption noted from the high standard deviation, which leads to a higher mean concentration. Despite the disruption, the Cu concentration in April was still the highest mean concentration recorded throughout the year.

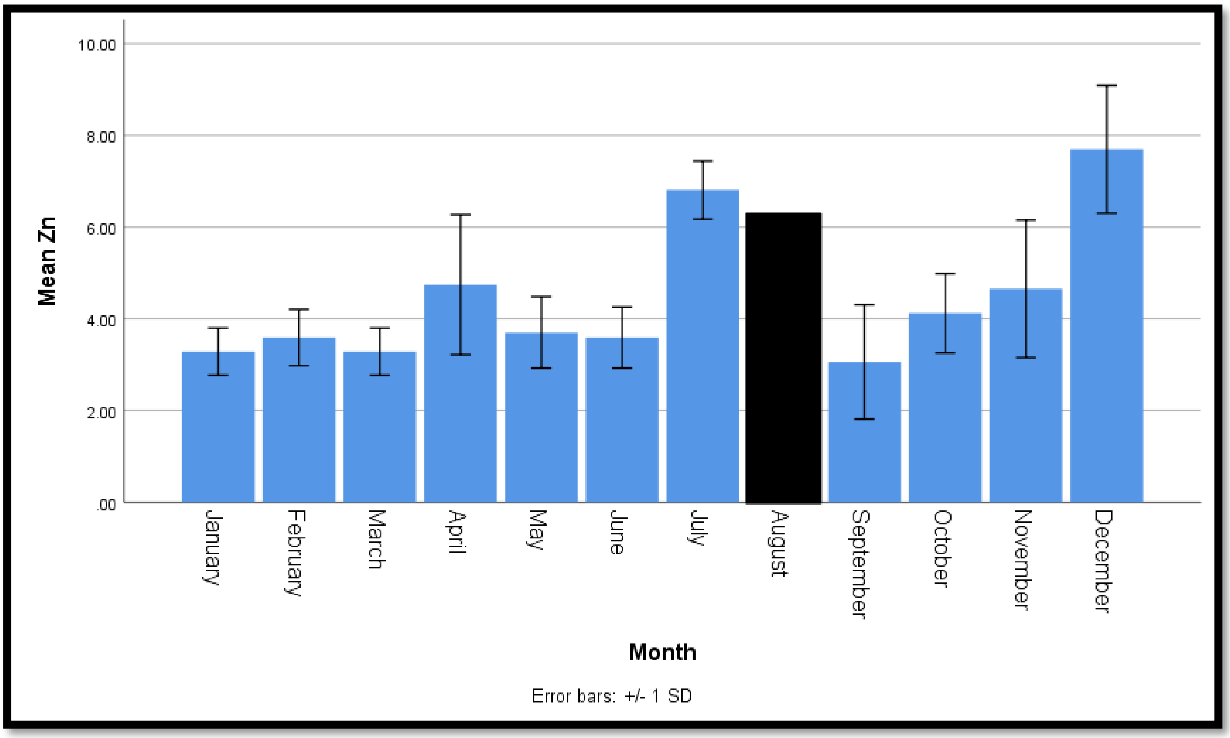

Figure 4.20: Error bar for Zn (mg/ L) by Month

It is clear from figure 4.20 for Zn, the highest mean concentration was reached during the month of December, whereas the lowest was present in September. Starting from January, the mean concentrations were relatively fluctuating until it started to increase during Summer, in July and August, but then decreased again in September. This was followed by a constant increase for the rest of the year, starting again in October, till it reached its highest level in Winter, December. It can be observed that the two highest findings were during July and December, concluding that there might be a possibility that the Zn concentrations are affected by either very high or very low temperatures.

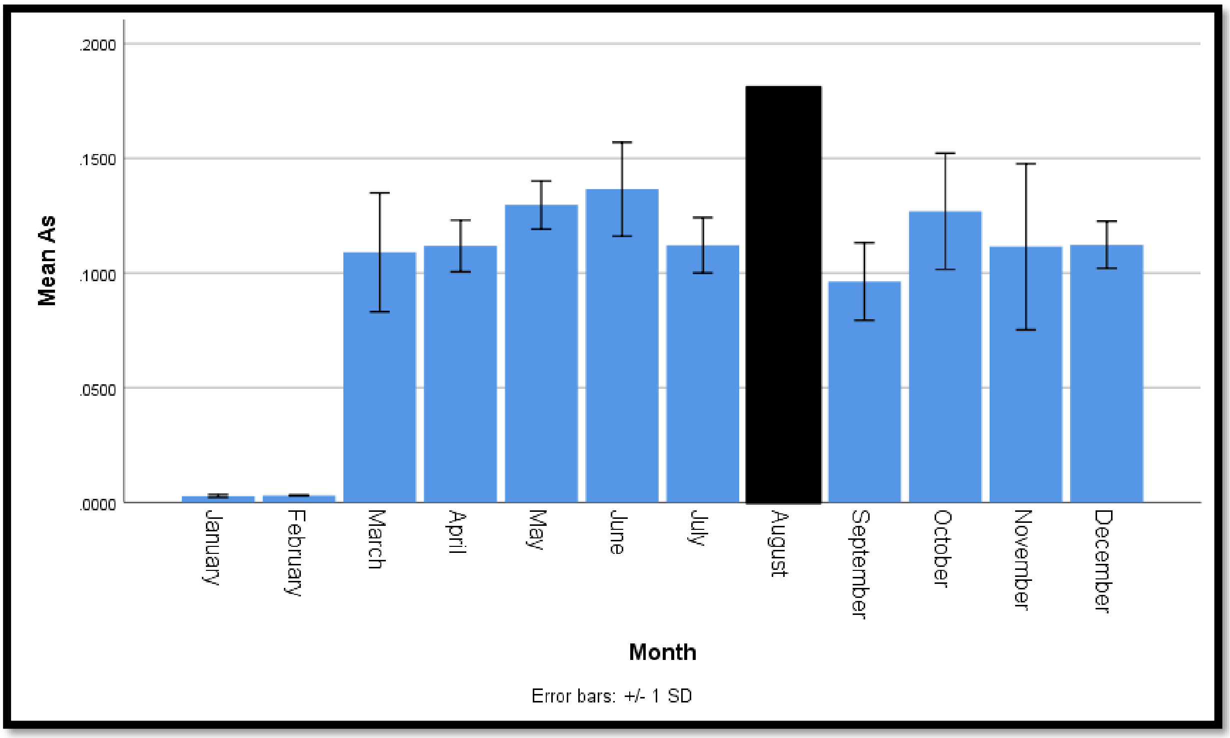

Figure 4.21: Error bar for As (mg/ L) by Month

Moving onto As the results recorded show that the highest mean concentration was found to be during the month of August, whilst the lowest mean concentration was recorded in January. Looking at the figure 4.21, as a whole, during the first two months of the year the concentrations were at a very low level, which were succeeded by much higher concentrations for the rest of the year, with slight wave-like fluctuations. Looking at this from the seasonality point of view, it seems that the higher mean concentrations were reached during the entire year with the exception of early Winter, in January and February.

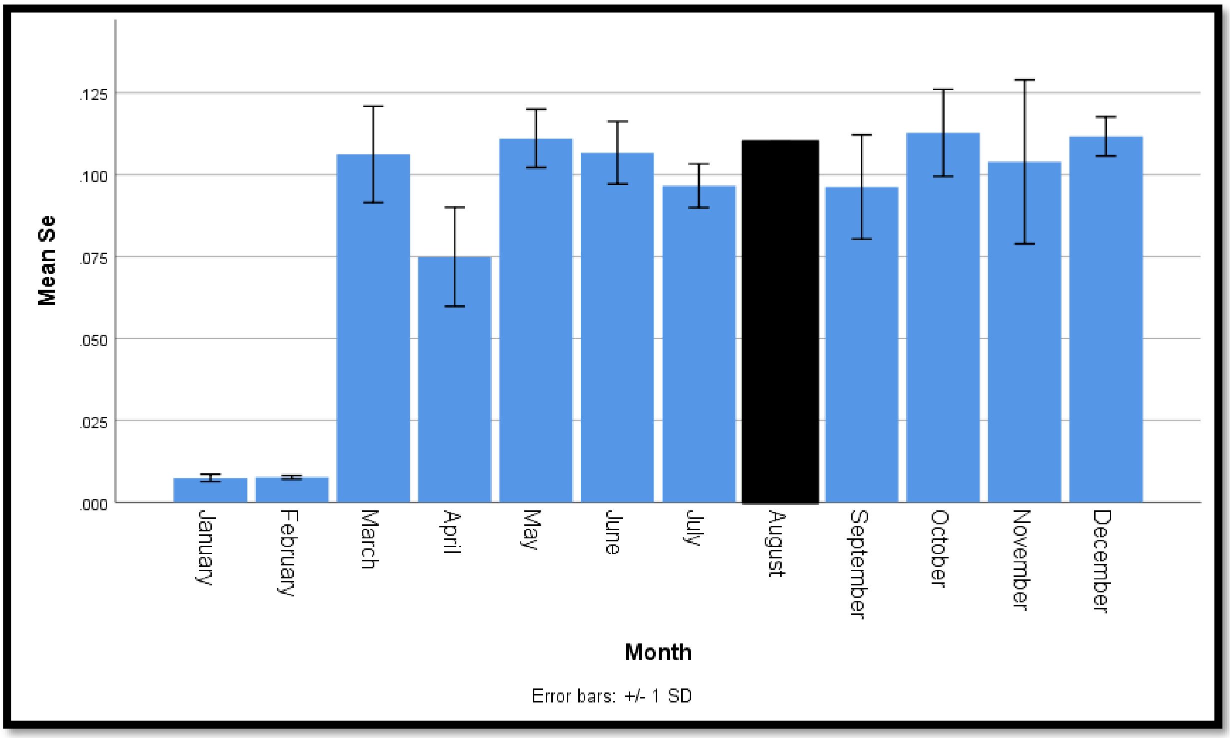

Figure 4.22: Error bar for Se (mg/ L) by Month

Figure 4.22 pertaining to Se, illustrates the highest mean concentration being in October, while the lowest mean concentration was noted to be in January, close to February. With the exception of April's reading which shows a lower concentration compared to March and May, the mean concentration remained high throughout the year with wave-like fluctuations, similar to As's results. Therefore, there seems to be a repetitive trend of concentration levels. Had the study been longer, there could have been the possibility that the concentration levels would start to decrease again following the month of December. Proof of this could be connected to seasonality since according to the figure, the months of January and February presented low mean concentrations. Further to seasonality effect, the higher concentrations were present during Spring, Summer, and Autumn. Therefore, the higher concentrations seem to be present during the entire year, except for the lower temperatures found in January and February.

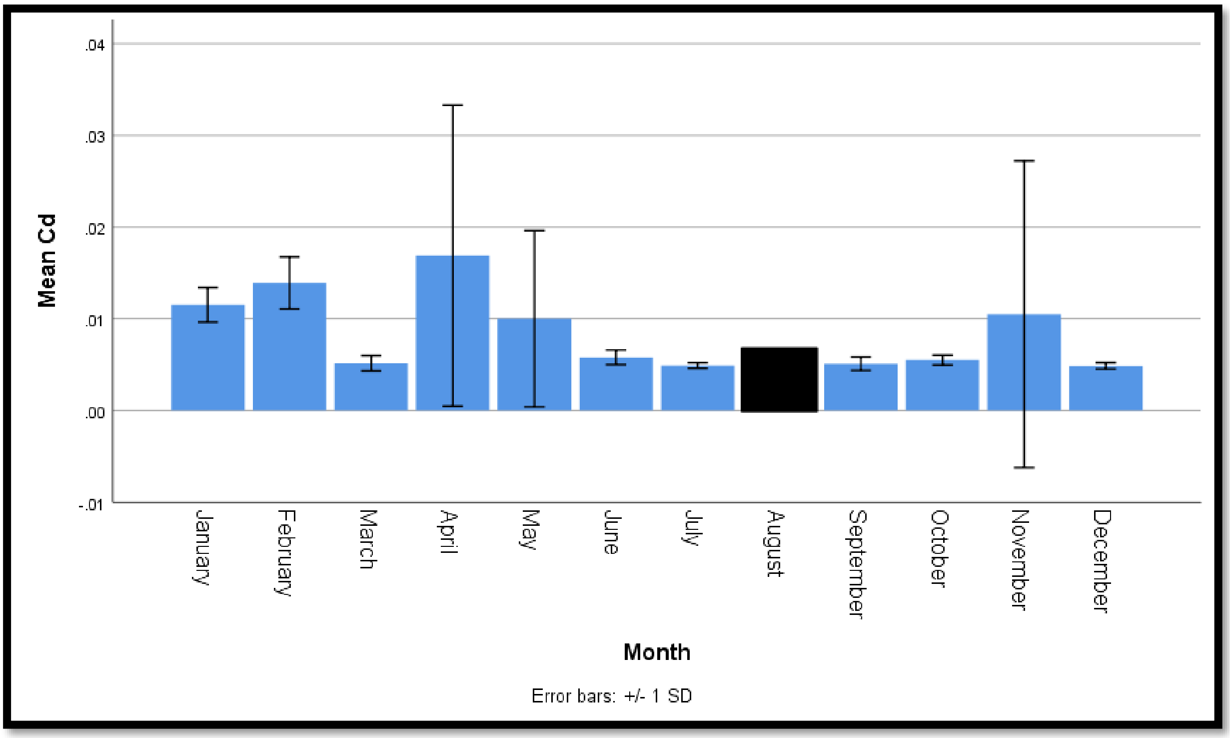

Figure 4.23: Error bar for Cd (mg/ L) by Month

In the above figure, it is clear to note that the highest mean concentration can be observed in April, whereas the lowest during the months of March, July, September and December. The mean concentration was high during the first four months of the year (January - April), with the exception of March, that is the Winter period. It then proceeded to a decline during the months of May – October (Spring, Summer and Autumn), except for August which presented a slightly higher concentration. Following that, the concentration went back to being similar to March, for the rest of the four months, with November having a slightly higher concentration (Autumn and early Winter). Therefore, in the case of Cd, seasonality seems to be the most effective during the months of Winter and Spring season, in this case those months being January, February and April, keeping in mind that April and November had disruption noted from the high standard deviation.

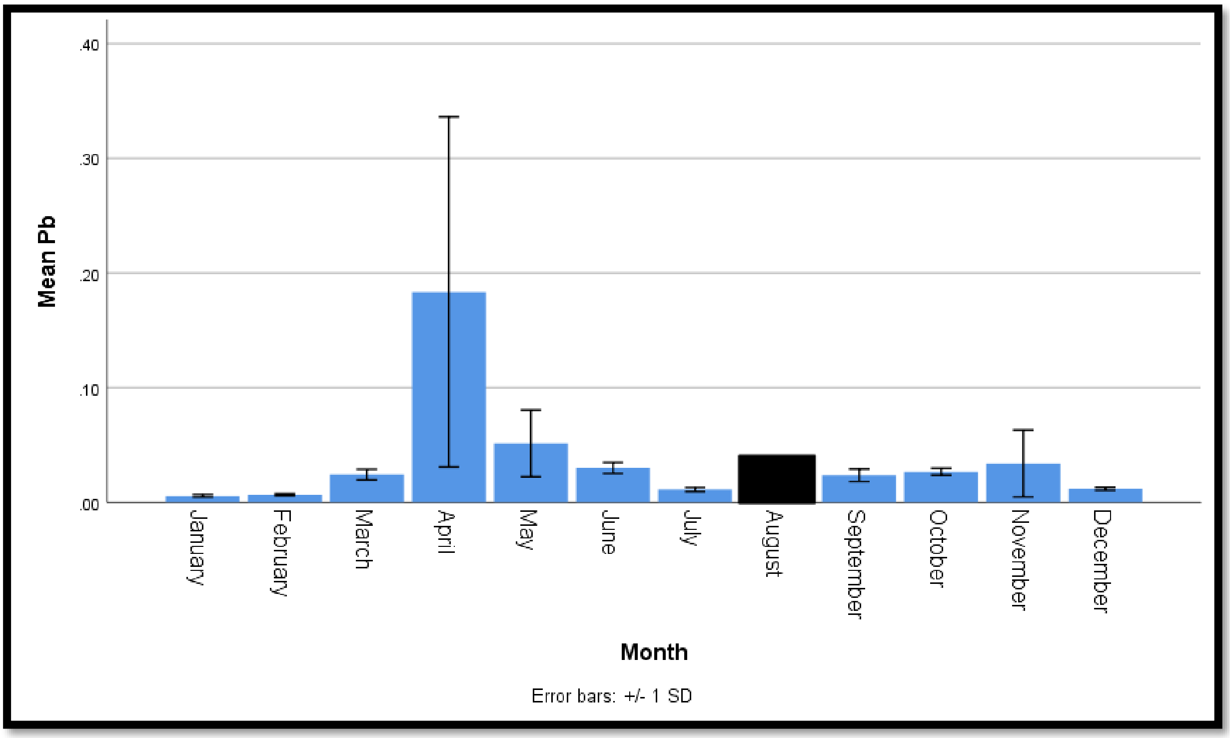

Figure 4.24: Error bar for Pb (mg/ L) by Month

Moving on to the last element, Pb, its highest mean concentration is present during April, while its lowest in January. It seems that the mean concentration remained at a low level throughout the year with the exception of April having the highest concentration, which was followed by May, August and November that had the second highest mean concentrations. It can be seen that the mean concentrations started at a low level in January in which then started to slightly increase until April. It then again declined to a lower level during the following three months (May – July), and then increased once more in August, though this time not as high as April. It then slightly decreased once more for the months of September – November, reaching an even lower concentration for the month of December. Therefore, there seems to be a repetitive trend of increasing and decreasing, with April having the highest mean concentration with high standard deviation. With regards to seasonality, Winter (December – February) had the lowest mean concentrations, therefore the least seasonal effect. Opposite to this, the seasons of Spring, Summer and Autumn had higher seasonal effect since they presented higher mean concentrations. As a last point to note, Pb faced the same problem as Cu when it came to April having a large gap which could be a result of the disruption noted from the high standard deviation, leading to a higher mean concentration.

Looking at the whole picture of all the elements, one can start to note similarities between their seasonal effect. Starting with Cu, Cd and Pb, all three elements had the highest mean concentration during the season of Spring, in the month of April, with mean concentration levels of 0.213, 0.017 and 0.183 mg/L, respectively. Moving onto the next similarity, Cr and Zn, they were noted to have the highest mean concentrations during Winter and Spring/Summer, with Cr having the highest levels in November and May, whilst Zn had its highest concentrations during the months of December and July. Cr had a mean concentration level of 0.030 mg/L in November and 0.027 mg/L. In addition to this, Zn was 7.70 mg/L in December and 6.81 mg/L in July. This means that both elements had high concentrations at one point during opposing seasons (warmer and colder seasons). The final similarity is between the two remaining elements of As and Se which both had relatively high concentrations during the entire year, with the exception of the months of January and February. This means that very low temperatures led for the concentrations to drop to a lower level.

In addition to this, comparisons can also be made with regards to the lowest mean concentration levels. Starting with As, Se and Pb, all of them had their lowest mean concentration levels during Winter, with all of them having their lowest concentrations in January and February. The elements' concentrations were approximately 0.003, 0.007, 0.006 mg/L respectively. Cu reached low levels of concentration during the same season as well, as well as reaching its lowest level during the month of August with a concentration of 0.007 mg/L, meaning that it had the least seasonal effect during Winter and Summer temperatures. The remaining three elements, Cr, Zn and Cd, presented similar low mean concentrations during September, which is the beginning of Autumn. Cr had a mean concentration level of 0.006 mg/L, Zn was 3.06 mg/L and lastly Cd had a mean concentration of 0.005 mg/L.

To examine the quality of the milk present in Malta, the results of some of the elements (Cu, Cd and Pb) are compared to the permissible limit according to WHO, FAO, and European Union Commission (EC). The remaining four elements (Cr, Zn, As and Se) lacked in information about their limit. In addition to this, Yahaya et al., *et al.* (2010), and Abd-El Aal *et al.*, (2012). pointed out that the content of heavy metal within milk changes depending on numerous factors, such as seasonal variations, climatic and health conditions, annual feed composition, the lactation period of cows, as well as environmental contamination (Yahaya, Ezeh, Musa, & Mohammad, 2010).

The first to be compared between this study's results and the permissible limit is Cu in which its concentrations were all higher than the permissible limit set of 0.010 ppm, throughout the year. As it is known, Cu is an essential element whose presence is important in milk, however it can be toxic if there are excessive concentration levels. In the case of this study, from the results obtained, all findings presented a higher concentration level of Cu than the recommended limit, which means that there might be a chance that the milk samples can cause harmful effects. According to Malhat *et al.,* (2012) the contamination of milk is a hazardous aspect that can lead to negative effects if present on a long-term. The excessive levels of Cu may, as a consequence, cause harmful effects such as irritation of the eyes, nose and mouth, vomiting, diarrhoea, stomach cramps, nausea, and even death (Agency for Toxic Substances and Disease Registry, 2015b).

The second element is Cd whose permissible limit is that of 0.05 ppm (Babu *et al.,* 2018) whilst this study's highest concentration was 0.017 ppm, meaning that Cd had much lower concentrations than the permissible limit throughout the year. Having acquired such low levels within this study is a positive outcome since Cd is one of the most toxic heavy metals. Therefore, this can be an indication of low contamination within cattle with regards to Cd. This is beneficial for a human's health since the consumption of milk is of good quality when compared to the small traces of Cd found.

The last to be compared is Pb. Its permissible limit is that of 0.020 ppm whereas the highest concentration obtained for this study was 0.183 ppm, much higher than the permissible limit set in the mentioned entities. The concentrations for Pb were either border-line or higher than the permissible limit mainly during Spring and Autumn, but lower in other months, mainly in Summer and Winter. Again, similar to Cu and Cd, a number of concentrations resulted in being higher than the permissible limit, meaning that not all Pb samples had acceptable concentrations when looking at the difference between the four seasons. The main source of Pb contamination in the environment came from leaded petrol. In connection to this, Malta has been using unleaded petrol for many years which could be a possible reason for the low concentration levels of Pb found in some of the findings. However, having said that, another important source of contamination of Pb is paint, which is still used nowadays in Malta. Therefore, this could be a result for the levels of concentration of Pb in milk samples, amongst other potential sources of anthropogenic origin.

In a recent study by Boudebbouz *et al.,* (2020), a review was conducted in different regions around the world, of research articles published over a period of 10 years (2010-2020) focusing on seven heavy metal levels (Pb, Cd, Ni, Hg, Fe, Cu, Al) and their presence in raw cow's milk. Linking to this current study, the study done by Boudebbouz *et al.* showed that the concentrations of Pb, Cd and Cu were different depending on their location. The study was done in 54 regions using different analysis techniques. Their results showed that the mean level of Cu in cow milk samples across ranged from 0.014 mg/L to 36 mg/L around the globe, (above the maximum limit, 0.010 mg/L). With regards to Cd, in 36 of the studied regions, the concentrations were above the standard limit of 0.0026 µg/g, in 18 of those regions were below standard limit (0.0026 µg/g), and in five regions, the Cd levels are below detectable limit of instrument. Finally, Pb was reported that its highest concentration level in all 10 years was that of 60 mg/L which was collected from matured grazing cows, which could have been strongly influenced by the seasonal variations, mainly in Winter, due to change in botanical composition of the herbage. In the same way, high Pb level was noted (23.2 ± 0.30 mg/L) during Summer in cow's milk collected from animals. This result is much higher than the permissible limit of 0.02 mg/L set by EC (2006). Egypt (4.40 ± 1.60 mg/L) and Slovak (3.80 ± 0.42 mg/L) too recorded high Pb content within cow milk when collected from an industrial air pollution area and from a local Nitra region, respectively. The Pb levels in cow's milk collected from several studies, highlighted the fact that rural regions such as those in Libya and Nigeria recorded samples with Below the Detectable Limit of contamination.

In comparison to this study being conducted in Malta, the concentration of Cu ranged from 0.007 to 0.200 mg/L, which is extremely lower than the range given by the connected study. With regards to Cd, all the results collected in Malta were below the detectable limit, the same as the five regions in the discussed study. Finally, Pb in Malta ranged between 0.006 mg/L and 0.183 mg/L which were either lower or higher than the permissible limit. In the study made by Boudebbouz *et al.,* (2020), both Cd and Pb were higher in concentration in developing and lower in developed countries which is in agreement with the results achieved in this study for Cd, since Malta is classified as a developed country. With regards to Pb, the results achieved by Boudebbouz *et al*., (2020), showed that the higher concentrations were present during Summer and Winter, whereas in Malta, these were found to be during Spring and Autumn.

According to the knowledge found in a research done by Muhib *et al.,* the concentration of Cr in dairy cow milk ranged from below the Detectable Limit and 1.233 ppm, with a mean concentration of 0.373 ppm (Muhib *et al.,* 2016). Linking to the study done in Malta, Cr ranged from 0.006 and 0.030 ppm with a mean concentration of 0.014 ppm, which is much lower than the linked study. Much lower results in comparison to Malta were found in a research done by Soldium *et al*., (2012) in the Philippines in which the range was 0.0008 to 0.001 ppm. It has been demonstrated by Spiteri and Attard (2017) that the Cr content was not detectable in cow milk, in Malta. Furthermore, in the study made by Spiteri and Attard (2017), Zn was found to have a ranging concentration of 2.08 to 4.87 ppm, whereas the present study had a range of 3.06 to 7.70 ppm, which is higher than the compared study.

Moving onto As, in a study done by (Zhou *et al.,* 2019), the mean value was 0.310μg/L much lower than the one observed in this study in Malta in which the mean value was 0.100 mg/L, as concentration ranged from 0.003 to 0.180 mg/L. Unfortunately, not many large-scale studies are found in relation to As and its contamination in raw milk. Last but not least, it is clear to note that the concentration on Se in Malta and its study were one of the lowest in comparison to most of the elements pertaining to this research, with a range of 0.007 – 0.111 mg/L. The lack of information written about Se in cow milk is due to missing resources, however, a reason for the low concentration noted can be possibly due to a psychological effect on the state of the cow, since the milk in early lactation tends to contain reduced Se in comparison to the milk later in lactation (Wichtel *et al.,* 2004).

One of the main functions of a cow is the production of milk, meaning that heavy metal content is of great importance with regards to the quality of life of the cow and the health of its consumers (humans). In order to better analyse this, a comparison between the heavy metal content in milk (as an output) with fodder and water (as intake) is made. In view of water and milk, Cu and As both had the highest concentrations during the same months for both intake and output. Starting with Cu, its concentration was the highest during the month of April both in water and milk. Similarly, As was noted to have high concentrations in both water and milk during Summer with water being highest in July and milk in the month of August. Similar to these two elements, Cr, Zn and Cd each had concentrations during the same months for both milk and water, however the milk concentrations were also high in other months. Starting with

Cr, the water concentration was highest in April, similar to milk, which was highest in May, however, was also high during the month of November. Secondly, Zn had high concentrations in both water and milk during the month of December, however, milk was also high during July. With regards to Cd, it had high concentrations in April for both water and milk, with milk being high during the winter season as well, from November till February. In addition to this, Pb was highest in April with regards to milk, and highest in the months of June and August for water. Despite the slight difference, both milk and water had high concentrations in months with similar temperatures. Finally, Se in milk was high throughout the year, except for the months of January and February, however in water, it had high concentrations during the months from June to August.

Moving on to the relations between fodder and milk, the average concentrations in fodder and milk are compared within the context of intake and output. Fodder resulted to have a higher average concentration than in milk in all the heavy metals during the entire year, keeping in mind that the average taken for fodder included all the types of feed used for this study.

4.5.1 Conclusion

The quantity and quality of the milk is dependent on the good health status of cows. The basic essential heavy metal requirements of the animals must be sufficiently met for better health. In spite of the fact that many minerals are described as essential for the animals when fed in surplus, the same mineral can be toxic and even deadly. Therefore, supplementation of amounts of minerals, particularly trace elements, must be performed with diligence. It is important to note as well that the increase in environmental pollution has led to an acceleration in problems of milk contamination, leading to uncertainties with regards to milk qualities (Farid and Baloch 2012).

All milk samples collected in this study were analysed by the most validated methods which resulted in Cd having lower concentrations than the permissible limit, when found. Cu and Pb were determined to have a higher concentration than the allowed limit. In the case of the metals analysed for (Cr, Zn, As and Se), these were found to be of similar quantities as in other studies. All the elements' concentration levels were then observed in order to comment on the effects of seasonality. This application was helpful for a better understanding of the changes in the cow's milk composition through the different seasons.

Besides this, the heavy metals concentrations in milk are not only affected by season, but also by water and fodder. Accompanied by other sources of intake, such as medical treatments and supplements, these methods of intake can shift the level of concentrations in milk. The conditions present for the cow's body processing of milk, such as digestion and metabolism, can also have an effective influence on the contents and may retain minerals in total composition of milk.

Although milk and dairy products contain many active biomolecules for human health, different factors, including seasonality, types of intake, and the environment can shift the concentration levels of the final production of milk. Therefore, through this study's findings, it is clear that all factors and processes are intertwined with each other since each one can have either a positive or negative effect on the other.

4.6 Hair Analysis

Following the analysis of fodder, water, manure and milk, this last section will focus on the analysis of hair. Unlike previous specimens that were collected over a period of twelve months, hair samples were collected once a month from ten different cows for a period of eight months during the year of 2019, starting in January until the month of August, as depicted in section 2.2.

The results achieved from these samples were then worked out statistically as in table 4.9, which presents the mean ± sd of all the elements for each month. The statistical significance with which each month is different from the other months when using Bonferroni post-hoc tests is denoted for each element with the appropriate symbols in the superscript.

Table 4.9: Contents for Cr, Cu, Zn As, Se, Cd and Pb (µg/g) along the months January 2019 – August 2019.

