

Assessing Fe and Zn content in *Egeria densa*: Sample treatment influences significantly the quantification of metals in *Egeria densa*

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Abstract

Wetlands are fragile ecosystems that support diverse habitats and are under constant environmental pressure. The "Carlos Anwandter" Ramsar Site located in Valdivia, Chile, is home to many waterfowl species, and it is the main reproductive site for black-necked swan in the world. The main food source for these swans, *Egeria densa*, has been affected by sediment deposits with high iron (Fe) and zinc (Zn) concentrations, however the methodology for the evaluation of such concentrations has not been standardised. In fact, results obtained from commercial labs showed significant differences in metal concentrations highlighting the need to develop better assessment protocols for improved reproducibility and comparability of metal content in aquatic plants. The study aimed to understand the effects of different sample treatments and plant section subsampling on Fe and Zn concentrations in *E. densa*. To address this aim, samples were collected at the Ramsar site and a control site. Results indicated that washing the samples in the field and in the laboratory significantly reduced reported Fe and Zn concentrations. These findings highlighted the need for establishing standard protocols for sampling and sample pre-treatment and their influence in interpreting metal pollution.

Introduction

Wetlands provide many ecosystem services, many modulated by aquatic plants (Mitsch et al., 2013; Muneeppeerakul et al., 2008; Spieles, 2005). They serve as water reservoirs, buffering floods, and droughts and provide habitat for many species and breeding areas for migratory birds (Salimi et al., 2021). The Cruces River Wetland is part of the "Carlos Anwandter" Ramsar site in southern Chile. This wetland has tidal influence and is within Valdivia River's estuarine system, of which the Cruces and Calle-Calle rivers are the main tributaries. This area presents a high diversity of birds and aquatic plants, and it is a crucial breeding area for the black-necked swan (*Cygnus melancoryphus*) and other birds due to the high abundance of the Brazilian waterweed -*Egeria densa*- (Jaramillo et al., 2007; Velásquez et al., 2019), which is the main food supply for this swan (Corti & Schlatter, 2002). *E. densa* is the most dominant submerged aquatic plant in the Cruces River (Velásquez et al., 2019).

In 2004, a significant reduction of *E. densa* caused a dramatic decrease in the population of *Cygnus melancoryphus* and other aquatic birds in this area due to mortality and emigration (Jaramillo et al., 2007; Lopetegui et al., 2007; Marín et al., 2009). The event was considered an environmental disaster and attributed to industrial pollution. Subsequent studies showed that stems and leaves of the *E. densa* collected in the wetland presented a dark coloration due to apparent necrotic tissue, characteristic of plants exposed to high iron concentrations (Yarrow et al., 2009). Plants also presented a fine sediment crust adhered to their surface, which may hinder sunlight absorption and their photosynthetic capacity (Sand-Jensen, 1989). Zinc was also determined to be one of the most concentrated metals on the Cruces River (Universidad Austral de Chile, 2015). Zinc is a relevant element of wetland biota physiology (Kosik-Bogacka & Łanocha-Arendarczyk, 2019; Maret, 2013) and can interact with iron, modulating its absorption (Doyle & Otte, 1997). In high levels, Zn can be toxic to wetlands biota (Carpenter et al., 2004; Eisler, 1993; Kamal et al., 2004; Sharma et al., 2025). There is a gap regarding standard methodologies

for metal quantification in aquatic plants, principally during sample collection and preparation, *E. densa*, which is no exception. Different sample treatment and sub-sampling procedures can reduce representativeness and reproducibility leading to different conclusions regarding the level of contamination and the potential impacts on herbivore species. Some standard protocols for metal analysis in agricultural plant material encourage eliminating any residual particulate matter on the plant's surface, since the target is to quantify a specific analyte within the plant tissue (Sadzawka et al., 2007). However, this methodology neglects the contribution of particulate contaminants deposited on the plant's surface that are likely to be ingested by herbivores. Accordingly, a comprehensive understanding of the potential variability due to sampling, sample processing, and analysis is crucial to understand the influence of deposited particles on reported metal concentrations in aquatic plants. Therefore, correctly quantifying metal concentration on aquatic plants is essential to understanding metal exposure and potential ecotoxicological effects on wetland herbivorous.

This research aims to compare different sample treatments and section subsampling of *E. densa* and to assess their effects on the quantification of Fe and Zn. In doing so, best practices for sampling, sample treatment and analysis are discussed for improved reproducibility and contaminant assessment.

Materials and methods

Sampling site and strategy

Samples were collected at two sites: 1) the study site, which corresponds to the Carlos Anwandter Ramsar site within the river Cruces catchment, and 2) the control site, located in the Calle-Calle River (Fig. 1).

Sampling campaigns were undertaken from May 2022 to April 2024 (see sample information in Table S1). Plant material was collected using an outboard motorboat equipped with an articulated anchor and a hook. The sampling procedure was performed in shallow waters (up to 3 m depth) using the boat's anchor and hook to carefully extract the plants minimising sediment resuspension. Subsequently, the collected *E. densa* was immersed three times in the river water to remove any potential loose sediment excess due to the sampling procedure. Sub-samples were taken by hand, separating green sections of the plants, without evidence of sediment deposition on the surface, and abnormal dark coloured sections (black or brown leaves and stems), sometimes with evident sediment crusts (see Fig. 2). Roots and other minor aquatic plants were discarded and only leaves and stems were analysed. The sub-samples were placed in clean polyethylene bags, labelled accordingly, and transported to the laboratory. Once in the lab, the samples were stored in a fridge at 4°C before treatment and analysis.

