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Title:Evaluation of the Impacts of Heavy Metal Enriched Agricultural Soil on Shoot Length and Dry Mass of *Lolium perenne* var. *Ballica* in Central Chile

Abstract

Nutrient and metal concentrations in soils are impacted by both natural and anthropogenetic processes, including mining and agrochemicals (Fagnano et al., 2020; Burgos, 2017). Build-ups of certain nutrients and metals can lead to long-term pollution and toxic effects on local plant production (Neaman et al. 2020; [Yruela](https://www.scielo.br/j/bjpp/a/F43kT7jYFPygVtd86sLGBYx/?format=pdf&lang=en), 2005). As a major Cu-mining and agricultural producing country, Chile contains soils that exhibit a wide variety of nutrient and metal concentrations. In this study, we collect data on the toxic impacts of several nutrients on plant production, using *Lolium perenne* var. *Ballica* as a bioindicator. We predict that high levels of these contaminants lead to reduced shoot length and dry mass. We find that high levels of Zn, As, and P had significant negative impacts on *Ballica* biomass, while Cu, Pb, and N have positive impacts. We find that high levels of Cu in soil pore waters have positive impacts on *Ballica* shoot length, while alkaline pH levels in soil pore waters had negative impacts. Our models are subject to error in several ways, leading to some results that contradict previous literature. Some solutions for these inconsistencies are explored in the Discussion.

Introduction & Literature Review

The dynamics of heavy metal content in soils and their uptake by plants are influenced by many different factors. These factors vary based on the physicochemical properties of the soil and the nature of the contaminant (Agrelli et al., 2020). Both natural and anthropogenetic processes impact metal and nutrient concentrations in soils. The bioavailability of naturally occurring metals and nutrients in soils for plant uptake is subject to environmental effects including temperature, microorganisms, and precipitation (Gadd, 2000; Alloway, 2013). Anthropogenic sources of metals in agricultural soils include historical mining activities and the use of agrochemicals, such as pesticides and fertilizers (Fagnano et al., 2020; Burgos, 2017). Such contaminants can build up in the soil over time, leading to long-term heavy metal pollution and toxic effects on local plant production (Neaman et al. 2020; [Yruela](https://www.scielo.br/j/bjpp/a/F43kT7jYFPygVtd86sLGBYx/?format=pdf&lang=en), 2005).

Copper (Cu), for example, is an essential element of many terrestrial and aquatic plants as it is required for many physiological processes, including protein and enzyme activities (Trevors & Cotter, 1990). However, excess concentrations of copper are toxic to many plants, inhibiting growth and impairing cellular processes (Yruela, 2005).

Chile is one of the leading producers of copper in the world (Comisión Chilena del Cobre, 2019). Therefore, there is a high risk of soil and water source copper enrichment near regions where copper production is carried out. This poses an environmental hazard to nearby ecosystems (Ginocchio, 2000; Kelm et al., 2009). Chile is also a key global agricultural producer (ODEPA, 2021), so excessive use of Cu-based fungicides in Chile results in toxic concentrations of Cu in agricultural soils. Poblete et al. (2017) report high Cu concentrations of roughly 250 mg/kg in an orchard in the central Chilean O’Higgins Region, which is primarily explained by the application of Cu-based pesticides. This indicates a positive relationship between the use of Cu-based pesticides and the copper enrichment of agricultural soils (Schoffer et al., 2020). Excessive levels of copper enrichment in agricultural soils can negatively impact local plant growth (Adrees et al., 2015; Gharbi et al., 2005; Yruela, 2005).

Here, we will examine the impacts of Cu and other nutrients found in central Chilean agricultural soils on ryegrass, using *Lolium perenne* var. *Ballica* as a bioindicator and measuring shoot length and dry mass as our variables. We hypothesize that the presence of high concentrations of these nutrients will negatively impact the shoot length and dry mass of our bioindicator. Collecting data from agricultural sites in the central Chilean O’Higgins Region, we will use linear regression analysis to examine whether the bioavailability of these nutrients impacts local ryegrass growth.

Research Methodology

In this study, we aimed to determine the effects of Cu and other nutrients abundant in central Chilean agricultural soils on ryegrass. By using *Lolium perenne* var. *Ballica* as a bioindicator, this study measures shoot length and dry mass as dependent variables.