	Cr	Cu	Zn	As	Se	Cd	Pb
January	0.350±0.060	10.9±1.33***	146 ±40.5**	0.04±0.000***	0.130±0.000***	0.19±0.010***	0.12±0.020***
February	0.360±0.050	9.81±4.07**	149±21.7***	0.04±0.010***	0.130±0.010***	0.18±0.010***	0.13±0.040***
March	0.390±0.140	6.06±1.24**	84.3±16.9**	1.05±0.100*	1.41±0.080***	0.07±0.020**	0.43±0.110*
April	0.340±0.050	5.92±0.99**	81.3±11.5	1.07±0.160†	1.41±0.040***	0.06±0.000**	0.38±0.080†
May	0.400±0.100**	7.56±2.32	98.2±16.2**	1.09±0.110*	1.39±0.100***	0.07±0.010*	0.59±0.130***
June	0.720±0.350***	10.8±4.36**	125±28.9†	1.22±0.180***	1.43±0.120***	0.06±0.010†	0.82±0.280***
July	0.440±0.240	7.13±6.70	80.6±16.4***	1.12±0.140**	1.21±0.090†	0.06±0.010***	0.42±0.150*
August	0.210±0.080***	5.16±4.78***	81.3±14.2***	0.90±0.540***	1.19±0.080*	0.09±0.010***	0.38±0.080†

*Statistically different from other monthly levels at p<0.001***, p<0.01**, p<0.05*, p<0.1*†

For better visualization and explanation of the above results, the following figures have been prepared:

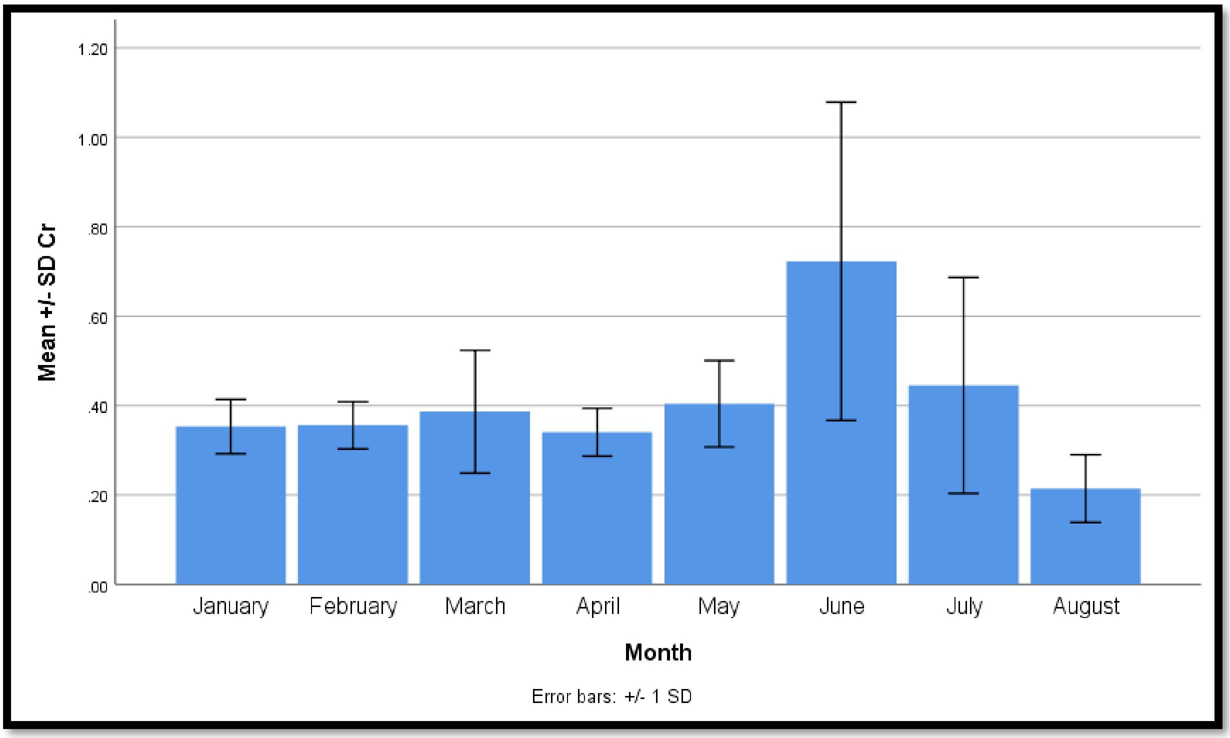

Figure 4.25: Error bar for Cr (µg/g) by Month

In figure 4.25, it can be noted that Cr had its highest mean concentration during the month of June, whereas its lowest mean concentration was during August. With regards to the month of June, it also had the highest standard deviation in comparison to the rest of the months. Looking at the figure, the mean concentrations kept on increasing from January to reach its highest concentration in June, with a slight dip in April. The concentrations were then at a decline, reaching the lowest level during August. A clearer picture might have been obtained had the research lasted longer, such as a whole year as opposed to eight months. With regards to seasonality effect, the mean concentration reached the highest during the beginning of Summer, whereas its lowest was during August, which is the middle of Summer, and it seems that the concentration would have kept on declining as time went by.

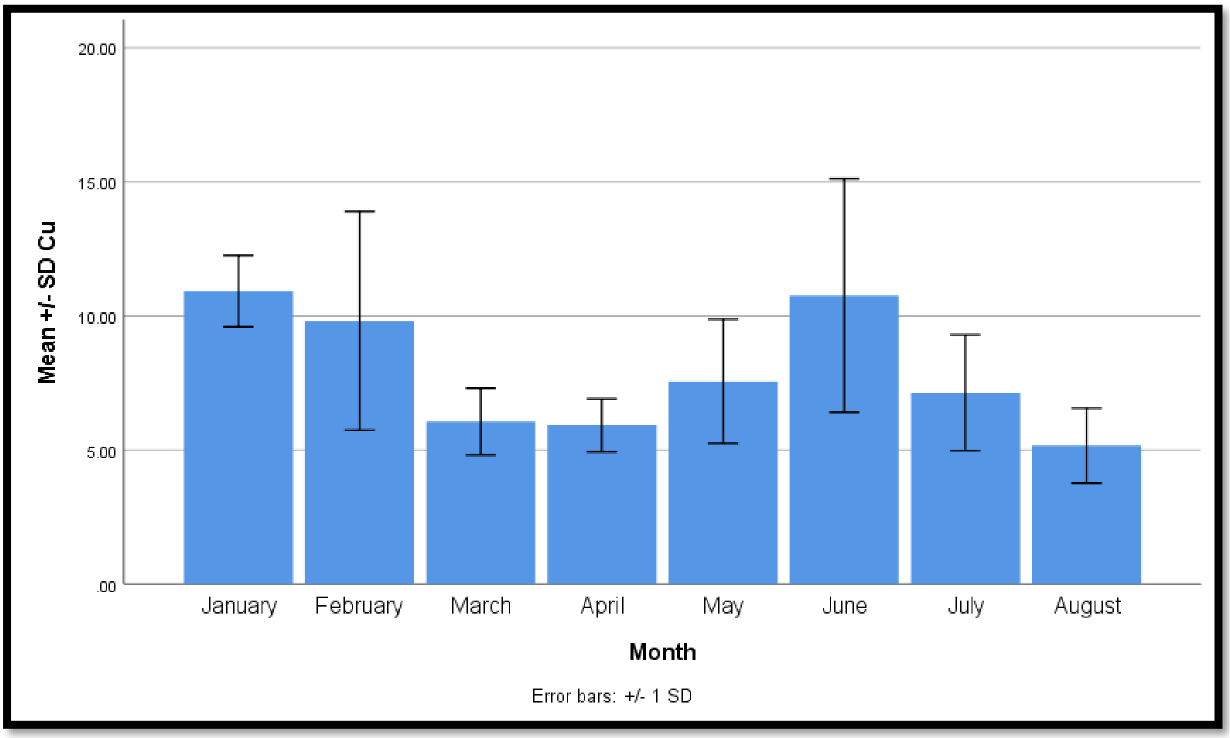

Figure 4.26: Error bar for Cu (µg/g) by Month

While observing figure 4.26, the highest mean concentration of Cu is during the month of January, similar to June's concentration. The lowest mean concentration was during the month of August, close to March and April. The concentrations had up and down fluctuations throughout the eight months of the sample collection. From the very first glance, it can be seen that there is a clear trend in the mean concentration during the eight months, since it can be noted that seasonality is most effective during the beginning of Winter and beginning of Summer. On the other hand, during Spring and end of Summer, the concentrations are lower, giving the impression that, same as Cr, had the sampling lasted longer, the concentrations might have decreased even further and started to increase once more as Winter approaches.

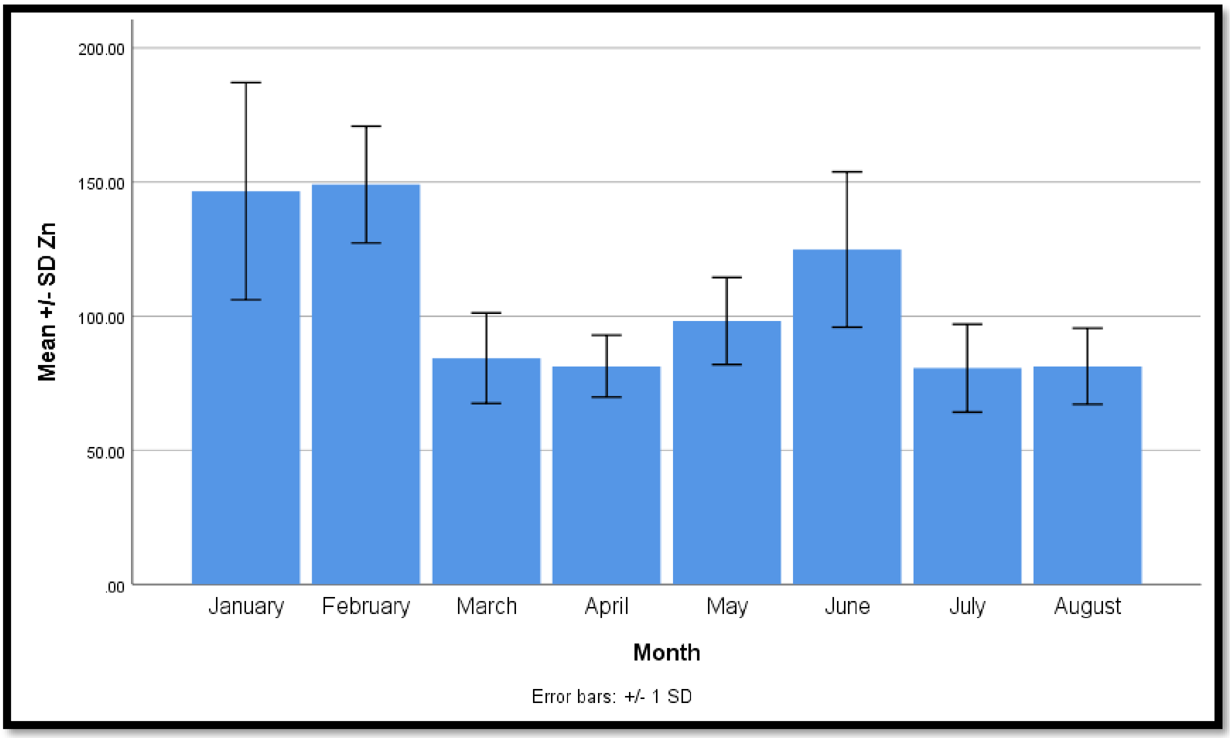

Figure 4.27: Error bar for Zn (µg/g) by Month

It is clear from figure 4.27 for Zn, the highest mean concentrations were reached during the beginning of the year (January and February), with February being the highest out of the two. The lowest concentrations were then reached during April and July – August, with the lowest pertaining to July. Once again, there is an upward and downward trend within the studied eight months. With regards to seasonality, it can be observed that Winter had the most effect on Zn, followed by the beginning of Summer in June.

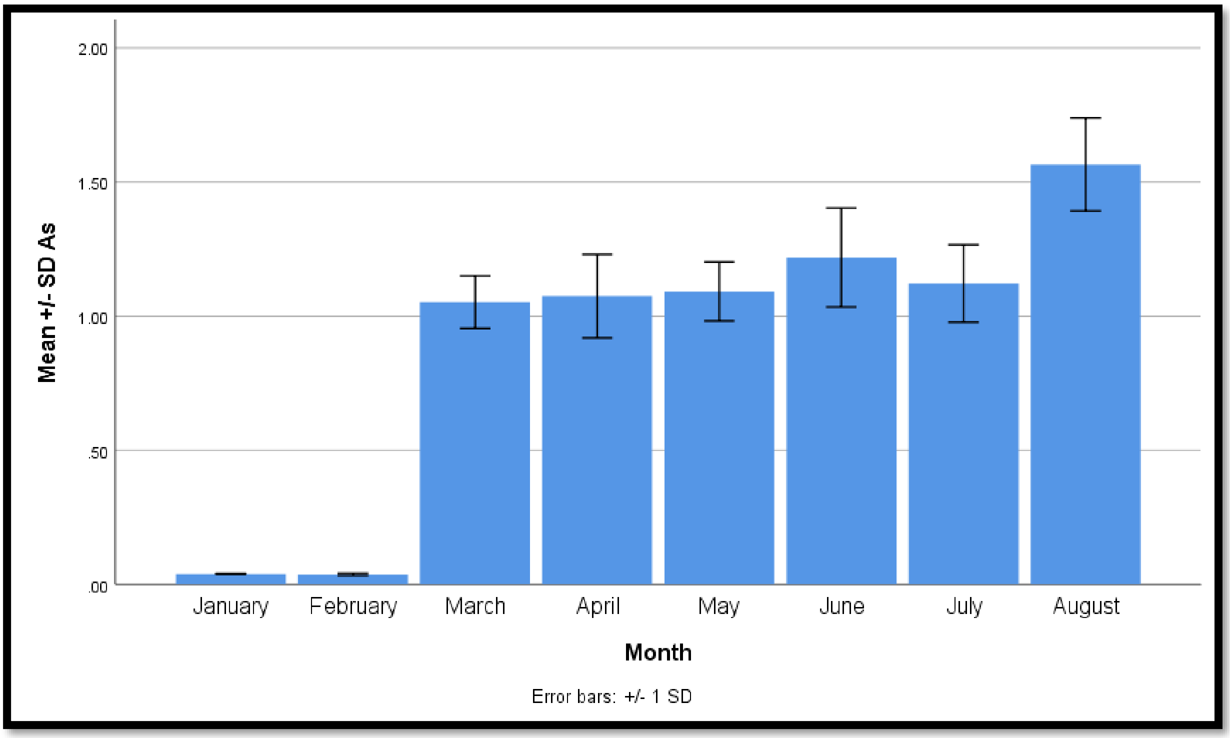

Figure 4.28: Error bar for As (µg/g) by Month

Moving to As, the results (figure 4.28) recorded demonstrate the huge difference between January and February and the rest of the months. The highest mean concentration was found during August, while the lowest concentration was during both January and February. Unlike the previous elements, As has had a constant upward movement throughout the eight months, with a slight drop in July. Even with a quick glance, it is clear that January and February had both low mean concentrations, as well as standard deviations with the latter meaning that the 10 random samples collected for each month were identical. From the point of view of seasonality, as temperatures increase, so does the mean concentration as proved by the above figure in which each month had a higher concentration than the one before it, reaching its highest during the peak of Summer.

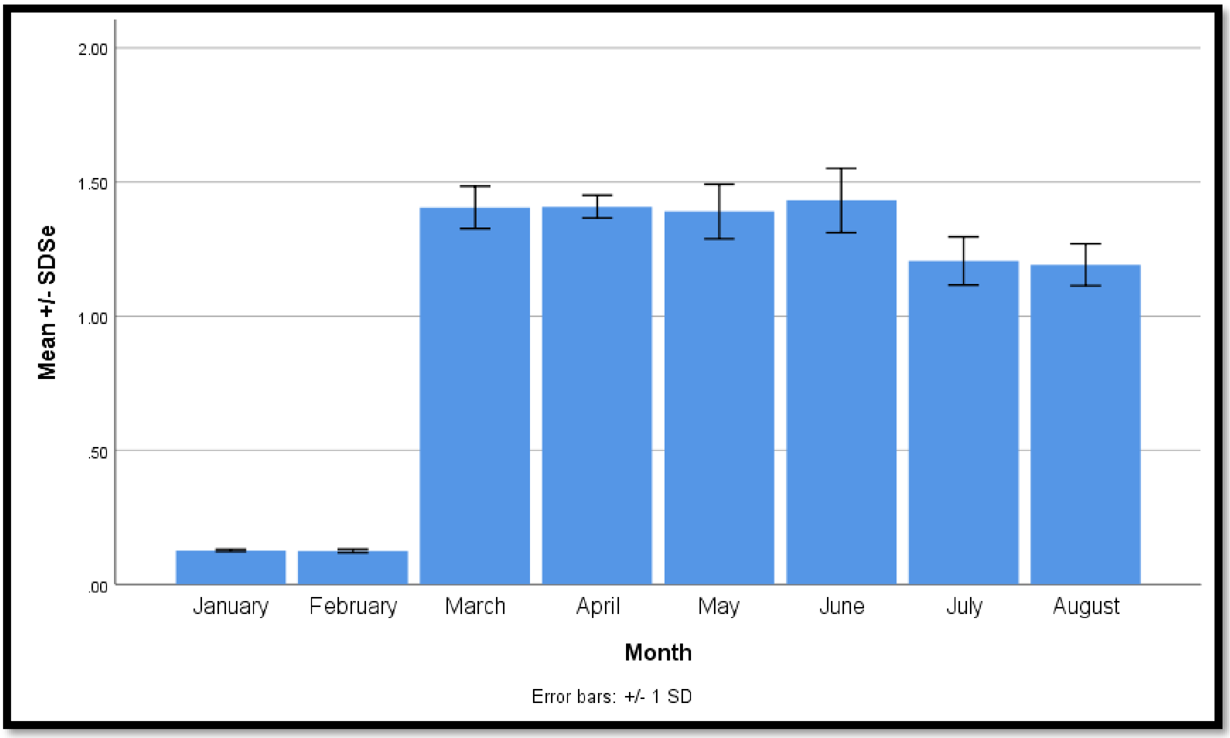

Figure 4.29: Error bar for Se (µg/g) by Month

In figure 4.29, the results noted down, once again, similar to As. There is a significant difference between January - February and the rest of the six months. As pointed out, January and February had the lowest concentrations and standard deviations, with the same reasoning as As, whereas the highest mean concentration was in June. When it comes to seasonality, temperature had a strong effect on the mean concentrations during all of the eight months. What is meant by this is that January and February which have alike temperatures had alike concentrations. The same happened in Spring and early Summer in which March – June had stable and similar concentrations, as well as in July and August which once again, had similar concentrations to one another due to similar temperatures.

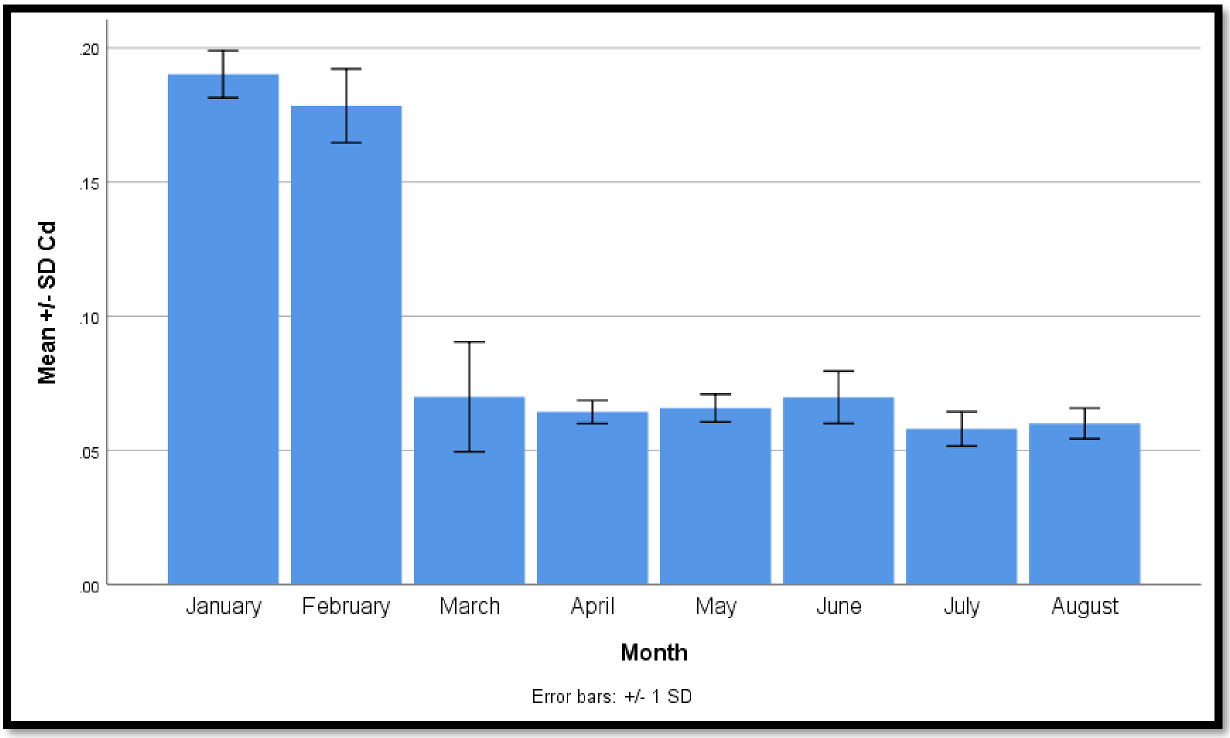

Figure 4.30: Error bar for Cd (µg/g) by Month

In figure 4.30, pertaining to Cd, it is clear to note that opposite to As and Se, Cd has higher mean concentrations in January and February, with January being the highest. The rest of the six months all had similar concentrations, with April, June and July having the lowest mean concentration out of all. Keeping in mind seasonal effects, Winter had the most influence on Cd, since it was the highest during January and February. On the other hand, both Spring and Summer (March – August) had lower yet alike concentrations. Contrary to As and Se, as temperatures get colder, the mean concentration levels increase, but then decrease when warmer temperatures approach.

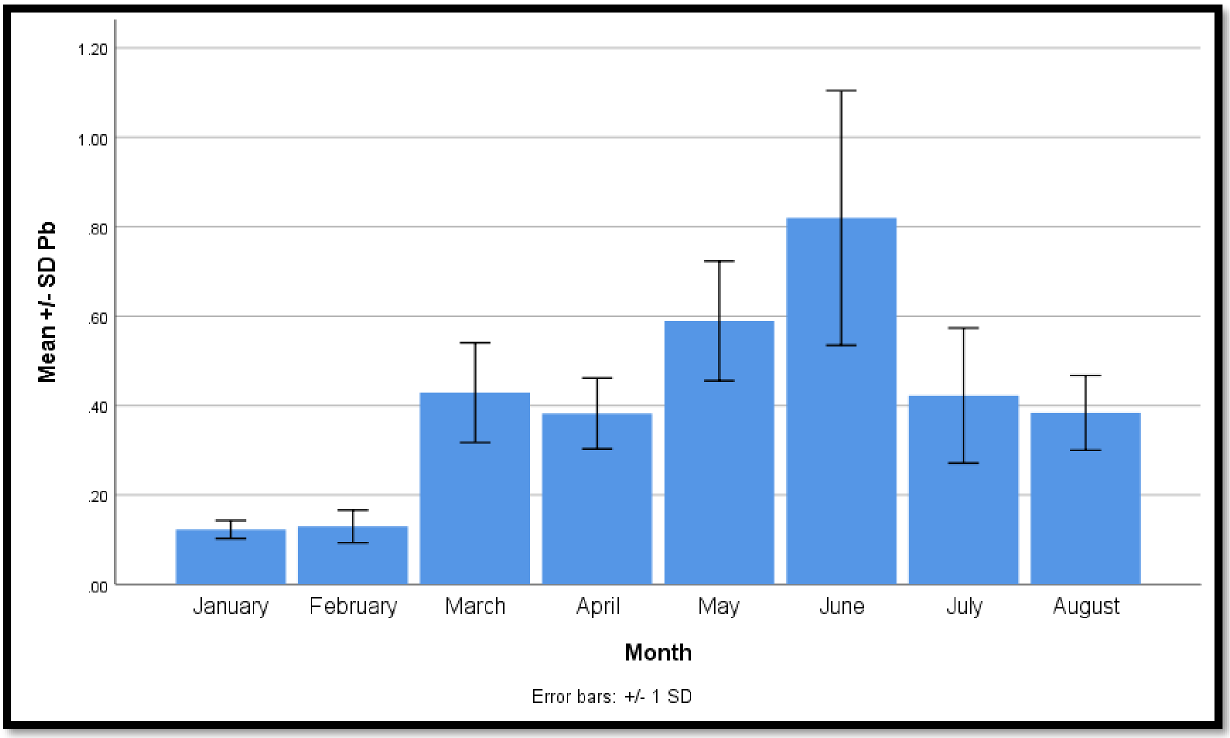

Figure 4.31: Error bar for Pb (µg/g) by Month

Finally, the last element, Pb, its highest mean concentration was present during June, while its lowest in January, closely followed by February (figure 4.31). Therefore, the year started off at low concentrations but then started to increase in March, reaching its highest in June which then proceeded to decrease once more to lower mean concentration levels in July and August. From what can be assumed, with the passing of time, the mean concentration levels would have likely continued decreasing and then increased again. Proof of this is the wave-like trend in the concentration levels. The beginning of Summer, specifically June, was the most affected period seasonally, whereas Winter (January – February) had the lowest mean concentration levels, therefore the least seasonal effect.

Table 4.10: Kruskal-Wallis test outcomes for each element

	Kruskal-Wallis H	df	Sig.
As	59.4	7	0.000
Cd	53.2	7	0.000
Cr	29.1	7	0.000
Cu	38.5	7	0.000
Pb	58.9	7	0.000
Se	65.0	7	0.000
Zn	44.2	7	0.000

The Kruskal-Wallis test was applied for all elements, with Bonferroni post-hoc tests. In Table 4.10, it can be seen that the p-value is 0.000 for all elements for the Kruskal Wallis test. This indicates that collecting monthly samples was the correct method since there resulted significant month-to-month variations for each element.

Looking at the overall perspective, starting with the higher concentrations, the majority of the elements (Cr, As, Se, and Pb) had the highest level during the beginning of Summer, in the month of June. In molecular order, Cr had a mean concentration of 0.720 µg/g, As was 1.22 µg/g, Se 1.43 µg/g and last but not lease, Pb had a mean concentration of 0.820 µg/g. As for the rest of the elements (Cu, Zn, and Cd), they ranked higher mean concentrations during the season of Winter opposite to the majority of the chosen elements for this study, with Cu and Cd ranking highest in January while Zn in February. Cu reached its highest concentration of 10.9 µg/g, Zn 149.0µg/g and Cd, 0.190 µg/g. In addition to this, once again, the majority of the elements reached their lowest concentration levels during the season of Summer as mentioned prior in the descriptions of the figures, those elements being Cr, Cu, Zn, Cd, and Pb. In molecular form, the elements had mean concentration levels of 0.210, 5.16, 80.6, 0.060 and 0.120 µg/g, respectively. The remaining two elements, As and Se, reached their lowest levels during Winter rather than Summer, in January and February with concentration levels reaching 0.040 and 0.130 µg/g, respectively.

In a recent study done in Russia by Miroshnikov *et al.*, (2017), trace element content was observed in cows and heifers. The study focused on many elements, including the heavy metals investigated for this study done in Malta, Cr, Cu, Zn, As, Se, Cd and Pb. For easier reference, both the Russian and the Maltese study's results have been noted down in the table 4.11:

T*able 4.11 Hair trace element content in cows in Malta and Russia μg/g*

Elements	Cows in Malta	Cows in Russia	Heifers in Russia
Cr	0.720	0.312	0.305
Cu	10.9	4.76	4.85
Zn	149	97.0	102
As	1.22	0.130	0.137
Se	1.43	0.330	0.572
Cd	0.190	0.043	0.034
Pb	0.820	0.338	0.288

In general, the study done in Malta resulted in higher heavy metals concentrations in cows than in Russia's cows and heifers as shown in table 4.11. The heavy metals concentrations in Malta's cows in Cr, Cu and Pb are relatively double the ones in Russia, whereas Zn, As, Se and Cd are higher than Russia's by a significant amount (Miroshnikov *et al.*, 2017).

Gabryszuk *et al.* (2010) studied the content of mineral elements in cow hair and milk in Poland, including the heavy metals chosen for the study in Malta. According to this author, the investigation of elements in hair could be useful for long-term monitoring of animals' mineral status. Similar to the results in the previous study, this study done by Gabryszuk *et al.* resulted in heavy metals concentrations being much lower than the ones in Malta, except for Cr which had similar concentrations in both Poland (0.758 μg/g) and Malta (0.720 μg/g) (Gabryszuk, Sloniewski, Metera, & Sakowski, 2010)

Therefore, looking at the results obtained from the study done in Malta as opposed to those done in Russia and Poland, it is clear that Malta has higher concentrations of heavy metals which can be seen as a caution of high levels of pollution.

