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Detecting copper toxicity in sediments: from the sub-individual level to the population level

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Running title: Comparing sub-individual, individual and population responses

Summary

1. Sediments accumulate chemicals that can be toxic to biota and often contribute to aquatic ecosystem decline. Measuring mortality in laboratory-bred organisms is a common way to assess sediment toxicity. However, mortality-based responses of resilient laboratory organisms may not reflect indigenous macroinvertebrate responses, which can be relatively more sensitive to sediment toxicants. A possible solution is to also measure responses at the sub-individual level.

2. Several organism responses to sediment copper toxicity were assessed in a field-based microcosm. Responses of laboratory-bred chironomids and snails deployed in microcosms were compared at sub-individual (metabolomic and gene expression), individual (survival and dry weight) and population (reproduction) levels, and contrasted to the abundance of colonizing macroinvertebrates in the microcosms.

3. Colonizing macroinvertebrate abundance showed a range of sensitivities based on EC50 (effect dose 50% change). Chironomidae made up 94.5% of the microcosm macroinvertebrates, with *Paratanytarsus* the most sensitive genus (EC50: 89 mg/kg copper) and *Procladius* the least sensitive (EC50: 681 mg/kg).

4. Survival of laboratory-bred organisms was the least sensitive response, comparable to decreased abundance of the least sensitive macroinvertebrate. Juvenile production in the snail, *Potamopyrgus antipodarum,* was the most sensitive population level response (EC50: 121 mg/kg), in contrast the snail *Physella acuta* was relatively more tolerant (EC50: 298 mg/kg).

5. Changes in sub-individual responses (gene expression and metabolite abundance) in laboratory-bred chironomid, *Chironomus tepperi,* were evident at 60 mg/kg. These changes likely reflect the direct effects of copper exposure and represent metal-specific responses.

6. *Synthesis and applications.* We showed that copper toxicity in sediments could be readily detected through changes in gene expression and metabolites in laboratory-bred chironomids exposed in field-based microcosms. These responses were more sensitive than mortality, and detected copper levels that caused microcosm chironomid populations to decline. These novel approaches will provide managers with new tools to better assess sediment toxicity.

Key-words: biomarkers, chironomids, copper toxicity, gene expression, metabolomics, microcosm, mesocosm, sediment toxicity, transsulfuration, cysteine metabolism

Introduction

Sediment pollution can adversely affect aquatic ecosystems because many aquatic organisms rely on sediment for food and protection (Burton 1991). Hydrophobic contaminants accumulate in sediment, and contaminants often occur at higher concentrations than in the surrounding surface waters. Natural systems are complex and it is common practice to isolate different factors (such as sediment contamination) to investigate the contribution of toxicants to ecosystem decline. While the relative toxicity of contaminants may not always reflect toxicity in complex natural systems (Wang, Goulet & Chapman 2004; Holmstrup *et al.* 2010), considering toxicants in isolation helps to establish causality and allows managers to prioritize remediation (Marshall *et al.* 2010).

Sediment toxicity is most often measured at the individual level (dry weight, survival), and sometimes at the sub-individual (through gene, metabolite or proteins) or population levels (reproduction, first generation responses) (Burton 1991; Forbes & Forbes 1994; Chapman 1995). These responses are often used as indicators of broader scale impacts (Picado *et al.* 2007; O'Brien & Keough 2014). For management, it is important to establish if these responses reflect changes at other levels of biological organization and relate to higher-order biological effects in populations or communities (Kefford *et al.* 2004; Forbes, Palmqvist & Bach 2006; O'Brien & Keough 2014). Individual and sub-individual responses provide important causal links to exposure but links with higher-order changes is required to establish environmental relevance (Beketov & Liess 2012; Rasmussen *et al.* 2013).

Microcosm and mesocosm approaches can be used to isolate the effects of specific factors, such as sediment contamination, and measure the response of indigenous macroinvertebrate communities (e.g. Pettigrove & Hoffmann 2005; Wagenhoff, Townsend & Matthaei 2012). These approaches involve exposing indigenous macroinvertebrates to a simplified ecosystem with particular stressors, which can give a more environmentally relevant assessment of sediment toxicity than laboratory-based tests (Dzialowski *et al.* 2014). The microcosm approach employed here has been successfully used to investigate the impacts of urban (Marshall *et al.* 2010) and agricultural sediments (Sharley, Hoffmann & Pettigrove 2008; Townsend *et al.* 2009) on indigenous macroinvertebrate populations. This approach provides

a useful platform for validating and comparing responses, because it includes replication and controls.

Individual and sub-individual level responses are widely used in toxicity testing (McCarthy & Shugart 1990; Peakall 1994; Huggett *et al.* 2002; Forbes, Palmqvist & Bach 2006). These endpoints require confounding factors, such as organism age, to be controlled, so field deployment of laboratory-bred organisms is often used (e.g. Morales-Casellesa *et al.* 2008; Kellar *et al.* 2014). Copper is an essential metal, as well as being present in fungicide, insecticide and herbicide formulations, and this metal may cause direct or indirect toxicity to macroinvertebrates depending on the concentration and organism considered. Sub-individual responses represent sensitive endpoints that can reflect direct rather than indirect effects of exposure to copper and other metals. The cysteine metabolism pathway plays a central role in detoxification and antioxidant responses. Several metabolites and genes involved in this pathway have been shown to respond to metal exposure including copper (Fig. 1) (Sugiura *et al.* 2005; Hughes *et al.* 2009; Jeppe *et al.* 2014; Long *et al.* 2015). Measuring metabolites and gene expression in the cysteine metabolism of laboratory organisms could provide sensitive indicators of exposure and help managers identify specific environmental toxicants.

This study uses field-based microcosms to compare the effects of copper exposure on changes in abundance of colonizing macroinvertebrates (mostly Chironomidae) with effects on microcosm deployed laboratory organisms at several levels of biological organization. The deployed laboratory-bred organisms included two snails, *Potamopyrgus antipodarum* (Gray, 1843) and *Physella acuta* (Draparnaud, 1805), and one chironomid, *Chironomus tepperi* (Skuse, 1889). These species have been used extensively in laboratory- and field-based toxicity testing (e.g. Duft *et al.* 2003; Choung *et al.* 2010; Seeland *et al.* 2013; Gust *et al.* 2014; Kellar *et al.* 2014; Ma *et al.* 2014). Population-level responses (reproduction and first generation survival) were measured in *P. antipodarum* and *P. acuta*. Individual (dry weight) and sub-individual level responses (metabolite abundance and gene expression in the cysteine metabolism pathway) were measured in *C. tepperi*. These responses were then compared to copper effects on the abundance of colonizing macroinvertebrates in the microcosms.

Materials and methods

SEDIMENT SPIKING

Sediment was collected from an uncontaminated wetland (Glynns wetland, Warrandyte); this wetland was also the location of the microcosm experiment. Sediment was sieved to 63 µm and allowed to settle for 24 hours. Then it was spiked using methods from Simpson *et al.* (2005) with adjustments to be 'dilution pH 7' spiking, whereby a high-spike sediment stock (6000 mg/kg copper) was diluted to improve binding and equilibration of metal to sediment (Hutchins *et al.* 2008) (Appendix S1 in Supporting Information). The stock sediment was diluted to reach five exposure concentrations of copper per dry weight sediment (62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg and 750 mg/kg). These concentrations were chosen to span the range of environmentally relevant concentrations (Vinot & Pihan 2005; Gardham, Chariton & Hose 2014).

Sediment from each treatment was analysed at the start and end of the experiment for total copper through ICP-AES method EG005T *(*Australian Laboratory Services (ALS), Scoresby, Australia). The concentrations detected did not differ from the expected concentrations by more than 10%, so the expected concentrations are reported. The reference sediment had a background copper concentration of 11 mg/kg but is reported as 0 mg/kg. Furthermore, overlying water copper concentrations were measured at the end of the experiment (Method EG020A-T, ALS). Copper concentrations in the overlying water are as follows: 0.008 mg/L for the reference, 0.048 mg/L for the 62.5 mg/kg and 125 mg/kg treatments, 0.099 mg/L copper for the 250 mg/kg treatment and 0.185 mg/L and 0.189 mg/L for the 500 mg/kg and 750 mg/kg treatments, respectively.

