Author(s): Vehniäinen, Eeva-Riikka; Kukkonen, Jussi

Title: Multixenobiotic resistance efflux activity in Daphnia magna and Lumbriculus variegatus

Year: 2015

Version:

Please cite the original version:

Vehniäinen, E.-R., & Kukkonen, J. (2015). Multixenobiotic resistance efflux activity in Daphnia magna and Lumbriculus variegatus. Chemosphere, 124(April 2015), 143-149. https://doi.org/10.1016/j.chemosphere.2014.12.014

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

2 Eeva-Riikka Vehniäinen[1]*, Jussi V.K. Kukkonen[1]

3

4 1 University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014

5 University of Jyväskylä, Finland

6

7 1st author e-mail address: eeva-riikka.vehniainen@jyu.fi; senior author e-mail address:

8 jussi.v.k.kukkonen@jyu.fi

9 1st author phone: +358-400-73 5959

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

Abstract

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

1 Introduction

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

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

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

102

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

108

109 2 Materials and methods

110 2.1 Daphnia culture

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

114

115 The daphnids used for the RhB and Ca-AM influx assays were 6 to 8 days old, and qPCR was
116 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

140 consisted of 5 µl of the diluted cDNA, 0.75 µl each of forward and reverse primers (final
141 concentration 300 nM), 6 µl sterile H_2O and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad). The
142 reactions were run in triplicates on clear 96-well PCR plates (Bio-Rad). The qPCR was run on a CFX96
143 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
144 °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
145 instead of cDNA) were always over 38. Melt curves showed a single peak, confirming formation of
146 only one PCR product.

147

148 2.4 ABC protein activity assays - dye uptake

149 The dye influx assays were modified from (Smital and Kurelec, 1997). Daphnids were exposed to 1
150 µM RhB or 0.5 µM Ca-AM in ISO test water (ISO 6341, 2012), with or without specific inhibitors at
151 various concentrations for two hours in the dark. The substrate concentrations were chosen after
152 conducting preliminary exposures in which several concentrations based on literature values were
153 tested. The model inhibitors were verapamil (unspecific ABC inhibitor), reversin 205 (ABCB1
154 inhibitor) and MK571 (ABCC inhibitor), and all the inhibitors were used at concentrations of 1, 5, and
155 10 µM, and verapamil and MK571 also at concentration of 20 µM. Treatments were replicated three
156 times, and each replicate consisted of eight daphnids in 8 ml of the exposure solution. After the
157 exposure, the daphnids were examined for mortality, sieved, blotted dry, and weighed to the
158 nearest µg. The eight daphnids of each vial were homogenized in a microcentrifuge vial in 200 µl
159 distilled water with a plastic homogenizing rod. The homogenate was centrifuged (13 000 x *g*, 5
160 min), and 150 µl of the supernatant was taken for fluorescence measurement (ex 584 nm, em 612
161 nm for RB and ex 485 nm, em 538 nm for Ca-AM; Fluoroskan Ascent fluorometer, Labsystems). The
162 fluorescence readings were proportionated to the fresh weights of the organisms (yielding
163 fluorescence unit / mg fresh weight), and these values were normalized to control (daphnids

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

166

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

170

171 2.6 Statistics

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

179

180 3 Results

181 3.1 *Daphnia magna* possesses active ABCB1

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

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

188

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

196

197 3.2 ABCB1 and ABCC inhibitors affect *Lumbriculus variegatus* ABC transporter activity

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

205

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

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

213

214 4 Discussion

215 4.1 mRNA levels and activity of ABCB and ABCC proteins in *Daphnia magna*

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

225

226 Surprisingly, the classical inhibitor verapamil increased neither RhB nor Ca-AM influx in daphnids.
227 Similar results have been obtained in western mosquitofish and bluegill sunfish, where no inhibition
228 with 10 µM verapamil could be observed (Damare, Kaddoumi et al. 2009). Thus it seems that even
229 though verapamil is a good inhibitor of the MXR system in many organisms, it is not a universal
230 inhibitor, and the lack of inhibition by verapamil in a species cannot be taken as a proof of that that
231 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%

256 similar to bacterial ABC transporter nucleotide-binding domains. As it has been noticed in the
257 oligochaete *Olavius algarvensis* that its bacterial symbionts have very strong expression of ABC
258 transporters for the uptake of nutrients (Kleiner, et al., 2012), it could be assumed that the protein
259 detected in *P. vexillosa* is synthetized by symbiotic bacteria of the polychaete and not the polychaete
260 itself. When comparing the MXR activity of *L. variegatus* to that of the other members of the phylum
261 *Annelida*, inhibition of the MXR by verapamil seems to be more pronounced in *U. caupo* and *E.*
262 *andrei* than *L. variegatus*. The maximal increase in fluorescence caused by verapamil was
263 approximately 400 % in *U. caupo* (22 µM ver) and 80 % in *E. andrei* (10 µM ver) (Hackenberger, et al.,
264 2012; Toomey and Epel, 1993), whereas in this study 20 µM ver caused only a 20 % rise in
265 fluorescence compared to control in *L. variegatus*.