Experimental design

The experimental design is summarised in Fig. 3. In brief, a preliminary experiment was performed to examine the effect on the concentrations of Fe and Zn when the collected samples were subjected to a field washing procedure with river water. For this purpose, two sample pre-treatments were evaluated: 1)

samples without a field washing step ($n = 19$) and 2) samples with an *in situ* field washing ($n = 13$). When testing the field washing, selected, and laboratory washed, green and black sections of the same sample of *E. densa* ($n = 15$) were sent to two independent laboratories the reported Fe and Zn concentrations. Samples were analysed using the protocols established by the National Accreditation Commission (CNA) – SCHCS for laboratory 1 and the AOAC 985.35 ed 2012, S. methods 3030 C, E and S. Methods 3120 of 2005 for laboratory 2.

A third set of samples were tested for laboratory pre-treatment. Here, three cleaning treatments were tested: 1) gentle rinsing with distilled water using a washing bottle, and 2) sonication in distilled water for 1 min and 3) sonication in distilled water for 5 min. The plant material and the resulting washing waters were visually inspected for colour and apparent water turbidity, respectively. After defining a suitable lab pre-treatment, a further experiment was performed to investigate the effect of laboratory washing on the type of leaf. In this case, plants from both sampling sites (control and study), that were already washed in the field, were classified as “Green, G” with no evidence of sediment deposition or damaged tissue, and “Black, B” with evident sediment deposition (sediment crusts) or black coloration.

Sample processing and analysis

The next stages were applied to all samples independent of the experimental design for sub-sampling and washing.

All labware employed during leaching, filtration, and analysis were placed in an acid bath (HNO_3 10%) for 24 h, rinsed thoroughly with ultrapure water (Milli-Q) and dried.

The samples were dried at 105°C for 24 h, grinded using a mortar and pestle, placed in plastic bags, and stored in a desiccator. Subsamples of about 0.5 g were weighed into Teflon beakers. The process was repeated to prepare at least three replicates per sample. Then, 7 mL of HNO_3 65% and 1 mL of H_2O_2 30% (for analysis, EMSURE®) were added to each sample and placed into a microwave digestion system (Milestone START-D) as follows: heating ramp to 220°C and maintained for 20 minutes and left to cool to room temperature for 1 h. The digests were quantitatively transferred to 10 mL volumetric flasks and made up to the mark with ultrapure water (Milli-Q). The samples were filtered and transferred to 50 mL HDPE bottles before analysis.

The samples were analysed for Fe and Zn content using an atomic absorption spectrophotometer (Thermo Scientific iCE 3000 series) operated with an air/acetylene gas mixture. Fe and Zn were analysed at the 248.3 and 213.9 nm absorption lines, respectively. Calibration curves were prepared from a certified standard solution (Titrisol® 1,000 mg L^{-1}) in the range from 0.3 to 10 mg L^{-1} for Fe and from 0.1 to 1.5 mg L^{-1} for Zn. Calibration checks were run before every analysis batch using a certified reference material (CRM) of wastewater (EnviroMAT QX102848, SCP Science). Method blanks were prepared in the same fashion as the samples and method performance was evaluated using the EnviroMAT SS-2, SCP Science, contaminated soil, CRM ($n = 4$) with recovery percentages of 96% and 87%, and % RSD of 2.0% and 5.5% for Fe and Zn respectively. Both CRMs provided ranges for confidence

and tolerance intervals that were used to inform the instrument and method performances (Table S2). Additionally, triplicates were run every 10 samples to assess method repeatability (% RSD = 0.5–13.2% for Fe and 0.1–8.3% for Zn), and a standard of known concentration was used to check instrument stability (% RSD = 0.1–1.8% for Fe and 0.2–2.7% for Zn).

Statistical Analysis

The sample pre-treatments, i.e. river and lab washing, and plant sections were compared statistically. For this purpose, the grouped data by sample pre-treatment was tested for normality and homoscedasticity. Depending on the outcome, a two-sample t-test or a Wilcoxon (Mann-Whitney U-test) was performed. The significance level was set at $\alpha = 0.05$.

Results and discussion

Comparison between commercial laboratory analyses

When this research started, the first samples collected were sent to two commercial laboratories to assess the concentration levels of Fe and Zn present in the samples. Nevertheless, Fe and Zn concentrations determined in commercial laboratories of the same fraction of the plant presented significant differences (Wilcoxon test, $p < 0.001$ for both elements), showing percentual differences ranging from 134 to 3,190% and 540 to 2,700% for Fe and Zn respectively (Fig. 4 and Table S3). These results highlighted that the lack of an established protocol for sample treatment in aquatic plants may lead to totally different results obtained from the same sample. Laboratory 1 performed a gentle wash with distilled water, and laboratory 2 did not wash the samples prior to analysis. Still, the results obtained by the laboratory that performed a sample wash are significantly higher than the one that did not wash the samples. Concluding that the differences reported may be due to other factors.

Field washing

The field-washing procedure significantly affected the quantified metals (Fig. 5 and Table S4). For example, Fe concentrations averaged $42,501 \pm 13,186 \text{ mg kg}^{-1}$ in the samples that were not washed in the field, while the samples that were washed in the river averaged $22,202 \pm 7,311 \text{ mg kg}^{-1}$, which was two times lower. The Fe concentration distributions between these two groups differed significantly (U-test, $p = 3.59 \times 10^{-4}$). On the other hand, Zn concentrations averaged $78.4 \pm 15.4 \text{ mg kg}^{-1}$ in the samples that were not subjected to washing compared to the ones washed in the field, which averaged $57.7 \pm 6.1 \text{ mg kg}^{-1}$, a ~ 1.5 -fold decrease. Zn concentration distribution between these two treatments was also significantly different (U-test, $p = 4.51 \times 10^{-6}$). These results highlighted that rinsing the sample in the field during sample collection is an important aspect to be considered. Neglecting this step can result in significantly higher concentrations in the analysed material.

The distilled water washing step is the common procedure before the quantification of metals in aquatic plants (Lopetegui et al., 2007; Pinochet et al., 2004). A qualitative test was performed on the samples to investigate the potential effects of distilled water washing. This test showed that sonication effectively eliminated the sediment adhered on the plant's surface (Fig. 6). However, the experiment also showed that after 1 and 5 min of sonication, the plants were not only free of recently deposited sediment during sampling but also from the older sediment crust (Fig. 5b and c). Complete cleaning of *E. densa*, therefore, is not representative of the real environmental conditions at which this food source is available to the swans and other herbivores.