MICROCOSM EXPERIMENT

The field-based microcosm experiment followed the methods of Pettigrove and Hoffmann (2005). The experiment was set up at the reference wetland (Glynns wetland, Warrandyte). This site has been used for previous microcosm experiments and supports a variety of aerial invertebrate species (Bahrndorff *et al.* 2006). Each treatment had 5 replicate microcosms, except the unspiked reference treatment, which had 20 replicates to provide a common baseline for comparing toxicity. Each replicate microcosm consisted of a 20 litre polypropylene plastic tub (Starmaid International, Melbourne) containing 500 grams of treatment sediment

158 and 15 litres < 63 µm sieved reference site water. Microcosm were covered with
159 polypropylene nets (2 cm^2 mesh) to allow colonization by small aerial invertebrates while
160 preventing large predators entering the microcosms. The microcosm experiment ran for 7
161 weeks during which the microcosms were colonized by indigenous invertebrates (Appendix
162 S1).
163

164 DEPLOYMENT OF LABORATORY-BRED ORGANISMS

165 Three laboratory-bred test organisms were deployed in the microcosm experiment; as this
166 was a novel process in the microcosm approach, 'no animal' controls were included (Appendix
167 S1). The laboratory-bred organisms included two snail species (*P. acuta* and *P. antipodarum*),
168 which were added in week one and exposed for 6 weeks. For *P. antipodarum,* 40 individuals
169 were added, but for *P. acuta*, which is a larger animal (15 to 20 mm shell height), 5 individuals
170 were added to each microcosm. Five days before the experiment concluded, 40 five-day old
171 *Chironomus tepperi* larvae (not present at the wetland) were added to each replicate to
172 simulate a five-day toxicity test (OECD 2004).
173

174 After 7 weeks, the experiment concluded and the microcosms were processed. Each
175 microcosm was sieved through a 250 µm screen to recover macroinvertebrates and added
176 laboratory organisms. Microcosm macroinvertebrates were stored in ethanol for identification
177 under the microscope. Chironomidae were the most common family and were identified to
178 species, but analysis was performed at genus level to increase statistical power. Other taxa
179 were identified to family, or lower where possible. Laboratory-bred organisms were also
180 retrieved and their survival recorded. A subset of 20-30 *C. tepperi* was snap frozen on dry ice
181 for biomarkers and the remaining individuals were stored in ethanol for larval dry weight
182 (Appendix S1). Surviving *P. acuta* were stored in ethanol and *P. antipodarum* were
183 anesthetized with 2.5% magnesium chloride for later assessment of embryo production
184 (Schmitt *et al.* 2006). Surviving juvenile snails, egg masses of *P. acuta* and embryo production
185 for *P. antipodarum* were assessed under a microscope. Empty snail shells were also counted
186 to confirm that reduced numbers were due to mortality and not migration out of the
187 microcosms.
188

GENE EXPRESSION ANALYSIS OF *CHIRONOMUS TEPPERI*

RNA was isolated with High Pure RNA Tissue Kit (Roche, Cat. no. 12033674001) according to the manufacturer's protocol with minor adjustments (Appendix S1). Extracted RNA was eluted in 50 µL RNase-free water. All RNA samples had a 260/280 ratio between 1.9 and 2.1 and were suitable for cDNA synthesis. Total RNA (0.5 µg) was reverse transcribed using an oligo dT primer and M-MLV reverse transcriptase (Promega Cat. no. M1705), following the manufacturer's protocol. The cDNA was then checked for gDNA contamination (Appendix S1). Template cDNA was diluted in 100 µL DPEC water and stored at –20 ºC.

Target genes in cysteine metabolism, their primers and amplicon sequences were previously described (Jeppe *et al.* 2014) (Table S1). Gene expression was measured using quantitative real-time PCR (qPCR) performed with a Roche LightCycler® 480 (Roche Applied Science, USA) in 10 µL reactions containing the components adapted from Takahashi et al. (2010). Exact recipes and protocols are described in Appendix S1.

METABOLOMIC ASSESSMENT OF *CHIRONOMUS TEPPERI*

Metabolites were extracted using a modified Bligh-Dyer extraction method (Bligh & Dyer 1959). Analysis of amine-containing metabolites was performed by LC-MS according to Boughton *et al.* (2011). Details metabolite extraction and analysis are described in Appendix S1. Amine-containing metabolites were quantified and analysed using multiple reaction monitoring (Agilent 1200-HPLC/Agilent 6460 Triple Quad MS).

STATISTICAL ANALYSIS

All statistical analysis was conducted in R (R version 3.3.0, The R Foundation for Statistical Computing). The packages used are presented in Appendix S1.

MICROCOSM MACROINVERTEBRATES

Multi-response permutation procedure (MRPP) was conducted to compare genera composition between copper treatments. All macroinvertebrate data was log-transformed before analysis, but were not parametric even after transformation. The response of total abundance, number of taxa and abundance of common (occurring in >10 microcosms and >200 individuals) genera to copper-spiked microcosms was modelled with a 3-parameter log-

logistic dose response model. The effect dose of 50% change (ED50) was calculated for comparison between responses.

LABORATORY-BRED ORGANISMS

The survival data of added organisms were arcsine transformed and dry weight data were log-transformed before analysis. The changes in each response to copper-spiked microcosms were modelled with a log-logistic model and the ED50 calculated.

GENE EXPRESSION OF *CHIRONOMUS TEPPERI*

Fluorescence data were normalized using the data-driven NORMAGene normalization method (Heckmann *et al.* 2011). Principal Component Analysis (PCA) was performed to observe differences in gene expression between the 6 copper treatments. Differences between copper treatments were then assessed using ANOVA after verifying normality and homogeneity of variances. Differences between copper treatments and the reference were then established with Dunnett's *post hoc* tests.

METABOLOMIC ASSESSMENT

Prior to statistical analysis, all metabolomic data were median normalized and log-transformed. Metabolites known to be associated with cysteine metabolism were selected from the LC-MS data (Fig. 1). Differences in abundance of these metabolites between copper treatments were identified using ANOVA after verifying normality and homogeneity of variances. Differences between copper treatments and the reference were then established with Dunnett's *post hoc* tests. Significant amine-containing metabolites not involved in cysteine metabolism were identified with ANOVA and their variance investigated with PCA.

For gene expression and metabolomics assessment, *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg correction.

Results

MICROCOSM MACROINVERTEBRATES

A total of 17,302 invertebrates from 45 taxa colonized the 55 microcosms, with 94.5% of individuals belonging to the family Chironomidae. Abundances and number of microcosms inhabited by common taxa are presented in Table 1. Genus composition between treatments was not significantly different when tested by an MRPP test (A = -0.003, P = 0.563). The average number of taxa did not differ between treatments (χ^2 = 6.53, df = 5, P = 0.25). However, total abundance declined with increasing copper, and a log-logistic model indicated that 50% of individuals were lost at 133 mg/kg (EC50) copper (Fig. 2a). Model parameters and significance are displayed in Table S2.

The five most common genera, which belonged to the family Chironomidae, decreased in abundance with increasing copper. *Paratanytarsus* displayed an EC50 of 89 mg/kg (Fig. 2b), *Procladius* had an EC50 of 681 mg/kg (Fig. 2c), *Chironomus* an EC50 of 97 mg/kg copper (Fig. 2d), and *Kiefferulus* an EC50 of 238 mg/kg copper (Fig. 2e). *Cladopelma* had an EC50 of 253 mg/kg copper (Fig. 2f) but due to low numbers and occurrence a log-logistic model could not establish significance (Table S2).

LABORATORY-BRED *POTAMOPYRGUS ANTIPODARUM*

The survival of adult *P. antipodarum* decreased with increasing copper and had an EC50 of 364 mg/kg (Fig. 3a). Reproduction was also affected, with juvenile production (EC50: 121 mg/kg) and embryo production (EC50: 172 mg/kg) decreasing at lower concentrations than adult survival (Fig. 3c, 3e). Furthermore, juveniles and embryos were not present in the 750 mg/kg treatment and embryos were not present in surviving adults from the 500 mg/kg treatment.

