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1 **First insight in element localisation in different body parts of the**
2 **acanthocephalan *Dentitruncus truttae* using TEM and NanoSIMS**

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37

38 **Abstract**

39

40 Acanthocephalans, intestinal parasites of vertebrates, are characterised by orders of magnitude
41 higher metal accumulation than free-living organisms, but the mechanism of such effective metal
42 accumulation is still unknown. The aim of our study was to gain a new knowledge on high-
43 resolution element localisation in acanthocephalans' body as an initial step in revealing metal
44 uptake and accumulation in organisms in real environmental conditions. For the first time,
45 nanoscale secondary ion mass spectrometry (NanoSIMS) was applied for high-resolution
46 mapping of 12 elements (C, Ca, Cu, Fe, N, Na, O, P, Pb, S, Se, and Tl) in the ultrastructure of
47 three selected body parts (trunk spines, inner part of the proboscis receptacle and inner surface of
48 the tegument) in *Dentitruncus truttae*, hosted in the brown trout (*Salmo trutta*) from the Krka
49 River in Croatia. In addition, the same body parts were analysed by transmission electron
50 microscopy (TEM) and correlated to NanoSIMS images. . Metal concentrations, determined by
51 HR ICP-MS, confirmed higher accumulation in *D. truttae* than in the fish intestine. The chemical
52 composition of the acanthocephalan body showed the highest density in all studied structures for
53 C, Ca, N, Na, O, S, as important and constitutive elements in living cells, while Fe was
54 predominant among trace elements. Generally, higher element density was recorded in trunk
55 spines and tegument, as body structures responsible for substance absorbance in parasites. The
56 results obtained by NanoSIMS and TEM-NanoSIMS correlative imaging represent pilot data on
57 element mapping with nanoscale resolution in the ultrastructure of different body parts of
58 acanthocephalans and generally, give contribution for the further application of this technique in
59 any parasite species.

60

61 **Keywords:** nanoscale secondary ion mass spectrometry, transmission electron microscopy,

62 correlative imaging, thorny-headed worms, body ultrastructure, chemical composition

63

64

65 **1. INTRODUCTION**

66

67 Nanoscale secondary ion mass spectrometry (NanoSIMS), as a type of imaging mass
68 spectrometry, represents a technique for elemental imaging of high sensitivity, high spatial and
69 mass resolution, and a powerful tool in tracking molecules and quantifying biological processes
70 at subcellular level (Guerquin-Kern et al., 2005). Although, NanoSIMS contributed significantly
71 in the last two decades to the field of biology, especially to microbiology (Kleinfeld et al., 2004),
72 it has not previously been applied for high-resolution multi-element mapping in the ultrastructure
73 of any parasite species. Such data can represent an important contribution for future application
74 in environmental studies. As parasitism is among most common lifestyles on the earth, there is
75 certainly a need for investigating parasites and their role in aquatic ecosystems, as well as their
76 impact on the environmental health. Investigation of parasites by NanoSIMS serves as a base for
77 future application in environmental parasitology and metal accumulation in biota. Further, as
78 acanthocephalans are already known as effective metal accumulators, they are successfully
79 applied as indicators of water quality in biomonitoring studies (Sures, 2004; Thielen et al., 2004;
80 Filipović Marijić et al., 2013, 2014, 2022) and a novel field, Environmental Parasitology, was
81 established regarding the linkage between contamination and parasitism (Sures et al., 2017).
82 Although present in fish intestines, acanthocephalans were confirmed as relevant bioindicators of
83 environmental conditions, reflecting metal exposure in accordance to fish soft and hard tissues
84 (Filipović Marijić et al., 2022). With respect to morphology, the ultrastructure of
85 acanthocephalans has been studied by scanning electron microscopy (SEM) (Taraschewski,
86 2000). However, the localization of chemical elements in their structure is limited so far to few
87 studies using energy dispersive X-ray analysis for the detection of major elements in

88 micrometer-sized spots, mainly in the hooks of the proboscis of the parasite (Heckmann et al.,
89 2007, 2012a, b). Therefore, our aim was to acquire the first data on high-resolution element and
90 metal localisation in the ultrastructure of acanthocephalans' body, which may help to understand
91 the mechanisms of metal uptake in environmental conditions.

92 Acanthocephalans is a small group of endoparasites, included in the taxon Syndermata
93 (Mauer et al., 2021). They use Mandibulata (Arthropoda) as intermediate hosts and jawed
94 vertebrates (Gnathostomata), often teleost fish, as definitive hosts (Kennedy, 2006). Although
95 endoparasites are not directly exposed to the environment, they are highly affected, indirectly via
96 their hosts and numerous studies have demonstrated utility of fish parasites to detect changes in
97 polluted environments (Thielen et al., 2004; Nachev et al., 2013; Sures, 2014; Filipović Marijić
98 et al., 2022). In addition, acanthocephalans were also shown to have a possible protective role
99 against metal accumulation in their hosts (Oyoo-Okoth et al., 2012, Filipović Marijić et al., 2013,
100 2014). Accordingly, it is important to investigate complex parasite-host relationship and metal
101 uptake, especially in environmental conditions where parasites can significantly affect responses
102 of their hosts, but are often neglected in research.

103 In the absence of data on metal accumulation or high-resolution elemental mapping in
104 different body parts of acanthocephalans, our data may provide a step forward in understanding
105 the successful metal uptake and storage and in observing which body parts are responsible for
106 effective metal absorption. For that purposes, the acanthocephalan *Dentitruncus truttae* Sinzar,
107 1955, a parasite of brown trout (*Salmo trutta* L.), which is native in the karst Krka River in
108 Croatia, was studied. Hence, the specific objective of the present study was to examine and
109 compare the ultrastructure and localisation of 12 elements (C, Ca, Cu, Fe, N, Na, O, P, Pb, S, Se
110 and Tl) at nanometer level in three representative acanthocephalan body parts (trunk spines,

111 proboscis receptacle and inner surface of the tegument) using transmission electron microscopy
112 (TEM) and NanoSIMS analyses, as well as the correlation of both imaging techniques. Non-
113 metals were selected as essential elements in all living organisms (C, N, O, P, S), while
114 metal(loid)s were chosen as essential macroelements (Ca, Na) and trace elements (Fe, Cu, Se),
115 and moreover non-essential metals (Pb, Tl), showing different roles and concentrations in the
116 organisms. As previously stated by Taraschewski (2000) and Heckmann et al. (2012a),
117 knowledge of the structure and chemical composition of acanthocephalans may help us to
118 understand effective mechanisms of uptake and storage of chemical substances, and to clarify
119 their roles and utilisation in the biology of these parasites.

