



Elemental quantification of adults zebrafish from different vivaria using X-ray fluorescence

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ABSTRACT

Zebrafish (*Danio rerio*) has been used as a model organism in several areas of research, including ecotoxicology, genetics and preclinical research. Investigating the elemental composition of adult zebrafish samples collected from different vivaria can provide valuable insights into various aspects of their physiology, influenced by intrinsic factors, such as gender, and extrinsic factors, including environmental conditions.

In this study, adult zebrafish specimens from three different vivaria in Portugal (Biology Department of University of Aveiro (UA), Instituto Gulbenkian de Ciência (IGC) and Instituto de Medicina Molecular João Lobo Antunes (iMM), Lisbon) were analysed using X-ray fluorescence (XRF). The XRF system used was the M4 Tornado (Bruker, Germany) and quantitative analysis was performed using the inbuilt software through the Fundamental Parameters method considering a matrix of 7 % H, 23 % C, 7 % N and 60 % O.

Twelve elements were detected and analysed, with Ca and P showing the highest concentrations across all vivaria. Fe, Zn, and Br exhibited significant gender differences in all vivaria, while Ca, Ni, and Cu showed no significant differences between genders or vivaria.

1. Introduction

The zebrafish (*Danio rerio*) is a freshwater species widely used as a model organism in various fields of scientific research, including developmental biology, genetics, and toxicology. Zebrafish are particularly valued in research due to their genetic similarity to humans, sharing approximately 70 % of their genes, which makes them a valuable model for studying human genetic diseases (Howe et al., 2013) (Yang et al., 2020). Their embryos develop rapidly and externally, offering easy access to observation and experimental manipulation (Yang et al., 2009) (Caliskan and Emekli-Alturfan, 2021). Additionally, the transparency of zebrafish embryos and larvae allows for direct visualization of organ and tissue development *in vivo*. Zebrafish are also highly fertile, small, and easy to maintain in large numbers, making them ideal

for large-scale experimental studies. These characteristics have established zebrafish as a crucial tool for investigating a wide range of biological processes, including organogenesis, tissue regeneration, and chemical toxicity assessments (Yang et al., 2020) (Beffagna, 2019) (Henke et al., 2023) (Ben Chabchoubi et al., 2023).

The X-ray Fluorescence (XRF) analysis technique offers several advantages, including the simultaneous identification of multiple elements, its non-destructive nature (i.e., samples remain intact for further analysis or treatments), the simplicity and speed of sample preparation, and the ability to perform quantitative analysis with sensitivity in the µg/g range. These characteristics make this technique a widely used tool for rapid analyses in various applications, including environmental analysis, geology, agriculture, biomedicine, cultural heritage studies, metallurgy, and more (Carvalho et al., 2020) (Barbosa et al., 2023)

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Table 1

Environmental conditions to which the fish were exposed.

	Water Source	pH	Temperature (°C)	Oxygen saturation	Photoperiod (light/dark)
UA	Reverse	7.5 ± 0.5	27 ± 1	≥95 %	12h/12h
IGC	Osmosis	7.2 ± 0.5			14h/10h
iMM		7.5 ± 0.5			14h/10h

(Santos et al., 2024) (Sales et al., 2020).

There are several studies in the literature analysing the accumulation of heavy metals and their toxicity in embryos and larvae of zebrafish as well as adult zebrafish using XRF techniques (Bilo et al., 2015) (Bourassa et al., 2014) (Leite et al., 2022). In 2006, Vives et al. (2006) studied metal contamination in a Brazilian river using fish as indicators, employing the Total Reflection X-ray Fluorescence (TXRF) technique for analysis. Limburg et al. (2007) used synchrotron microbeam x-ray fluorescence to map multiple trace elements in otoliths, offering a non-destructive alternative to the methods previously used for this type of analysis. De Samber et al. (De Samber et al., 2008) explore the use of both synchrotron and laboratory-based X-ray techniques to analyse the tissue-specific distribution of trace-level metals in *Daphnia magna*, a freshwater crustacean commonly used in ecotoxicology.

The increasing production and use of nanomaterials have raised concerns about their potential risks to humans and the environment. Da Silva et al. (Da Silva et al., 2018) conducted a study to assess the toxicity of titanium dioxide nanoparticles (TiO₂NP) and TiO₂ multi-walled carbon nanotubes using Zebrafish embryos. A μ -XRF technique was employed to evaluate nanoparticle uptake and accumulation within the organisms. The results showed no Ti presence in the control group, while in zebrafish exposed to TiO₂NPs, titanium was detected only in the gut region. Santos-Rasera et al. (Ribeiro Santos-Rasera et al., 2022) used micro-XRF, atomic absorption spectroscopy (GF-AAS), and Synchrotron X-ray absorption spectroscopy (XAS) in adult zebrafish to investigate the toxicity of copper oxide nanoparticles and their distribution/concentration, concluding that most of the copper was concentrated in the gastric system. Leitão et al. (Leitao et al., 2022) investigated the distribution of mercury (Hg) and other trace elements, including zinc (Zn), calcium (Ca) and iron (Fe), in zebrafish exposed to mercury (II) chloride. Using a micro energy dispersive X-ray fluorescence (μ -EDXRF) spectrometer, they assessed the toxicity, bioaccumulation, and distribution of these elements across various organs, while confirming the absence of mercury in the control group.

