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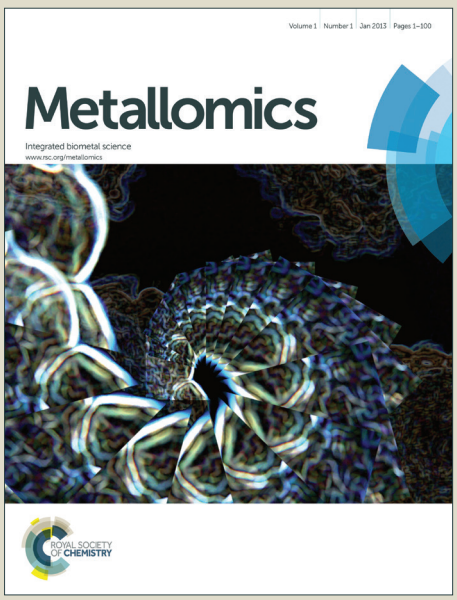

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Elemental mapping inventory of the fish *Liza aurata* brain: a biomarker of metal pollution vulnerability

Rita Godinho[1,2,3*], Patricia Pereira[2,4,5], Joana Raimundo[2,3], Mário Pacheco[4], Teresa Pinheiro[1,6]

[1] Instituto Superior Técnico, Universidade de Lisboa, EN 10, 2686-953 Sacavém, Portugal

[2] Instituto Português do Mar e da Atmosfera (IPMA), Av. de Brasília, 1449-006 Lisboa, Portugal

[3] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIMAR/CIIMAR), Universidade do Porto, Porto, Portugal

[4] Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro, 3810-193 Aveiro, Portugal

[5] Instituto de Ciências da Vida e Saúde (ICVS), Escola de Ciências da Saúde, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[6] Instituto de Bioengenharia e Biociências, Instituto Superior Técnico, Universidade de Lisboa, Portugal

[*]Corresponding author:

Rita Mendes Godinho

E-mail: rmgodinho@yahoo.com

Phone: +351-213027000

Fax : +351-213015

email: rmgodinho@yahoo.com

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Abstract

The elemental distributions in *optic tectum* of brains of wild *Liza aurata* a teleost fish captured in polluted and reference coastal areas were assessed quantitatively by nuclear microscopy providing insights into brain vulnerability to metal pollution. Elemental maps enabled to visualize *optic tectum* layers and identify cellular arrangements. Whereas Cl, K and Ca contents identify meninges, the Ca, Fe and Zn concentrations distinguish the underneath grey matter, white matter and inner cellular layers. Exposed animals showed significantly decreased P concentrations and increased contents of Cu, Zn and Ni in all brain structures. These changes highlight homeostasis modification, altered permeability of the blood-brain barrier and suggest risk for neurological toxicity. Our study initiated for the first time an inventory of physiological measures containing images and elemental compositions of brain regions of fish exposed to different environmental conditions. This will help defining total and local brain vulnerability to metals and pollution levels.

Keywords: Trace elements mapping; Brain; *Liza aurata*; Environmental exposure neurotoxicity

1. Introduction

Fish are key components of the trophic chains and also play an important role signalling water pollution, once they react with great sensitivity to changes in the aquatic systems.[1] Recently brain was pointed as a potential target tissue of environmental metal contamination[2,3,4,5] but the influence of trace elements in fish neurophysiology has been scarcely addressed.[6] The metal imbalance in fish is thought to play a critical role in

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neurodegeneration and neuronal dysfunctions.[7,8] The health of neuronal tissue is important in cognitive processes, vision and locomotion among other functions. Therefore, alterations in brain function due to metal imbalances may endanger not only the individual but also the population. Physiological measures can provide valuable indicators for monitoring, implement restoration strategies and improve fisheries management and conservation. Research in the biology of elements in fish brain may shed light into potential risks of metal exposure considering population dynamics.