266

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

281 aware of other systems in which reversin 205 stimulates ABC protein transfer activity. As the gene
282 and protein sequences of *L. variegatus* ABC proteins remain unresolved, the structure of the active
283 site of them cannot be modeled.

284

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

293

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

302

303 Acknowledgement

The authors would like to thank Mervi Koistinen and Leena Siitonen for technical assistance, and Sari Kakko for preliminary testing of the exposure system. The research was funded by the University of Jyväskylä.

References

Achard, M., Baudrimont, M., Boudou, A., Bourdineaud, J., 2004. Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam *Corbicula fluminea* after heavy metals exposure. Aquatic Toxicology. 67, 347-357.

Achard-Joris, M., van Saparoea, H., Driessen, A., Bourdineaud, J., 2005. Heterologously expressed bacterial and human multidrug resistance proteins confer cadmium resistance to *Escherichia coli*. Biochemistry (N. Y.). 44, 5916-5922.

Anselmo, H.M.R., van den Berg, J.H.J., Rietjens, I.M.C.M., Murk, A.J., 2012. Inhibition of cellular efflux pumps involved in multi xenobiotic resistance (MXR) in echinoid larvae as a possible mode of action for increased ecotoxicological risk of mixtures. Ecotoxicology. 21, 2276-2287.

Bošnjak, I., Bielen, A., Babić, S., Šver, L., Popović, N.T., Strunjak-Perović, I., Čož, R.R., Klobuč ar, R.S., 2014. First evidence of the P-glycoprotein gene expression and multixenobiotic resistance modulation in earthworm. Archives of Industrial Hygiene and Toxicology. 65, 67.

Campos, B., Altenburger, R., Gómez, C., Lacorte, S., Piña, B., Barata, C., Luckenbach, T., 2014. First evidence for toxic defense based on the multixenobiotic resistance (MXR) mechanism in *Daphnia magna*. Aquatic Toxicology. 148, 139-151.

Chandramouli, K.H., Mok, F.S.Y., Wang, H., Qian, P., 2011. Phosphoproteome analysis during larval development and metamorphosis in the spionid polychaete *Pseudopolydora vexillosa*. Bmc Developmental Biology. 11, 31.

Chao Yeh, G., Lopaczynska, J., Poore, C.M., Phang, J.M., 1992. A new functional role for P-glycoprotein: Efflux pump for benzo(a)pyrene in human breast cancer MCF-7 cells. Cancer Research. 52, 6692-6695.

Epel, D., 1998. Use of multidrug transporters as first lines of defense against toxins in aquatic organisms. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology. 120, 23-28.

Epel, D., Luckenbach, T., Stevenson, C.N., Macmanus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: Newly appreciated roles in protection against pollutants. Environ. Sci. Technol. 42, 3914-3920.

Eufemia, N., Epel, D., 1998. The multixenobiotic defense mechanism in mussels is induced by substrates and non-substrates: Implications for a general stress response. Mar. Environ. Res. 46, 401-405.

339 Faria, M., Navarro, A., Luckenbach, T., Pina, B., Barata, C., 2011. Characterization of the
340 multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena*
341 *polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants.
342 Aquatic Toxicology. 101, 78-87.

343 Ferreira, M., Costa, J., Reis-Henriques, M.A., 2014. ABC transporters in fish species: a review.
344 Frontiers in Physiology. 5.

345 Hackenberger, B.K., Velki, M., Stepic, S., Hackenberger, D.K., 2012. First evidence for the presence of
346 efflux pump in the earthworm *Eisenia andrei*. Ecotoxicol. Environ. Saf. 75, 40-45.

347 Heckmann, L., Connon, R., Hutchinson, T.H., Maund, S.J., Sibly, R.M., Callaghan, A., 2006. Expression
348 of target and reference genes in *Daphnia magna* exposed to ibuprofen. BMC Genomics. 7, 175.

349 ISO, 2012. Water quality Determination of the inhibition of the mobility of *Daphnia magna* Straus
350 (*Cladocera*, *Crustacea*)—acute toxicity test. ISO 6341:2012.

