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1 Multixenobiotic resistance efflux activity in *Daphnia magna* and *Lumbriculus variegatus*

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10

11 Abbreviations:

12 ABC protein ATP-binding cassette transfer protein

13 Ca-AM calcein-AM

14 MDR multidrug resistance protein

15 MRP multidrug resistance related protein

16 MXR multixenobiotic resistance (protein)

17 NBD nucleotide binding domain

18 P-gp permeability glycoprotein

19 qRT-PCR quantitative reverse transcription PCR

20 RhB rhodamine B

21

22

23 Abstract

24 Multixenobiotic resistance is a phenomenon in which ATP-binding cassette (ABC) family proteins
25 transfer harmful compounds out of cells. *Daphnia magna* and *Lumbriculus variegatus* are model
26 species in aquatic ecotoxicology, but the presence and activity of ABC proteins have not been well
27 described in these species. The aim of this work was to study the presence, activity, and inhibition of
28 ABC transport proteins in *Daphnia magna* and *Lumbriculus variegatus*. The presence of *abcb1* and
29 *abcc* transcripts in 8 - 9-day-old *D. magna* was investigated by qRT-PCR. The activity of MXR in *D.*
30 *magna* and *L. variegatus* was explored by influx of the fluorescent ABC protein substrates rhodamine
31 B and calcein-AM, with and without the model inhibitors verapamil (unspecific ABC inhibitor),
32 reversin 205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor). Juvenile *Daphnia magna* possessed all
33 examined *abcb* and *abcc* transcripts, but only reversin 205 inhibited MXR activity. The MXR activity
34 in *Lumbriculus variegatus* was inhibited by MK571, and to a lesser extent by verapamil, whereas
35 reversin 205 seemed to stimulate the transport activity. Whereas calcein-AM worked better as an
36 MXR substrate in *D. magna*, rhodamine B was a better substrate for *L. variegatus* MXR activity
37 measurements. This is the first report on MXR activity in the order *Lumbriculida*, subclass
38 *Oligochaeta*, and class *Clitellata*.

39

40 1 Introduction

41 The ATP-binding cassette (ABC) proteins are transmembrane proteins that transfer a wide range of
42 substrates across membranes against a concentration gradient. The transport is energetically driven
43 by the hydrolysis of ATP. The ABC proteins are present in all organisms, including plants, vertebrates,
44 invertebrates, and microbes (Licht and Schneider, 2011).

45

46 The ABC proteins are divided into seven classes (A - G) according to their sequence homology. The
47 cytosolic nucleotide binding domain (NBD) is highly conserved among the classes, and the
48 membrane domain is the one that confers the substrate specificity to the protein. The ABC proteins
49 typically involved in transferring xenobiotic compounds belong to the classes of ABCB, ABCC, and
50 ABCG. The ones best studied are the Abcb1 protein, which is also called MDR (multidrug resistance
51 protein), MXR (multixenobiotic resistance protein), or P-gp (permeability glycoprotein); and Abcc1,
52 which is also called MRP (multidrug resistance related protein). Both of these proteins have wide
53 substrate specificity, and many compounds may be substrates of both of them. The substrates are
54 usually amphiphilic compounds with separate hydrophilic and hydrophobic moieties. The Abcb1
55 substrates are neutral or weakly positive lipophilic compounds whereas those of Abcc1 are often
56 lipophilic anions, such as Phase II metabolites (Schinkel and Jonker, 2003).

57

58 Abcb1 and Abcc proteins have a role in defense against xenobiotics: As they transfer certain groups
59 of xenobiotics out of the cell, they keep the intracellular concentration of these compounds low.
60 Known Abcb1 or Abcc substrates include metals such as cadmium and mercury; pesticides such as
61 dacthal and pentachlorophenol; and polycyclic aromatic hydrocarbons (Achard-Joris, et al., 2005;
62 Campos, et al., 2014; Chao Yeh, et al., 1992; Epel, 1998). Abcb1 and Abcc proteins, and their activity,
63 are induced by environmental chemicals in various aquatic organisms (Achard, et al., 2004; Eufemia
64 and Epel, 1998; Ferreira, et al., 2014; Kurelec, 1997; Luckenbach, et al., 2014; Prevodnik, et al., 2007;
65 Smital, et al., 2004). There are differences between species and strains, and between populations
66 from clean and polluted sites, in the activity of the ABC proteins, and this correlates with sensitivity
67 to chemicals (Kurelec, et al., 1996; Kurelec, 1997; Smital, et al., 2000; Smital, et al., 2004; Velki and
68 Hackenberger, 2012).