Laboratory washing

The laboratory washing procedure was assessed in samples previously washed in the field. Fe and Zn were quantified in samples that were washed and not washed in the laboratory from both the control and study sites (Fig. 7 and Table S5). Fe concentrations in the control site significantly differed between the washed and non-washed samples, regardless of the plant section (t-test, $p = 1.1 \times 10^{-7}$ and Wilcoxon test, $p = 0.0013$, respectively). However, in the study site, there were no statistically significant differences between both treatments (Wilcoxon test, $p = 0.94$; and t-test, $p = 0.45$ for the green and black sections, respectively). The difference might be attributed to the fact that the samples collected in the control site did not show significant amount of fine sediment deposited, and if it were, the washing process might have eliminated most of it. On the contrary, in the study site, the plants have been exposed to significant sediment deposition, and the crusts were firmly adhered to the leaves not removed during the field and laboratory wash.

Zinc concentrations for lab-washed and non-washed samples in the green and black sections in the control site were significantly different (Wilcoxon, $p = 7.4 \times 10^{-7}$ and t-test, $p = 0.033$, respectively, Fig. 6). In addition, washing treatment in the green sections of the plant was not significantly different in the study site (Wilcoxon test, $p = 0.7$). In contrast, the black sections showed significant differences (t-test, $p = 0.0013$). Zn concentrations followed similar trends as Fe for the green and black sections in the control site. However, in the study site, no clear trend is observed.

The laboratory washing procedure with distilled water significantly influenced the metal quantification, particularly for the control site. In an area that might be more influenced by fine sediment pollution, such as the study site, the difference might not be as significant due to sediment incrustation into the plant tissue. Consequently, the laboratory washing step should be carefully considered before carrying out an environmental impact assessment of metals on submerged aquatic plants.

Fe and Zn content in green and black sections of the plant

The concentration of Fe in both green and black sections of the plant (Fig. 8 and Table S6) showed significant differences in the control and in the study sites (Wilcoxon test, $p = 0.028$; and Wilcoxon test, $p = 8.3 \times 10^{-5}$, for the control and study sites, respectively). Zn concentrations in both green and black sections of the plants in both sites were not significantly different (Wilcoxon test $p = 0.38$ and $p = 0.57$

for control and study sites, respectively). This suggests that the subsampling procedure might influence reported Fe concentrations. In addition, Fe concentrations varied from 57–95% in the study site and from 25–41% for the control site. Zinc, on the other hand, presented lower variation (from 37 to 42% and 38 to 50% for the study and control sites, respectively). High variability in reported Fe and Zn concentrations might be associated with different spatially riverine dynamics influencing particle deposition e.g. meandering versus straight channel areas. Nevertheless, the presence of outliers might have influenced these values.

Concentrations of Fe and Zn in *E. densa* in the literature

Our results were compared with those reported in a previous study in the same wetland. Fe concentrations in damaged plants (Black in our study) averaged $40,090 \text{ mg kg}^{-1}$, and healthy plants (Green In our study) averaged $13,251 \text{ mg kg}^{-1}$ in a control site (Pinochet et al., 2004). Another study presented average Fe concentrations of $31,000 \text{ mg kg}^{-1}$ in the sanctuary and $9,800 \text{ mg kg}^{-1}$ in a control site outside the sanctuary (Saldivia, 2005). Our average Fe concentrations in the sanctuary were similar to those reported for the non-damaged tissue (Green) collected from plants outside the sanctuary by these authors. However, the comparison is not straightforward as details regarding the year of sampling and because field washing were not given, although it does report that the samples were washed with distilled water in the laboratory. According to our results, concentrations in the study site without river and/or lab washing might raise the iron content to approximately $42,000 \text{ mg kg}^{-1}$ and consequently become more similar to those reported for damaged tissue (Pinochet et al., 2004; Saldivia, 2005). The same was true for zinc, where average concentrations in the plants were 53 mg kg^{-1} and 108 mg kg^{-1} in the control and study sites, respectively. Values reported in 2004 presented average Zn concentrations of 66 mg kg^{-1} in the Cruces River (Pinochet et al., 2004), which were similar to those reported in the control site. However, as stated above, Zn concentrations may not be as affected by the sample treatment as Fe. Furthermore, the lack of a standardised protocol challenges fair comparisons with concentrations reported in the literature. Our findings suggest that metal concentrations in this aquatic plant are significantly influenced by the sample treatment employed (which often is not thoroughly reported), and hence, different conclusions regarding pollution status in aquatic plants can be made. This should also be the case for other aquatic plants, at least for Fe, since its deposition occurs by precipitation. Our results have implications for the study of wetland plants' metal bioaccumulation since bioaccumulation is the accumulation of a contaminant in (and occasionally on) an individual organism (Newman & Clements, 2007). The lack of standardisation makes difficult the possible differences caused by factors unrelated to sampling (i.e., rain pattern, pollution inputs) in different years.

Methodological recommendations for future studies

To better assess the field concentrations that accurately represent metal exposure to herbivorous wetland biota, future studies should consider the following procedures when assessing concentrations of metals in aquatic plants:

- Truly clean the aquatic plants in the field with river/lake water before subsampling.
- Plant section selection should address the sample heterogeneity by collecting representative sections of healthy and non-healthy plants.
- Once in the laboratory, a gentle wash with distilled water or, preferentially, Milli-Q water should be used to eliminate the excess loose sediment potentially added by field sampling procedures.
- Sample collection from different areas should also be considered to account for spatial variability.
- Follow strict quality assurance and quality control procedures.