To date, there has yet to be a comprehensive, atlas of the distribution and function of metals in fish brain. Mapping elemental distributions in animal tissues requires techniques with multi-elemental capabilities, high-spatial resolution and sufficient sensitivity to detect vestigial concentrations of metals. Nuclear microscopy technique combines most of these features.[9,10,11] It allows imaging and quantitative determination of the distribution of elements with sub-cellular resolution, thus enabling to understand the link between metal localization and function. The nuclear microscopy has been used for the analysis of thin tissue sections, in both environmental[12,13] and biomedical research.[14,15,16]

We have thus begun providing information on the distribution and functional significance of metals in the brain of fish *Liza aurata* using nuclear microscopy mapping. *Liza aurata* is a ubiquitous species in coastal ecosystems that is often used as a bioindicator of water quality. In this first report results from optic *tectum* region are described. The optic *tectum* is one of the fundamental components of the vertebrate brain, existing across the full range of species from hagfish to humans, being considered a homologue of the mammalian superior *colliculus*. In teleost fish it is greatly expanded and as been used as study model for the vertebrate species.[17,18,19] Beyond it is

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considered the main visual centre is involved in a wide variety of activities such as the goal-directed locomotion.[17]

This study evaluates major and trace elemental contents, essential and toxic, and their compartmentalization in optic lobe structures of *Liza aurata* captured from polluted and clean areas of a coastal system providing insights into species physiological requirements and vulnerability to metal toxicity. This is the first multi-elemental mapping of neuronal tissue of teleost fish.

2. Experimental

2.1. Sampling area and characterization of fish pollution profile

Native *Liza aurata* individuals were captured at two locations, a polluted and a reference area, of Aveiro coastal lagoon, in the northwest of Portugal.

Laranjo, the polluted area, is an inner and enclosed contaminated basin that has received effluents from a chloro-alkali plant during around five decades (1950-1994). Its contamination with Hg has been largely studied[3,5] however this area also presents high levels of other metals in sediments.[20] São Jacinto, the reference site, situates near the lagoon entrance, distancing around 10 km from the most contaminated area (Laranjo). Sediment metal concentration from Laranjo were higher than from reference area, namely: 21 $\mu g g^{-1}$ Ni, 29 $\mu g g^{-1}$ Cu, 119 $\mu g g^{-1}$ Zn and 0.33 $\mu g g^{-1}$Cd at Laranjo and 2.4 $\mu g g^{-1}$Ni, 14 $\mu g g^{-1}$Cu, 2.9 $\mu g g^{-1}$ Zn and 0.02 $\mu g g^{-1}$ Cd at S. Jacinto (unpublished data). Liver metal (Ni, Cu, Zn, As, Se, Cd and Pb) concentrations from n=10 fish captured at the same survey also were significantly different (Wilcoxon Mann-Whitney test, $p<0.05$) between the two areas, respectively: 1.7 ± 0.43 $\mu g g^{-1}$Ni, 275 ± 137 $\mu g g^{-1}$Cu, 73 ± 18 $\mu g g^{-1}$ Zn, 11 ± 2.7 $\mu g g^{-1}$As, 0.6 ± 0.13 $\mu g g^{-1}$ Cd and 0.7 ± 0.25 Pb for S. Jacinto,

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and 3± 0.75 μgg^{-1}Ni 1106 ± 191 μgg^{-1}Cu, 94 ± 19 μgg^{-1}Zn, 14 ± 2 μgg^{-1}As, 0.96 ± 0.29 μgg^{-1}Cd and 0.60 ± 0.3 μgg^{-1}Pb for Laranjo (unpublished data). Mercury levels in the brain, eye wall and lens of fish from the same areas were recently published and pointed out to significantly higher accumulation at Laranjo than reference area.[5]

2.2. Sampling

Golden grey mullet (*L. aurata*) were collected using a traditional beach-seine net. Immediately after catching, fish brain was dissected and deep-frozen in liquid nitrogen. Optical lobe transversal sections of 20 µm were obtained at midbrain level from the frozen material in a cryo-microtome. The sections were deposited on 1.5 µm polycarbonate foils and freeze-dried before analysis. The cellular integrity was checked under the light microscope, previous to analysis.

