Trace element and metal sequestration in vitellaria and sclerites, and reactive oxygen intermediates in a freshwater monogenean, *Paradiplozoon ichthyoxanthon*

Beric M. Gilbert, Annemari Avenant-Oldewage

Department of Zoology, University of Johannesburg, Johannesburg, Gauteng, South Africa

These authors contributed equally to this work.

* aoldewage@uj.ac.za

Abstract

Exposure to metals and other trace elements negatively affects infection dynamics of monogeneans, including diplozoids, but, physiological mechanisms linked to exposure have yet to be documented. In this study sequestration of trace elements and reactive oxygen intermediate production in the monogenean, *Paradiplozoon ichthyoxanthon*, was demonstrated. During dissection of host fish, *Labeobarbus aeneus*, the gills were excised and assessed for *P. ichthyoxanthon*, which were removed and frozen for fluorescence microscopy or fixed for transmission electron microscopy. Trace elements were sequestered in the vitellaria and sclerites in *P. ichthyoxanthon*, and the presence of reactive oxygen intermediates was observed predominantly in the tegument of the parasite. Trace elements and metals identified and ranked according to weight percentages (wt%) in the vitellaria were Cu > C > Au > O > Cr > Fe > Si while for the sclerites C > Cu > O > Au > Fe > Cr > Si were identified. For most element detected, readings were higher in the vitellaria than the sclerites, except for C and O which were higher in sclerites. Specifically for metals, all levels detected in the vitellaria were greater than in sclerites. Based on the proportion of trace elements present in the vitellaria and sclerites it appears that most trace elements including metals were sequestered in the vitellaria. The results of reactive oxygen intermediate production in the tegument of the parasite suggests either trace element accumulation takes place across the tegument or results from the action of the host’s immune response on the parasite. The results serve as the first demonstration of trace element sequestration and reactive oxygen intermediates in a freshwater monogenean parasite.

Introduction

Aquatic organisms are exposed to and accumulate a wide variety of naturally occurring trace elements which become stored in inert tissues and subcellular compartments. Toxic effects associated with accumulated metals and some trace elements become evident in organisms...
when these elements bind inappropriately to sensitive organelles and molecules [1–3]. To control toxic effects associated with the accumulation of trace elements, biota have evolved mechanisms to minimize and regulate the interaction of reactive trace elements with proteins and other cellular components, and optimally utilise essential elements [4]. This is achieved through binding to metalloproteins which have an affinity for binding metals and other trace elements and effectively decrease excess levels of trace elements. Vitelloproteins, are such proteins, have been found to be useful biomarkers of exposure in invertebrates and have been shown to bind a number of trace elements [5–7]. In this way, the levels of free trace elements including metals which can potentially form free radicals within cells are reduced [8].

Trace element accumulation in parasites and their use as bioindicators in aquatic ecosystems has received increased attention [9]. Most of these investigations have analysed metals and other trace element concentrations in endohelminths of fishes, particularly, cestodes [10–15], acanthocephalans [16–19], nematodes [20–25] and digeneans [26]. Of the groups studied, acanthocephalans demonstrate the highest accumulation potential followed by cestodes. However, little is understood about the fate of metals and trace elements in parasites. Studies on Bothriocephalus scorpii and Schyzocotyle (formerly Bothriocephalus) achiagnathi [27] indicated that gravid, egg laden proglottids accumulate trace elements to higher levels compared to the anterior mature and immature proglottids [10,28]. Similarly in the acanthocephalan, Moniliformis moniliformis, metal burdens in female worms have been shown to be higher than in males [29]. For both cestodes and acanthocephalans, this was related to the egg shells functioning as sequestration sites for trace element including metals [10,30,31].

Exposure of parasites to metals and metalloids has also been shown to result in elevated stress responses or biomarker responses in parasites. Exposure to arsenic (As\text{III}) and antimony (Sb\text{III}) was found to result in cell death of Leishmania spp. but through different mechanisms [32]. Palladium exposure resulted in elevated production of heat shock proteins in the cysticanths [33] of Polymorphus minutus.

Globally, metal and trace element accumulation studies in ectoparasites have lagged behind those for endoparasites, and have mostly focused on the effects at a population level [34–37]. Laboratory studies on gyrodactylids [38–42] and larvae of Paradiplozoon ichthyoxanthon [43] documented sensitivity toward exposure to a number of metals. The exact mechanism by which these metals exert their toxic effects on the parasites studied is still largely unknown. The aims of this study were therefore to determine where in the body of adults and diporps of P. ichthyoxanthon metals are sequestered and which metals are present at these sites in adult worms, and to document events of oxidative stress though visualisation of reactive oxygen intermediates in adult parasites, which may be due to metals becoming accumulated.

