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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. Introduction

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

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

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

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

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

2 Materials and methods

2.1 Test Organisms and Culture Procedure

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

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

2.2 Exposures to nickel

The certified loamy sand soil LUFA 2.2 (Speyer, Germany) was used as test soil. The main properties of this soil include a pH = 5.5 ± 0.2 (0.01 M CaCl2), WHC = 41.8 ± 3.0 (g/100g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand. LUFA 2.2 soil was spiked with nickel (II) sulphate hexahydrate with 50 mg and 250 mg Ni/kg soil, with a final moisture content equivalent to ±50% of the soil water holding capacity. The concentration of 50 mg Ni/kg soil represents the maximum allowed by the Canadian framework guideline (CBP, 2010) and 250 mg Ni/kg soil, represents 5x this maximum concentration. Both concentrations can be found in European soils (Figueira et al., 2002).

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

Organisms were exposed for a 96 h period with 16:8h (light:dark) photoperiod, at 22ºC±1°C, after which they were transferred to TRIzol ® and stored at -20ºC for total RNA extraction, for a period not longer than a week. Animals were all euthanised at the same time in the day, to avoid any bias due to the circadian cycle. For each treatment a total of five organisms were individually exposed and the best three RNA extractions selected for the next steps.

2.3 RNA extraction

Total RNA was isolated from individuals using TRIzol® (Ambion) extraction and followed by a column purification step using RNAeasy Mini Kit® (Qiagen - performed according to the manufacturers manual) with the eluted DNA being stored at -80°C. Prior to freezing, RNA concentration and purity was assessed using Nanodrop 2000c

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

2.4 Library constructions and sequencing

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

2.5 Transcriptome assembly and redundancy assessment

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

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

2.6 Homology search and functional annotation

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

2.7 Differential Expression Analysis

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

3 Results

3.1 Transcriptome sequencing output and assembly

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

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

3.2 Homology search and functional annotation

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

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

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

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

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

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

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

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

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

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

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

3.4 RNA-Seq analysis: nickel treatment exposures comparison

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

4 Discussion

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

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

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

4.1 Genetic and epigenetic impact

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

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

4.2 Ion trafficking and storage

Terrestrial isopods are organisms that can assimilate high amounts of metals from the environment (Donker et al., 1990; Drobne, 1997; Hopkin, 1990) by their specific compartmentalisation of metals into specialised '*B*' and '*S*' cells of the hepatopancreas (Hopkin and Martin, 1982). However, although one or both cells will be the destination for Ni storage and/exclusion in terrestrial isopods, specific proteins responsible for up taking, trafficking and distribution and/or Ni storage are not known. Nickel is described as a metal that will use pre-existent (and specific) proteins already used by other transition metals, as zinc transporters, for its own transport (Sterling et al., 2007). Within our study, we found two separate but well-identified patterns that can be related to Ni trafficking, storage and/or excretion, which fits within the proposed 'Ahearn Model' described by Ahearn (2010). In brief, the trafficking of Zn into lysosomes is performed by a pH gradient (low pH inside, high pH outside) that will involve membrane-bound, ATP-dependent H^+ or Zn^{2+}-ATPases or an anion exchanger (Ahearn, 2010), and the last one can also exchange SO_4^{2-}, Ox^{2-} or even Cl^-. There is a definite impact on genes related to the storage of metals as Ni induces genes that are potentially related to lysosomes. The zinc finger proteins and their related RING finger proteins showed approx. 40 transcripts all being at least 2-fold upregulated. Although these proteins are abundant and their functions are diverse (e.g. DNA recognition or regulation of apoptosis), they are also essential for lipid binding or zinc transport (Laity et al., 2001). Another important transcript is haemocyanin, a protein that transports oxygen in some invertebrate species that contain copper atoms that bind to oxygen (van Holde and Miller, 1995). Haemocyanin can also serve as a transporter for other metals such as zinc as described in the work of Zatta (1984), this fact allied with the upregulated expression observed in our study (regulations up to 6-fold in both alpha and beta subunits) indicates that it may also similarly transport Ni as it transports Zn.