2.3. Elemental distribution determination

The samples were examined at the proton microprobe facility of Centro Tecnológico e Nuclear/IST.[21,22] A proton beam of 2 MeV energy with a current of 100 pA and of 3µm resolution was used to scan the samples. Particle induced X-ray emission (PIXE), Rutherford backscattering spectrometry (RBS), and scanning transmission ion microscopy (STIM) were used simultaneously to obtain morphological and quantitative elemental distribution data. The PIXE technique is capable of simultaneous multielementary analysis providing information on both major and trace elements, although in the conditions used in the this work Hg was not detectable. The RBS enables the measurement of matrix composition, depth variations and sample stoichiometry to normalize PIXE spectra for calculation of elemental concentrations.[23]

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STIM provides measures of areal density variations, and high-resolution images (<0.5 μm) of the sample morphology.[24] Maps of tissue sections were generated assigning the various detector signals to a digital X–Y positional coordinate. Selected areas ranging between approximately 250x250 μm^2 and 1000x1000 μm^2 were scanned. The relative amount measured was represented by a colour gradient. Concentration profiles were produced using point analyses in selected transepts across scanned areas.

2.4 Data analysis

The combination of PIXE and RBS data was used to quantify elemental concentrations as described elsewhere.[25] The areal density information obtained through STIM was used to identify tissue morphology in scanned areas and correlate those details with elemental distribution obtained by PIXE.[24] Data acquisition and elemental quantitative analysis were performed using OMDAQ and DAN32 software.[26,23] Elemental concentration data in the different optic *tectum* regions result from the average of, at least, five replicates of each point analysis in various sections from 2 fish brains at both polluted and reference areas. Elemental concentrations of reference and exposed brains were compared applying Mann-Whitney non-parametric test. Tests were considered significant when $p \leq 0.05$.

3. Results

3.1. *Liza* aurata optic *tectum* morphology and elemental mapping

Figure 1 and 2 illustrate how it was possible to characterize and distinguish the different brain tissues and cellular structures based on areal density and elemental distribution maps. Figure 1 show a transversal section of the optical lobe of *Liza aurata* from the reference area. The analysed area of superior optic *tectum* is signed on the optical

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microscopy photo on the left [Figure 1_1]. The layered organization could be visualized using STIM maps of areal mass density variations (Fig.1_2) and having optical stained sections as reference. Four main areas were identified from the outer exterior layer: (i) meninges (M); (ii) peripheral zone of grey matter (PG); (iii) central zone of white matter (CW); (iv) inner cellular layer (CL). Also the lobular arrangement of optic *tectum* could be visualized (Fig. 1_5). The meninges membranes are clearly evidenced in density maps (Fig. 1_3 and 1_4). Bellow the meninges different density layers of grey and white matter were distinguished (Fig. 1_5). The inner cellular layer showed a characteristic granular-like morphology (Fig. 1_ 6).

Elemental distribution maps obtained with PIXE also differentiate these four regions (meninges, grey matter, white matter and cellular layer), as illustrated in Fig. 2 for optic *tectum* superior layers. The Cl, K and Ca contents identify the outer layer, corresponding to the meninges (Fig. 2A), whereas Ca, Fe and Zn enabled to distinguish between deeper layers, such as the peripheral grey and white matter (Fig. 2B). The inner cellular layer showed a homogeneous distribution for most elements detected as illustrated for K in Fig. 2C. The granular structure evidenced in the density maps can be also visualized by Ca distribution. However, the details of both density and Ca images did not exactly match. Elemental distribution maps are useful to identify the brain architecture but they only provide qualitative information. To obtain quantitative data on elemental concentrations, point analyses have to be performed on selected transepts across the tissue sections[24,25,26]. This procedure enabled to accurately associate elemental concentrations to the different brain structures and therefore to compare animals caught at control and polluted areas as described below.

