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# Unravelling the molecular mechanisms of nickel in woodlice.

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**Abstract:**

During the last few years, there has been an alarming increase in the amount of nickel (Ni) being released into the environment, primarily due to its use in the production of stainless steel but also from other sources such as batteries manufacturing and consequent disposal. The established biotic ligand models provide precise estimates for Ni bioavailability, in contrast, studies describing the mechanisms underpinning toxicological effect of Ni are scarce. This study exploits RNA-seq to determine the transcriptomic responses of isopods using *Porcellionides pruinosus* as an example of a terrestrial metal-resistant woodlouse. Furthermore, the recently proposed model for Ni adverse outcome pathways (Ni-AOP) presents an unprecedented opportunity to fit isopod responses to Ni toxicity and define *Porcellionides pruinosus* as a metalomic model.

Prior to this study, *P. pruinosus* represented an important environmental sentinel, though lacking genetic/omic data. The reference transcriptome generated here thus represents a major advance and a novel resource. A detailed annotation of the transcripts obtained is presented together with the homology to genes/gene products from Metazoan and Arthropoda phylum, Gene Ontology (GO) classification, clusters of orthologous groups (COG) and assignment to KEGG metabolic pathways.

The differential gene expression comparison was determined in response to nickel (Ni) exposure and used to derive the enriched pathways and processes. It revealed a significant impact on ion trafficking and storage, oxidative stress, neurotoxicity, reproduction impairment, genetics and epigenetics. Many of the processes observed support the current Ni-AOP although the data highlights that the current model can be improved by including epigenetic endpoints, which represents key chronic risks under a scenario of Ni toxicity.

**Keywords:** Transcriptome, RNA-Seq analysis, terrestrial isopods, metal trafficking

## 1 **1. Introduction**

2 Metal ions remain a group of pollutants that present a significant risk to ecosystems and  
3 human health, with exposure resulting from variation in their natural distribution which  
4 is exacerbated with the contribution from anthropogenic sources. Anthropogenic  
5 activities such as mining, smelting, foundries, transport and storage yards or even waste  
6 incineration activities produce a high input of metals, such as nickel (Ni), into aquatic and  
7 terrestrial ecosystems. Global usage of Ni in 2016 exceeded 2 Million tonnes (NN, 2015).  
8 Its use correlates with economic development, being exploited in a range of industrial  
9 practices including stainless steel production, alloys, plating, casting and batteries (NN,  
10 2015). It is the latter of these applications that pose an acute environmental hazard. With  
11 the integration of batteries into short-lived consumer electronics begot the challenge for  
12 recycling. Moreover, the increased battery use in locations without an appropriate  
13 recycling-chain escalates the volume of Ni-based batteries being deposited to landfills.  
14 Nickel is considered a carcinogenic metal and has been proven to impact the transcription  
15 of genes related to oxygen transport, transcriptional and translational processes and even  
16 the phosphate cycle (Lee et al., 1995; Pane et al., 2003; Vandenbrouck et al., 2009).  
17 Nickel legal limits in the environment and its legislation are highly variable, for example  
18 in Europe a range of 300-400 mg/kg is used for soil (86/278/EEC) although, in some  
19 countries like the Netherlands, thresholds are as low as 30 mg Ni/kg soil (Inglezakis et  
20 al., 2011). Nevertheless, the heavy metal survey and geochemical database for European  
21 soils (Lado et al., 2008) showed that natural occurring Ni levels could range up to 2,565  
22 mg/kg in soil. It is thus paramount to evaluate the impact of elevated Ni levels on  
23 terrestrial ecosystems especially using transcriptomic data, to support the already existing  
24 data for higher organisational levels (Ferreira et al., 2015; Ferreira et al., 2016).

25  
26 The terrestrial isopod *Porcellionides pruinosus* represents an ideal sentinel species for  
27 evaluating terrestrial ecosystem since it is a crucial component of terrestrial food chains,  
28 acts as a macro-decomposer mediating nutrient recycling, and is essential to maintain the  
29 soil structure (Ferreira et al., 2010; Loureiro et al., 2006; Zimmer, 2002; Zimmer et al.,  
30 2003). Isopods have been adopted as ecotoxicological models because they are  
31 considered sensitive organisms to organic compounds but conversely have a high  
32 capacity to handle exposure to high metal ions concentrations (Morgan et al., 1990).  
33 Interestingly, there is also no defined international standard for its use as indicator species

34 under a pollution scenario, although widely used as an ecotoxicological model (van  
35 Gestel et al., 2018).

36

37 In ecotoxicology, the use of transcriptomic and gene expression analyses can supply  
38 essential information about the molecular mechanisms underlying toxic response and  
39 complementing traditional assays (e.g. reproduction, mortality). The integration of data  
40 from different organizational levels can thus provide additional evidence to legislators  
41 and environmental regulators. Even in exposure scenarios where low toxicity is observed,  
42 alterations may occur at the transcriptional level that is predictive of sublethal or early  
43 indicators of longer-term impact (Gibb et al., 2011; Szabo, 2014). For the order Isopoda,  
44 and most specifically terrestrial isopods, a limited amount of genomic resources exist that  
45 can serve as a base for molecular studies. Only a recently sequenced, poor quality (low  
46 n50 and highly fragmented) genome of *Armadillidium vulgare* (Leclercq et al., 2016) was  
47 released along with other RNA-Seq analysis of 19 species of terrestrial isopods (Becking  
48 et al., 2017). However, the recent advances in Next Generation Sequencing (NGS) allow  
49 us to deploy genomics approaches in neglected and poorly studied organisms, such as *P.*  
50 *pruinosis* (Grabherr et al., 2011b; Simpson et al., 2009).

51

52 The principal aim of this research was to unravel the molecular mechanisms of nickel in  
53 woodlice. This was achieved by performing a global descriptive transcriptome for the  
54 terrestrial isopod species *P. pruinosis* (gene annotation and pathway mapping) and by  
55 analysing the global transcriptome of isopods exposed to Ni. The results obtained will  
56 bring new insights on Ni mechanisms of action and its potential effects on terrestrial  
57 organisms.

58

## 59 **2 Materials and methods**

60

### 61 **2.1 Test Organisms and Culture Procedure**

62 The organisms used in this study belong to the species *Porcellionides pruinosis* and were  
63 collected from a horse manure heap (Coimbra, Portugal - 40°13'12.9"N 8°28'20.8"W) and  
64 maintained for several generations in laboratory cultures at the Biology Department,  
65 University of Aveiro, Portugal in polypropylene (PP) plastic boxes. In culture, isopods  
66 were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at 22±1°C, with

67 a 16:8 h (light:dark) photoperiod in garden soil at 40-60% of its water holding capacity  
68 (WHC).

