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1 **Mixtures of aluminum and indium induce more than additive**
2 **phenotypic and toxicogenomic responses in *Daphnia magna***

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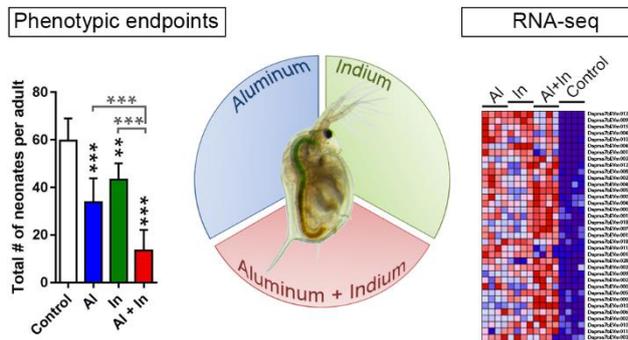
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23



25

26 **Abstract**

27 Aquatic systems are contaminated by many metals but their effects as mixtures on organisms are not well
 28 understood. Here, we assessed effects of aluminum with fairly well-known modes of actions and indium, an
 29 understudied emerging contaminant from electronics, followed by studying equi-effective mixtures thereof.
 30 We report acute and adverse phenotypic effects in *Daphnia magna* adults and global transcriptomic effects
 31 employing RNA sequencing in neonates. The mixture induced more than additive activity in mortality, and in
 32 physiological effects, including growth and reproduction. Similarly, transcriptomic effects were more than
 33 additive, as indicated by a markedly higher number of 463 differentially expressed transcripts in the mixture
 34 and by distinct classes of genes assigned to several biological functions, including metabolic processes,
 35 suggesting depleted energy reserves, which may be responsible for the observed impaired reproduction and
 36 growth. A gene set enrichment analysis (GSEA) of *a priori* known response pathways for aluminum confirmed
 37 activation of distinct molecular pathways by indium. Our study is highlighting more than additive effects at the
 38 transcriptional and physiological level and is providing a state-of-the art approach to mixture analysis, which is
 39 important for risk assessment of these metals and metal mixtures.

40

41

42 Introduction

43 Aquatic organisms are continuously exposed to multiple chemicals from different sources in polluted
44 environments. Among them are metals, including aluminum (Al) mobilized from bedrock in acidified waters
45 and indium (In) originating from the production, use, and disposal of electronic devices.¹⁻³ In risk assessment,
46 the joint activity associated with mixtures of chemicals are still not fully considered.⁴ However, increasing
47 evidence indicates that joint activity of mixtures matters and mixtures can even have more than additive
48 effects.⁵

49 Mixture activity can be described by two concepts. Effects of chemical mixtures with a similar mode of
50 action (MoA) such as binding to the same receptors are assessed using the concept of concentration addition
51 (CA),⁶ in which it is assumed that one compound can be replaced by an equal fraction of an equally effective
52 concentration of another. Thus, each component of the mixture contributes to the combined effects in
53 proportion to its concentration and individual potency. In case of compounds with dissimilar MoAs in a
54 mixture, and thus, interacting with different target sites, the concept of independent action is applied.⁷ Thus,
55 joint toxic effects can be estimated multiplying the probabilities of responses.⁸ Both concepts are applicable to
56 metals, with the CA concept being the most conservative.⁹⁻¹¹ Both concepts rely on compound additivity in a
57 mixture. However, mixtures can also lead to an overall more than additive (synergistic) or lower than additive
58 (antagonistic) effect.⁹ Synergistic interactions were previously found for endocrine active organic compounds
59 *in vitro*^{12,13} and *in vivo*^{14,15} in fish and for metal mixtures in *Daphnia*¹⁶⁻¹⁸ and may be explained by combined
60 activated molecular pathways that converge, and thus, potentiate the response.

61 Here, we study the effects of binary mixtures of Al and In to better understand mixture interactions of trivalent
62 metals and shed light on the barely known aquatic toxicity of the emerging contaminant In. In principal, these
63 two trivalent metals may coexist and interactions may occur, as Al is commonly found in the aquatic
64 environment. Contamination by In is more restricted to contamination sites, such as industrial production of
65 electronics, but also to disposal and recycling sites of electronic waste due to its application in modern
66 electronic equipment including smart phones, flat panel displays, and light emitting diodes (LEDs).¹⁹
67 Particularly high concentrations are measured in lakes influenced by acidic deposition with up to 396.3 $\mu\text{g L}^{-1}$
68 Al^{20} and in river sediments near smelters with up to 75 mg kg^{-1} In^3 . Due to the prevalence of Al in acidified

69 waters but also in manufacturing and as in industrial catalysts,²¹ the toxic potential of Al has been studied. The
70 MoAs include immune system responses,^{22,23} oxidative stress,²⁴ and hypoxia and apoptosis²² among others. In
71 contrast, ecotoxicological effects of In are poorly known.^{25,26}

72 In our study, we applied the CA concept to assess the joint activity of Al and In. Our rationale for
73 applying the CA concept is the fact, that first, there are clear dose-response relationships in the endpoint
74 (mortality) on which our equi-effective concentrations is based upon. This is necessary in contrast to the IA
75 concept. Second, In and Al are trivalent metals and thus might have a similar uptake into cells of *Daphnia*.
76 Third, we hypothesized *a priori* similar MoAs of Al and In. Moreover, when assessing CA responses of binary
77 mixtures at phenotypic and transcriptional levels, no previous knowledge other than an effective
78 concentration for both compounds is needed setting the stage for including emerging compounds in mixture
79 toxicity assessment.

80 The aim of our present study was to assess the mixture activity of binary mixtures of Al and In for
81 additive action using equi-effective mixtures focusing on mortality, growth, and reproduction, and for
82 transcriptome signatures. *Daphnia* reproduce by cyclical parthenogenesis, thereby allowing the molecular
83 responses between compounds to be measured without the confounding effects of genetic variation among
84 strains in their sensitivity and in their regulatory pathways. A causal relationship between metal exposure and
85 adverse effect outcomes on somatic growth, reproduction, and transcriptional responses has been suggested,
86 with some knowledge of the underlying molecular mechanisms.²⁷ The completion of the *D. magna* reference
87 transcriptome now enables global gene regulation profiling by RNA sequencing (RNA-seq).^{28,29} We elucidate
88 and compare *de novo* co-regulatory gene networks of Al, In, and their mixture to explore shared or distinct
89 functional biological processes. By comparing evolutionary-conserved *a priori* known adverse response
90 pathways for Al, we explore the application of this approach to assess combined effects at transcriptional
91 level. Ultimately, we discuss the utility of transcriptional responses for a chemical read-across, and whether
92 transcriptional responses may be linked to the chronic adverse outcomes, such as reproduction and growth.

93

94 **Materials and Methods**

95 **Metals.** Aluminum sulfate ($\text{Al}_2(\text{SO}_4)_3$), 99.99% trace metals basis, catalog no. 202614) and indium (III) chloride
96 (InCl_3 ; anhydrous, 99.999% trace metals basis, catalog no. 429414) were obtained from Sigma Aldrich
97 (Gillingham, UK).