In another study done by Tadayon, *et al.* (2014), the heavy metals concentrations for Pb, Cd and Al were compared between polluted and non-polluted areas. In comparison to the study done in Malta, Pb and Cd concentrations were both much lower in Malta than in Tehran (polluted) and Semnam (non-polluted). Pb in Tehran had a concentration of 13090 µg/g and 6980 µg/g in Semnam, whilst on the other hand had a concentration of 0.820µg/g in Malta. Similarly, Cd had a concentration of 3450 µg/g in Tehran, 2780 µg/g in Semnam and 0.190 µg/g in Malta. With regards to the study done in Iran, the researchers noted that the concentrations of Cd and Pb differed between the two studied areas, with strong positive correlation present (Tadayon, Jamshidi, Tadayon, & Ostovar, 2013). Therefore, this presents the possibility that had this comparison between polluted (urban) and non-polluted (rural) areas in Malta been done, the heavy metals concentrations might have presented with different concentrations as well.

In the same manner that the heavy metal content in milk as an output, in section 4.5, was compared to fodder and water as intakes, the hair content too can be linked. Regarding the comparison between water and hair, Cr and As both had their highest concentrations for water from April to June resulting in the hair concentration being highest in June for Cr whereas the latter being in August, both during the season of Summer. Opposite to this, however with the same predicament, Zn had its highest concentration for water during December, which resulted in Hair having a high concentration during January and February. Therefore, a possible reason for this result is that since the intake (water) had a high concentration in one month, the month that followed had a high concentration in the output (hair).

Moving onto the next link between the elements' results, Se and Pb both had high concentrations in hair during the month of June. Starting with Se, its highest concentration in water lasted for six months, between the months of April to September, with June being the highest for hair. In addition, Pb had high concentrations during June to August for water and same as Se, high concentrations in June for hair. Therefore, in this case it may be possible that hair was not affected by the intake concentrations for Se and Pb in water and what links the two elements together is that they both had their highest concentration for hair in June.

In view of the last two remaining elements, Cu and Cd, their concentration in water was both at its highest during the warmer seasons of the year, specifically around the end of Spring, beginning of Summer, April for Cu and April - June for Cd. In contrast to this, their highest concentration for hair was found to be at the beginning of Winter, in January. Therefore, a final possibility for these results is that water does not leave much of an effect on the concentration of hair, same as in the case of Se and Pb.

Moving onto the comparison between fodder (intake) and hair (output), the results showed that fodder had higher average heavy metal concentrations than hair, in Cr and Cu. Cr was found to have a concentration of 0.550 µg/g in fodder and 0.400 µg/g in hair while Cu had concentrations of 8.20 and 7.92 µg/g, respectively. A possible reason for the decrease between the intake and output concentration could be that when consumed, both elements were digested and metabolised in the body and distributed to various tissues and milk, therefore it is used for the body's own benefit.

On the other hand, the remaining elements investigated for this study had lower average concentrations in fodder than in hair. Starting with the most significant difference, Zn, As and Pb, had a concentration of 105.8 µg/g in hair and 32.8 µg/g in fodder. Secondly, As's concentrations were 0.816 µg/g in hair and 0.580 µg/g in fodder, whereas Pb, its concentrations were 0.410 and 0.190 µg/g, respectively. In addition to this, Se and Cd registered approximately double the concentration in hair than in fodder. While Se had concentrations of 1.04 and 0.510 µg/g, Cd had concentrations of 0.098 and 0.040 µ/g, respectively. The fact that Se, Cd and Pb are toxic elements for cows, it is acceptable and beneficial that hair had higher levels than fodder in this study.

In general, it is favourable for hair (output) to result in higher levels of any heavy metal than in fodder (intake), due to the risks both toxic and essential heavy metals carry with them since in excess they become toxic which can bring about repercussions. Respectively, hair is a monitor for valuable proof that the intake of heavy metals may vary the concentrations of the outputs depending on the metabolism of the cattle, as well as seasonal effects.

4.6.1 Conclusion

The analysis of heavy metals in hair tissue is widely practiced as it is beneficial to monitor the animal's health status, since it can be proof of environmental contamination caused by pollution that seeps into water and food. Small-scale traces of these heavy metals are common and are necessary for good health, however if any of them are present excessively, they can cause harm since they become toxic.

Focusing on this study’s results, in general, all the heavy metals investigated had detectable concentrations. Since hair extracts the extra heavy metals within the body, finding high levels of concentration can be seen as an advantage in order to reduce the chances of contamination and toxicity within the body. Another reason high levels of heavy metals in hair can be seen in a positive light is that hair is redundant in connection to human’s health, as opposed to manure which is used as a fertilizer for growing crops that later form part of the human food chain.

Narrowing down this section of hair, seasonality had a strong effect on all elements with some of them having their highest concentrations in Summer, whereas others in Winter, likewise with regards to the lowest concentrations recorded. As seen from the aforementioned figures, as temperatures increased or decreased, so did the mean concentration. The results obtained reached positive outcomes since the effect of seasonality was prominent, however, had the sampling collection period been longer than eight months, even more accurate results might have been reached.

Further to this, the effect of intake (in both water and fodder) varied depending on the element since the concentrations of intake did not always have a significant effect on that of the output (hair). Metabolism, digestion and absorption, amongst other factors such as other intakes, including medication and treatment, all correlate to the levels of heavy metals in hair. In addition to this, another important possibility for the high levels of concentration in hair, are external factors, such as direct contact with outdoor metal, gates and other barriers.

The distribution of heavy metal concentrations within the cow’s body and out is not uniform since the tissue selects elements specifically, depending on the body’s necessities and needs. Studying this distribution and establishing reference values within hair for a better understanding of the elements’ concentrations, depending on both internal and external factors, can aid in making a contribution to better the animal’s health as part of its life cycle, which would in turn benefit a human’s health as well.

4.7 Conclusion

In conclusion, to give an overall perspective on all the heavy metals concentrations' contributions within feed, water, manure, milk and hair, with feed and water acting as input and manure, milk and hair acting as output parameters with regards to the cow's system (Udo, 1978) (McDowell, Lee, McMullan, Fohrman, & Swett, 1954), the estimated daily intake and output were calculated as precented in the appendix 2.2. This was done by taking each month's daily intake and output and calculating the year's overall average for both intake and output to generate the following figures for each heavy metal. It is important to note that in the case of hair, the imputed method was used for the last 4 months, as shown in appendix2.1-ICP table A.23 to calculate the hair output. Starting with Cr, the following figures have been done and explained.

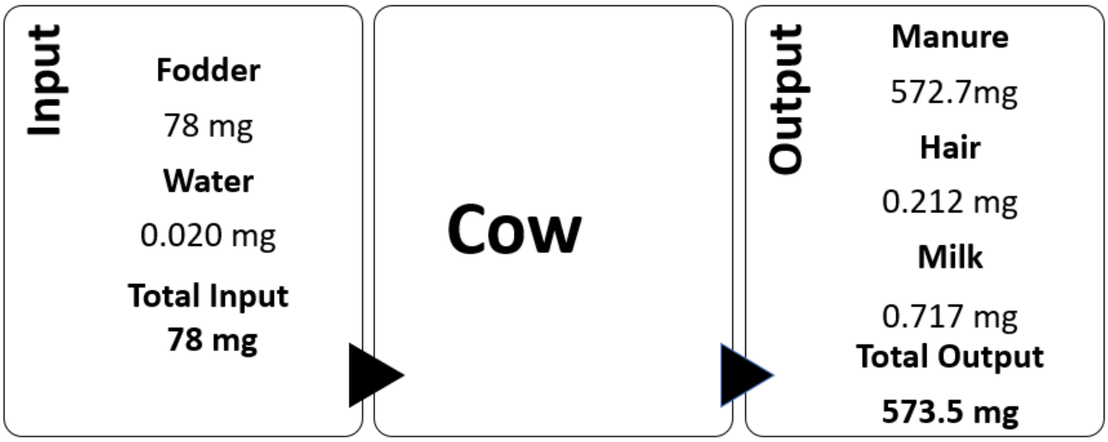

Figure 4.32 The input and output parameters considered in this study, and the chromium contribution of each parameter on a daily basis.

It can be noted, from figure 4.32, that the Cr input coming from feed and water does not tally to the Cr output through manure, milk and hair.

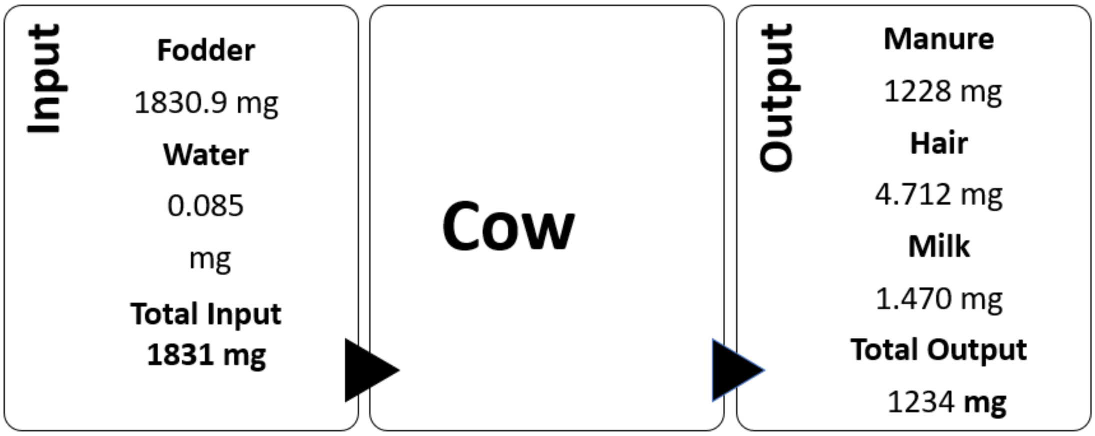

Figure 4.33 The input and output parameters considered in this study, and the copper contribution of each parameter on a daily basis.

From figure 4.33 for Cu, it can be observed that the Input concentration was higher than the Output, which is opposite to the result obtained in the AAS analysis (in Section 3.3.3).

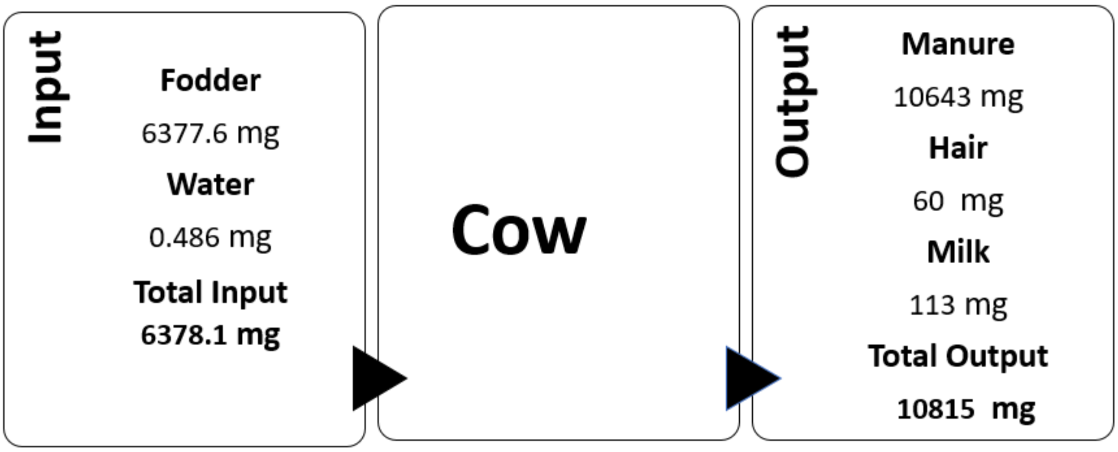

Figure 4.34The input and output parameters considered in this study, and the zinc contribution of each parameter on a daily basis.

With regards to Zn, the output, as illustrated in figure 4.34, resulted higher than the total input concentration.

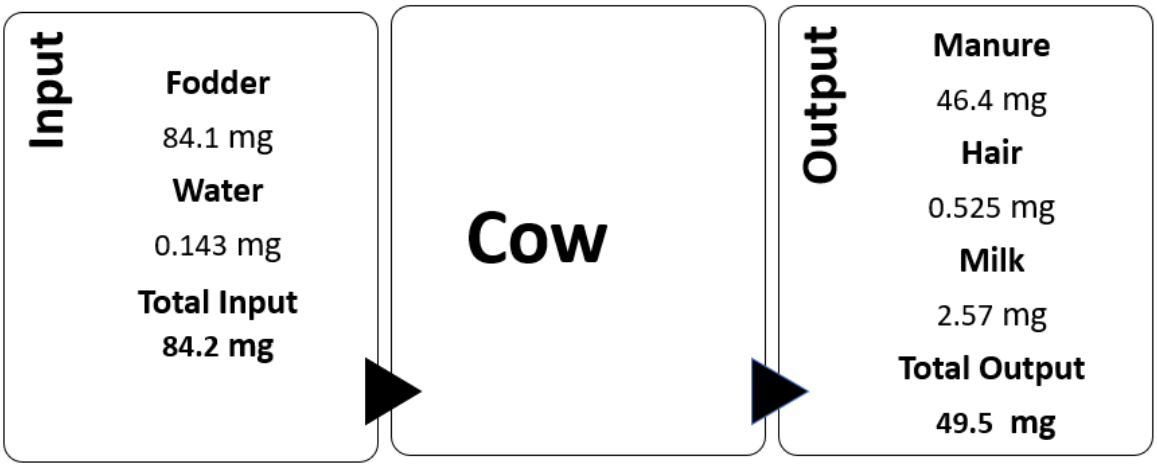

Figure 4.35 The input and output parameters considered in this study, and the arsenic contribution of each parameter on a daily basis.

When it comes to figure 4.35 for As, it can be observed that the total input was higher than the output released.

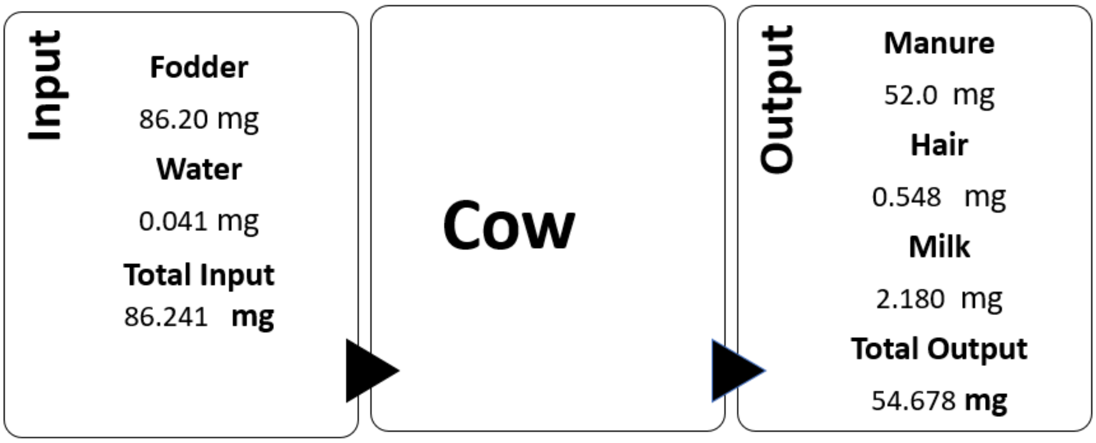

Figure 4.36 The input and output parameters considered in this study, and the selenium contribution of each parameter on a daily basis.

As illustrated in figure 4.36 for Se, the input had higher concentrations than the output.

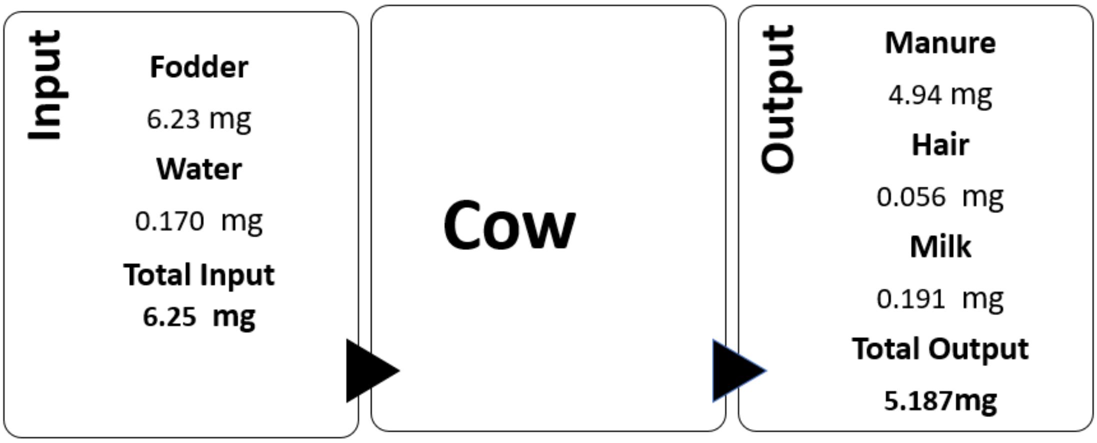

Figure 4.37 The input and output parameters considered in this study, and the cadmium contribution of each parameter on a daily basis.

As observed in the figure 4.37 for Cr, although it had similar concentrations, input was still found to have higher concentration than the output.

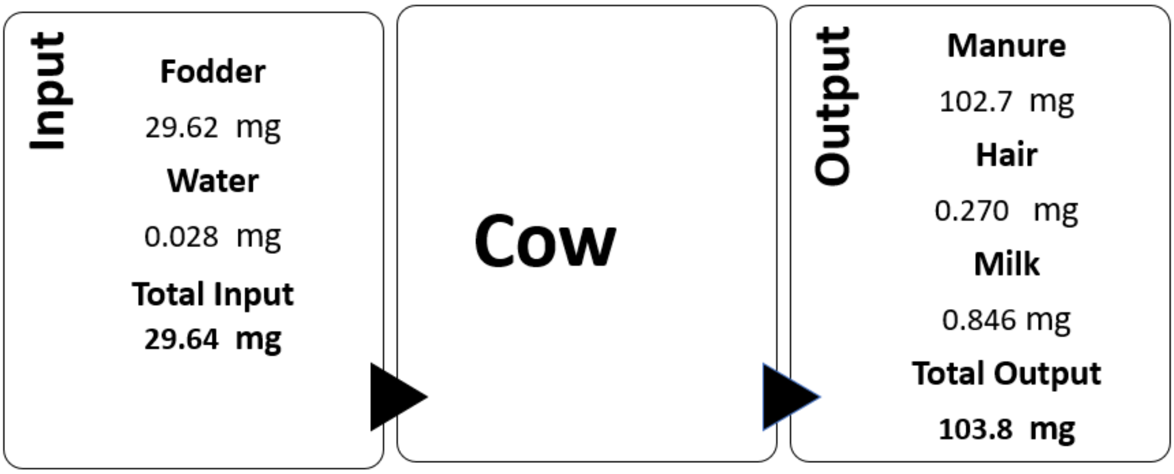

Figure 4.38 The input and output parameters considered in this study, and the lead contribution of each parameter on a daily basis.

Finally, in figure 4,38 for Pb, there was a much higher concentration from the outputs than in the initial Pb inputs.

In conclusion of all the above figures, similarities and differences were noted between all the heavy metals. It is firstly interesting to note the difference in concentrations between the inputs, feed and water in all the heavy metals’ figures. The reasoning behind the higher concentration in feed than in water is that, as discussed in section 4.3, the drinking water used in this study was tap water, which resulted to be much lower than the permissible limit set by the WHO guidelines, which, from the point of view of heavy metals, tap water is not harmful. On the other hand, most of the feed used for this study was brought from abroad, with little to no information about how it was grown. Combining this with factors such as the water used for watering the fodder, the fertilizer used for its enrichment, and the level of pollution in the area that it came from, led to the acceptable high levels of heavy metals in fodder.

In addition to this, Cr, Zn and Pb reached higher concentrations in the total output than the total input, whereas Cu, As, Se and Cd were opposite to this in which the total input was higher than the total output. Despite this, all the heavy metals resulted in manure having much higher concentrations than hair and milk, when comparing the three outputs together.

Breaking it down, the reasoning behind the higher concentration found in the output, mainly in manure, in Cr, Zn and Pb, can be a result of each heavy metal entering the system with the help of other input parameters, heavy metal accumulation and outputs release, such as the cow possibly being under treatment or taking supplements, as explained further on.

On the other hand, the input's higher concentration found in Cu, As, Se and Cd than the output may be due to each heavy metal depositing in the cow's organs, such as the kidneys, liver, amongst others, according to a study done by Narozhnykh *et al. (2018)*. In addition to this, it might find its way into blood, urine or sweat glands.

A study done Lead Content in Soil, Water, Forage, Grains, Organs and the Muscle Tissue of Cattle

The concentration ranges of the different elements found in fodder are similar to work by other researchers such as Zhang *et al.* (2012). For example, in their study As compounds were used in order to improve weight gain in animals. This could also be a reason to the concentrations of fodder as an intake, resulting with higher concentrations in manure as an output, since the cow absorbed supplements and compounds that could possibly enhance the productivity. According to present knowledge, unlike As, Cd is not used for weight gain, however, it is often present in mineral supplements, for example phosphates. Therefore, Cd, as well as Se and Pb, enter the cow's production process accompanied with these supplements which can lead to dietary contamination (Zhang *et al.* 2012), explaining as to why in this case the manure concentration is higher than the one in fodder. Furthermore, another possible reason could be through blood contamination, which another study done by Chirinos-Peinado & Castro-Bedrinana (2020) analysed how Cd and Pb concentrations were higher in milk than in the original intake. Concentrations in manure vary depending on, not only the concentration of fodder, but any other type of intake as well, since it can all affect the cow's production process. Therefore, the average concentration can be higher in manure than in fodder and vice versa, regardless of the month in which the sample was taken from.

To sum up, the previous calculations and heavy metals contributions within parameters have been analysed. This led to the decision of finding correlations between all the parameters present in the study. To achieve this the Pearson Correlation test, which has been defined in section 2.3.3.5, has been carried out between the different parameters for each element. The first element analysed is Cr, followed by the other elements.

4.7.1 Chromium

Table 4.12 Pearson Correlations between the different samples for Cr

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	.006	.816**	-.139	-.329
Water	Pearson Correlation	.006	1	.211	.702*	.313
Manure	Pearson Correlation	.816**	.211	1	.039	-.298
Milk	Pearson Correlation	-.139	.702*	.039	1	-.042
Hair	Pearson Correlation	-.329	.313	-.298	-.042	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

In the table above, table 4.12, the correlation is very strong between feed and manure since the value is (0.816), therefore there is enough evidence at 0.01 level of significance, for this correlation to be deemed significantly different from zero. In addition to this the correlation between milk and water provides enough evidence, at 0.05 level of significance, for this correlation to be deemed significantly different from zero. As for the other correlations, there is not enough evidence that they are significantly different from zero at either the 0.01 or 0.05 level. It must be noted that this does not mean that a correlation does not exist, but that there is not enough evidence for one due to small sample sizes. Thus, here, and elsewhere, some of these correlations will also be discussed.

Therefore, with regards to the correlation in Cr between inputs and outputs in figure 4.32, should the daily feed average intake of 78 mg increase, this would result in the daily manure average output to increase above the level of 573.7 mg. On the other hand, should feed increase above 78mg, the average daily output of milk and hair would decrease to lower than 0.717 and 0.212 mg, respectively, since they are negatively correlated.

Moreover, the second input, water, should it increase above 0.02 mg, then the average daily results for all outputs will also increase above 572.7 mg for manure, 0.212 mg for hair and 0.717 mg for milk, since they resulted in a positive correlation.

The outcome achieved is advantageous since due to the positive correlation, the high level of feed as input and the high level of manure, as output, was reached, which led to a positive effect since manure cannot directly enter in a human's food chain, unless used as a manure for crops. On the other hand, in accordance with the negative correlation achieved, as feed's level increased, milk's level decreased, again leaving a positive mark since milk can enter the human food chain directly, however since it resulted in a weak negative correlation, it would only make a slight effect.

Moreover, water with manure and milk had a positive correlation, however this should not have any negative effect since water was found to be within the permissible limit. Therefore, as long as the water keeps on being controlled, the high levels will not be of harm to the human food chain.

4.7.2 Copper

Table 4.13 Pearson Correlations between the different samples for Cu

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	-.196	-.789**	-.350	-.098
Water	Pearson Correlation	-.196	1	.522	.389	-.202
Manure	Pearson Correlation	-.789**	.522	1	.385	.370
Milk	Pearson Correlation	-.350	.389	.385	1	-.293
Hair	Pearson Correlation	-.098	-.202	.370	-.293	1

**. Correlation is significant at the 0.01 level (2-tailed).

In table 4.13, the significant correlation between feed with manure presented enough evidence at 0.01 level of significance, for this correlation to be deemed significantly different from zero.

Therefore, in terms of the correlation in Cu between inputs and outputs in figure 4.33, should the daily feed average intake of 1830.9 mg increase, this would result in the daily manure, hair and milk average output to decrease below than 1228, 4.712, and 1.470 mg, respectively.

Moreover, with regards to the second input, water, should it increase above 0.085 mg, then the average daily output for manure and milk would also increase above 1234 and 1.470 mg, respectively since they had a positive correlation. On the other hand, when it comes to water with hair, if water increases over 0.085 mg, then hair would decrease below 4.712 mg, as it resulted in a negative correlation.

The outcome achieved can be seen from a positive light with regards to feed with manure and milk. The reasoning behind this is that low Cu levels in manure and milk can result in a healthier outcome, since milk enters the human food chain directly. As for manure, Malta uses it as a fertilizer, therefore, although indirectly, should it have high levels of Cu, it can also affect the human food chain. However, the negative correlation also raises a red flag because if the Cu level in the output was less than the input, this means that the heavy metal should have deposited in different organs or as mentioned before, in blood or urine.

In addition to this, water with manure and milk achieved a positive correlation, however this should not result in a negative effect since water was found to be within the permissible limit. Therefore, as far as water stays controlled, the high levels will not harm the human food chain.

4.7.3 Zinc

Table 4.14 Pearson Correlations between the different samples for Zn

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	.441	.491	-.126	.030
Water	Pearson Correlation	.441	1	.309	.226	.708**
Manure	Pearson Correlation	.491	.309	1	-.113	.370
Milk	Pearson Correlation	-.126	.226	-.113	1	.199
Hair	Pearson Correlation	.030	.708**	.370	.199	1

**. Correlation is significant at the 0.01 level (2-tailed).

In table 4.14 above, the correlation between hair and water presented enough evidence, at 0.01 level of significance, for this correlation to be deemed significantly different from zero.

Furthermore, in terms of the correlation in Zn between inputs and outputs in figure 4.34, should the daily feed average intake of 6377.6 mg increase, this would result in the daily manure and hair to increase as well above 20643 and 60 mg, respectively. As for feed with milk, should the feed increase, then milk will consequently decrease to less than 113 mg. Alike feed, should water (0.486 mg) increase, then all parameters in output would also increase above their average.

The results obtained gave a positive outcome in terms of feed with manure and hair. The reasoning behind this is that the higher the concentrations in manure and hair output, the better since the Zn levels have moved out of the cow’s body, consequently no direct contact would be present with the human food chain. as previously mentioned, Malta uses manure as a fertilizer, meaning that the Zn levels can still leave a mark within the food chain in an indirect manner through crops. On the other hand, with regards to feed with milk, and its negative correlation results, are an indication of the presence of other intake parameters which can affect Zn levels in milk, such as the medical treatments, supplements or air pollution.

Moreover, water with all output parameters resulted in a positive correlation, however this should not raise any alarms as water was found to be within the permissible limit. Thus, as long as the water is kept under control, the high levels will not be harmful to the human food chain.

4.7.4 Arsenic

Table 4.15 Pearson Correlations between the different samples for As

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	-.279	-.033	-.665*	-.547
Water	Pearson Correlation	-.279	1	-.277	.358	.500
Manure	Pearson Correlation	-.033	-.277	1	-.205	-.308
Milk	Pearson Correlation	-.665*	.358	-.205	1	.973**
Hair	Pearson Correlation	-.547	.500	-.308	.973**	1

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

In table 4.15 the correlation between feed with milk has enough evidence at 0.05 level of significance, for this correlation to be deemed significantly different from zero. On the other hand, the correlation between milk and hair has enough evidence, at 0.01 level of significance, for this correlation to be deemed significantly different from zero.

Additionally, in terms of the correlation between As inputs and outputs in figure 4.35, when the daily feed average intake of 84.1 mg increases, the daily manure, hair and milk would in turn decrease below the levels of 46.4, 0.525 and 2.56 mg, respectively. Same as feed, as water increases (0.143 mg), all other parameters decrease, except for milk which increases as well due to its positive correlation.

What can be observed through these results is that, despite the negative correlation found between feed and the output parameters, positive results were still achieved since manure had the highest level out of all the outputs, therefore the higher percentage of As occurrence was present within manure which leads to a positive outcome as manure does not have a direct contact with the human food chain either. The lower levels of the output in comparison to the input leads to the question as to whether the heavy metal's concentration decreased by precipitation or other means such as through sweat glands or urine, as previously discussed. Unlike the rest, the only input-output As relation that resulted in a positive correlation was water with milk. Since water had no high levels, this allows for the search of other input parameters that could possibly affect the As levels in milk.

4.7.5 Selenium

Table 4.16 Pearson Correlations between the different samples for Se

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	-.375	.102	-.728**	-.236
Water	Pearson Correlation	-.375	1	.109	.404	.743**
Manure	Pearson Correlation	.102	.109	1	-.227	.133
Milk	Pearson Correlation	-.728**	.404	-.227	1	.691*
Hair	Pearson Correlation	-.236	.743**	.133	.691*	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

In table 4.16 above, the correlation between feed and milk, and water and hair, have enough evidence at 0.01 level of significance, for this correlation to be deemed significantly different from zero, whereas the correlation between milk and hair has enough evidence, at 0.05 level of significance, for this correlation to be deemed significantly different from zero.