Despite the widespread use of zebrafish in various fields of research, limited information exists regarding its elemental composition. To address this gap, the present work aims to provide a more detailed and comprehensive analysis of zebrafish elemental profiles. For this purpose, micro-Energy Dispersive X-ray Fluorescence (μ -EDXRF) was employed, having demonstrated efficacy in similar studies, as previously described.

In this work, the μ -XRF M4 TORNADO spectrometer was employed to analyse the elemental composition of Zebrafish. Specimens from three different vivaria were examined to determine whether there were significant differences in elemental concentrations between males and females within the same vivarium, as well as to assess any significant

variations in element concentrations across the different vivaria.

2. Materials and methods

2.1. Zebrafish assay

For this study, Zebrafish (*Danio rerio*) maintained at three Zebrafish facilities in Portugal (Biology Department of the University of Aveiro (UA, Aveiro), Instituto Gulbenkian de Ciência (IGC, Lisbon) and Instituto de Medicina Molecular João Lobo Antunes (iMM, Lisbon)), were used. The specimens were kept under controlled environmental conditions, as defined in Table 1.

Tap water is first filtered through a sand filter and then goes through reverse osmosis filtration. The water is then adjusted for conductivity using artificial Sea Salt (Instant Ocean) and for pH with calcium carbonate. Finally, it is maintained at a temperature of 27 ± 1 °C.

A total of 36 adult Zebrafish specimens (twelve to twenty-four months old) were selected for the experimental assay: 6 females and 6 males of each zebrafish facility. The genetic background of the zebrafish from UA was AB, while those from IGC and iMM were TU. The sample preparation protocol is shown in Fig. 1. Each of the individuals was removed from the filtered water of the recirculation life support system, euthanized by anaesthetic overdose (tricaine methanesulfonate – MS222 at a concentration of 2 g/L), snap-frozen at –80 °C, and freeze-dried for approximately 48 h before being prepared into pellets. The samples were ground using mortar and pestle into small fragments and then pressed into pellets using a 10 ton hydraulic press.

Each pellet consists of 2 Zebrafish, with thickness between 1.5 and 2 mm, meaning that each zebrafish facility included six pellets, three of which were composed of female zebrafish and three of male zebrafish. The obtained thickness was a compromise between the needed thickness to consider the pellets infinitely thick considering the system's geometry, average energy of incoming radiation, the considered matrix and the characteristic lines of the elements, and the use of enough material to have a stable pellet. After sample preparation, each Zebrafish pellet was analysed with the M4 TORNADO micro-XRF system (M4 TORNADO Bruker).

2.2. Micro – EDXRF system – M4TORNADO

The analysis of the pellets was performed using the M4 TORNADO (Bruker®, Germany), a commercial micro-XRF spectrometer. This imaging spectrometer operates on a scanning basis, where the sample is irradiated point by point using a micro-focused X-ray beam. The accumulated spectrum is then used for analysis and deconvolution.

The M4 TORNADO is equipped with an X-ray tube with a rhodium (Rh) target, operating at 50 kV and 300 μ A, high voltage and tube filament current, respectively, to excite the samples. A polycapillary is employed to achieve a spot size as small as 25 μ m for Mo-K α radiation. The resulting fluorescence radiation is detected by an energy-dispersive silicon drift detector (SDD) with a 30 mm² sensitive area and an energy resolution of 142 eV for Mn-K α (5.9 keV).

Elemental analysis was performed under vacuum (20 mbar), using a

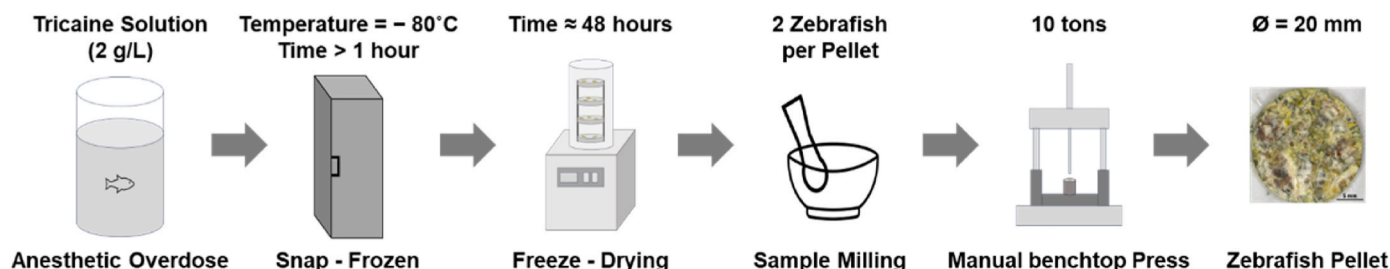


Fig. 1. – Scheme of sample preparation.

Table 2
Comparison of the values obtained for certified reference materials.

	Fish Flesh MA-A-2		Oyster Tissue 1566b	
	Obtained	Certified	Obtained	Certified
	Mean ± Unc (µg/g)	Mean ± Unc (µg/g)	Mean ± Unc (µg/g)	Mean ± Unc (µg/g)
S			6425 ± 4	6890 ± 140
Cl			6048 ± 11	5140 ± 100
K			7907 ± 25	6520 ± 90
Ca			972 ± 4	838 ± 20
Mn			21.47 ± 0.01	18.5 ± 0.2
Fe	44 ± 2	54 ± 1	262 ± 3	205.8 ± 6.8
Cu	3.08 ± 0.04	4 ± 0.1	71 ± 1	71.6 ± 1.6
Zn	27.6 ± 0.2	33 ± 1	1402 ± 22	1424 ± 46

Table 3
Limits of Detection (LoD) and Limits of Quantification (LoQ) calculated.