120

121 **2. MATERIALS AND METHODS**

122

123 **2.1 Study area and sampling procedure**

124

125 Samples of trout and their acanthocephalan parasites were taken from the Krka River, a
126 typical karstic river flowing through the Dinaric carbonate area of the Republic of Croatia.
127 Detailed map of the sampling area and physico-chemical water characteristics were provided by
128 Filipović Marijić et al. (2018) and Sertić Perić et al. (2018). Brown trout were sampled in the
129 river source to estimate physiological conditions without pollution disturbances and in May 2016
130 to avoid physiology-related metal variability during fish spawning period, which occurs in late
131 autumn. At the same time, water was sampled for metal analyses, pointing to increasing trend in
132 metal concentrations downstream due to contamination from industrial (Fe, Se, Pb) and
133 municipal (Cu) effluents, except for comparable or higher Tl levels at the river source (Dragun et

134 al., 2018, Filipović Marijić et al., 2018). Sampling was performed by electrofishing, in
135 accordance with the Croatian standard legislation of fish sampling by electric power (HRN EN
136 14011, 2005). In total, 13 fish individuals were sampled and all of them were infected with
137 acanthocephalans, containing 269 acanthocephalans and resulting with a mean intensity of
138 infection 20.7 ± 8.9 . Detailed data on fish biometry and acanthocephalan epidemiological
139 characteristics can be found in Mijošek et al. (2022). Captured fish were kept alive in aerated
140 water until further processing in the laboratory.

141 After trouts were anesthetized with tricaine methanesulfonate (MS 222 Sigma Aldrich) in
142 accordance with the Ordinance on the Protection of Animals used for scientific purposes (NN
143 55/2013) and sacrificed, the gastrointestinal tract (stomach, pyloric caeca and the anterior,
144 middle and posterior portions of the intestine) was removed and opened along its entire length
145 and carefully checked for the presence of intestinal parasites. Acanthocephalans found were
146 gently detached from the intestine using tweezers, counted per each specimen and isolated for
147 the further NanoSIMS and metal analyses. Collected acanthocephalans were identified as
148 *Dentitruncus truttae* following Manilla et al. (1976), Dezfuli et al. (2008b) and Vardić Smrzlić et
149 al. (2013) This species represents the only member of the genus *Dentitruncus* Sinzar, 1955 of the
150 family Illiosentidae Golvan, 1960 and occurs only in the limited areas of Bosnia and
151 Herzegovina (Šinžar, 1955), Italy (Dezfuli et al., 2008b) and Croatia (Topić Popović et al., 1999;
152 Vardić Smrzlić et al., 2013). It was reported that *D. truttae* can cause serious intestinal damage to
153 its fish host (Dezfuli et al., 2008a) and, similar to other acanthocephalans, it can effectively
154 accumulate metals and be considered as an indicator of metal exposure in the aquatic
155 environment (Filipović Marijić et al., 2022; Mijošek et al., 2022).

156 Specimens used for TEM and NanoSIMS analyses were initially chemically fixed by the
157 procedure explained in section 2.3. To compare metal localisation in different body parts of the
158 parasite, all analyses were performed in the same parasite individual of *D. truttae*. Other
159 acanthocephalan individuals (n = 10) isolated from the same host as those used for NanoSIMS
160 analyses were immediately stored at -80 °C for metal analyses. In addition, the posterior intestine
161 of the host fish (total length 22.1 cm, total body mass 107.2 g) was dissected, carefully removing
162 gut content and parasites, then washed with phosphate buffered saline and stored at -80°C for
163 further metal analysis.

164

165 **2.2 Determination of total metal concentrations in acanthocephalans and fish intestine**

166

167 Prior to the metal measurements, the wet tissue of the posterior part of intestine (0.06 g)
168 of the brown trout hosting acanthocephalan intended for NanoSIMS analyses and whole
169 acanthocephalans (composite sample of 0.02 g) were digested using the oxidation mixture of
170 concentrated HNO₃ (Rotipurán® Supra 69%, Carl Roth, Germany) and 30% H₂O₂ (Suprapur®,
171 Merck, Germany) in the volume ratio of 3 : 1. The digestions were performed at 85 °C for 3.5 h
172 in the dry oven. Metal concentrations were determined in the appropriately diluted samples using
173 high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2; Thermo
174 Finnigan, Bremen, Germany), equipped with an autosampler SC-2 DX FAST (Elemental
175 Scientific, USA). Measurements of ²⁰⁸Pb, ⁸⁰Se and ²⁰⁵Tl were performed in low-resolution mode
176 and of ²³Na, ⁴²Ca, ⁵⁶Fe, ⁶³Cu in medium-resolution mode. Indium (In, 1 µg L⁻¹, Atomic
177 Spectroscopy Standard Solution, Fluka, Seelze, Germany) was added, prior to the measurements,
178 to all solutions as an internal standard.

179 Two external calibrations were performed, one for trace elements using multielement
180 stock standard solution (Analytika, Prague, Czech Republic) and the other for the macroelements
181 using standard containing Na (1.0 g L⁻¹) and Ca (2.0 g L⁻¹) (Fluka, Seelze, Germany Germany).
182 The accuracy and precision of metal determination by HR ICP-MS was controlled by the
183 analysis of the certified reference material DORM-2 (National Research Council, Canada),
184 previously digested in the same way as parasites and fish intestine. Agreement between
185 performed measurements and certified values was generally good, resulting in the following
186 recovery values (% , mean ± S.D.): Cu 100±3.1, Fe 101±5.0, Se 102±5.1, Tl 101±12.3, Pb
187 118.5±15.2. The ratio of metal levels in parasites to that in the fish intestine was calculated and
188 considered as bioconcentration factor (BCF) according to Sures et al. (1999), in order to be
189 consistent with other studies dealing with similar topics.

190

191 **2.3 Sample preparation for TEM and NanoSIMS analyses**

192

193 Samples were fixed in two steps due to the long geographic distance between the
194 sampling site in Croatia and the sample preparation laboratory at the Bordeaux Imaging Centre
195 (BIC) in France. Pre-fixation of acanthocephalan tissue was performed at the nearby veterinary
196 ambulance in the town of Knin, close to the Krka River sampling site, using 2 %
197 paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M of phosphate buffer according to a
198 standard procedure (Hayat, 1982; Graham and Orenstein, 2007). After 4 hours at room
199 temperature, samples were cooled down to 4 °C (Graham and Orenstein, 2007). The mixture of
200 paraformaldehyde and glutaraldehyde was employed at room temperature allowed thus rapid and
201 efficient fixation. Then, the pre-fixed tissue samples were shipped by express mail to BIC.

202 Acanthocephalans stored in buffer solution were refrigerated during shipping and the sample pre-
203 fixation process took less than 2 days.

204 At BIC, samples were rinsed with 0.1 M phosphate buffer and post-fixed with 1%
205 osmium tetroxide in 0.1 M phosphate buffer for 2 h. Afterwards, they were dehydrated by a
206 stepwise gradient of ethanol (50, 70, 90, 100 %, at 37 °C for 19 min) and impregnated with
207 epoxy resin (EPON 812, Delta Microscopies, Mauressac, France) with a graded series of ethanol
208 : resin mixtures with increasing resin fraction (3 : 1, 1 : 1, 1 : 3, pure resin, at 40–50 °C for a total
209 of 55 min) using an automatic microwave tissue processor Leica EM AMW (Leica
210 Microsystems, Vienna, Austria). Finally, the resin was polymerized at 83 °C for 105
211 min.(Schroeder et al., 2006).