Furthermore, looking at figure 4.36 for Se inputs and outputs, as the daily feed average intake of 86.20 mg increases, the rest of the daily average of all outputs, except for manure, will decrease due to the negative correlations, leading for the milk, hair and water averages to decrease less than 2.18, 0.548 and 0.041 mg, respectively. As for the correlations between water and all outputs, the daily water average did not have a sufficient level that was high enough to affect the output parameters. Manure was once more found to have the highest level between all outputs having the same positive outcome and reasoning as previous heavy metals. Since the feed input had a negative correlation with the majority of the outputs, the question of why the output parameters decrease instead of increase is brought to light. A possible reason for this can be due to other input parameters that can leave a mark on the Se levels such as mentioned previously, medical treatments, supplements and air pollution.

4.7.6 Cadmium

Table 4.17 Pearson Correlations between the different samples for Cd

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	-.016	-.712**	-.701*	-.410
Water	Pearson Correlation	-.016	1	.049	.449	-.317
Manure	Pearson Correlation	-.712**	.049	1	.573	.831**
Milk	Pearson Correlation	-.701*	.449	.573	1	.322
Hair	Pearson Correlation	-.410	-.317	.831**	.322	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

With respect to table 4.17, the correlation between feed and manure, and manure and hair, have enough evidence at 0.01 level of significance, for this correlation to be deemed significantly different from zero, whereas the correlation between milk and feed has enough evidence, at 0.05 level of significance, for this correlation to be deemed significantly different from zero.

Additionally, in terms of the correlation between Cd and its inputs and outputs in figure 4.37, as the daily feed average intake of 6.23 mg increases, the daily manure, hair and milk would in turn decrease below the levels of 4.94, 0.055 and 0.198 mg, respectively. Similar to feed, as water (0.170 mg) increases, hair decreases. However opposite to this, as water increases, manure and milk increase, due to their positive correlations.

The achieved outcome has led to positive results since despite the negative correlation between feed and all outputs, manure has resulted in obtaining the highest output level, meaning that the majority of the Cd input level exited the cow’s body. This allows lesser chances for Cd to enter the human food chain through milk. It is important to note that despite the negative correlation between feed and all outputs, the input level still managed to leave an effect, specifically in manure, one way or another. A possible reasoning for this is that Cd is the heavy metal that is mostly present in air pollution, due to factors such as smoking cigarettes, as well as due to the correlation between water with milk and manure. Since the levels of input were higher than those in output, this again points towards the possible reasoning that Cd precipitated in other organs or exited the body by means of other outputs.

4.7.7 Lead

Table 4.18 Pearson Correlations between the different samples for Pb

		Feed	Water	Manure	Milk	Hair
Feed	Pearson Correlation	1	-.331	.527	.271	-.720**
Water	Pearson Correlation	-.331	1	-.383	-.050	.257
Manure	Pearson Correlation	.527	-.383	1	-.431	-.555
Milk	Pearson Correlation	.271	-.050	-.431	1	.057
Hair	Pearson Correlation	-.720**	.257	-.555	.057	1

**. Correlation is significant at the 0.01 level (2-tailed).

Looking at the presented results in table 4.18, the correlation between feed and hair, has enough evidence at 0.01 level of significance, for this correlation to be deemed significantly different from zero.

Therefore, another positive result was achieved due to the fact that the manure level was the highest out of all the outputs when looking at figure 4.38. However, its levels of 102.7 mg was much higher than the initial Pb level in fodder (29.62 mg) and water (0.028 mg). This means that besides fodder and water, other input parameters may have been present that led to the manure levels in the output to increase as much as they did. Similar to previous heavy metals, the high levels of Pb in manure as an output decreases the chances of direct Pb exposure to the human food chain. In addition to this, since a positive correlation was achieved between feed with manure and milk, it would be most beneficial to make sure that the Pb levels in feed remain controlled, mainly because of milk. The reasoning for this is that milk can have a direct effect on the human food chain, whereas Pb levels in manure can only affect it indirectly through crops due to its presence in fertilizers.

5. Comparison Chapter

5.1 Conventional Method and Microwave Digestions

In this particular chapter, the study between two different methods on preparing samples was analysed. Hot plate (conventional method) and microwave digestion were applied to digest the sample matrices, ensuring digests to be subject to inductively coupled plasma optical emission spectroscopy (ICP-OES). Ten different milk samples were collected and digested using the conventional method (Method 1), and the microwave digestions (Method 2), as explained in Chapter 2, sections 2.3.2.3 and 2.3.3.1 respectively. For comparative sample preparation method, the two methods were performed in order to see if there is any difference between the heavy metal concentrations of each method. The analysis of the heavy metals chosen in this study Cu, Cr, Zn, As, Se, Cd and Pb are presented and discussed, with respect to the two techniques.

Following that, the flowing statistical methods were applied. From the ten samples collected, the results of the mean concentration and the standard deviation for all the elements in this study, are shown in table 5.1 below:

Table 5.1 The mean concentration and the standard deviation for all the elements

Descriptive Statistics

Method	Element	Mean	Std. Deviation	N
Method 1	Cr	1.69	.298	10
	Cu	10.4	1.65	10
	Zn	478	216.0	10
	As	11.1	.324	10
	Se	8.263	.392	10
	Cd	1.20	.331	10
	Pb	7.44	2.42	10
	Total	74.0	184	70
Method 2	Cr	.840	.140	10
	Cu	5.64	.804	10
	Zn	472	72.8	10
	As	13.4	1.76	10
	Se	12.5	1.34	10
	Cd	.652	.063	10
	Pb	3.08	.271	10
	Total	72.6	167	70

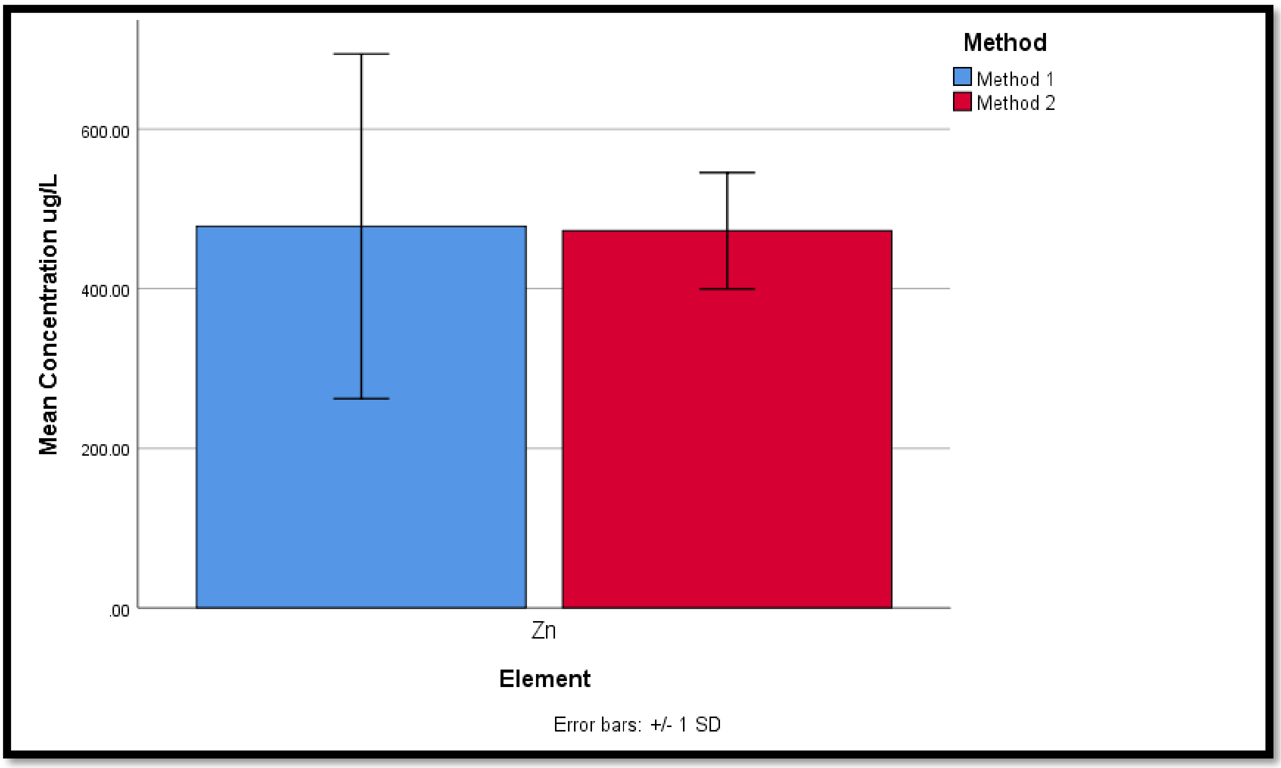

Figure 5.1: Mean Bar Graphs with Error Bar (SD) across milk between method 1 and 2 for Zn.

Figure 5.1 above, represent mean bar plot for the concentration (µg/L) of Zn. From the bar plot there appears to be no difference in the mean between method 1 and method 2. However, the standard deviation of the readings from method 1 is considerably higher than method 2.

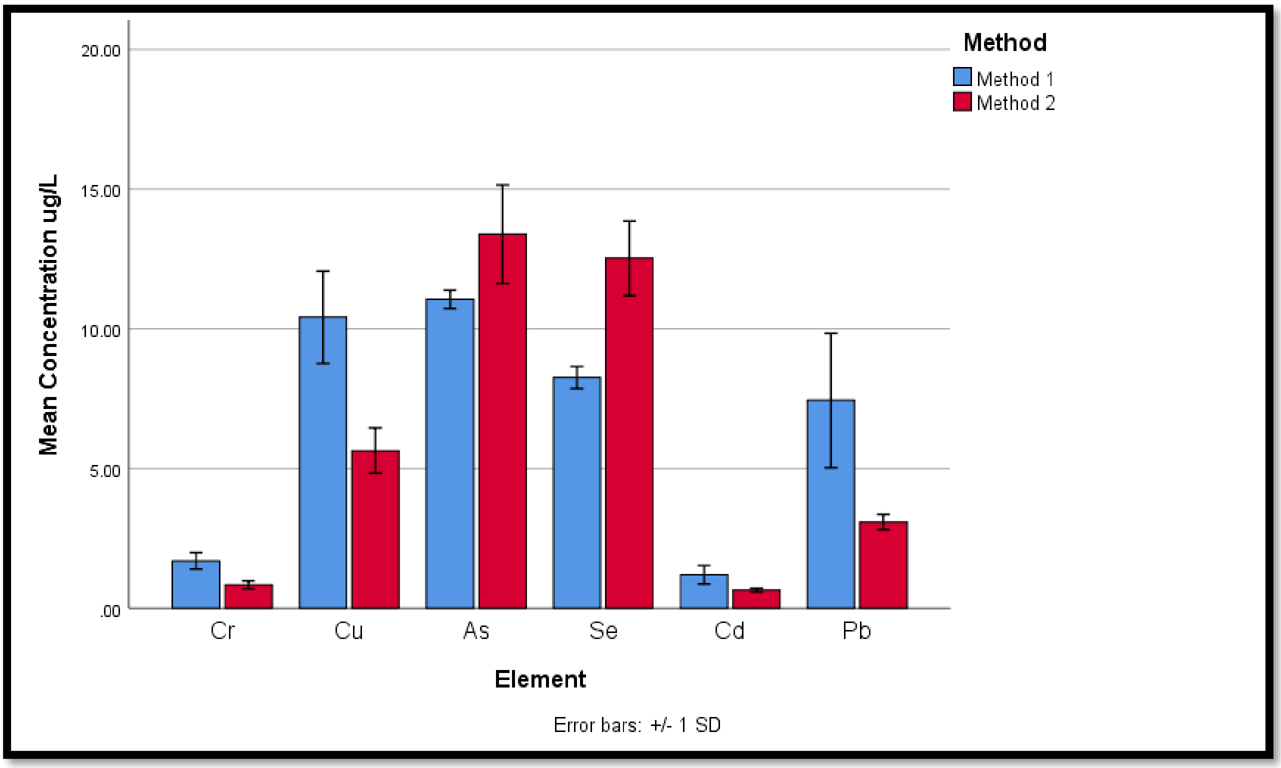

Figure 5.2: Mean Bar Graphs with Error Bar (SD) across milk between method 1 and 2 for Cr, Cu, As, Se, Cd and Pb.

In Figure 5.2, showing the means of Cr, Cu, As, Se, Cd and Pb concentrations and standards deviation across milk between method 1 and method 2, it is noticeable that there is a significant difference between method 1 and 2 with regards to the mean concentrations of each element in this graph. However, one can observe that the mean concentrations of Cr, Cu, Cd and Pb are higher in method 1 than in method 2. Opposite to this, As and Se show higher levels of mean concentration in method 2 than method 1.

Table 5.2 Tests of normality using Kolmogorov Smirnov tests and Shapiro Wilk test for differences between the two methods for different elements.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	Df	Sig.	Statistic	df	Sig.
diffCrReading	.167	10	.200*	.938	10	.535
diffCuReading	.123	10	.200*	.973	10	.920
diffZnReading	.192	10	.200*	.917	10	.331
diffAsReading	.145	10	.200*	.972	10	.908
diffSeReading	.233	10	.131	.846	10	.052
diffCdReading	.271	10	.037	.809	10	.019
diffPbReading	.225	10	.161	.843	10	.048

*. This is a lower bound of the true significance.

Table 5.2 shows normality tests using Kolmogorov Smirnov tests and Shapiro Wilk test for differences between the two methods for different elements. The null hypothesis of normality is not rejected for both tests, at the 0.05 level, for Cr, Cu, Zn, As, Se. On the other hand, it is rejected for both tests for Cd at the 0.05 level. In the case of Pb, the Kolmogorov Smirnov test accepts the null hypothesis of normality at the 0.05 level, but the opposite happens in the Shapiro-Wilk test. For this reason, the paired sample t-test was considered for Cr, Cu, Zn, As, Se. For Cd and Pb the Wilcoxon signed ranks test was considered.

Table 5.3Tests of normality using paired samples t-test for differences between the two methods for Cr, Cu, Zn, As and Se

paired samples t-test

		Mean	Std. Deviation	t	df	Sig. (2-tailed)
Pair 1	Cr µg/L (1) - Cr µg/L (2)	.852	.267	10.1	9	.000
Pair 2	Cu µg/L (1) - Cu µg/L (2)	4.77	2.04	7.42	9	.000
Pair 3	Zn µg/L (1) - Zn µg/L (2)	5.65	241	.074	9	.942
Pair 4	As µg/L (1) - As µg/L (2)	-2.32	1.86	-3.97	9	.003
Pair 5	Se µg/L (1) - Se µg/L (2)	-4.26	1.49	-9.02	9	.000

Table 5.3 above, shows that the paired samples t-test rejects the null hypothesis of no difference at the 0.05 level of significance for Cr, Cu, As and Se (*p*-values less than 0.05). For Zn, the null hypothesis of no difference was failed to be rejected, at the 0.05 level of significance (t=0.074, df=9, p=0.942). The conclusion is that the methods differ significantly for all the aforementioned elements except Zn. With regards to Cr and Cu, method 1 gives significantly higher readings, (t=10.07, df=9, p≈0.000) and (t=7.42, df=9, p≈0.000) respectively. On the other hand, significantly higher readings were given by method 2 for As (t=-3.96, df=9, p=0.003) and (t=-9.02, df=9, p≈0.000) for Se.

Table 5.4Tests of normality using Wilcoxon Signed Ranks tests for differences between the two methods for Cd and Pb

Test Statistics^a

	Cd µg/L (2) - Cd ug/L (1)	Pb µg/L (2) - Pb µg/L (1)
Z	-2.80^b	-2.80^b
Asymp. Sig. (2-tailed)	.005	.005

a. Wilcoxon Signed Ranks Test
b. Based on positive ranks.
c. Based on negative ranks.

Following Cr, Cu, As and Se, the Wilcoxon signed ranks test was applied for Cd and Pb. The above table 5.4, shows that the Wilcoxon signed ranks test rejects the null hypothesis of no difference at the 0.05 level of significance for Cd and Pb (p-values less than 0.05). In both cases, Method 1 is significantly higher than Method 2. Both Cd and Pb resulted with (Z=-2.803, p=0.005).

All the tests above performed in order to find the difference between the two methods, serve as proof for the results obtained in figures 5.1 and 5.2. Starting with Zn, no difference was found between the two methods, even though the first method, the Conventional Method, has a higher chance of being exposed to pollution during preparation at the lab since basic lab tools, such as hot-plate, beakers and watch-glasses are used. Moving onto Cr, Cu, Cd and Pb had concentrations higher in Method 1 than 2, which could be a positive result from the contamination accumulated during preparation. A number of factors contribute to the high levels of Cd and Pd, mainly air pollution. As with regards to Cr and Cu, other factors could have played a role in obtaining high concentrations.

On the other hand, As and Se had higher concentrations in Method 2 than 1, despite the use of microwave digestion closed-vessels under very high temperatures and pressure in comparison to the first method. This sealed process of Method 2 ensures that there is no exposure to any contamination during the preparation of samples. Microwave digestion systems can give greater extraction efficiency whereas in the conventional digestion extraction can be incomplete due to evaporation. The different results obtained might be an indicator of different characteristic of each element.

5.1.1 Conclusion

For better understanding how elemental concentrations vary with digestion method, concentrations of seven elements in milk were investigated using the two digestion methods; the performance of conventional method (hot plate) was compared to that of microwave digestion.

According to (Geana, Iordache, Voica, Culea, & Ionete, 2011), the use of the traditional open system presents risks of contamination within the atmosphere and volatilization losses of volatile elements during the extraction procedure. That is why the second method, microwave digestion, has become more popular among researchers due to its safe, efficient and fast performance, since it reduces the risk of external contamination giving more accurate results. A microwave digestion procedure is also less time consuming than conventional digestions since it is achieved with a shorter digestion time. However, one of the possible disadvantages of microwave digestion system could be the explosion or damage of digestion tubes due to the increase of pressure and temperature simultaneously. Another disadvantage is its high price which in turn limits labs from being able to acquire it, opposite to the hot plate which is found in every lab.

Good recovery was obtained for both conventional digestion and microwave digestion procedure. The results reported in this work highlight that method 1 showed higher concentrations in all elements, with the exception of As and Se, which in their case, method 2 was higher. No change was observed for the concentration of Zn. Being knowledgeable about the results of these two methods allows the researchers to be guided towards choosing the best method for each individual element.

5.2 White vs Black Hair Analysis

In the last few decades, hair has been studied as it allows the determination of trace elements in the body and can be done through non-invasive tools. An old study done by Miller *et al.* (1965) found that Zn was eminent in white tail hair rather than in either black of white body hair which can be a possible reason to the stiffness of hair in rats and humans (O'Mary, Bell, Sneed, & Butts Jr, 1970), also had an interesting study in which higher levels of Na, Ca and K were found in white hair from Holstein cattle than the black-haired Hereford cattle. Another study by the same researchers (O'Mary, Butts Jr, Reynolds, & Bell, 1969)had analysed Na, Cu, Zn, Ca, P, Mg, Mn, Fe and K content within Red and White hair in Hereford and calves. The results obtained presented a high significance in variances between seasons in seven out of the nine elements studied. August had resulted with hair containing greater amounts of Na, Cu, Ca, Mg, Mn and K in comparison to samples taken in March. On the other hand, the samples taken in March had a higher content of Fe. The Red hair was found to contain more Ca, P, Mg, Mn, Fe and K but less Cu than in white hair. However, it was also found that Cu in white hair was higher than in red hair in August but little difference in March. These studies, along with others, led to the formation of this second and last part of this chapter, which will deal with the analysis of heavy metals (Cr, Cu, Zn, As, Se, Cd, and Pb) in white vs black hair.

Five random samples were each collected for white and other 5 for black hair, in order to check if there are any significant differences between them. Each element presented its own set of results, as seen in the table 5.5 below:

Table 5.5 Tests of normality using Kolmogorov Smirnov tests and Shapiro Wilk test for differences between the two white and black hair for different elements.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	Df	Sig.	Statistic	Df	Sig.
Cr (µg/g)	.211	10	.200*	.873	10	.110
Cu (µg/g)	.159	10	.200*	.921	10	.368
Zn (µg/g)	.249	10	.080	.945	10	.605
As (µg/g)	.400	10	.000	.537	10	.000
Se (µg/g)	.256	10	.063	.743	10	.003
Cd (µg/g)	.233	10	.131	.818	10	.024
Pb (µg/g)	.179	10	.200*	.920	10	.355

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests for normality (Kolmogorov-Smirnov and Shapiro-Wilk) were applied across the two groups (white and black hair) for all the heavy metals. Potential violation of normality was detected in As, Se, and Cd since the p-value of at least one of the tests is less than 0.05. Due to this, Cr, Cu, Zn and Pb were tested with the independent samples t-test, while As, Se, and Cd with the Mann-Whitney test.

Table 5.6 The independent samples t-test for white and black hair for Cr, Cu, Zn and Pb

		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Cr (µg/g)	Equal variances assumed	1.67	.232	-2.46	8	.040	-.094	.038
	Equal variances not assumed			-2.46	5.550	.053	-.094	.038
Cu (µg/g)	Equal variances assumed	.039	.848	-.875	8	.407	-.172	.197
	Equal variances not assumed			-.875	7.733	.408	-.172	.197
Zn (µg/g)	Equal variances assumed	4.07	.078	.173	8	.867	.886	5.13
	Equal variances not assumed			.173	6.011	.869	.886	5.13
Pb (µg/g)	Equal variances assumed	.002	.967	-1.13	8	.293	-.025	.022
	Equal variances not assumed			-1.13	7.979	.293	-.025	.022

In table 5.6, Levene's test p-value indicates whether equal variance (p>0.05) should be assumed or whether it should not (p<0.05). Either way, in all cases, p-value is greater than 0.05, so it shall be assumed equal variances throughout. The p-values for Cr, Cu, Zn, Pb, in which equal variances have been assumed, indicate that there is no significant difference at the 0.05 level for Cu, Zn and Pb, since the p-values is greater than 0.05. For Cr, it is seen that the p-value is less than 0.05 which indicates that levels of Cr between black and white hair are different.

Table 5.7the Mann-Whitney U test for white and black hair for As, Se and Cd

Test Statistics[a]

	As (ug/g)	Se (µg/g)	Cd (µg/g)
Mann-Whitney U	5.50	5.00	9.00
Wilcoxon W	20.5	20.0	24.0
Z	-1.47	-1.57	-.745
Asymp. Sig. (2-tailed)	.142	.116	.456
Exact Sig. [2*(1-tailed Sig.)]	.151[b]	.151[b]	.548[b]

a. Grouping Variable: Hair Colour

b. Not corrected for ties.

Looking at table 5.7 above, the Mann-Whitney U test is used for As, Se and Cd which indicates that there is no significant difference between white- and black-haired cows for all three elements due to p-values all being greater than 0.05.

For further clarification, the following figures have been done for reference:

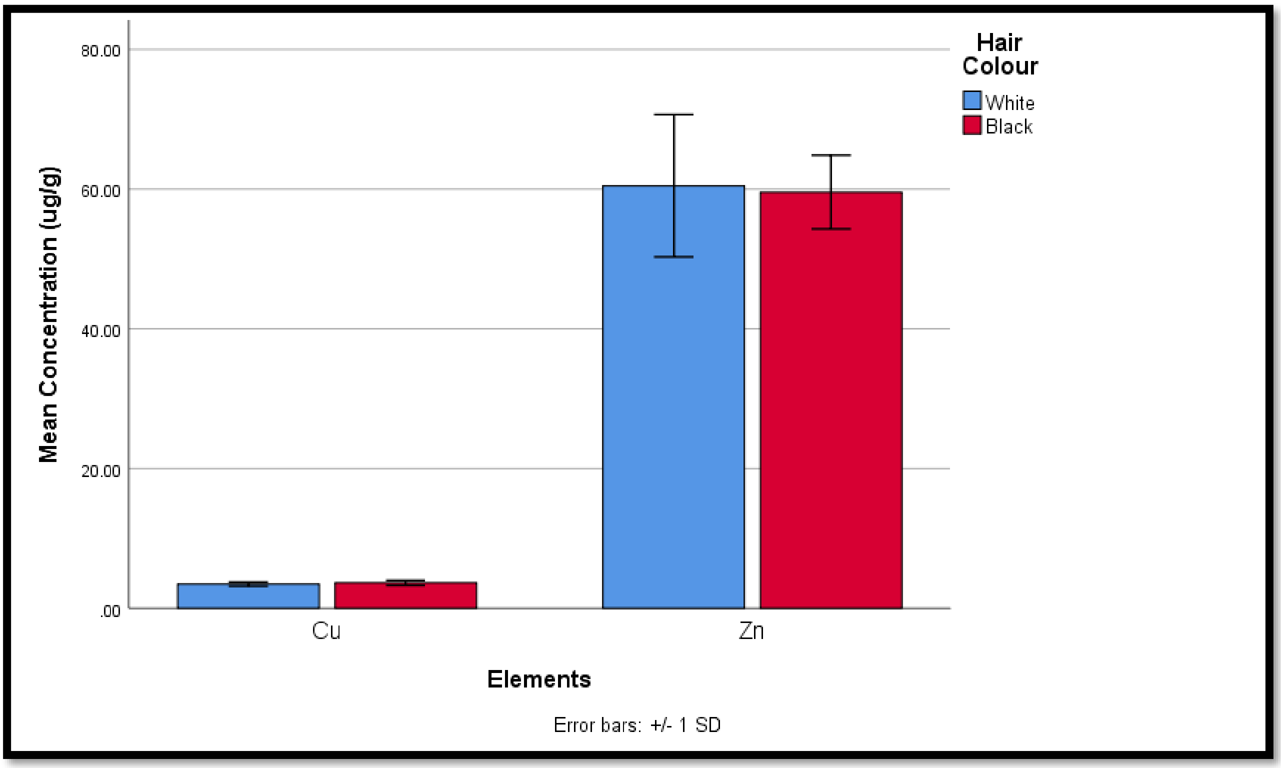

Figure 5.3: Mean Bar Graphs with Error Bar (SD) between white and black hair for Cu and Zn.

Figure 5.3 above, shows a mean bar plot for the concentrations (µg/g) of Cu and Zn. From the bar plot there appears to be no difference in the mean between black and white hair for both Cu and Zn. However, in the case of Zn the standard deviation of the readings from white hair is considerably higher than in black hair.

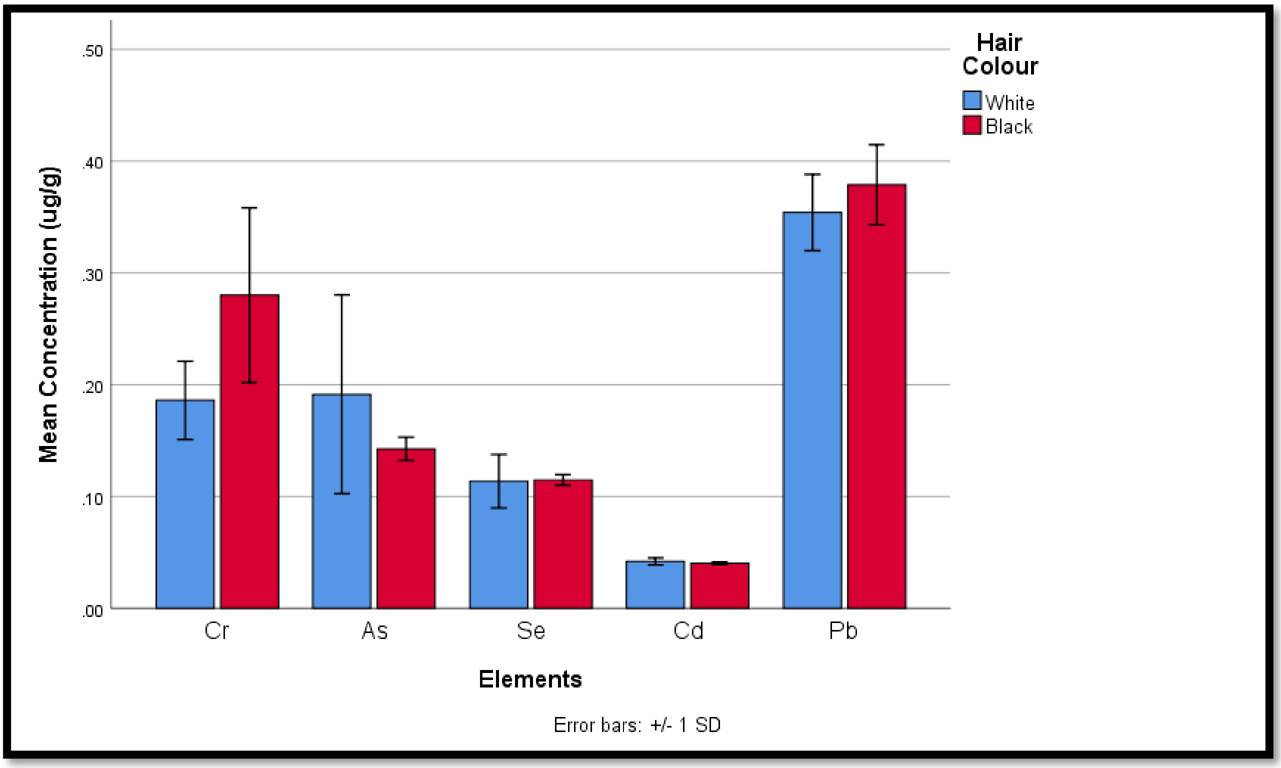

Figure 5.4: Mean Bar Graphs with Error Bar (SD) between white and black hair for Cr, As, Se, Cd, and Pb.

Moving onto the means of Cr, Cu, As, Se, Cd and Pb concentrations and standards deviation between white and black hair were compared. From first glance onto figure 5,4, it is noted that there is no significant difference between white and black hair, in Se and Cd. In addition, with regards to As, it was the only element in which the concentration of white hair was higher than the one in black hair. Opposite to this however, Cr and Pb resulted in higher concentrations in black than in white hair. Despite this, Cr is the only element that resulted in any significant difference between white and black hair, statistically.