	LoD (µg/g)	LoQ (µg/g)
S	5.3 ± 0.1	15.9 ± 0.3
Cl	4.2 ± 0.1	12.6 ± 0.3
K	3.4 ± 0.1	10.2 ± 0.3
Ca	2.8 ± 0.2	8.4 ± 0.6
Fe	1.7 ± 0.1	5.1 ± 0.3
Cu	0.80 ± 0.05	2.4 ± 0.1
Zn	0.90 ± 0.06	2.7 ± 0.1

composition of filters 100 µm Al/50 µm Ti/25 µm Cu for detecting heavier elements and 12.5 µm Al filter for lighter elements, at the X-ray tube’s output helping to reduce background. The microbeam scans the entire pellet (20 mm in diameter) in 100 µm steps, with an acquisition time of 10 ms per pixel in a serpentine pattern. This pattern was repeated three times, resulting in a total acquisition time of approximately 1 h. Each pellet was analysed four times in total, with both the heavier and lighter filters applied to each surface. Quantitative analysis was performed using the inbuilt software - MQuant – based on the Fundamental Parameters method. This software requires an input of the sample’s matrix. This was determined by using a calibration curve previously developed (Pessanha et al., 2019) (Machado et al., 2020) (Pessanha et al., 2023) that allows to determine the average Z of the sample by comparing the average Compton-to-Rayleigh ratio of the characteristic

peaks of the X-ray tube in the samples with the calibration curve and make it correspond to a given matrix composed of H, C, N and O with similar average Z. Considering the obtained average Compton-to-Rayleigh ratios of the different samples (10.6 ± 0.3) and tentative approximations to the composition of the matrix, a matrix of 7 % H, 23 % C, 7 % N and 60 % O was chosen. This was the combination that rendered the most accurate quantification for similar Z (Z ≈ 6.7) certified reference materials, namely Fish Flesh MA-A-2 and Oyster Tissue 1566b, as shown in Table 2.

Limits of Detection (LoD) were calculated according to the following formula:

$$LoD = 3 C_i \sqrt{\frac{N_B}{N_P}}$$

where C_i is the certified concentration, N_P is the intensity of the peak and N_B is the intensity of the corresponding background. The Limit of Quantification (LoQ) was calculated according to the formula (Pessanha et al., 2017):

$$LoQ \approx 3 LoD$$

The LoDs and LoQs for the analysed elements are presented in Table 3.

2.3. Statistical analysis

The goal of the statistical analysis performed is to determine whether 1) there are differences between males and females within each vivarium, and 2) whether there are differences among vivaria in the quantified elements for each gender. In Shapiro-Wilk Tests, the null hypothesis (H0) is rejected for significance values $\alpha \geq 0.05$ which means that in these conditions, each group of samples to be compared can be represented by a normal (or close to normal) distribution, and ANOVA test is applicable to test differences (variability). In such conditions, ANOVA statistical results were selected for a significance level equal to or less than 0.05 (that is, 0.05, 0.01, 0.001, and 0.0001). This means that, for these levels of confidence ($\alpha \leq 0.05$), the statistical result (p-value) of ANOVA tests is accepted, and the null hypothesis (H0) is rejected meaning that, under these conditions, the groups of samples compared are distinct, and have differences. In turn, when Kruskal-Wallis tests were applicable, the null hypothesis H0 was rejected for statistical values (p-values) close to zero.

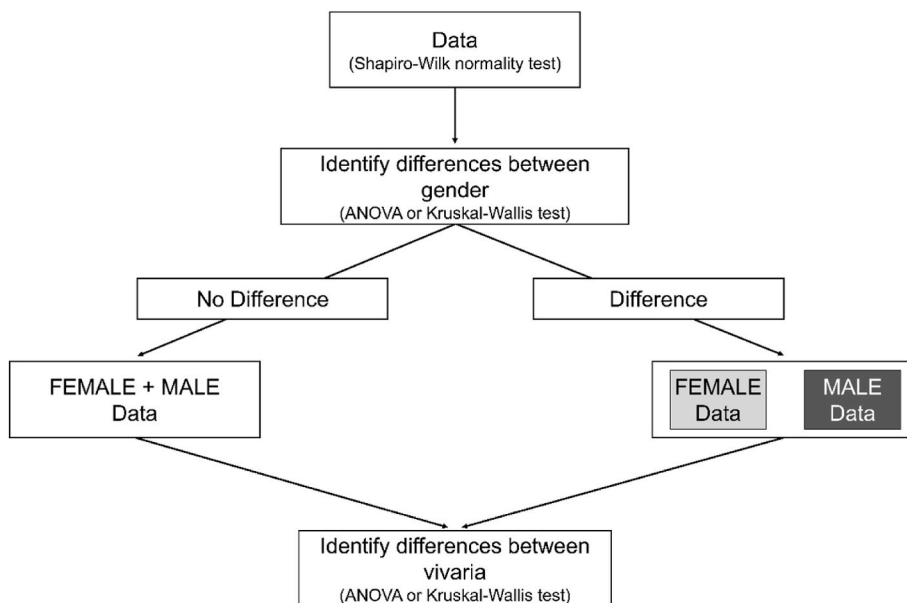


Fig. 2. – Diagram of the statistical analysis process used in this work for each element identified.