69

## 70 **2.2 Exposures to nickel**

71 The certified loamy sand soil LUFA 2.2 (Speyer, Germany) was used as test soil. The  
72 main properties of this soil include a pH =  $5.5 \pm 0.2$  (0.01 M CaCl<sub>2</sub>), WHC =  $41.8 \pm 3.0$   
73 (g/100g), organic C =  $1.77 \pm 0.2$  (%), nitrogen =  $0.17 \pm 0.02$ , texture =  $7.3 \pm 1.2$  (%) clay;  
74  $13.8 \pm 2.7$  (%) silt and  $78.9 \pm 3.5$  (%) sand. LUFA 2.2 soil was spiked with nickel (II)  
75 sulphate hexahydrate with 50 mg and 250 mg Ni/kg soil, with a final moisture content  
76 equivalent to  $\pm 50\%$  of the soil water holding capacity. The concentration of 50 mg Ni/kg  
77 soil represents the maximum allowed by the Canadian framework guideline (CBP, 2010)  
78 and 250 mg Ni/kg soil, represents 5x this maximum concentration. Both concentrations  
79 can be found in European soils (Figueira et al., 2002).

80 Toxicity tests were performed in polystyrene (PS) plastic boxes (14 length x 9 width x 5  
81 height cm), containing approx. 2 cm height of LUFA 2.2 soil layer and five isopods (per  
82 box). Test organisms from both genders and different ages (weighing 15-25 mg) were  
83 collected from culture boxes and placed in each test-box. Females with marsupium,  
84 animals with abnormalities and apparent moulting were excluded from trials. Alder leaf  
85 disks ( $\varnothing$  10 mm,  $\pm$  20 mg) were supplied *ad libitum* as food but limiting it to a quantity  
86 and size that prevented organisms from remaining on top, avoiding contaminated soil.

87 Organisms were exposed for a 96 h period with 16:8h (light:dark) photoperiod, at  
88  $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , after which they were transferred to TRIzol<sup>®</sup> and stored at  $-20^{\circ}\text{C}$  for total  
89 RNA extraction, for a period not longer than a week. Animals were all euthanised at the  
90 same time in the day, to avoid any bias due to the circadian cycle. For each treatment a  
91 total of five organisms were individually exposed and the best three RNA extractions  
92 selected for the next steps.

93

## 94 **2.3 RNA extraction**

95 Total RNA was isolated from individuals using TRIzol<sup>®</sup> (Ambion) extraction and  
96 followed by a column purification step using RNeasy Mini Kit<sup>®</sup> (Qiagen - performed  
97 according to the manufacturers manual) with the eluted DNA being stored at  $-80^{\circ}\text{C}$ . Prior  
98 to freezing, RNA concentration and purity was assessed using Nanodrop 2000c

99 spectrophotometer (Nanodrop Technologies, USA) and RNA integrity was checked using  
100 Bioanalyzer (RNA Nano Chip).

101

#### 102 **2.4 Library constructions and sequencing**

103 Whole body RNA-seq libraries were prepared from *P. pruinosis*. A reference  
104 transcriptome library was prepared to contain equal amounts of total RNA extracted  
105 separately from organisms from different life stages: mancae, juveniles and adults (males  
106 and pregnant/non-pregnant females). Libraries representing treatments were prepared  
107 from pooled adults (n=3) harvested from organisms maintained in control soil and those  
108 exposed to soil spiked containing 50 mg and 250 mg Ni/kg soil. Each treatment library  
109 was made by pooling equal amounts of total RNA extracted separately from three  
110 organisms where the RNA integrity had been validated. Libraries were prepared  
111 following Illumina TrueSeq protocol with an average insert length of 100-200 bp and  
112 sequencing was performed on an Illumina HiSeq 2000, these processes completed by  
113 Baseclear (BioSciencePark of Leiden, Netherlands). Paired-end sequences were produced  
114 to derive the reference transcriptome while single-end data was generated for the  
115 treatment libraries.

116

#### 117 **2.5 Transcriptome assembly and redundancy assessment**

118 The transcriptome was assembled according to the procedures described by Jain et al.  
119 (2013) and Zeng et al. (2011). Sequence quality check was performed using FastQC  
120 (v.0.10.1 Babraham Bioinformatics). For the multi-assembly approach the pipelines used  
121 were: Velvet/Oases with  $k$ -mers ranging from 21-39 nt (Schulz et al., 2012; Zerbino and  
122 Birney, 2008), Trans-ABYSS, with  $k$ -mers ranging from 19-35 nt (Simpson et al., 2009),  
123 SOAPdenovo-Trans, with  $k$ -mers ranging from 17-33 nt (Xie et al., 2013), Trinity  
124 (Grabherr et al., 2011a) and CLC Genomics®. Apart from different  $k$ -mer sizes, the  
125 default assembly parameters were kept in all used pipelines.

126 The redundancy of the individual assemblies was initially removed using CD-HIT-EST  
127 (Fu et al., 2012) using default parameters applied to the pooled set of contigs. The  
128 resultant non-redundant contigs were validated using the EvidentialGene package  
129 (<http://arthropods.eugenesis.org/EvidentialGene/>), and a final assembly was defined as the  
130 Model Assembly (MA). To infer the completeness of the assembled transcriptome an  
131 analysis using BUSCO was performed (Simão et al., 2015).

132

## 133 **2.6 Homology search and functional annotation**

134 Homology search was carried out in 2019 by query of the NCBI non-redundant (nr)  
135 database using the Blastx algorithm (v.2.7.0 E-value cut-off of  $1e^{-5}$  - Altschul et al., 1997).  
136 Blast2GO was used (v.2.7.0 - Conesa et al., 2005) to obtain the Gene Ontology (GO)  
137 annotation, Enzyme Commission number (EC) terms and biochemical pathway  
138 information from the KEGG database (Kanehisa and Goto, 2000). The same procedure  
139 was followed individually against the Metazoa and Viridiplantae kingdom, the phyla:  
140 Porifera, Cnidaria, Ctenophora, Nematoda, Arthropoda, Platyhelminthes, Annelida,  
141 Mollusca, Echinodermata and Chordata. Within the phylum Arthropoda annotations were  
142 performed against the subphyla Chelicerata, Crustacea, Hexapoda and Myriapoda and the  
143 clades from the subphylum Crustacea: Amphipoda, Cladocera, Copepoda and Isopoda.  
144 The classification presented before was based in ITIS (<http://www.itis.gov/>).

145

146

## 147 **2.7 Differential Expression Analysis**

148 RNA-Seq analysis was performed by mapping the control and treatments against the MA,  
149 followed by reads counting, normalisation and statistical identification of fold-change  
150 regulation for treatments when compared between each other and the control. A cut-off  
151 value was defined based in the study by Dalman et al. (2012), where they show that the  
152 chosen arbitrary cut-off of fold-change  $>2$  can provide valuable information not obtained  
153 using only *p* values. Since our study presents for each treatment a pool of organisms, the  
154 use of cut-offs will be appropriated for the RNA-Seq analysis. Additionally, the dispersal  
155 estimation of variance across replicates was assessed using the DESeq package (Anders  
156 and Huber, 2012) and is presented in the Supplementary Data.