98 **Cultivation of *Daphnia magna* and Experimental Design.** We used a *D. magna* genotype with inherited alleles
99 from parents with different phenotypic and environmental backgrounds, such as the Xinb3 and linb1
100 genotype. Genotyping, breeding, and cultivation are described in the Supporting Information (SI).

101 During acute exposures, *Daphnia* neonates were kept at a density of ten organisms per 200 mL in artificial
102 *Daphnia* medium (ADaM) without feeding. For each treatment group, four biological replicates were set up. At
103 higher concentrations, $\text{Al}_2(\text{SO}_4)_3$ led to lowering of the pH due to the production of H_2SO_4 . To maintain a stable
104 pH during exposure, pH was adjusted using sodium hydroxide before starting the exposures to In and Al and
105 mixtures.

106 Acute mixture experiments were designed according to an equi-effective protocol, where two metals were
107 combined at concentrations producing equal mortality. This allows the comparison of responses of the single
108 metals with those of equi-effective mixtures. Effect concentrations (ECs) were based on concentration-related
109 48 h mortality curves with single metals (4 replicates, $n = 10$).

110 Equi-effective concentrations of both metals were applied in mixtures to determine the activity of $\text{EC}_{0.625}$,
111 $\text{EC}_{1.25}$, $\text{EC}_{2.5}$, EC_5 , EC_{10} , EC_{20} , EC_{40} , EC_{80} mixtures. The effects were analyzed according to the CA concept, and
112 based on the assumption that, for example, the mixture of $\text{EC}_{5(\text{compound A})}$ and $\text{EC}_{5(\text{compound B})}$ would lead to an
113 overall additive effect of 10% in the mixture. To determine the onset of mortality, it was recorded continuously
114 over 48 h. More than 80% of *Daphnia* survived the first 10 h in the $\text{EC}_5 + \text{EC}_5$ mixture and consequently,
115 transcriptional effects of an equi-effective mixture of $\text{EC}_5 + \text{EC}_5$ and the single metal concentration of EC_{10} were
116 assessed at this time-point. Following exposure, surviving *Daphnia* were immediately snap frozen in liquid
117 nitrogen and stored at -80°C until RNA extraction.

118 Chronic exposure experiments were conducted using a 5-, 10-, and 20-fold dilution concentration of the 48 h
119 10% effect concentrations (EC_{10}) of the single metals (6.062, 3.031, 1.516 mg L^{-1} Al, 9.168, 4.584, 2.292 mg L^{-1}
120 In), and $\text{EC}_5 + \text{EC}_5$ of the metal mixture. *Daphnia* were exposed until the release of the third brood or 21 days

121 at maximum. One organism was kept in a volume of 60 mL ADaM in glass beakers under 16:8 h light:dark
122 photoperiod and fed daily. A total of ten replicates per concentration group was set up and refreshed every
123 third day.

124 **Inductively-Coupled Plasma Mass-Spectrometry (ICP-MS) Analyses.** Concentrations of the soluble fraction of
125 aluminum (^{26}Al) and indium (^{115}In) isotopes in exposure media were quantified by inductively-coupled-plasma
126 mass-spectrometry (ICP-MS; Agilent 7500cx, Switzerland) equipped with an Octopole Reaction System,
127 pressurized with an optimized helium flow of 5 mL min^{-1} . Medium samples were filtered through a $0.45 \mu\text{m}$
128 membrane, acidified to 1 % HNO_3 before analysis. Rubidium was used as internal standard.

129 **Molting, Growth, and Reproduction.** Endpoints for chronic exposures were assessed according to established
130 methods and described in SI including their statistical analyses.

131 **Bio-imaging.** The Laser Ablation ICP-MS (LA-ICP-MS) technique was applied for bio-imaging the elemental
132 distribution within *Daphnia* as previously described³⁰ and outlined in the SI.

133 **RNA Extraction, Library Preparation, and Sequencing.** Transcriptome analysis using RNA-seq was performed
134 of neonates exposed for 10 h to EC_{10} for Al or In, respectively, and EC_5 for Al and In in the mixture exposure.
135 RNA of 20 exposed neonates of each of the four replicates was extracted using the RNeasy Mini Kit (Qiagen)
136 following the manufacturer's instructions including RNase-free DNase I treatment. *Daphnia* were homogenized
137 using the 2010 Geno/Grinder (SPEX SamplePrep, UK; 1750 rpm for 10 s). RNA quantity was measured using a
138 Nanodrop 8000 (Thermo Scientific, US), and integrity verified on a 2200 TapeStation system (Agilent
139 Technologies, US). Only samples with an RNA integrity number (RIN) higher than 7 were used for further
140 analysis. Poly(A)+ RNA was enriched using a NEBNext Poly(A) mRNA Magnetic Isolation Module. After reverse
141 transcription, a complementary cDNA library was constructed using the NEBnext Ultra Directional RNA Library
142 Prep Kit (New England Biolabs, U.S.).

143 Briefly, mRNA was further purified by exploiting the poly-A tails using NEBNext Oligo d(T) beads. Then, mRNA
144 was fragmented to suitable lengths, which were subsequently converted into cDNA by reverse transcriptase.
145 The fragmented cDNA was purified using AMPure XP beads, bound by oligonucleotide adaptor, followed by
146 PCR library enrichment. After library production, QC was performed using the TapeStation system with a High-

147 Sensitivity D1000 tape, confirming the size of the library. Quantitation was performed using the Kapa Library
148 Quantitation Kit (Kapa Biosystems Ltd, UK) for Illumina Platforms, and equal molar quantities of each library
149 mixed to produce a pooled library sample, which was tested again by the same procedures. A 2 nM pooled
150 library sample was denatured using NaOH (per Illumina protocols), and loaded onto a Rapid-Run v2 slide using
151 the Illumina cBot instrument at a 12 pM concentration. The cDNA was sequenced in paired-end sequencing-
152 mode with 50 bp read length on an Illumina HiSeq 2500 machine using a v2 Rapid-Run SBS kit.

153 **Bioinformatic analysis of RNA-seq data.** A detailed description of the analysis is given in the SI. Briefly, several
154 quality check steps were performed before resulting high-quality reads were mapped to the *D. magna de novo*
155 transcriptome.²⁹ The transcript counts (number of mapped reads per transcript per sample) were summarized
156 using Bioconductor package tximport (v1.6.0)³¹ then normalized. We used DESeq2³² to conduct differential
157 gene expression analysis. Genes were considered differentially expressed (DE) if the resultant adjusted *p*-value
158 < 0.1 (False discovery rate, FDR = 10%) for the purpose of reducing a Type II error (to falsely infer that there is
159 no overlap of genes across treatments), and for discovering shared co-responsive gene networks in *Daphnia's*
160 response of the three treatments. We annotated DE gene sets based on their responses to each of the three
161 trivalent metal treatments. Class-1 genes were differentially expressed in only 1/3 of the treatments. Class-2
162 genes were differentially expressed in 2/3 of the treatments. Class-3 genes were differentially expressed for Al,
163 In and their mixture (3/3 of the treatments). An *ab initio* search for enriched gene sets among the treatments
164 and shared co-regulated gene networks was also conducted.