**Materials and methods**

**Parasite collection**

During a single survey to the Vaal Dam, South Africa, in winter (August 2014), Paradiplozoon ichthyoxanthon specimens were collected from Labeobarbus aeneus. The host fish were caught by means of gill nets (mesh size: 45–190 mm) around UJ Island (26°52’33.62"S; 28°10’25.76"E) and 36 live fish were removed from the nets, in accordance to permits from the Gauteng Department of Agriculture for collection of fish (permit number: CPE3000123). Live fish were transported back to a field laboratory and maintained in aerated live wells containing dam water. Thereafter, fish were weighed, measured and euthanized by spinal severance posterior to the head. All procedures involving animals in this study were done in accordance with ethics guidelines as set out by the South African National Animal Ethics Council and approval by the University of Johannesburg Ethics committee (Protocol number: 9 April 2013). The gills
were excised and assessed for *P. ichthyoxanthon*. Seventy seven parasites were removed from the gills and 38 were flash frozen in liquid nitrogen while the remainder were fixed in 2.5% glutaraldehyde in 1 M sodium cacodylate buffer for 1 hour and then post fixed in 1% OsO₄ (w/v) in 1 M sodium cacodylate buffer (pH 7.2) at 4°C.

**Cryomicrotomy and fluorescence microscopy**

Whole mounts and sections of flash frozen parasites were prepared for fluorescence microscopy. Microscope slides were cleaned in acidic alcohol and dried at 60°C and coated with poly-L-lysine (Sigma-Aldrich, Missouri, United States of America). A working solution of poly-L-lysine was prepared by diluting 100 mL stock with 1000 mL deionized water, and used to coat slides that were dried over night at room temperature. Cryosections of 10 frozen *P. ichthyoxanthon* were prepared by embedding whole worms in optimal cutting temperature (OCT) compound (Sakura Finetek, California, United States of America) and freezing in a bath of dry ice and 2-methyl butane (Sigma-Aldrich, Missouri, United States of America) until the compound turned white [44]. The blocks were removed and stored at -80°C until sectioning on a Reichert—Jung CryoCut E cryomicrotome at 5 μm. Sections were mounted on the coated slides, air dried for 60 minutes and stored at -80°C until staining.

The fluorochromes used for visualising metal ions and reactive oxygen intermediates (ROI) in sections were Phen-Green™ FL cell-permant diacetate (Molecular Probes, Eugene, Oregon) and CellROX® deep red (Molecular Probes, Eugene, Oregon) respectively. NucBlue (Molecular Probes, Eugene, Oregon), a derivative of DAPI, was used as a counter stain to visualise nucleic acids. Working solutions of the fluorochromes were prepared by dissolving 1 mg Phen-Green in 1 mL dimethyl sulphoxide (Merck, South Africa) and then diluting 5 μL of the solution in 495 μL milli-Q water. CellROX deep red stock solution was diluted to 5 μM in the Phen-green solution. Finally 50 μL of NucBlue stock solution was added to the Phen-Green—CellROX mixture and stored at 4°C until treatment of sections. Slides were removed from the freezer and air dried for 60 minutes beneath a UV lamp to photobleach. The sections were then mounted in the fluorochrome mixture, covered with a coverslip, sealed with clear nail varnish and incubated in the dark at room temperature for 30 minutes. Whole mounts of adult parasites and diporps were similarly prepared by photobleaching, and mounting in the fluorochrome mixture. Slides were sealed with clear nail varnish. Sections and whole mounts were studied with a Zeiss Axioplan 2 epifluorescence microscope operated with Axiovision 4.3 software.

**Ultra-microtomey and EDS analysis**

Ten paired adult parasites were separated at the fusion region and each individual in the pair were then post fixed in osmium tetroxide and then washed three times in fresh 1 M sodium cacodylate buffer (pH 7.2) for 10 minutes each. Thereafter specimens were dehydrated through an ascending series of ethanol (70, 80, 90, 96 and 100% for 15 minutes each) concentrations, infiltrated and embedded in Spurr’s resin. Resin blocks were polymerized in an oven for two days at 60°C. Semi-thin sections (70 nm) of *P. ichthyoxanthon* were made using a Reichter-Jung Ultracut E ultramicrotome, mounted on formvar coated copper grids and contrasted using 5% (w/v) uranyl acetate (Agar Scientific, Essex, United Kingdom) and lead citrate. Sections were studied and x-ray microanalysis was performed using a Jeol JEM 2100 transmission electron microscope (TEM) equipped with an 80 T X-Max™ SSD energy-dispersive x-ray spectrometer (Oxford Instruments, City, Country), operated at 200 kV in spot size 1 mode. Concentrations of trace elements and metals determined at the points in sections where scans were performed re expressed as normalised concentrations in weight percentage (wt%) of specific
elements according to the setup of the instrument. The weight percentage is therefore a measure of the number of atoms at the analysis point in the section and accounts for the atomic mass of the specific elements detected. As osmium, lead and uranium are incorporated during preparation of samples for analysis these elements were included in the scans of sections but have been excluded from the results as they have no bearing on the levels of elements which are incorporated into adult parasites.

Results

In whole mounts of adults and diporpas of *P. ichthyoxanthon* the presence of metals was confirmed by use of Phen-Green (Fig 1A–1C). In adult parasites, the sclerites of the clamps were found to fluoresce a bright green colour, compared to the body of the worms. This green fluorescence is indicative of the presence of metals, and the intensity correlates with the concentration within different body compartments [45]. Furthermore, the fluorescence of clamp sclerites of the adults was brighter when compared to diporpas. In adults, the vitellaria produced an intensely bright green fluorescence (Fig 1C and 1D) while the egg showed a negative reaction for the fluorochrome, as seen by the brown colour. Vitellaria were absent in diporpas.