5.2.1 Conclusion

In conclusion, the results presented showed no significant difference between the light and dark hairs, except for a slight variance in Cr. Having said that, in connection to the whole study, it seems that there is no need to focus and highlight the variances between different-coloured hair since the results obtained did not prove to have any effect on the heavy metal concentrations. Proof of this is that no recent studies were found specifically orbiting around cattle coloured hair. Should this be further pursued, it is suggested to collect more samples in order to have more accurate results that may possibly present some sort of difference between coloured hair, leading to a variance in heavy metal concentration.

6. Conclusion

The main objective for this research was to investigate how the seasonal pollution level and accumulation of heavy metals within the cows' life cycle in Malta occur by studying fodder, water, milk, hair and manure. These, with the exception of water, were first studied using AAS, and then using ICP-OES, this time including water as well. It is of noticeable importance that heavy metals, in nature, are bio-degradable and therefore accumulate in the food chain through three processes; biotransformation, bioaccumulation and biomagnification (Aslam, Javed, & Khan, 2011; Muhib *et al.,* 2016). Cd, Cr, Cu, Fe, Pb and Zn are among the most common heavy metals known as environmental contaminants and thus come affront as hazardous substances to both human and animal health (Roberts, 1999). Only one other study is known to have been done regarding this topic, however, it only focused on heavy metals found in different types of cattle milk in Malta, which was done by Spiteri & Attard (2017). This current study was the first research done worldwide that focused on the concentration of heavy metals (Cr, Cu, Zn, As, Se, Cd, and Pb) in the life cycle of cows, starting with the intake (fodder and water), as well as the output (milk, hair and manure) for the entirety of the research's period.

6.1 Atomic Absorption Spectrometry

For the first section of this research, AAS was used to analyse the heavy metals of interest in fodder, milk, hair and manure samples that were collected for a period of 11 weeks, starting in September until December 2016. The Cu concentration obtained in the results testify to a substantial amount of inconsistency in accumulation from week-to-week, which was even more clear through post-hoc tests after treating outliers in which there was a significant difference from one week to another. Furthermore, outliers were the reason for the rejection of the multivariate normality hypothesis. In the samples analysed it was noted that collecting from the same cows led to numerous outliers to be created. This is proved from the milk and hair sample of one of the cows, referred to as Cow 1, in which it was recognised as an outlier six times. That is why in the following section, when ICP was used, it was decided that samples would be collected randomly so as not to perpetuate any anomalies coming from the subject frequently in the research. In addition to this, the study was extended from 11 weeks to a year, in order to reach more accurate results, especially with regards to seasonality, for most of the samples.

As time passed, the concentration of Cu in milk samples kept on decreasing, starting at a mean concentration of 0.077 and ending at 0.018 ppm, with fluctuations in between. For this reason, the study of seasonality was pursued. The concentrations of Cu found were higher than the permissible limit of 0.01 ppm, which raises a red flag when compared to the results found in this research. Therefore, this raises concern and gives reason to look further into the matter. as an excess of Cu concentration can lead to high levels of pollution in the Maltese environment.

As opposed to the rest of the samples, hair samples did not have any significant difference with regards to seasonality. However, though not significant, it did depict an increase in the first three weeks and then a decrease in concentrations in weeks 6, 7, 8 and 9 possibly due to seasonal impact. Furthermore, different hair colours were taken into account for this research since the colour of hair influences the results of heavy metal content. Despite the limited number of ten cows studied for this section made it difficult to realise the effects of hair colour, it was still noted that black hair had slightly higher Cu concentrations than the lighter hair. In fact, black hair had Cu concentrations of 6.07 and 6.43 ppm, while the lighter hair had concentrations of 4.07, 3.95, 3.92 and 3.59 ppm. This difference led to furthering the study by focusing on the differences between black and white hair in Chapter 5.

When it comes to fodder, seven types were purchased for the dairy farm, most of which were imported from abroad. According to the results obtained, the Cu concentration was not affected by the seasons. This can be proved by Maltese Hay, in which concentrations of 3.80 and 3.91 ppm were similar, even though they were collected two months apart. Due to a lack of information regarding the origins of the fodder, the results obtained could not be explained in detail, however, since all types were imported from EU countries, they fall under the auspices of the European Union, allowing for the assumption that each type was monitored in a strictly manner before being presented to Malta.

Similar to the fodder, manure was found to have no significant difference when based on seasonal effect. However, it was found that there was a significant variation of concentrations during the study period which had no relation to the change in temperature. It can be observed that the Cu input coming from feed did not tally to the Cu output through manure, milk and hair. In fact, manure was found to have a higher concentration of Cu, than other input and output parameters. A possible reason for this may be due to Cu entering the system with the help of other input parameters, through accumulation and outputs release. Nevertheless, the higher concentration in manure is a positive outcome since it exited the cattle's body instead of accumulating in the cattle's organs and its milk. In turn, it is used as a fertilizer for crops which subsequently filters into the human's food chain.

To conclude, the collected results help shed light on the heavy metal contamination coming from intensive dairy farms. Certain increases in heavy metals being observed raise the possibility that the consumer population of Malta could face significant health threats in the long term from consuming contaminated products that come from outputs, such as milk. That is why it is useful to understand these processes in a schematic manner that indicates this cycle. The samples analysed allow for the basis of further studies whilst taking into consideration more inputs, such as atmospheric pollution and drinking water, as well as urine in liquid manure as outputs. Going hand in hand with this, it would also be ideal if further research done on a long-term basis, would be carried out in order to identify whether any trends of seasonality are present when it comes to the presence of Cu in cows, as well as other possible pollutants.

6.2 Inductively Coupled Plasma – Optical Emission Spectrometry

Moving onto this next section, ICP-OES was used to analyse the heavy metals of interest (Cr, Cu, Zn, As, Se, Cd, and Pb) in fodder, water, manure, hair and milk for 12 months, except for hair which was analysed for 8 months instead. The reason why ICP-OES was chosen, as mentioned before, was due to the fact that the AAS equipment was damaged and therefore it was brought to the lab in replacement. ICP-OES is similar to AAS, however as it is more developed, it brought with it further benefits.

Starting with fodder, this study, which took place during two periods with different seasons, Cu and Zn resulted with the highest concentrations throughout both periods, which is expected since they are essential elements and non-toxic for the human body, as long as they do not exceed the permissible limit. Moreover, the other two essential elements, Cr and Se, and the toxic once As, were the next set that had the highest concentrations in fodder, which was then followed by the two most toxic elements, Cd and Pb which had lower concentration levels, in which As, Cd and Pb were all under the permissible limit. In the end, only Cr and Zn were found to have variances between seasons in which the Winter season had higher concentrations than in the end of Spring and beginning of Summer. In addition to this, Cu, Zn and Se were found to have the highest concentration in Maize small pellet with concentrations of 17.9, 63.1 and 0.740 ppm, respectively. As and Pb had the highest level in Imported hay with concentration levels of 0.780 and 0.380 ppm, respectively. Cr was highest in Maltese hay with 0.880 ppm, and Cd was highest in Mixed seeds with 0.080 ppm. The concentration levels of heavy metals found in fodder are normal for farms and cattle feed since they are within the permissible limit, however more accuracy might have been possible had there been more information regarding EU guidelines on all studied elements in this study.

Following fodder, with regards to water, it was deciphered that despite the variance in concentrations according to season, none were found to have concentrations higher than the permissible limit, as set by the WHO guidelines. Therefore, it can be concluded that the heavy metal concentration found in water is not high enough to have a harmful effect neither on the cattle’s drinking water quality, nor on a human’s health.

Moreover, manure resulted as having the highest level of concentrations in comparison to fodder, water, milk and hair, in all the heavy metals. Similar to the results when using AAS, the high concentrations found in manure may be regarded as a positive outcome since the majority of the heavy metals are toxic; therefore, their exit equals to a healthier body both for the cattle and its consumer.

With regards to hair, Cr, As, Se and Pb reached the highest levels of concentration during the beginning of Summer with levels of 0.720, 1.22, 1.43, 0.820 µg/g respectively. Moving onto Cu, Zn, and Cd their highest concentrations were 10.9, 149 µg/g and 0.190 µg/g, respectively, which were reached during the season of Winter. On the other hand, Cr, Cu, Zn, Cd, and Pb were lowest during Summer with concentration levels of 0.210, 5.16, 80.6, 0.060 and 0.120 µg/g, respectively, whilst As and Se were lowest in Winter with 0.040 and 0.130 µg/g, respectively. The results obtained all reached the detectable level of concentrations. The high levels found can be seen as an advantage since it reduces the chances of contamination within the body, similar to manure. In addition to this, the results had positive outcomes since the effect of seasonality was prominent with concentrations changing depending on the temperature levels in different seasons.

The last type of samples analysed for this study, milk, concluded that Cr, Cu, Cd and Pb all reached the high seasonality effect during Spring with concentrations of 0.027, 0.213, 0.017 and 0.183 ppm, respectively. In addition to this, Cr also reached high seasonality during late Autumn, in November. With regards to Zn and As, they both reached high seasonality during Summer with concentrations of 6.808 and 0.181 ppm, respectively, with Zn reaching high seasonality in Winter as well with a concentration of 7.70 ppm. The last heavy metal, Se, reached its highest seasonality in Autumn with a concentration of 0.113 ppm. Looking at the lowest concentrations, As, Se, Cd and Pb all resulted lowest during Winter with concentrations of 0.003, 0.007, 0.005 and 0.006, respectively. On the other hand, looking at the lowest concentrations of each heavy metal, it can be noted that As, Se and Pb all reached their lowest seasonal effect during Winter with concentrations as low as 0.003, 0.007 and 0.006 ppm, respectively. Cu resulted in having the lowest effect during Summer with a concentration of 0.007 ppm and Cr resulted lowest during Autumn and early Winter with the lowest concentrations reaching 0.006 ppm. As for the last two remaining heavy metals, Zn and Cd had low concentrations throughout the year, therefore low seasonal effect was present in different seasons, with the concentrations being as low as 3.06 ppm for Zn and 0.005 ppm for Cd. It is of importance to note that all heavy metals experienced up and down fluctuations throughout the year. Looking at the overall results for each heavy metal, it is concluded that Cd was found to have concentrations lower than the permissible limit, whereas Cu and Pb both resulted in having higher concentrations than the allowed limit. With regards to Cr, Zn, As and Se, their results were compared to those of other studies to determine the outcome of their concentration levels.

The proper way of destroying the organic matter portion of a sample has created a series of debates within the sample preparation aspect prior to metals determination. The two most common, and the two analysed for this study, have been the wet acid digesting using hot plate (conventional) and microwave oven procedures (Gorsuch *et al.*, 2017). By analysing concentrations of seven elements in milk using these two digestion methods and comparing them, it was concluded that good recovery was achieved for both conventional digestion and microwave digestion procedure, in which method 1 presented higher concentrations in all elements, except for As and Se which had higher concentrations in method 2. With regards to Zn, no changes were presented. Understanding the results of these two methods will allow the researchers to be knowledgeable and guided towards choosing the best method out of the two for each individual element.

When it comes to the analysis and comparison between black and white hair done for this study, it was concluded that the results obtained had no significant difference between the light and dark hairs, except for a mild difference in Cr. With regards to the whole study, it seemed that it was not required to focus and highlight the differences between different-coloured hair since the results achieved proved to have no effect on the heavy metal concentrations. Proof of this is that there have been no recent studies specifically orbiting around cattle coloured hair.

To summarise all the above ICP-OES results that have been concluded in this study, table 6.1 summarises the correlation between the highest and lowest concentrations of each element and the seasonal effect within a cow's life cycle:

Table 6.1: Correlation between the highest and lowest monthly concentrations for each type of sample

	Fodder		**Water***		**Manure***		**Milk**		**Hair**	
	Highest	Lowest	Highest	Lowest	Highest	Lowest	Highest	Lowest	Highest	Lowest
Cr	January	May	May	November	March	August	November	Sep/Oct/Dec	June	August
Cu	January	May	May	November	February	September	April	August	January	August
Zn	January	May	February	August	February	September	December	Jan/Mar	February	April
As	January	May	June	November	October	August	August	Jan/Feb	August	January
Se	May	January	July	February	May	October	October	January	June	January
Cd	**	**	May	November	February	August	April	Mar/Jul/Sept/Dec	January	July
Pb	January	May	July	November	February	August	April	Jan/Feb	June	January

**Moving average results.*

*** Fodder has no difference between the two periods.*

From table 6.1 above, it can be concluded that:

6.2.1 Chromium

- Chromium in fodder was found to be highest in during the first period of the study, in January. Therefore, a seasonal effect was mostly present in Winter.
- There was clear seasonal effect for chromium in water which was at an incline till it reached its highest during Spring, in May, and then proceeding to decline throughout the rest of the year.
- Chromium in manure was mostly affected seasonally during Winter, proceeding to decline for the rest of the year.
- The highest seasonal effect for chromium in milk was found in early Winter, in November. However, it was closely followed by the next highest concentration in late Spring, early Summer. Therefore, it seems that mild warm or cold temperatures have the most seasonal effect on Cr.
- Chromium in hair had the highest seasonal effect at the beginning of the Summer Season, in June, however had the lowest effect during August, during the strongest temperatures of Summer.

Therefore, in all the samples, Cr seems to be mostly affected during Winter, Spring and early Summer, concluding that its concentrations increase during cold or mild temperatures, but remain low during high temperatures during the year.

6.2.2 Copper

- Copper in fodder was found to be highest concentration during the first period of the study, in January. Therefore,a seasonal effect was mostly present in Winter.
- Copper in water had high concentrations throughout the first few months of the year, reaching its highest seasonal effect during Spring, in the month of May. This was succeeded by a decline in concentration for the remainder of the year.
- Seasonal effect of copper in manure was highest in Winter during February which was followed by a constant decline for the rest of the year.

- Seasonal effect of copper in milk was stable throughout the year, with the exception of the middle of Spring, reaching a higher concentration than the rest.
- The copper's seasonal affect in hair had up and down fluctuations with the highest affects found in Winter and early Summer.

Hence, Cu mostly had the highest seasonal effect during the seasons of Winter and Spring for all samples and was also high in early Summer in hair. It is interesting to note as well, that all the concentrations of milk were higher than the permissible limit of 0.01 ppm in accordance with WHO and FAO.

6.2.3 Zinc

- Zinc in fodder was found to be highest concentration during the first period of the study, in January. Therefore, a seasonal effect was mostly present in Winter.
- Seasonal effect of zinc in water started with the highest concentration during Winter, in February. It then proceeded to decline up until the middle of Summer, in August, in which it started to increase again up until it reached the second highest concentration in November. Therefore, seasonal effect was mostly present during the colder season of the year.
- Seasonal effect of zinc in Manure was almost at a stable concentration throughout the year, however seemed to have a significant effect during the end of Winter, in February.
- Zinc in milk had a wave-like seasonal effect throughout the year, with the highest concentration during early Winter, in December, followed by the middle of Summer, in July.
- Zinc in hair was found to be highest seasonal effect during Winter. The remainder of the year had stable levels of seasonal effect, with early Summer (June), having a slightly higher concentration.

Summarising, seasonal effect was predominant in all samples during the season of Winter, along with Milk and Hair having high levels during Summer as well.

6.2.4 Arsenic

- Arsenic in fodder was found to have the highest concentration during the first period of the study, in January. Hence, a seasonal effect was mostly present in Winter.
- Arsenic in water resulted in the concentration increasing from the beginning of the year, reaching its highest concentration during June, in which it then proceeded to decline till the end of year reaching its lowest in November. Therefore, seasonal effect was mostly present during Summer.
- Arsenic in manure had high seasonal effect from the beginning of the year which declined continuously until it reached its lowest point on August. Following this, it started to increase again, reaching its highest seasonal effect during October. Therefore, it can be concluded that a seasonal effect was higher during the colder seasons of the year, but declined as temperatures become warmer.
- Arsenic in milk had a stable seasonal effect throughout the year, mainly in Summer, in August, with the exception of the middle of Winter, in January and February in which concentrations were low.
- Arsenic in hair kept on increasing until it reached its highest seasonal effect in Summer, in August.

Hence, As, depending on the sample, had the most seasonal effect during Summer or Winter, with the majority being in Summer.

6.2.5 Selenium

- Selenium in fodder was found to have the highest concentration during the second period of the study, in May. Hence, a seasonal effect was mostly present in Spring.
- Selenium in water resulted in the concentration increasing from the lowest concentration in February, reaching its highest concentration in July, and then proceeded to decline till the end of year. Therefore, seasonal effect was mostly present during Summer.
- The concentration of selenium in manure kept on increasing from the beginning of the year, reaching its highest seasonal effects during Spring, in May, and then proceeded

to decline, reaching its lowest point in Autumn, in October. Therefore, seasonal effect in manure was mostly present during the mild warm temperatures of Spring.

- Selenium in milk presented with fluctuations throughout the year reaching its highest seasonal effect in Autumn, in October, whereas its lowest was in Winter, in January.
- Selenium in hair had mainly a higher seasonal effect during Spring and early Summer, reaching its highest concentration in June.

Hence, Se reached a seasonal effect during each season depending on the sample analysed, except for the season of Winter. The majority of elements were mostly seasonally affected in the warmer seasons of the year of Spring and Summer.

6.2.6 Cadmium

- With regards to cadmium in fodder, the results appeared to have no difference between the two periods, which means seasonal effect could not be clearly identified.
- Cadmium concentration in Water kept on increasing, reaching its highest seasonal effect during Spring, in May, to which it then proceeded to decline again, reaching its lowest during the end of Autumn, in November. Therefore, it was mostly present during the warmer temperatures of Spring.
- Cadmium in manure had the most seasonal effect starting with the highest concentration in February and then started to decline reaching its lowest in August. This was then succeeded with the concentration increasing again. Therefore, this can conclude that seasonal effect is present at its most during the colder seasons of the year, especially Winter, but decreases when temperatures get warmer, being the lowest during Summer.
- Milk had concentration fluctuations throughout the year, with the highest seasonal effect being present during Spring, in April. It reached low concentrations during the second half of the year, with the exception of March which also resulted with low concentrations.
- Hair was found to have the highest seasonal effect during the cold season of Winter, which then decreased as temperatures being warmer during the seasons of Spring and Summer.

Therefore, it can be concluded that a seasonal effect in Cd was mostly present during the season of Spring, with the exception of Manure which was highest during Winter.

6.2.7 Lead

- Lead in fodder was found to be highest concentration during the first period of the study, in January. Hence, a seasonal effect was mostly present in Winter.
- Lead's concentration in water increased from the beginning of the year, reaching its highest seasonal effect in Summer, July, and then proceeded to decline reaching its lowest at the end of Autumn, in November. Hence, Pb seems to be mostly affected in Summer.
- Lead in manure reached an outcome of the highest seasonal effect being present in Winter, in February, whereas the lowest was during Summer, in August. The concentration was fluctuating in a wave-like trend throughout the year; therefore, it can be concluded that seasonal effect in manure is mostly present during the colder seasons of the year.
- Seasonal effect of lead in milk was low throughout the year with the lowest being in Winter, in January and February. However, it was still affective in Spring, during April, in which its concertation was higher than the rest.
- Hair's seasonal effect kept on increasing during the year, starting from the lowest concentration in Winter, January, till it reached its highest effect in Summer, June, to which it then declined again.

Therefore, Pb resulted in being affected all seasons, except for Autumn, depending on the sample analysed.

The proper way of digesting the organic matter portion of a sample has created a series of debates within the sample preparation aspect prior to metals determination. The two most common, and the two analysed for this study, have been the wet acid digesting using hot plate (conventional) and microwave oven procedures (Gorsuch *et al.*, 2017). By analysing concentrations of seven elements in milk using these two digestion methods and comparing them, it was concluded that good recovery was achieved for both conventional digestion and microwave digestion procedure, in which method 1 presented higher concentrations in all elements, except for As and Se which had higher concentrations in method 2. With regards to Zn, no changes were presented. Understanding the results of these two methods will allow the researchers to be knowledgeable and guided towards choosing the best method out of the two for each individual element.

When it comes to the analysis and comparison between black and white hair done for this study, it was concluded that the results obtained had no significant difference between the light and dark hairs, except for a mild difference in Cr. With regards to the whole study, it seemed that it was not required to focus and highlight the differences between different-coloured hair since the results achieved proved to have no effect on the heavy metal concentrations. Proof of this is that there have been no recent studies specifically orbiting around cattle coloured hair.

By focusing on the life cycle of cattle, all the methods used, and results achieved in this study have concluded that the fodder purchased for this farm contained the highest levels of heavy metal concentrations for all elements which resulted with high levels of concentration in the output as a whole, with the majority being present in manure, followed by hair and lastly, milk. Despite the fact that milk resulted with some of the elements having higher concentrations than the permissible limit, the overall outcome is positive, since the majority of the heavy metal concentrations settled in manure and hair, which do not affect the human food chain directly, whereas milk, which had the lowest concentration out of the three, does. In addition to this, water concluded to have the most beneficial results since those heavy metals that have an established permissible limit, were found to have a concentration that does not exceed that limit. Therefore, from this study, it can be concluded, that the life cycle of cattle was investigated to determine the presence of pollution in Malta. Although the majority of the fodder used for this study was imported from abroad, it was still slightly affected by air pollution in Malta. In addition to this, milk was also found to have concentrations higher than the permissible limit. The presence of such heavy metals indicates a high levels of pollution in Malta. Furthermore, despite the fact that manure having the highest concentrations for all the elements is a positive outcome for this study, it also raises a red flag towards the possibility of there being another type of pollution that is directly affecting Maltese crops, since non-treated manure is used as a fertilizer for these crops.

6.3 Future Works:

One of the main issues faced during the build-up of this study is the lack of time that was available. The results for manure samples faced fluctuations, while in the case of hair, the collection of samples could only last for eight months. Had the research had a longer period, more accurate results could have been noted for both manure and hair, which would have aided in achieving a more comprehensive result. This would have also been an advantage towards seasonal effect as more samples could have been compared between the different seasons rather than a comparison done within a more limited time space of approximately one year.

According to the results obtained from the samples of both the essential and toxic elements used for this study, some heavy metals were found to have a higher concentration in the intake than in the output samples, which is suggestive that for future reference, other parts of the cattle can be looked into, such as the liver, kidneys and meat, as well as blood and urine, providing a possible explanation as to why the concentration levels were higher in intake than in output samples.

In addition to this, some elements were found to have high levels of concentration, which is of concern, and therefore would be beneficial if a study involving the air pollution in Malta is done.

Furthermore, it was noticed that manure has been directly used as a fertilizer without any previous treatment which could lead to high levels of heavy metals. Therefore, it is recommended that a research should be carried out in Maltese crops farms in order to identify if crops in Malta contain any excessive concentrations of heavy metals.

When it comes to the high concentrations in milk samples, the Malta Dairy Products Ltd., who is in charge of Benna, and therefore the only milk provider in Malta, were contacted to understand in what way are the heavy metals being detected in milk. It was found out that they do not measure the heavy metal concentrations in milk. It would be interesting to determine the heavy metals concentrations in Benna milk since the majority of Malta’s resident are consumers of their products.

According to a study done by Li *et al*. (2013), it has shown that a difference between ICP-OES and AAS is present in which ICP-OES proved to have better sensitivity in comparison to the AAS instrument used for the study. This study done in Malta, was initially started using the AAS instrument, however due to a lack of resources, there was a switch to use of an ICP-OES had to start being used in order to proceed with the research. Keeping this in perspective, it is suggested that a comparative study is done between the two before starting the analysis of the samples in order to define which instrument would be more ideal for this type of analysis, in the case that both instruments are available at hand.

With regards to the two methods of sample preparation, Conventional and Micro-wave digestions methods, method 1 was found to be suitable for some elements, whereas method 2 was better for other elements. This leads to the suggestion that more elements should be examined in order to establish the best method for each individual element.

Appendix

Appendix 1-AAS

Table A.1 Copper concentration (milk, mg/L) for each week for 10 cows

Week	**Cow1**	**Cow2**	**Cow3**	**Cow4**	**Cow5**	**Cow6**	**Cow7**	**Cow8**	**Cow9**	**Cow10**
1	0.341	0.038	0.059	0.035	0.062	0.034	0.079	0.071	0.027	0.023
2	0.076	0.042	0.048	0.065	0.062	0.071	0.135	0.069	0.068	0.091
3	0.047	0.035	0.053	0.045	0.062	0.061	0.155	0.037	0.083	0.043
4	0.073	0.088	0.042	0.037	0.062	0.027	0.022	0.104	0.036	0.029
5	0.097	0.034	0.054	0.057	0.062	0.044	0.025	0.025	0.028	0.020
6	0.021	0.024	0.020	0.014	0.062	0.077	0.031	0.033	0.039	0.029
7	0.142	0.006	0.022	0.015	0.062	0.030	0.011	0.023	0.019	0.030
8	0.002	0.027	0.005	0.057	0.062	0.013	0.015	0.019	0.010	0.005
9	0.014	0.022	0.015	0.018	0.062	0.026	0.005	0.018	0.022	0.029
10	0.044	0.016	0.017	0.012	0.062	0.011	0.012	0.014	0.015	0.022
11	0.025	0.010	0.019	0.012	0.062	0.016	0.018	0.019	0.012	0.007

Table A.2 Copper concentration (hair, mg/Kg) for each week for 10 cows

Week	**Cow1**	**Cow2**	**Cow3**	**Cow4**	**Cow5**	**Cow6**	**Cow7**	**Cow8**	**Cow9**	**Cow10**
1	1.85	1.13	4.17	1.01	1.61	1.49	2.72	3.20	1.53	1.01
2	3.23	3.17	4.11	0.722	6.04	5.03	3.75	7.37	3.72	3.88
3	4.10	4.05	6.24	4.77	11.00	3.87	3.84	3.92	33.6	5.56
4	2.62	6.88	7.02	5.69	6.51	6.92	5.22	6.35	4.14	5.24
5	20.91	5.86	4.22	6.93	5.45	4.91	6.56	1.94	3.99	3.72
6	2.68	6.08	4.12	7.22	2.60	4.04	5.44	9.87	6.33	3.73
7	4.42	3.01	3.45	4.38	6.16	4.14	5.47	5.62	2.97	5.18
8	4.20	2.73	6.24	3.82	5.53	3.42	2.64	4.97	5.06	2.47
9	6.98	3.34	4.63	0.416	0.494	3.27	4.74	4.40	3.86	2.10
10	12.7	5.50	3.36	6.34	2.97	3.44	3.05	2.39	2.70	3.05
11	3.12	2.98	3.61	2.97	2.04	2.60	3.07	3.18	2.86	2.71

Copper concentration results of various types of feed are listed by feed type and weeks in Table A.3 The numbers allocated to weeks in the table refers to the number of weeks when fodder was collected. The period of a week was adopted to illustrate the time of collection of the samples and relate them to the seasonal effect.

Table A. 3 Mean Cu concentration in fodder (ppm)

Fodder type	Weeks	Cu concentration (mg/kg)	Mean ± SD
Imported hay	**2**	**5.79**	**4.93±0.751**
	9	**4.49**	
	10	**4.50**	
Maltese hay	**1**	**3.80**	**3.73±0.224**
	3	**3.48**	
	8	**3.91**	
Grounded maize (mixed corn)	**1**	**10.4**	**9.81±0.774**
	7	**9.27**	
Maize (small pellets)	**8**	**6.74**	**7.33±0.837**
	11	**7.93**	
Large pellets	**2**	**3.43**	**2.83±0.844**
	6	**2.24**	
Mixed seeds	**3**	**1.03**	**1.08±0.069**
	5	**1.13**	
Dairy pellets	**4**	**16.1**	**16.1±0.000**

Appendix 2.1- ICP

The following Figures show a plot for concentration of (Cr, Cu, Zn, As, Se, Cd and Pb) for the ICP.