Soil samples were first collected from 13 agricultural sites in the O’Higgins Region of central Chile. These sites were chosen based on evidence from previous studies which found high concentrations of Cu in these regions (Burgos, 2017; Badilla-Ohlbaum et al., 2017, Ginocchio et al., 2002). The information for sampling sites was found at the Restoration, Soils and Metals Laboratory (RESUME) of the Faculty of Agronomy and Forestry Engineering (FAIF) at the Pontifical Catholic University of Chile.

There is evidence showing heavy metal contamination in these crops is partially a result of mining waste from a historical copper mining site located upstream in the valley (Burgos, 2017). This suggests the long-term impacts of mining activities are not locally confined. Rather, they can be diffused throughout the landscape over time, leading to an excess buildup in non-local regions (Ginocchio et al., 2000). There is also evidence suggesting heavy metal contamination in these crops is partially derived from the use of agrochemicals (Burgos, 2017).

At each site, a composite sample of topsoil (0-20 cm) of roughly 40-60 kg was collected with stainless steel shovels. The samples were stored in plastic bags and transported to the RESUME Laboratory of the FAIF at the Pontifical Catholic University of Chile for processing and analysis. There, the soils were cleaned of organic matter and stones. The samples were then sieved to particles of less than 2 mm in diameter with nylon mesh and dried in a forced-air laboratory oven at 72° C for 24 hours. They were then homogenized and stored.

A small fraction of these samples were taken for physiochemical analysis by the Soil and Foliar Analysis Laboratory of the Faculty of Agronomy and Food Sciences at the Pontifical Catholic University of Valparaiso. Key results of the physicochemical analysis, including nutrient levels, pH, and soil types of each sample, are shown in Table 1. The rest of the soils were used in the experimental trial as described below.

To analyze the impacts of heavy metal contamination on *Lolium perenne* shoot length and dry mass in central Chilean agricultural soil, a bioassay was conducted in a controlled greenhouse environment. Five replicates were measured for each of the 13 sites sampled, for a total of 65 experimental pots. The plastic pots used were 680 grams each and had drainage at the base. Each pot was filled with 480 grams of soil.

A portion of the samples was used to determine the quantity of water needed to reach 100% field capacity, using the method outlined by Klute (1986). For this process, pre-weighed 140 cc plastic test tubes were used. First, the lower part of each tube was filled with pre-weighed synthetic cotton. Next, each tube was filled with a sample, one for each replicate. The tubes were then positioned on a rack submerged in a container filled with deionized water. This allowed the water to rise through the substrate through capillary action until the surface was saturated. The container with deionized water was then removed, allowing gravity drainage. The tops of the test tubes were covered with plastic film to avoid evaporation. After 12 hours of gravity drainage, the cotton was removed from the test tubes and reweighed, to obtain the weight at 100% field capacity. Next, the difference was calculated between the weight of the test tube at 100% field capacity and the test tube weight with dry substrate and the water quantity in the substrate at 100% field capacity. This data was used to determine the irrigation volume at 70% field capacity for each experimental pot with 480 grams of substrate.

The experimental pots were placed randomly on counters in a FAIF greenhouse, where they remained under controlled temperature conditions (24 - 28° C) for six weeks during typical central Chilean summer sunlight conditions. Once a week, the placement of the pots was randomly rearranged to avoid confounding variables.

The first three weeks were classified as the stabilization phase. During the stabilization phase, soil moisture was monitored daily by weighing each pot with a digital balance accurate to the nearest hundredth. Deionized water was added to replenish the missing volume needed to maintain 70% field capacity in each replica. At the beginning of the fourth week, each pot was sown with a dose of 0.6 g of seeds from the bioindicator species used in this study, *Lolium perenne* var. *Ballica*. This species was chosen because of its high sensitivity to acidification and metal toxicity (Grigorita et al., 2020). The seeds were covered with approximately 1 cm of the same experimental substrate, and irrigation was maintained at 70% field capacity. Moisture was monitored and controlled daily, as during the stabilization phase.

During the sixth week of the trial period, soil pore water extractions of 5-9 mL were taken from three of the five replicates for each sampled site, for a total of 39 soil pore water extractions. These extractions were taken with 5 cm long Rhizon® pore water samplers (Rhizosphere Research Products, Wageningen), following the methodology outlined by Vulkan et al. (2000). The soil pore water extractions were stored in polyethylene vials of 15 mL which had been pre-washed with acid. Measurements of pH were taken from the extractions, using a combined pH electrode (Sensorex 120C). Measures of soil pore water copper (Cu) and zinc (Zn) levels were also obtained. The results of these evaluations are shown in Table 2 in the Results.