351 Kleiner, M., Wentrup, C., Lott, C., Teeling, H., Wetzel, S., Young, J., Chang, Y., Shah, M., VerBerkmoes,
352 N.C., Zarzycki, J., Fuchs, G., Markert, S., Hempel, K., Voigt, B., Becher, D., Liebeke, M., Lalk, M.,
353 Albrecht, D., Hecker, M., Schweder, T., Dubilier, N., 2012. Metaproteomics of a gutless marine worm
354 and its symbiotic microbial community reveal unusual pathways for carbon and energy use. Proc.
355 Natl. Acad. Sci. U. S. A. 109, E1173-E1182.

356 Kurelec, B., 1997. A new type of hazardous chemical: The chemosensitizers of multixenobiotic
357 resistance. Environ. Health Perspect. 105, 855-860.

358 Kurelec, B., Krca, S., Lucic, D., 1996. Expression of multixenobiotic resistance mechanism in a marine
359 mussel *Mytilus galloprovincialis* as a biomarker of exposure to polluted environments. Comparative
360 Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology. 113, 283-289.

361 Licht, A., Schneider, E., 2011. ATP binding cassette systems: structures, mechanisms, and functions.
362 Central European Journal of Biology. 6, 785-801.

363 Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish.
364 Comparative Biochemistry and Physiology C-Toxicology & Pharmacology. 165, 28-52.

365 Prevodnik, A., Lija, K., Bollner, T., 2007. Benzo[a]pyrene up-regulates the expression of the
366 proliferating cell nuclear antigen (PCNA) and multixenobiotic resistance polyglycoprotein (P-gp) in
367 Baltic Sea blue mussels (Mytilus edulis L.). Comparative Biochemistry and Physiology C-Toxicology &
368 Pharmacology. 145, 265-274.

369 Schinkel, A.H., Jonker, J.W., 2003. Mammalian drug efflux transporters of the ATP binding cassette
370 (ABC) family: an overview. Adv. Drug Deliv. Rev. 55, 3-29.

371 Shapiro, A.B., Ling, V., 1997. Positively cooperative sites for drug transport by P-glycoprotein with
372 distinct drug specificities. European Journal of Biochemistry. 250, 130-137.

373 Smital, T., Kurelec, B., 1997. Inhibitors of the multixenobiotic resistance mechanism in natural
374 waters: In vivo demonstration of their effects. Environmental Toxicology and Chemistry. 16, 2164-
375 2170.

376 Smital, T., Luckenbach, T., Sauerborn, R., Hamdoun, A., Vega, R., Epel, D., 2004. Emerging
377 contaminants - pesticides, PPCPs, microbial degradation products and natural substances as
378 inhibitors of multixenobiotic defense in aquatic organisms. Mutat. Res. -Fundam. Mol. Mech. Mutag.
379 552, 101-117.

380 Smital, T., Sauerborn, R., Pivcevic, B., Krca, S., Kurelec, B., 2000. Interspecies differences in P-
381 glycoprotein mediated activity of multixenobiotic resistance mechanism in several marine and
382 freshwater invertebrates. Comparative Biochemistry and Physiology C-Pharmacology Toxicology &
383 Endocrinology. 126, 175-186.

384 Srivalli, K.M.R., Lakshmi, P.K., 2012. Overview of P-glycoprotein inhibitors: a rational outlook.
385 Brazilian Journal of Pharmaceutical Sciences. 48, 353-367.

386 Sturm, A., Cunningham, P., Dean, M., 2009. The ABC transporter gene family of Daphnia pulex. BMC
387 Genomics. 10, 170.

388 Toomey, B., Epel, D., 1993. Multixenobiotic resistance in *Urechis caupo* embryos - protection from
389 environmental toxins. Biol. Bull. 185, 355-364.

390 Velki, M., Hackenberger, B.K., 2013. Inhibition and recovery of molecular biomarkers of earthworm
391 *Eisenia andrei* after exposure to organophosphate dimethoate. Soil Biol. Biochem. 57, 100-108.

392 Velki, M., Hackenberger, B.K., 2012. Species-specific differences in biomarker responses in two
393 ecologically different earthworms exposed to the insecticide dimethoate. Comparative Biochemistry
394 and Physiology Part C: Toxicology & Pharmacology. 156, 104-112.

395 Velki, M., Stepić, S., K. Hackenberger, B., 2013. Effects of formalin on some biomarker activities of
396 earthworms pre-exposed to temephos. Chemosphere. 90, 2690-2696.

397 Waldmann, P., Pivcevic, B., Muller, W., Zahn, R., Kurelec, B., 1995. Increased genotoxicity of
398 acetylaminofluorene by modulators of multixenobiotic resistance mechanism - studies with the
399 fresh-water clam *Corbicula fluminea*. Mutation Research-Genetic Toxicology. 342, 113-123.