LABORATORY-BRED *PHYSELLA ACUTA*

The survival of *P. acuta* decreased slightly at the two highest concentrations of copper with a predicted EC50 of 823 mg/kg (Fig. 3b). Reproduction of *P. acuta* declined with increasing copper, with juvenile production (EC50: 298 mg/kg) responding at a lower concentration than egg mass production (EC50: 428 mg/kg) or adult survival (Fig. 3d, f). The low number of

individuals combined with the tolerance of *P. acuta* limited power to detect patterns in these data.

LABORATORY-BRED *CHIRONOMUS TEPPERI*

INDIVIDUAL RESPONSES

The survival of *C. tepperi* was not affected by copper exposure (data not shown). However, larval dry weight decreased with increasing copper, with an EC50 of 238 mg/kg (Fig. 3g).

SUB-INDIVIDUAL RESPONSES

GENE EXPRESSION

Gene expression profiles associated with cysteine metabolism genes were investigated in *C. tepperi*. The PCA showed that copper influenced gene expression profiles (Fig. 4). Copper treatments separated along two axes, PC1 (52.35% variance explained) and PC2 (21.7% variance explained). All copper treatments separated from the reference, with the 500 mg/kg treatment displaying the largest variance (Fig. 4a). When considered independently, the lowest copper treatments (60 and 125 mg/kg) separated from the reference PC1 (41.1% variance explained) and PC2 (28.5% variance explained), and variance increased with copper concentration (Fig. 4b).

The expression of *S-adenosylmethionine synthetase* (*SAM*) was significantly altered by the copper treatments. The expression of *SAM* was upregulated at least 3-fold in all treatments, however, this was only significant in the 125 mg/kg and 500 mg/kg treatments (Fig. 5a, Table S3). A similar pattern occurred for *S-adenosylhomocysteine hydrolase* (*SAH*) with upregulation evident in the 125, 250 and 500 mg/kg treatments, and 500 mg/kg being significantly different from the reference. In the 750 mg/kg treatment, *SAH* expression had returned to reference levels (Fig. 5b, Table S3).

The expression of *cystathionine-β-synthase* (*CβS*) did not significantly change due to copper exposure (Fig. 5c, Table S3), while *cystathionine-γ-lyase* (*CγL*) expression was significantly different between copper treatments. *Cystathionine-γ-lyase* expression upregulated with increasing copper, reaching a maximum of 3-fold upregulation in the 500 mg/kg treatment. In the 750 mg/kg treatment, upregulation of *CγL* expression reduced and was not significantly

different from the reference (Fig. 5d, Table S3). The expression of *metallothionein Mtn* upregulated 10- to 150-fold in all treatments and was significantly different in the 125 mg/kg treatment and above (Fig. 5e, Table S3).

The expression of *γ-glutamylcysteine synthase* (*GCS*) differed significantly between copper treatments (Fig. 5f, Table S3). The expression of *GCS* was upregulated in all copper treatments but was only significant in the 500 mg/kg treatment, with a 2.5-fold increase relative to the reference. For *glutathione synthetase* (*GS*), *glutathione-S-transferase d1* (*GST*) and *thioredoxin glutathione reductase* (*TGR*) expression, there was no significant effect (Fig. 5g, h and i, respectively, Table S3).

METABOLOMICS

CYSTEINE METABOLISM METABOLITES

From the LC-MS analysis, nine metabolites involved in cysteine metabolism were selected to link with gene expression, and tested for differences compared to the reference treatment (Figs 1 & 6, Table S4). The concentration of methionine did not differ between treatments although there was an increase in concentration in the 250 mg/kg treatments and above (Fig. 6a). For homocysteine, concentration was significantly altered by copper treatments. Homocysteine concentration increased with increasing copper and was significantly different from reference in the 500 mg/kg and the 750 mg/kg treatments (Fig. 6b). Concentrations of serine, cysteine and glutamate, were not altered by copper treatments (Fig. 6c, d and f, respectively). The concentration of lanthionine was significantly altered by copper treatments. However, although lanthionine concentration increased in the 250 mg/kg treatments and above, Dunnett's *post hoc* comparisons found no treatment significantly differed from the reference (Fig. 6e). The concentration of γ-glutamylcysteine was marginally altered by copper treatments. Dunnett's *post hoc* comparisons indicated that the concentration of γ-glutamylcysteine increased at higher copper concentrations and was significantly different from the reference in the 500 mg/kg treatment (Fig. 6g). Glycine concentration increased in treatments over 125 mg/kg and Dunnett's *post hoc* indicated that 500 mg/kg and 750 mg/kg treatments were significantly different from reference exposures (Fig. 6h). Copper had no effect on glutathione concentration (Fig. 6i).

OTHER AMINE-CONTAINING METABOLITES

Of the 37 amine-containing metabolites identified by LC-MS that were not selected based on their association with cysteine metabolism, 18 were altered significantly in copper treatments after Benjamini-Hochberg correction (Table S5). A PCA of these metabolites separated primarily along one axis (variance explained 70.33%), with a second axis explaining 9.57% (Fig. 7a, b). The PCA clearly separated copper treatments from the reference, with highest copper concentrations (500 and 750 mg/kg) displaying the largest deviation from the reference. Most (11 of 18) of the significantly altered amine-containing metabolites increased in concentration in copper exposures of 125 mg/kg and above. Asparagine, proline and dihydroxyphenylalanine increased in the 60 mg/kg treatment and above compared to the reference, while norepinephrine and kynurenine increased in the 250 mg/kg treatment and above. Citrulline was the only amine-containing metabolite to display a decrease in concentration, which occurred in 500 and 750 mg/kg copper treatments.

Discussion

This research detected invertebrate responses to copper exposure at multiple levels of biological organization. Our results indicated that mortality in laboratory-bred organisms was the least sensitive response measured, declining at concentrations comparable to those at which abundance of the least sensitive macroinvertebrates declined in the field-based microcosms. Sub-individual responses appeared to better reflect population-level changes in the microcosm and may be more useful indicators of decline than mortality of laboratory-bred organisms.

MICROCOSM MACROINVERTEBRATES

Changes in abundance of microcosm macroinvertebrates (specifically Chironomidae) occurred at a range of copper concentrations. This supports previous research that highlights the different sensitivities of Chironomidae genera to pollution (Mousavi, Primicerio & Amundsen 2003; Carew *et al.* 2007; Gresens *et al.* 2007). This range of pollution sensitivities is likely a result of inherent resistance, different feeding traits and/or avoidance behaviour of different groups. The genus *Procladius* that was tolerant to copper is a carnivorous group that feeds on other Chironomidae species (Takacs & Tokeshi 1993). This trait could allow avoidance of copper in sediment as prey could detoxify it before being consumed. In contrast, other metals (e. g. cadmium) may be more toxic to carnivores due to bioaccumulation

(Goodyear & McNeill 1999). Toxicity could also make prey more available, by slowing them and reducing burrowing behaviour in toxic sediments (Callaghan *et al.* 2001). Species relying on sediment and algae for food, such as *Paratanytarsus* or *Chironomus* spp., were comparatively more sensitive to copper. This could be due to reduced food supply or direct exposure through ingestion of copper-containing sediment (Armitage, Cranston & Pinder 1995).

LABORATORY-BRED ORGANISMS

Measured responses in laboratory-bred organisms altered at several copper concentrations. The survival of laboratory-bred organisms was not a sensitive indicator of copper toxicity. The traits that make organisms appropriate for laboratory testing, such as being able to survive, breed and be handled in the laboratory, could also make them tolerant of contaminants. The three species tested here have been recorded in contaminated and modified habitats, so it is unsurprising that survival of these species was not a sensitive indicator of copper (Carew *et al.* 2007; Gust *et al.* 2011). The short exposure time of late instar *C. tepperi* added to the microcosms could also help explain the lack of any impact on survival, in contrast to *Chironomus* spp. colonizing the microcosms, particularly because earlier instars are more sensitive to metal exposure (Williams *et al.* 1986). The tolerance of the snails may reflect their ability to avoid sediments, as they were observed on the sides of the microcosms and sometimes above the waterline.