157

158

## 159 **3 Results**

### 160 **3.1 Transcriptome sequencing output and assembly**

161 The high throughput sequencing produced 88,315,479 reads each containing 50 bp,  
162 representing approx. 4.50Gb of sequence data. It generated a total of 535,772 transcripts  
163 of which only 32.4% (173,843) were unique. EvidentialGene pipeline was used to remove  
164 redundant and artefactual assembly products (detailed output statistics are shown in Table

165 1SD). The resulting model assembly (MA) had a total of 21,053 primary transcripts  
166 (Table 2SD). A BUSCO analysis (Simão et al., 2015) using the Arthropoda reference  
167 sequence database indicated that 70% of the core genes were present as full-length  
168 isoforms with no duplicates, 7.2% were present as fragments, and 22% were missing.

169

### 170 **3.2 Homology search and functional annotation**

171 Primary functional annotation exploited blastx (threshold  $1e^{-5}$ ) against the nr protein  
172 database successfully annotated a total of 16,177 of the MA transcripts (76.84%). It is  
173 worthy of note that recent NGS data added to the databases associated with Isopoda have  
174 not been reflected in accompanying increase non-redundant protein database. Despite the  
175 recent uploaded genome of *Armadillidium vulgare* (Chebbi et al., 2019), its lack of  
176 annotation increased the number of hits as a hypothetical protein, thus impacting the  
177 number of GO hits. The resultant analysis of the top-hits species distribution shows  
178 representation from across the whole animal kingdom (Fig. 1). When analysing the top  
179 ten species providing significant (threshold  $1e^{-5}$ ) hits belong to Arthropoda members,  
180 except for a minor contribution resulting from matches with *Lingula anatina*  
181 (Brachiopoda).

182

183 To assess gene function and biological pathways in the transcriptome, we proceed with a  
184 homology Gene Ontology (GO) classification and KEGG metabolic pathways analysis.  
185 GO terms could be assigned to 11,280 transcripts (69.73%) of the initial 16,177  
186 transcripts who displayed a significant Blast-hit (Fig. 2). The transcripts were associated  
187 with 119 predicted KEGG pathways (Fig. 1SD and Fig. 2SD), and the number of  
188 transcripts associated with each pathway ranged from single gene up to 203. The top 25  
189 pathways with highest transcript numbers are shown on Table 3SD.

190

### 191 **3.3 RNA-Seq analysis for the exposure to 50 mg Ni/kg soil**

192 No mortality was observed for isopods exposed to 50 mg Ni/kg soil. As for the KEGG  
193 pathway analysis, it revealed that upregulated genes were related to 46 pathways and  
194 downregulated genes were related to 42 pathways (Fig. 3SD and Fig. 4SD).  
195 Downregulated and upregulated genes showed a range between -6.0 fold to control in the  
196 case of Minor spike protein H and 7.6 fold increase to control for Maltase-glucoamylase

197 (a list of the genes whose expression level is most impacted is given in Table 4SD). The  
198 distribution into GO classifications is presented in Fig. 3.

199

200 To contextualise the underlying network of genes impacted by Ni exposure we performed  
201 a semantic network analysis of the GO terms enriched in genes up and down-regulated  
202 by the treatment (a summary is provided in Table 1; detailed networks are given in Fig.  
203 5SD to 7SD). Changes in expression of genes regulating ion transport/symport together  
204 with alterations in ion binding proteins, contrasting impact on Zn and Na binders, may be  
205 straightforwardly linked to Ni disrupting ion trafficking pathways. The upregulation of  
206 genes associated with incorporation/reduction of oxygen may suggest that Ni has  
207 disrupting redox potential, again a process observed when toxic metals displace redox  
208 active essential heavy metals such as Fe. It is therefore interesting to see the upregulation  
209 of heme-binding activity that may further support the impact of Ni on the Fe metabolism.  
210 The simulation of the endocytic pathways and downregulation of plasma membrane and  
211 cilium associated axoneme may be related with the organism modifying its  
212 uptake/excretion pathways to reduce the Ni challenge. The up-regulation of  
213 carboxypeptidase activity may be associated with catabolism, a process that may provide  
214 additional energy to respond to the toxic challenge. The more intriguing responses are  
215 associated with the major upregulation of genes linked to sexual reproduction and  
216 nucleosome assembly. These processes may represent the higher-order impacts caused  
217 by Ni challenge while the disruption of mRNA stability/RNA binding and kinase  
218 activities may provide limited insight as for the key events that may be driving the broader  
219 impact.

220

### 221 **3.4 RNA-Seq analysis for the exposure to 250 mg Ni/kg soil**

222

223 No mortality was observed for organisms exposed to 250 mg Ni/kg. The upregulated  
224 genes were related to 41 pathways and the downregulated genes to 28. A complete list of  
225 genes that present a 2-fold change is presented in the Supplementary Data.

226

227 We analysed the downregulated and upregulated genes in response to the higher Ni  
228 exposure, showing a range of change of expression between -6.5 fold to control (Major  
229 spike protein G) and 8.8 fold to control (Transmembrane protease serine 11B – Table

230 5SD). The distribution into GO classifications is presented in Fig. 3 and shows a similar  
231 distribution between up and downregulated processes

232

233 A semantic network analysis of the GO terms enriched in genes up and down-regulated  
234 by the treatment was performed to reveal an overview of the impact of the exposure to an  
235 increase in Ni concentration (a summary is provided in Table 1; detailed networks are  
236 given in Fig. 12SD and 13SD). A striking concordance was found when comparing the  
237 networks for 50 and 250 mg Ni/kg soil. In either case, we found a prevalence of processes  
238 associated with metal homeostasis (ion transport, symport and ion binding). Besides, at  
239 250 mg Ni/kg soil there are also impacts to the endocytic, plasma membrane and cilia  
240 processes, indicating that handling with Ni leads to profound alterations in membrane  
241 trafficking processes. The changes in histidine family metabolism, p-granule and serine  
242 hydrolase activity are more challenging to link to Ni exposure transparently. However,  
243 consistent with the lower level Ni exposure we observe an upregulation of processes  
244 associated with reproduction and nucleosome activity reinforcing the link between Ni and  
245 these major biological processes.

246

### 247 **3.4 RNA-Seq analysis: nickel treatment exposures comparison**

248 Integration of results showing the overlapping responses between the two exposure  
249 conditions is presented in Fig. 4, along with an analysis of the conserved GO  
250 classifications for these shared responses. The distribution between up and downregulated  
251 transcriptions shows similar distribution across treatments, even though some differences  
252 were identified for specific processes. The KEGG pathway analysis revealed that common  
253 up and downregulated genes impacted 29 and 13 pathways, respectively (Fig. 10SD and  
254 11SD).