165 The responding genes were functionally analysed by a targeted approach using gene set enrichment analysis
166 (GSEA) for an *a priori* gene set containing genes that are members of known pathways associated with Al
167 exposure. Thus, the CA concept was tested by testing for additivity in similar gene sets for both compounds.
168 This gene set was composed of all the known *D. magna* homologs to genes of the following seven pathways³³⁻
169 ³⁶ identified for *D. pulex* using the Panther database³⁷: Oxidative stress response, apoptosis signaling pathway,
170 hypoxia response via HIF activation, p53 pathway, p53 pathway via glucose deprivation, p53 pathway feedback
171 loop 1, p53 pathway feedback loop 2. We tested whether the genes from these pathways were enriching the
172 top-ranked genes for all four conditions (Al *versus* control, In *versus* control, Al + In *versus* control, Al *versus* In).
173 based on expression levels across all conditions. *Daphnia pulex* genes that belong to candidate general stress
174 response pathways were retrieved from PROWLER³⁸ and mapped to their respective *D. magna* orthologues

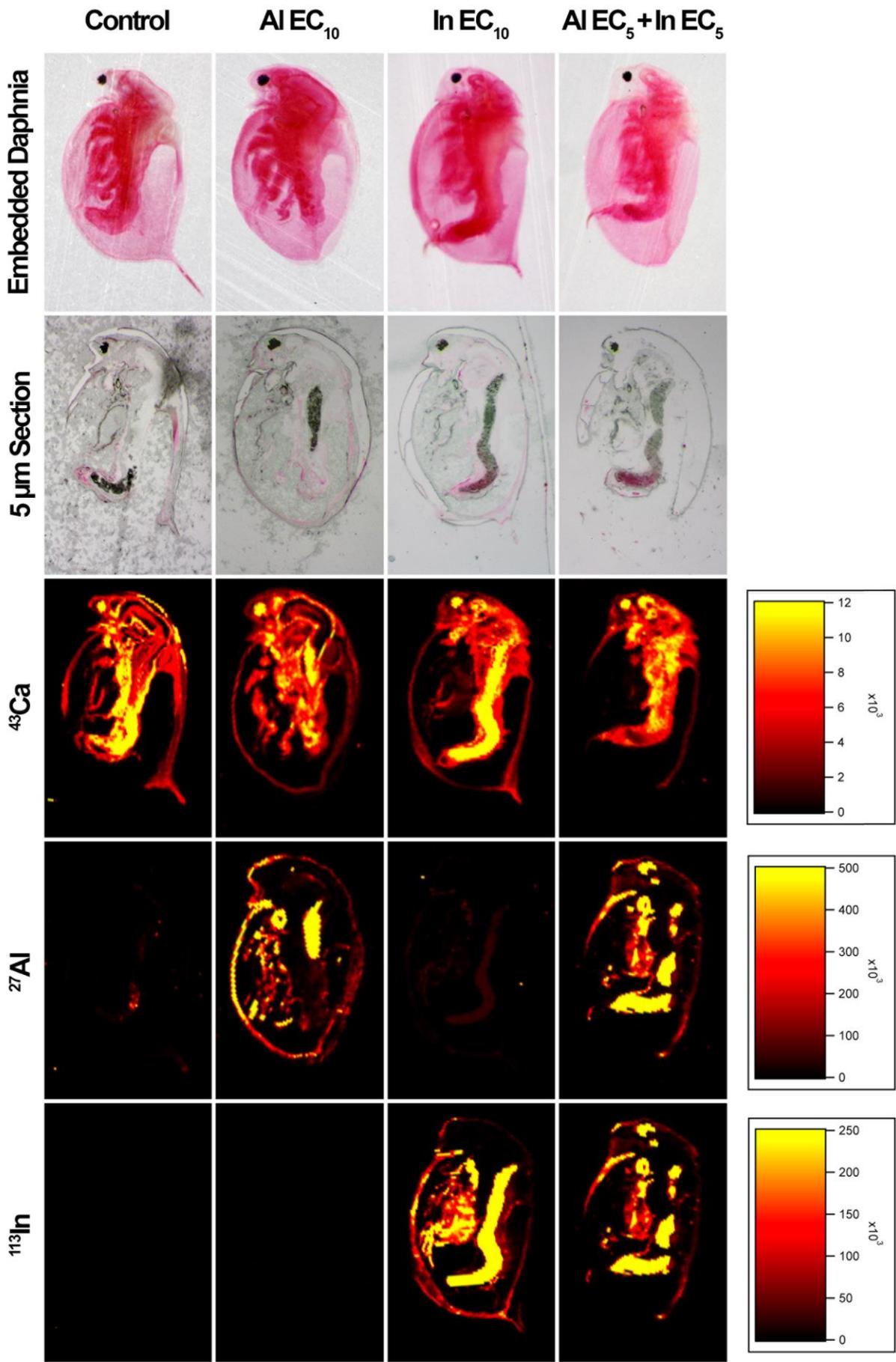
175 using a custom python script and OrthoDB³⁹. Gene differential expression matrices from DESeq2 were pre-
176 ranked based on average LFC in gene expression. GSEA was then conducted using the GSEA tool (v3.0) of the
177 candidate pathway genes against the pre-ranked gene list, using default^{40,41} Reports containing enrichment
178 scores, normalised enrichment scores and FDR for each analysis were generated, highlighting enrichment of
179 genes from candidate pathways within the pre-ranked gene lists.

180

181 **Results**

182 **Exposure concentrations.** The measured soluble fraction of the Al and In concentrations in the transcriptomic
183 experiment was lower than nominal and was 0.635 mg L⁻¹ and 11.535 mg L⁻¹, respectively. During the 10 h
184 exposure, the concentrations fell between 8.7 % (Al) and 11.6 % (In). Details are given in the SI including values
185 (Table S1, SI).