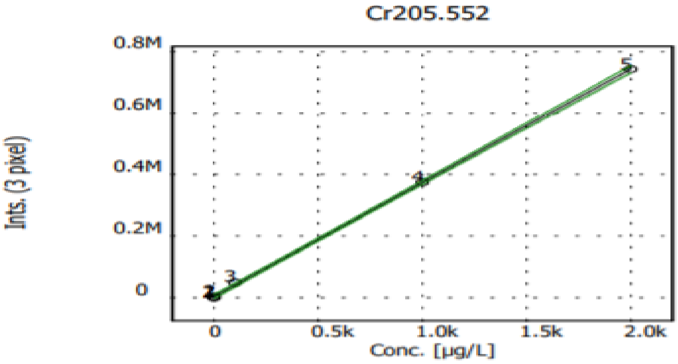

Figure 6Calibration curve of Cr

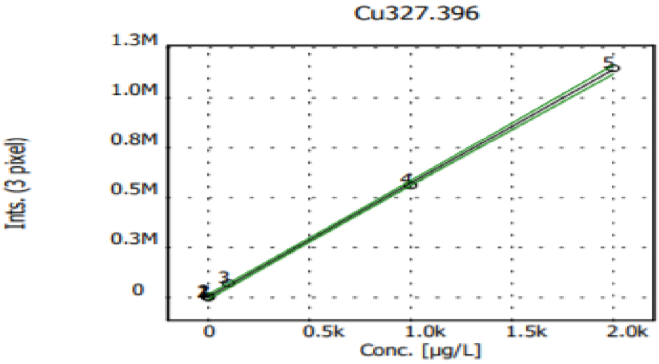

Figure A.2 Calibration curve of Cu

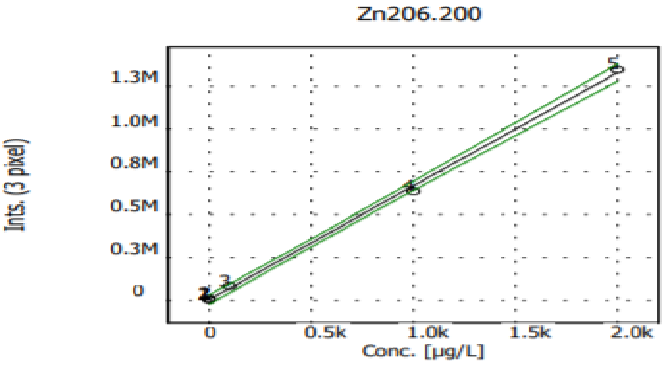

Figure 7Calibration curve of Zn

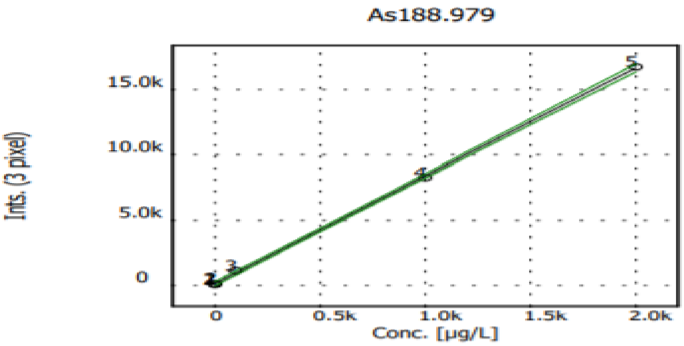

Figure A.4 Calibration curve of As

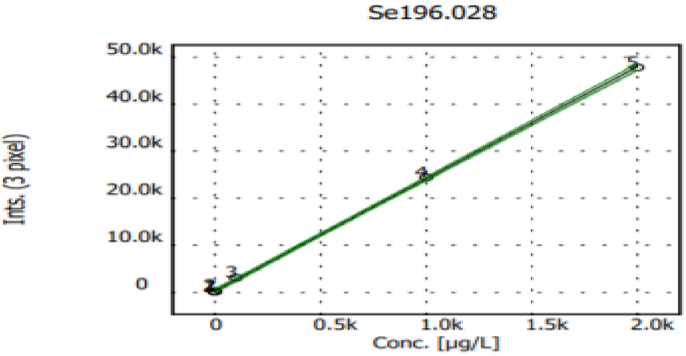

Figure A.5 Calibration curve of Se

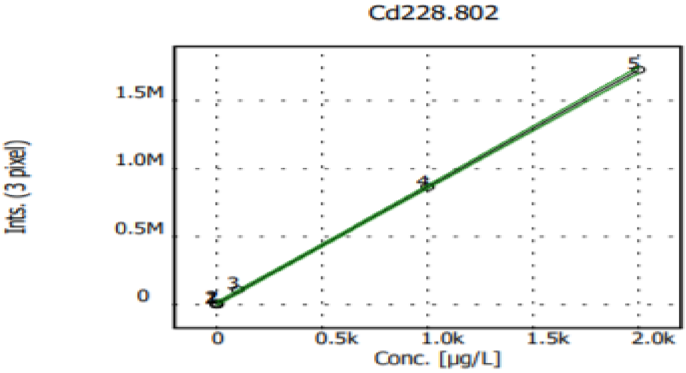

Figure A.6 Calibration curve of Cd

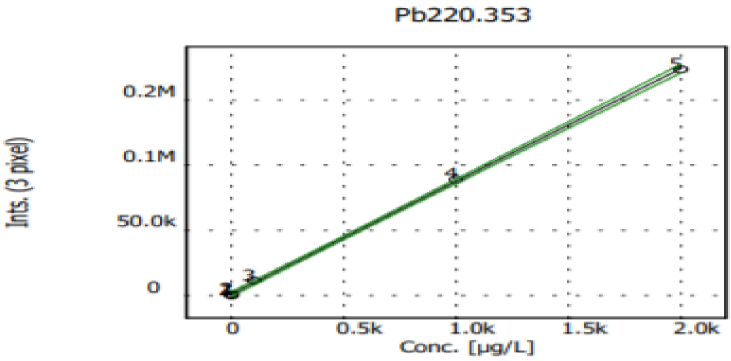

Figure A.7 Calibration curve of Pb

Table A.4 Milk concentrations for all elements in January (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
1.1*	0.007	0.017	2.83	0.002	0.007	0.011	0.006
1.2	0.007	0.014	2.84	0.002	0.006	0.009	0.006
1.3	0.007	0.031	3.97	0.002	0.007	0.011	0.004
1.4	0.007	0.016	3.25	0.002	0.006	0.010	0.005
1.5	0.011	0.023	2.84	0.003	0.009	0.016	0.005
1.6	0.012	0.015	3.67	0.004	0.007	0.013	0.007
1.7	0.006	0.031	4.03	0.004	0.009	0.012	0.007
1.8	0.007	0.030	3.09	0.002	0.007	0.012	0.007
1.9	0.006	0.020	2.65	0.003	0.008	0.011	0.005
1.10	0.006	0.024	3.66	0.003	0.008	0.012	0.004

*1.1: January first sample.

Table A.5 Milk concentrations for all elements in February (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
2.1*	0.010	0.021	4.17	0.0028	0.008	0.014	0.006
2.2	0.009	0.027	3.50	0.0029	0.008	0.014	0.006
2.3	0.026	0.045	2.43	0.0029	0.007	0.021	0.008
2.4	0.010	0.034	3.66	0.0031	0.008	0.016	0.007
2.5	0.008	0.013	4.02	0.0029	0.007	0.013	0.007
2.6	0.009	0.032	3.04	0.0027	0.007	0.012	0.005
2.7	0.014	0.035	4.23	0.0029	0.007	0.013	0.006
2.8	0.007	0.016	3.76	0.0032	0.008	0.012	0.007
2.9	0.007	0.025	2.93	0.0030	0.008	0.013	0.007
2.10	0.006	0.020	4.17	0.0034	0.008	0.012	0.006

*2.1: February first sample.

Table A.6 Milk concentrations for all elements in March (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
3.1*	0.007	0.017	2.83	0.082	0.095	0.005	0.025
3.2	0.007	0.014	2.84	0.079	0.086	0.004	0.018
3.3	0.007	0.031	3.97	0.081	0.102	0.005	0.023
3.4	0.007	0.016	3.25	0.092	0.089	0.004	0.020
3.5	0.011	0.023	2.84	0.139	0.126	0.007	0.030
3.6	0.012	0.015	3.67	0.128	0.108	0.006	0.030
3.7	0.006	0.031	4.03	0.149	0.129	0.006	0.028
3.8	0.007	0.030	3.09	0.097	0.101	0.005	0.022
3.9	0.006	0.020	2.65	0.121	0.109	0.005	0.018
3.10	0.006	0.024	3.66	0.121	0.118	0.005	0.027

*3.1: March first sample.

Table A.7 Milk concentrations for all elements in April (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
4.1*	0.012	0.151	5.89	0.106	0.068	0.011	0.155
4.2	0.013	0.116	3.72	0.102	0.070	0.007	0.083
4.3	0.013	0.148	4.17	0.097	0.064	0.009	0.122
4.4	0.020	0.155	4.53	0.110	0.074	0.009	0.108
4.5	0.023	0.230	8.60	0.138	0.116	0.057	0.569
4.6	0.027	0.224	4.20	0.115	0.069	0.010	0.114
4.7	0.032	0.424	4.06	0.104	0.070	0.019	0.202
4.8	0.024	0.161	3.19	0.116	0.064	0.006	0.076
4.9	0.024	0.277	4.75	0.118	0.079	0.034	0.311
4.10	0.032	0.243	4.30	0.111	0.076	0.007	0.094

*4.1: April first sample.

Table A.8 Milk concentrations for all elements in May (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
5.1*	0.010	0.027	3.77	0.127	0.105	0.006	0.037
5.2	0.009	0.022	4.04	0.133	0.122	0.006	0.038
5.3	0.007	0.046	5.54	0.120	0.110	0.005	0.035
5.4	0.009	0.024	3.49	0.130	0.110	0.007	0.045
5.5	0.009	0.040	3.19	0.150	0.114	0.005	0.038
5.6	0.006	0.094	3.30	0.115	0.093	0.005	0.031
5.7	0.077	0.048	3.26	0.138	0.109	0.022	0.084
5.8	0.117	0.051	3.04	0.123	0.125	0.033	0.122
5.9	0.014	0.021	3.03	0.138	0.108	0.006	0.046
5.10	0.010	0.159	4.34	0.122	0.114	0.005	0.036

*5.1: May first sample

Table A.9 Milk concentrations for all elements in June(mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
6.1*	0.013	0.021	3.27	0.139	0.102	0.007	0.026
6.2	0.022	0.036	3.34	0.155	0.114	0.007	0.038
6.3	0.011	0.022	3.86	0.124	0.112	0.006	0.031
6.4	0.016	0.034	2.60	0.122	0.099	0.006	0.025
6.5	0.133	0.087	3.38	0.182	0.129	0.006	0.036
6.6	0.010	0.020	3.42	0.114	0.100	0.005	0.025
6.7	0.014	0.015	4.12	0.138	0.103	0.006	0.029
6.8	0.009	0.022	4.35	0.146	0.108	0.005	0.035
6.9	0.006	0.018	2.83	0.118	0.096	0.005	0.027
6.10	0.008	0.019	4.72	0.135	0.104	0.005	0.027

*6.1: June first sample

Table A.10 Milk concentrations for all elements in July(mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
7.1*	0.010	0.023	6.08	0.115	0.099	0.005	0.010
7.2	0.007	0.034	5.99	0.117	0.103	0.005	0.012
7.3	0.010	0.035	6.75	0.131	0.100	0.005	0.013
7.4	0.006	0.022	6.86	0.111	0.087	0.005	0.010
7.5	0.007	0.087	6.21	0.127	0.101	0.005	0.015
7.6	0.008	0.037	7.92	0.109	0.102	0.005	0.010
7.7	0.006	0.027	7.33	0.087	0.090	0.005	0.010
7.8	0.009	0.027	7.54	0.114	0.098	0.005	0.010
7.9	0.006	0.017	6.65	0.103	0.102	0.005	0.010
7.10	0.008	0.019	6.75	0.106	0.085	0.004	0.010

*7.1: July first sample.

Table A.11 Milk concentrations for all elements in August (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
8.1*	0.002	0.003	0.313	0.139	0.009	0.0004	0.002
8.2	0.001	0.002	0.331	0.010	0.007	0.0004	0.002
8.3	0.001	0.003	0.419	0.011	0.006	0.0003	0.002
8.4	0.001	0.002	0.214	0.018	0.009	0.0004	0.002
8.5	0.001	0.002	0.373	0.018	0.009	0.0004	0.002
8.6	0.001	0.007	0.443	0.018	0.008	0.0004	0.002
8.7	0.001	0.001	0.291	0.012	0.010	0.0004	0.002
8.8	0.001	0.002	0.290	0.010	0.006	0.0004	0.002
8.9	0.001	0.003	0.337	0.017	0.009	0.0004	0.002
8.10	0.001	0.003	0.251	0.012	0.008	0.0004	0.002

*8.1: August first sample

Table A.12 Milk concentrations for all elements in September (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
9.1*	0.007	0.058	0.313	0.099	0.089	0.005	0.026
9.2	0.007	0.023	4.73	0.125	0.113	0.006	0.026
9.3	0.005	0.003	3.15	0.011	0.095	0.005	0.023
9.4	0.006	0.036	3.28	0.090	0.097	0.005	0.028
9.5	0.007	0.066	2.37	0.118	0.111	0.006	0.026
9.6	0.004	0.008	0.05	0.071	0.058	0.004	0.009
9.7	0.007	0.028	2.94	0.095	0.096	0.004	0.022
9.8	0.005	0.017	4.14	0.077	0.112	0.005	0.025
9.9	0.008	0.029	2.92	0.096	0.092	0.006	0.027
9.10	0.005	0.014	3.65	0.107	0.097	0.005	0.023

*9.1: September first sample

Table A.13 Milk concentrations for all elements in October (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
10.1*	0.006	0.032	4.08	0.106	0.109	0.006	0.027
10.2	0.005	0.024	3.66	0.090	0.095	0.005	0.019
10.3	0.006	0.058	4.46	0.175	0.141	0.006	0.029
10.4	0.006	0.023	3.45	0.090	0.116	0.006	0.027
10.5	0.005	0.045	2.55	0.110	0.102	0.005	0.028
10.6	0.005	0.065	5.50	0.124	0.114	0.005	0.028
10.7	0.006	0.020	4.74	0.156	0.128	0.006	0.029
10.8	0.006	0.038	3.43	0.107	0.103	0.006	0.027
10.9	0.006	0.018	4.90	0.133	0.113	0.005	0.028
10.10	0.005	0.023	4.45	0.135	0.107	0.005	0.024

*10.1: October first sample

Table A.14 Milk concentrations for all elements in November (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
11.1*	0.011	0.023	5.15	0.129	0.130	0.006	0.025
11.2	0.003	0.050	6.49	0.055	0.044	0.003	0.012
11.3	0.008	0.100	3.20	0.091	0.103	0.004	0.019
11.4	0.215	0.032	3.77	0.193	0.109	0.058	0.115
11.5	0.011	0.087	3.11	0.112	0.125	0.007	0.034
11.6	0.015	0.021	3.92	0.088	0.086	0.006	0.021
11.7	0.010	0.020	4.74	0.123	0.125	0.006	0.031
11.8	0.008	0.040	4.01	0.119	0.118	0.006	0.029
11.9	0.007	0.020	5.22	0.087	0.100	0.004	0.028
11.10	0.007	0.062	3.92	0.117	0.101	0.005	0.024

*11.1: November first sample

Table A.15 Milk concentrations for all elements in December (mg/L).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
12.1*	0.006	0.058	7.74	0.126	0.115	0.004	0.013
12.2	0.009	0.021	7.11	0.100	0.100	0.005	0.012
12.3	0.005	0.037	5.79	0.097	0.108	0.005	0.009
12.4	0.005	0.021	7.29	0.114	0.111	0.005	0.011
12.5	0.005	0.020	8.32	0.116	0.120	0.005	0.013
12.6	0.006	0.014	7.87	0.121	0.111	0.005	0.011
12.7	0.006	0.009	10.31	0.118	0.113	0.005	0.011
12.8	0.006	0.014	7.19	0.105	0.116	0.005	0.013

*12.1 December first sample

Looking at the milk figures below, the first matter to note is that all elements had the lowest concentration during the month of August. However, it is important to note that during the analysis of the August sample, the equipment used (ICP- OES), was damaged and repaired. Therefore, there might be a possible difference in the analysis; hence why all elements resulted in low concentrations in August.