400

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		AACGCCCATGATTTTATCCA	GCAGAAGGATTTTGGGTTGA	96.3	139
ABCC1/3†	abcc1/3	KC122922		TAGCTCGCGCTCTACTGAGAA	GATCGTCGGTCTCCAGATCG	101.9	100
ABCC4†	abcc4	KC122923		CCCGATCCCTTTACGTCGAT	GGTGGCGTCCTACATGAGTGT	97.3	100
ABCC5†	abcc5	KC122924		CAGTCCAGTCATCGAGAACGG	TGACGCAACAGAGCTCGG	101.4	100

403 *Heckmann et al. BMC Genomics. 2006; 7: 175.
404 † Campos et al. Aquat Toxicol 2014; 148: 139-151.
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

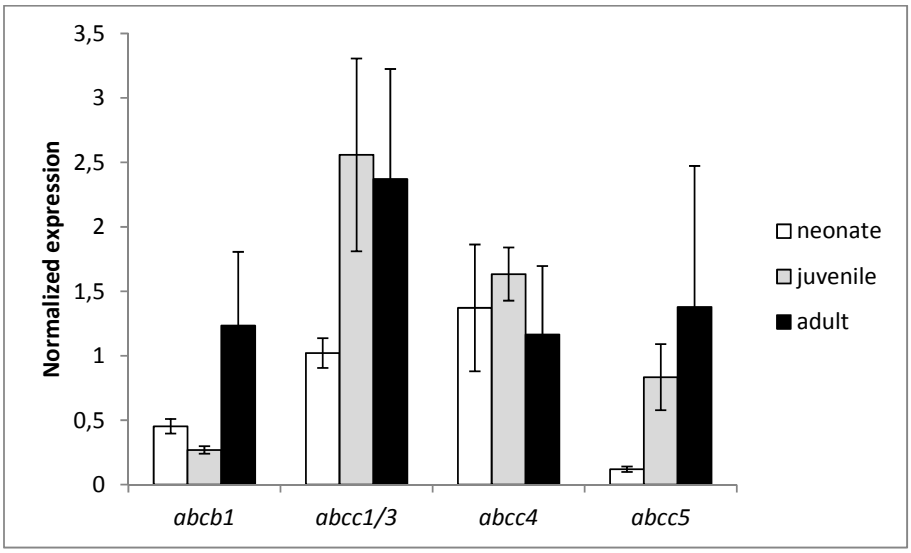
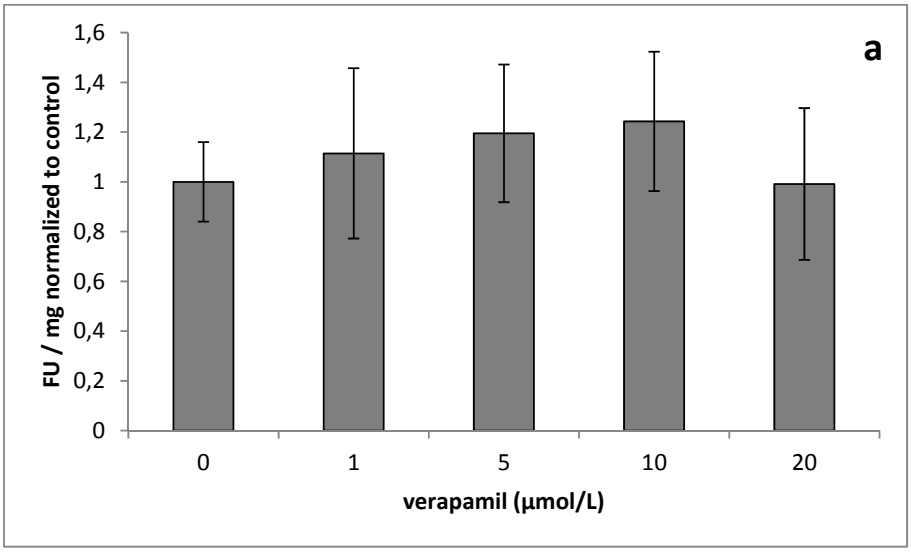
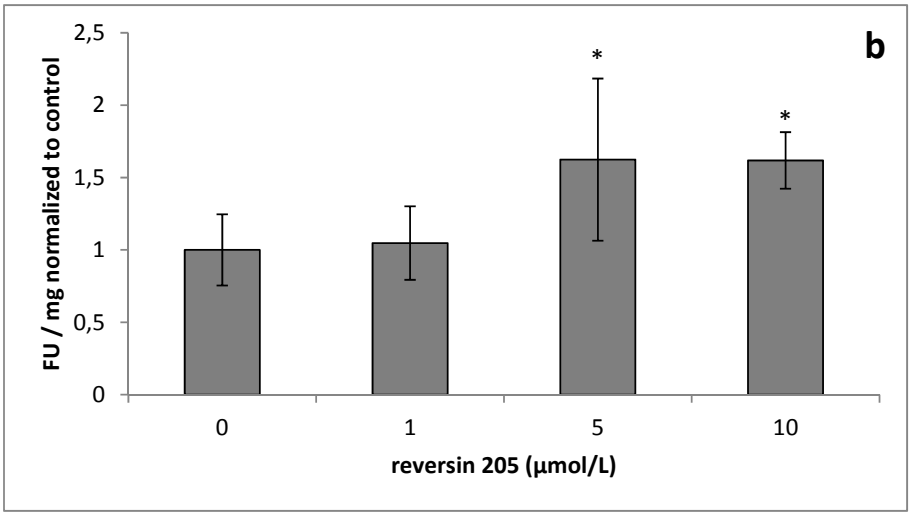