The trial was stopped at the end of the sixth week. The aerial biomass of each pot was then harvested at ground level, and the shoots were washed with deionized water. Excess water was removed with absorbent paper, and the fresh aerial biomass was weighed. To obtain the dry weight of the aerial biomass, a drying procedure was carried out at 45° C in a forced-air laboratory oven for 72 hours, until a constant weight was obtained. The dry weight was then recorded.

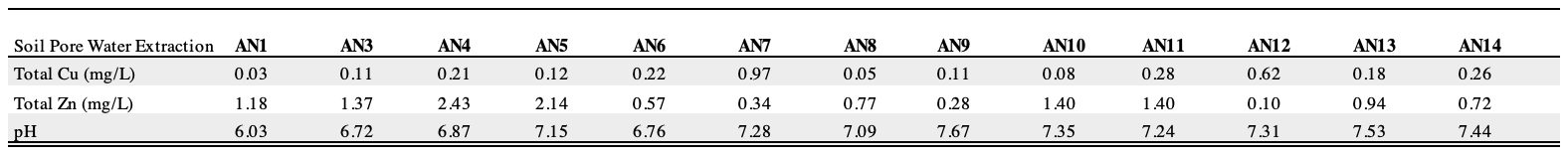
The statistical software Rstudio version 1.3.959 was used to analyze the relationship between the dry mass and shoot length of the biomass obtained and the independent variables, for both the soil and the soil pore water extractions. Linear regression analysis was performed in Microsoft Excel version 16.61.1 to statistically model the impacts of soil samples and pore water extractions on *Ballica* shoot length and dry mass. Hypothesis testing was used to detect the statistical significance of correlations found between independent and response variables (α = 0.05). To check the normality and homoscedasticity of the relationships found in the data, Shapiro’s test and Levene’s test were performed, respectively.

Data Analysis & Results

Each sampled soil is comprised of different textural classes, different types of soil particles, different nutrient balances, and different pH levels. All of these factors may influence *Lolium perenne* var. *Ballica* shoot length and dry mass and are therefore relevant to the statistical models presented in this paper. Table 1 shows these measured physiochemical differences for each soil sample collected. Table 2 shows the measured physiochemical differences for each collected soil sample’s pore water extraction.

Table

Description automatically generated**Table 1.** Physiochemical descriptions of the 13 agricultural soil samples (AN1, AN3, …) collected in the O’Higgins Region of central Chile, including pH, textural class, clay, silt, sand, total copper (Cu), total zinc (Zn), total lead (Pb), total arsenic (As), total phosphorus (P), total potassium (K), and total nitrogen (N) concentrations.

**Table 2.** Measurements of pH, total copper (Cu), and total zinc (Zn) concentrations in 5-9 mL pore water extractions of each soil sample (AN1, AN3, …) collected in the O’Higgins Region of central Chile for each of the 13 agricultural soils collected.

In Table 3, the adjusted *R*2 value implies that 63.6% of the variance in *Ballica* dry mass can be explained by the variables in this model, including soil pH and total concentrations of the nutrients copper, zinc, lead, arsenic, phosphorus, potassium, and nitrogen. The observed *F8, 56*-statistic value of 14.95 is much larger than the critical value of the *F*-distribution at a 95% probability level (*F* = 2.11), meaning we can reject the null hypothesis. This suggests that high concentrations of the modelled nutrients in these agricultural soils have a significant effect on *Ballica* dry mass. Looking at the coefficients of each predictor variable, we can see that copper, lead, nitrogen, and soil pH are positively correlated with *Ballica* dry mass. This implies that increases in pH and concentrations of those nutrients increase dry mass. The probability values of copper (0.00), lead (0.00), and pH (0.00) show us that these correlations are highly statistically significant at a 95% probability level, meaning we can reject the null hypothesis for these variables. Zinc, arsenic, phosphorus, and potassium are negatively correlated with *Ballica* dry mass, implying that increases in these nutrients decrease dry mass. The probability values of zinc (0.00), arsenic (0.00), and phosphorus (0.00) are highly statistically significant at a 95% probability level, meaning we can reject the null hypothesis for these variables.

Table

Description automatically generated**Table 3.** Multiple linear regression model – summary output: Impacts of measured soil nutrient levels and soil pH on *Lolium perenne* var. *Ballica* dry mass in grams (g).