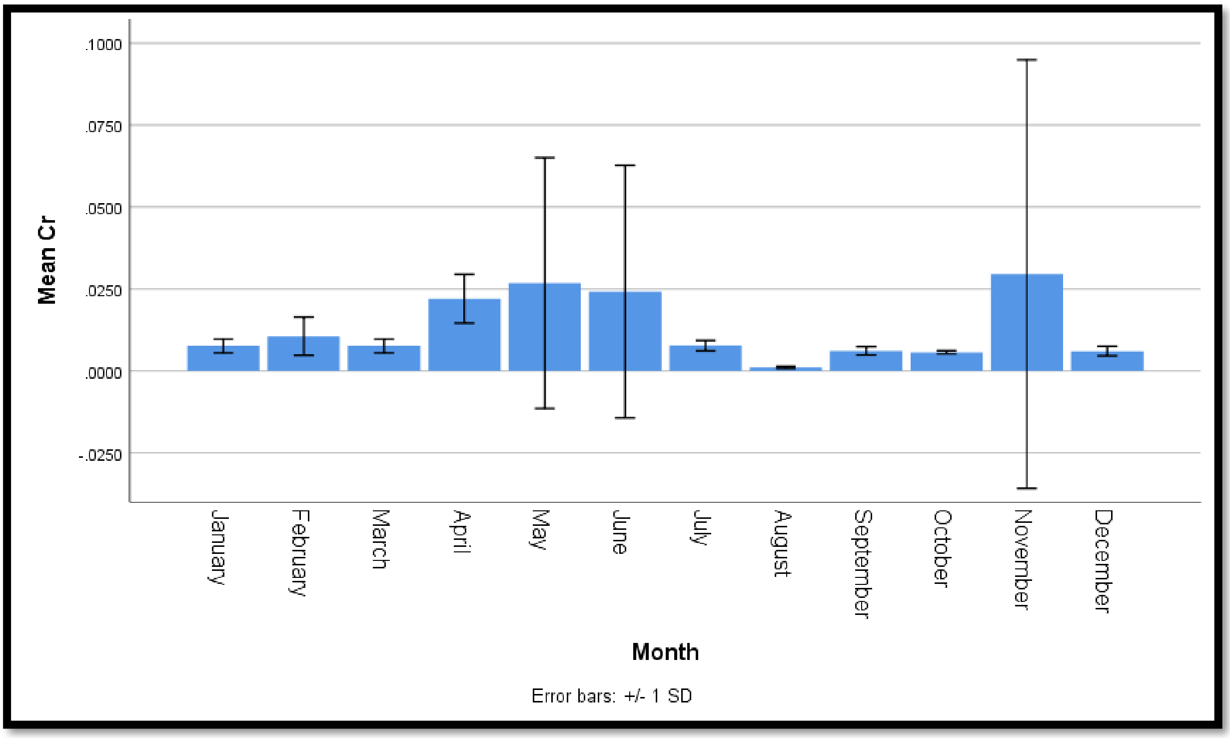

Figure A.8 Error bar for Cr (mg/L) by Month

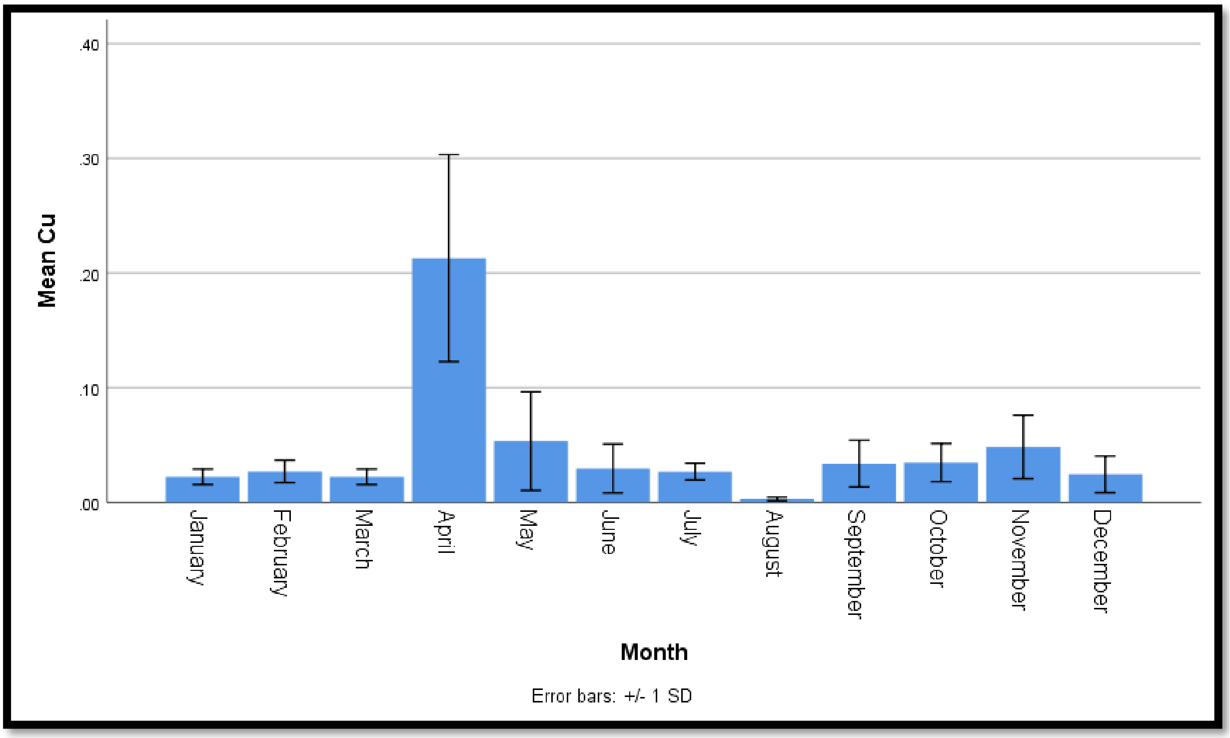

Figure A.9: Error bar for Cu (mg/L) by Month

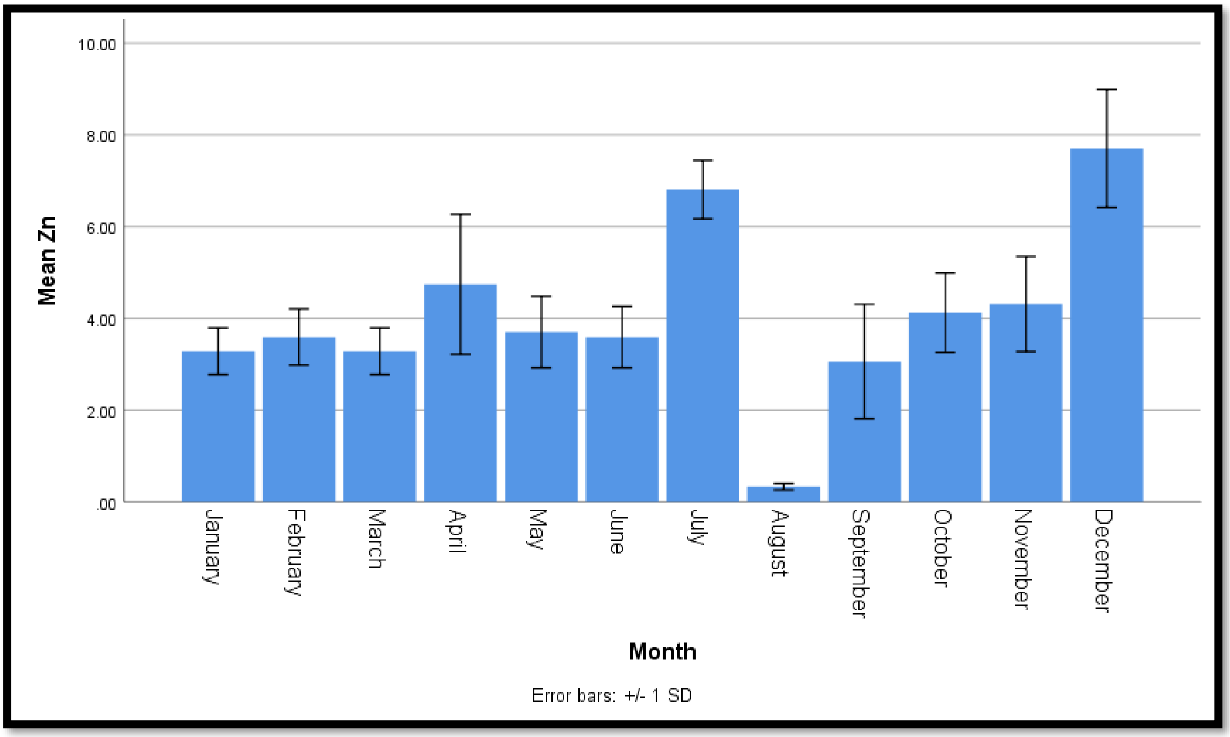

Figure A.10: Error bar for Zn (mg/ L) by Month

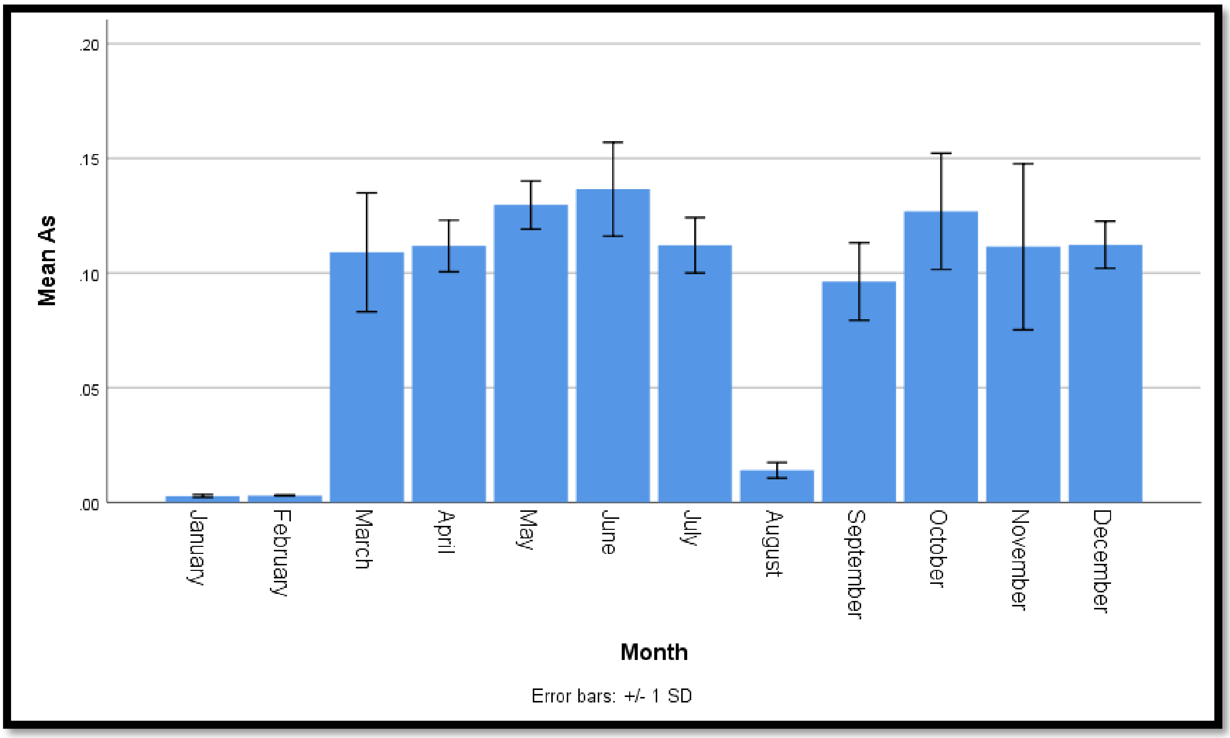

Figure A.11 Error bar for As (mg/ L) by Month

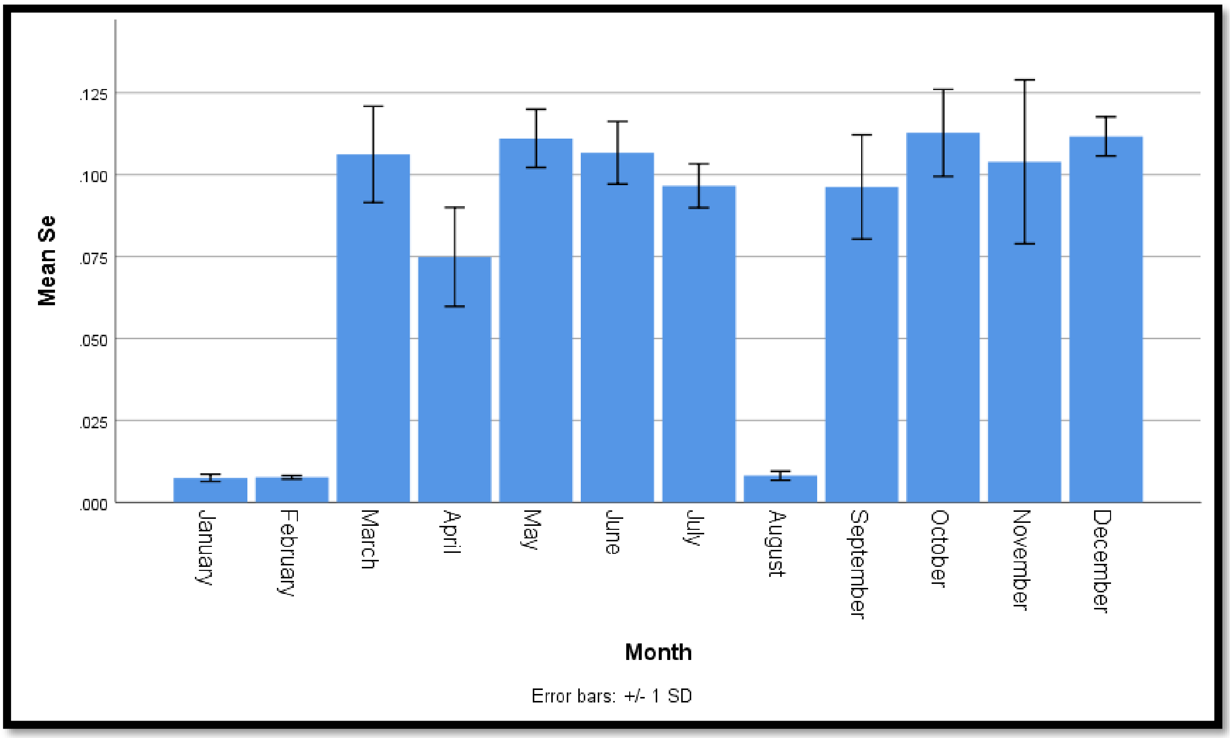

Figure A.12 Error bar for Se (mg/ L) by Month

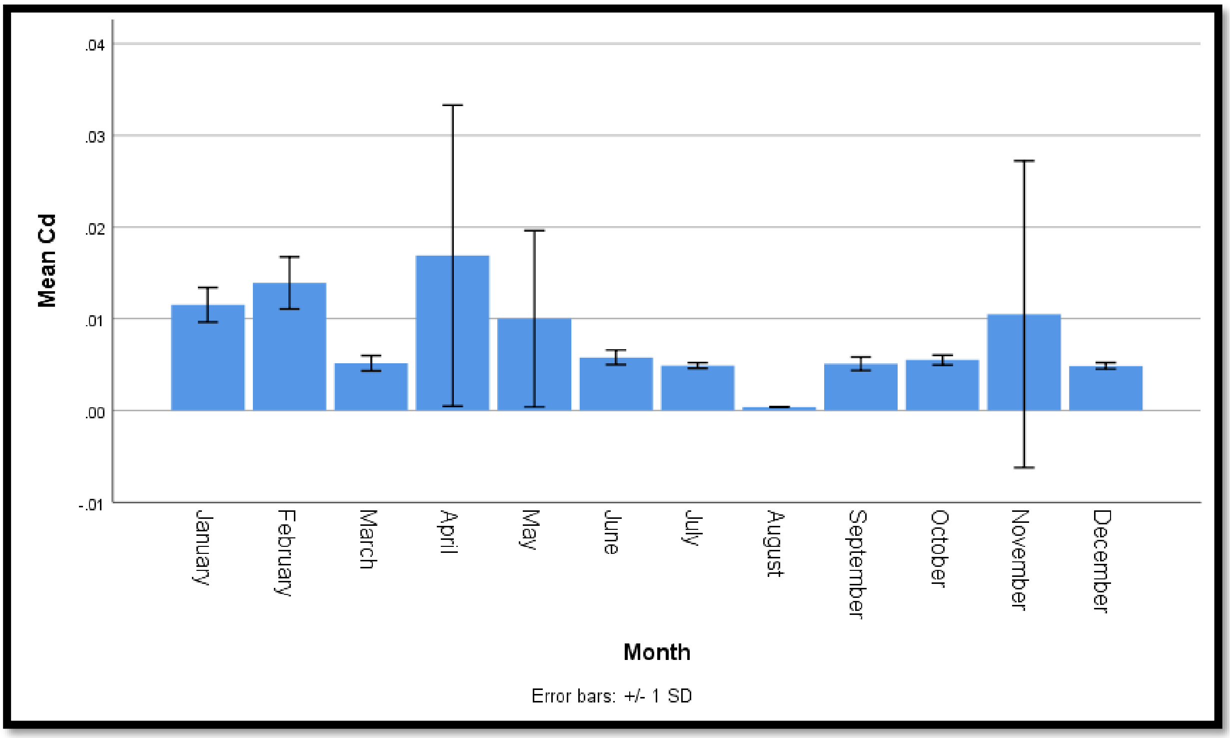

Figure A.13 Error bar for Cd (mg/ L) by Month

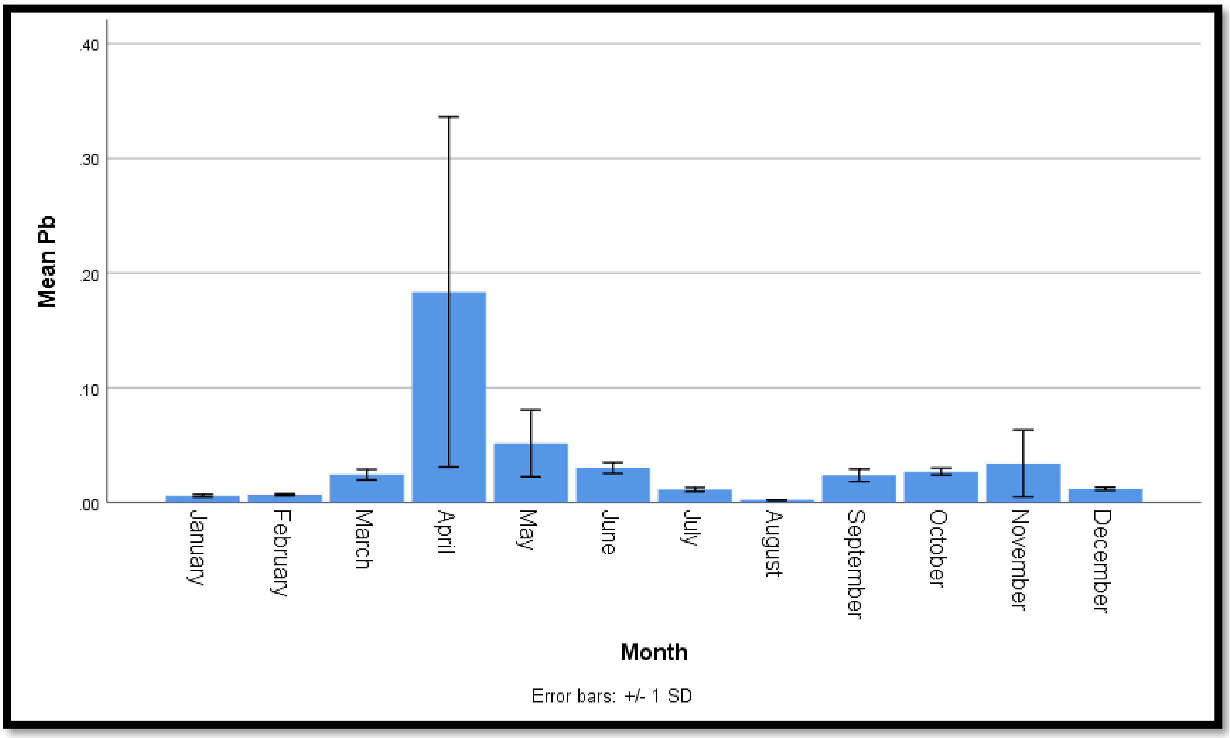

Figure A.14 Error bar for Pb (mg/ L) by Month

Table A.16 Hair concentrations for all elements in January (μg/g).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
1.1*	0.461	11.9	165	0.040	0.133	0.191	0.142
1.2	0.442	11.8	181	0.038	0.125	0.191	0.167
1.3	0.329	10.0	124	0.039	0.130	0.176	0.110
1.4	0.384	10.8	147	0.039	0.120	0.179	0.117
1.5	0.341	10.0	45.3	0.042	0.129	0.201	0.111
1.6	0.295	9.72	135	0.038	0.119	0.202	0.110
1.7	0.286	10.2	150	0.039	0.128	0.187	0.109
1.8	0.313	11.2	164	0.038	0.127	0.183	0.101
1.9	0.373	13.5	185	0.037	0.129	0.194	0.135
1.10	0.306	11.2	171	0.041	0.130	0.195	0.127

*1.1: January first sample.

Table A.17 Hair concentrations for all elements in February (μg/g).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
2.1*	0.412	15.0	188	0.033	0.121	0.198	0.131
2.2	0.352	8.05	120	0.032	0.121	0.162	0.085
2.3	0.327	9.25	159	0.040	0.126	0.165	0.157
2.4	0.283	9.10	136	0.041	0.118	0.187	0.122
2.5	0.424	13.5	179	0.031	0.129	0.168	0.206
2.6	0.365	10.0	13	0.043	0.116	0.187	0.095
2.7	0.310	9.10	127	0.041	0.120	0.163	0.095
2.8	0.291	10.0	149	0.031	0.127	0.178	0.120
2.9	0.418	13. 8	150	0.036	0.136	0.199	0.131
2.10	0.378	0.380	149	0.041	0.134	0.127	0.154

*2.1: February first sample.

Table A.18 Hair concentrations for all elements in March (µg/g).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
3.1*	0.007	0.017	2.83	0.082	0.095	0.005	0.025
3.2	0.007	0.014	2.84	0.079	0.086	0.004	0.018
3.3	0.007	0.031	3.97	0.081	0.102	0.005	0.023
3.4	0.007	0.016	3.25	0.092	0.089	0.004	0.020
3.5	0.011	0.023	2.84	0.139	0.126	0.007	0.030
3.6	0.012	0.015	3.67	0.128	0.108	0.006	0.030
3.7	0.006	0.031	4.03	0.149	0.130	0.006	0.028
3.8	0.007	0.030	3.09	0.097	0.102	0.005	0.022
3.9	0.006	0.020	2.65	0.121	0.109	0.005	0.018
3.10	0.006	0.024	3.66	0.122	0.118	0.005	0.027

*3.1: March first sample

Table A.19 Hair concentrations for all elements in April (µg/g).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
4.1*	0.312	6.13	87.2	0.951	1.33	0.065	0.408
4.2	0.337	5.52	78.2	0.984	1.40	0.061	0.414
4.3	0.394	5.21	72.2	1.02	1.44	0.068	0.448
4.4	0.356	6.79	94.2	0.890	1.38	0.068	0.108
4.5	0.240	4.85	65.8	1.21	1.46	0.065	0.297
4.6	0.296	4.88	62.9	1.11	1.37	0.057	0.288
4.7	0.334	5.28	87.7	1.09	1.44	0.065	0.202
4.8	0.378	6.25	79.3	0.951	1.46	0.061	0.284
4.9	0.328	6.24	92.9	1.42	1.42	0.062	0.518
4.10	0.430	8.03	93.1	1.11	1.39	0.066	0.385

*4.1: April first sample

Table A.20 Hair concentrations for all elements in May (µg/g).

Samples number	Cr	Cu	Zn	As	Se	Cd	Pb
5.1*	0.436	8.53	188	1.28	1.65	0.063	0.560
5.2	0.336	5.92	120	1.10	1.33	0.061	0.532
5.3	0.346	6.15	159	1.01	1.34	0.065	0.583
5.4	0.511	11.8	136	1.29	1.40	0.074	0.876
5.5	0.494	9.44	179	1.04	1.37	0.069	0.718
5.6	0.286	5.41	134	1.02	1.31	0.062	0.493
5.7	0.305	4.20	127	1.12	1.33	0.058	0.410
5.8	0.359	6.47	149	1. 10	1.32	0.068	0.567
5.9	0.575	9.55	150	1.06	1.43	0.073	0.662
5.10	0.392	8.07	149	0.100	1.45	0.067	0.488

*5.1: May first sample.

Table A.21 Hair concentrations for all elements in June (µg/g).

Samples Number	Cr	Cu	Zn	As	Se	Cd	Pb
6.1*	0.682	14.0	131	1.22	1.39	0.077	0.944
6.2	0.558	6.74	114	1.06	1.68	0.073	0.868
6.3	0.337	7.20	88.8	1.11	1.40	0.057	0.414
6.4	0.942	16.2	140	1.40	1.46	0.086	1.27
6.5	0.850	8.74	140	0.950	1.43	0.067	0.876
6.6	0.358	7.26	98.6	1.14	1.46	0.064	0.509
6.7	0.739	15.4	140	1.18	1.50	0.078	1.02
6.8	0.361	4.29	76.8	1.20	1.42	0.055	0.402
6.9	0.897	15.2	164	1.60	1.39	0.073	0.927
6.10	1.50	12.6	154	1.34	1.19	0.070	0.966

*6.1: June first sample

Table A.22Hair concentrations for all elements in July (µg/g).

Samples number	**Cr**	**Cu**	**Zn**	**As**	**Se**	**Cd**	**Pb**
7.1*	0.354	7.19	80.5	0.913	1.30	0.059	0.401
7.2	0.305	7.75	74.3	0.910	1.32	0.057	0.336
7.3	0.987	10.0	110	1.19	1.34	0.066	0.683
7.4	0.629	8.14	92.6	1.21	1.16	0.055	0.476
7.5	0.388	5.12	60.3	0.927	1.22	0.057	0.349
7.6	0.667	11.0	99.7	1.26	1.13	0.062	0.646
7.7	0.228	4.24	57.2	1.25	1.23	0.054	0.241
7.8	0.277	6.28	74.8	1.17	1.17	0.052	0.316
7.9	0.273	5.33	77.0	1.18	1.11	0.057	0.276
7.10	0.339	6.76	79.5	1.21	1.08	0.050	0.497

*7.1: July first sample

Table A.23 Hair concentrations for all elements in August (µg/g).

Samples number	**Cr**	**Cu**	**Zn**	**As**	**Se**	**Cd**	**Pb**
8.1*	0.325	7.71	106	1.20	1.36	0.066	0.381
8.2	0.357	7.39	106	1.51	1.15	0.061	0.454
8.3	0.235	4.78	76.5	1.52	1.20	0.063	0.346
8.4	0.193	5.34	80.6	1.78	1.15	0.062	0.491
8.5	0.233	5.33	81.8	1.37	1.22	0.068	0.513
8.6	0.181	5.33	77.0	1.63	1.28	0.062	0.434
8.7	0.190	5.33	66.1	1.67	1.11	0.051	0.307
8.8	0.157	3.90	79.2	1.72	1.15	0.055	0.292
8.9	0.127	3.44	75.0	1.66	1.16	0.059	0.324
8.10	0.144	4.20	64.9	1.58	1.12	0.053	0.294

*8.1: August first sample

Table24 Contents for As, Cd, Cr, Cu, Pb, Se and Zn along the months (January– August) 2019, also imputed readings for (September – December) 2019.

	Cr	Cu	Zn	As	Se	Cd	Pb
January	0.350±0.060	10.9±1.33***	147±40.5**	0.040±0.000***	0.130±0.000***	0.190±0.010***	0.120±0.020***
February	0.360±0.050	9.81±4.07**	149±21.7***	0.040±0.001***	0.130±0.010***	0.180±0.010***	0.130±0.040***
March	0.390±0.140	6.06±1.24**	84.3±16.9**	1.05±0.100*	1.41±0.080***	0.070±0.002**	0.430±0.110*
April	0.340±0.050	5.92±0.99**	81.3±11.5	1.07±0.160†	1.41±0.040***	0.060±0.000**	0.380±0.080†
May	0.400±0.100**	7.56±2.32	98.2±16.2**	1.09±0.110*	1.39±0.100***	0.070±0.010*	0.590±0.130***
June	0.720±0.350***	10.8±4.36**	125±29.0†	1.22±0.180***	1.43±0.120***	0.060±0.010†	0.820±0.280***
July	0.440±0.240	7.13±6.70	80.6±16.4***	1.12±0.140**	1.21±0.090†	0.060±0.010***	0.420±0.150*
August	0.210±0.080***	5.16±1.39***	81.3±14.2***	1.56±0.170***	1.19±0.080*	0.060±0.010***	0.380±0.080†
September	**0.300**	**4.33**	**60.3**	**0.470**	**0.400**	**0.020**	**0.330**
October	**0.300**	**4.18**	**90.8**	**0.580**	**0.530**	**0.100**	**0.320**
November	**0.160**	**5.16**	**89.0**	**0.490**	**0.410**	**0.040**	**0.340**
December	**0.300**	**5.20**	**117**	**0.480**	**0.320**	**0.050**	**0.310**

*Statistically different from other monthly levels at $p<0.001$***, $p<0.01$**, $p<0.05$*, $p<0.1^{\dagger}$*
values in bold are means imputed through regression imputation for months not included in the analysis

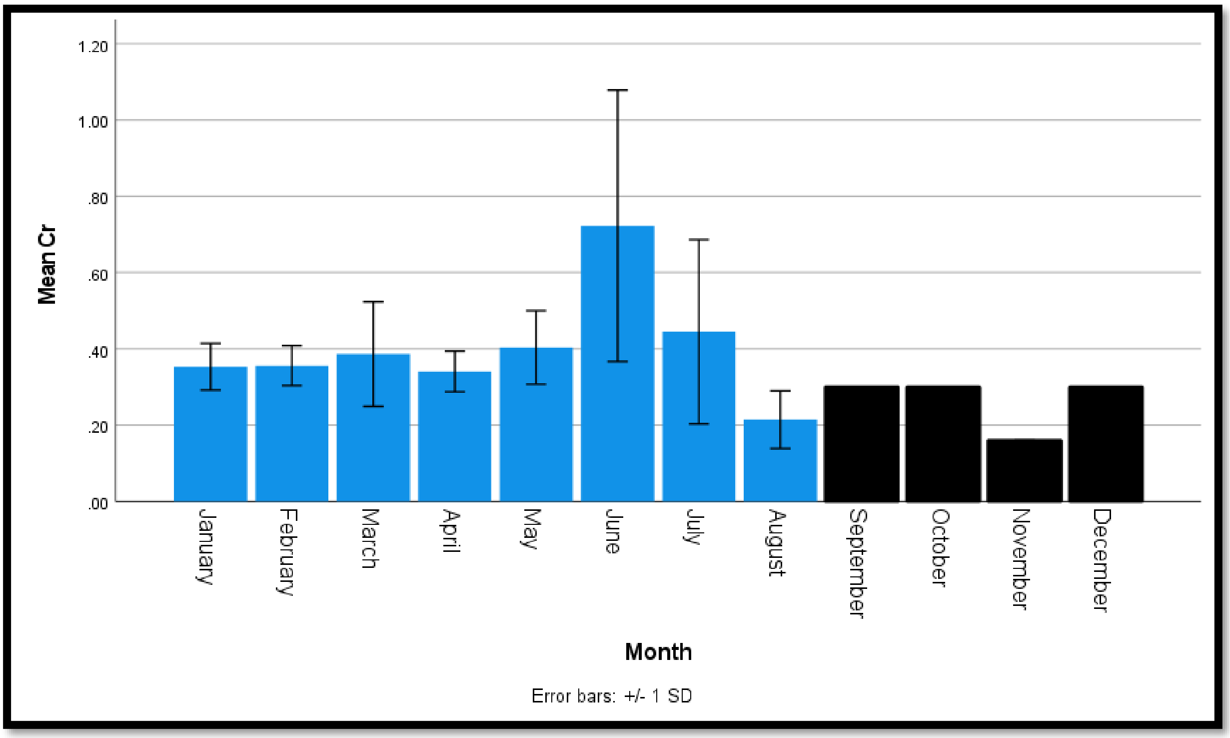

Figure A.14: Error bar for Cr (µg/g) in hair by Month included imputed readings for (September – December) 2019.

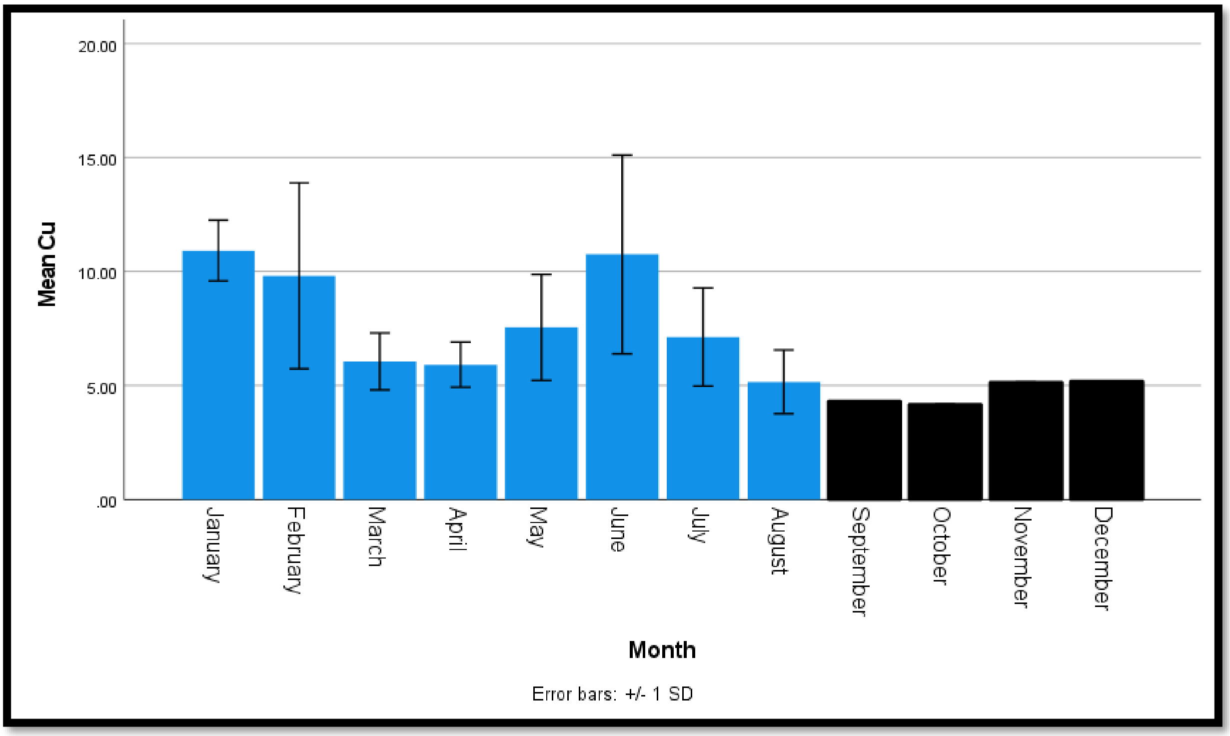

Figure A.15: Error bar for Cu (µg/g) in hair by Month included imputed readings for (September – December) 2019.

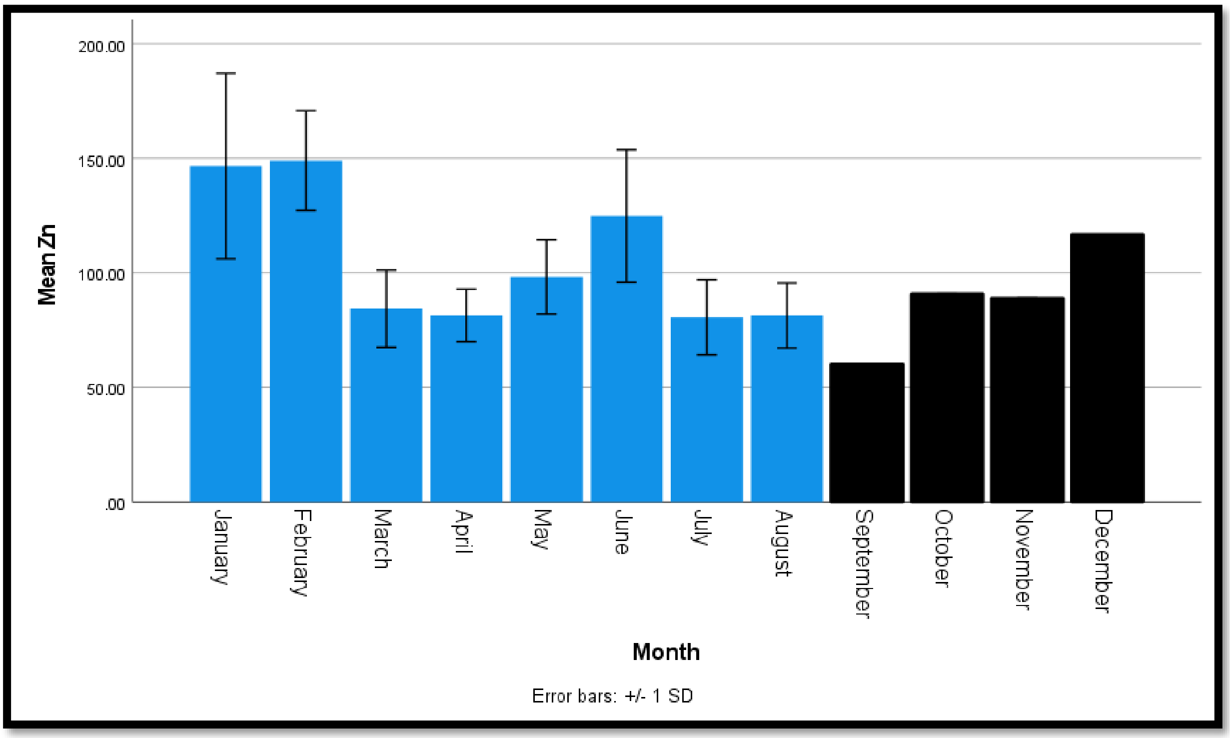

Figure A.16: Error bar for Zn (µg/g) in hair by Month included imputed readings for (September – December) 2019.

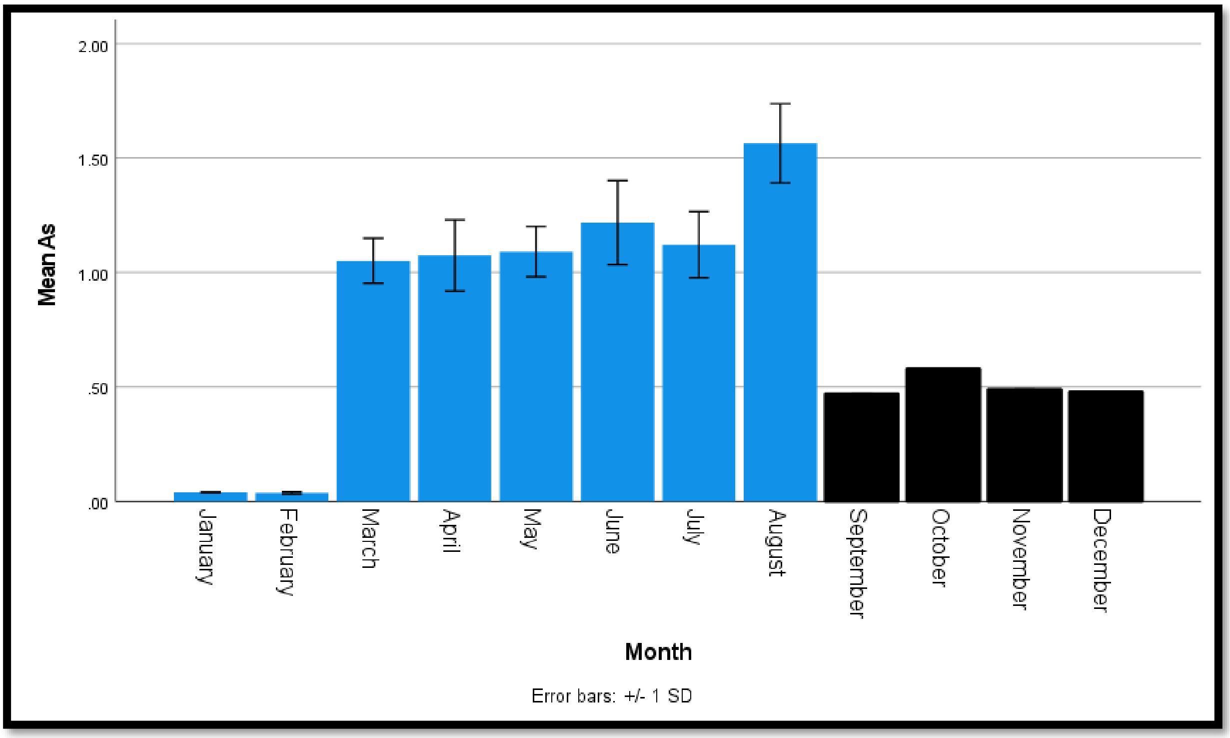

Figure A.17: Error bar for As(µg/g) in hair by Month included imputed readings for (September – December) 2019.

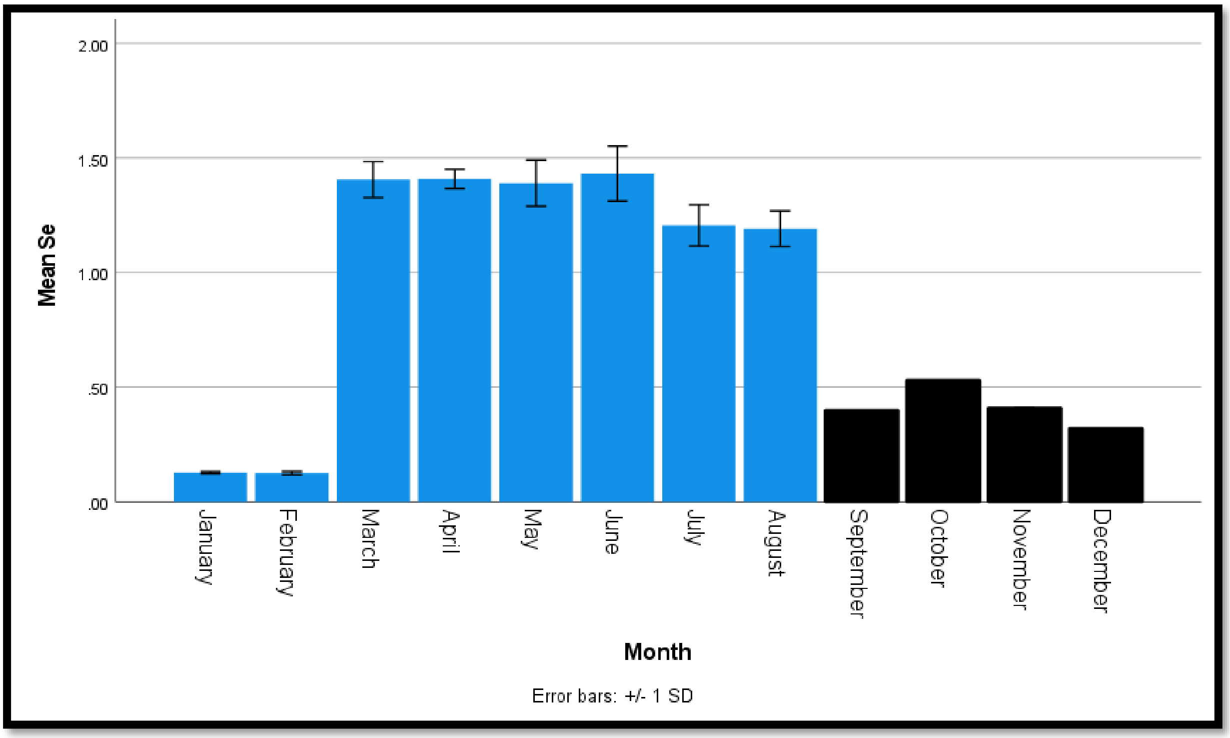

Figure A.18: Error bar for Se(µg/g) in hair by Month included imputed readings for (September – December) 2019.