255

## 256 **4 Discussion**

257

258 To our knowledge, this is the first reference transcriptome for the terrestrial isopod  
259 species *P. pruinosus*, with >77% representation of the core genes of Arthropoda. The GO  
260 analysis of this new transcriptome shows approx. 80% similarity to other Arthropoda  
261 members, which defines the robustness of the assembly. Caution should be taken when  
262 observing the top-hit species similarity distribution to our transcriptome (since better-  
263 represented species in the database will have more hits). The high number of hits to the

264 crustacean *Daphnia pulex* (a widely used species for aquatic ecotoxicology, along with  
265 *Daphnia magna*) suggests a huge potential for homology comparison between these two  
266 ecologically relevant species, aiding development and a better understanding of  
267 toxicological pathways in these invertebrates. Importantly, this reference transcriptome  
268 includes more information than other transcriptomes already published for other  
269 terrestrial isopods (Becking et al., 2017) as it was generated using data from both sexes  
270 and all developmental stages.

271

272 The second part of our study focused on the toxicological effects of the exposure to Ni.  
273 As expected, most of the analysed transcripts showed a dose-dependency among Ni  
274 concentrations, and the transcription level observed. Although Ni is a recognised  
275 carcinogenic metal, its molecular mechanisms and physiological alterations are unclear.  
276 Here we show evidence of several distinct toxicological impacts ranging from genetic to  
277 epigenetic changes, ion trafficking and storage and even neural and physiological  
278 impairment. A special note should also be included regarding the lack of strong activation  
279 of hypoxia signalling (mediated by the hypoxia-inducible factor 1 - HIF). Although a vast  
280 number of studies reported Ni as a metal producing precise gene expression patterns  
281 similar to those in response to hypoxia (Maxwell and Salnikow, 2004; Salnikow et al.,  
282 2000; Salnikow et al., 2003), our results showed no evident alteration for hypoxia related  
283 genes. In the previous work by Ferreira et al. (2015), performed with the same conditions  
284 of this study, respiration rates were measured and no hypoxia was observed, thus  
285 supporting our findings. This suggests that, at least, in terrestrial isopods this response is  
286 not present or that the used Ni concentrations did not trigger a response.

#### 287 **4.1 Genetic and epigenetic impact**

288 DNA damage, cell cycle impairment and cell death were processes highlighted as induced  
289 by Ni exposure and had already been previously reported (Ahamed et al., 2011; Hartwig  
290 et al., 2002; Kasprzak et al., 2003; Shiao et al., 1998). The main mechanism underlying  
291 these impairments are related to DNA repair inhibition and/or pro-apoptosis systems as  
292 DNA polymerase malfunction causes base miss-incorporation into the newly synthesised  
293 oligonucleotides (Sirover and Loeb, 1976). Identical processes were identified within our  
294 study, the DNA repair and recombination proteins RAD54 and DNA double-strand break  
295 repair RAD50 appear 2 to 4-fold upregulated for both exposure concentrations. The E3  
296 ubiquitin-protein ligase RAD18 required for postreplication repair of UV-damaged DNA

297 appears more than 2-fold upregulated, which underlies the impact of reactive oxygen  
298 species (ROS) as a similar toxicological mechanism involved in Ni and UV radiation  
299 toxicity described below. Along with the previous types of proteins, the mismatch repair  
300 endonuclease PMS2 also appears 2.09 and 0.74 upregulated respectively for 50 mg and  
301 250 mg Ni/kg soil exposures. PMS2 not only acts within DNA repair but is also  
302 implicated in DNA damage signalling, a process which induces cell cycle arrest and can  
303 lead to apoptosis in case of major DNA damages (Jenkins, 2009). This can explain the  
304 lower upregulation for organisms exposed to the higher concentration of Ni. An inhibition  
305 of apoptosis and impairment in cell cycle would not be surprising as suggested by the  
306 data, thus showing that Ni not only alters and mismatches DNA, but also prevents  
307 important “checkpoint mechanisms” like the replication of these abnormal cells (Jenkins,  
308 2009). The apoptosis inhibitor IAP, responsible for the inhibition of cell apoptosis also  
309 involved in copper homeostasis, appears 2.5 to 3.5-fold upregulated in both exposure  
310 concentrations with some level of increased response to cell death and potential disruption  
311 of copper metabolism. G2 mitotic-specific cyclin-A and cyclin-B types along with mitotic  
312 spindle assembly checkpoint MAD2A and the mitotic checkpoint serine/threonine protein  
313 kinase BUB1 (related with several forms of cancer: gastric cancer (Grabsch et al., 2003),  
314 breast cancer (Myrie et al., 2000), lung cancer (Haruki et al., 2001) and thyroid cancer  
315 (Ouyang et al., 2002)), are also impacted appearing 2 to 3-fold upregulated, again tightly  
316 associated with cell cycle (growth/death).

317

318 The results obtained also imply epigenetic changes that are closely connected to oxidative  
319 stress, a unifying process across different metal exposures explained below (Valko et al.,  
320 2005). Ni has been proposed to trigger *de novo* DNA methylation, induce  
321 hypermethylation, enhance chromatin condensation and may even replace magnesium in  
322 DNA interactions (Baccarelli and Bollati, 2009; Lee et al., 1995; Salnikow and  
323 Zhitkovich, 2007). Here upregulation in genes encoding histones was observed ranging  
324 from 2 to 3.5-fold change and mainly in histone H1 and H2 (A/B) but also the histone  
325 lysine *N*-methyltransferase SETD7 used as a specific tag for epigenetic transcriptional  
326 activation. Another important upregulated transcript is the chromatin assembly factor 1,  
327 subunit B (CHAF1B) which is required for the assembly of histone octamers onto newly-  
328 replicated DNA, but also plays a role in mediate chromatin assembly, DNA replication  
329 and repair. Finally, DNA (cytosine-5-)-methyltransferase 1 (DNMT1) or methylated-  
330 DNA-protein-cysteine methyltransferase transcripts also appeared 2 to 3-fold upregulated

331 which supports the hypermethylation already observed in other studies (Hermann et al.,  
332 2004; Sutherland et al., 2001).