69

70 Various compounds can inhibit the ABC protein transfer activity: pharmaceuticals such as verapamil,
71 reserpine, anthracycline, and cyclosporins; synthetic musks; longchain perfluoroalkyl acids;
72 pesticides such as endosulfan, malathion, and dichlorvos; microbial degradation products; and
73 natural products such as algal extracts (Epel, et al., 2008; Kurelec, 1997; Smital, et al., 2004).

74 If the activity of the ABC proteins that keep xenobiotics out of cells is inhibited, the intracellular
75 concentration of those compounds increases. This may lead to toxic effects at environmental
76 concentrations that are not normally toxic to the organism (Anselmo, et al., 2012; Epel, et al., 2008;
77 Faria, et al., 2011; Smital, et al., 2004; Waldmann, et al., 1995). It has been speculated that this may
78 be one of the mechanisms behind mixture effects (Anselmo, et al., 2012; Epel, et al., 2008; Faria, et
79 al., 2011).

80

81 *Daphnia magna* and *Lumbriculus variegatus* are model species in aquatic ecotoxicology, and they are
82 widely used for ecotoxicological testing of compounds and environmental samples. The presence
83 and activity of ABC proteins have not been well described in these species. The genes of the ABC
84 transporter family members have been characterized in *Daphnia pulex* (Sturm, et al., 2009), and
85 partial sequences have been cloned in the genome of *D. magna* (NCBI), but no gene sequences are
86 available for *L. variegatus*. ABC transporter activity in *D. magna* has been characterized in one study
87 (Campos, et al., 2014), but no published studies exist for *L. variegatus* MXR activity. In the phylum
88 *Annelida* MXR activity has only been characterized in the echiuroid *Urechis caupo*, and in the
89 earthworms *Eisenia andrei*, *Eisenia fetida*, *Lumbricus rubellus*, *Octolasion lacteum* and *Dendrobaena*
90 *octaedra* (Bošnjak, et al., 2014; Hackenberger, et al., 2012; Toomey and Epel, 1993; Velki and
91 Hackenberger, 2012; Velki, et al., 2013). These organisms possess verapamil-sensitive MXR system
92 that uses rhodamine B as a substrate.

93

The activity of the MXR system can be examined with the help of fluorescent substrates. Rhodamine B (RhB) is a fluorescent substrate of the MXR system that readily crosses cell membranes. If the MXR system is inhibited, the concentration of the substrate in the cells increases, and this can be seen as a rise in fluorescence. Calcein-AM (Ca-AM) is a cell-permeable substance that is transformed to fluorescent calcein by esterases inside the cells. The ABC proteins transfer Ca-AM out of cells before the esterase action takes place, and thus, the more ABC protein activity there is, the less fluorescence is produced. When ABC proteins are inhibited, Ca-AM reaches the cytoplasm, gets transformed to calcein, and there is a rise in fluorescence.

The aim of this work was to study the presence, activity, and inhibition of ABC transport proteins in *Daphnia magna* and *Lumbriculus variegatus*. The presence of *abcb1* and *abcc* transcripts in *D. magna* was investigated by qRT-PCR. The activity of MXR in *D. magna* and *L. variegatus* was explored by influx of RhB and Ca-AM with and without the model inhibitors verapamil (unspecific ABC inhibitor), reversin 205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor).

2 Materials and methods

2.1 Daphnia culture

Daphnia were cultured in Elendt M7 medium at the density of 50 animals per liter in constant temperature of 20 ± 1 °C and a photoperiod of 18 h light/6 h darkness. They were daily fed with *Scenedesmus* green alga (about 1.2×10^5 cells/mL).

The daphnids used for the RhB and Ca-AM influx assays were 6 to 8 days old, and qPCR was conducted on newborn (<24 h), juvenile (6 - 8 d), and adult (28 d) daphnids.

117

118 2.2 *Lumbriculus* culture

119 The *Lumbriculus* were cultured in ISO test water (ISO, 2012) the total hardness of which was 1 mM,
 120 in constant temperature of 20 ± 1 °C and a photoperiod of 18 h light/6 h darkness. The bottom of the
 121 aquarium was covered with strips of paper towels, and the animals were fed with fish food flakes
 122 (Sera mikropan). The mean wet weight (SD) of the organisms used in the study was 4.6 (0.8) mg.