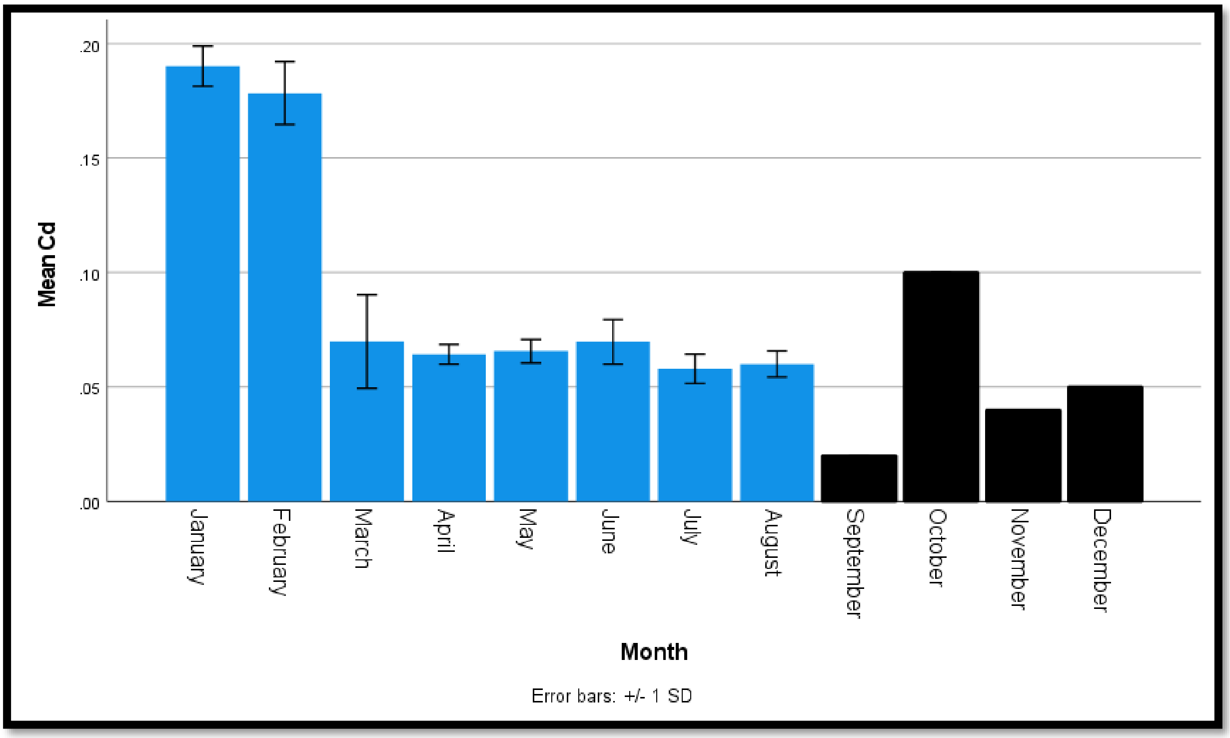

Figure A.19: Error bar for Cd (µg/g) in hair by Month included imputed readings for (September – December) 2019.

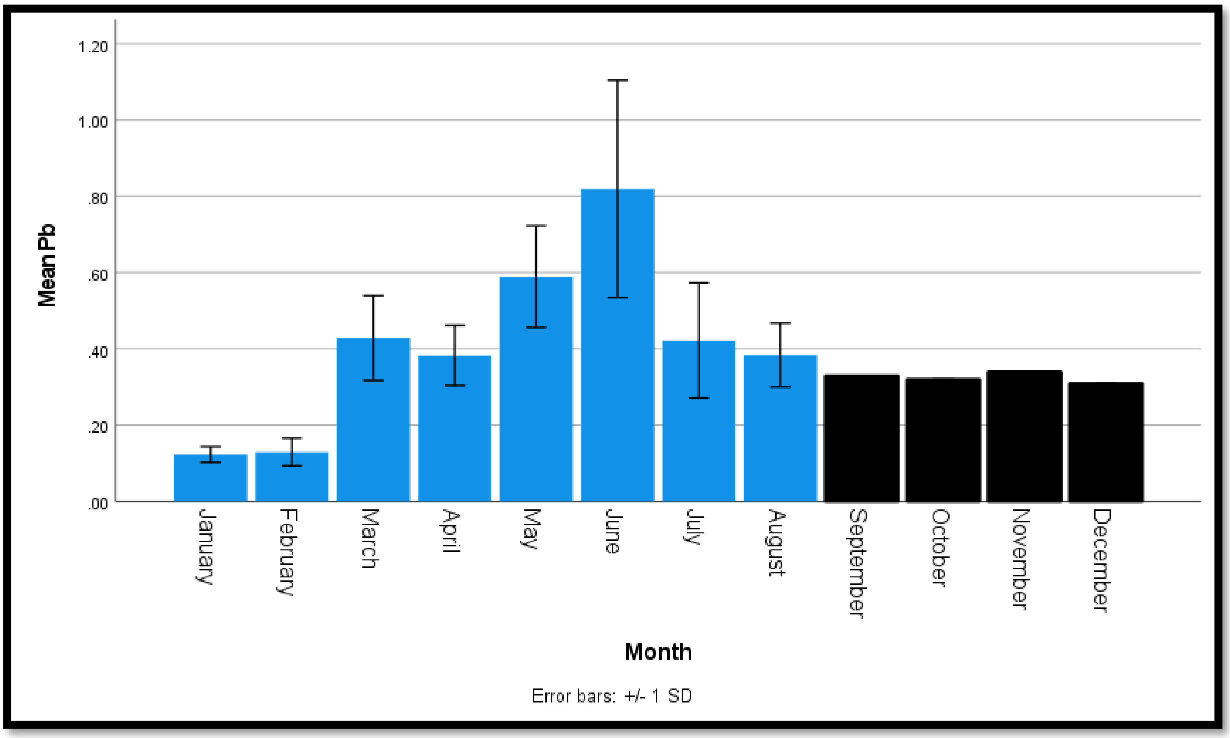

Figure A.20: Error bar for Pb (µg/g) in hair by Month included imputed readings for (September – December) 2019.

Appendix 2.2

The estimated daily intake and output were calculated from the instrument's software the concentration of the respective metal in a matrix was obtained. The values were then transformed into the actual intake or output per day per cow for each mineral.

- Mineral contents in manure samples were expressed as µg/g or mg/kg. This was obtained from

$$Mineral\ Content_{manure} = \left[\frac{Conc_{final} \times 25\ ml}{1000\ ml}\right] \times \frac{1\ g}{0.2\ g}\ \mu g/g$$

25 ml of final solution expressed as µg/L by the ICP, prepared from a 0.2 g sample.
An average cow produces 29.5 kg of manure per day (Van Horn *et al.*, 1994). The values were therefore multiplied by 29.5, and expressed as mg of mineral in manure per cow.

- Mineral contents in milk samples were expressed as mg/L. This was obtained from

$$Mineral\ Content_{milk} = \left[\frac{Conc_{final} \times 25\ ml}{1000\ ml}\right] \times \frac{1}{1000} \times \frac{1000ml}{3\ ml}\ mg/L$$

25 ml of final solution expressed as µg/L by the ICP, prepared from a 3 ml sample.
An average cow produces 25 L of milk per day (Agius *et al.*, 2019). The values were therefore multiplied by 25, and expressed as mg of mineral in milk produced per cow.

- Mineral contents in hair samples were expressed as µg/g. This was obtained from

$$Mineral\ Content_{hair} = \left[\frac{Conc_{final} \times 25\ \mathrm{ml}}{1000\ \mathrm{ml}}\right] \times \frac{1\ g}{0.2\ g}\ \mu\mathrm{g/g}$$

25 ml of final solution expressed as µg/L by the ICP, prepared from a 0.2 g sample.
A cow's surface area on average is 4.26 m^2 (i.e. 42,600 cm^2). (McDowell *et al.*, 1954). In different breeds, hair content varies. In Fresian and Holstein cows these are 9.7 and 18.2 mg/cm^2, respectively (Udo, 1978). An average was taken as these are common breeds in Malta. This leads to a total weight of hair per cow ranging between 0.4132 – 0.7753 kg. The metal contents were therefore multiplied by 0.5943 to obtain the content of the mineral, and expressed as mg of mineral in hair per cow.

- Mineral contents in water samples were expressed as µg/L. This was obtained from

$$Mineral\ Content_{water} = Conc_{final}\ \mu\text{g/L}$$

An average cow consumes 105 l of water (Ominski *et al.*, 2002). The values were therefore multiplied by 105 and divided by 1000, and expressed as mg of mineral in water consumed per cow.

- The dairy cows were provided a ration of normal dairy pellets (56.8%), mixed seeds and ground maize (9.4%), local hay (7.5%) and imported hay (26.6%). The metal contents in the individual feed/fodder types were expressed as mg/Kg.

$$Mineral\ Content_{feed\ or\ fodder} = \left[\frac{Conc_{final} \times 25\ \text{ml}}{1000\ \text{ml}}\right] \times \frac{1\ g}{0.5\ g}\ \mu\text{g/g (or mg/Kg)}$$

Each feed/fodder, the % dry matter was multiplied by percentage of feed/fodder in ration divided by 100 and then multiplied by 10 to obtain the content per kg, as follows:

$$Mineral\ Content_{feed\&fodder} = \left[\left(\frac{Mineral_{feed\ 1} \times \%\ in\ ration}{100}\right) + \left(\frac{Mineral_{fodder2} \times \%\ in\ ration}{100}\right) + \cdots\right] \times 10\ \text{mg/kg}$$

Feed/fodder intake is 13.5kg on average (NRC, 2001). The metal contents were therefore multiplied by 13.5 to obtain the content of the mineral and expressed as mg of mineral in feed per cow.

- Chromium concentrations and calcalation as explaned above in the appendix 2.2.

Table A. 25 The averags of Chromium concentrations in Fodder, water, manure, Milk and Hair for a whole year.

	January	February	March	April	May	June	July	August	September	October	November	December
Dairy pellets	0.449	0.449	0.449	0.449	0.425	0.425	0.425	0.425	0.425	0.425	0.425	0.425
Ground maize	0.097	0.097	0.097	0.097	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106
Mixed seeds	0.489	0.489	0.489	0.489	0.373	0.373	0.373	0.373	0.373	0.373	0.373	0.373
Maltese hey	0.961	0.961	0.961	0.961	0.868	0.868	0.868	0.868	0.868	0.868	0.868	0.868
Imported hey	1.03	1.03	1.034	1.03	0.721	0.721	0.721	0.721	0.721	0.721	0.721	0.721
Water μg/L	0.037	0.041	0.045	0.633	0.625	0.616	0.056	0.043	0.043	0.040	0.036	0.029
Manure mg/kg	60.9	59.7	5.517	81.0	4.18	3.13	1.644	1.09	2.74	6.106	3.012	3.969
Milk mg/L	0.007	0.009	0.004	0.018	0.009	0.012	0.008	0.251	0.006	0.006	0.009	0.006
Hair μg/g	0.353	0.356	0.387	0.340	0.404	0.722	0.445	0.214	0.302	0.301	0.165	0.299

Table A.26 Chromium content mg per cow per day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	88.3	88.3	88.3	88.3	73.0	73.0	73.0	73.0	73.0	73.0	73.0	73.0
Water	0.004	0.004	0.005	0.066	0.066	0.065	0.006	0.005	0.004	0.004	0.004	0.003
Manure	1797	1761	163	2389	123	92	48	32	81	180	89	117
Milk	0.164	0.223	0.092	0.462	0.234	0.300	0.193	6.279	0.153	0.146	0.221	0.139
Hair	0.210	0.212	0.230	0.202	0.240	0.429	0.264	0.127	0.179	0.179	0.098	0.178

Table A.27 Chromium inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	88.3	88.3	88.3	88.4	73.1	73.1	73.0	73.0	73.0	73.0	73.0	73.0
OUTPUTS	1797	1761	163	2390	124	93.0	49.0	38.4	81.3	181	89.2	117

- Copper concentrations and calcalation as explaned above in the appendix 2.2

Table A. 28 The averags of Copper concentrations in Fodder, water, manure, Milk and Hair for a whole year.

	January	February	March	April	May	June	July	August	September	October	November	December
Dairy pellets	17.3	17.3	17.3	17.3	18.6	18.6	18.6	18.6	18.6	18.6	18.6	18.6
Ground maize	2.48	2.48	2.48	2.48	1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12
Mixed seeds	11.2	11.2	11.2	11.2	7.34	7.34	7.34	7.34	7.34	7.34	7.34	7.34
Maltese hey	4.71	4.71	4.71	4.71	4.21	4.21	4.21	4.21	4.21	4.21	4.21	4.21
Imported hey	7.90	7.90	7.90	7.90	7.17	7.17	7.17	7.17	7.17	7.17	7.17	7.17
Water μg/L	0.76	0.77	0.796	1.30	1.26	1.20	0.991	0.931	0.828	0.565	0.134	0.126
Manure mg/kg	67.7	55.6	52.1	61.7	50.3	48.5	38.6	17.2	31.44	24.8	26.3	25.2
Milk mg/L	0.022	0.027	0.022	0.190	0.040	0.023	0.027	0.185	0.046	0.030	0.062	0.038
Hair μg/g	10.9	9.82	6.06	5.92	7.56	10.8	7.19	5.16	4.33	4.18	5.16	5.21

Table A. 29 Copper content mg per cow/ day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	85.8	85.8	85.8	85.8	83.2	83.2	83.2	83.2	83.2	83.2	83.2	83.2
Water	0.119	0.131	0.134	0.154	0.158	0.187	0.202	0.154	0.143	0.129	0.110	0.105
Manure	47.3	52.6	47.7	37.0	49.5	50.1	39.9	41.3	39.9	57.1	43.0	51.6
Milk	0.075	0.075	2.73	2.80	3.25	3.43	2.80	4.52	2.40	3.18	2.76	2.80
Hair	0.023	0.022	0.625	0.638	0.649	0.724	0.667	0.930	0.470	0.580	0.490	0.480

Table A. 30 Copper inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	86.0	86.0	86.0	86.0	83.4	83.4	83.4	83.4	83.4	83.4	83.3	83.3
OUTPUTS	47.4	52.7	51.0	40.5	53.4	54.3	43.4	46.8	42.8	60.9	46.2	54.9

- Zinc concentrations and calculation as explaned above in the appendix 2.2.

Table A.31 The average of Zinc concentrations in Fodder, water, manure, Milk and Hair for a whole year.

	January	February	March	April	May	June	July	August	September	October	November	December
Dairy pellets	70.6	70.6	70.6	70.6	55.7	55.7	55.7	55.7	55.7	55.7	55.7	55.7
Ground maize	12.1	12.1	12.1	12.1	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5
Mixed seeds	56.1	56.1	56.1	56.1	45.2	45.2	45.2	45.2	45.2	45.2	45.2	45.2
Maltese hey	15.3	15.3	15.3	15.3	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
Imported hey	23.8	23.8	23.8	23.8	21.9	21.9	21.9	21.9	21.9	21.9	21.9	21.9
Water µg/L	6.20	6.27	6.43	4.04	3.89	3.92	1.44	1.43	3.76	5.92	5.60	6.65
Manure mg/kg	224	280	276	230	181	214	179	91.0	147	107	224	160
Milk mg/L	3.28	3.59	3.28	4.74	3.70	3.35	6.81	6.26	3.39	4.12	4.31	7.33
Hair µg/g	147	149	84.3	81.3	98.2	125	80.6	81.3	60.3	90.8	89.0	117

Table A. 32 Zinc content mg per cow/ day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	7280	7280	7280	7280	5927	5927	5927	5927	5927	5927	5927	5927
Water	0.651	0.659	0.675	0.424	0.409	0.411	0.151	0.150	0.395	0.621	0.588	0.698
Manure	66170	8244	8128	6783	5327	6310	5270	2684	4336	3142	6608	4711
Milk	82.1	89.8	82.1	119	92.5	83.9	170	157	84.8	103	108	183
Hair	87.1	88.6	50.1	48.3	58.3	74.2	47.9	48.3	35.8	54.0	52.9	69.4

Table A. 33 Zinc inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	7280	7280	7280	7280	5927	5927	5927	5927	5927	5927	5927	5927
OUTPUTS	66339	8422	8261	6950	5478	6468	5488	2889	4456	3299	6768	4964

- Arsenic concentrations and calcalation as explaned above in the appendix 2.2.

Table A.34 The averags of Arsenic concentrations in Fodder, water, manure, Milk and Hair for a whole year

	January	February	March	April	May	June	July	August	September	October	November	December
Dairy pellets	0.503	0.503	0.503	0.503	0.483	0.483	0.483	0.483	0.483	0.483	0.483	0.483
Ground maize	0.464	0.464	0.464	0.464	0.533	0.533	0.533	0.533	0.533	0.533	0.533	0.533
Mixed seeds	0.580	0.580	0.580	0.580	0.503	0.503	0.503	0.503	0.503	0.503	0.503	0.503
Maltese hey	0.541	0.541	0.541	0.541	0.580	0.580	0.580	0.580	0.580	0.580	0.580	0.580
Imported hey	0.802	0.802	0.802	0.802	0.764	0.764	0.764	0.764	0.764	0.764	0.764	0.764
Water μg/L	1.14	1.24	1.28	1.46	1.51	1.78	1.93	1.46	1.36	1.23	1.04	1.00
Manure mg/kg	1.60	1.78	1.62	1.26	1.68	1.70	1.35	1.40	1.35	1.94	1.46	1.75
Milk mg/L	0.003	0.003	0.109	0.112	0.130	0.137	0.112	0.181	0.096	0.127	0.111	0.112
Hair μg/g	0.039	0.037	1.05	1.07	1.09	1.22	1.12	1.57				

Table A. 35 Arsenic content mg per cow/ day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	85.8	85.8	85.8	85.8	83.2	83.2	83.2	83.2	83.2	83.2	83.2	83.2
Water	0.119	0.131	0.134	0.154	0.158	0.187	0.202	0.154	0.143	0.129	0.110	0.105
Manure	47.3	52.6	47.7	37.0	49.5	50.1	39.9	41.3	39.9	57.1	43.0	51.6
Milk	0.075	0.075	2.73	2.80	3.25	3.43	2.80	4.52	2.40	3.18	2.78	2.80
Hair	0.023	0.022	0.625	0.638	0.649	0.724	0.667	0.930	0.470	0.580	0.490	0.480

Table A. 36Arsenic inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	86.0	86.0	86.0	86.0	83.4	83.4	83.4	83.4	83.4	83.4	83.3	83.3
OUTPUTS	47.4	52.7	51.0	40.5	53.4	54.3	43.4	46.8	42. 8	60.9	46.2	54.9

- Selenium concentrations and calcalation as explaned above in section 2.2.

Table A. 37The average of Arsenic concentrations in Fodder, water, manure, Milk and Hair for a whole year

	January	February	March	April	May	June	July	August	September	October	November	December
Dairy pellets	0.779	0.779	0.779	0.779	0.696	0.696	0.696	0.696	0.696	0.696	0.696	0.696
Ground maize	0.387	0.387	0.387	0.387	0.487	0.487	0.487	0.487	0.487	0.487	0.487	0.487
Mixed seeds	0.579	0.579	0.579	0.579	0.566	0.566	0.566	0.566	0.566	0.566	0.566	0.566
Maltese hey	0.388	0.388	0.388	0.388	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44
Imported hey	0.351	0.351	0.351	0.351	0.394	0.394	0.394	0.394	0.394	0.394	0.394	0.394
Water µg/L	0.294	0.312	0.319	0.450	0.468	0.472	0.497	0.501	0.338	0.352	0.315	0.316
Manure mg/kg	2.00	1.78	1.76	1.65	2.18	2.08	1.90	1.28	1.81	1.71	1.82	1.13
Milk mg/L	0.007	0.008	0.106	0.075	0.111	0.107	0.097	0.009	0.096	0.113	0.104	0.112
Hair µg/g	0.127	0.125	1.41	1.41	1.39	1.43	1.21	1.19				

Table A. 38 Selenium content mg per cow/ day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	88.3	88.3	88.3	88.3	85.1	85.1	85.1	85.1	85.1	85.1	85.1	85.1
Water	0.031	0.033	0.034	0.047	0.049	0.050	0.052	0.053	0.035	0.037	0.033	0.033
Manure	59.0	53.0	52.0	48.8	64.4	61.3	56.2	37.8	53.4	50.6	53.8	33.2
Milk	0.175	0.200	2.65	1.88	2.78	2.68	2.43	2.76	2.40	2.83	2.60	2.80
Hair	0.076	0.074	0.835	0.837	0.826	0.851	0.716	0.708	0.395	0.530	0.412	0.319

Table A.39 Selenium inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	88.4	88.4	88.4	88.4	85.2	85.2	85.2	85.2	85.2	85.2	85.2	85.2
OUTPUTS	59.3	53.3	55.4	51.5	68.0	64.9	59.3	41.2	56.2	53.9	56.8	36.3

- Cadmium concentrations and calcalation as explaned above in the appendix 2.2.

Table A.40 The average of Cadmium concentrations in Fodder, water, manure, Milk and Hair for a whole year

	January	February	March	April	May	June	July	August	September	October	November	December
Dairy pellets	0.046	0.046	0.046	0.046	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.048
Ground maize	0.017	0.017	0.017	0.017	0.023	0.023	0.023	0.023	0.023	0.023	0.023	0.023
Mixed seeds	0.087	0.087	0.087	0.087	0.077	0.077	0.077	0.077	0.077	0.077	0.077	0.077
Maltese hey	0.027	0.027	0.027	0.027	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026
Imported hey	0.028	0.028	0.028	0.028	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031
Water µg/L	0.015	0.017	0.019	0.620	0.585	0.570	0.021	0.021	0.022	0.019	0.019	0.018
Manure mg/kg	0.258	0.226	0.181	0.190	0.169	0.154	0.128	0.092	0.119	0.211	0.134	0.147
Milk mg/L	0.012	0.014	0.005	0.012	0.010	0.006	0.005	0.007	0.005	0.006	0.005	0.005
Hair µg/g	0.190	0.174	0.064	0.064	0.066	0.070	0.057	0.060				

Table A. 41 Cadmium content mg per cow/ day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	6.08	6.08	6.08	6.08	6.30	6.30	6.30	6.30	6.30	6.30	6.30	6.30
Water	0.002	0.002	0.002	0.065	0.061	0.060	0.002	0.002	0.002	0.002	0.002	0.002
Manure	7.60	6.68	5.35	5.62	5.00	4.55	3.76	2.71	3.52	6.22	3.95	4.34
Milk	0.300	0.350	0.125	0.300	0.250	0.150	0.125	0.167	0.125	0.150	0.125	0.125
Hair	0.113	0.103	0.038	0.038	0.039	0.042	0.034	0.036	0.029	0.104	0.041	0.052

Table A.42 Cadmium inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	6.09	6.09	6.09	6.15	6.36	6.36	6.30	6.30	6.30	6.30	6.30	6.30
OUTPUTS	8.02	7.13	5.52	5.96	5.29	4.74	3.92	2.91	3.67	6.47	4.11	4.52

- Lead concentrations and calcalation as explaned above in the appendix 2.2.

Table A.43 The averages of Lead concentrations in Fodder, water, manure, Milk and Hair for a whole year

	January	February	March	April	May	June	July	August	September	October	November	December
Fodder 1.1	0.174	0.174	0.174	0.174	0.142	0.142	0.142	0.142	0.142	0.142	0.142	0.142
Fodder 1.2	0.078	0.078	0.078	0.078	0.114	0.114	0.114	0.114	0.114	0.114	0.114	0.114
Fodder 1.3	0.181	0.181	0.181	0.181	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152
Fodder 1.4	0.152	0.152	0.152	0.152	0.141	0.141	0.141	0.141	0.141	0.141	0.141	0.141
Fodder 1.5	0.429	0.429	0.429	0.429	0.337	0.337	0.337	0.337	0.337	0.337	0.337	0.337
Water µg/L	0.111	0.134	0.172	0.187	0.172	0.702	0.714	0.657	0.099	0.089	0.084	0.081
Manure mg/kg	8.04	7.22	3.14	1.72	2.59	3.77	1.69	1.01	2.54	3.31	2.21	4.53
Milk mg/L	0.006	0.007	0.024	0.141	0.051	0.03	0.011	0.040	0.023	0.027	0.034	0.012
Hair µg/g	0.123	0.129	0.429	0.382	0.589	0.819	0.422	0.384				

Table A.44 Lead content mg per cow/ day for each month

	January	February	March	April	May	June	July	August	September	October	November	December
Feed	33.5	33.5	33.5	33.5	27.7	27.7	27.7	27.7	27.7	27.7	27.7	27.7
Water	0.012	0.014	0.018	0.020	0.018	0.074	0.075	0.069	0.010	0.009	0.009	0.009
Manure	237	213	92.7	50.7	76.5	111	49.8	30.0	74.8	97.8	65.1	134
Milk	0.150	0.175	0.600	3.525	1.275	0.750	0.275	1.008	0.575	0.675	0.850	0.300
Hair	0.073	0.077	0.255	0.227	0.350	0.487	0.251	0.228	0.331	0.324	0.338	0.304

Table A.45 Lead inputs and outputs for the whole year (mg per cow)

	January	February	March	April	May	June	July	August	September	October	November	December
INPUTS	33.5	33.5	33.5	33.5	27.8	27.8	27.8	27.8	27.7	27.7	27.7	27.7
OUTPUTS	238	213	93.6	54.5	78.2	112	50.3	31.2	75.7	98.8	66.3	134

Appendix 2.3. Validation method for ICP

The first step was to prepare a mixture as mentioned in section 2.3.3.4, then the following calculations were carried out for each element to ensure that the method used is valid. Milk was chosen for the validation method in this research. However, the matrix effect was eliminated as after digestion, matrices from different sources provided solely a solution of minerals. All samples were prepared in the form of a mineral solution, which were then analysed by either ICP or AAS. The F test was used to compare the theoretical value of the mean concentration of each element in W:QC with the actual concentration, from the results, it can be seen there is no significant difference at the 0.05 level are obtained between the two values for all the minerals in the research. Starting with Cr.

1. Chromium

Table A.55: Mean ± SD of Cr for M:W, M:QC and W:QC

		ug/L	ug/L	mean-SD	mean+SD	ug/ml	ug/ml	Average	Combined Average
75	M:W	72.9	76.4			0.608	0.636	0.622	
83	M:QC	88.3	88.5	77.8	99.0	0.648	0.825	0.737	0.784
149	W:QC	114	113			0.953	0.940	0.947	

Table A.56: LOD and LOQ for Cr

SD	LOD	LOQ
3.535534	0.03	0.094
SLOPE	384	

*F test P value = 0.893

2. Copper

Table A.57: Mean ± SD of Cu for M:W, M:QC and W:QC

	ug/L	ug/L	mean-SD	mean+SD	ug/ml	ug/ml		Combined Average
M:W	62.7	65.0			0.523	0.542	0.532	
M:QC	84.2	85.7	65.8	104	0.549	*0.867*	*0.708*	*0.771*
W:QC	121	122			1.00	1.02	1.01	

Table A.58: LOD and LOQ for Cu

SD	**LOD**	**LOQ**
6.36	0.034	0.112
SLOPE	580	

*F test P value = 1.000

3. Zinc

Table A.59: Mean ± SD of Zn for M:W, M:QC and W:QC

	ug/L	**ug/L**	**mean-SD**	**mean+SD**	**ug/ml**	**ug/ml**		**Combined Average**
M:W	1538	1570			12.8	13.1	13.0	
M:QC	875	878	702	1050	5.85	*8.75*	*7.30*	*7.01*
W:QC	130	128			1.09	1.06	1.07	

Table A.60: LOD and LOQ for Zn

SD	**LOD**	**LOQ**
58.0	0.251	0.836
SLOPE	709	

*F test P value = 0.431

4. Arsenic

Table A.61: Mean ± SD of AS for M:W, M:QC and W:QC

	ug/L	**ug/L**	**mean-SD**	**mean+SD**	**ug/ml**	**ug/ml**		**Combined Average**
M:W	64.2	79.0			0.535	0.658	0.597	
M:QC	89.9	86.0	47.6	128	0.397	*1.07*	*0.732*	*0.920*
W:QC	150	148			1.25	1.23	1.24	

Table A.62: LOD and LOQ for As

SD	**LOD**	**LOQ**
13.4	4.68	15.6
SLOPE	8.78	

*F test P value = 0.592

5. Selenium

Table A.63: Mean ± SD of Se for M:W, M:QC and W:QC

	ug/L	ug/L	mean-SD	mean+SD	ug/ml	ug/ml		Combined Average
M:W	94.4	89.6			0.787	0.746	0.767	
M:QC	128	129	111	145	0.928	*1.21*	*1.07*	*1.01*
W:QC	149	153			1.24	1.28	1.26	

Table A.64: LOD and LOQ for Se

SD	LOD	LOQ
5.66	0.643	2.14
SLOPE	26.8	

*F test P value = 0.932

6. Cadmium

Table A.65: Mean ± SD of Cd for M:W, M:QC and W:QC

	ug/L	ug/L	mean-SD	mean+SD	ug/ml	ug/ml		Combined Average
M:W	40.7	40.4			0.339	0.337	0.338	
M:QC	74.9	71.3	32.8	113	0.274	*0.945*	*0.609*	*0.575*
W:QC	97.9	97.0			0.816	0.808	0.812	

Table A.66: LOD and LOQ for Cd

SD	LOD	LOQ
13.43503	0.045	0.14161
SLOPE	916	

*F test P value = 0.361

7. Lead

Table A.67: Mean ± SD of Pb for M:W, M:QC and W:QC

	ug/L	ug/L	mean-SD	mean+SD	ug/ml	ug/ml		Combined Average
M:W	81.35	82.3			0.678	0.686	0.682	
M:QC	111	106	97.7	119	0.814	*0.991*	*0.903*	*0.840*
W:QC	123	117			1.02	0.973	0.997	

Table A.68: LOD and LOQ for Pb

SD	LOD	LOQ
3.536	0.117	0.391\
SLOPE	92.2	

*F test P value = 0.912

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