#### 333 **4.2 Ion trafficking and storage**

334 Terrestrial isopods are organisms that can assimilate high amounts of metals from the  
335 environment (Donker et al., 1990; Drobne, 1997; Hopkin, 1990) by their specific  
336 compartmentalisation of metals into specialised ‘*B*’ and ‘*S*’ cells of the hepatopancreas  
337 (Hopkin and Martin, 1982). However, although one or both cells will be the destination  
338 for Ni storage and/exclusion in terrestrial isopods, specific proteins responsible for up  
339 taking, trafficking and distribution and/or Ni storage are not known. Nickel is described  
340 as a metal that will use pre-existent (and specific) proteins already used by other transition  
341 metals, as zinc transporters, for its own transport (Sterling et al., 2007). Within our study,  
342 we found two separate but well-identified patterns that can be related to Ni trafficking,  
343 storage and/or excretion, which fits within the proposed ‘Ahearn Model’ described by  
344 Ahearn (2010). In brief, the trafficking of Zn into lysosomes is performed by a pH  
345 gradient (low pH inside, high pH outside) that will involve membrane-bound, ATP-  
346 dependent H<sup>+</sup> or Zn<sup>2+</sup>-ATPases or an anion exchanger (Ahearn, 2010), and the last one  
347 can also exchange SO<sub>4</sub><sup>2-</sup>, Ox<sup>2-</sup> or even Cl<sup>-</sup>. There is a definite impact on genes related to  
348 the storage of metals as Ni induces genes that are potentially related to lysosomes. The  
349 zinc finger proteins and their related RING finger proteins showed approx. 40 transcripts  
350 all being at least 2-fold upregulated. Although these proteins are abundant and their  
351 functions are diverse (e.g. DNA recognition or regulation of apoptosis), they are also  
352 essential for lipid binding or zinc transport (Laity et al., 2001). Another important  
353 transcript is haemocyanin, a protein that transports oxygen in some invertebrate species  
354 that contain copper atoms that bind to oxygen (van Holde and Miller, 1995).  
355 Haemocyanin can also serve as a transporter for other metals such as zinc as described in  
356 the work of Zatta (1984), this fact allied with the upregulated expression observed in our  
357 study (regulations up to 6-fold in both alpha and beta subunits) indicates that it may also  
358 similarly transport Ni as it transports Zn.

359

360 Inhibition by Ni on other pathways is also known, in particular on the epithelial sodium  
361 channels (ENaC), which are responsible for mediating Na<sup>+</sup> transport across high electrical  
362 resistant epithelia and participate in the ionic regulation (Sheng et al., 2002). A high  
363 number of sodium-solute transporters such as the sodium-coupled monocarboxylate

364 transporter 2 (SLC5A12) were found to be 3-fold upregulated. Although the function of  
365 these secondary transporters are not associated with Na<sup>+</sup> transport, they play an essential  
366 role in carbohydrates and lipids transport, re-uptake and accumulation. Identically, solute  
367 carrier transporters for amino acids and lipids were also 2 to 4.5-fold upregulated in our  
368 study (e.g. SLC36A1, SLC16A1, etc.). Accumulation and trafficking of Ni into 'B' cells  
369 may occur through a co-transport and since the mechanism of metal uptake is unclear,  
370 one should not disregard a potential detoxification role by 'B' hepatopancreas cells  
371 through a daily cycle of accumulation and release of carbohydrates and lipids into the  
372 midgut lumen (Hames and Hopkin 1991). The co-transport of Ni may also occur during  
373 moult as it requires the accumulation of carbohydrates, lipids and proteins already  
374 identified in previous biochemical (Ferreira et al., 2015) and metabolomic studies  
375 (Ferreira et al., 2016).

### 376 **4.3 Oxidative stress and reactive oxygen species (ROS)**

377 An essential and well-known toxicity mechanism in metal response is oxidative stress  
378 and the related ROS production. Ferreira et al. (2015), using enzymatic assays, showed  
379 that Ni induced oxidative stress in *P. pruinosis*, with responses similar to the ones found  
380 in this study. For the exposure to 50 mg Ni/kg soil, glutathione *S*-transferases (GST)  
381 transcripts showed almost no differences to control, whereas at the highest exposure,  
382 transcripts showed upregulations of 2-4 fold. Similar upregulation was found for  
383 glutathione peroxidase (GPx) at the lowest exposure concentration, whereas at the higher  
384 exposure a downregulation was observed (-2 fold change to control). Other related  
385 oxidative stress enzymes appeared as downregulated like the superoxide dismutase Cu-  
386 Zn and Mn (-1 to -1.5 fold).

### 387 **4.4 Neurotoxicity**

388 Mechanisms of neurotoxicity are mainly related with the inhibition of the  
389 acetylcholinesterase (AChE) and have also been depicted in a previous study performed  
390 in similar conditions and with the same species (Lee et al., 1995), where a small inhibition  
391 of AChE was reported. The affected transcripts in the present study are related to the  
392 encoding of cholinergic and neuro-cholinergic receptors but also other essential co-  
393 factors (e.g. *N*-acetyltransferase necessary for the incorporation of acetyl coenzyme A  
394 specific for the formation of acetylcholine, the substrate to be used by AChE) all  
395 appearing downregulated up to 11-fold. Another downregulated neuro-mechanism in the  
396 present study was the neurotransmitter gamma-aminobutyric acid (GABA) receptors

397 (Kuffler and Edwards, 1958), which is in line with a previous metabolomics study  
398 performed for the same species in the same conditions (Ferreira et al., 2016).

#### 399 **4.5 Reproduction**

400 Although literature is still scarce on the effects of Ni in reproduction pathways, the study  
401 performed by Vandebrouck et al. (2011) using the aquatic crustacean *D. magna*, showed  
402 downregulation of genes involved in reproduction such as vitellogenin, PFK2-FBPase2  
403 or apolipoprotein *d*. Furthermore, Evens et al. (2009) report a fitness decrease of 33% in  
404 *D. magna* together with the earlier release of the first brood release and lower numbers  
405 of offspring, persistent in subsequent broods kept at higher Ni concentrations. The study  
406 suggested that a variety of mechanisms could be involved, such as altered resource  
407 allocation or targeted reproductive inhibition. Our data shows an impact in the  
408 reproductive pathways, mainly related to cellular division. These transcripts include  
409 centromere protein I/L, tightly involved in the response of gonadal tissues to follicle-  
410 stimulating hormone (Uren et al., 2000) or the hormone vitellogenin (45-fold up) a  
411 biomarker used for endocrine disruption (Hansen et al., 1998).

412

#### 413 **5 Conclusions**

414

415 This work provides the first full body transcriptome of a terrestrial isopod *P. pruinosus*,  
416 thus representing an important source of molecular information for this group of  
417 organisms. The transcriptome that we present here includes a high similarity to the  
418 transcriptome of the aquatic crustacean *D. pulex* and also provides information on the  
419 main shared pathways in these organisms that can be used as a starting point for  
420 comparative research.

421

422 The second part of the study was based on the RNA-Seq analysis of the impaired  
423 pathways induced by Ni exposure. The main results could be mainly grouped into  
424 epigenetic responses, ion trafficking and storage, cellular cycle, oxidative metabolism,  
425 neural functions and reproduction. Although similar effects have been observed for other  
426 species, the results presented here contribute for a better understanding of the links  
427 between the mechanistic molecular basis and higher organisational levels.

428

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430

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447

448 **Ethics**

449 Research complied with the EU ethics guidelines.

450 **Data accessibility**

451 The assembled transcriptome and reads from each sample supporting the results of this  
452 article are available in the NCBI Transcriptome Shotgun Assembly and Sequence Read  
453 Archive databases under BioProject accession number SUB2540610 (ongoing  
454 submission).