Results of the fluorescence microscopy of sections of *P. ichthyoxanthon* are presented in Fig 2 and section planes are indicated in the line diagram of an adult *P. ichthyoxanthon*. Fluorescence micrographs of sections through adult *P. ichthyoxanthon* indicate positive reactions for metals and reactive oxygen intermediates. Positive reactions for metals are indicated by green fluorescence (I) and show clear internal partitioning of metals within the body of the parasite. Bright green fluorescence can be seen for the vitellaria (Fig 2A) and sclerites (Fig 2B), whereas, a weaker reaction for metals occurred for the tegument (Fig 2A) and egg shell (Fig 2C and 2D) of the parasite. Blue fluorescence indicates the presence of nuclear material within sections.

**Fig 1.** Micrographs showing whole mount of an adult (A and C) and diporpa (B), and a sectioned egg (D) of *Paradiplozoon ichthyoxanthon* stained only with Phen-Green. Sclerites of the clamps of the adult parasite and diporpa (white arrows) and vitellaria (white triangle) can be seen fluorescing, whereas, negative reaction to the fluorochrome by an in utero egg (encircled by dotted line) can be seen situated between posterior regions of the two individuals.

https://doi.org/10.1371/journal.pone.0177558.g001
Nuclei appear to be aggregated along the periphery of the organism (ie. within the tegument) and to a lesser extent around viscera. Reactive oxygen intermediate production is indicated by red fluorescence (III) and was identified in all sections through *P. ichthyoxanthon*. Brighter ROI fluorescence was found for the tegument, while weaker reactions could be seen for the visceral organs and parenchyma, and where bright fluorescence for metals was present. Differences in production of ROI were further noted for the anterior and posterior regions of the parasites. Bright fluorescence was noted in the posterior aspect of the parasites compared to the anterior, this is particularly evident by localised fluorescence of ROI in the longitudinal section through the anterior and posterior regions of the parasite (Fig 2B). Furthermore, areas where metals appear to be accumulated do not indicate a correspondingly intense fluorescence for ROI.

Identification of metals at sites in parasites where bright fluorescence was identified, was achieved through analysis of semi-thin sections by EDS. Although sections were contrasted using lead citrate and uranyl acetate it was possible to identify elements which did not have overlapping excitation wavelengths with elements in contrasting solutions. In both vitellaria and sclerites C, O, Si, Cr, Fe, Cu, and Au were detected (Table 1). Comparison of the detectable levels of the metals between vitellaria and sclerites of adult *P. ichthyoxanthon* differed, with higher element percentages detected in vitellaria compared to the sclerites. This was especially apparent when comparing the levels of metals (Cr, Fe, Cu, and Au), which were present in

**Table 1.** Weight percentages (wt%) for trace elements and metals detected at points within in the sclerites and vitellaria of *Paradiplozoon ichthyoxanthon* from the Vaal Dam, South Africa.

| Element | Sclerites n = 20 | Vitellaria n = 20 |
|---------|-----------------|-----------------|
|         | Mean (wt%)      | Standard deviation | Mean (wt%) | Standard deviation |
| Carbon  | 49.95           | 21.70           | 28.20      | 23.47               |
| Oxygen  | 3.79            | 2.14            | 1.34       | 1.10                |
| Silicon | 0.21            | 0.23            | 0.19       | 0.13                |
| Chromium | 0.43             | 0.51           | 1.24       | 0.80                |
| Iron    | 0.45            | 0.39            | 0.61       | 0.47                |
| Copper  | 24.41           | 16.52           | 44.49      | 18.06               |
| Gold    | 2.48            | 4.04            | 5.66       | 3.36                |

n—number of observations in sections of adult *P. ichthyoxanthon*

https://doi.org/10.1371/journal.pone.0177558.t001
both organs. According to the weight percentages of the metals detected, in the vitellaria Cu was highest (44.49 wt%) and was followed by Au > Cr > Fe > Si. For the sclerites, all metals detected were lower than in the vitellaria and Cu (24.41 wt%) was similarly highest, followed by Au > Fe > Cr > Si being lowest (0.21 wt%).

**Discussion**

This study indicates that trace elements accumulated by adults of *P. ichthyoxanthon* differentially bind to organs, and particularly the vitellaria and sclerites. X-ray analysis of the element composition of the vitellaria and sclerites of *P. ichthyoxanthon* indicated elements such as C, O, Si, Cr, Fe, Cu and Au. Comparison of the amount of each element detected in the vitellaria and sclerites indicates more metals and trace elements are sequestered to the vitellaria than the sclerites, except for carbon and oxygen which were higher in sclerites. Comparability of the element values between the sclerites and the vitellaria was possible due to the fact that structures were sectioned and mounted on copper grids. The point where readings were taken was done in the centre of the grids so as to further minimise the interference with the copper wire forming the grids. This meant that the sections were the same thickness and therefore interference from background levels of elements was the same for both structures. The interference of background levels with carbon stubs and sample thickness in x-ray analysis was also indicated by Shinn et al. [46] in a study on the element composition of the sclerotised haptoral elements in three species of *Gyrodactylus*. The differences in element values between the sclerites and the vitellaria further indicates that vitellaria has greater binding capacity for metals compared to the sclerites. The sequestration of metals to vitelline has been shown to be an effective mechanism for regulating the levels of trace metals in the adult organism [47]. This however, may have further implications with regard to the development of embryos as these may become transferred to developing juvenile parasites via the yolk from adults. Erasmus [48] further indicated that antimony in vitellaria of *Schistosoma mansoni* was strongly associated with the vitelline droplets in vitelline cells. Vitellogenin has further been shown to be a suitable biomarker for monitoring exposure to pollutants, such as metals [49,50] and from the current results the same may be possible for *P. ichthyoxanthon*. However, from the lack of fluorescence of the eggs, it appears that metals are not associated with the contents of the egg. This needs further clarification as there is no information regarding the vitelline ultrastructure in diplozoids. Other studies have documented similarities in ultrastructural development of vitelline cells between other monogeneans [51,52] and some digeneans [48,51].