Conclusions

This study aimed to compare different sample treatments and plant section subsampling of *E. densa* to assess their effects on the quantification of Fe and Zn. For this purpose, sample treatments were compared, and samples analysed for Fe and Zn concentrations. Results indicated that washing the samples in the field and laboratory significantly reduced Fe and Zn concentrations. These findings highlighted the importance of establishing standard methodologies for sample treatment in aquatic plants' metal content analysis. The same is true when sampling different sections of the plant, particularly for samples collected in areas under anthropogenic stress. The plant section sampling and the further washing procedures directly impacted metal pollution assessment, particularly the accuracy of estimation of metal exposure concentration to herbivorous wetland biota. For future studies, consider the implications demonstrated in this study. Therefore, it allows unbiased comparison between studies and better assessment of aquatic plant pollution and subsequent consequences to herbivores.

Declarations

Declaration of Competing Interest

All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors"

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Author Contribution

C.B.L: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. E.D.: Data curation, Formal analysis, Methodology, Software. M.C.Z.: Data curation, Formal analysis, Methodology, Software. E.M.A: Data curation; Formal analysis, Writing-review & editing. J.A. T.: Investigation, Methodology, Sampling,

Data collection, Field Work, Formal analysis, Visualisation, Writing-review & editing. A. N.: Writing-review & editing. I.R.J.: Conceptualization; Formal analysis; Funding acquisition; Investigation; Sampling; Data collection, Field Work, Methodology; Project administration; Resources; Writing-review & editing.