212 The resin blocs were cut with an ultramicrotome (EM Ultracut-UC7, Leica
213 Microsystems, Vienna, Austria) using a diamond knife (Diatome, Biel-Bienne, Switzerland). For
214 TEM-NanoSIMS correlative imaging, subsequent sections of 70 nm thickness for TEM and of
215 300 nm for NanoSIMS were cut. The TEM sections were placed on copper grids and NanoSIMS
216 sections were placed on silicon wafers (Ted Pella, Redding, USA). Samples for NanoSIMS
217 analyses were shipped from the BIC to the Institut des Sciences Analytiques et de Physico-
218 Chimie pour l'Environnement et les Matériaux (IPREM), Université de Pau et des Pays de
219 l'Adour, France.

220

221 **2.4 TEM analysis**

222

223 Localisation of the subcellular structures and definition of regions of interest of *D. truttae*
224 at nanometer (nm) level was performed by TEM (Hitachi H7650, Tokyo, Japan; ×3000 to

225 ×20000). This allowed imaging of subcellular structures (three regions of the anterior metasoma,
226 i.e., trunk spine, proboscis receptacle and the inner surface of the tegument) and superposing
227 them to NanoSIMS images. In this way, subcellular structures recorded by TEM were explained
228 in detail in the acanthocephalan body and were correlated to elemental images obtained by
229 NanoSIMS. Terminology of subcellular structures follows Beermann et al. (1974), Dezfuli and
230 Sbrenna (1990), Taraschewski (2000), Herlyn and Ehlers (2001) and Herlyn and Taraschewski
231 (2017).

232

233 **2.5 NanoSIMS analysis**

234

235 High resolution secondary ion mass spectrometry analysis with a NanoSIMS 50L
236 (Cameca, Gennevilliers, France) was used to examine the elemental distribution in the
237 acanthocephalan tissues. The recent implementation of a bright radio frequency plasma O⁻
238 primary ion source in NanoSIMS for the generation of positive secondary ions allows the
239 mapping of major and trace metals in tissue and cells with a spatial resolution down to 40 nm
240 (Malherbe et al., 2016). In our study, the HyperionTM RF plasma primary oxygen ion source was
241 used for the mapping of the electropositive elements: sodium (²³Na⁺), calcium (⁴⁰Ca⁺), iron
242 (⁵⁶Fe⁺), copper (⁶³Cu⁺), thallium (²⁰⁵Tl⁺), lead (²⁰⁸Pb⁺), and carbon (¹²C⁺)⁶. Subsequently, a
243 primary Cs⁺ ion source was used for the mapping in the same spots of electronegative elements:
244 carbon (¹²C₂⁻), nitrogen (¹²C¹⁴N⁻), oxygen (¹⁶O⁻), sulphur (³²S⁻), selenium (⁸⁰Se⁻) and phosphorus
245 (³¹P¹⁶O₂⁻), with the cluster atoms selected to achieve higher signal intensity. The elements were
246 measured in multicollection mode with seven parallel electron multiplier detectors and the mass

247 resolution was adjusted to resolve possible interferences. For both ion sources, the carbon signal
248 was used for data normalization.

249 Regions of interest in the tissue were selected using an integrated Charged Coupled
250 Device (CCD) camera, and then analyzed by NanoSIMS with a raster size of $55 \times 55 \mu\text{m}$, with
251 256×256 pixels, with a dwell time of 10 ms per pixel. A beam of 8.5 pA was applied on the
252 sample with the oxygen ion source, and of 1.0 pA with the cesium ion source, after a pre-
253 sputtering of the sample surface during 10 min at about 100 pA. Image processing was
254 performed using WinImage (Cameca). A total of 50 cycles of the same area were acquired and
255 they were stacked to obtain the final image. Normalization over the carbon signal was also
256 performed, as well as overlays of several elements for better visualization of data.

257

258 **3. RESULTS AND DISCUSSION**

259

260 **3.1 Metal concentrations in acanthocephalans and trout intestine**

261

262 Concentrations of measured elements in acanthocephalans followed the order: $\text{Ca} > \text{Na} >$
263 $\text{Cu} > \text{Fe} > \text{Tl} > \text{Se} > \text{Pb}$, while in intestine of fish host the order was slightly different: $\text{Na} > \text{Ca} >$
264 $\text{Fe} > \text{Cu} > \text{Se} > \text{Pb} > \text{Tl}$ (Table 1). The ratio of metal concentrations in the parasites to those in
265 the fish intestine, called bioconcentration factor (BCF), confirmed more effective metal
266 accumulation of metals in the parasites than in the fish, following the order $\text{Tl} (69.5) > \text{Ca} (34.5) >$
267 $\text{Cu} (23.0) > \text{Pb} (12.6) > \text{Fe} (1.58) > \text{Se} (1.37) = \text{Na} (1.37)$, which mostly confirmed already
268 reported BCF data for the whole population of fish from the Krka River source in spring
269 (Mijošek et al., 2020, Mijošek et al., 2022). This order of BCF values is consistent with literature

270 data, which also confirmed higher BCFs in different acanthocephalan species than their fish
 271 hosts (Sures et al., 1999; Thielen et al., 2004; Filipović Marijić et al., 2013, 2014), again
 272 suggesting possible protective role for the host when dealing with enhanced accumulation of
 273 non-essential elements. The higher absorption of non-essential elements was explained by the
 274 parasites' dependence on host micronutrients, as they lack a gastrointestinal system (Kennedy,
 275 1985). Accordingly, the parasites compete for essential elements from the host intestine, as
 276 elements of metabolic importance, but their chemical similarity with toxic metals results in
 277 parallel absorption and increased accumulation of non-essential elements (Sures, 2002). Due to
 278 such variable concentrations, accumulation pattern and BCFs of metals in acanthocephalans,
 279 these trace and macroelements were therefore selected for NanoSIMS analysis to compare their
 280 possible variations in metal localisation.

281

282 **Table 1.** Metal concentrations of analysed metals in *Dentitruncus truttae* and intestinal tissue of
 283 brown trout specimens from the Krka River (mean \pm S.D., n=10)

	Microelements/ $\mu\text{g g}^{-1}$					Macroelements/ mg g^{-1}	
	Cu	Fe	Pb	Se	Tl	Ca	Na
Parasite	15.9 \pm 0.4	11.9 \pm 0.8	0.63 \pm 0.01	0.92 \pm 0.06	2.78 \pm 0.04	4.48 \pm 0.15	1.56 \pm 0.79
Intestinal tissue	0.69 \pm 0.21	7.54 \pm 2.94	0.05 \pm 0.01	0.67 \pm 0.11	0.04 \pm 0.02	0.13 \pm 0.06	1.14 \pm 0.14