Terrestrial and aquatic invertebrates sequester metals to the exoskeleton which is shed during ecdysis and thus functions as a possible method of regulating metals in the body [53–57]. In monogeneans, Shinn et al. [46] indicated vanadium was incorporated into the ventral bar of *Gyrodactylus caledoniensis* and the hamuli of *G. salaris* and *G. colemanensis*. Shinn et al. [46] indicated a high sulphur content in the sclerites of *G. caledoniensis*, *G. salaris* and *G. colemanensis*. The high affinity of trace elements including metal ions for SH–groups has been indicated in a number of proteins, particularly the metallothioneins which have been extensively studied in the role of trace element detoxification organisms. Kritsky et al. [58] indicated through staining the haptor of *Gyrodactylus* with Gomori’s trichrome, the ventral bar is composed of collagen while the composition of the hamuli comprise another protein which did not react with the stain. Lyons [59] and Shinn et al. [60] suggested that the sclerotized haptoral elements of gyrodactylids are composed of keratin–like proteins. Regarding the protein composition of the sclerites of diplozoids, two studies have suggested that these structures are composed of either chitin [61] or resilin–like proteins [62], which is similar to the composition of arthropod exoskeletons. Terrestrial and marine marcoinvertebrates have been shown to
incorporate elements into their exoskeletons as a means of detoxification [63–65] and structural support [53–56]. As the clamps of diplozoids are possibly similar to the composition of arthropod exoskeletons, it is likely that elements incorporated into the sclerites may serve a similar structural supportive purpose. Element incorporation into the sclerites of *P. ichthyoxanthon* is evident from comparison of the intensity of the fluorescence of the clamps in adult parasites and diporps. Such fluorescence intensity variance is indicative that the amount of metal and trace elements present in these structures [45] and variations between the developmental stages of the parasite could further relate to the length of exposure of the parasite. Schofield et al. [53] indicated that the amount of Zn present in the mandibles of leaf cutter ants differed between different developmental stages.

Negative fluorescence of the egg shell of *P. ichthyoxanthon* for metals was indicated and contrasts the results found for cestodes. In endoparasites, Sures et al. [10], Scheef et al. [29], Degger et al. [30] and Khalil et al. [31] collectively found that the eggs of the cestodes *B. scorpii* and *S. aechiloragnathi* function as sequestration sites for metals. Scheef et al. [27] suggested that metals were sequestered in eggs of the acanthocephalan, *M. moniliformis*, as female parasites contained greater metal concentrations than males. Sures et al. [10] found that the concentration of Pb and Cd were higher in the posterior, egg containing segments of *B. scorpii* compared to the anterior ones. Degger et al. [28] indicated that metals were associated with the egg shells of *S. aechiloragnathi* with the use of Phen-Green stain. Degger et al. [30] attributed negative results for metals in shells of *S. aechiloragnathi* to the age of the eggs, with younger eggs which did not contain metals and therefore producing a similar orange fluorescence as found in the present study for eggs of *P. ichthyoxanthon*. The negative result obtained in the present study can be linked to differences in the composition and formation of cestode and monogenean egg shells. According to Ramalingam [66] the monogenean egg shell is composed of dityrosine stabilised by sulphur cross-linkages and shells are not formed through a tanning process which occurs in cestodes [67], as they found that quinones were absent in egg shells of the monogeneans, *Pseudomicrocotyle* and *Pricea multae* after incubation with heated catechol [62]. The hardening of monogenean eggs is, therefore, achieved through dehydration of the shell matrix and not enzymatic polymerisation [68]. Metals have also been shown to sequester to the egg cases of sharks and skates [69,70], which are composed of similar proteins and harden via quinone tanning as in cestodes. It is thought that metals are incorporated into the shell matrix of skates, rays, dogfish sharks and cestodes during the formation of the egg shell.