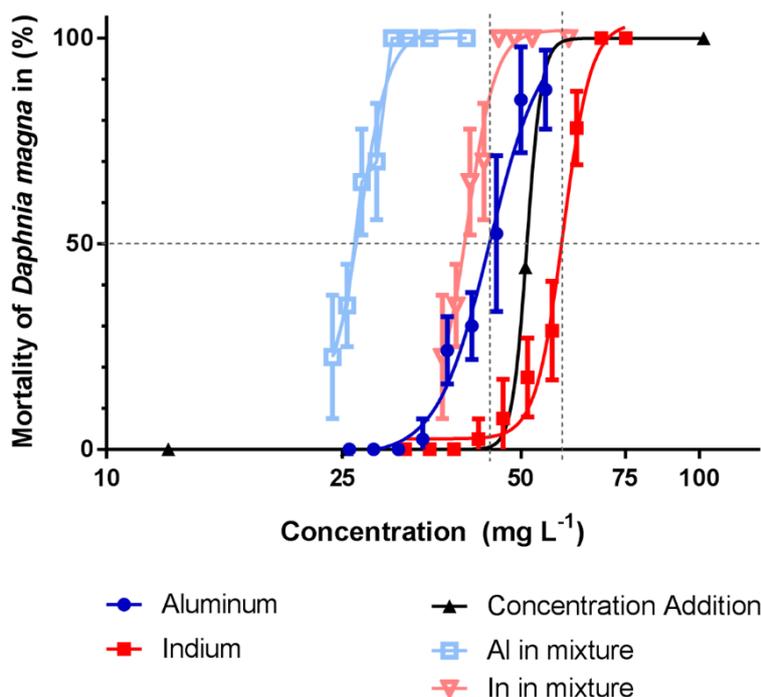
186 **Metal uptake.** The LA-ICP-MS profiles of embedded *Daphnia* showed the distribution of Al and In in the
187 organisms after ten hours (Figure 1). Al as an essential trace element⁴² showed accumulation mainly in the
188 carapace, which is also observed in the mixture, but also in the hind gut and midgut, as well as in the thoracic
189 limbs, carrying the filtering screens. In controls, only minor levels of Al and In occurred. In showed a similar, if
190 not almost identical accumulation as Al in the carapace, thoracic limbs, and gut. The metal pattern is distinctly
191 different from calcium (Figure 1) and phosphorus (Figure S1, SI) delineating the carapace and gut, but also
192 eyes, and blind gut (caecum) in case of calcium. ¹¹³In to ¹¹⁵In intensity is displayed according to its isotope ratio
193 of 22 with both isotopes revealing similar images and intensity, therefore interferences can be ruled out
194 (Figure S1, SI).



196

197 **Figure 1.** Laser-ablation ICP-MS elemental mapping of calcium (Ca), aluminum (Al), and indium (In) in 6 days
198 old *Daphnia magna* after a ten-hour exposure to measured concentrations of 0.635 mg L⁻¹ Al, 11.535 mg L⁻¹ In
199 (EC₁₀ for neonates) and the equi-effective mixture of Al and In (0.596 and 9.096 mg L⁻¹), First row: paraffin
200 embedded *Daphnia magna*; second row: 5 µm section; third to the fifth row: LA-ICP-MS images of elements
201 indicated on the left. Colour bars show the intensity in counts per seconds. The variation in the profile of the
202 animals is a result of the dehydration, embedding, and slicing procedure and thus images cannot be used as
203 species identification.

204 **Effects on Survival - Acute Exposures.** The mortality dose-response curve produced a steep hill slope (Al:
205 9.838, In: 18.62) for both metals after 24 h with nominal EC₅₀ values of 44.27 mg L⁻¹ (Al) and 58.93 mg L⁻¹ (In;
206 Figure 2). The effects of equi-effective mixtures were greater than additive, as the curves were shifted left to
207 the predicted CA curve. Even a low concentration of EC₅ + EC₅ lead to about 70% mortality (Figure S2, SI). After
208 48 h, the EC₅₀ value for Al and In was 35.91 mg L⁻¹ and 54.49 mg L⁻¹, respectively, with a hill slope of 12.73 for
209 Al and 12.62 for In. At both exposure times, the EC₅ and EC₁₀ values were relatively close to each other due to
210 the steep hill slope. Dose-response curves at 48 h were then taken as a basis to define nominal equi-effective
211 concentrations to be used for mixture experiments. Due to high mortality at 48 h, no comparison of single
212 metals and mixture exposures was possible. Mortality started to increase after ten hours for both metals at
213 lower concentrations, and even earlier (6 h) at higher concentrations (Figure S2, SI). The mixture of EC₅ + EC₅
214 induced an average mortality rate of 10% after ten hours. Consequently, for subsequent transcription analysis,
215 a ten-hour exposure and a combination of EC₅ + EC₅ and EC₁₀ were chosen. All nominal EC values are listed in
216 Table S2 in the SI.



217

218 **Figure 2.** Mortality of *Daphnia magna* neonates exposed for 24 h to aluminum ($\text{Al}_2(\text{SO}_4)_3$) or indium (InCl_3)
 219 illustrating synergistic activity in the mixture. Hill slope for aluminum and indium is 9.838 and 18.62,
 220 respectively. For the subsequent transcriptomic experiments, a mixture exposure of EC5 + EC5 for 10 h was
 221 chosen allowing for survival of on average 90% of *Daphnia magna* (for time course study from 0 to 48 h see
 222 Figure S2, SI). Error bars represent the standard deviation (SD) of each exposure group (four replicates, n = 10).

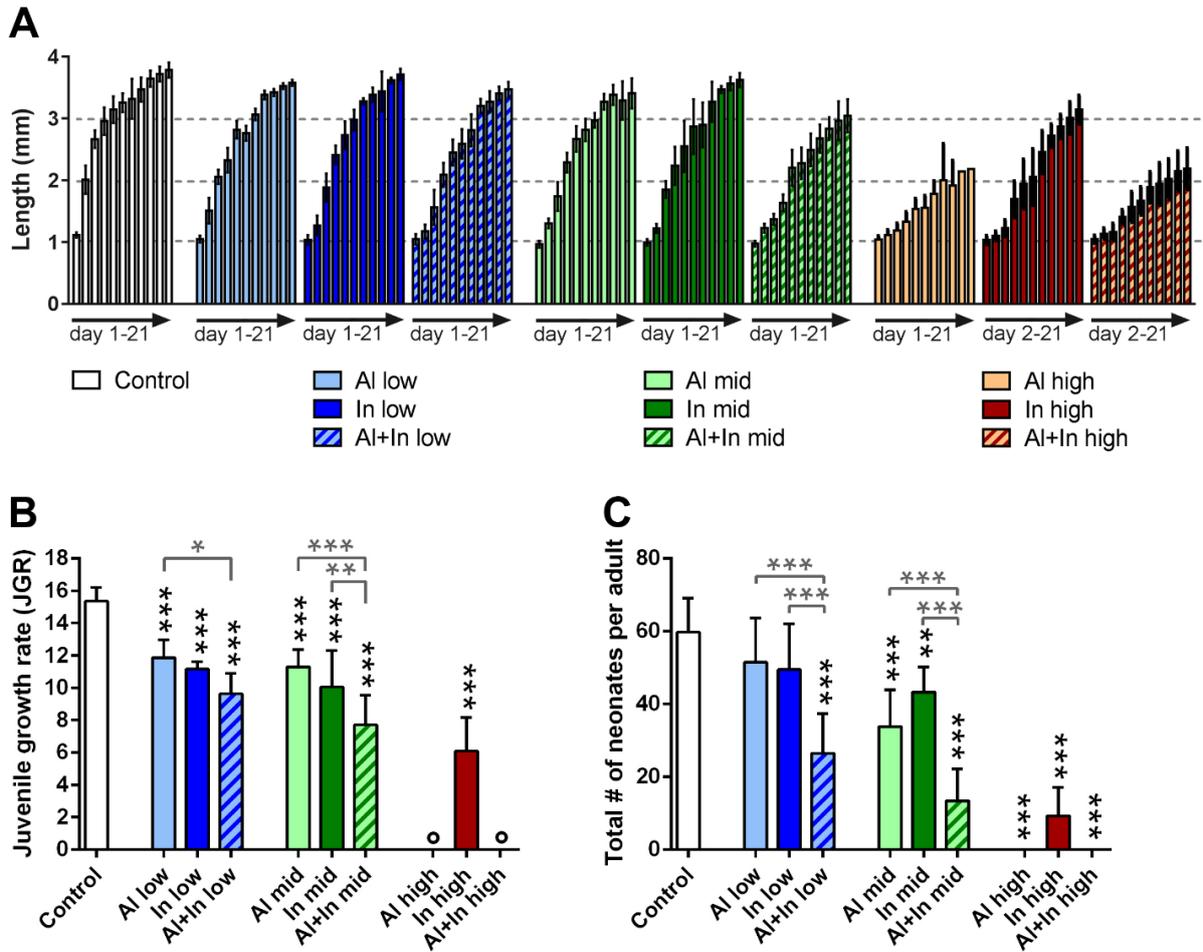
223 **Effects on molting, growth, and reproduction – Chronic exposures.** Concentrations used in chronic exposures
 224 were for Al 1.52 (low), 3.03 (mid), 6.06 (high) mg L^{-1} , for In 2.29 (low), 4.58 (mid), 9.17 mg L^{-1} (high), and for Al
 225 + In 1.43 + 2.17 (low), 2.85 + 4.33 (mid), 5.71 + 8.66 mg L^{-1} (high). Molting was recorded daily and plotted
 226 cumulative against time (Figure S3, SI). High Al concentration significantly decreased molting that did not
 227 appear in the binary mixture.

