Energy dispersive X-Ray microanalysis in conjunction with scanning electron micrography to establish nematodes as bioindicators in marine fish environment

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Abstract Energy Dispersive X-Ray Microanalysis has been used as the non-invasive technique on Indian helminthes to explore the role of nematode parasites as bioindicators in the marine ecosystem of Central West coast of India for the first time. The accumulation of sulphur and iron were analysed from a raphidascaridoid roundworm, Rostellascaris spinicaudatum (Malhotra and Anas) parasitizing marine catfish, Arius maculatus from the Central West coast of India at Goa. Quantitatively, the cuticle on oral armature comprised as much as ten times more sulphur than iron content in the roundworm under study. However, only carbon and oxygen were detected over caudal papillae, where no metals or other elements were recorded. The utility of a raphidascaridoid nematode to act as a bioindicator, that had the potential of a bioaccumulator effector, is highlighted.

Keywords Rostellascaris spinicaudatum · Bioindicators · Metal · Bioremediation · Environmental impact assessment · Goa

Introduction

Helminth parasites as Accumulator Effectors have been gaining ground in the role of Bioindicators in India. Bioremediation using live organisms is a challenging task. However, utilizing naturally available tools as predictors is even a greater sensitive challenge. Several earlier reports have illustrated that the findings of toxicological investigations could be falsified if parasitic organisms are ignored (Turčekova et al., 2002). The accumulation bio-indication studies were involved in such studies conducted oncestodes and acanthocephalans. Some of the commonly occurring pollutants that have been acting in a natural environment directly or synergistically are inorganic metals viz. Fe, Zn, Pb, Cd, Cu and Mn (Baby et al. 2010). Since past three decades considerable interest has been generated in parasitic bioindicatorsto monitor aquatic biota to thrash out intricacies in the host-parasite interactions Geetanjali et al. (2002). The contribution to the discovery of parasites as water quality indicators was rightly acknowledged, but until recently the potential of bio-adaptability of endoparasites to accumulate biologically available metals in their bodies, when parasitized in fish and other aquatic biotas, was little understood. Certain contributions (Galli et al. 1998) appreciated the utility of parasites to assert the possibility of their role as a specialist bio-indicator that were competent to evade detection by the ordinarily available methods of hydrological monitoring. The role of cysteine as both catalyst and precursor in prebiotic peptide synthesis has been illustrated by Foden et al. (2020). The noticeable binding affinity of cysteine with iron to generate complexes with the potential to regulate protein function was elaborated. The amino acid, Cysteine interacted with environmentally abundant iron. These conclusions were also supported by the assertion by Baby et al. (2010).
Methodology

The roundworms *Rostellascaris spinicaudatum* (Malhotra and Anas, 2001) were extracted from the marine catfish, *Arius maculatus* at the Central Westcoast of India at Goa were washed with Hank’s medium and then fixed in 4% (w/v) Gluteraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) containing 3% (w/v) sucrose for 4.5 h at 4 °C. to conduct X-Ray Scanning Electron Microanalysis. The fishes were collected from the fresh landings at Jetti at Panjim, Goa. The tissues used for these investigations were head of *R. spinicaudatum* and the region of pre-caudal papillae. SEM photomicrographs were prepared with the specimens attached to the stubs, forelaborate analysis of the anterior (head) and the posterior (pre-caudal papilla) regions of the worm. Each body organ of three worms of the same stage of development were subjected for analysis, and these were impinged twice to extract data on elements as well as metals. EDXMA (Energy Dispersive X-Ray Microanalysis) was conducted using JSM6510LV JEOL (Japan) equipped with Oxford Instrument INCAx-act energy-dispersive x-ray analyzer, in conjunction with SEM analysis at USIF (University Sophisticated Instrumentations Facility), Aligarh Muslim University, Aligarh, U.P., India, and the results presented as Figs. 1, 2, 3, 4 along with quantitative data on elements analysed from the tissues. KRUSKAL WALLIS Test and

![Fig. 1](image1.png)  
*Fig. 1* SEM of head of Rostellascaris spinicaudatum (Ventral view) (Purple square represents measured area). Typical chart recording elemental composition using energy–dispersive X-ray Microanalysis (EDXMA) in conjunction with a Scanning Electron Microscope

![Fig. 2](image2.png)  
*Fig. 2* Head of Rostellascaris spinicaudatum for observed chemical elements after a 50-Sec run include specific peaks of different elements

![Fig. 3](image3.png)  
*Fig. 3* Bar diagrams to show quantitative illustration of peaks as depicted in Fig. 2

Student’s t-test were calculated and ANOVA was applied work out levels of significance of the metals accumulated.