The production of ROI by *P. ichthyoxanthon* was observed and localised predominantly to the tegument of the parasite, particularly in the region of the haptor. The production of ROI in relation of metals has been studied in a number of organisms, from protozoans to vertebrates. Exposure to environmental pollution has been found to produce biomarker responses in parasites. Mehta and Shaha [32] reported that exposure of *L. donovani* to As\(^{III}\) and Sb\(^{III}\) resulted in increased cell death due to oxidative stress. With accumulation of the metalloids resulting in an influx of Ca\(^{2+}\) ions and unavailability of glutathione (GSH) in reducing ROI in the cell due to the metals binding to the sulphhydryl groups of the protein. Not only did *P. ichthyoxanthon* produce ROI but this was localised to the tegument of the haptor. Sures and Radszuweit [33] found that exposure to elevated levels of Pd resulted in increased levels of heat shock protein (Hsp) 70 in *P. minutus* cysticercans infecting the haemocoel of *Gammarus roeseli*. Furthermore, the production of Hsp70 in response to Pd exposure was higher in the acanthocephalan cysticercans than both uninfected and parasitised gammarid intermediate hosts. Rico et al. [71] indicated localized production of ROI in certain areas of the cytoplasm and organelles of four ciliate species exposed to Cd and Cu. Even though it was shown that *P. ichthyoxanthon* produce ROI, it is unclear if this is specifically related to the accumulation of metals. The fact that bright ROI signals were localised in the posterior of the parasite does not necessarily indicated
that production is related to the uptake of metals and other trace elements, but rather may be related to the interaction of the host immune response toward the parasite. Further investigation into this aspect is required and should constitute exposure of the parasites to metals under laboratory conditions.

Supporting information

S1 Table. Raw data of EDS scans for sections of parasites analysed by TEM. The data can be accessed from the following citation: The data supporting the conclusions drawn in this article can be accessed via Gilbert, Beric; Avenant-Oldewage, Annemarie (2017): EDS raw data. figshare. https://doi.org/10.6084/m9.figshare.4733320 (DOCX)

Acknowledgments

University of Johannesburg is thanked for providing infrastructure and Spectrum analytical facility for the use of equipment. Dr S Peters is thanked for his advice on sectioning techniques for cryomicrotomy of the parasite material and Mr QM Dos Santos for assistance in the field, collecting and transporting fish to the laboratory. Dr F Jirsa is thanked for assistance with interpretation of x-ray spectra.

Author Contributions

Conceptualization: AAO BMG.
Data curation: AAO BMG.
Formal analysis: AAO BMG.
Funding acquisition: AAO.
Investigation: AAO BMG.
Methodology: AAO BMG.
Project administration: AAO BMG.
Resources: AAO.
Supervision: AAO.
Validation: AAO BMG.
Visualization: AAO BMG.
Writing – original draft: BMG.
Writing – review & editing: AAO BMG.

References

1. Monserrat JM, Geracitano LA, Bianchini A. Current and future perspectives using biomarkers to assess pollution in aquatic ecosystems. Comments Toxicol. 2003; 9:255–69.
2. Wallace WG, Lee B-G, Luoma SN. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Mar Ecol Prog Ser. 2003; 249:189–97.
3. Wang WX, Rainbow PS. Influence of metal exposure history on trace metal uptake and accumulation by marine invertebrates. Ecotoxicol Environ Saf. 2005; 61 (2):145–59. https://doi.org/10.1016/j.ecoenv.2005.01.008 PMID: 15883088

4. Vijver MG, van Gestel CAM, Lanno RP, van Straalen NM, Peijnenburg WJGM. Internal metal sequestration and its ecotoxicological relevance: A review. Environ Sci Technol. 2004; 38:4705–12. PMID: 15487776

5. Gagné F, Blaise C, Hellow J. Endocrine disruption and health effects of caged mussels, *Ellipitio complanata*, placed downstream from a primary-treated municipal effluent plume for 1 year. Comp Biochem Physiol—C Toxicol Pharmacol. 2004; 138 (1):33–44. https://doi.org/10.1016/j.cbpc.2004.04.006 PMID: 15313444

6. Gagné F, Blaise C, Pellerin J. Altered exoskeleton composition and vitellogenesis in the crustacean *Gammarus pulex* sp. collected at polluted sites in the Saguenay Fjord, Quebec, Canada. Environ Res. 2005; 98 (1):89–99. https://doi.org/10.1016/j.envres.2004.09.008 PMID: 15721888

7. Hwang DS, Lee KW, Han J, Park HG, Lee J, Lee YM, et al. Molecular characterization and expression of vitellogenin (Vg) genes from the cyclopoid copepod, *Paracyclopinina nana* exposed to heavy metals. Comp Biochem Physiol—C Toxicol Pharmacol. 2010; 151:360–8. https://doi.org/10.1016/j.cbpc.2009.12.010 PMID: 20045491

8. Oliva M, Vicente JJ, Gravato C, Guilhermino L, Galindo-Riaño MD. Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): Seasonal and spatial variation. Ecotoxicol Environ Saf. 2012; 75:151–62. https://doi.org/10.1016/j.ecoenv.2011.08.017 PMID: 21937114

9. Sures B. The use of fish parasites as bioindicators of heavy metals in aquatic ecosystems: A review. Aquat Ecol. 2001; 35 (2):245–55.

10. Sures B, Taraschewski H, Rokicki J. Lead and cadmium content of two cestodes, *Monobothrium wageneri* and *Bothriocephalus scorpii* and their fish hosts. Parasitol Res. 1997; 83 (6):618–23. PMID: 9211516

11. Sures B, Grube K, Taraschewski H. Experimental studies on the lead accumulation in the cestode *Hymenolepis diminuta* and its final host, *Rattus norvegicus*. Ecotoxicology. 2002; 11 (5):365–8. PMID: 12463683

12. Jirsa F, Leodolter-Dvorak M, Krachler R, Frank C. Heavy metals in the nase, *Labeobarbus kimberleyensis* (Gilchrist and Thompson, 1913) in the Vaal Dam, South Africa as indicators of heavy metal bioaccumulation. Phys Chem Earth. 2006; 31 (15–18):840–7.