228 In the low concentration, both single metals and mixture led to a similar growth reduction in the first ten days.
 229 A strong reduction occurred in the middle concentration of the mixture, while in the highest concentration,
 230 growth was already minimal in the Al exposure after two days. Over all time points, exposed *Daphnia* showed
 231 a concentration-dependent decrease in growth (Figure 3A).

232 Age at maturity (Figure S4A, SI) was significantly delayed in all exposure groups (except in AI low) suggesting an
233 effect on early life stages. This is even more evident when comparing juvenile growth rate (considering growth
234 until sexual maturity; Figure 3B) with specific growth rate (considering growth until day 21; Figure S5A, SI). The
235 juvenile growth rate was reduced in all concentrations and treatments, whereas the specific growth rate was
236 reduced in the highest concentration only. The population growth rate was significantly reduced in all mid and
237 high concentrations (Figure S5B, SI).

238 Mid and high concentrations of AI and In and all mixture concentrations decreased the total number of
239 produced juveniles (Figure 3C). Effects on fecundity were higher in the mixtures than in single metal exposures
240 and in high concentrations, any neonates were produced. Brood sizes increased from the first to the third
241 brood in controls and single metals exposures (low and mid concentrations), whereas the high In and mid
242 mixture concentration resulted in a decrease in the third brood size (Figure S4B, SI).

243 AI and In exhibited similar phenotypic responses in all the endpoints, except in population growth rate and age
244 at maturity, where In induced a stronger effect than AI at low concentration, while AI had a stronger effect
245 than In at high concentrations in molting and growth reduction.



246

247

Figure 3. Length, growth rate, and fecundity of *Daphnia magna* from day 1 (after 24 h of exposure) to 21

248

exposed to three concentrations of aluminum (Al), indium (In), and their equi-effective mixture concentration

249

(Al+In). (A) Carapace length over 21 days. Bars for day 1, 3, 5, 7, to 21 are shown. (B) Juvenile somatic growth

250

rates from day 2 to maturity. Circles indicate that, due to toxicity, none of the individuals in the group reached

251

maturity within 21 days, and therefore, were not included in statistical analysis. (C) Fecundity of *Daphnia* in

252

controls and exposure groups. The total number of neonates includes 3 brood releases or 21 experimental

253

days maximal. Mean \pm SD. Black asterisks indicate significantly different to control, gray asterisks indicate the

254

difference between single compound and equi-effective mixture (n=10). Nominal concentrations are for Al

255

1.52 (low), 3.03 (mid), 6.06 (high) mg L^{-1} , for In 2.29 (low), 4.58 (mid), 9.17 mg L^{-1} (high), and for Al + In 1.43 +

256

2.17 (low), 2.85 + 4.33 (mid), 5.71 + 8.66 mg L^{-1} (high).

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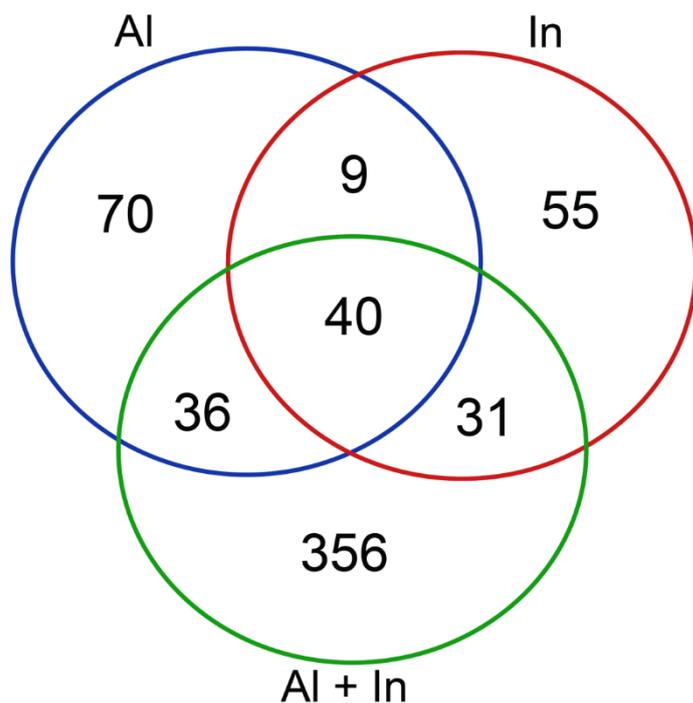
258 **Transcriptomic profiles of *D. magna* at similar effective concentrations.** The genotype of the IXF1 *D. magna*
259 clone used in our study is confirmed by PCA analysis of the genetic variation (Figure S6, SI). Normalized counts
260 for each gene of every replicate and the statistically significant differentially expressed (DE) genes across the
261 three treatments compared against the control are given in Table S3, SI, the volcano plot in Figure S7, SI, and
262 the heat map in Figure S8, SI. An adjusted p-value of 0.1 was applied, to broaden the gene list at reducing a
263 Type II error, more stringent adjusted p-value of 0.05 are also given in Table S3, SI. The functional annotation
264 of this gene set, including gene family from OrthoDB, InterPro, and derived Gene Ontology, as well as more
265 comprehensive annotation using the 'Panther database' is provided in Table S3, SI. The same table also lists
266 the inferred orthologs of all of the *Daphnia magna* loci in *D. pulex*, *Drosophila*, *Danio rerio*, and *Mus musculus*,
267 including the *Daphnia* orthologs to AI responding genes from the previously published zebrafish study⁴³.