Results:

The histograms evidently illustrated micro-quantities of C, O, S and Fe detected from Figs. 1,2 elaborating comparative assessment of contents of C, O, S and Fe. Though in microquantities iron content was higher than sulphur in different parts of the body, as analysed, but no metal deposition on papilla was detected but for the universal presence of C and O in both the regions studied. The values of iron element in trace quantities obtained from the
The present analysis as well as reported from other studies have been summarized in Table 1. The differentiating pattern of elemental distribution was notably specific with regard to body organs of the worm; iron and sulphur being encountered over the cuticle of oral armature (Figs. 1, 2, 3; Table 2), while no sulphur occurred over caudal papilla that was scrutinized for element analysis (Figs. 4, 5, 6; Table 3). Quantitatively, the content of sulphur analysed was at least ten times higher than iron content in the cuticle of the roundworm under study, that were expressed as weight percent.

The level of significance as assessed by the application of Kruskal-Wallis Test and Analysis of Variance between the iron content measured for the larval Vs adult roundworms analysed during the study, were as follows:-

| Source of Variation   | Df | Sum-of-Squares | Mean-Square | F Ratio |
|-----------------------|----|----------------|-------------|---------|
| Metal absorption by   | 1  | 1.541          | 1.541       | F_{1,2} = 0.561 |
| Nematodes             |    |                |             |         |
| Larva vis-à-vis Adult | 2  | 5.498          | 2.749       |         |
| Regression            | 3  | 7.039          |             |         |
| Total                 | 3  | 7.039          |             |         |

F_{1,2} = 0.561; P < 0.005

Discussion

Non-invasive detection of metals

The differential element’s accumulator response from the varied nematode body region’s measurements of *R. spini-caudatum* was in evidence. The evidence of occurrence of sulphur and iron was available in anterior part of body in the region of the head of the worm (Figs. 1–3). But no deposition of any trace metal or other elements were recorded (Figs. 4–6) from the surface of the caudal papilla in the hinder region of the body. These observations support the role of these worms as Accumulation Effector as a component of the environmental impact assessment (EIA).

Heckmann et al. (2007) enumerated advantages to avoid multiplicity of elemental run for detection and diagnosis of chemical elements by application of Energy Dispersive X-Ray Microanalysis (EDXMA). The sulphur ion contributors, disulphide bonds in the twin amino acids, Cysteine and Cystine that were the part of protein infrastructure of mammalian hair and horns as well as their probable association with acanthocephalan egg shells and hooks were emphasized by Heckmann et al. (2007). These amino acids also contributed to the strength of microstructure within the outer cuticle that also facilitated movement of fluids across to further smoothen the physiological functioning of worm’s various systems. These macro and micro elements are important structural and functional factors, as they are involved in the architecture of many enzymes and other complex molecules (Sures 2004). The predominant accumulation of essential metals like Cu, Fe, Zn and Se occurred with inhalation of food by the adult roundworms (Bird and Bird 1991; Szefer et al. 1998). The parallels in the morphological infrastructure and biology of the nematodes could be drawn vis-a-vis the requirements of essential elements enumerated above. Their larvae equip themselves well before encapsulation to feed on blood and connective tissues available during migration through tissues of their fish hosts. The revelations by Bird and Bird (1991) and Szefer et al. (1998) on the microstructure that assigned specific properties to the cuticle of nematodes made it obvious that the infrastructure around wall of larvae was much more simpler than the complex cuticularized skin of their adult counterparts. This indeed facilitated much more smoother movement of required fluids across physiological systems of larvae.

The bio-remediating effect of helminth parasites of fish in polluted water bodies has been a serious subject of study in recent years (Khalaj et al. 2007). It has been claimed that...
the nematode infra communities were remediating (pollutant is in priority because of its persistence) by triggering inherent accumulating instincts to absorb chemical elements inside soft body tissues of the fish. Indeed the survival of the fish in aquatic bodies laden with metal polluted loads were adequately supported. Physiologically, the developmental phase of parasitized helminthes and secondly, the time period spent while passing through the tissues of internal organs of its fish host virtually equipped the worm with an enriched potential to accumulate metal. Other supplemental factors could be the metal characterized by specific chemical elements, site of worm infesting within the host as well as the habitat of fish.

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**Table 1** Mean metal concentrations (µg/g wet weight) in the parasites (mean value ± SE) of infected fishes reported from worldover

| Family                  | Fe (µg/g) | Source                      |
|------------------------|-----------|-----------------------------|
| Raphidascaridae        | 0.15 ± 0.003* | Present study               |
| Rostellascaris spinicaudatum |          |                              |
| Anisakidae             | 0.102 ± 0.054* | Dural et al., 2011         |
| Hysterothylacium aduncum | 0.0234* | Morsy et al. (2012)         |
| Anisakid larva         | 0.1260* | Leite et al. (2017)         |
| Contracaecum           | 0.14* | Le et al. (2016)            |
| Contracaecum larvae    |          |                              |
| Camallanidae           | 2.95 ± 1.50** | Akansaya and Kuton (2016a) |
| Procamallanus spp.     | 1.66 ± 1.05* | Akansaya and Kuton (2016b) |
| Dioctophymatidae       |          |                              |
| Eustrongylides larva   | 0.0250* | Nachev et al. (2013)        |