3,5
3
2,5
2
1,5
1
0,5
0
Normalized expression
neonate
juvenile
adult
abcb1
abcc1/3
abcc4
abcc5

Figure 1.


a
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
FU / mg normalized to control
0
1
5
10
20
verapamil (µmol/L)


b
2,5
2
1,5
1
0,5
0
FU / mg normalized to control
*
*
0
1
5
10
reversin 205 (µmol/L)

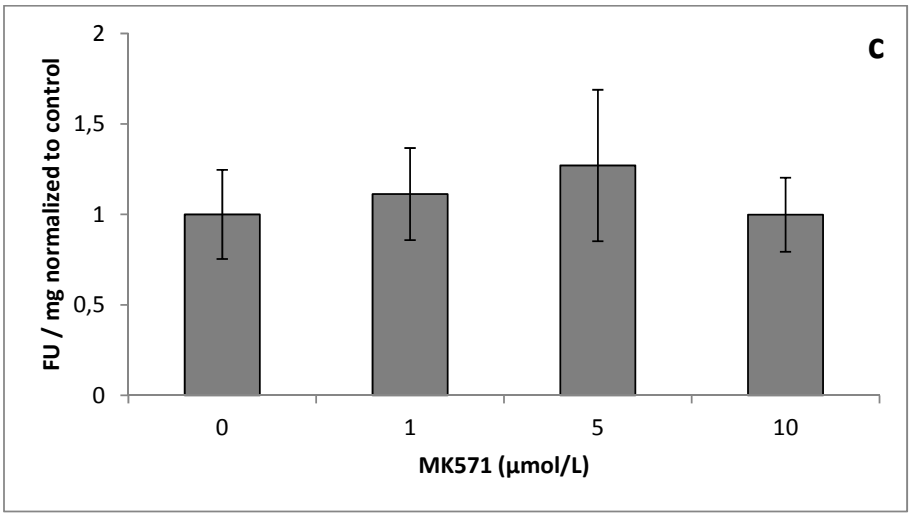
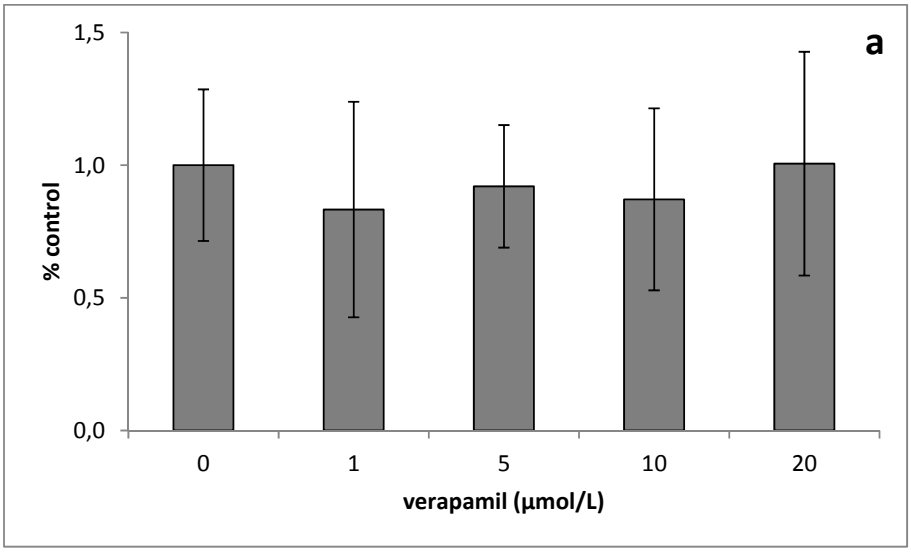
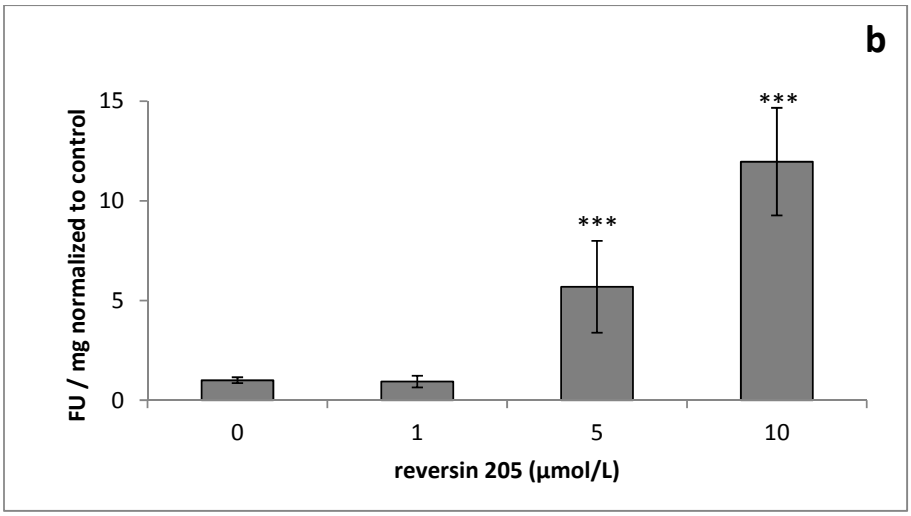
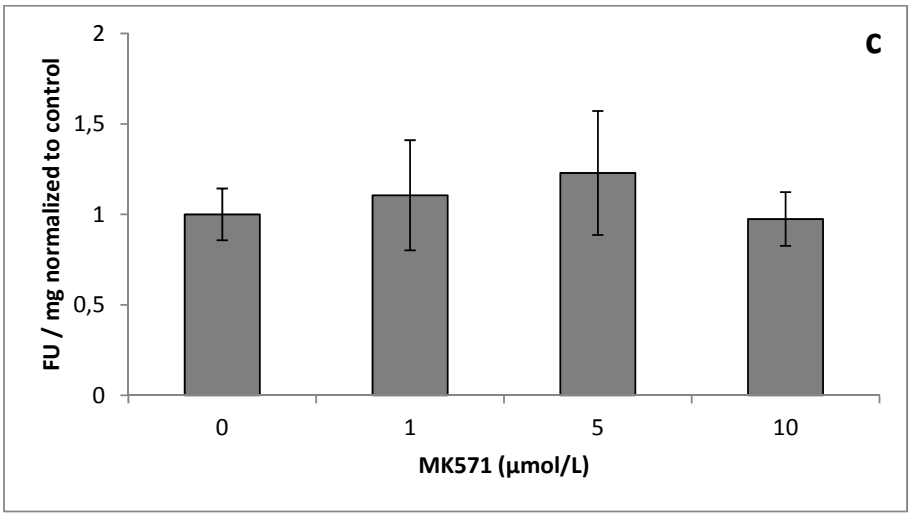

c
FU / mg normalized to control
2
1,5
1
0,5
0
0
1
5
10
MK571 (µmol/L)

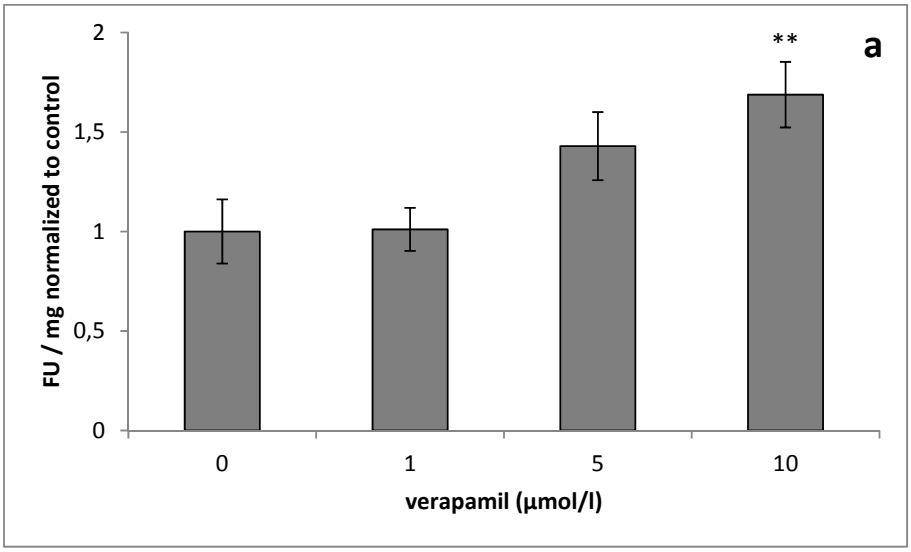
Figure 2.