268 From among all the 597 DE genes in at least one treatment, the two most prominent molecular
269 functions are antioxidant activity (GO:0016209) followed by catalytic activity (GO:0003824; Figure S9, SI).
270 Although only nine genes are DE for both metals at the exclusion of their mixture (Figure 4), shared molecular
271 functions are found for the class-1 gene sets that are DE for either the AI or In treatments, including binding,
272 glutamate receptor activity, ligand-gated ion channel activity, signal transducer activity and transferase
273 activity, transferring glycosyl groups (Table S3, Table S4, SI). Our experiments revealed only 40 class-3 genes
274 that are DE across all three treatments (Figure 4, Table S3, SI). Inferred orthologs for other model species can
275 be found in Table S3 (SI).

276 Exposure to AI and In resulted in differential expression of 155 and 135 genes, respectively. Among
277 the fraction of uniquely expressed genes in each of the two conditions (45% and 41%), AI down-regulates the
278 majority of responding genes (28 up *versus* 42 down), while In upregulates the majority of respective
279 responding genes (37 up *versus* 18 down). Among the 463 genes that are DE when *Daphnia* were exposed to
280 the mixture, a substantially larger fraction of genes are uniquely altered (77%), which suggests distinct gene
281 responses compared to those induced by each component of the mixture. Clustering of the treatment groups
282 highlights two characteristics of their DE genes (Figure S10, SI): (i) the AI and In replicated treatments cluster
283 together; (ii) the mixture treatments cluster independently of the AI and In treatments. Therefore, the
284 transcriptomes of AI and In are more alike than the transcriptome of the mixture treatment. Functional
285 analysis of transcripts according to biological process, revealed processes that are unique to the mixture such

286 as calcium-mediated signalling, carbohydrate transport, cholesterol metabolic process, DNA recombination,
287 DNA repair, endocytosis, ectoderm development, glycogen metabolic process, phospholipid metabolic
288 process, protein lipidation, protein phosphorylation, response to abiotic stimulus, segment specification, and
289 translation (for full list see Table S4, S1).

290



291

292 **Figure 4.** Venn diagram of all differentially expressed (DE) genes (p -adjusted < 0.1 , $n=4$) determined by
293 transcriptome sequencing of *Daphnia magna* exposed to measured concentrations of 0.635 mg L^{-1} aluminum
294 (Al), 11.535 mg L^{-1} indium (In), and an equi-effective mixture of Al and In (0.596 and 9.096 mg L^{-1}), respectively,
295 compared to non-exposed controls.

296

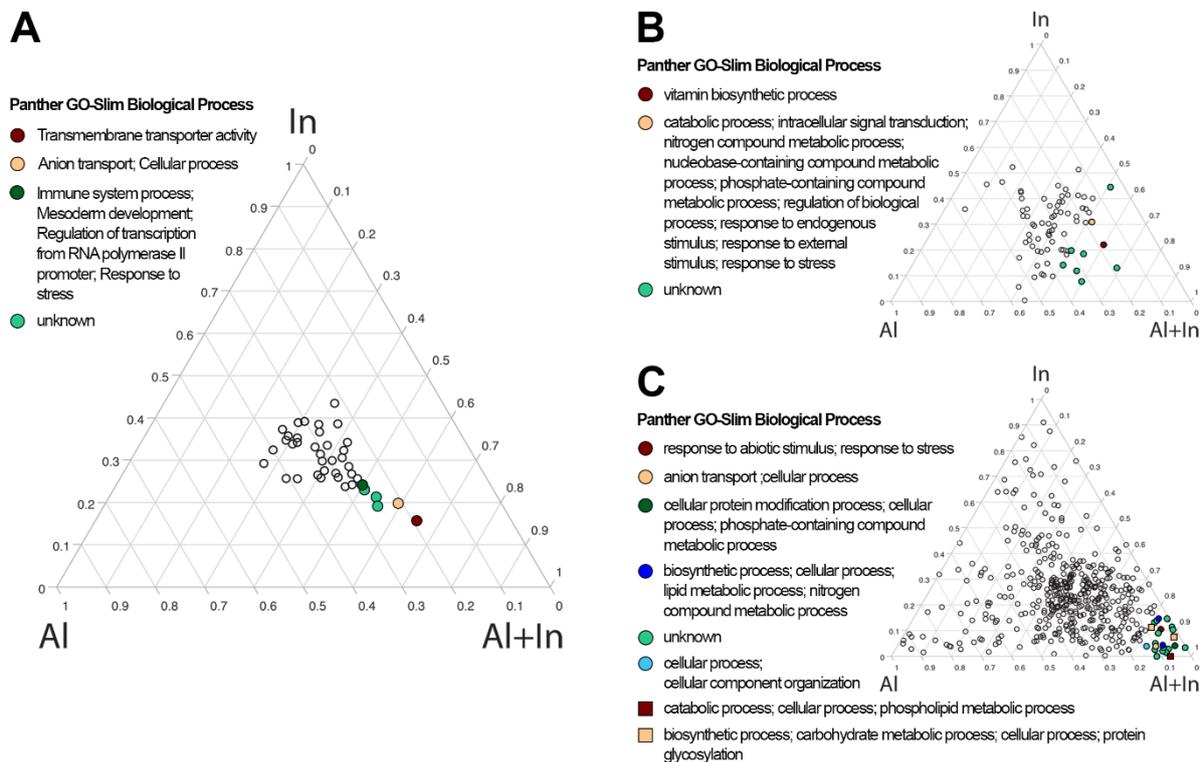
297 Only 49 DE genes are shared between Al and In from among 241 DE genes across both conditions and
298 40 of them are also DE in the mixture, which has an even larger number of responding genes at any p -adjusted
299 threshold. Although the transcriptomes of Al and In exposure are more alike than that of the mixture, there

300 are few detectable functional classes of genes (biosynthetic process, catabolic process, cellular process,
301 nitrogen compound metabolic process) that are shared among all the treatment groups (Table S3, SI).

302 **Synergistic Equi-Effective Mixture Effects.** To explore potential synergistic effects, all class-3, class-2 and class-
303 1 DE genes were plotted comparing mixture *versus* single metal treatments (Figure 5). The ternary plot of
304 class-3 DE genes (Figure 5A) revealed six genes out of 40 (15%) with expression levels that are amplified by a
305 synergistic effect of Al and In as a mixture. These include a RH-associated glycoprotein (Dapma7bEVm004715)
306 that also functions as a transmembrane (cation) transporter, a sodium-independent sulphate anion
307 transmembrane transporter (Dapma7bEVm029187) with the annotated biological process of anion transport,
308 and a basic leucine zipper transcription factor (Dapma7bEVm005752) associated with many biological
309 processes including immune system processes. The remaining three genes have no known orthologs in other
310 model species (Table S3, SI).

311 The ternary plot of class-2 DE genes (Figure 5B) revealed 9 genes out of 76 (12%) with amplified
312 expression levels by interactions between the two metals. These include a gammy-butyrobetaine dioxygenase
313 (Dapma7bEVm002353), an extracellular matrix protein (Dapma7bEVm000277), a C1qdc1 protein
314 (Dapma7bEVm010318), and a small GTPase (Dapma7bEVm011784). For completeness, we identify 26 genes in
315 *Daphnia* that are most responsive to the mixture treatment (Figure 5C). Of these 41 genes, none are known
316 orthologs to Al or In responsive genes in other species. When the expected expression level of DE genes (by
317 the additive model) is regressed against the observed expression level under the equi-effective mixture
318 condition (Figure S11, SI), there is a statistically significant signal that the global effect of the mixture on gene
319 transcription is twice the predicted value under the additive CA model, irrespective of class-3 (r^2 value = 0.85)
320 or class-2 (r^2 value = 0.89) DE genes (Figure S11, SI). Thus, the annotated DE genes deviate from additivity.