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3.2. Elemental concentrations in the optic *tectum* of *Liza aurata*

In animals captured in the reference area, the concentrations of P, S and Cl were relatively constant in all brain regions across the optic *tectum* (Fig. 3). The levels of K, Ca, Fe, Cu and Zn were mostly useful to discriminate outer and inner layers (from meninges to the interior cellular layer). Peripheral grey matter regions contrasted to other brain regions by a two-fold higher Fe content (41 ± 8 μg/g). On the other hand, the white matter regions showed the lowest concentrations of Cu and Zn, close to the detection limit (5-8 μg/g). Higher contents of K, Ca, Cu and Zn were measured in the inner cellular layer and meninges, relative to other brain regions, as can be inferred from Fig. 3.

3.3. Vulnerability of brain to environmental pollution

Brain of animals captured in the polluted area showed remarkable concentration differences compared with the brain of ones captured at the reference area (see Fig. 3) namely for P, Ni, Cu and Zn. All analysed optic *tectum* regions of fish from the polluted area were depleted in P and showed an increase of Cu and Zn. The meningeal layer, peripheral grey and central white regions of pollution exposed fish brain showed a ten-fold increase of Cu and Zn concentrations, while in the inner cellular layer smaller changes were observed (see Fig. 3). The most striking alteration found was the presence of Ni in all brain regions of fish from polluted site, contrasting with the fish from the reference site, where Ni was not detected. These alterations were more evident in the meninges and inner cellular layer. In these two regions a 25% increase of Ca concentration was also observed in fish from the polluted site, opposite to the other regions where Ca diminished, although significance was only verified in CW region.

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4. Discussion

The meningeal layers cover the teleost central nervous system, give protection and have protein secretion function. They are an interface between the vascular tissue and the cerebrospinal fluid and are active in the blood brain barrier.[27,28] In this context it is relevant the presence of electrolytes and essential trace elements that guarantee the cellular metabolism and neuronal communication.

The central white matter layer contains many fibres, mainly the axons and efferent nerve fibres of cells that originate at grey matter layer. The very low contents of Cu and Zn in the central white matter probably relates to the physiologic characteristics of this brain region. The axon has a reduced metabolic activity compared with the one achieved in the cell body.[29] Consequently higher elemental concentration would be expected in the layers associated with cellular bodies, such as the peripheral grey, containing neuronal cell bodies, fibres and synapses, and the inner cellular layers, mainly composed of neuronal cell bodies. In fact, relevant concentrations of Fe, Cu and Zn were associated with these regions. These three elements are essential to many cellular functions and enzymatic reactions such as protein synthesis, metabolism, and cellular energetics. Prohaska and Bailey[30] reported on high Zn and Cu concentrations in brain compared to other organs of fish highlighting its responsibility in normal central nervous system development and function.

Major changes in elemental contents were observed in the brain of animals from the contaminated Laranjo area. Increasing concentrations of Cl and K in the meninges observed in fish from the polluted area may influence the osmotic potential, altering cell permeability to metals. The enhanced concentrations of S, Fe, Ni, Cu and Zn at the

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meningeal layers were reflected in different magnitudes, in the underneath layers of optic *tectum*. Therefore, the meninges metal enrichment suggests a role of this brain structure in the control of elemental pools and the passage of those elements to and from blood vessels.[31]