Compared with survival, population responses of laboratory-bred organisms (number of juveniles, embryo production) aligned better with abundance changes of the colonizing Chironomidae in the microcosms. For snails, *P. antipodarum* was more sensitive than *P. acuta,* with the number of juveniles being the most sensitive response. The ED50 for *P. antipodarum* juvenile number aligned with moderately sensitive Chironomidae populations, as did embryo production of *P. antipodarum* and *C. tepperi* dry weight. The three laboratory-bred organisms also exhibited a possible beneficial effect of copper at low levels (60-125 mg/kg). This response may be caused by the contaminant being an essential element, or by reduced competition and increased resource availability due to mortality of more sensitive organisms (Pettigrove 2006).

SUB-INDIVIDUAL RESPONSES

CYSTEINE METABOLISM

Sub-individual responses in laboratory-bred organisms occurred at concentrations where sensitive Chironomidae populations were declining in the microcosms. Trends in gene expression and metabolite concentrations in the cysteine metabolism of *C. tepperi* were observed at 60 mg/kg copper and above. The upregulation of *SAM* and *SAH* expression are likely responsible for the observed increase in homocysteine under copper exposure. The upregulation of *CγL* could increase the transfer of homocysteine to cysteine via cystathionine. Although cysteine and serine levels were not altered, their production and transfer to lanthionine (which was increased) and metallothionein through increased *Mtn* expression could explain the stable levels of these metabolites. Downstream of cysteine, increased expression of *GCS* corresponded with increased γ-glutamylcysteine concentration, and an increase in glycine concentration indicated the beginning of antioxidant responses, although *GS, GST* and glutathione levels were stable. This antioxidant response induced by copper could be metal specific, as previous studies with zinc found no change in the concentration of intermediates downstream of cystathionine (Long *et al.* 2015).

OTHER AMINE-CONTAINING METABOLITES

Several amine-containing metabolites that were not involved in cysteine metabolism were also affected by copper exposure, with all increasing in concentration except citrulline. Metabolites that responded to copper exposure were involved in several cellular functions. Ammonia metabolism intermediates were affected (through glutamine, citrulline, arginine and asparagine), as was polyamine biosynthesis (through cadaverine and putrescine) and neurotransmission and modulation (3.hydroxytyramine, 3.methoxytyramine, phenethylamine, tyramine, octopamine and dihydroxyphenylalanine). Tyramine and octopamine can be involved in melanin production (Fuchs *et al.* 2014). As well as protecting against ultraviolet exposure, melanin can bind to metal ions (Gallas *et al.* 1999; Szpoganicz *et al.* 2002). Recently, melanin concentration was found to increase in *C. riparius* exposed to copper (Loayza-Muro *et al.* 2013). The increase in pyridoxamine could also be linked to copper toxicity as it forms complexes with Cu^{2+} and Fe^{2+} and is involved in hydroxyl radical scavenging, indicating another possible antioxidant response (Casasnovas *et al.* 2013). The amine-containing metabolites that have responded here offer pathways that could prove useful to further investigate contaminant and toxicity responses in *C. tepperi.*

This research used an integrated approach to detect differences in macroinvertebrate responses under field conditions. Responses to copper exposure were found at all levels of biological organization, with sub-individual responses the most sensitive. Furthermore, metal specific responses were observed and pathways of interest for further investigation identified through LC-MS. These multiple responses provide managers with ways to assess the contribution of sediment toxicity to ecosystem decline. The sub-individual responses in laboratory-bred organisms provide managers with sensitive endpoints and information on the nature of the chemicals of concern, highlighting exposure pathways that can be investigated to improve remediation. **Authors' Contributions**

KJ, JY, SL, MC, XZ, VP and AH conceived the ideas and designed the methodology; KJ, JY and SL collected the data; KJ analysed the data; KJ lead the writing of the manuscript. All authors contributed critically to the manuscript drafts and gave final approval for publication.

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Data accessibility

Chironomus tepperi dry weight, metabolite and gene expression data, *Physella acuta* survival and reproduction data, *Potamopyrgus antipodarum* survival and reproduction data and microcosm colonizing species identification and abundance data uploaded to Dryad Digital Repository http://dx.doi.org/10.5061/dryad.3kf74 (Jeppe *et al.* 2016).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1. Detailed materials and methods

Table S1. Primers used for measuring gene expression

Table S2. Statistics from the log-logistic dose response models

Table S3. Statistics from the ANOVA and Dunnett's tests for gene expression

Table S4. Statistics from ANOVA and Dunnett's tests for metabolite concentration

Table S5. Significance of amine-containing metabolites

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Tables

Table 1 Macroinvertebrates representing more than 0.3% of the total number of individuals colonizing the microcosm experiment. Taxa are ranked from most to least abundant.

Taxon	Family	Number of individuals	% total abundance	Number of microcosms
Paratanytarsus	Chironomidae	5940	35.1	46
Procladius	Chironomidae	3707	21.9	46
Chironomus	Chironomidae	3635	21.5	33
Kiefferulus	Chironomidae	2356	13.9	25
Cladopelma	Chironomidae	279	1.7	11
Chaoboridae	Chaoboridae	211	1.2	7
Parachironomus	Chironomidae	194	1.1	11
Berosus	Hydrophilidae	182	1.1	11
Polypedilum	Chironomidae	104	0.6	29
Oribatida	Not identified	99	0.6	27
Microvelia	Veliidae	48	0.3	27
Ceratopogonidae	Ceratopogonidae	45	0.3	8
Ephydridae	Ephydridae	44	0.3	18

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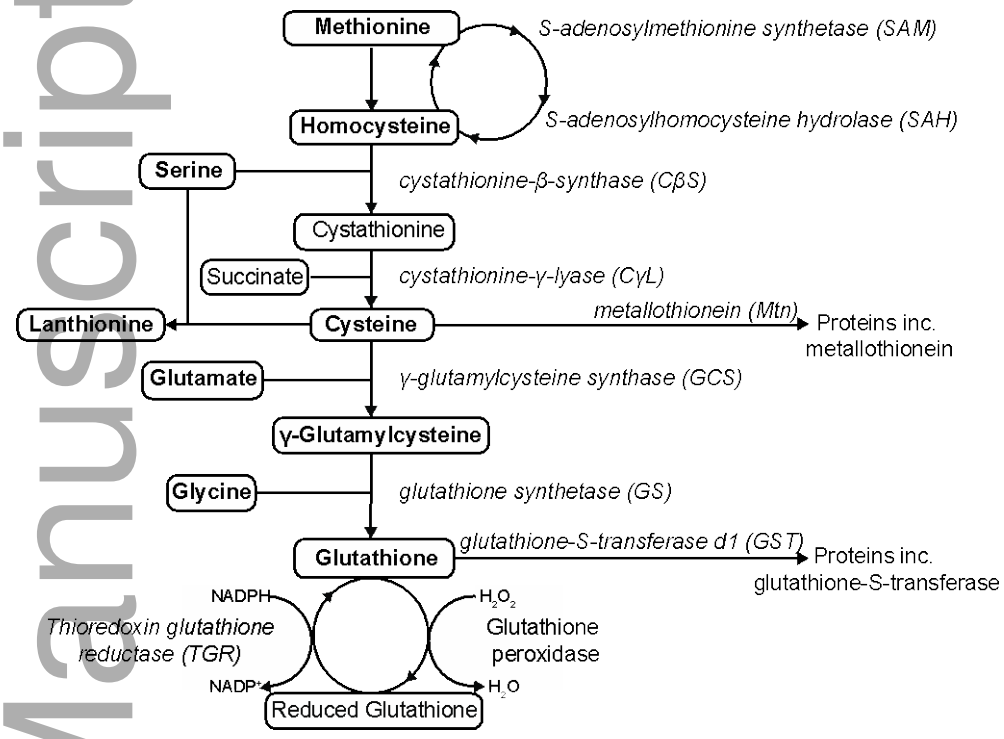
662 **Figures**



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664 Figure 1 Predicted cysteine metabolism of *Chironomus tepperi* adapted from Hughes *et al.* (2009).
665 Genes that were quantified with qPCR are indicated in italic text and metabolites measured with LC-
666 MS are indicated in bold text.