a
% control
1,5
1,0
0,5
0,0
0
1
5
10
20
verapamil (µmol/L)

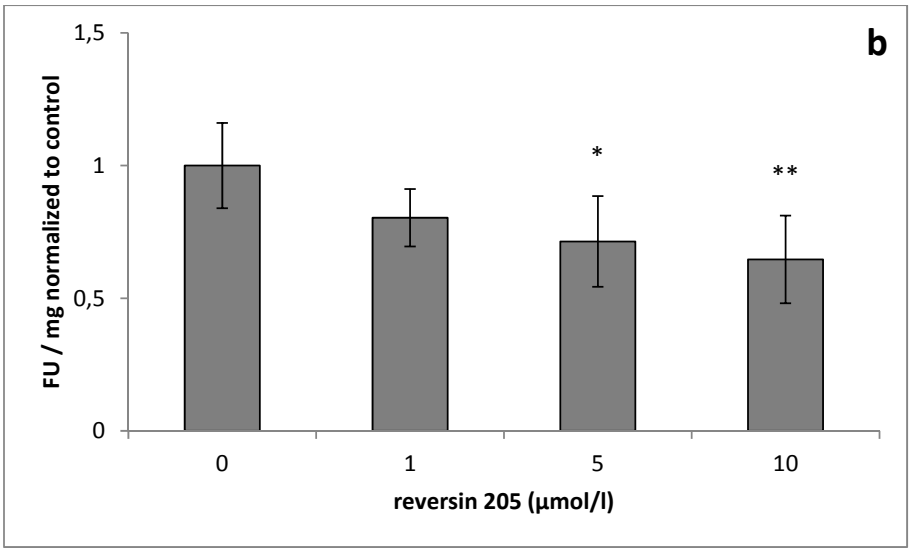

b
FU / mg normalized to control
15
10
5
0
***
***
0
1
5
10
reversin 205 (µmol/L)