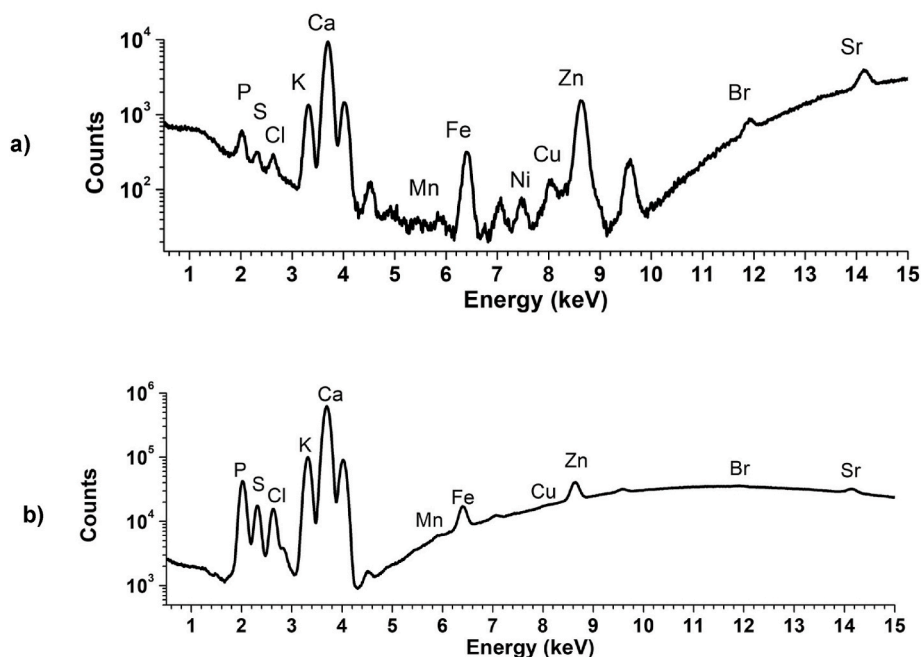


Fig. 3. – Representative spectrum obtained from the analysis of one face of a single Zebrafish pellet, showing all the elements identified. The tube operated at 50 kV and 300 μA filament current, with an acquisition time of approximately 1 h: a) with the heavier filter (100 μm Al/50 μm Ti/25 μm Cu) and b) with the lighter filter (12.5 μm Al).

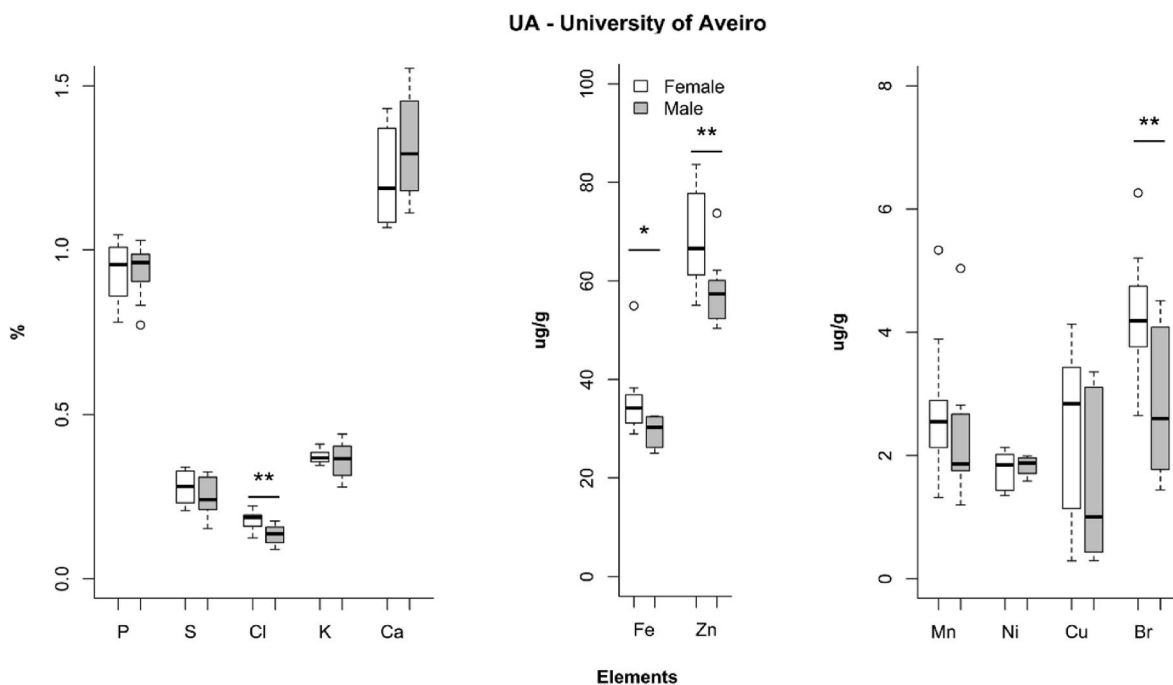


Fig. 4a. Quantification of each of the elements identified in the UA (University of Aveiro) vivaria in grey, and results of the analysis between females and males (* p-value <0.05 ** p-value <0.01 *** p-value <0.001 **** p-value <0.0001).