The adjusted *R*2 value in Table 4 implies that 67.3% of the variance in *Ballica* shoot length can be explained by the variables in this model, including soil pH and total concentrations of the nutrients copper, zinc, lead, arsenic, phosphorus, potassium, and nitrogen. The observed *F8, 56*-statistic value of 17.45 is much larger than the critical value of the *F*-distribution at a 95% probability level (*F* = 2.11), meaning we can reject the null hypothesis. This implies high concentrations of the nutrients shown in this model have a significant effect on *Ballica* shoot length. Observing the coefficients of the predictor variables, we can see that copper, lead, nitrogen, and soil pH are positively correlated with *Ballica* shoot length. This suggests that increases in pH and modelled nutrient concentrations increase shoot length. The probability values of copper (0.04), lead (0.00), and nitrogen (0.03) show us that these positive correlations are highly statistically significant at a 95% probability level, meaning we can reject the null hypothesis for these variables. Zinc, arsenic, phosphorus, and potassium are negatively correlated with *Ballica* shoot length. This suggests that increases in these nutrients decrease shoot length. The probability values of zinc (0.01), arsenic (0.00), and phosphorus (0.00) are highly statistically significant at a 95% probability level, meaning we can reject the null hypothesis for these variables.

**Table 4.** Multiple linear regression model – summary output: Impacts of measured soil nutrient levels and soil pH on *Lolium perenne* var. *Ballica* shoot length in centimeters (cm).

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The adjusted *R*2 value in Table 5 implies that a negligible amount of the variance in *Ballica* dry mass can be explained by the variables in this model, pH, total copper, and total zinc concentrations of the collected soil pore water extractions. The observed *F3, 35*-statistic value of 0.52 is smaller than the critical value of the *F*-distribution at a 95% probability level (*F* = 2.87), meaning we cannot reject the null hypothesis. This implies that high concentrations of these nutrients in soil pore waters do not have any significant effect on *Ballica* dry mass. The coefficients of pH, total copper, and total zinc concentrations are not statistically significant.

Table

Description automatically generated**Table 5.** Multiple linear regression model – summary output: Impacts of measured soil pore water extraction nutrient levels and pore water pH on *Lolium perenne* var. *Ballica* dry mass in grams (g).

In Table 6, the adjusted *R*2 value implies that 57.3% of the variance in *Ballica* shoot length can be explained by the variables in this model, including soil pore water pH, total copper, and total zinc concentrations. The observed *F3, 35*-statistic value of 18.02 is much larger than the *F*-distribution’s critical value at a 95% probability level (*F* = 2.87), meaning we can reject the null hypothesis for the model. This suggests that high levels of pH, copper, and zinc concentrations have a significant effect on *Ballica* shoot length. Looking at the coefficients of the predictor variables, we can see that copper and zinc are positively correlated with *Ballica* shoot length. This suggests that increases in zinc and copper concentrations positively impact shoot length. The probability values of copper (0.00) show us that this positive correlation is highly statistically significant at a 95% probability level, meaning we can reject the null hypothesis for copper. pH is negatively correlated with *Ballica* shoot length. This suggests that higher pH decreases shoot length. The probability value of this correlation (0.00) is highly statistically significant at a 95% probability level, meaning we can reject the null hypothesis for pH.

Table

Description automatically generated**Table 6.** Multiple linear regression model – summary output: Impacts of measured soil pore water extraction nutrient levels and pore water pH on *Lolium perenne* var. *Ballica* shoot length in centimeters (cm).

Discussion

We found that high concentrations of Zn, As, and P in central Chilean agricultural soils decrease *Ballica* shoot length and dry mass. While Zn is an essential plant nutrient, high soil concentrations of the element have been found to stunt the uptake of iron in certain crops (O’Sullivan et al., 1997). In Mediterranean subtropical climates like the O’Higgins region, Zn concentrations above 150 mg/kg are considered high and may result in iron deficiency and plant necrosis (Landon, 1991). As shown in Table 1, the majority of samples collected had high concentrations of Zn, which could explain the negative relationship found in our results. Arsenic is generally toxic to plants, suppressing growth and causing necrosis in many crops (Garg & Singla, 2011). High concentrations of P have also been found to cause necrosis in plants through the inhibition of photosynthesis and other metabolic processes (Takagi et al., 2020). Our results support this previous research, implying that the agricultural soils in the O’Higgins region of Chile have excess concentrations of these nutrients. While further research must be conducted to measure healthy nutrient levels and explore the extent of these contaminations, caution should be exercised when exposing soils in this region to Zn, As, and P from agrochemicals, mining, or other activities.