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Figures

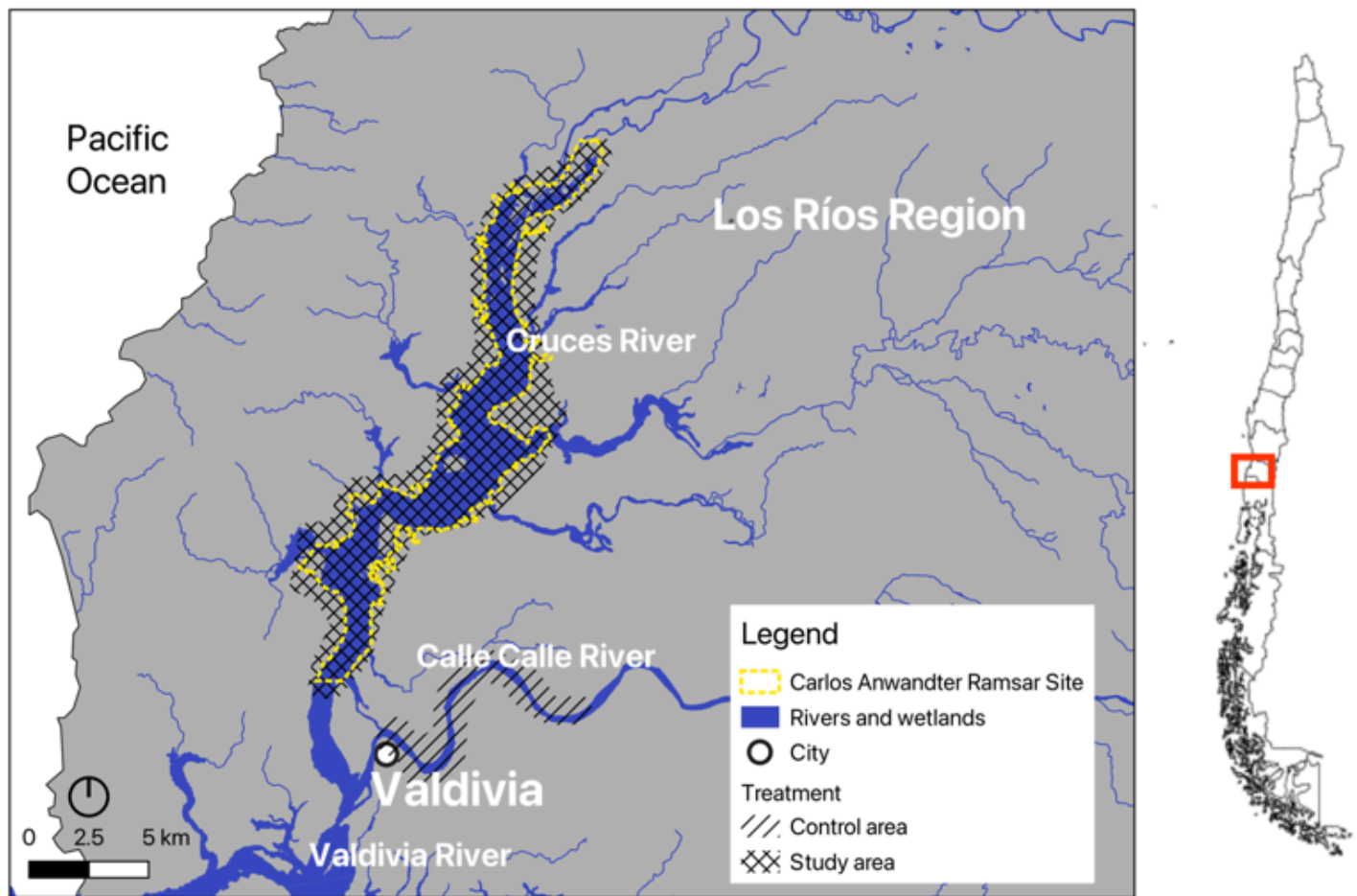


Figure 1

Map of the study and control areas within the Carlos Anwandter Ramsar Site

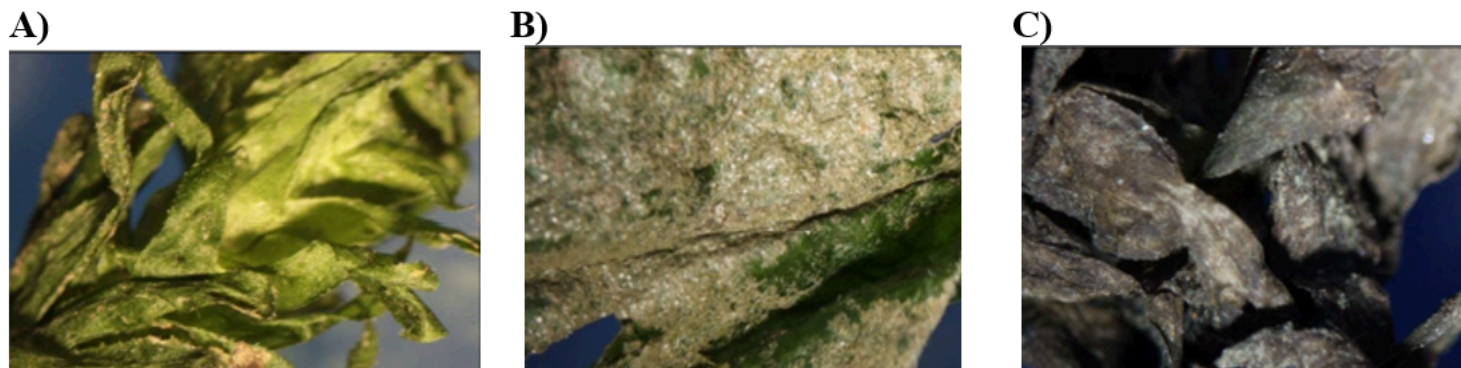


Figure 2

Images of the different dry collected material of *E. densa* A) Green leaves, B) Leaves with sediment crusts C) damaged (black) tissue

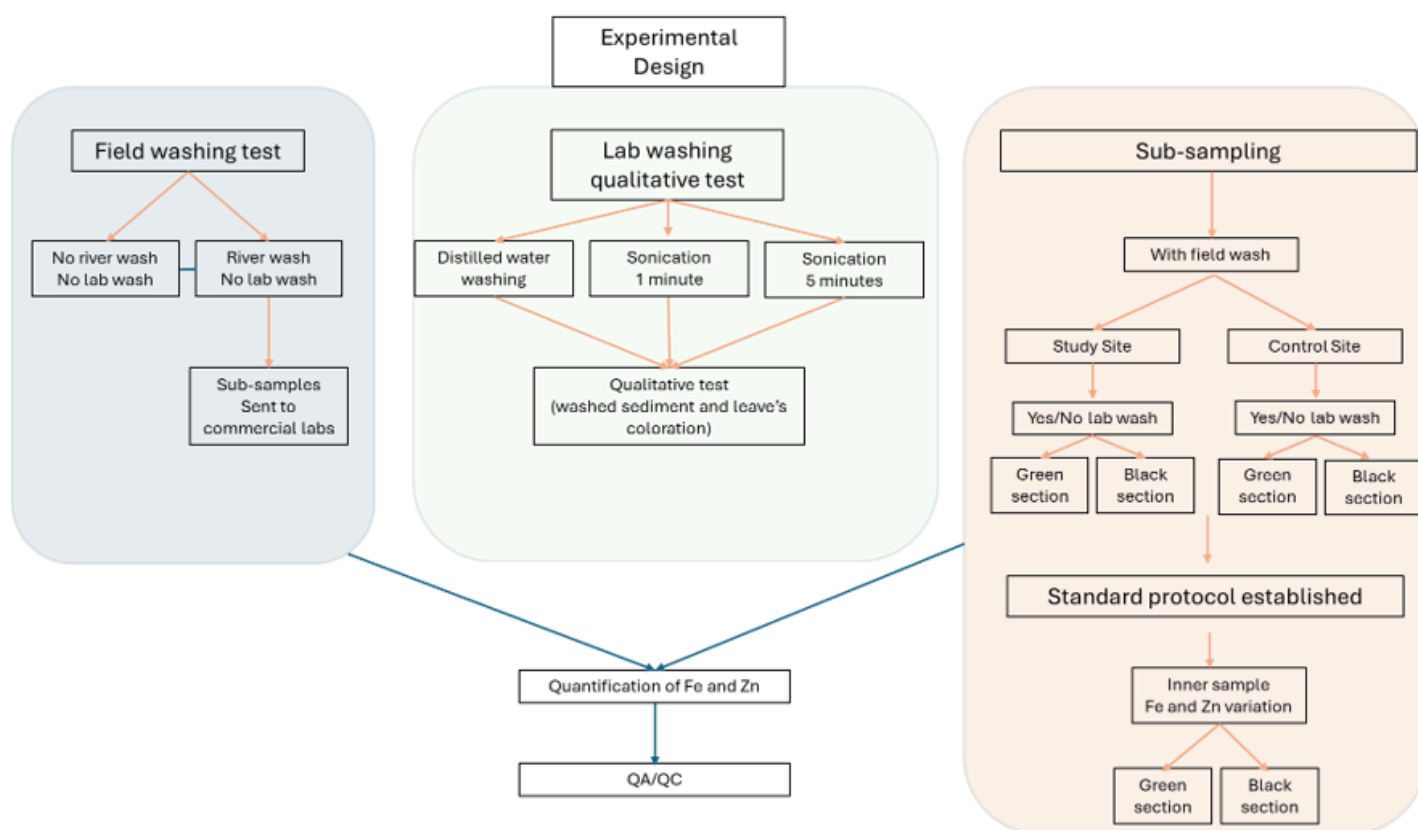


Figure 3

The experimental design proposed in this research to study the influence of different sample treatments in *E. densa*