The statistical analysis was carried out using the program R©v.4.4.0 2024 - The R Foundation for Statistical Computing Platform. Fig. 2 shows a general diagram of the tests applied to the data.

3. Results and discussion

Results of the analysis of the Zebrafish pellets showed that 12 elements can be found in zebrafish organisms – Phosphorus (P); Sulfur (S); Chlorine (Cl); Potassium (K); Calcium (Ca); Manganese (Mn); Iron (Fe);

Nickel (Ni); Copper (Cu); Zinc (Zn); Bromine (Br); Strontium (Sr) – as can be seen in Fig. 3. Sr was also detected in the samples, however, our quantitative method is not validated for Sr so it was not considered for statistical comparison.

In the study by Leitão et al. (Leitao et al., 2022), the presence of Ca, Fe and Zn in zebrafish specimens was also observed. The elemental maps presented show calcium predominantly in the skeleton and fins of zebrafish, as bones are known to be rich in minerals such as Calcium and Phosphorus. Toppe et al. (2007) investigated the chemical composition

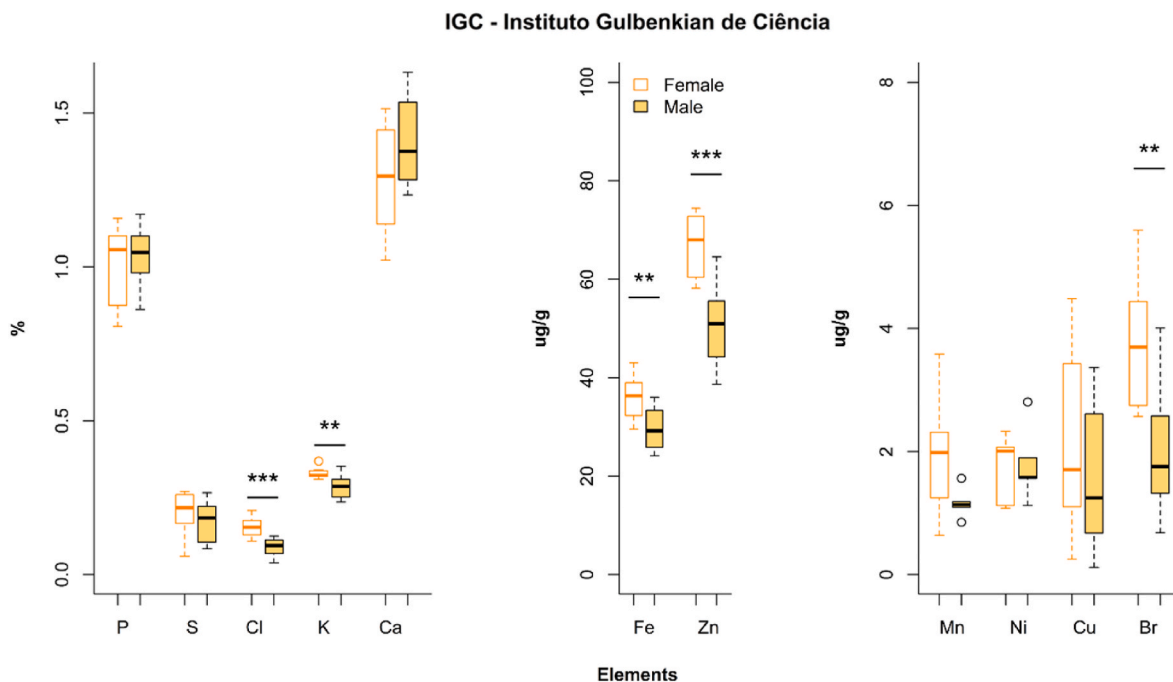


Fig. 4b. Quantification of each of the elements identified in the IGC (Instituto Gulbenkian de Ciência) vivaria in yellow, and results of the analysis between females and males (* p-value <0.05 ** p-value <0.01 *** p-value <0.001 **** p-value <0.0001).

