(Elemental characterization of injuries in fish liver

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1. Introduction

Coastal environments have been transformed by human activity, especially with increasing addition of substances in marine and estuarine environments characterizing contamination [1]. Fish can be used as monitors of environmental quality, both, in terms of the biodiversity of species in the community [2], as well as the healthiness of individuals [3]. Fish biomarkers are biological responses measured on organization levels below individual, namely cells, body fluids, tissues or organs. In this way, they provide an indirect measure of exposure and/or effects of contaminants [4].

Histopathological biomarkers can detect modifications or injuries in several tissues and organs and constitute a very important tool because they present a relatively fast response to the sublethal stressors. These biomarkers are highly sensitive and ecologically relevant, but have low specificities to contaminants, since certain injuries may be derived from different sources. Therefore, the diagnostics is not reliable and useful for resolving questions about the causative agents of injuries [1].

The liver is considered the first organ to identify histological biomarkers due to its central role in many metabolic functions like protein synthesis, gall secretion, metabolites accumulation, intoxication and detoxification. These functions make the liver bioaccumulate higher levels of toxic substances up to several orders of magnitude higher than the environment itself or even other organs [1]. One of the tissue’s most characteristic alterations are the melanomacrophage centers (MMCs), commonly associate with chronic inflammatory injuries and cell degeneration. The MMCs are clusters of monocytes containing melanosomes, lysosomes, and an accumulation of ceroid and lipofuscin. Several studies have demonstrated positive correlations between liver injuries and bottom-living fishes exposed to contaminated areas [5].

As histological studies are time-consuming and present low specificity, the present study was focused on using a new tool to evaluate modifications on micro-structures of fish liver with micro-PIXE along with broad PIXE as techniques to determine the presence of heavy metals on MMCs and their respective distribution on the liver tissue [6].

The micro-PIXE technique, among other ion beam analysis techniques, has some potentialities for environmental studies. Particularly in this case, besides the non-destructive character of the
analysis, it can indicate in which way some elements are distributed along the modifications. Moreover, the microscopic characteristic of the microprobe together with the capability of scanning certain areas of the sample open the possibility of correlating different elements by producing maps of each one of them [7]. This enables a deep analysis on which elements are present on healthy tissues, on MMCs and on other organ alterations.

2. Materials and methods

Specimens used in this study were caught in the Santos-São Vicente estuarine system, located in a tropical area of Southeastern

Fig. 1. Photomicrography of fish liver. Panels A–D represent liver tissues in the following conditions: (A) healthy liver with its morphology preserved and without apparent injuries or MMCs; (B) liver with injuries with MMCs (red arrows), showing disorganization and small focus steatosis (green arrows); (C) liver with injuries and no apparent MMCs, showing small focus of necrosis (blue arrows) and steatosis (green arrows); (D) liver with injuries but without apparent MMCs, showing focus of steatosis (green arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Elemental maps showing the distribution of Fe in the liver tissues. The scanned area is 1500 × 1500 µm². The panels show liver tissues in the following condition: (A) healthy liver; (B) liver with injuries and MMCs; (C) liver with injuries but no apparent MMCs as revealed by the histopathological study. The MMCs both in panels B and C are indicated with white arrows.
Brazilian coast. This area presents a history of contamination by several compounds due to the intense industrial and harbor activities and the disposal of sewage in the estuarine system [8].

For the present study, three cases were selected according to their liver condition classified as follows: healthy liver tissue; injured liver tissue with MMCS; and injured liver tissue without MMCS. From each case, three fish specimens were chosen and, of those, two liver tissue samples were extracted for analysis, resulting in 18 samples (of those, 9 liver replicates). In general, micro-PIXE measurements were carried out for a few samples in order to obtain elemental maps. On the other hand, PIXE measurements of all eighteen samples were performed for the quantitative analysis.

2.1. Sample preparation

For the histopathological characterization, well known protocols were used. The livers were extracted right after the fish’s death and were fixed on a 10% buffered formalin solution. Then, the livers were dehydrated on crescent alcoholic solutions from 70% to 100% for 60 min. Subsequently, they were submitted to diafanization in two xylene baths of 60 min and then embedded in paraffin. After cooling down, the blocks were placed in a microtome and 4–5 μm thick liver slices were produced. Finally, they were mounted on glass slides and dyed with hematoxylin/eosin for light microscopy analysis.

For the analysis of fish liver by micro-PIXE technique, some modifications in the standard protocol had to be made. First of all, glass slides are undesirable substrates for PIXE analysis because of the presence of silica and other elements that interfere on the X-ray energy spectra, causing a misinterpretation of the data. Therefore, it was used a polymer as a substrate in order to avoid such problems. In this case, a 2 μm thick Mylar® substrate was used for fixing the fish tissue.

Another problem on the traditional histopathological analysis is the use of dyes that may contain metals in their composition which could show up in the X-ray spectra. Because of that, the samples for PIXE and micro-PIXE analyses were not dyed. However, tissue modifications without dye are very difficult to be observed under an optical microscope. To solve this problem, a two-step process was devised. Initially, a tissue slice was cut from the paraffin block, fixed in a glass slide, and finally dyed. The stained tissue injuries were located under an optical microscope and mapped in the glass slide. Secondly, the injured regions were re-drawn over the paraffin block and through the use of a microarrayer, cylinders containing only modified tissue were cut and new tissue slices were fixed on Mylar® substrates.