284

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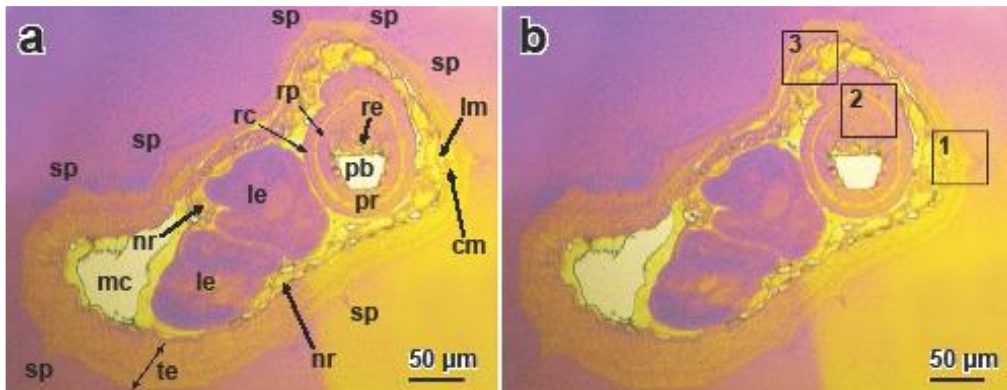
286

287 **3.2 Ultrastructure of the anterior metasoma of *D. truttae***

288

289 The selected acanthocephalan body part, anterior metasoma, is covered by tegument and
290 surrounded by trunk spines, as seen in Fig. 1a. Since hooks (or spines when found on the
291 metasoma) are the most representative structure of acanthocephalans, we selected trunk spine,
292 proboscis retractor (inner part of the parasite body) and inner surface of the tegument (Fig. 1b),
293 as three specific spots for comparison of element localisation in the parasite body. Additionally,
294 the ultrastructure of each of the three selected areas of the anterior portion of trunk of *D. truttae*
295 was for the first time analysed using transmission electron microscopy.

296



297

298

299 **Figure 1.** Micrographs of a cross-section of the anterior metasoma of *Dentitruncus truttae*
300 obtained with the optical CCD charge-coupled device (CCD) camera of NanoSIMS. a) Internal
301 and external structures; b) Overview and orientation of the three areas of 50 µm × 50 µm chosen
302 for TEM analysis and element localisation by NanoSIMS: 1 – trunk spine; 2 – proboscis
303 receptacle; 3 –inner surface of the tegument. *Abbreviations:* cm, circular musculature; le,
304 lemniscus; lm; longitudinal musculature; mc, metasoma cavity; nr, neck retractor; pb, body

305 cavity of the proboscis receptacle; pr, double-walled proboscis receptacle; rc, receptacle
306 constrictor; re, proboscis retractor; rp, receptacle; sp, trunk spine; te, tegument.

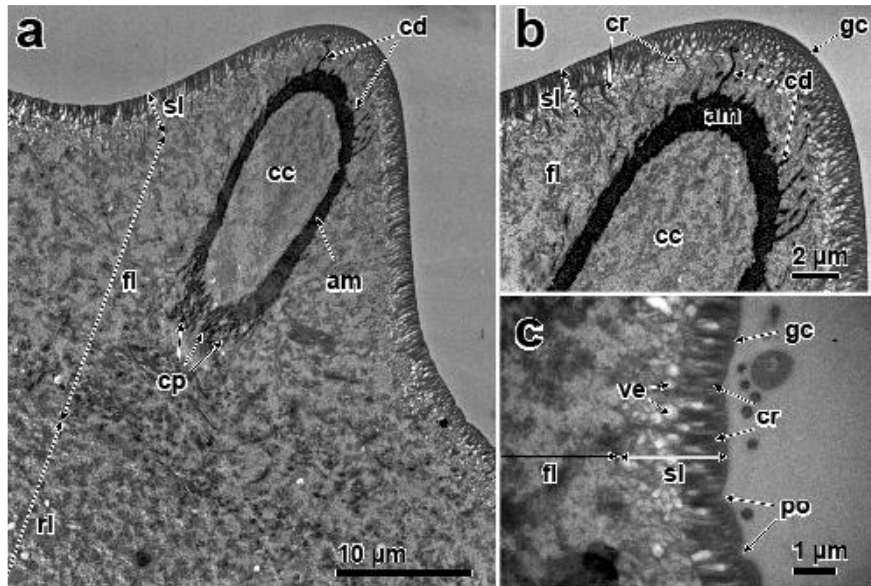
307

308 **3.2.1 Trunk spines**

309

310 High magnification with TEM micrographs shows three specific layers, i.e., striped,
311 feltwork and radial, in the tegument surrounding the trunk spines of *D. truttae* (Fig. 2a). The
312 striped layer, with an approximate thickness variable from 1.3 to 3.7 μm , is covered by a thin
313 glycocalyx (Fig. 2b, c). Parallel crypts (or channels) immersed in an electron-dense,
314 homogeneous substance, can be distinguished in the striped layer (Fig. 2b, c). These crypts
315 penetrate the outer part of the tegument (glycocalyx) forming numerous pores (Fig. 2c). The
316 TEM micrographs suggest that the crypts of the striped layer may be connected to vesicle-like
317 structures (third layer *sensu* Dezfuli and Sbrenna, 1990) (Fig. 2c), and extend to the outer part of
318 the feltwork layer (Fig. 2b). The feltwork layer cover and support the trunk spines and is
319 composed by a mesh of fibres running in all directions, (Fig. 2a). The spines of *D. truttae*
320 comprise a central core of connective tissue which is surrounding by an exterior layer of
321 electron-dense amorphous matter of 0.7 to 1.7 μm in thickness (Fig. 2a, b). Numerous crypts
322 containing electron-dense amorphous matter are observed in the distal and proximal extremities of
323 the layer of amorphous matter (Figs. 2a, b). The crypts of amorphous matter of the distal extremity
324 are less densely set than those of the proximal extremity (Fig. 2a), but are somewhat thicker, and
325 may reach the posterior part of the striped layer (Fig. 2a, b). Beneath the feltwork layer the radial
326 layer is observed (Fig. 2a).

327



328

329

330 **Figure 2.** Transmission electron micrographs of cross-section of a trunk spine and tegument
 331 surface of *Dentitruncus truttae* (see Fig.1b, area 1). a) Trunk spine and surrounding tegument; b)
 332 Enlargement of the shaft of a spine and crypts of the layer of amorphous matter; c) Section of
 333 tegument surface showing the striped layer above the feltwork layer. *Abbreviations:* am, layer of
 334 electron-dense amorphous matter; cc, central core of trunk spine; cd, distal crypts of the layer of
 335 amorphous matter; cp, proximal crypts of the layer of amorphous matter; cr, crypts within striped
 336 layer; fi, feltwork layer; gc, glycocalyx; po, pore openings; rl, radial layer; sl, striped layer; ve,
 337 vesicle-like structures.