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

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

4.3 Oxidative stress and reactive oxygen species (ROS)

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

4.4 Neurotoxicity

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

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

4.5 Reproduction

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

5 Conclusions

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

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

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Ethics

Research complied with the EU ethics guidelines.

Data accessibility

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

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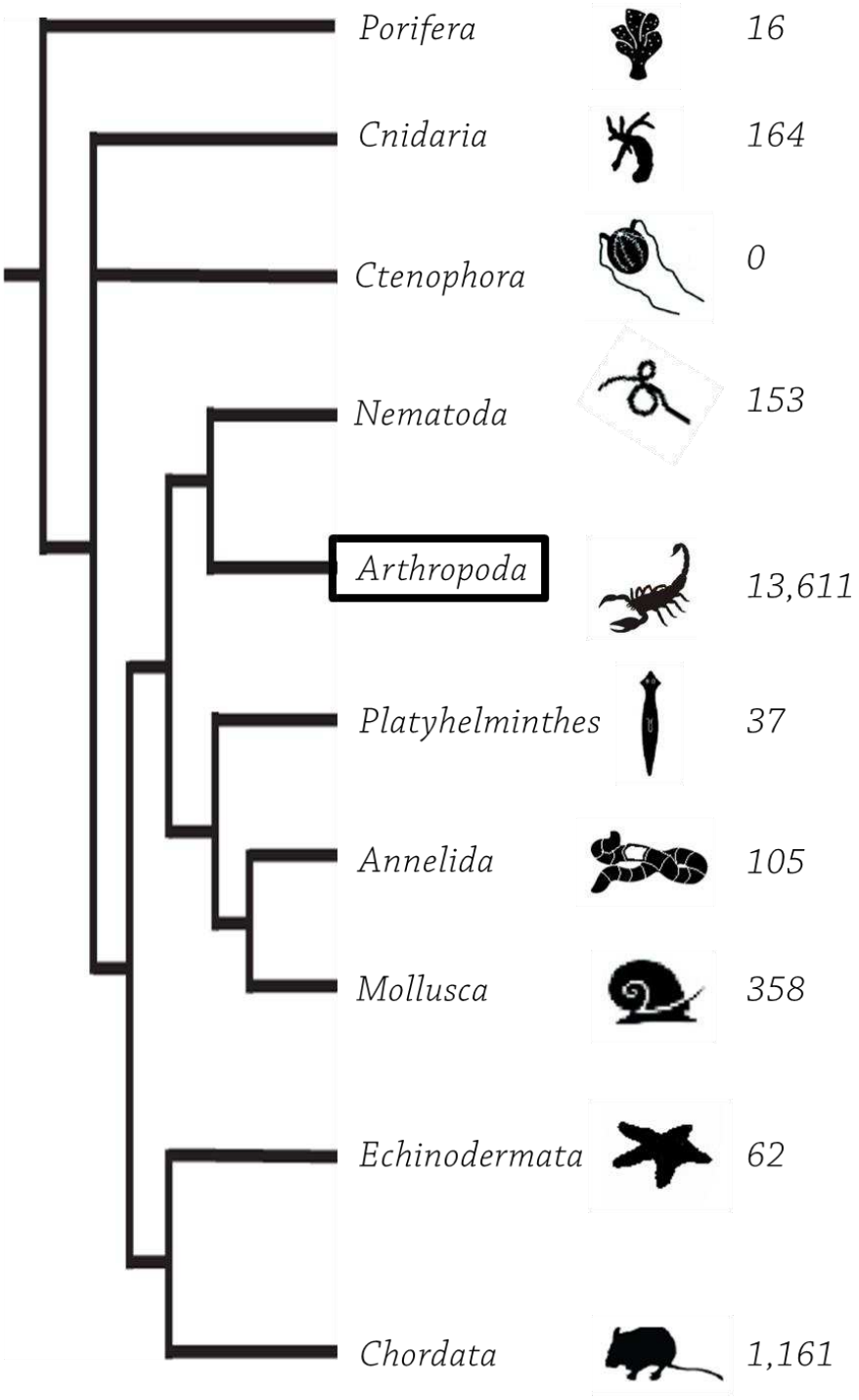
A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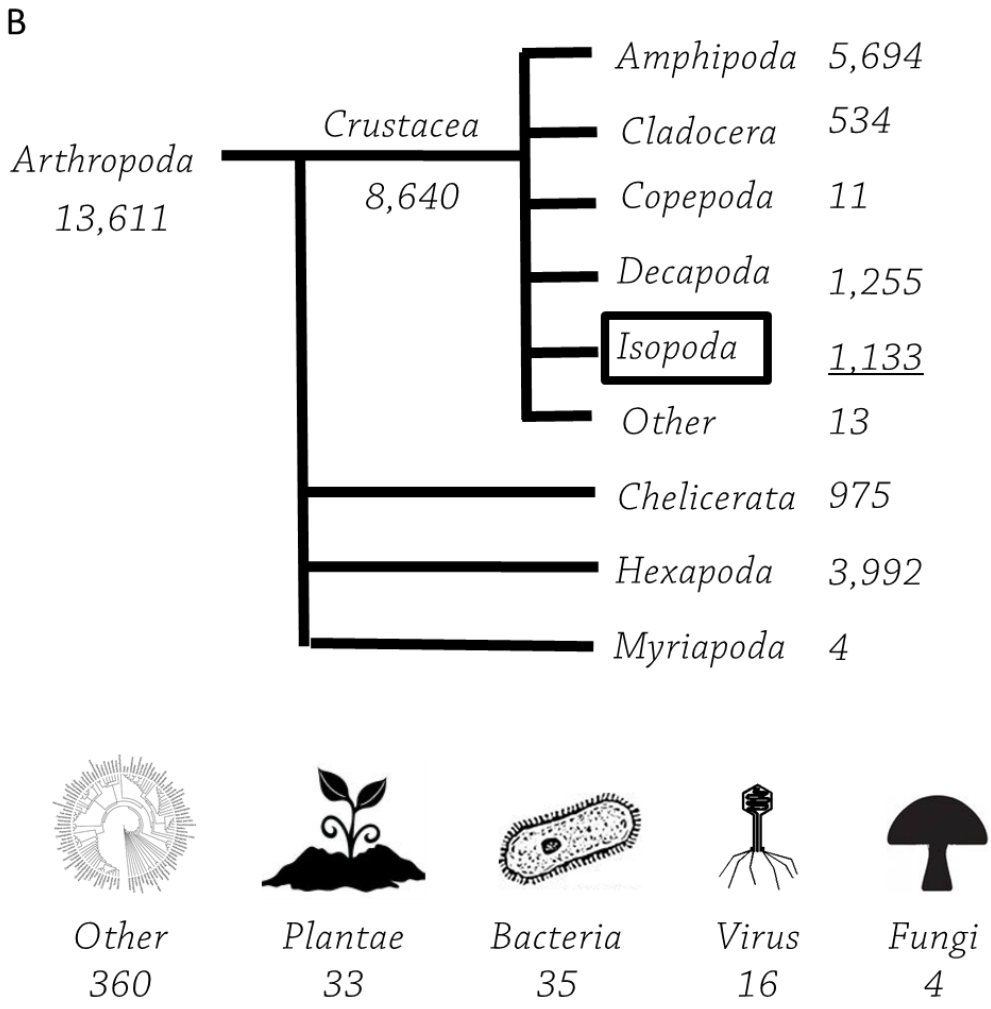