2.2. Micro-PIXE measurements

Micro-PIXE measurements were performed using the Oxford Microbeams® system operating in triplet mode. The proton beam current varied between 20 and 150 pA while the beam energy was kept fixed at 3 MeV. The spot size was about 2.5 × 2.5 μm². Measurements were performed with different scan sizes, from 75 × 75 μm² to 1000 × 1000 μm². X-rays induced in the samples by the proton beam were detected with a Si(Li) detector placed a 135° with respect to the beam direction. The energy resolution of

![Elemental maps of a MMC depicting the following elements: (A) chlorine; (B) phosphorous; (C) sulfur; (D) iron; (E) copper; (F) zinc; and (G) nickel. The scan area is 300 × 300 μm². The scale bar is relative to all maps.](image-url)
the X-ray detector was 165 eV at 5.9 keV. Elemental maps were obtained for several tissues.

2.3. PIXE measurements

For quantitative analysis, PIXE measurements were performed with 2.0 MeV proton beam. Typical beam currents were about 1 nA. The beam spot size of $1 \times 1 \, \text{cm}^2$ was large enough to cover the entire sample. X-rays were detected by a Si(Li) detector with an energy resolution of 155 eV at 5.9 keV. The detector was placed at 135° with respect to the beam direction. The quantitative analysis of the X-ray spectra was carried out with the GUPIXWIN software in the thin target approximation. Finally, statistical analyses were performed as well using ANOVA One Way and Tukey's post hoc tests in order to check differences among the groups.

3. Results and discussion

Fig. 1 shows the histopathological analysis of fish liver showing a healthy liver, an injured liver without melanomacrophage centers (MMCs) and an injured liver with MMCs. A $1500 \times 1500 \, \text{mm}^2$ scan of the samples showed that there are no irregularities on the iron concentration of healthy tissue samples (Fig. 2). On the other hand, liver samples that presented melanomacrophage centers on the histopathological study showed iron spots on the micro-PIXE analysis. Moreover, samples with injuries but without apparent melanomacrophage centers on the histopathological study presented smaller spots of iron, suggesting that despite they were not detectable by the light microscope, the samples presented small melanomacrophage centers.

Fig. 3 depicts a more detailed scan ($300 \times 300 \, \mu\text{m}^2$) of a melanomacrophage center. The elemental maps reveal that chlorine is homogeneously distributed over the tissue, while elements such as phosphorus and sulfur are a little more concentrated in the MMC. Iron occurred in the MMC with higher concentrations in comparison to other metals like copper, zinc and nickel, suggesting that larger amounts of metals are indeed located in this structure. As iron is an essential erythrocytes component, the higher concentration can be expected in such structures. Concerning copper, zinc and nickel, a much longer measurement would have to be made to achieve enough statistics to produce better elemental. However, our results indicate clearly that these elements correlate with the MMC. Although some metals like iron, copper, zinc and cobalt are considered essential to biological processes, they can be toxic when present in higher concentrations [9].

Concerning the samples containing smaller MMCs, scans of $150 \times 150 \, \mu\text{m}^2$ were performed in order to obtain more detailed information of these structures. According to the results shown in Fig. 4, the concentration of iron is much higher in the MMCs. Moreover, a faint but still visible correlation between the MMCs and copper, zinc and to a lesser extent nickel was observed.

Results of the broad PIXE analyses can be seen in Fig. 5. As can be seen, some elements like Ti and Fe have higher concentrations in the injured tissue with bigger MMCs. This suggests that these elements are correlated to the MMCs rather than to the injury itself. On the other hand, elements such as Cr, Mn and Ni present larger concentrations in the liver tissue with small MMCs (not visible

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![Elemental maps of a MMC depicting the following elements: (A) chlorine; (B) phosphorous; (C) sulfur; (D) iron; (E) copper; (F) zinc; and (G) nickel. The scan area is $150 \times 150 \, \mu\text{m}^2$. The scale bar is relative to all maps.](image-url)
in the histopathological analysis), suggesting that these elements are related to the injury rather than with the MMCs. However, it must be stressed that from the statistical point of view, no significant differences were observed for most of the elements among the groups. Further studies with larger number of samples are needed in order to have a better picture relating the elements with the injuries studied in this work.

4. Conclusions

In this work we performed the elemental characterization of fish liver tissues using PIXE and micro-PIXE techniques. The results show that melanomacrophage centers (MMCs) are rich in Fe, while other metals like Ni, Cu and Zn appear in smaller concentrations. Elements like Ti and Fe appear to correlate with the presence of MMCs, while Mn and Ni could be related to the injuries themselves. However, no significant differences were observed for most elements from injured tissues with MMCs visible or not under histological analyses. Further studies could shed some light in this issue.

In spite of being a sensitive tool to diagnose toxic effects that affect animal tissues, the routine histological techniques are limited and cannot provide further details of lesions generated in tissues as a biological response to injury and stress. However, when associated to other methods of analysis such as PIXE and micro-PIXE, a comprehensive understanding of lesions in organic tissues can be achieved.

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