455

456

457

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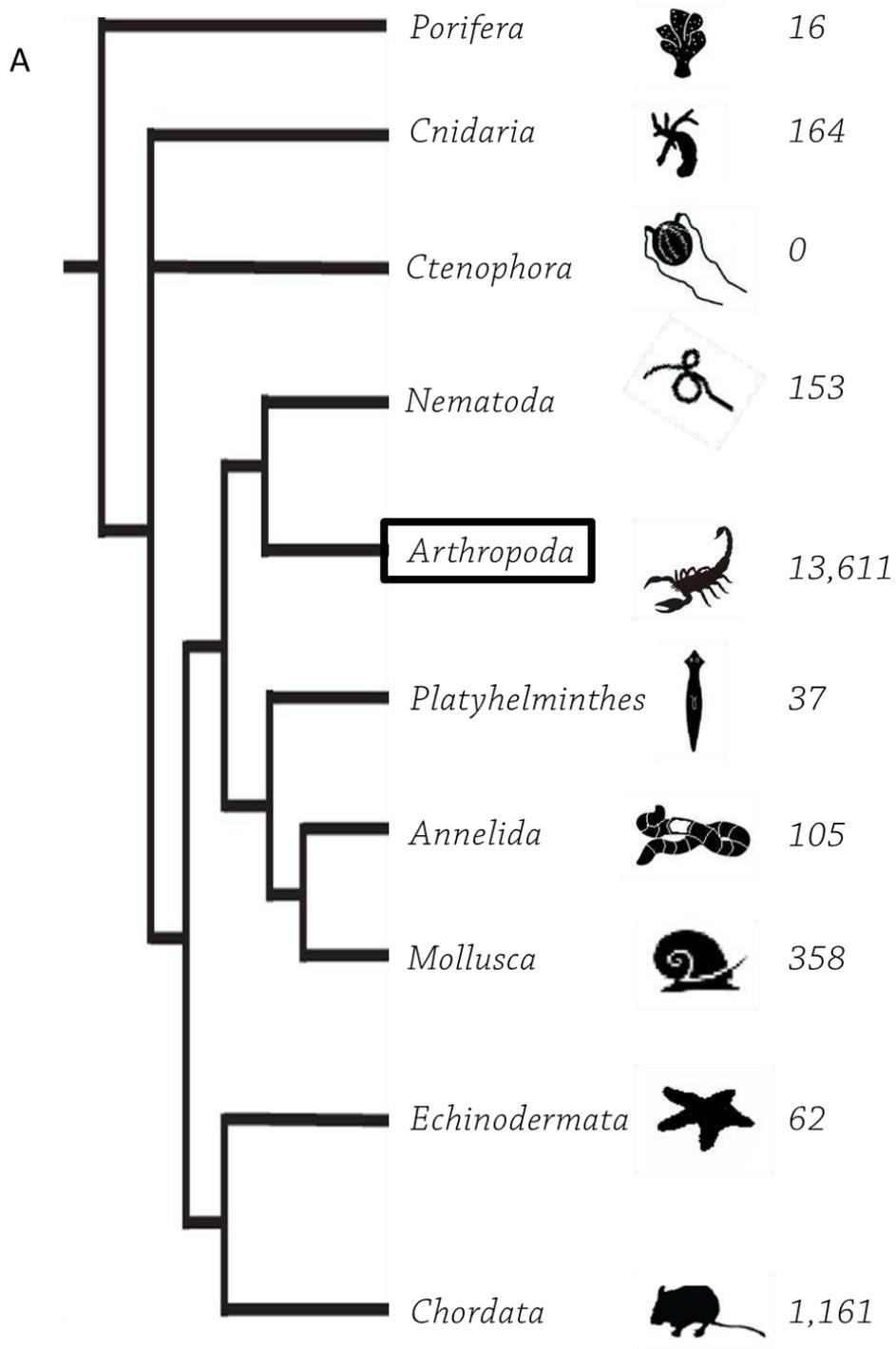
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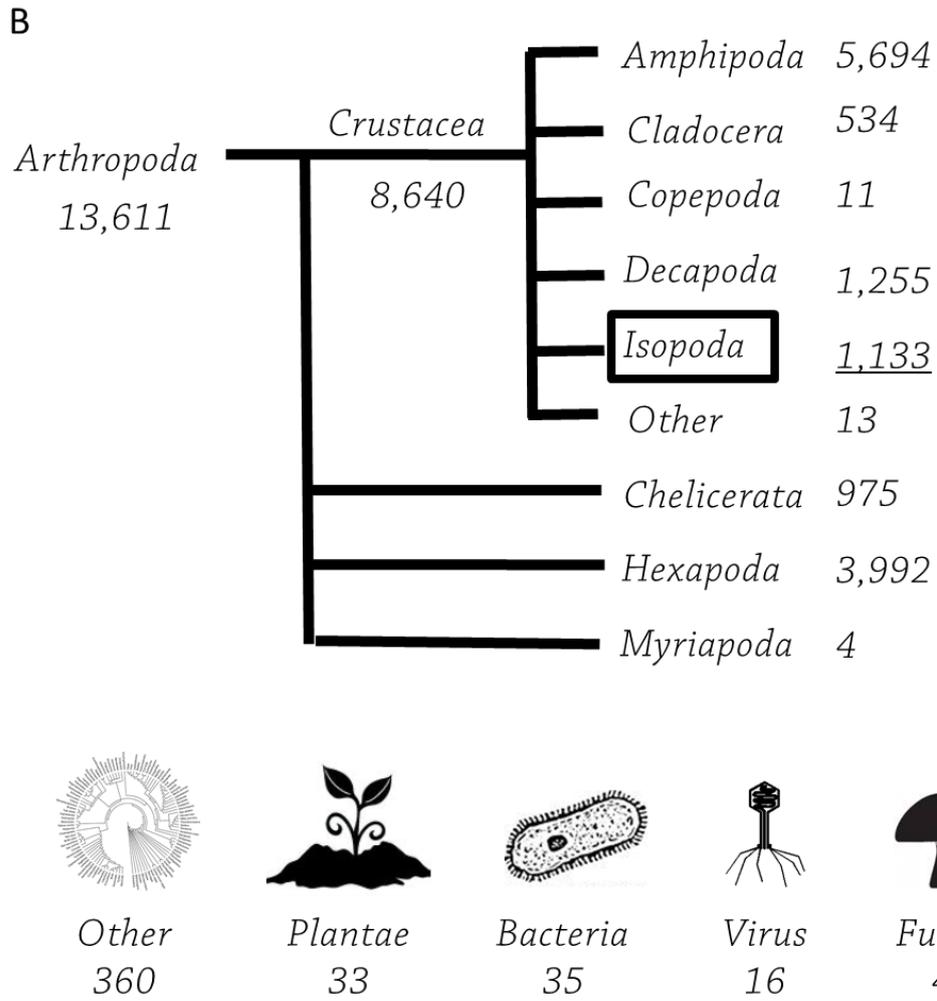