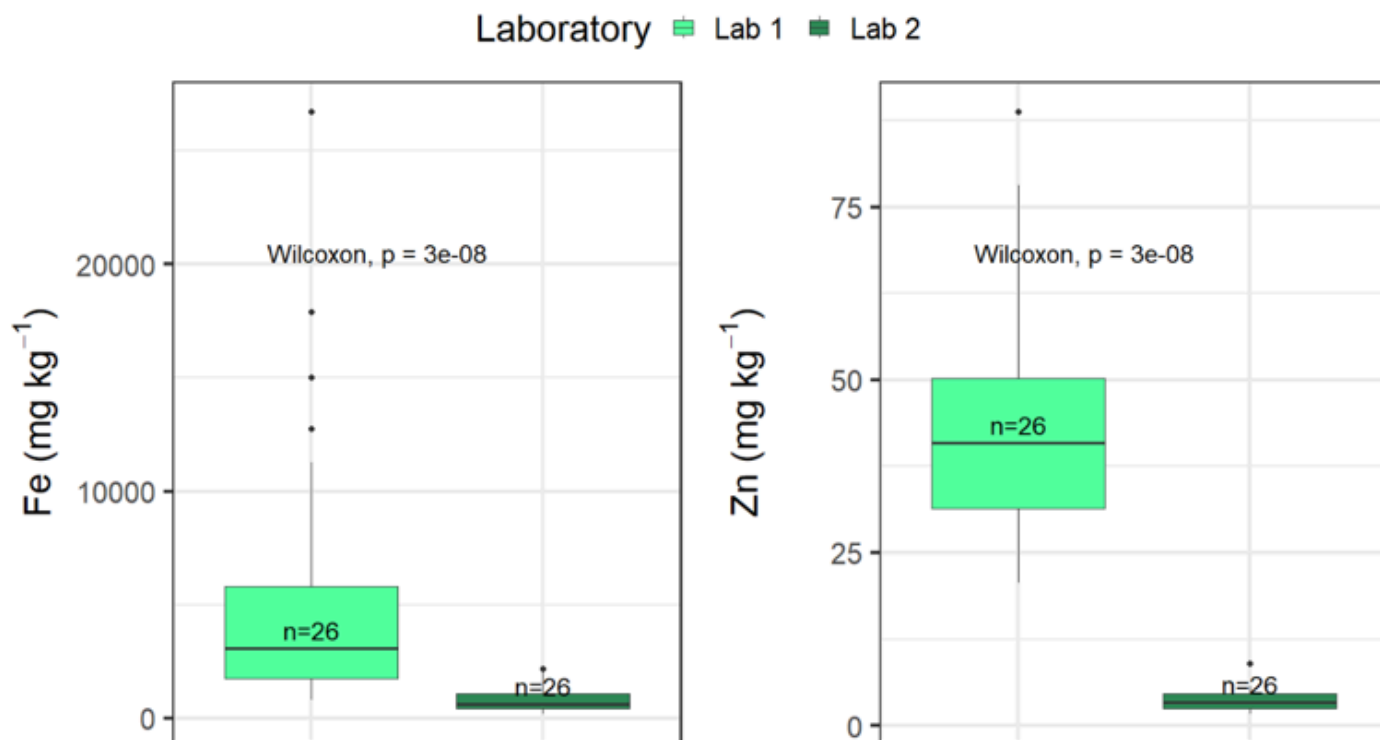


Figure 4

Box-plots for Fe (left) and Zn (right) showing the differences between the two commercial labs reported results

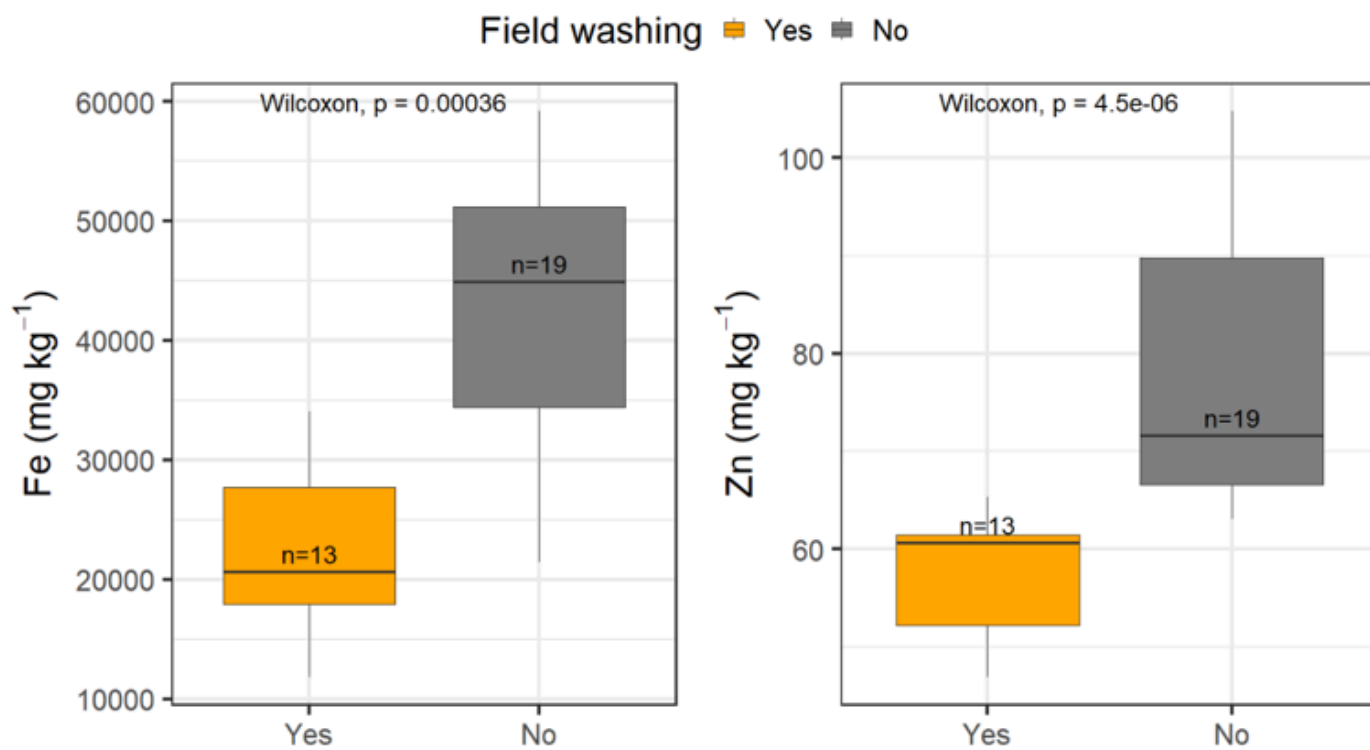


Figure 5

Concentrations of Fe and Zn (mg kg^{-1}) for the field washing procedure: field washed (orange) and not field washed (grey)