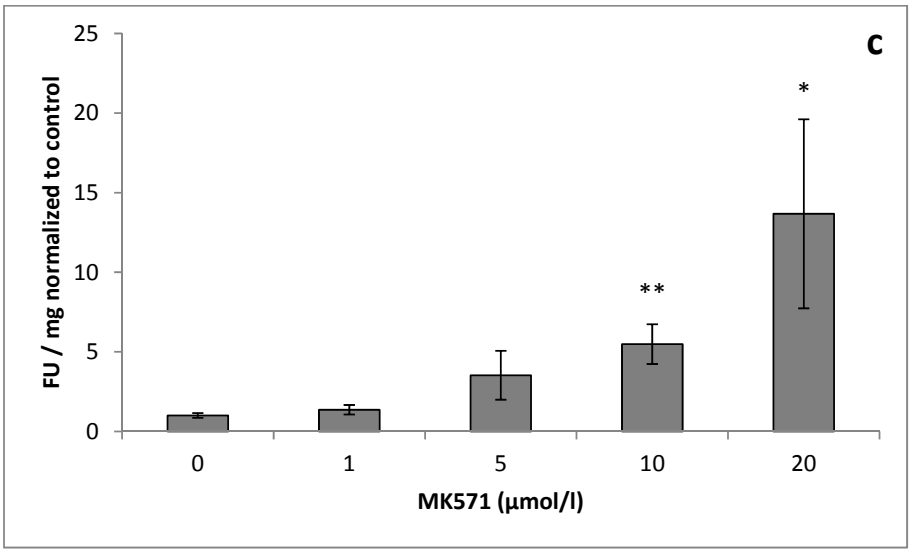
448
449
450 Figure 3.
451



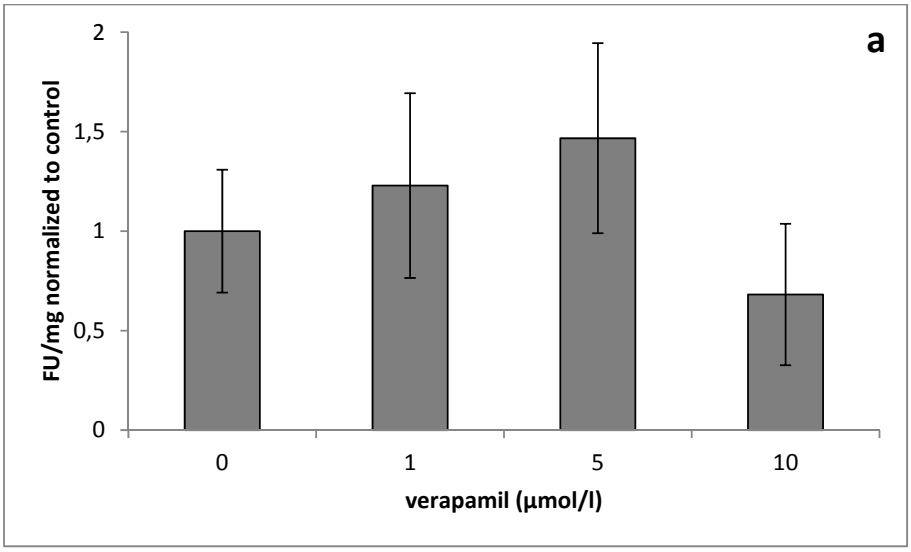
452
453



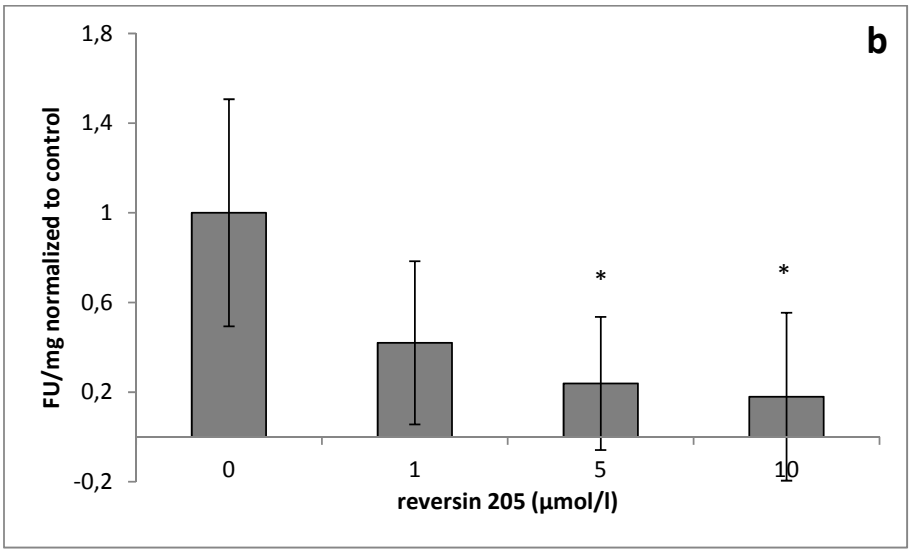
454



455
456
457 Figure 4.
458



459
460


b
1,8
1,4
1
0,6
0,2
-0,2
FU/mg normalized to control
*
*
0
1
5
10
reversin 205 (µmol/l)

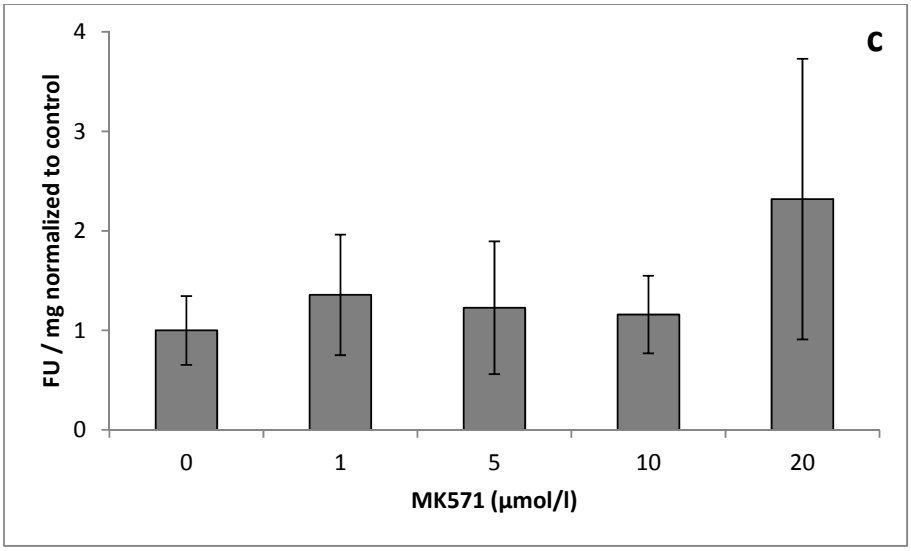

c
4
3
2
1
0
FU / mg normalized to control
0
1
5
10
20
MK571 (µmol/l)

Figure 5.