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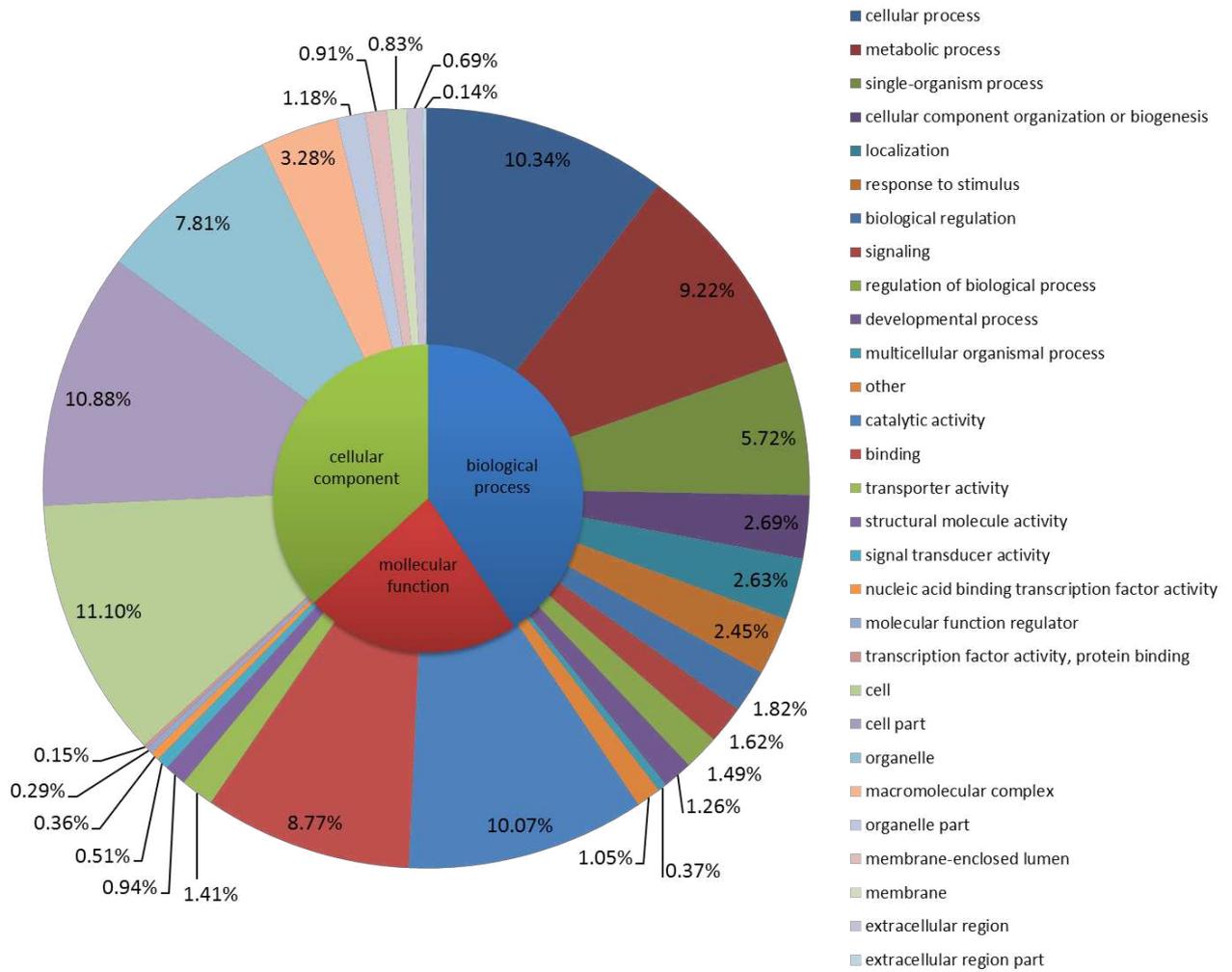
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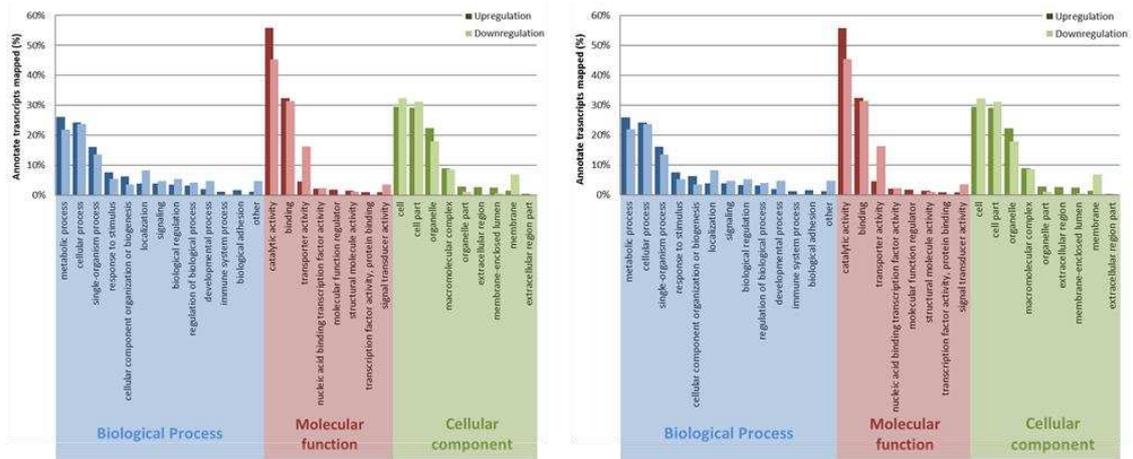