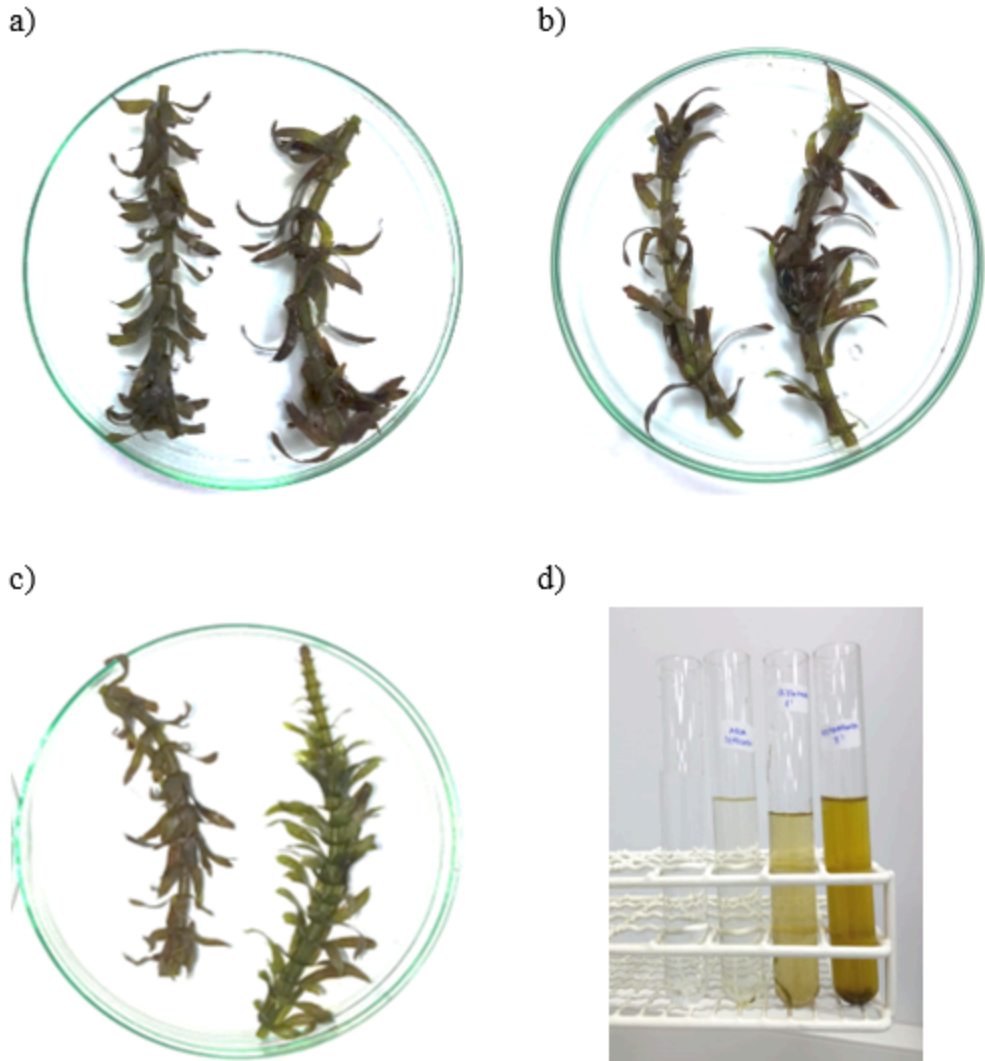


Figure 6

Photographs of the plants before (left image) and after (right image) treatment with a) gentle streams of distilled water, b) 1 min of sonication, c) 5 min of sonication and d) photograph of the appearance of water collected after plant washings in a, b and c. The first tube is distilled water (control)