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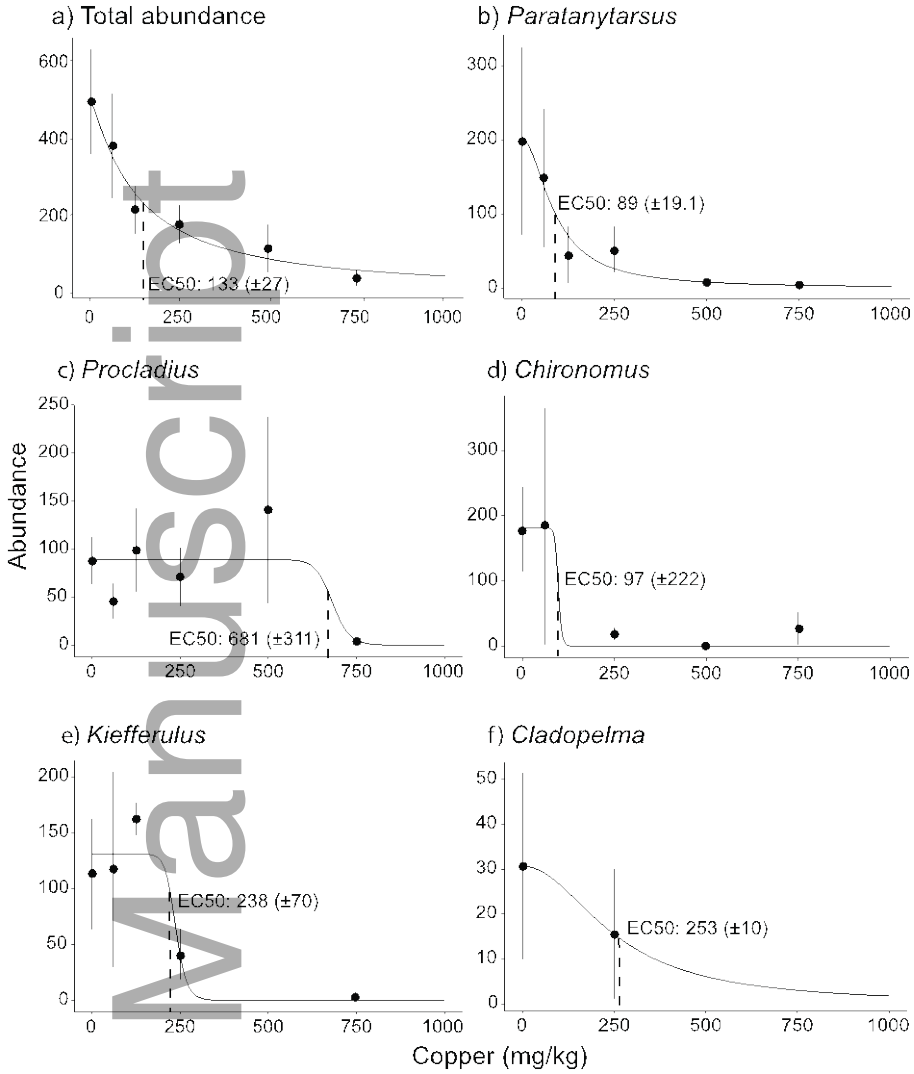

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670 Figure 2 Abundance of common indigenous macroinvertebrates retrieved from copper (mg/kg) spiked
671 microcosm sediments: a) total macroinvertebrates, b) *Paratanytarsus*, c) *Procladius* and d)
672 *Chironomus*, e) *Kiefferulus* and f) *Cladopelma*. Lines represent 3-parameter log logistic models of the
673 data with EC50 (± standard errors) indicated by a dashed line. Points represent mean abundance and
674 error bars are standard errors of the mean.
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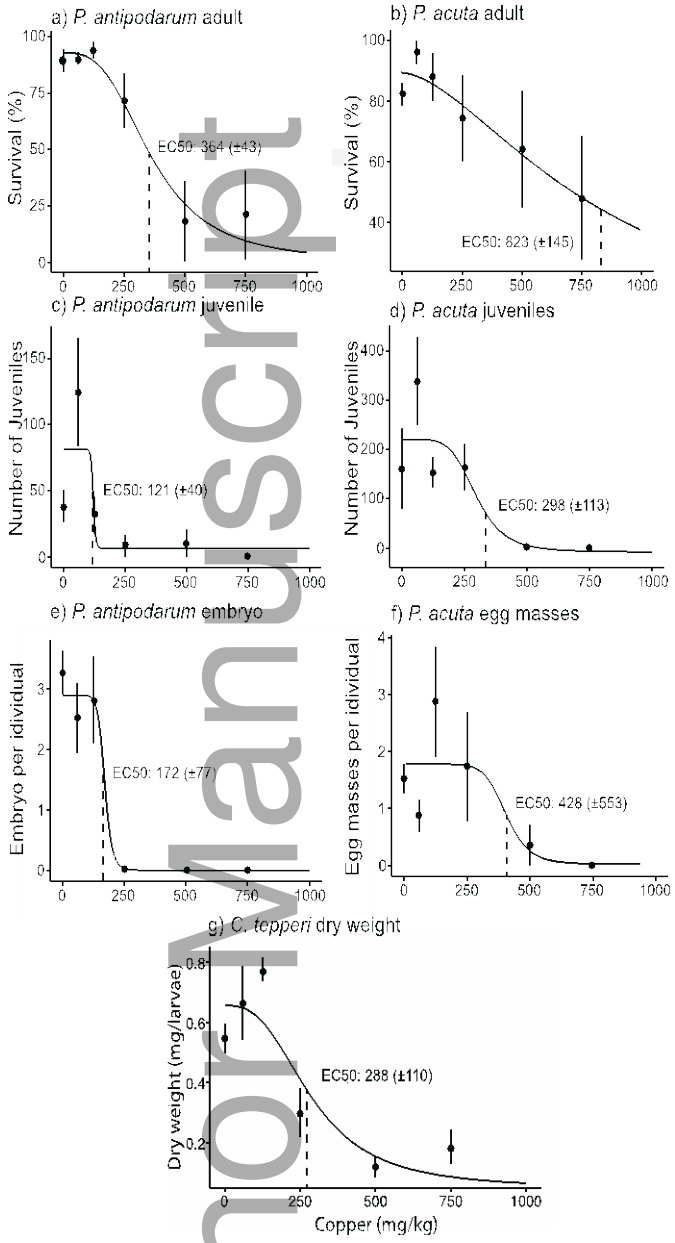

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Figure 3 Individual-level responses of laboratory-bred organisms: Survival of a) *Potamopyrgus antipodarum* and b) *Physella acuta* exposed to copper (mg/kg) in spiked microcosms for 6 weeks. Juvenile production of c) *P. antipodarum* and d) *P. acuta*, e) *P. antipodarum* embryo production and f) *P. acuta* egg mass production per surviving individual. g) Dry weight of *Chironomus tepperi* after 5 days. Lines represent 3-parameter log-logistic models of the data with EC50 (± standard errors) indicated by a dashed line. Points represent mean values and error bars are standard errors of the mean.

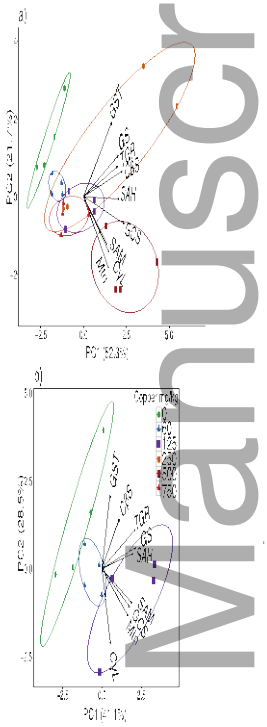


Figure 4 Sub-individual level responses of laboratory-bred organisms. Plots of principal components analysis (PCA) scores of 9 *Chironomus tepperi* cysteine metabolism genes after 5-day exposure to sediment copper concentrations (mg/kg) in spiked microcosms: a) all copper treatments and b) sub-lethal copper treatments (n=4).