**Fig. 1** Distribution of taxa homologues to *Porcellionides pruinosus* transcripts. A – BLAST hits for Metazoa phyla. B – Total number of BLAST hits for some clades of the Arthropoda phylum along with Plantae, Bacteria, Virus and Fungi. The BLAST hits were performed with an e value of  $1e^{-5}$  within the NCBI nr database.



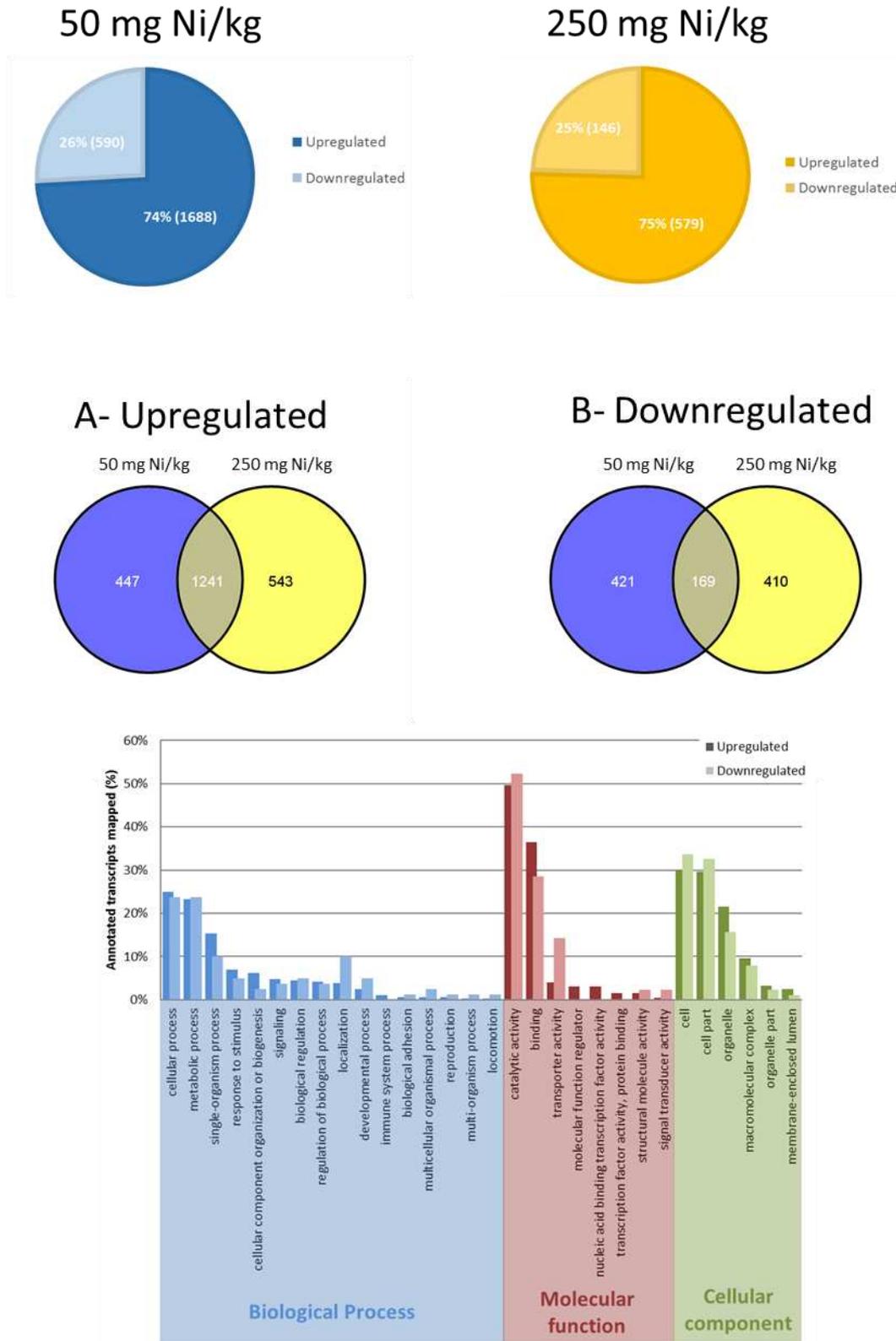
**Fig. 2** Distribution of GO classifications of *Porcellionides pruinosus* into the three main categories: biological process, cellular component and molecular function and their subcategories.



**Fig. 3** Distribution of GO classifications of *Porcellionides pruinosus* regarding significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their subcategories. Column heights represent the percentage of annotated transcripts that mapped to each correspondent GO term. Darker bars represent upregulated transcripts and lighter bars represent downregulated transcripts. Left side – 50 mg Ni/kg; right side – 250 mg Ni/kg.

Table 1- GO enrichment analysis table of major upregulated and downregulated transcripts for *Porcellionides pruinosus* exposed to 50 mg and 250 mg Ni/kg soil divided into Biological Process, Cellular Component and Molecular Function.

| <b>Upregulation</b>                           |      |                                  |                     |
|---|------|----------------------------------|---------------------|
| <b>Ni 50 mg/kg</b>                            |      |                                  | <b>Ni 250 mg/kg</b> |
| <b>Biological Process</b>                     |      |                                  |                     |
| Nucleosome assembly                           | 64 % | Nucleosome assembly              | 37 %                |
| Sexual reproduction                           | 20 % | Sexual Reproduction              | 45 %                |
| Membrane lipid metabolism                     | 10 % | Histidine family metabolism      | 10 %                |
| Regulation of mRNA stability                  | 4 %  | Regulation of mRNA stability     | 5 %                 |
| Other   | 2 %  | Other                            | 3 %                 |
| <b>Cellular Component</b>                     |      |                                  |                     |
| Lysosome                                      | 43 % | Anchored to membrane             | 29 %                |
| External encapsulating structure              | 15 % | External encapsulating structure | 24 %                |
|   |      | P granule                        | 17 %                |
| Other   | 42 % | Other                            | 30 %                |
| <b>Molecular Function</b>                     |      |                                  |                     |
| Carboxypeptidase activity                     | 30 % | Serine Hydrolase activity        | 21 %                |
| Zinc ion binding                              | 19 % | Zinc ion binding                 | 31 %                |
| Incorporation/reduction of oxygen             | 16 % | Symporter activity               | 10 %                |
| Heme binding                                  | 7 %  |                                  |                     |
| Cyclin binding                                | 6 %  |                                  |                     |
| Single-stranded RNA binding                   | 6 %  |                                  |                     |
| Other   | 16 % | Other                            | 38 %                |
| <b>Downregulation</b>                         |      |                                  |                     |
| <b>Ni 50 mg/kg</b>                            |      |                                  | <b>Ni 250 mg/kg</b> |
| <b>Biological Process</b>                     |      |                                  |                     |
| Ion transport                                 | 79 % | Ion transport                    | 73 %                |
| Positive regulation of protein kinase active. | 17 % | Homeostatic process              | 21 %                |
| Other   | 4 %  | Other                            | 6 %                 |
| <b>Cellular Component</b>                     |      |                                  |                     |
| Integral to plasma membrane                   | 17 % | Cilium part                      | 52 %                |
| Axoneme                                       | 16 % | Plasma membrane part             | 27 %                |
| Extracellular region part                     | 5 %  |                                  |                     |
| Other   | 62 % | Other                            | 21 %                |
| <b>Molecular Function</b>                     |      |                                  |                     |
| Symporter activity                            | 37 % | Symporter activity               | 32 %                |
| Sodium ion binding                            | 23 % | Serine type peptidase activity   | 25 %                |
| Ion binding                                   | 5 %  |                                  |                     |
| Other   | 35 % | Other                            | 43 %                |



**Fig. 4** Up and downregulated transcripts for *Porcellionides pruinosus* exposed to nickel at 50 and 250 mg/kg soil, along with Venn diagrams showing the differentially expressed transcripts that are unique and common between treatments (A- upregulated transcripts; B- downregulated transcripts). Diagram with the distribution of GO classifications of significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their subcategories. Upregulated transcripts are assigned into dark colours and downregulated transcripts are assigned into lighter colours. Transcript numbers always represent annotated transcripts.