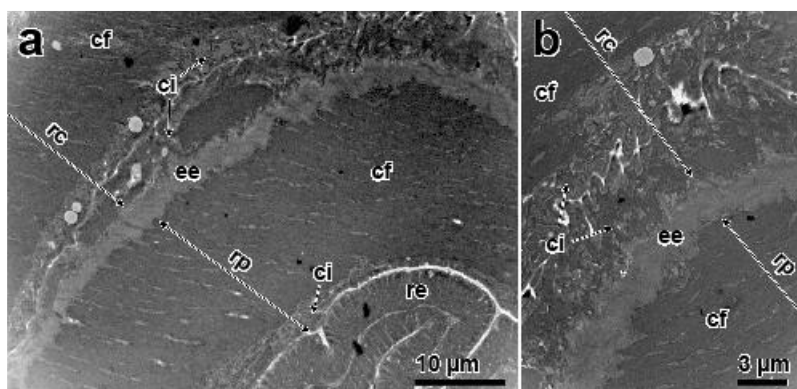
338

339 3.2.2 Proboscis receptacle

340

341 Similar to other members of the class Palaeacanthocephala, the proboscis receptacle of *D.*
 342 *truttae* is double walled. This organ is formed by two muscles, the receptacle constrictor and the
 343 receptacle (Fig. 3a, b), which are symmetrically arranged in the proboscis receptacle (Fig. 1a).

344 The receptacle constrictor and the receptacle have somewhat similar organization (Fig. 3a), both
345 comprises of a strong peripheral layer of contractile filaments and a thin cytoplasmic inner
346 lining (Fig. 3a, b); however, the cytoplasmic lining of the receptacle constrictor is divided in two
347 sublayers (Fig. 3b). A layer of extracellular matrix separates the receptacle constrictor from the
348 receptacle. Finally, the proboscis retractor consists of transversally oriented muscle fibres located
349 within the proboscis receptacle (Fig. 3a). These muscles have an asymmetrical arrangement,
350 since they appear confined only to one-half of the inner space of the proboscis receptacle (Fig.
351 1a).



352
353
354 **Figure 3.** Transmission electron micrographs of cross-section of the proboscis receptacle of
355 *Dentitruncus truttiae* (see Fig.1b, area 2). a) Higher magnification of the proboscis receptacle
356 musculature; b) Detail of the cytoplasmic lining and the extracellular matrix between the inner
357 and outer walls of the proboscis receptacle. *Abbreviation:* cf, contractile filaments; ci,
358 cytoplasmic lining; ee, extracellular matrix; rc, receptacle constrictor; re, proboscis retractor; rp,
359 receptacle.

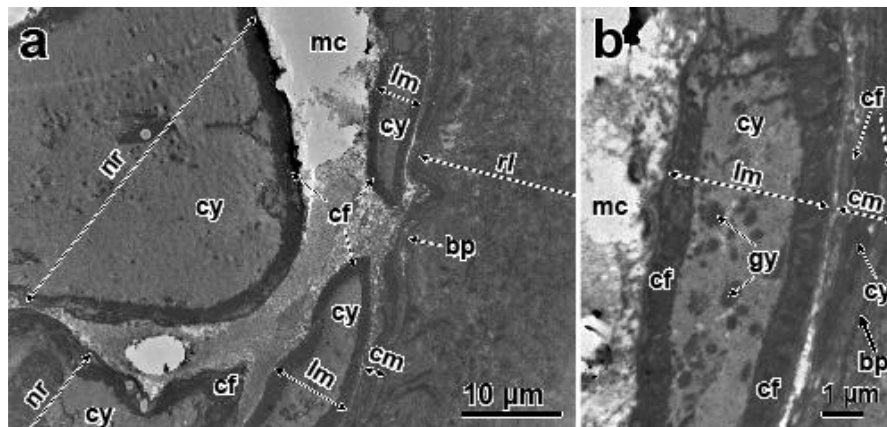
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363 **3.2.3 Inner surface of the tegument**

364

365 The inner surface of the tegument of the anterior metasoma of *D. truttiae* is mainly composed
366 by the radial layer, which possess numerous fibres without an apparent definite arrangement
367 (Fig. 4a). A very thin electro-dense basal plate lies beneath the radial layer (Figs. 4a, b). Under
368 the metasoma tegument, an outer coat of circular musculature and an inner coat of longitudinal
369 musculature (Fig. 4b) are observed, both of which are supported by connective tissue. Circular
370 and longitudinal musculature are divided into a peripheral layer of contractile filaments
371 surrounding a central cytoplasm core (Fig. 4a, b), which contained glycogen particles (Fig. 4b).
372 Similar to the musculature beneath the tegument, the neck retractor consists of the contractile
373 layer that surrounds the cytoplasm (Fig. 4a, b). The neck retractor is located in the metasoma
374 cavity, somewhat continuous with the longitudinal musculature (Figs. 1a, 4a).

375



376

377

378 **Figure 4.** Transmission electron micrographs of cross-section of the inner surface of the
379 tegument of *Dentitruncus truttiae* (see Fig.1b, area 3). a) Musculature beneath the tegument; b)
380 Detail of longitudinal and circular musculature. *Abbreviation:* bp, basal plate; cf, contractile

381 filaments; cm, circular musculature; cy, central cytoplasmic core; gy, glycogen particles; lm,
382 longitudinal muscle; mc, metasoma cavity; nr, neck retractor; rl, radial layer.

383

384 **3.3 NanoSIMS mapping of chemical elements in the anterior metasoma of *D. truttae*** 385 **and correlative TEM-NanoSIMS imaging**

386

387 In contrast to previous studies, which used less sensitive energy dispersive X-ray analysis
388 for low spatially-resolved elemental detection in micrometer-sized spots, our study presents for
389 the first time NanoSIMS data for high-resolution mapping of chemical elements in three
390 acanthocephalan body parts. In addition, NanoSIMS images were correlated to TEM of the same
391 sample areas in order to assign NanoSIMS data to the ultrastructure of *D. truttae*. Moreover, the
392 high sensitivity of NanoSIMS allowed the detection of some trace elements. Analysed chemical
393 elements, ^{12}C , ^{40}Ca , ^{63}Cu , ^{56}Fe , N (as $^{12}\text{C}^{14}\text{N}^-$), ^{23}Na , ^{16}O , P (as $^{31}\text{P}^{16}\text{O}_2^-$), ^{208}Pb , ^{32}S , ^{80}Se , and
394 ^{205}Tl showed different localisation regarding specific body structures. The presence of Na, Ca, S,
395 C, N, O was the highest, confirming their importance as essential elements for living cells (Figs.
396 5, 6, 7). Some non-essential elements, like Tl and Pb, were barely detected by NanoSIMS,
397 indicating their lower content in the analysed structures. This observation was also confirmed by
398 metal levels in tissue, which were the lowest for Tl and Pb in parasites and the intestine of their
399 fish hosts (Table 1). Such results are in accordance to their very low concentration in river water
400 during the same sampling campaign which confirms their low availability for organisms (Dragun
401 et al., 2018; Sertić Perić et al., 2018). Selenium signals appeared in all samples to be within the
402 noise level and did not show clear pattern (data not shown). All samples were firstly analysed by
403 the O⁻ primary source and afterwards with the Cs⁺ source. The analysis of the same area using a

404 second ion source, after a previous analysis by the opposite source, is known to decrease the
405 sensitivity for the elements of choice. However, this does not hinder the detection of major
406 elements such as C, N, S and P, but it significantly affects microelements like Se, resulting in
407 low Se secondary ion yield and thus low Se visibility. Apart from that, Se concentration in water
408 samples was also low, even below LOD which was $<0.059 \mu\text{g L}^{-1}$ (Dragun et al., 2018). Specific
409 accumulation and localisation of elements in three different areas, namely the trunk spines,
410 tegument and proboscis receptacle of *D. truttae*, are further shown and discussed in Figs. 5, 6
411 and 7.