321



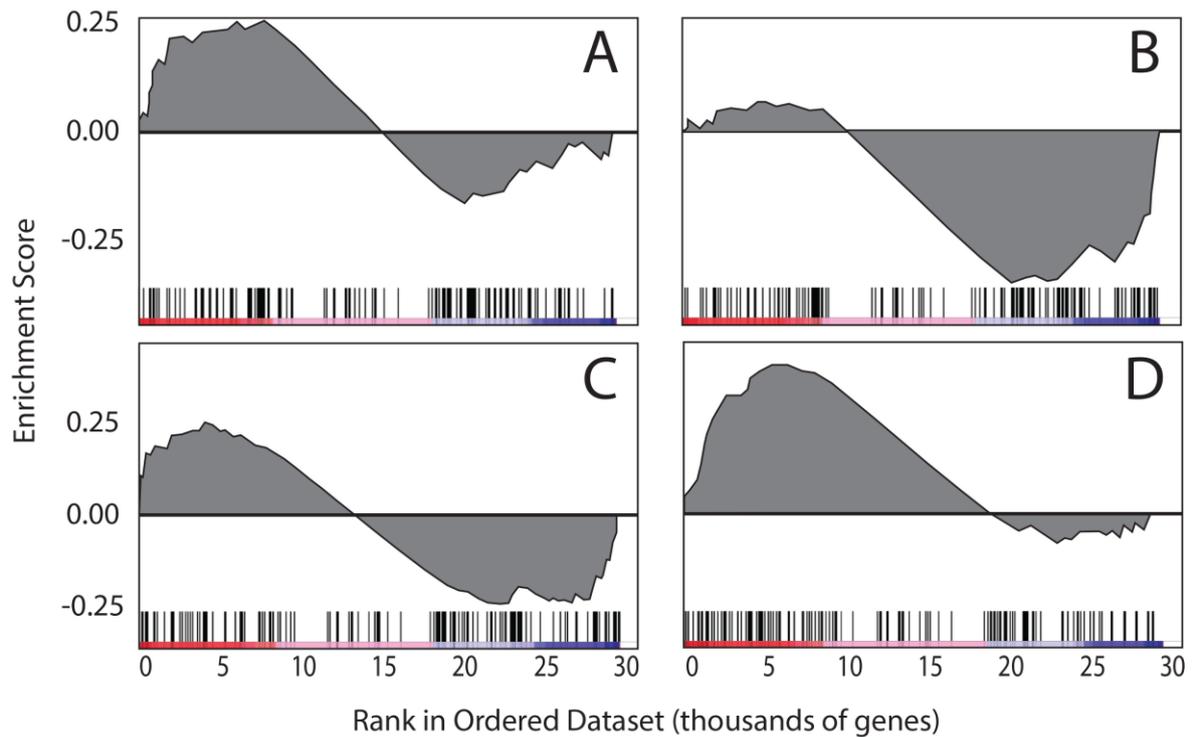
322

323 **Figure 5.** Comparison of response of all DE genes in the mixture exposure *versus* the two single metal
 324 treatments. Ternary plots depict differentially expressed (DE) genes for (A) all three conditions (class-3 DE
 325 genes), (B) two out of three conditions (class-2 DE genes), and (C) class-1 DE genes (n=4). The genes edging
 326 towards 'Al + In' correspond to the genes that have the largest residues and whose expression levels are most
 327 amplified in the mixture, whereas genes in the centre respond additively. The majority of genes respond
 328 independently in the mixture condition, as seen in (C). The functions of the genes are derived from Table S3
 329 (SI) giving the residuals for each of the DE genes measured against the orthogonal regression lines in Figure
 330 S11 in the SI, which takes in account error estimates from both the expected and observed values.

331

332 **Comparing Known Responsive Genes.** The gene set enrichment analysis (GSEA) of stress pathways associated
 333 with Al exposures revealed that these *a priori* selected stress response genes from different organisms are also
 334 significantly *up-regulated* in *Daphnia's* response to Al exposure (Figure 6A). By contrast, these stress response
 335 genes from within the same gene set significantly enrich the *down-regulated* genes in response to In exposure
 336 (Figure 6B), while in the mixture (Figure 6C), these genes enrich *both the up-regulated and down-regulated*
 337 *genes*. A direct comparison of Al *versus* In (Figure 6D) reinforces this finding of a near opposite response

338 between the two metals, by almost doubling the enrichment score for overexpressed Al genes, from 0.25 to
 339 >0.4. The mixture shows the same enrichment score as seen in the Al exposure (Figure S12, SI), indicating that
 340 a separate set of known gene responders are differentially expressed by exposure to Al and In present in the
 341 mixture, via either characteristic up-regulation or down-regulation associated with Al and In exposure
 342 respectively.



343

344

345 **Figure 6: Gene set enrichment analysis (GSEA) of stress response pathways.** GSEA on differentially expressed
 346 (DE) genes to (A) aluminum, (B) indium, (C) mixture, calculated through comparison *versus* control, or (D)
 347 between aluminum *versus* indium. *Daphnia magna* genes were aligned to their respective *D. pulex* orthologues
 348 and ordered based on their fold difference in expression. These genes were then analysed against *D. pulex*
 349 genes of candidate pathways retrieved from PANTHER. Candidate genes belonged to ontologies oxidative
 350 stress response, apoptosis signalling pathway, hypoxia response via HIF activation, p53 pathway, p53 pathway
 351 via glucose deprivation, p53 pathway feedback loop and p53 pathway feedback loop 2. Black bars form part of
 352 leading-edge analysis, highlighting genes that contribute most to enrichment scores. The coloured bars
 353 indicate the fold change in expression between compared datasets (red; increased expression, blue; decreased

354 expression). Enrichment score is an indication of how frequently candidate genes are found together (i.e. have
355 similar expressional changes) in the rank-ordered dataset. Normalised enrichment scores (NES) and associated
356 false discovery rate (FDR) are shown for each analysis with the exception of (C).

357 **Comparing Unknown Responsive Genes.** Untargeted differential expression analyses were employed in order
358 to characterise putatively conserved unknown DE genes. Qualitative comparison of DE genes with the highest
359 differences in treatment conditions (Figure S12, SI) highlights an overall disparity between each condition.
360 Responses on individual DE genes vary substantially for all but a few genes, with no obvious similarity.
361 Therefore, genes with the greatest differences in expression between the control and treatments show very
362 little commonality in expression on individual gene level.