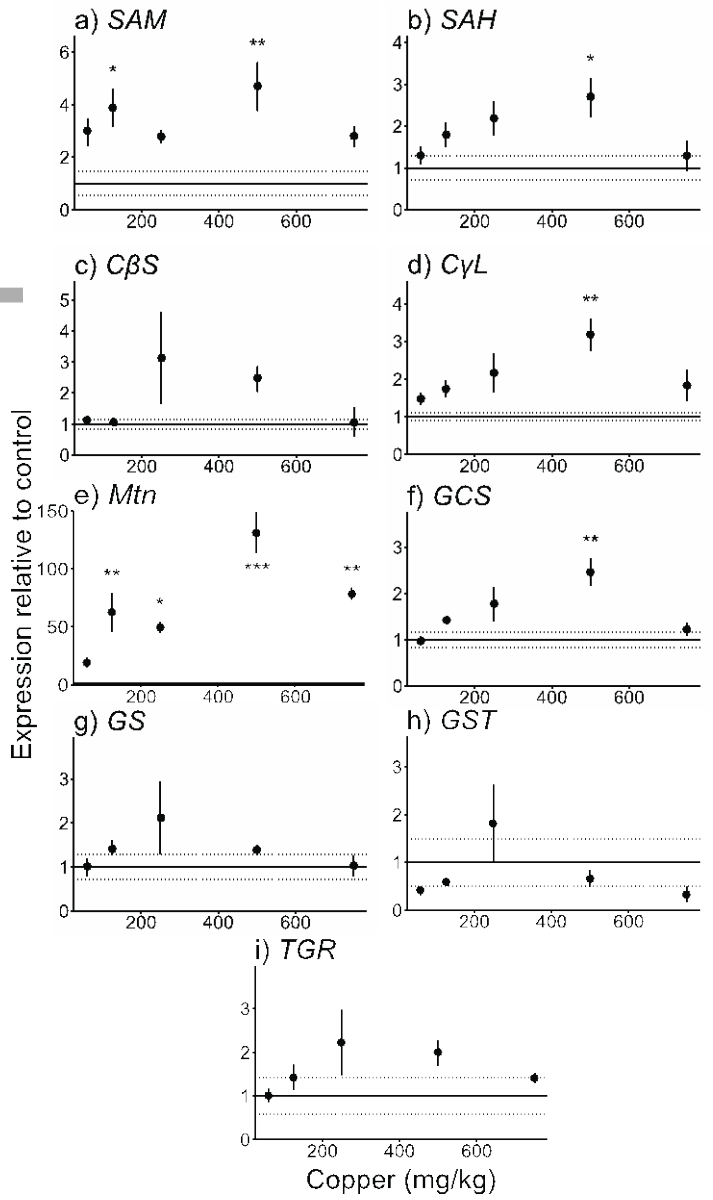


Figure 5 Sub-individual level responses of laboratory-bred organisms. Expression profile of *Chironomus tepperi* cysteine metabolism genes after 5-day sediment exposure in copper-spiked microcosms: a) *S-adenosylmethionine synthetase* (*SAM*), b) *S-adenosylhomocysteine hydrolase* (*SAH*), c) *cystathionine-β-synthase* (*CβS*), d) *cystathionine-γ-lyase* (*CγL*), e) *metallothionein* (*Mtn*), f) *γ-glutamylcysteine synthase* (*GCS*), g) *glutathione synthetase* (*GS*), h) *glutathione-S-transferase d1* (*GST*), and i) *thioredoxin glutathione reductase* (*TGR*). Expression was measured using RT qPCR and normalized using the data-driven algorithm NORMAgene. Data are displayed relative to reference exposures (black line) ± SEM (black dashed lines). Significance was determined with ANOVA and Dunnett's *post hoc* tests with Benjamini-Hochberg adjustment for multiple comparisons (*$P < 0.05$, ** $P < 0.01$). Error bars indicate standard errors of the mean.

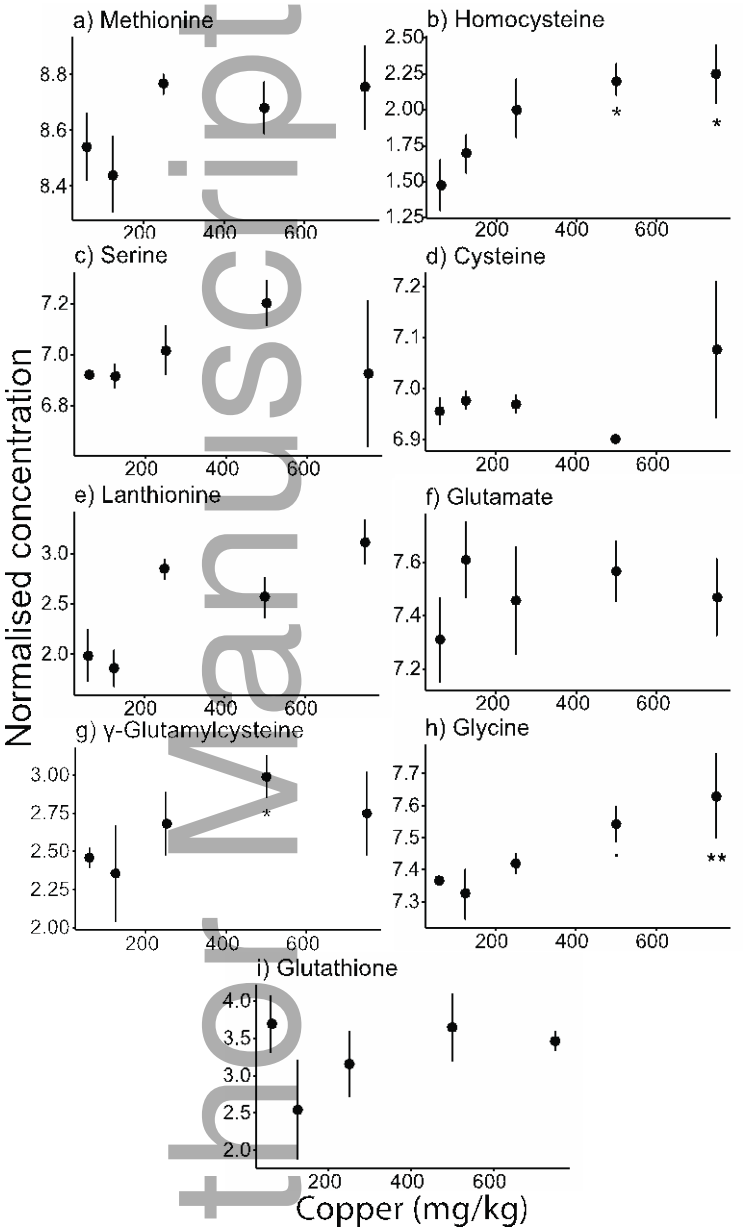


Figure 6 Sub-individual level responses of laboratory-bred organisms. Concentration of *Chironomus tepperi* metabolites associated with cysteine metabolism after 5-day sediment exposure in copper-spiked microcosms: a) methionine, b) homocysteine, c) serine, d) cysteine, e) lanthionine, f) glutamate, g) γ-glutamylcysteine, h) glycine and i) glutathione. Significance was determined with ANOVA and Dunnett's *post hoc* tests with Benjamini-Hochberg adjustment for multiple comparisons (. $P < 0.1$, *$P < 0.05$, ** $P < 0.01$). Error bars indicate standard errors of the mean.