13. Retief NR, Avenant-Oldewage A, du Preez H. The use of cestode parasites from the largemouth yellowfish, *Labeobarbus kimberleyensis* (Gilchrist and Thompson, 1913) in the Vaal Dam, South Africa as indicators of heavy metal bioaccumulation. Phys Chem Earth. 2006; 31 (15–18):840–7.

14. Retief NR, Avenant-Oldewage A, du Preez HH. Ecological aspects of the occurrence of asian tape-worm, *Bothriocephalus aecilognathi* Yamaguti, 1934 infection in the largemouth yellowfish, *Labeobarbus kimberleyensis* (Gilchrist and Thompson, 1913) in the Vaal Dam, South Africa. Phys Chem Earth. 2007; 32 (15–18):1384–90.

15. Abdel-Ga ber R, Abdel-Gh affar F, Bashtar A-R, Morsy K, Saleh R. Interactio ns between the intestinal nematodes, *Pseuda lius inflexus* and their fish hosts. Parasitol Res. 1997; 83 (6):618–23. PMID: 9211516

16. Sures B, Siddall R. *Pomphorhynchus laevis*: the intestinal acanthophanthalan as a lead sink for its fish host, *chub* (*Leuciscus cephalus*). Exp Parasitol. 1999; 93:66–72. https://doi.org/10.1006/expr.1999.4437 PMID: 10502468

17. Sures B, Siddall R. *Pomphorhynchus laevis* (Palaecanthocephala) in the intestine of chub (*Leuciscus cephalus*) as an indicator of metal pollution. Int J Parasitol. 2003; 33:65–70. PMID: 12547347

18. Sures B, Taraschewski H, Rydlo M. Intestinal fish parasites as heavy metal bioindicat ors: A comparison between *Acanthocephalus lucii* (Palaecanthocephala) and the Zebra Mussel, *Dreissena polymorpha*. Bull Environ Contam Toxicol. 1997; 59(1):14–21. PMID: 9184035

19. Sures B, Dezfuli BS, Krug HF. The intestinal parasite *Pomphorhynchus laevis* (Acanthocephala) interferes with the uptake and accumulation of lead (210Pb) in its fish host chub (*Leuciscus cephalus*). Int J Parasitol. 2003; 33 (14):1617–22. PMID: 14636677

20. Sures B, Taraschewski H, Jackwerth E. Lead content of *Paratenonisentis ambigua* (Acanthocephala), *Anguillicola crassus* (Nematodes) and their host *Anguilla anguilla*. Dis Aquat Organ. 1994; 19:105–7.

21. Szefer P, Rokicki J, Frelak K, Skora K, Malinga M. Bioaccumulation of selected trace elements in lung nematodes, *Pseudulus inflexus*, of harbor porpoise (*Phocoena phocoena*) in a Polish zone of the Baltic Sea. Sci Total Environ. 1998; 220(1):19–24. PMID: 9800383
22. Baruš V, Jarkovský J, Prokeš M. Philometra ovata (Nematoda: Phascolarctidae): A potential sentinel species of heavy metal accumulation. Parasitol Res. 2007; 100(5):929–33. https://doi.org/10.1007/s00436-006-0384-8 PMID: 17149604

23. Nachev M, Schertzinger G, Sures B. Comparison of the metal accumulation capacity between the acanthocephalan Pomphorhynchus laevis and larval nematodes of the genus Eustrongylides sp. infecting barbel (Barbus barbus). Parasit Vectors. 2013; 6 (1):21.

24. Otachi EO, Körner W, Avenant-Oldewage A, Fellner-Frank C, Jirsa F. Trace elements in sediments, blue spotted tilapia Oreochromis leucostictus (Trewavas, 1933) and its parasite Contracaecum multipapillatum from Lake Naivasha, Kenya, including a comprehensive health risk analysis. Environ Sci Pollut Res. 2014; 21 (12):7339–49.

25. Abdel-Ghaffar F, Abdel-Gaber R, Bashtar A-R, Morsy K, Mehlhorn H, Al-Quraishy S, et al. Khalil M, Furness D, Polwart A, Hoole D. X-ray microanalysis (EDXMA) of cadmium-exposed eggs of M. mosquitofish (Gambusia holbrooki) from Nador, Morocco. Int J Parasitol. 2009; 39 (10):1093–8. https://doi.org/10.1016/j.ijpara.2009.02.023 PMID: 19341741

26. Torres J, Kacem H, Eira C, Neifar L, Miquel J. Total mercury and selenium concentrations in the blue-spotted tilapia Oreochromis leucostictus (Trewavas, 1933) and its role as a biological indicator of pollution. Parasitol Res. 2014; 114:513–22. https://doi.org/10.1007/s00436-014-4213-1 PMID: 25468378

27. Brabec J, Waeschenbach A, Scholz T, Littlewood DTJ, Kuchta R. Molecular phylogeny of the Bothriocephalidea (Cestoda): Molecular data challenge morphological classification. Int J Parasitol. 2015; 45:761–71. https://doi.org/10.1016/j.ijpara.2015.05.006 PMID: 26185667