The increases of Cu, Zn and Ni contents in optic *tectum* regions reflect the permeability of the blood-brain barrier for these metals. Copper and Zn have multiple essential physiological roles and therefore need to be readily available to brain cells. However, an overload of these elements in the brain tissue may influence metabolism. Copper may be involved in oxidative pathways with damaging consequences being observed to have neurological effects.[32] Although deleterious effects associated with Zn are negligible, since it can be chelated by many proteins and is not involved in oxidative reactions, exposure to excessive concentrations was observed to cause behavioural alterations in fish.[33] Nickel was nil in brain tissues of reference area animals. In exposed animals all brain regions showed increased Ni concentrations indicating a permeability of the blood-brain barrier to Ni and suggesting risk for neurological toxicity due to this teratogenic element. A well-known metal detoxification strategy consists of inducing metallothioneins, proteins to which several metals and metalloids have a high affinity.[34,35] However, a similar role for fish brain metallothioneins has not been established yet, results have been reported to be species, metal and condition specific.[36,37,38] Increased sulphur concentration in cellular layer of exposed fish may be related with enhanced protein content suggesting high relevance of further studies on this matter.

The Ca decrease in central white mater region (axons and efferent nerve fibres) and Cl increase in cellular layers (cell bodies) may have physiological effects in neuronal

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function. However, no significant changes of K were observed in our study, opposite to reported studies on fish brain exposed to pesticides and cadmium.[39,40]

The decrease of P concentrations observed in exposed organisms may indicate a down regulation of metabolic activity. The lower availability or retention of P may have drastic consequences as neuronal activity is highly dependent on ATP consumption.[41] This may be caused by excess of Fe, Ni and Cu as these elements can modulate oxidation-reduction reactions, influence or impair metabolic pathways, transport across cell membranes, axonal transport, and metal-responsive transcription factors, among other features.[42]

Further data is required to link these elemental concentration changes to specific cellular environments. In this context, detailed information of the reference brain would be useful. Both elemental maps of multiple planes and detailed elemental distribution at cellular level would be helpful to interpret the role of the elements in brain physiology and the effects of their concentration changes in fish behaviour and disease.

4. Conclusion

To our knowledge, this study was the first performing elemental mapping and initiating a quantitative elemental inventory of neuronal tissue of teleost fish.

Nuclear microscopy mapping and quantification of elements proved to be appropriate to spatially resolve brain morphological structures and detect diminutive variations of elemental concentrations. In the reference brain elemental signatures characterized different brain structures enabling to associate these signatures with the tissues physiological roles. Whereas Cl, K and Ca contents identify meninges, the Ca, Fe and Zn distinguish optical *tectum* layers. Brains of exposed animals showed altered

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elemental concentrations in the meninges and underneath optic *tectum* layers. These changes highlight homeostasis modification, altered permeability of the blood-brain barrier and suggest risk for neurological toxicity and behaviour alteration.

The elemental distributions of optic *tectum* of *L. aurata* brain reflected environmental burden, which can be useful for biomonitoring. In addition, cataloguing fish brain will have a significant impact in estimating neuronal health effects and will pave the way to further neurotoxicity studies. This is of particular relevance as teleost fish has been used as a model to study the central nervous system of vertebrate species

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Figure captions

Fig 1. Transversal section of the *Liza aurata* brain at midbrain level. 1) Optical microscopy image on the left identifies the region of the superior optic *tectum* analysed by nuclear microscopy (rectangle). 2) Nuclear microscopy areal mass density (STIM) map of the scanned region identifying optic *tectum* layers. 3), 4), 5) and 6) Zoomed STIM maps of on the different layers: M – Meningeal layers; PG – peripheral zone of grey matter; CW – central zone of white matter; CL – inner cellular layer. Density gradient represented by a dynamic colour scale: high density - white, to low density – black. Horizontal line denotes 100 μm

Fig 2. Elemental distribution in the superior optic *tectum* of the brain of *Liza aurata*. A- Meninges layer. B- Grey and white matter layers. C- Cellular layers. Areal mass density (STIM), on the left, and elemental distribution maps obtained by PIXE on the right: M – Meningeal layers; PG – peripheral zone of grey matter; CW – central zone of white matter; CL – inner cellular layer. Density and amount gradient represented by a dynamic colour scale: low – black/ blue to high – white/ red. Horizontal line denotes 100 μm