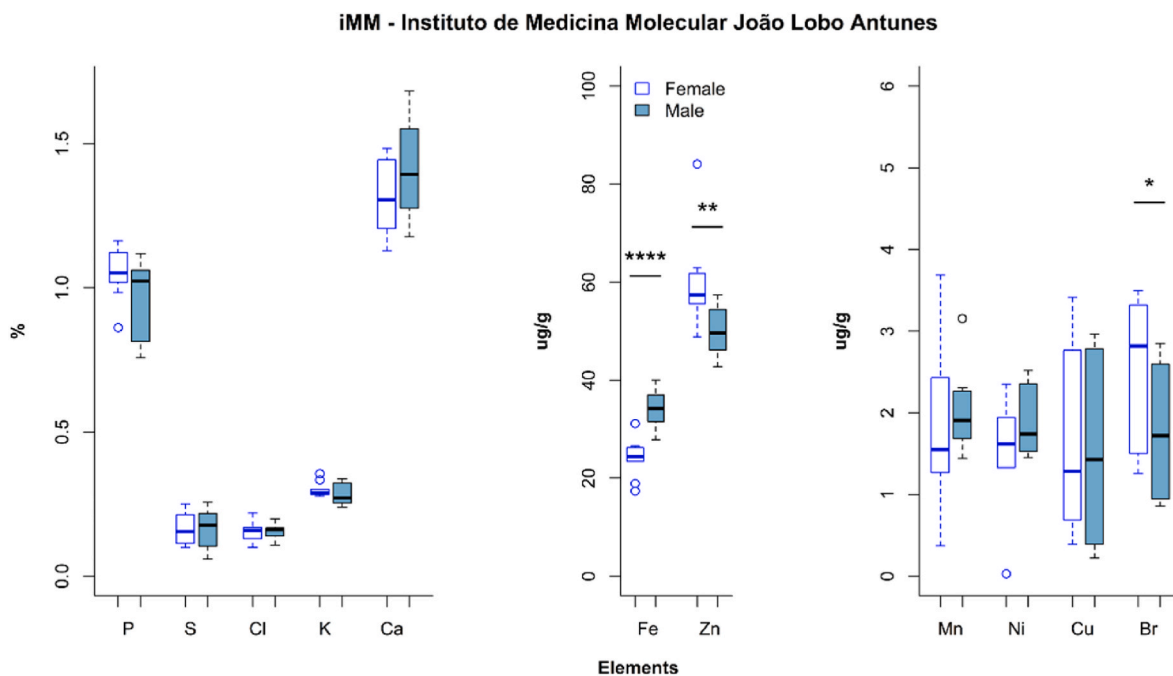


Fig. 4c. Quantification of each of the elements identified in the iMM (Instituto de Medicina Molecular João Lobo Antunes) vivaria in blue, and results of the analysis between females and males (* p-value <0.05 ** p-value <0.01 *** p-value <0.001 **** p-value <0.0001).

and mineral content in the bones of various fish species, identifying Calcium as the primary element.

To determine whether there were differences in the elemental composition between females and males, a statistical analysis was conducted. The results of this analysis are presented in Fig. 4. Data points without an asterisk indicate no significant difference, while asterisks represent increasing levels of significance as follows: * p-value <0.05, ** p-value <0.01, *** p-value <0.001, **** p-value <0.0001.

In the vivarium of the University of Aveiro (UA, grey), significant

differences between genders were observed for four elements: Cl, Fe, Zn and Br (p-value <0.05). At the Instituto Gulbenkian de Ciência (IGC, yellow), significant differences were identified in Cl, K, Fe, Zn and Br. In the third vivarium, Instituto de Medicina Molecular João Lobo Antunes (iMM, blue), only Fe, Zn and Br exhibited differences between females and males.

This comparison aimed to determine whether the variations in elemental concentrations between females and males were consistent across different vivaria or specific to each environment. It is evident that