28. Riggs MR, Lemly AD, Esch GW. The growth, biomass, and fecundity of Bothriocephalus acheilognathi in a North Carolina cooling reservoir. J Parasitol. 1987; 73 (5):893–900. PMID: 3656010

29. Scheef G, Sures B, Taraschewski H. Cadmium accumulation in Paraphysocotyle auriculata (Cestoda: Hymenolepididea) and its influence of this heavy metal on mitotic activity. Acta Parasitol. 2000; 45:761–71. https://doi.org/10.1017/S0022149X07751465 PMID: 17578999

30. Dušek L, Gelnar M, Šeblová Š. Biodiversity of parasites in a freshwater environment with respect to pollution: Metazoan parasites of chub (Leuciscus cephalus L.) as a model for statistical evaluation. Int J Parasitol. 1998; 28(10):1555–71. PMID: 9801915

31. Khan RA, Thulin J. Influence of pollution on parasites of aquatic animals. Adv Parasitol. 1991; 30:201–39. PMID: 2069073

32. Valtonen ET, Holmes JC, Aronen J, Rautalahti I. Parasite communities as indicators of recovery from pollution: parasites of roach (Rutilus rutilus) and perch (Perca fluviatilis) in central Finland. Parasitol. 2003; 126:S43–52. PMID: 14667171

33. Gilbert BM, Avenant-Oldewage A. Effects of altered water quality and trace elements on the infection variables of Paradiplozoon ichthyoxanthon (Monogenea: Diplozoidae) from two sites in the Vaal River system, South Africa. Acta Parasitol. 2016; 61 (1):52–62. https://doi.org/10.1515/ap-2016-0005 PMID: 26751871

34. Poléo ABS, Schjolden J, Hansen H, Bakke TA, Mo TA, Rosseland BO, et al. The effect of various metals on Gyrodactylus salaris (Platyhelminthes, Monogenea) infections in Atlantic salmon (Salmo salar). Parasitology. 2004; 128 (2):169–77.

35. Soleng A, Poléo ABS, Bakke TA. Toxicity of aqueous aluminium to the ectoparasitic monogenean Gyrodactylus salaris. Aquaculture. 2005; 250:616–20.

36. Pettersen RA, Vellestad LA, Flodmark LEW, Poléo ABS. Effects of aqueous aluminium on four fish ectoparasites. Sci Total Environ. 2006; 369 (1–3):129–38. https://doi.org/10.1016/j.scitotenv.2006.05.024 PMID: 16904736
41. Gheorghiu C, Cable J, Marcogliese DJ, Scott ME. Effects of waterborne zinc on reproduction, survival and morphometrics of _Gyrodactylus turnbulli_ (Monogenea) on guppies (_Poecilia reticulata_). Int J Parasitol. 2007; 37(3–4):375–81. https://doi.org/10.1016/j.ijpara.2006.09.004 PMID: 17049530

42. Ohashi H, Umeda N, Hirazawa N, Ozaki Y, Miura C, Miura T. Antiparasitic effect of calcium and magnesium ion-free buffer treatments against a common monogenean _Neobenedenina girellae_. Parasitology. 2007; 134:229–36. https://doi.org/10.1017/S0031182006001430 PMID: 17032471

43. Gilbert BM, Avenant-Oldewage A. Hatchability and survival of oncomiracidia of _Paradiplozoon ichthyoxanthus_ (Monogenea: Diplozoidae) exposed to aqueous aluminium. Parasit Vectors. 2016; 9:420. https://doi.org/10.1186/s13071-016-1706-z PMID: 27464982

44. Cohen M, Varki NM, Jankowski MD, Gagneux P. Using unfixed, frozen tissues to study natural mucin and changes induced by Astiban. Exp Parasitol. 1975; 38:240–56. PMID: 1175727

45. Montorzi M, Falchuk KH, Vallee BL. Vitellogenin and lipovitel lin 1 is a Zn$^{2+}$- and Cd$^{2+}$-binding protein. Mol Reprod Dev. 1995; 42:180–7. https://doi.org/10.1002/mrd.1080420207 PMID: 8562063

46. Shinn AP, Gibson DI, Sommerville C. A study of the composition of the sclerites of _Gyrodactylus_ Nordmann, 1832 (Monogenea) using X-ray elemental analysis. Int J Parasitol. 1995; 25 (7):797–805. PMID: 7558655

47. Sakai H, Ichihashi H, Suganuma H, Tatsukawa R. Heavy metal monitoring in sea turtles using eggs. Mar Pollut Bull. 1995; 30:347–53.

48. Erasmus DA. _Schistosoma mansoni_: Development of the vitelline cell, its role in drug sequestration, and changes induced by Astiban. Exp Parasitol. 1975; 38:240–56. PMID: 1175727

49. Schofield RMS, Nesson MH, Richardson KA. Tooth hardness increases with zinc-content in mandibles of young adult leaf-cutter ants. Naturwissenschaften. 2002; 89:579–83. https://doi.org/10.1007/s00114-002-0381-4 PMID: 12536282

50. Schofield RMS, Nesson MH, Richardson KA, Wyeth P. Zinc is incorporated into cuticular “tools” after ecdysis: The time course of the zinc distribution in “tools” and whole bodies of an ant and a scorpion. J Insect Physiol. 2003; 49:31–44. PMID: 12770014

51. Broomell CC, Zok FW, Walte JH. Role of transition metals in sclerotization of biological tissue. Acta Biomater. 2008; 4 (6):2045–51. https://doi.org/10.1016/j.actbio.2008.06.017 PMID: 18653388

52. Baptista-Farias MD, Kohn A. Ultrastructural observations of the vitelline cells of _Metamicrocotylina macracantha_ (Monogenea, Microcotylidae). Mem Inst Oswaldo Cruz. 1998; 93(4):543–8.