123

124 2.3 Quantitative reverse transcription PCR (qRT-PCR)

125 The primers for alpha-tubulin were adopted from (Heckmann, et al., 2006) and abcc1/3, abcc4 and
 126 abcc5 from (Campos, et al., 2014). The primers for glyceraldehyde-3-phosphate dehydrogenase and
 127 p-glycoprotein (ABCB/mdr) were designed with Primer3 (version 4.0.0. at
 128 <http://primer3.wi.mit.edu/>), and checked for specificity with Primer-BLAST
 129 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The features of the primers are presented in
 130 Table 1.

131

132 Total RNA was extracted from pools of ca. 70 newborn (<24 h), or 8-10 juvenile (6-8 d) or adult (28 d)
 133 *D. magna* using Tri reagent (Molecular Research Center) following manufacturer's instructions.
 134 Extractions were performed on 3 independent biological replicates. NanoDrop 1000 (Thermo Fisher
 135 Scientific) was used to estimate the RNA concentration and purity, and Agilent 2100 BioAnalyzer
 136 (Agilent) to assess RNA integrity, using Eukaryote total RNA 6000 nano kit (Agilent).

137

138 After DNase treatment (DNase I, Thermo), 1 µg total RNA was reverse transcribed to cDNA (iScript
 139 cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1+9 with nuclease-free water. One 25 µl qPCR reaction

consisted of 5 µl of the diluted cDNA, 0.75 µl each of forward and reverse primers (final concentration 300 nM), 6 µl sterile H₂O and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad). The reactions were run in triplicates on clear 96-well PCR plates (Bio-Rad). The qPCR was run on a CFX96 Real-Time PCR cycler (Bio-Rad). The protocol was 3 min at 95 °C; 40 cycles (10 s at 95 °C, 30 s at 58 °C); 10 s at 95 °C and a melt curve from 55 °C to 95 °C. The C_t values of no template controls (water instead of cDNA) were always over 38. Melt curves showed a single peak, confirming formation of only one PCR product.

2.4 ABC protein activity assays - dye uptake

The dye influx assays were modified from (Smital and Kurelec, 1997). Daphnids were exposed to 1 µM RhB or 0.5 µM Ca-AM in ISO test water (ISO 6341, 2012), with or without specific inhibitors at various concentrations for two hours in the dark. The substrate concentrations were chosen after conducting preliminary exposures in which several concentrations based on literature values were tested. The model inhibitors were verapamil (unspecific ABC inhibitor), reversin 205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor), and all the inhibitors were used at concentrations of 1, 5, and 10 µM, and verapamil and MK571 also at concentration of 20 µM. Treatments were replicated three times, and each replicate consisted of eight daphnids in 8 ml of the exposure solution. After the exposure, the daphnids were examined for mortality, sieved, blotted dry, and weighed to the nearest µg. The eight daphnids of each vial were homogenized in a microcentrifuge vial in 200 µl distilled water with a plastic homogenizing rod. The homogenate was centrifuged (13 000 x g, 5 min), and 150 µl of the supernatant was taken for fluorescence measurement (ex 584 nm, em 612 nm for RB and ex 485 nm, em 538 nm for Ca-AM; Fluoroskan Ascent fluorometer, Labsystems). The fluorescence readings were proportionated to the fresh weights of the organisms (yielding fluorescence unit / mg fresh weight), and these values were normalized to control (daphnids

exposed to 1 μ M RhB or 0.5 μ M Ca-AM). The experiments were conducted twice, each with a different batch of organisms, yielding six replicates for fluorescence measurements altogether.

The *Lumbriculus* assays were conducted similarly, but four organisms were used per 8 ml of exposure solution, and the total hardness of the artificial fresh water used in the assays was 1 mM. The total number of samples for fluorescence measurements was six.

2.6 Statistics

Kolmogorov-Smirnov test was used to test if the data was normally distributed, and Levene's test to test if the variances were equal. For those compounds that met the prerequisites of ANOVA, one-way ANOVA with Tukey's test was used to test if the fluorescence of tissue homogenate differed between inhibitor concentrations. Independent samples Kruskal-Wallis test was used for those compounds that were not normally distributed, and Dunnett's T3 test was used for the pairwise comparisons if variances of the groups were unequal. IBM SPSS Statistics 20 was used for the statistical analyses.