We found that high concentrations of Cu and Pb in central Chilean agricultural soils increase *Ballica* shoot length and dry mass. Our results in Tables 1, 3 and 4 suggest that Cu concentrations of 50-1245 mg/kg are non-toxic for ryegrass growth in central Chilean agricultural soils. However, it is important to note that the bioavailability of Cu in soil depends on many factors, including soil organic matter and salinity, which may also impact plant growth through other mechanisms (Matijevic, 2014). These factors are not included in our regression analyses, so they are potential confounding variables. Previous research has found that Pb is highly toxic for plants, which contradicts our results (Pourrut, 2011). This discrepancy may be due to confounding variables not included in our models, such as particle size and cation exchange capacity, both of which are shown to regulate Pb uptake (Sharma & Dubey, 2005). Future research should incorporate these potential confounding variables to improve model accuracy.

Additionally, we found that soils with higher concentrations of N increase *Ballica* shoot length. While the relationship between N and *Ballica* dry mass is also positive, the relationship is not significant at a 95% probability level. Nitrogen is an essential nutrient for the vital processes of all plants, as it is a necessary component of proteins (Leghart et al., 2016). Many studies have found that soils with higher N concentrations positively impact both shoot length and dry mass (Hardwick, 1987; Leghart et al., 2016). The lack of significance in Table 4 could be due to the small sample size. Further research should be conducted on the impacts of Cu, Pb, and N soil bioavailability on crop growth, using larger sample sizes and considering variables including particle size, climate, plant species, and cation exchange capacity.

We found that soils with a more alkaline pH increase *Ballica* shoot length. Previous research has found that the ideal soil pH range for *Lolium perenne* is 6.00-7.00 (Hall, 1996). Our sampled soil pH exhibited a positive relationship, generally within this ideal range (6.04-7.02). Our results imply that the optimal soil pH for *Ballica* shoot length in central Chilean climate conditions is close to 7.00. This relationship should be explored in further research.

Concerning the soil pore water extractions collected, we found that a multiple regression analysis using pore water pH, Cu, and Zn concentrations are not good predictors of *Ballica* dry mass. To extract more information on this, further research should be conducted, including regression analyses with additional explanatory variables, larger sample sizes, and more variation in samples.

We found that high Cu concentrations in soil pore waters increase *Ballica* shoot length. As shown in Table 2, sampled Cu soil pore water concentrations range from 0.03-0.97 mg/L. Our results imply that these levels of Cu in soil pore waters are non-toxic for ryegrass growth in this region. We found that high soil pore water pH levels decrease *Ballica* shoot length. Previous research has found that soil pore water pH depends on the saturation level and aerobic/anaerobic conditions found within the soil profile (Wijdeveld, 2022). These factors also impact plant growth and therefore are potential confounding variables in our model (Tiedje, 1984). The variables tested in this model only account for 57.3% of the variation in shoot length. Future research should incorporate additional explanatory variables to further explain the variation in shoot length and to control for potential confounding effects.

Conclusion

In this study, we examined the toxic impacts of several soil nutrients on plant production in the O’Higgins region of central Chile, using *Lolium perenne* var. *Ballica* as a bioindicator. We predicted that excessive concentrations of these nutrients would lead to reduced dry mass and shoot length. We found that high concentrations of Zn, As, and P had significant negative impacts on *Ballica* biomass. Therefore, caution is recommended when exposing central Chilean soils to agrochemicals with high concentrations of these nutrients. We found that high concentrations of Cu, Pb, and N have positive impacts on *Ballica* biomass. These results oppose findings from previous literature and should be examined in more detail in future studies. We found that high levels of Cu in soil pore waters have positive impacts on *Ballica* shoot length, while alkaline pH levels in soil pore waters had negative impacts. These results should be confirmed in future studies, as the variables included in our model explains less than 60% of the variation in *Ballica* shoot length. In general, our models are subject to error in several ways, resulting in inconsistencies with previous literature. In order to gain a more comprehensive understanding of the impacts of soil contaminants on plant production, future models should include additional explanatory variables, larger sample sizes, and more variation in sample nutrient concentrations.

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