53. Schofield RMS, Nesson MH, Richardson KA, Wyeth P. Zinc is incorporated into cuticular “tools” after ecdysis: The time course of the zinc distribution in “tools” and whole bodies of an ant and a scorpion. J Insect Physiol. 2003; 49:31–44. PMID: 12770014

54. Broome LL, Lin CL, Rintoul L, Rasch R, Hasenpusch J, Huang H. Hardness in arthropod exoskeletons in the absence of transition metals. Acta Biomater. 2008; 6 (8):2045–51. https://doi.org/10.1016/j.actbio.2008.06.017 PMID: 18653388

55. Kritsky DC, Leiby PD, Kayton RJ. A rapid stain technique for the haptoral bars of _Gyrodactylus_ species (Monogenea). J Parasitol. 1998; 84(2):315–8. PMID: 9502837

56. Lyons KM. The chemical nature and evolutionary significance of monogenean attachment sclerites. Parasitology. 1966; 56:63–100. PMID: 4161766

57. Shinn AP, Gibson DI, Sommerville C. An SEM study of the haptoral sclerites of the genus _Gyrodactylus_ Nordmann, 1832 (Monogenea) following extraction by digestion and sonication techniques. Syst Parasitol. 1993; 25:135–44.

58. Cribb BW, Lin CL, Rintoul L, Rasch R, Hasenpusch J, Huang H. Hardness in arthropod exoskeletons in the absence of transition metals. Acta Biomater. 2008; 6 (8):2045–51. https://doi.org/10.1016/j.actbio.2008.06.017 PMID: 18653388

59. Khan FR, Bury NR, Hogstrand C. Cadmium bound to metal rich granules and exoskeleton from _Gambusia pulex_ causes increased gut lipid peroxidation in zebrafish following single dietary exposure. Aquat Toxicol. 2010; 96 (2):124–9. https://doi.org/10.1016/j.aquatox.2009.10.010 PMID: 19883947

60. Shinn AP, Gibson DI, Sommerville C. An SEM study of the haptoral sclerites of the genus _Gyrodactylus_ Nordmann, 1832 (Monogenea) following extraction by digestion and sonication techniques. Syst Parasitol. 1993; 25:135–44.

61. Milne SJ, Avenant-Oldewage A. The fluorescent detection of _Paradiplozoon_ sp. (Monogenea: Diplozoidea) attachment clamps' sclerites and integumental proteins. Onderstepoort J Vet Res. 2006; 73 (2):149–52. PMID: 16958267

62. Wong WL, Michels J, Gorb SN. Resilin-like protein in the clamp sclerites of the gill monogenean _Diplozoon paradoxum_ Nordmann, 1832. Parasitology. 2012; (1963):1–4.

63. Wu X-Y, Yang Y-F. Heavy metal (Pb, Co, Cd, Cr, Cu, Fe, Mn and Zn) concentrations in harvest-size white shrimp _Litopenaeus vannamei_ tissues from aquaculture and wild source. J Food Compos Anal. 2011; 24:62–5.
64. Hook SE, Fisher NS. Reproductive toxicity of metals in calanoid copepods. Mar Biol. 2001; 138:1131–40.

65. Ahearn GA, Mandal PK, Mandal A. Mechanisms of heavy-metal sequestration and detoxification in crustaceans: A review. J Comp Physiol B Biochem Syst Environ Physiol. 2004; 174 (6):439–52.

66. Ramalingam K. Chemical nature of the egg shell in helminths: II. Mode of stabilization of egg shells on monogenetic trematodes. Exp Parasitol. 1973; 34:115–22. PMID: 4124670

67. Smyth JD, McManus DP. The physiology and biochemistry of cestodes. Cambridge, UK: Cambridge University Press; 1989. 156–187 p.

68. Wharton DA. The production and functional morphology of helminth egg-shells. Parasitology. 1983; 86:85–97. PMID: 6346235

69. Knight DP, Feng D, Stewart M. Structure and function of the selachian egg case. Biol Rev. 1996; 71:81–91.

70. Jeffree RA, Oberhansli F, Teyssie J-L. The accumulation of lead and mercury from seawater and their depuration by eggs of the spotted dogfish Scyllorhinus canicula (Chondrichthys). Arch Environ Contam Toxicol. 2008; 55:451–61. https://doi.org/10.1007/s00244-007-9103-4 PMID: 18214579

71. Rico D, Martín-González A, Díaz S, de Lucas P, Gutiérrez J-C. Heavy metals generate reactive oxygen species in terrestrial and aquatic ciliated protozoa. Comp Biochem Physiol Part C Toxicol Pharmacol. 2009; 149:90–6.