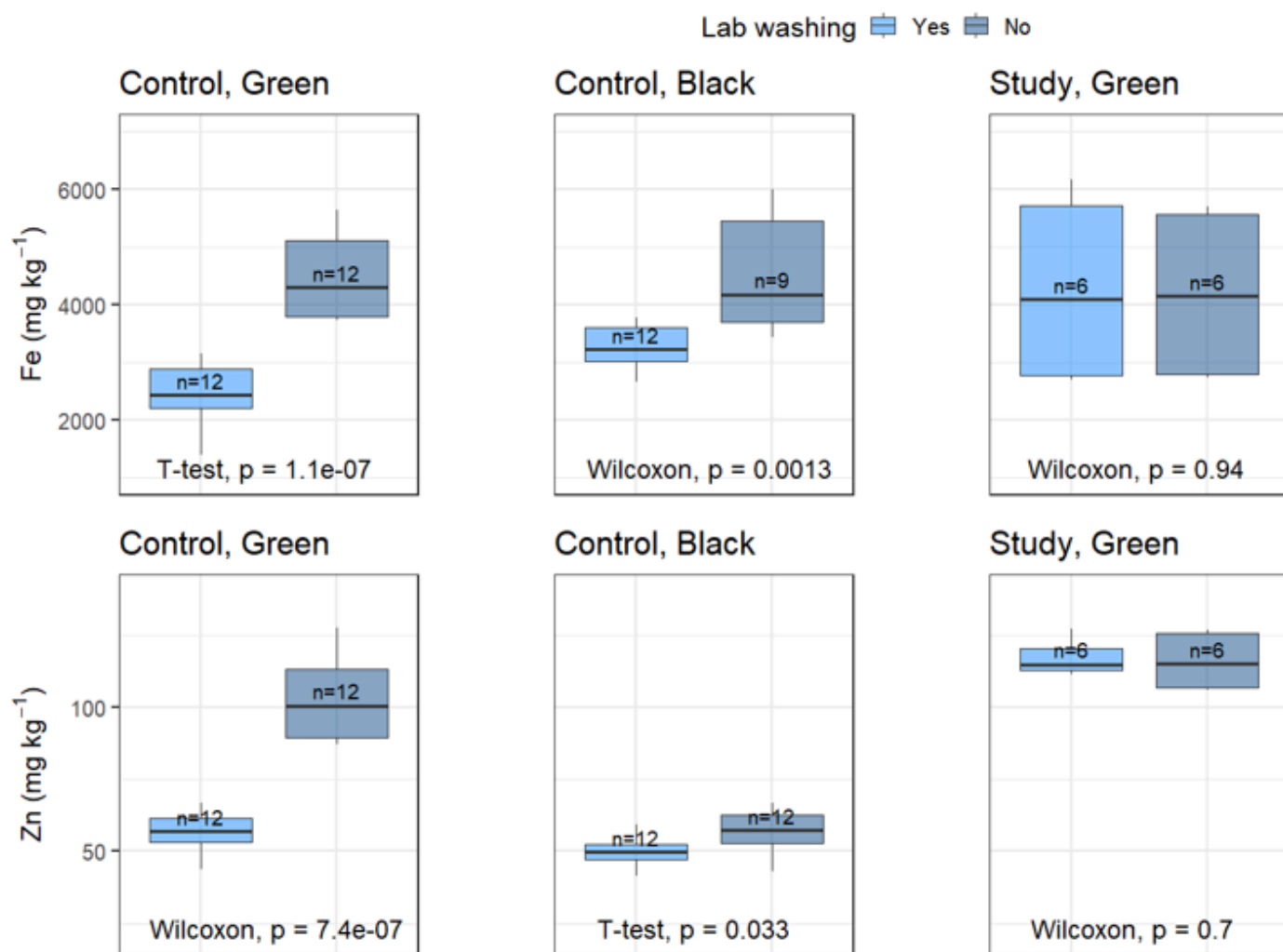


Figure 7

Comparison of Fe and Zn concentrations analysed in the different plant sections when washed “Yes “and not washed “No” in the laboratory from both sites i.e. control and study sites

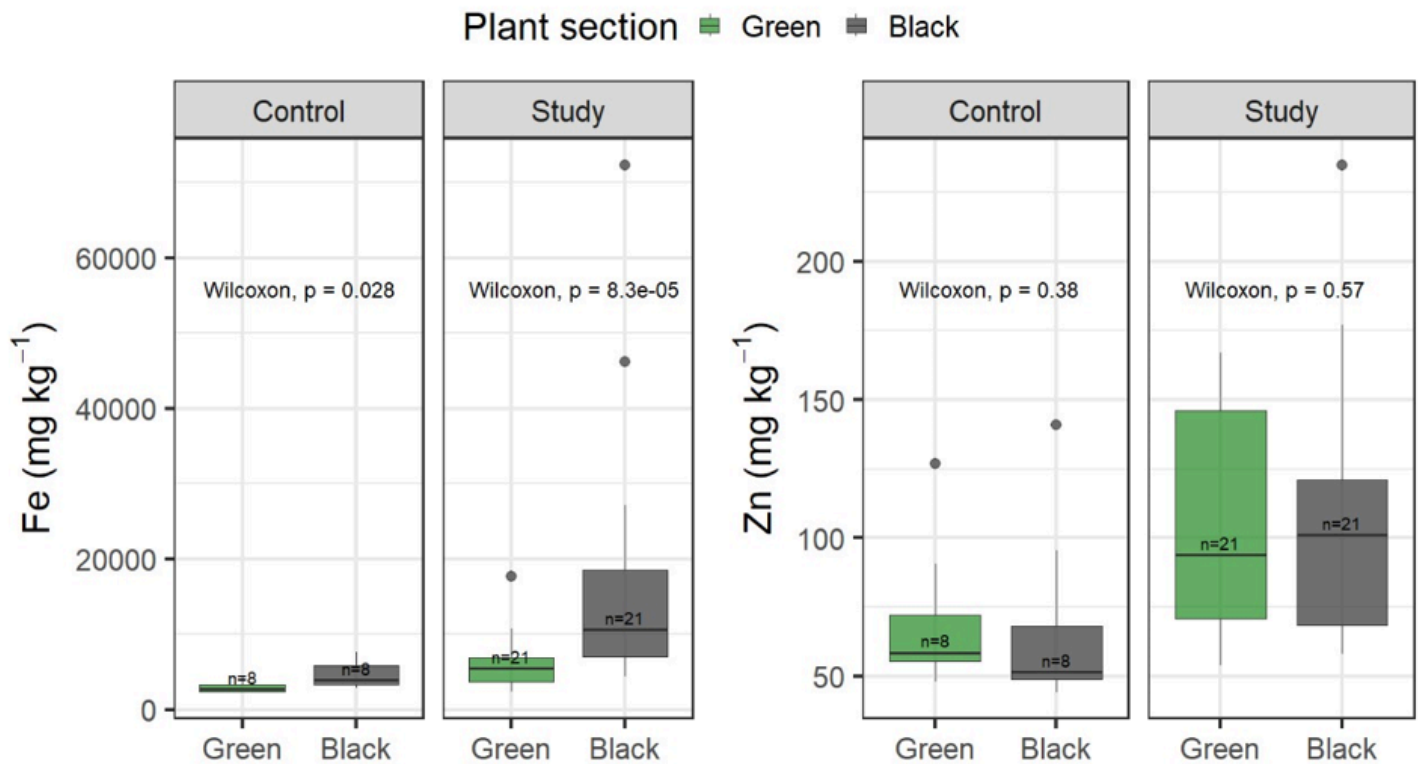


Figure 8

Box-plots for Fe (left) and Zn (right) for green and black sections of the *E. densa* in the different sampling sites. Green bars: Green section. Grey bars: Black section

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryinformationpaper1.docx](#)