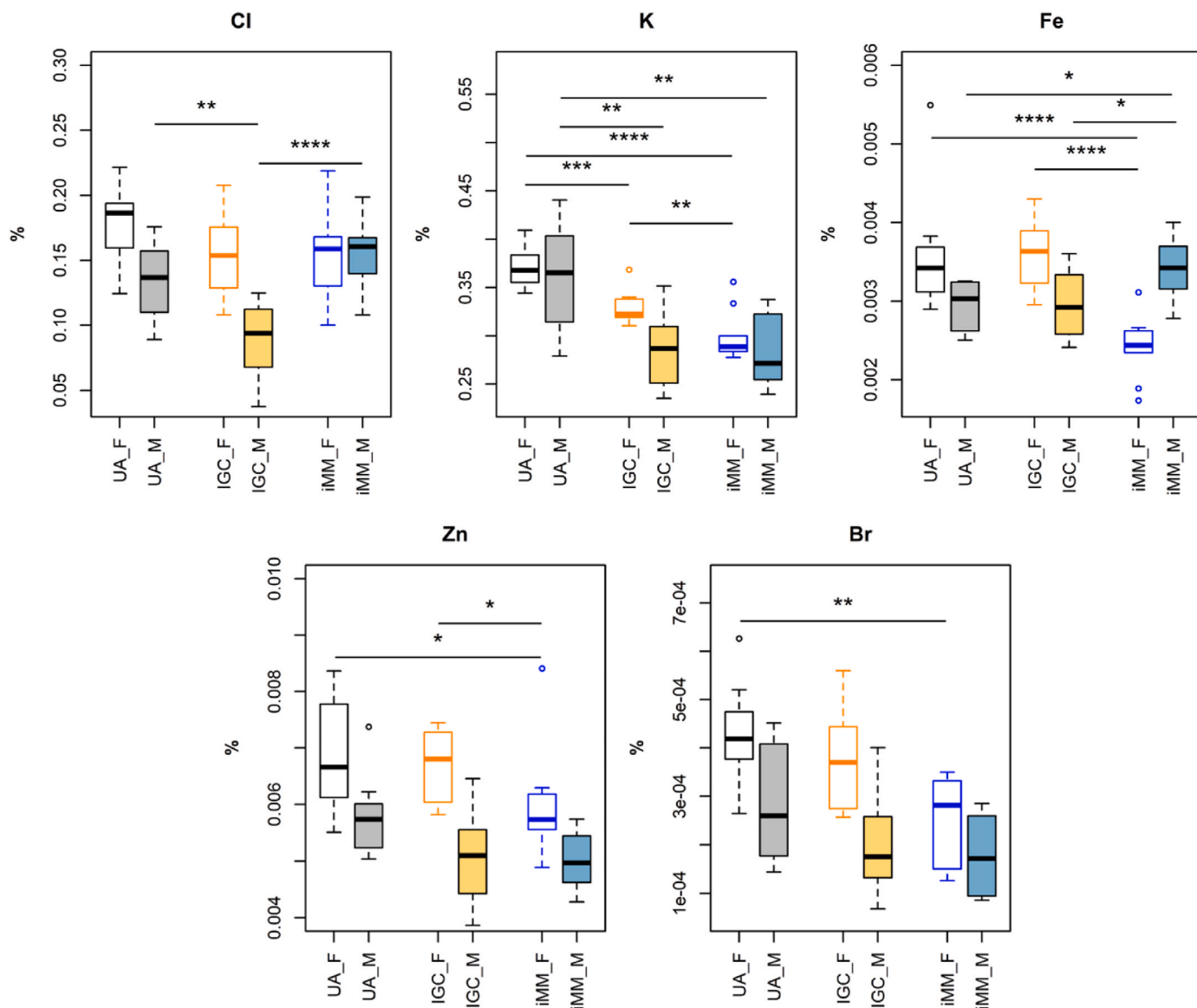


Fig. 5. – Statistical analysis between vivaria for the elements that presented differences between genders (* p-value <0.05 ** p-value <0.01 *** p-value <0.001 **** p-value <0.0001).

Fe, Zn and Br are common elements across all three vivaria, consistently showing differences between genders. In the UA and IGC vivaria, these elements were found in higher concentrations in females, whereas in the IMM vivarium, Fe exhibited higher levels in males.

Based on the results of the statistical analysis presented, the elements were categorized into two groups. The first group includes elements that exhibited significant differences between genders (Cl, K, Fe, Zn and Br), while the second group (P, S, Ca, Mn, Ni and Cu) comprises those that did not. For the latter group, the data from both females and males were combined and analysed collectively. A new analysis was conducted for the groups to determine if there were any significant differences between the three vivaria. For elements that showed significant gender differences, males were compared with males and females with females across the different vivaria. The results obtained are shown in Figs. 5 and 6.

As shown in Fig. 5, K exhibits the most differences across vivaria, with zebrafish from UA, of both genders, showing higher concentrations compared to the other vivaria and presenting significant differences with them. For Cl, only the males from IGC show significant differences compared to the males at UA and IMM. Fe presents significant

differences between both females and males from IMM when compared to UA and IGC zebrafish. Zn concentrations are notably lower in female zebrafish from IMM, leading to significant differences between female IMM and those from UA and IGC, while Br shows significant differences between female UA and female IMM.

As in other vertebrates, Fe plays a crucial role in zebrafish, as it is essential for various biological processes, including oxygen transport, development and growth, cellular respiration (Zhao et al., 2014) and immune response (Mao et al., 2024). Similarly, Zn plays a critical role in cellular function, immune response or reproduction and fertility (Zhao et al., 2014). Br may be an essential trace element, as it plays a role in the formation of collagen IV and basement membrane structures in some organisms (McCall et al., 2014). However, there is limited information on its presence and function in fish.

The significant difference in Zn or Fe levels between genders may be due to higher Zn and Fe requirements in females for reproduction. Zn and Fe play a crucial role in oocyte maturation and embryonic development. Since females produce and store a large number of eggs, their bodies may naturally accumulate more Zn compared to males (Galbraith et al., 2019) (Converse and Thomas, 2020).