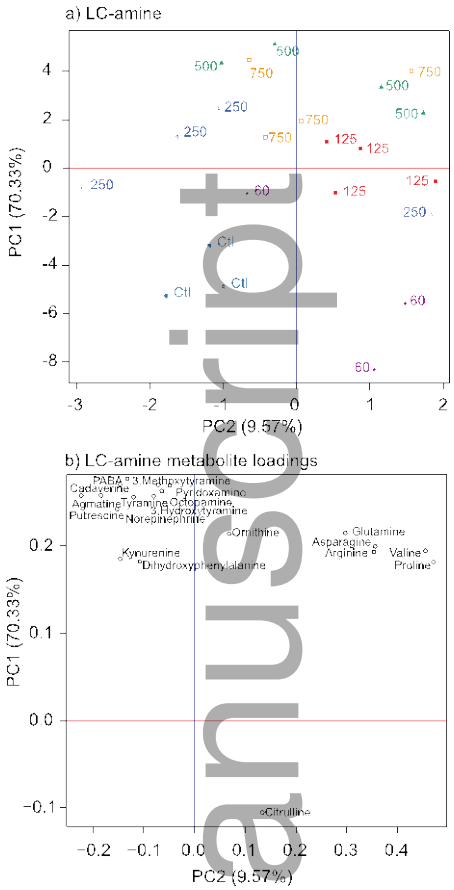
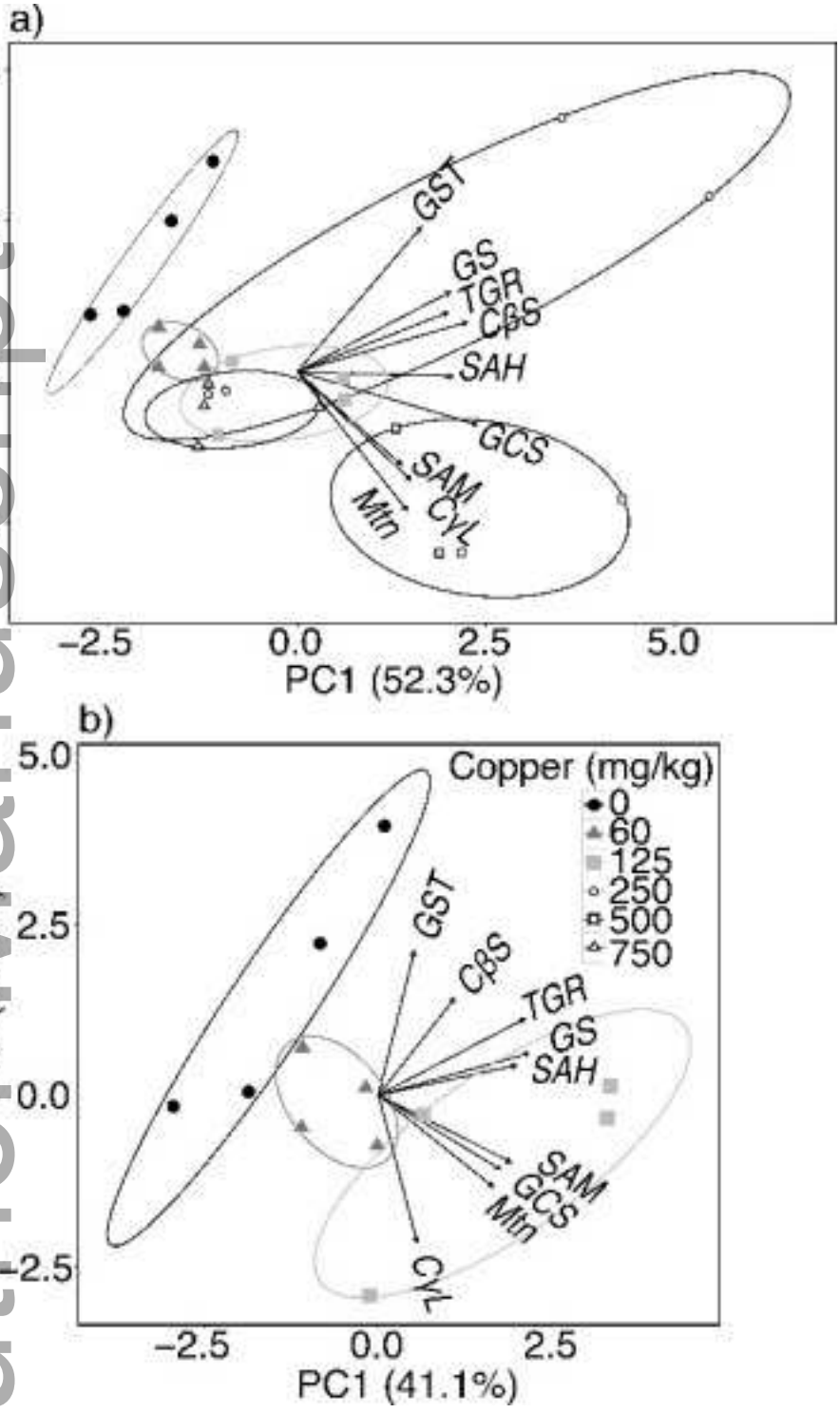


Figure 7 Plot of principal components analysis (PCA) scores for 19 significant amine-containing metabolites using targeted LC-MS approach, displaying a) sample indicated by copper concentration (mg/kg) and b) metabolite loadings. (n=4, except 60 mg/kg and unspiked control (Ctl) n=3).



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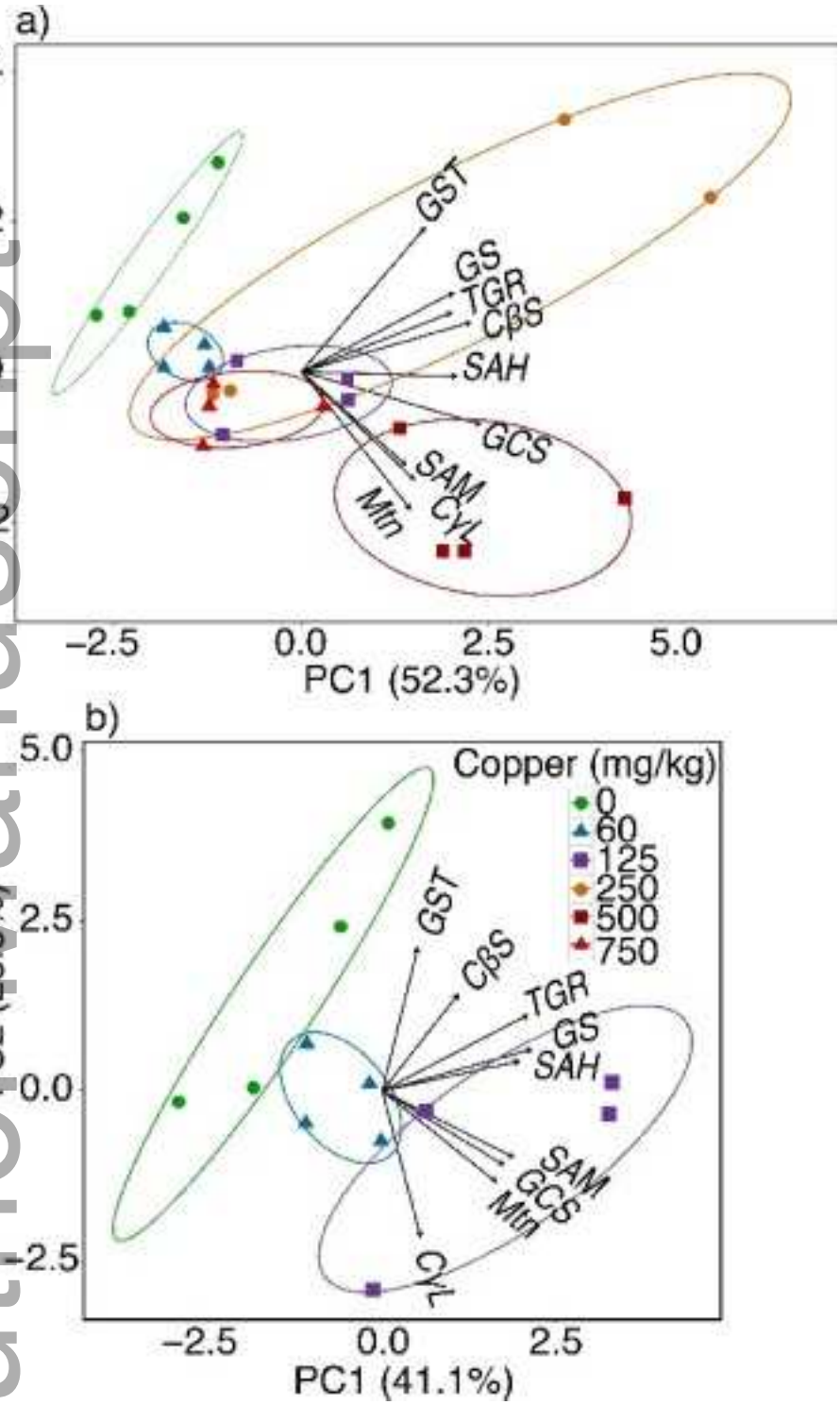