Fig 3 Elemental concentrations (expressed as mean and standard deviation) in the different layers, from the outer to the interior, of optic *tectum* of *Liza aurata* captured in the reference (white bars) and polluted site (black bars). M – Meningeal layers; PG – peripheral zone of grey matter; CW – central zone of white matter; CL – inner cellular layer. "nd" means not detected; * indicates significant difference to controls ($p<0.05$)

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Graphical abstract

Elemental mapping of fish brain exposed to metal pollution revealed altered elemental concentrations that highlight homeostasis modification, altered permeability of the blood-brain barrier and risk for neurological toxicity and behaviour impairments

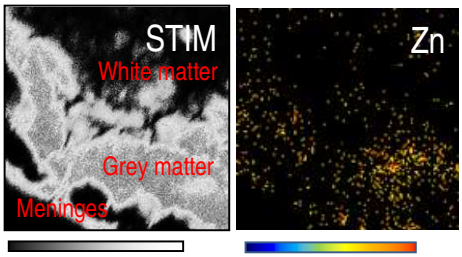

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Figure 1

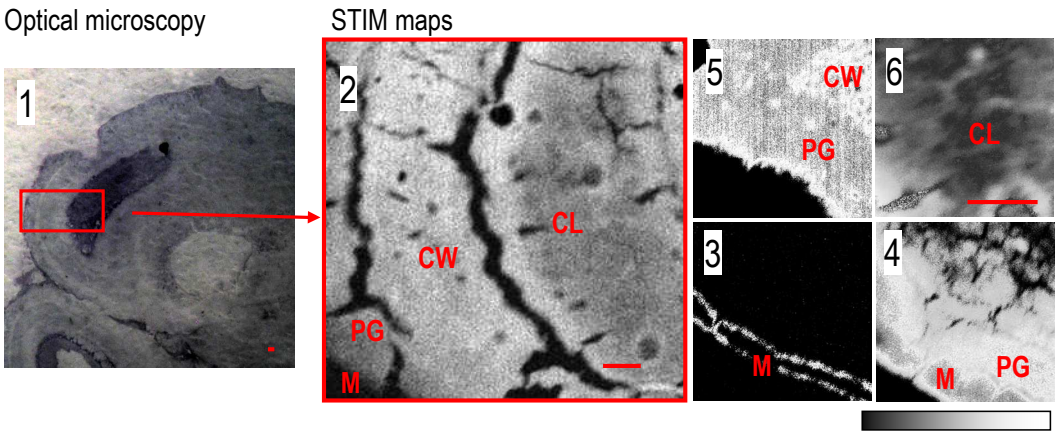

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Figure 2

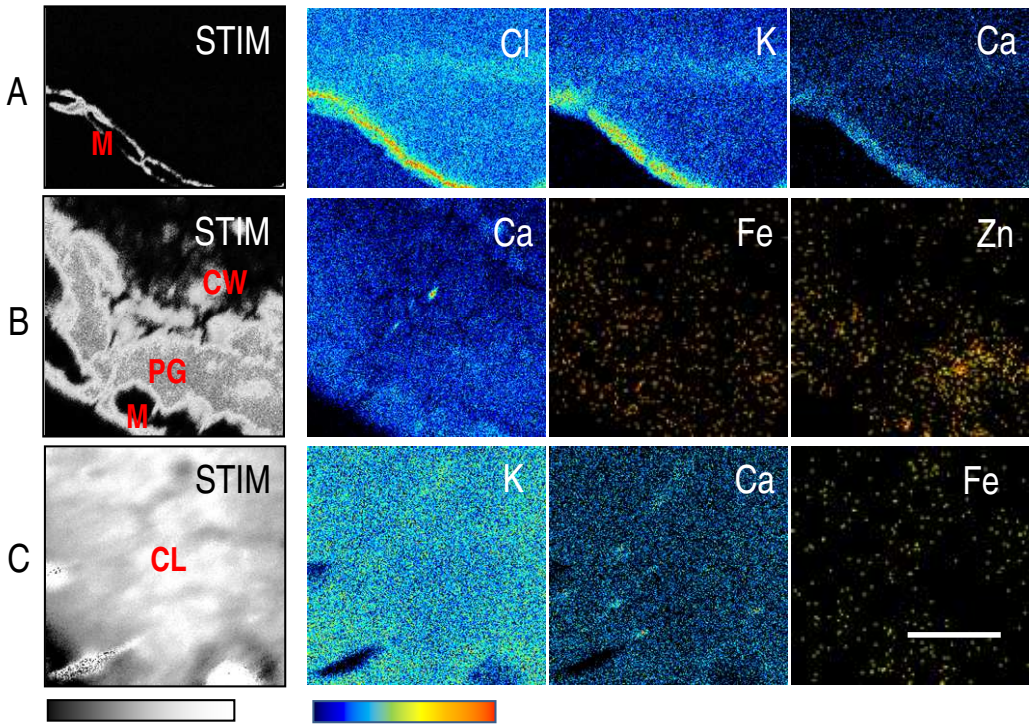

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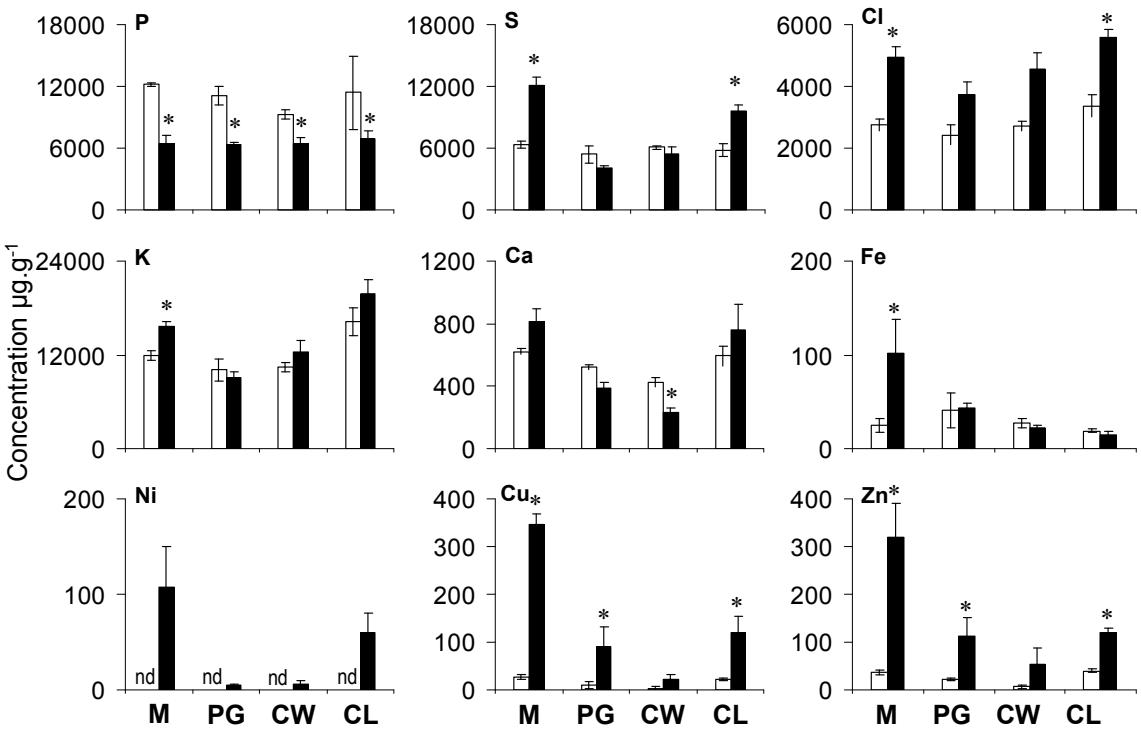
Figure 3



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