*µg metal/g wet weight, **mg/g [Mean values are significantly different (P < 0.05)]

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**Table 2** The experimental data on elemental composition analysis using energy-dispersive x-ray microanalysis (EDXMA) in conjunction with scanning electron microscope (SEM)

| Spectrum Processing | Number of iterations = 3 |
|---------------------|--------------------------|
| Processing option:  | All elements analyzed    |
|                     | (Normalised)             |
| Standard:           | Caco 3                   |
|                     | SiO 2                    |
|                     | FeS 2                    |
|                     | Fe                       |
| Element             | Weight%      | Atomic %     |
| C K                 | 63.71        | 70.56        |
| O K                 | 34.60        | 28.77        |
| S K                 | 1.54         | 0.64         |
| Fe K                | 0.15         | 0.04         |

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**Fig. 5** Precloacalpapilla (Dorsal view) of Rostellascaris spinicaudatum for observed chemical elements after a 50-sec run include specific peaks of different elements

**Fig. 6** The area on sunflower pre-anal papillae posterior of Rostellascaris spinicaudatum exposed to x-ray microanalysis that yielded histogram for an iron and a sulfur peak after a 50-s run
The inorganic elements inherent in the complex long-chain fatty acids are not available to round worms through their de novo synthesis, therefore, their compulsive dependence on hosts to acquire these for completion of their metabolic activities is obvious. The essential inorganic elements required for development and growth of roundworms have to be obtained from their host’s body organs (Wang et al. 2020). The elements of primary significance from digested and absorbed, dietary nutrients are important precursors for the synthesis and metabolism of cells, most of which need be extracted from those hosts of nematodes parasitizing them (Wang et al. 2020; Khalaj et al. 2007).

The efficiency of the technique facilitating attainment of peak height to detect even trace amount of chemical elements in the tissues of fish after a 50-s run for each specimen (ANOVA: \( F_{1,2} = 0.561; \ P < 0.005; \text{ KRUSKAL WALLIS Probability 0.50} \) have been reliable (Heckmann 1996). The emphasis given on technology oriented surveillance of metals assisted in the detection of iron as well as sulphur only in the region of head and in no other part of body of the round worm in the present study. Thus the strength of cephalic armature is apparently specifically identical in the same way as the hardness functioned in a manner as to give strength to the hooks and proboscis in the anterior part of body of acanthocephalan (Heckmann et al. 2007). In earlier literature, the outcome of the surveillance strategy adopted for organisms of the aquatic ecosystems by the application of atomic absorption spectrometer was reviewed by Sures (2003). Stedman (2001) emphasized that the disulfide bonds coupled with sulphur ions that are the essential constituents of the chemical bond infrastructure of the amino acids, viz. cystine and cysteine, are the common structural constituents of keratin too in the horns of mammalian hosts which are placed higher in the ladder of evolution contrary to the parasitized host fishes which were placed at the lowest order in the series of evolution.

On similar lines the strength assigned to the head of <i>R. spinicaudatum</i> due to sulphur and iron detected in the region could be similar to the hardness of the mammalian tissue constituents described above, to assist the former in attachment and penetration through the tissues during worm migration, as similar in function of proboscis hooks and egg shells of acanthocephalan, earlier concluded by Heckmann et al., (2007). In further studies, Azmat et al., (2008) emphasized that nematode parasites like - <i>Echinocephalus</i> sp. and <i>Ascaris</i> sp. are sensitive indicator of heavy metals in aquatic ecosystems. Heckmann et al. (2007) enumerated advantages to avoid multiplicity of elemental run for detection and diagnosis of chemical elements by application of Energy Dispersive X-Ray Microanalysis (EDXMA). The sulphur ion contributors, disulphide bonds in the twin amino acids, cysteine and cystine that comprised protein infrastructure of mammalian hair and horns as well as their probable association with acanthocephalan egg shells and hooks were emphasized by Heckmann et al., (2007). The hardness, strength and rigidity provided by Carbon and Sulphur to the chitinized components around armature equipped head of a variety of Anisakid and Raphidascaridoid worms draws parallel to the conclusions of similarity drawn with constituents of keratin i.e. the infrastructure components of hairs, horns, hooves, beaks, shells, nails, claws of higher vertebrates by Radwan et al., (2012). It was significant that the study by Antoniou and Tselentis (1993) held the constitution of cuticle with specified elements like S, Ca and P accountable for the strength in acuarid roundworms in addition to the specialized cordons. The examples of the marine nematodes, *Tripyloides marinus*, <i>Ascaris suum</i> and *Xiphinema vuittenezi* were also cited. The site specific distribution of Sulphur at the tips, mid and basal parts of spines was also encountered by the application of Energy Dispersive X-Ray Microanalysis (EDXMA) method by Heckmann et al. (2017).