3 Results

3.1 *Daphnia magna* possesses active ABCB1

abcb/mdr transcripts were present in *Daphnia magna* of all ages (Figure 1). Using RhB as the fluorescent substrate, it could be seen that verapamil (ANOVA $p=0.439$) did not have an effect on the amount of fluorescence in the daphnids (Fig. 2 a). Reversin 205 clearly caused an effect on fluorescence (ANOVA $p=0.006$), 10 μ M increasing the fluorescence compared to 0 μ M (Dunnett T3,

p=0.008) and 1 μ M (Dunnett T3, p=0.013) (Fig. 2 b). MK571 did not affect RhB accumulation (ANOVA p=0.382) (Fig. 2c).

Similar results were obtained with Ca-AM as the substrate: The fluorescence in daphnids treated with verapamil (Kruskal-Wallis, p=0.255) did not differ from that of controls (Fig. 3 a). The daphnids treated with reversin 205 possessed more fluorescence than controls (Kruskal-Wallis, p <0.001), and some dose-response could be seen, as both 5 and 10 μ M reversin 205 increased fluorescence compared to 0 and 1 μ M (Dunnett T3, p=0.001 for all), and daphnids exposed to 10 μ M reversin 205 had higher fluorescence than those exposed to 5 μ M (Dunnett T3 p=0.05) (Fig. 3 b). MK571 had no effect on fluorescence (Kruskal-Wallis, p=0.378) (Fig. 3 c)

3.2 ABCB1 and ABCC inhibitors affect *Lumbriculus variegatus* ABC transporter activity

Verapamil had an effect on RhB fluorescence (Kruskal-Wallis, p=0.002) in *Lumbriculus variegatus*, and the fluorescence in organisms treated with 10 μ M verapamil was higher than in 0 (Dunnett T3, p= 0.006) and 1 μ M (Dunnett T3, p=0.009) (Fig. 4 a). Reversin 205 reduced RhB fluorescence (ANOVA p=0.004), with 5 and 10 μ M being significantly different from control (Tukey, p=0.020 and p=0.004, respectively) (Fig. 4 b). Rhodamine B fluorescence in *L. variegatus* increased upon MK571 exposure (Kruskal-Wallis p<0.001), and 10 and 20 μ M MK571 caused a statistically significant increase (Dunnett T3, p=0.002 and p=0.023, respectively) (Fig. 4 c).

Contrary to the results obtained with RhB, verapamil did not increase Ca-AM fluorescence in *L. variegatus*. Though the ANOVA showed that there were differences between treatments (p=0.018), none of the treatments differed from control, and the post-hoc test confirmed significant difference

only between 5 and 10 μ M verapamil (Tukey $p=0.011$) (Fig. 5 a). Reversin 205 reduced Ca-AM fluorescence (ANOVA $p=0.011$), 5 and 10 μ M having significantly lower fluorescence than controls (Tukey, $p=0.024$ and $p=0.015$ for 5 and 10 μ M, respectively) (Fig. 5 b). MK571 had no effect (Kruskal-Wallis $p=0.325$) on fluorescence (Fig. 5 c).

4 Discussion

4.1 mRNA levels and activity of ABCB and ABCC proteins in *Daphnia magna*

This work shows that *abcb1*, *abcc1/3*, *abcc4*, and *abcc5* transcripts are present in *Daphnia magna* neonates, 7 - 8-day-old juveniles, and 28-day-old adults. The work confirms and adds to the findings of Campos et al., who showed that eggs, embryos, neonates, and 5-day-old juveniles possess these transcripts (Campos, et al., 2014). There is a difference in the transcript profile between the studies in the common life stage examined, the neonates. In the study of Campos et al. the transcript levels of all *abcs* were approximately similar to each other, whereas in the present study the neonates possessed much lower transcript levels of *abcc5* than other *abcs* (Campos, et al., 2014). There may be either a genetic difference between the populations, or some environmental factor may have caused the difference.

Surprisingly, the classical inhibitor verapamil increased neither RhB nor Ca-AM influx in daphnids. Similar results have been obtained in western mosquitofish and bluegill sunfish, where no inhibition with 10 μ M verapamil could be observed (Damare, Kaddoumi et al. 2009). Thus it seems that even though verapamil is a good inhibitor of the MXR system in many organisms, it is not a universal inhibitor, and the lack of inhibition by verapamil in a species cannot be taken as a proof of that that the organism does not possess MXR transfer activity.