Fig. 1 Distribution of taxa homologues to *Porcellionides pruinosus* transcripts. A – BLAST hits for Metazoa phyla. B – Total number of BLAST hits for some clades of the Arthropoda phylum along with Plantae, Bacteria, Virus and Fungi. The BLAST hits were performed with an e value of $1e^{-5}$ within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

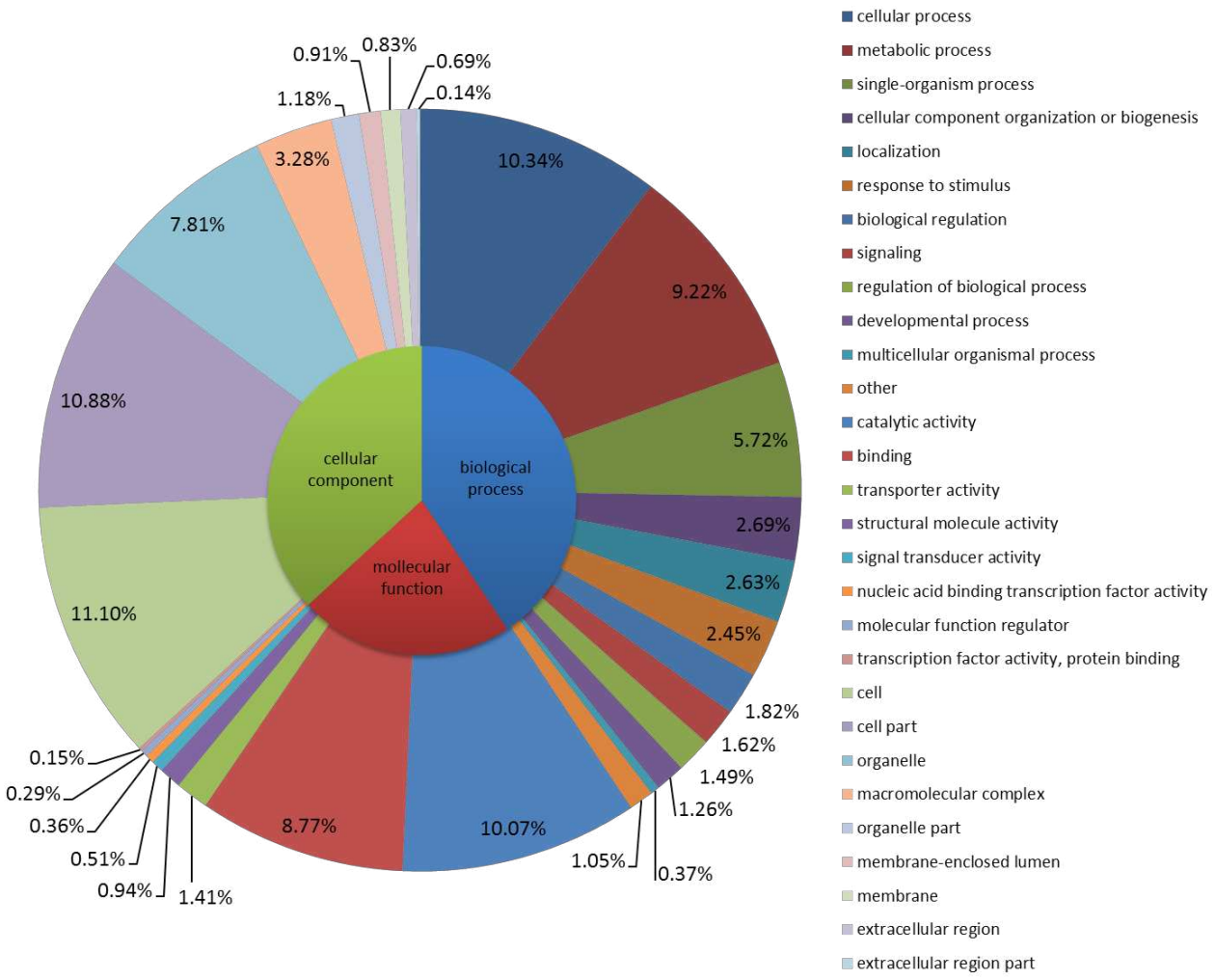

Fig. 2 Distribution of GO classifications of *Porcellionides pruinosus* into the three main categories: biological process, cellular component and molecular function and their subcategories.