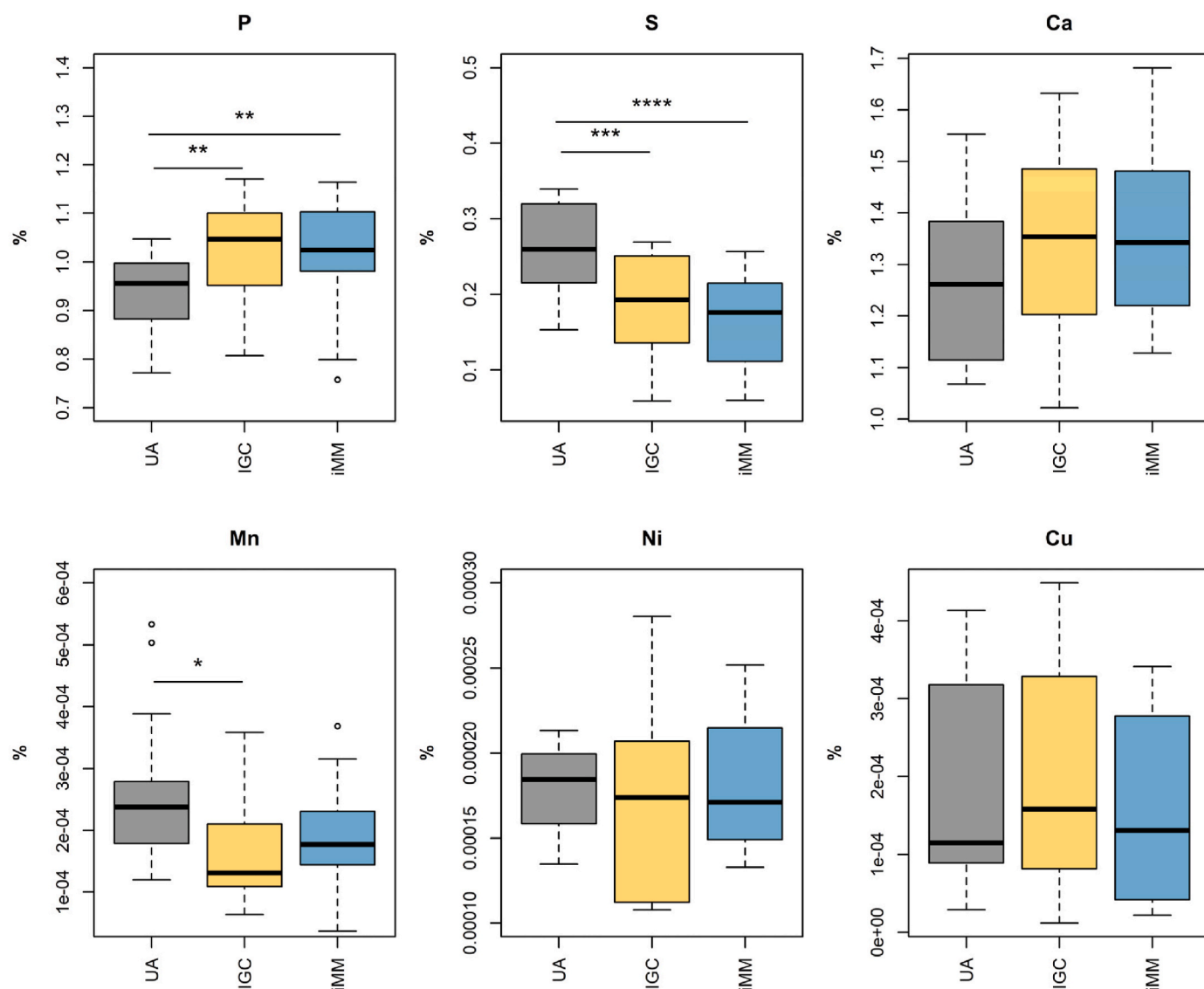


Fig. 6. Statistical analysis between vivaria for the elements that do not present differences between genders (* p-value <0.05 ** p-value <0.01 *** p-value <0.001 **** p-value <0.0001).

Additionally, variations in Fe, Zn and Br content among different fish food brands may also contribute to the differences observed. At iMM, a different processed diet was used compared to IGC and Aveiro, which both used the same brand. While all three vivaria rely on the same live feed, differences exist in rotifer feeding, a zooplankton component cultured in-house. UA does not use rotifers, whereas the other vivaria do.

Rotifer feeding can have several potential implications for the water/fish elemental composition, as it involves reconstituted marine saltwater and concentrated algae products. However, the elemental composition of these constituents is not fully available.

Fig. 6 shows the results of the statistical analysis for elements where the female and male data were combined. Out of the six elements analysed, three – Ca, Ni and Cu – do not show significant differences among the different vivaria, with similar concentrations observed in all three. For P and S, significant differences are found between UA and both IGC and iMM. In contrast, Mn shows a significant difference only between UA and IGC.

However, since the vivaria are located in different geographical regions, variations in the municipal water supply cannot be ruled out. Although the fish water is prepared similarly, differences in the

elemental composition of the municipal water may persist and may not be fully normalized by reverse osmosis treatment. Also, differences among commercial sea salt brands may contribute to the results obtained.

It is worth noting that the zebrafish specimens differ in genetic background, age, diet, and water chemistry parameters across the three vivaria. Any of these factors may potentially contribute to the variability in the results obtained for the different elements analysed.

4. Conclusions

In this work, Zebrafish that had been housed in a recirculation system with filtered water for their entire lifespan were analysed to identify and quantify the constituent elements. A commercial spectrometer, the micro-XRF system M4 TORNADO, was used for this purpose. Quantification of the identified elements was performed using the system's quantification software, based on the Fundamental Parameters method. The analysis used a matrix composition consisting of 7 % H, 23 % C, 7 % N and 60 % O.

Twelve elements were identified, with the highest concentrations being Ca and P for the three vivaria. Based on the obtained results, a

statistical analysis was conducted to determine if any elements exhibited significant differences between females and males. Following this, depending on the findings, the analysis was extended to evaluate potential differences between the vivaria. For the differences between genders, it was found that the elements Fe, Zn and Br show significant differences in the three vivaria. For elements such as Ca, Ni and Cu, no significant differences were found between females and males or between vivaria.

This exploratory study, despite its small sample size, provides valuable insights from the statistical analysis procedure that was applied. To enhance the robustness of these findings, conducting further analyses with a larger number of samples would be beneficial. In conclusion, the applicability of the micro-XRF system for studying the elemental constitution and quantification was shown, being capable of detecting reduced amounts of these elements.

CRedit authorship contribution statement

F.D. Leite: Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **S. Pessanha:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **S. Barbosa:** Writing – review & editing, Software. **A.C. Borges:** Writing – review & editing, Resources. **N. Pereira:** Writing – review & editing, Resources. **I. Domingues:** Resources. **A.L.M. Silva:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.radphyschem.2025.113139>.

Data availability

Data will be made available on request.

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