232

233 In addition to *abcb1* mRNA, *D. magna* possesses ABCB1 protein activity, as exposure to the specific
 234 inhibitor of ABCB1, reversin 205, lead to increased fluorescence of RhB and Ca-AM. The effect was
 235 more pronounced with Ca-AM, which may thus be considered a better substrate for *D. magna*
 236 ABCB1 than RhB. The rise in fluorescence in this work was higher than in the work of Campos *et al.*
 237 probably due to differences in the exposure. Whereas in this work the daphnids were exposed *in*
 238 *vivo* for two hours, Campos *et al.* exposed their organisms *ex vivo* for one hour to overcome the
 239 toxic effects of the inhibitors on filtrating activity (Campos, et al., 2014). In our work, no toxic effects
 240 of the inhibitors were seen.

241

242 Exposure to the ABCC inhibitor MK571 resulted in no effect on fluorescence in 7 - 8-day-old juvenile
 243 daphnids. This result is unexpected taken that these daphnids possess *abcc1/3*, *abcc4*, and *abcc5*
 244 transcripts, and that Campos *et al.* reported small but significant inhibition of MXR transport activity
 245 by MK571 in 4 - 5-day-old juvenile daphnids (Campos, et al., 2014). One possible explanation for the
 246 observation is that the activity of ABCB1 is so high in the daphnids of the present study that the
 247 fluorescent substrates are transferred out of the cells even when the ABCC activity is inhibited. RhB
 248 and Ca-AM are substrates of both ABCB1 and ABCC, and therefore both are transferred by both
 249 proteins.

250

251 4.2 ABC transport protein activity in *Lumbriculus variegatus*

252 The study clearly shows that *L. variegatus* has an active ABC transport protein system. To our
 253 knowledge this is the first report on MXR activity in the order *Lumbriculida*, subclass *Oligochaeta*, or
 254 class *Clitellata*. The presence of an ABC transporter protein (gi|149912747) has been detected in the
 255 spionid polychaete *Pseudopolydora vexillosa* (Chandramouli, et al., 2011), but this protein is 100%

similar to bacterial ABC transporter nucleotide-binding domains. As it has been noticed in the oligochaete *Olavius algarvensis* that its bacterial symbionts have very strong expression of ABC transporters for the uptake of nutrients (Kleiner, et al., 2012), it could be assumed that the protein detected in *P. vexillosa* is synthesized by symbiotic bacteria of the polychaete and not the polychaete itself. When comparing the MXR activity of *L. variegatus* to that of the other members of the phylum *Annelida*, inhibition of the MXR by verapamil seems to be more pronounced in *U. caupo* and *E. andrei* than *L. variegatus*. The maximal increase in fluorescence caused by verapamil was approximately 400 % in *U. caupo* (22 μ M ver) and 80 % in *E. andrei* (10 μ M ver) (Hackenberger, et al., 2012; Toomey and Epel, 1993), whereas in this study 20 μ M ver caused only a 20 % rise in fluorescence compared to control in *L. variegatus*.

If reversin 205 had inhibited the MXR system in *L. variegatus*, the RhB and Ca-AM content in the tissue would have increased, and an increase in fluorescence would have occurred. What was seen was the opposite: there was a drop in fluorescence with both substrates. There are two possible explanations to this observation. First, reversin 205 could be toxic to *L. variegatus*. This toxicity would be seen in Ca-AM exposures as a drop in calcein fluorescence as esterase activity would be inhibited. As the membrane integrity would be compromised, the RhB content in the cells would diminish. However, acute toxicity tests (72 h) confirmed that reversin 205 at the tested concentrations (together with the substrates) was not toxic to *L. variegatus* (Vehniäinen, unpublished). The other explanation to the decreased accumulation of substrates is that in *L. variegatus* reversin 205 stimulates the activity of the MXR system. Some ABC proteins possess multiple binding sites with different substrate specificities, and binding of one substrate at one binding site may stimulate the transfer of the other substrate (Shapiro and Ling, 1997). Whereas it is possible that the same compound acts as a substrate and an inhibitor, and even as an inhibitor and an inducer of MXR activity (Srivalli and Lakshmi, 2012; Velki and Hackenberger, 2013), we are not

aware of other systems in which reversin stimulates ABC protein transfer activity. As the gene and protein sequences of *L. variegatus* ABC proteins remain unresolved, the structure of the active site of them cannot be modeled.