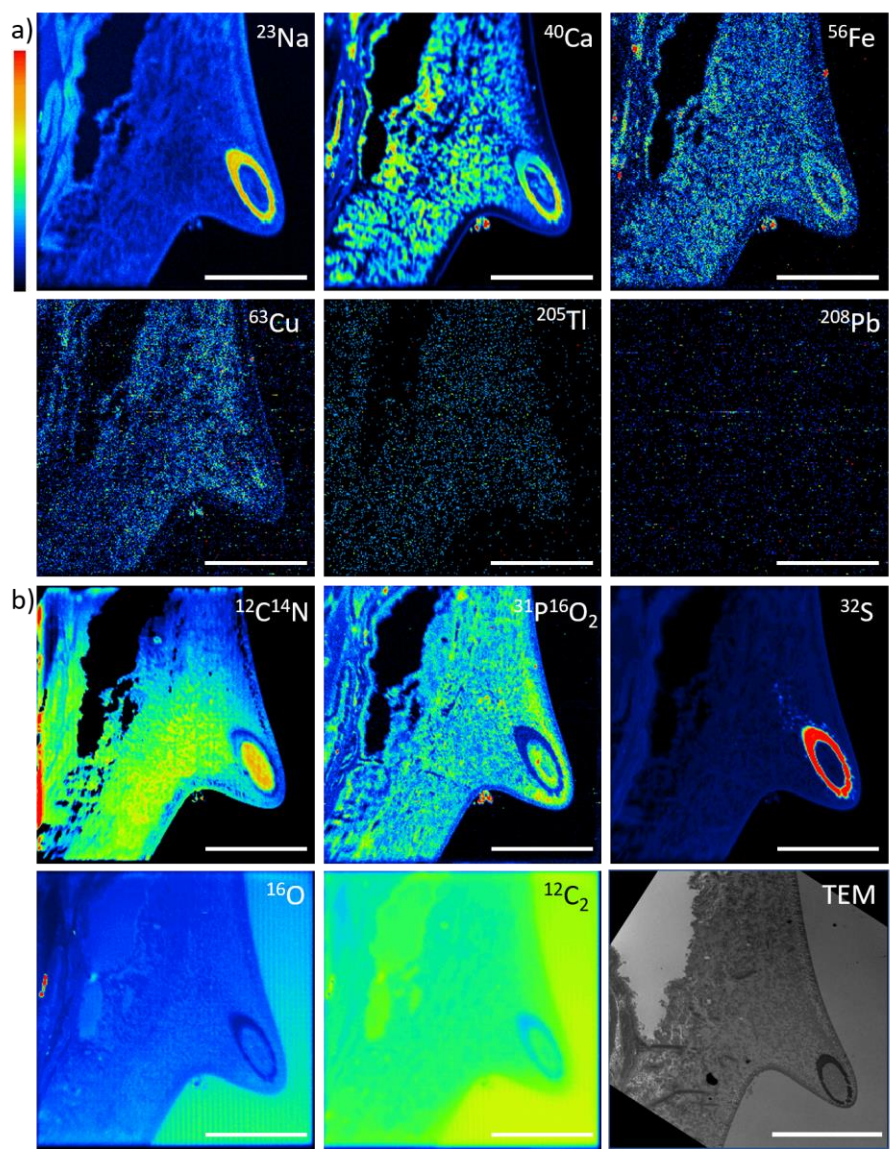
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413 **3.3.1 Trunk spines**

414

415 NanoSIMS analyses showed the highest element accumulation in the central part of the
416 trunk spines, involving skeletal fibres and surrounding substances concentrated around them.
417 Carbon is an element found in all biological structures, as reflected by its high presence in all
418 structures, but is also present in the epoxy resin, and so a high signal was detected, too, outside
419 the body (Fig. 5b). Sulfur, Na and Ca were highly concentrated in a circular structure (Fig. 5)
420 corresponding to the amorphous matter identified by TEM, and to a lower extent Fe, while N and
421 P were concentrated in the central core of the trunk spine, but not in the circular structure.
422 Moreover, Ca, N and P were found, too, in high concentration in the whole spine area,
423 confirming the presence of these essential elements characteristic for living cells. Copper was
424 detected in lower concentration, as well as the non-essential metal, Tl, which was detected in a
425 very low concentration, distributed homogeneously in the body. Trunk spines represent the only
426 area of acanthocephalans where we could observe some Tl signal. The presence of Pb was

427 negligible in this area, although some previous preliminary studies indicated the presence of Pb
428 in the hooks of acanthocephalans (as mentioned by Sures et al., 1999). However, concentrations
429 of these non-essential metals were low in the investigated acanthocephalan species from the
430 Krka River (Fig. 5).



431
432 **Figure 5.** Elemental mapping of the trunk spines (see Fig.1b, area 1) of *Dentitruncus truttae* by
433 NanoSIMS using: a) O⁻ source: ²³Na⁺, ⁴⁰Ca⁺, ⁵⁶Fe⁺, ⁶³Cu⁺, ²⁰⁵Tl⁺, ²⁰⁸Pb⁺; b) Cs⁺ source: ¹²C¹⁴N⁻,
434 ³¹PO₂⁻, ³²S⁻, ¹⁶O⁻, ¹²C₂⁻, as well as correlative imaging with TEM of the same sample area from a

435 subsequent ultramicrotome section. Color scale on the top left represents relative intensities of
436 the isotopes count rates. Scale bar: 20 μm .

437

438 Our data are in accordance with previous studies using X-ray spot analyses such as Brázová
439 et al. (2014) who found Ca as the dominant element in all parts of the proboscis hooks of another
440 spiny-headed worm with aquatic life cycle, *Acanthocephalus lucii* (Müller, 1776), followed by S
441 and P. The authors explained the high presence of Ca and P by forming a rigid calcium
442 phosphate apatite with disulfide bonds (thiol groups of the amino acids cysteine and cystine),
443 which represents the hardest tissue found in invertebrates (Luz and Mano, 2010). Moreover, the
444 lower content of S, Ca and P in the proximal region of the hook of *A. lucii* was explained by
445 providing flexibility to hooks, while presence of S in the outermost layer of the hook enhances
446 its hardness and attachment to the epithelium of the host's intestine (Brázová et al., 2014). A
447 similar observation was reported by Heckmann et al. (2012b) who localised Ca and P in basal
448 hooks and S in apical part of the hooks, and reported element order $S > Ca > P$ in the proboscis
449 hooks of *Rhadinorhynchus ornatus* Van Cleave, 1918 using Energy Dispersive X-Ray Analysis
450 (EDXA). High concentrations of S at proboscis and hooks of five acanthocephalans, namely
451 *Neoechinorhynchus qatarensis* (Amin et al., 2002); *Acanthocephalus dirus* Van Cleave, 1931,
452 *Neoechinorhynchus idahoensis* (Amin and Heckmann, 1992), *Echinorhynchus salmonis* Müller,
453 1784, and *Pseudoacanthocephalus* sp., were also reported by Heckmann et al. (2007). Our data
454 for *D. truttae* provide a first high-resolution insight to the distribution of chemical elements.
455 Owing to TEM-NanoSIMS correlative imaging, the high S, Na and Ca concentrations in hooks
456 described in previous studies can now be unambiguously assigned to a circular structure of
457 amorphous matter that contains also the trace metal Fe. In contrast, this circular amorphous matter

458 lacks of P, thus the high-resolved element map revealed that Ca and P are not co-localised in this
459 structure, which contests the possible formation of calcium phosphate apatite hypothesized by
460 Brázová et al. (2014).