The critical role of sulphur in several life processes including the protective role of sulphur-coupled ligands that occurred in natural environment has been on record (Colovic et al. 2018). The adverse toxic effect and accumulation of metal ions is prevented on the body of higher mammals due to the active intervention by naturally occurring sulphur in the biological systems. The chelating sites generated by the sulphur-containing amino acids provide a protective platform for eliminating toxic metals. In view of this, it is significant that such amino acids represent an effective constituent of cell antioxidant system. On similar lines Nozaki et al. (1998) also illustrated...
the critical role played in the antioxidative defense of enteric parasites by the cysteine biosynthetic pathway.

The highly recognized macro-bioelement of the heavy metals has been iron, as emphasized by Nies (1999). In his opinion, it acted as a heavy metal filter to facilitate improved health conditions of the host. They may also have a beneficial effect on the health of their hosts by acting as heavy metal filters. Resultantly iron was considered to be the biologically most important cation. Oscar et al. (2003) concluded that the accumulator organisms reflect the element contamination level of their environment. The enhanced affinity of nematodes parasitizing alligators to Fe, Pb and Cd within the aquatic environment was concluded by Tellez and Merchant (2015) in their investigations. Conclusively, a lot variety of investigations conducted worldover on fish parasitic organisms have so far focused on cestodes (Turckova et al. 2002), acanthocephalans (Galli et al. 1998; Thielen et al. 2004; Heckmann et al., 2007) and other minor organisms (Galli et al. 1998). The deposition of metals, including iron on the body of another marine nematode of Anisakidae viz., Contracaecum sp. (third stage larvae) was reported by Leite et al. (2017), while members of Camallanidae namely, Procamallanus reportedly accumulate dinorganic metal, Fe on its body (Akinsanya and Kuton 2016b).

The reason of high concentration of iron analysed by Sures and Siddall (1999) in the experiments conducted on Hysterothylacium reliquens was explained by Sures et al. (1999a). He assigned this to the enhanced bio-availability of metals within the physiological system of the experimental organism, i.e. fish due to the presence of bile acid inside the alimentary canal at the site of infection. Calculated bio-availability of the iron amino acid chelate was 75.0% compared to 27.8% for FeSO4. As suggested by Fairweather-Tait et al. (1992), this study also demonstrated that the greater bio-availability of the iron amino acid chelate allows for the rapid incorporation of iron into hemoglobin first, followed by a quicker repletion of depleted iron pools (i.e. serum ferritin) than is possible with FeSO4 (Pineda and Ashmead 2001). This study may well open up a new area to intricately explore the possible amending effects of parasites on the biomarker responses particularly because most of the time moderate levels of iron in water or nutrients provide a supportive strength for the survival, growth and development of nematodes parasitic in fish (Sures et al. 2017). This conclusion emanated from the fact that the mixing of nitrates and iron in the upwelling zones of riverine stretch, the mixing of dietary feed with the available iron in the euphotic zone habitation of A. maculatus, could easily provide enhanced iron rich dietary constituents to this fish host through which the cuticle of nematodes, R. spinicaudatum availed opportunity to absorb extra iron available.

CONCLUSIONS: An elaborate narrative has been developed by Parasitologists to assert (Sures and Siddall 1999) that the environmental value of certain endoparasites should credibly be required a more emphatic attention. The approaches, methodology and academic prowess of ecological parasitologists and aquatic ecologists must address to common goals at large to develop skills to substantiate parasitic bioindicators. The earlier studies have faltered to ignore the significance of helminth parasites as contributors to sinks for heavy metals (Sures and Siddall, 1999) within a fish host. It is thus quite likely that a host fish could have much more potential to tolerate heavy metals in the aquatic ecosystems than that have been actually reported (Hofer and Lackner 1995; Gunkel 1994; Köck 1996) till recent times notwithstanding the heavier amount of contaminants that helminthes parasitizing such fish could have been known (Sures 2001). Therefore, this is the first validated attempt in the Gangetic freshwater ecosystems to confirm the role of nematodes as sensitive bioindicators that could prove to be vital as a viable tool for environmental monitoring. In a way, like the response of organisms to abiotic factors, such organisms/animals/plants that were susceptible to a wide range of parasitic organisms or their developmental stages could be termed as Euryparasitic. Morsy et al. (2012) elaborated sustainability potential of anisakid worms, a closer taxonomic ally of rhabdiscaridoid roundworms that are the subject of present study, as potential instruments to monitor environmental pollution which had the capacity to help in the survival of their fish hosts by removing heavy metals from within the aquatic ecosystems.

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