Whereas the inhibitory effect of verapamil in *L. variegatus* was rather weak, a strong inhibition was observed with the ABCC1 inhibitor MK571. This suggests that the ABC transport protein activity in *L. variegatus* is mainly of ABCC-type. Neither verapamil nor MK571 caused a rise in Ca-AM fluorescence, which could mean that Ca-AM is not as good a substrate as RhB for *L. variegatus* ABC transport proteins. The autofluorescence of *L. variegatus* tissues at the wavelengths used to quantify Ca-AM may have reduced the sensitivity of the assay, but the rise in RhB fluorescence upon MK571 exposure was so substantial (13 x) that it should have been seen also with Ca-AM, had it worked similarly well as a substrate.

This study shows that the two invertebrate model species in ecotoxicology, *Daphnia magna* and *Lumbriculus variegatus*, possess MXR activity, but the substrate and inhibitor specificity seems to differ greatly between these species from different phyla. It is likely that also xenobiotics in nature will act differently as substrates and inhibitors of the MXR system in these species. As MXR activity affects both bioaccumulation and toxicity of chemicals, modulation of the activity may have ecotoxicological consequences. MXR activity measurements in *D. magna* and *L. variegatus* may provide valuable information about the effects of aquatic contaminants and contaminant mixtures in these organisms.

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307

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401

402 Table 1. Primers used in the qPCR.

Gene name	Symbol	GenBank accession no.	wFleaBase EST no.	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency	Amplicon size (bp)
alpha-tubulin*	tbl		WFes0007807	TGGAGGTGGTGACGACT	CCAAGTCGACAAAGACAGCA	103.1	89
glyceraldehyde-3-phosphate dehydrogenase	gadph	AJ292555		GTCTTCAGTGAACGAGACCC	GCATGGGCCTTTTCAAGAGT	101.2	104
p-glycoprotein (ABCB/mdr)	abcb1	KC172920		AACGCCATGATTTTATCCA	GCAGAAGGATTTTGGTTGA	96.3	139
ABCC1/3†	abcc1/3	KC122922		TAGCTCGCGCTCTACTGAGAA	GATCGTCGGTCTCCAGATCG	101.9	100
ABCC4†	abcc4	KC122923		CCCGATCCCTTTACGTCGAT	GGTGGCGTCCTACATGAGTGT	97.3	100
ABCC5†	abcc5	KC122924		CAGTCCAGTCATCGAAGCGG	TGACGCAACAGAGCTCGG	101.4	100

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405

406 Figure captions

407 Figure 1. Daphnids of all ages possess *abcb1*, *abcc1/3*, *abcc4*, and *abcc5* mRNA. *abc* mRNA
 408 expression was studied in newborn (< 24 h), juvenile (7 - 8 d) and adult (28 d) daphnids, using *gadph*
 409 and *α-tubulin* as reference genes. Data represent mean ± SE of the mean of three replicates, each
 410 pooled of ca. 70 (< 24 h) or 8 - 10 (7 - 8 d and 28 d) organisms.

411 Figure 2. ABCB1 but not ABCC1 transporter inhibition increases rhodamine B accumulation in
 412 *Daphnia magna* tissues. *Daphnia magna* juveniles were exposed to 1 μM rhodamine B with or
 413 without verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of
 414 the mean of two independent experiments performed in triplicate. *P < 0.05 when compared to
 415 control (one-way ANOVA, followed by Dunnett's T3 test).

416 Figure 3. ABCB1 but not ABCC1 transporter inhibition increases calcein-AM accumulation in *Daphnia*
 417 *magna* tissues. *Daphnia magna* juveniles were exposed to 0.5 μM calcein-AM with or without
 418 verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of the mean
 419 of two independent experiments performed in triplicate. *** P < 0.001 when compared to control
 420 (independent samples Kruskal-Wallis test, followed by Dunnett's T3 test).

421 Figure 4. ABCB1 and ABCC1 transporter inhibition affects rhodamine B accumulation in *Lumbriculus*
 422 *variegatus* tissues. *Lumbriculus variegatus* were exposed to 1 μM rhodamine B with or without
 423 verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of the mean
 424 of two independent experiments performed in triplicate. *P < 0.05 and ** P < 0.01 when compared
 425 to control.

426 Figure 5. ABCB1 but not ABCC1 transporter inhibition affects calcein-AM accumulation in
 427 *Lumbriculus variegatus* tissues. *Lumbriculus variegatus* were exposed to 0.5 μM calcein-AM with or
 428 without verapamil (a), reversin 205 (b) or MK571 (c). Data represent mean ± standard deviation of
 429 the mean of two independent experiments performed in triplicate. *P < 0.05 when compared to
 430 control (one-way ANOVA followed by Tukey's test).

431

432

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.