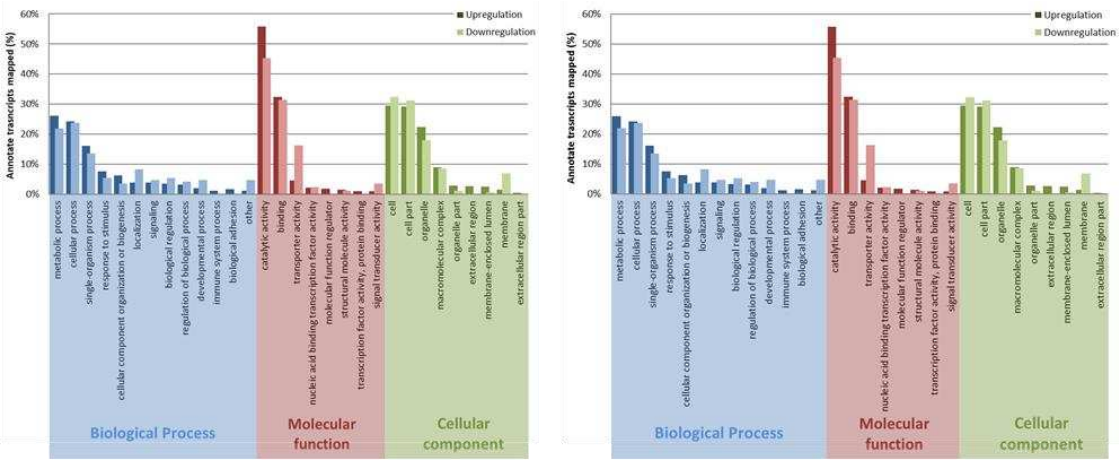

Fig. 3 Distribution of GO classifications of *Porcellionides pruinosus* regarding significant up and downregulated transcripts into three main categories: biological process, cellular component and molecular function and their subcategories. Column heights represent the percentage of annotated transcripts 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.

Upregulation			
Ni 50 mg/kg			**Ni 250 mg/kg**
Biological Process			
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 %
Cellular Component			
Lysosome	43 %	Anchored to membrane	29 %
External encapsulating structure	15 %	External encapsulating structure	24 %
		P granule	17 %
Other	42 %	Other	30 %
Molecular Function			
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 %

Downregulation			
Ni 50 mg/kg			**Ni 250 mg/kg**
Biological Process			
Ion transport	79 %	Ion transport	73 %
Positive regulation of protein kinase active.	17 %	Homeostatic process	21 %
Other	4 %	Other	6 %
Cellular Component			
Integral to plasma membrane	17 %	Cilium part	52 %
Axoneme	16 %	Plasma membrane part	27 %
Extracellular region part	5 %		
Other	62 %	Other	21 %
Molecular Function			
Symporter activity	37 %	Symporter activity	32 %
Sodium ion binding	23 %	Serine type peptidase activity	25 %
Ion binding	5 %		
Other	35 %	Other	43 %