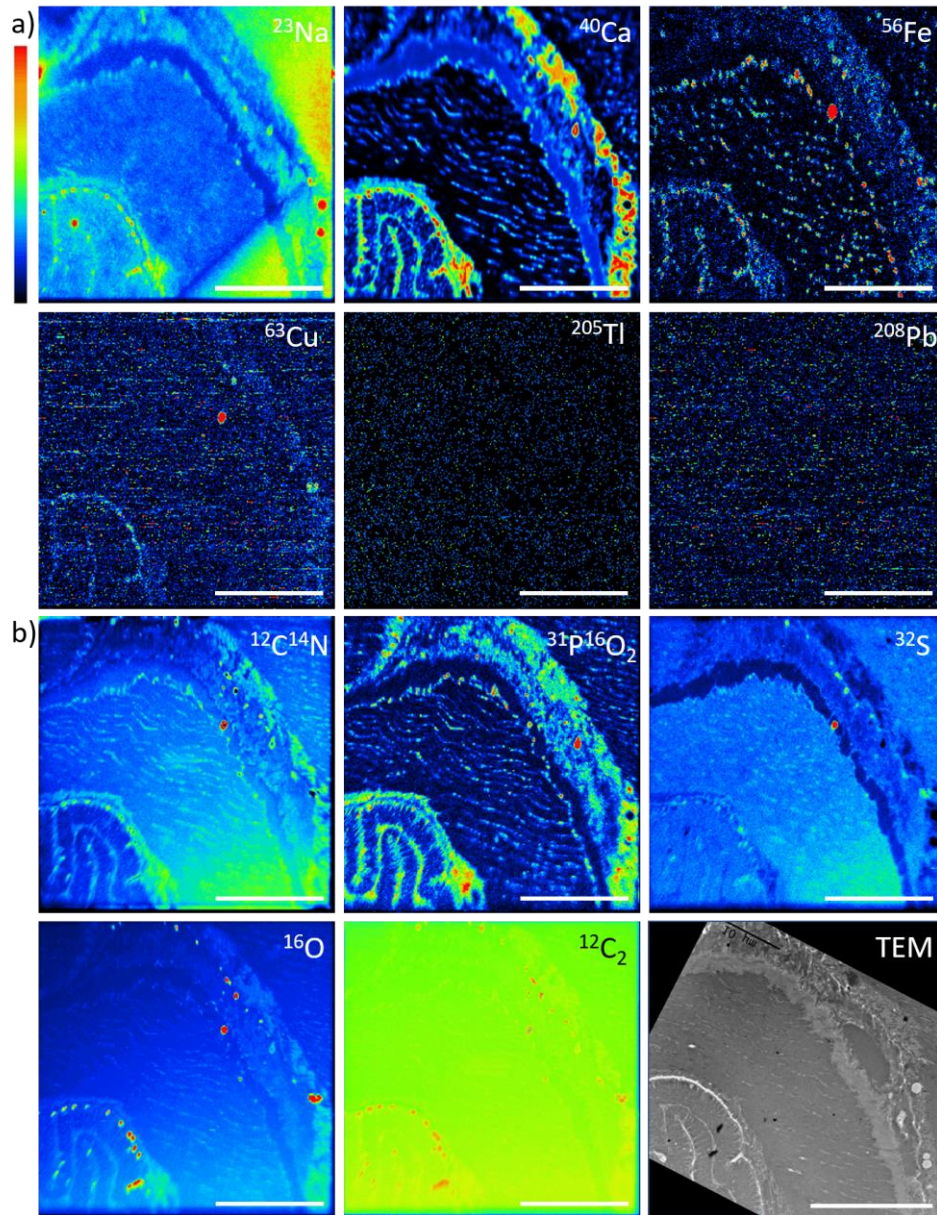
461

462 **3.3.2 Proboscis receptacle**

463

464 Till now, only the morphology but not the element distribution of the proboscis was only
465 studied using light, transmission and scanning electron microscopy (Taraschewski, 2000).
466 NanoSIMS imaging provided insight into the distribution of chemical elements in the
467 ultrastructure of the proboscis receptacle of *D. truttae* (Fig. 6.). The most dominant element in
468 the proboscis receptacle was Ca, mostly located in the cytoplasmic inner lining and the proboscis
469 retractor muscles, but its presence could be observed in other parts as well. Similarly, Na, Fe, Cu,
470 S, N and P occurred mostly in the proboscis retractor muscle, but these elements varied in their
471 intensities and distribution (Fig. 6). Further, Fe and Cu showed an overlay with O in few
472 hotspots, possible indicating the presence of metallic oxides deposits. As in trunk spine, Tl, Pb
473 and Se (not shown) mapping did not show any pattern (Fig. 6).

474



475

476

477 **Figure 6.** Elemental mapping of the proboscis receptacle (see Fig.1b, area 2) of *Dentitruncus*
 478 *truttae* by NanoSIMS using: a) O^- source: $^{23}Na^+$, $^{40}Ca^+$, $^{56}Fe^+$, $^{63}Cu^+$, $^{205}Tl^+$, $^{208}Pb^+$; b) Cs^+ source:
 479 $^{12}C^{14}N^-$, $^{31}PO_2^-$, $^{32}S^-$, $^{16}O^-$, $^{12}C_2^-$, as well as correlative imaging with TEM of the same sample area
 480 from a subsequent ultramicrotome section. Color scale on the top left represents relative

481 intensities of the isotopes count rates. Scale bar: 20 μm . Colour scale corresponds to relative
482 concentration of elements.