363 To test for potential conservation in DE genes, a network-based approach was employed, which
364 groups similarly expressing genes in co-expression modules that can then be compared across metal
365 treatments to identify unknown responders associated with novel expressional interactions. Networks
366 pertaining to each metal treatment were compared using PCA (Figure S13, SI) based on the gene content of
367 each module. Comparing three principle components highlights four distinct clusters of co-expressional
368 modules across treatments; Module 1 (Al 30, In 14, Alln 13), Module 2 (Al 14, In 3, Alln 9), Module 3 (Al 4, In
369 32, Alln 2), Module 4 (Al 23, In 24, Alln 27). A majority of co-expression modules do not cluster independently,
370 therefore the significance of these clusters is in the potential novel and conserved functionality served by the
371 composite genes responding in similar manners across the treatments. Taken together, comparison of DE
372 genes across treatments highlights low conservation in highest responding DE genes and at a transcriptional
373 network level, with shared responses at a network level associating novel gene interactions with Al, In and
374 mixture exposures.

375 **DISCUSSION**

376 In our study, we observe synergistic effects when combining two metals. By performing the first global
377 transcriptome analysis in the ecologically relevant *Daphnia* on In and its mixture with Al we substantiate the
378 notion that different target MoAs can potentiate the response. We provide a straightforward transcriptional

379 analysis of mixtures, and observe that the transcriptional data are paralleled with synergistic effects on growth
380 and reproduction.

381 More than additive interaction was found in adult *Daphnia* on mortality and chronic toxicity, including
382 reduced growth and fecundity. In neonates, the global transcription analysis revealed that the number of
383 differentially expressed genes in the mixture clearly exceeded the number of each single metal treatment,
384 which is in accordance with microarray studies describing transcriptional activity of mixtures.^{18,44} Here, we
385 took a novel approach using RNA-seq and compared the DE genes of the single metal exposures with their
386 mixture, revealing distinct classes of genes that deviate from the expected additive effect of the two similarly
387 acting compounds. Synergistically acting *de novo* co-regulatory genes were identified and assigned to
388 biological processes such as immune system process, transmembrane transporter activity, and several
389 metabolic processes, including lipid and carbohydrate metabolism and catabolic processes (Figure 5, Table S3,
390 SI).

391 The disruption in metabolic processes likely mirror the organisms elevated need for energy coping
392 with the stress situation. Disruption of energy homeostasis causing depleted lipid reserves is directly related to
393 reduced growth in *Daphnia*,⁴⁵⁻⁴⁷ which is a common adverse effect of metals.⁴⁸ In our study, growth reduction
394 occurred already after two days resulting in a reduced juvenile growth rate and delayed age at maturity, both
395 stronger affected in the equi-effective mixture. Consequently, the depleted energy reserves may impair
396 reproduction. Especially reduced clutch size in the third brood is indicative for exhausted energy stores.
397 However, the link of gene expression to organismal level responses is currently limited.⁴⁹

398 In addition, depleted energy sources may also originate from reduced food intake.⁵⁰ Bioimaging
399 showed that both metals were accumulated on the carapace and in the filtering screens, possibly physically
400 hindering a constant flow of algae and thus reduce net energy and growth. Reduced ingestion rate in *Daphnia*
401 after cadmium exposure was suggested to be a key mechanism impairing energy uptake and thus reproduction
402 and growth.⁵¹ Despite potentially reduced ingestion rate, the highest concentrations of Al and In were
403 detected in the midgut, which is in line with other studies on metal uptake in *D. magna*.⁵² Al crossing cell
404 membranes by endocytosis are found accumulated in lysosomes.^{52,53} The lysosome cellular component (Table
405 S3, SI) was enriched in all the treatment groups and endocytosis in the mixture, suggesting their involvement

406 in Al and In accumulation and effects. Furthermore, Al may be transported by yet unknown transporters in
407 *Daphnia*, similar to animal transporters, or transporters of the ABC or MATE family.^{54,55}

408 In our study, we demonstrate the utility of genome-wide transcriptional responses to assess
409 additivity. We propose the use of GSEA to test for enrichment of *a priori* defined set of genes across
410 treatments, whereby compounds that differ in their MoA also differ in their profiles. This requirement differs
411 from the more stringent use of informative molecular profiles as a basis for reporting chemical's MoA, where
412 additional evidence is needed to establish causation between chemicals and their adverse effects. The GSEA
413 result suggests that the gene set known to be involved in stress response pathways to Al respond as predicted
414 in *Daphnia*, yet the same gene set is expressing an opposite effect when *Daphnia* are exposed to In. The
415 response to In, as well as to the mixture, indicates that expressions of these same genes are affected in
416 disparate and putatively opposite manners between the two metals. The deviation from additivity in the
417 transcriptional alterations in mixtures is likely a complex gene regulation and evidence from toxicogenomic
418 studies pile up, showing that mixture expression profiles represent not merely the additive sum of individual
419 compounds fingerprint and converging pathways can be activated leading to a faster or stronger response.^{18,56-}
420⁵⁸ Therefore, this straightforward analysis elucidating gene sets deviating from additivity can be a powerful
421 tool in the mixture assessment.

422 Chemical safety legislations are often relying on chemical structure-base 'read-across' and
423 'quantitative structure-activity relationship' (QSAR) predictions at the expense of reporting actual toxicity data.
424 In the read-across approach, toxicity information for one well-studied chemical is used to predict the same
425 toxicological endpoint for another, i.e. emerging, chemical by virtue of their structural similarity or on the basis
426 of shared molecular responses.⁵⁹ However, the level of certainty of these read-across and QSAR predictions is
427 unsatisfactorily low thereby undermining the purpose of chemical safety legislation. Our approach provides a
428 simple test for similarity in transcriptional fingerprints and thereby support chemical safety assessment.

429 Together, our study leads to the conclusion that Al and In mixtures have more than additive
430 phenotypic effects in equi-effective concentrations, which is paralleled by expressional alterations of
431 substantially more genes, and more importantly, by synergistically responding DE genes. Reduced growth and
432 reproduction may be related to altered genes in energy metabolism processes which has important ecological

433 implications. We show that genome-wide transcriptional fingerprints provide a tool for reasonably rapid
434 assessment for additivity of two compounds.

435 **ASSOCIATED CONTENT**

436 Supporting Information includes the description of cultivation of *Daphnia magna*, experimental design, chronic
437 toxicity data, statistical analysis of phenotypic responses, bioimaging using laser-ablation ICP-MS, as well as
438 results of chemical analysis, effective concentrations of single compounds, and biological processes assigned to
439 Al and In treatment as well as figures on laser-ablation ICP-MS elemental mapping of ¹¹⁵In and ³¹P, time-course
440 study of single compounds, number of molts, clutch sizes and age at maturity, specific growth rate and
441 population growth rate, PCA analysis of genetic variation, volcano plots, heat map of all transcripts, functional
442 annotations of DE genes, MDS, regression analysis, top 100 ranked genes, and PCA of co-expressional modules.
443 The XLSX table S3 contains information on normalized mapping, DESeq results, annotations, orthologs, and
444 residuals. All Supporting Information is available free of charge on the ACS Publications website at
445 <http://pubs.acs.org>. The sequence data are available at the NCBI BioProject database under accession number
446 PRJNA508640.

447
448

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