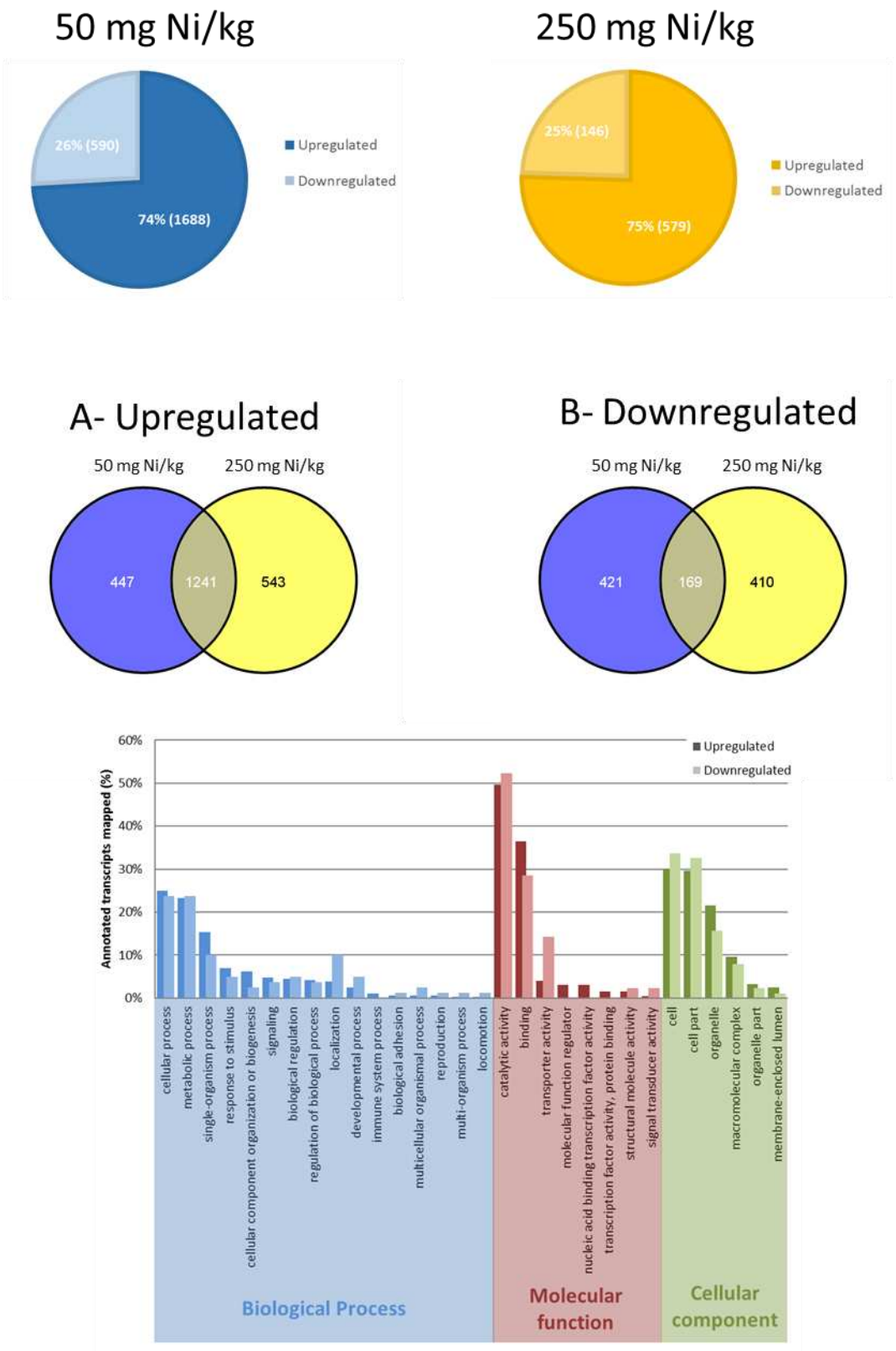

Fig. 4 Up and downregulated transcripts for *Porcellionides pruinosus* exposed to nickel at 50 and 250 mg/kg soil, along with Venn diagrams showing the differentially expressed transcripts that are unique and common between treatments (A- upregulated transcripts; B- downregulated transcripts). Diagram 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.