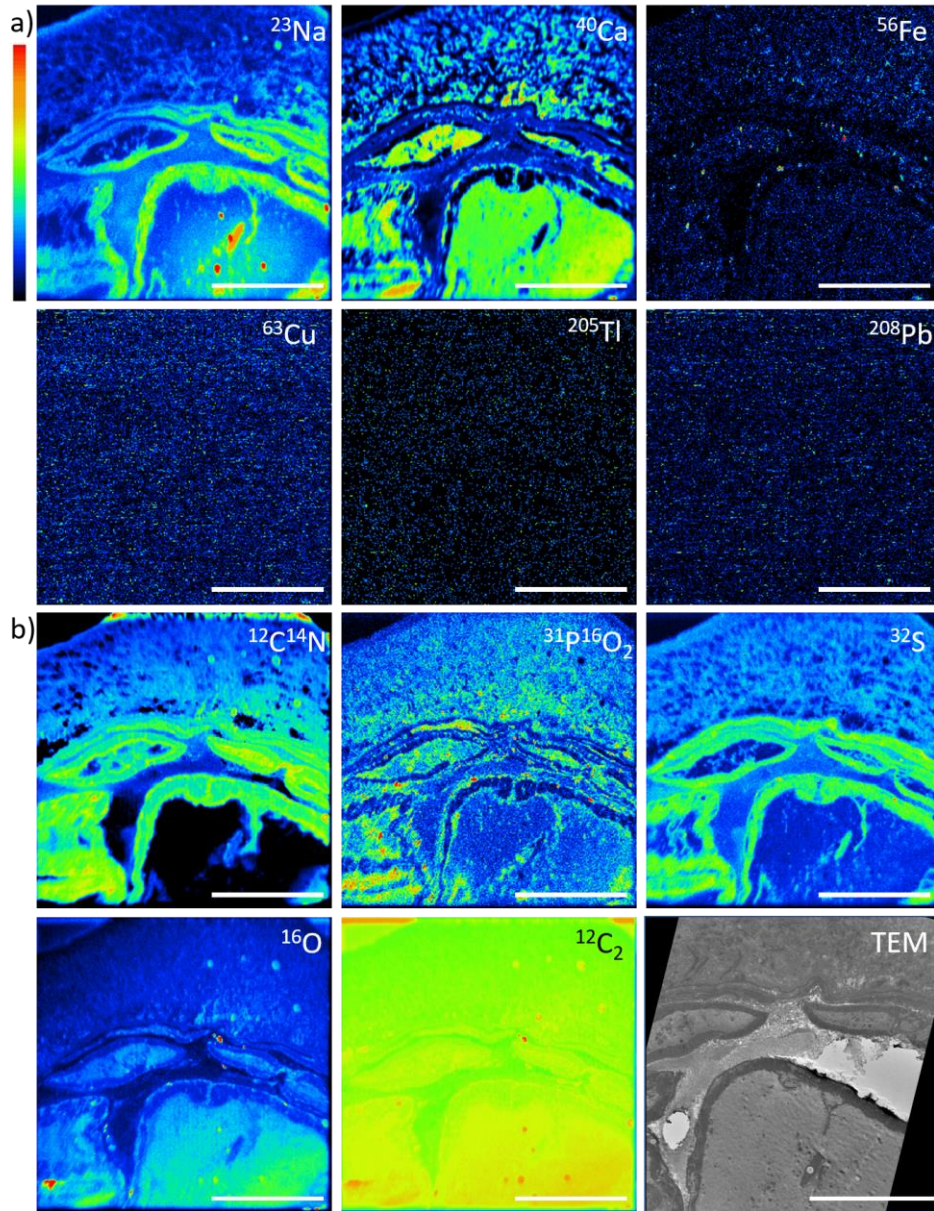
483

484 **3.3.3 Inner surface of the tegument**

485

486 In addition, NanoSIMS provided first data on elemental distribution in the ultrastructure of
487 the tegument of *D. truttae* (Fig. 7). The superficial layer of the acanthocephalan tegument also
488 had a higher concentration of S, Na and Ca than the circular and longitudinal musculature. Sulfur
489 and Na were mostly localised in contractile filaments of the muscle, while Ca was found mostly
490 in the central cytoplasmic core of longitudinal muscles and the neck retractor muscles (Fig. 7).
491 Sodium was also partially present in the same areas, showing few hotspots in the neck retractor
492 area (Fig. 7). Among other measured elements, only Fe and P were observed in the circular and
493 longitudinal musculature. Similar to the trunk spines (outer tegument), Fe and Cu were
494 predominant metals in the acanthocephalan inner tegument, whereas Se (not shown), Tl and Pb
495 were not detected in this part either (Fig. 7).

496



497

498

499 **Figure 7.** Elemental mapping of the tegument (see Fig.1b, area 3) of *Dentitruncus truttae* by
 500 NanoSIMS using: a) O⁻ source: ²³Na⁺, ⁴⁰Ca⁺, ⁵⁶Fe⁺, ⁶³Cu⁺, ²⁰⁵Tl⁺, ²⁰⁸Pb⁺; b) Cs⁺ source: ¹²C¹⁴N⁻,
 501 ³¹PO₂⁻, ³²S⁻, ¹⁶O⁻, ¹²C₂⁻, as well as correlative imaging with TEM of the same sample area from a
 502 subsequent ultramicrotome section. Color scale on the top left represents relative intensities of

503 the isotopes count rates. Scale bar: 20 μ m. Colour scale corresponds to relative concentration of
504 elements.

505

506 **4. CONCLUSIONS**

507

508 Our results represent important initial data with a perspective for the use of NanoSIMS in
509 parasitology and environmental studies in general, in spite of few limitations of our study with
510 respect to the methodology and low environmental metal concentrations. The first description of
511 the chemical composition of the acanthocephalan body at nanometer level indicates complex
512 structures that varies in element localisation. It was furthermore possible to link the distribution
513 of chemical elements to the acanthocephalan ultrastructure to TEM-NanoSIMS correlative
514 imaging. The highest concentration in all studied structures was confirmed for Na, Ca, S, C, N,
515 O, as essential and constitutive elements in living cells. The high amount of S found in trunk
516 spines could be explained for the first time by linking its presence to a circular structure of
517 amorphous matter. A high amount of Na was localised, too, in this circular structure of the spines,
518 and as well in contractile filaments of the muscles. Calcium was dominantly found in the
519 cytoplasmic inner lining and the proboscis retractor muscles and as well in the spine circular
520 structure. As for trace metals, Fe was predominant in all studied structures, while Cu showed the
521 same pattern in the proboscis receptacle and trunk spines, but no concentration in the tegument.
522 Lead and Tl concentrations were generally low in the environment and the parasite so its
523 detection by NanoSIMS was not efficient, but we could still observe some shapes of Tl
524 localisation in the region of the trunk spines compared with other two areas. Generally, non-

525 essential metals were mostly distributed in small concentrations along investigated structures and
526 did not show distinctive locations for accumulation in the parasite's body.

527

528 High-resolution localisation of chemical elements by NanoSIMS confirmed the highest
529 density of essential elements characteristic for living cells and their specific incorporation in
530 different body parts of the acanthocephalan *D. truttae*. Trunk spines and tegument showed
531 mostly higher element accumulation than the proboscis receptacle, probably reflecting their role
532 in substance absorbance in the organisms. Further analyses on element localisation are needed to
533 compare element distribution in parasites found in hosts exposed to different environmental
534 conditions, such as unpolluted and pollution-impacted sites, as well as to compare NanoSIMS
535 data between different acanthocephalan species.

536

537

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542

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549 Aquatic Organisms (Ruđer Bošković Institute, Croatia).

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