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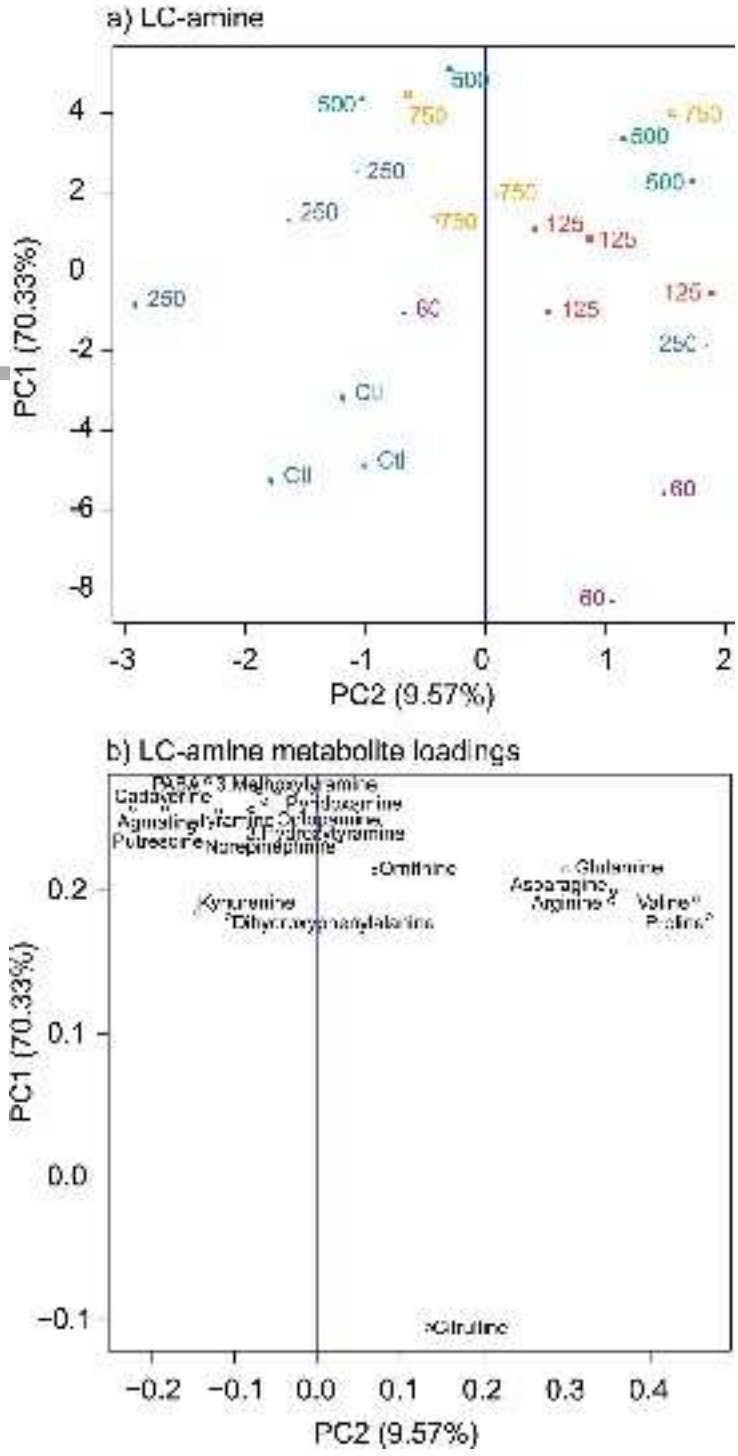

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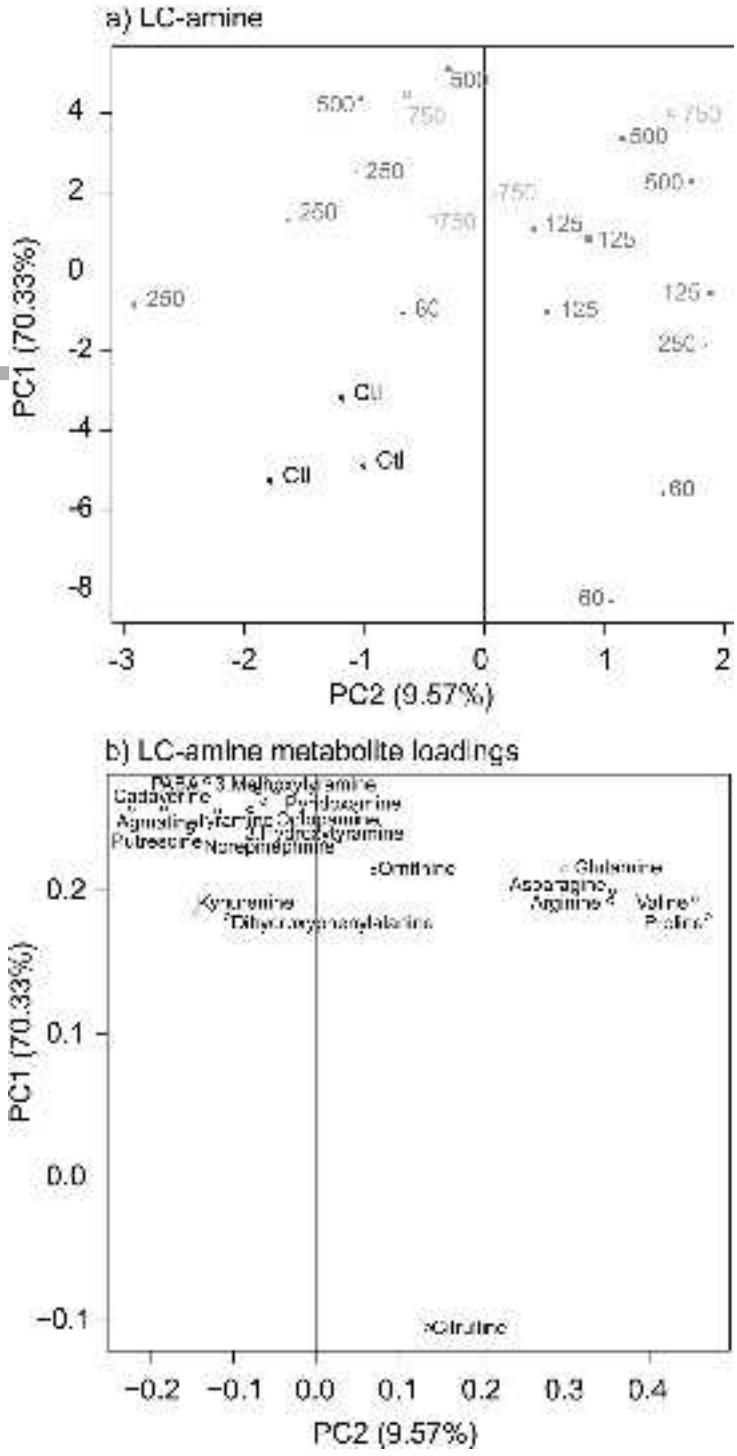

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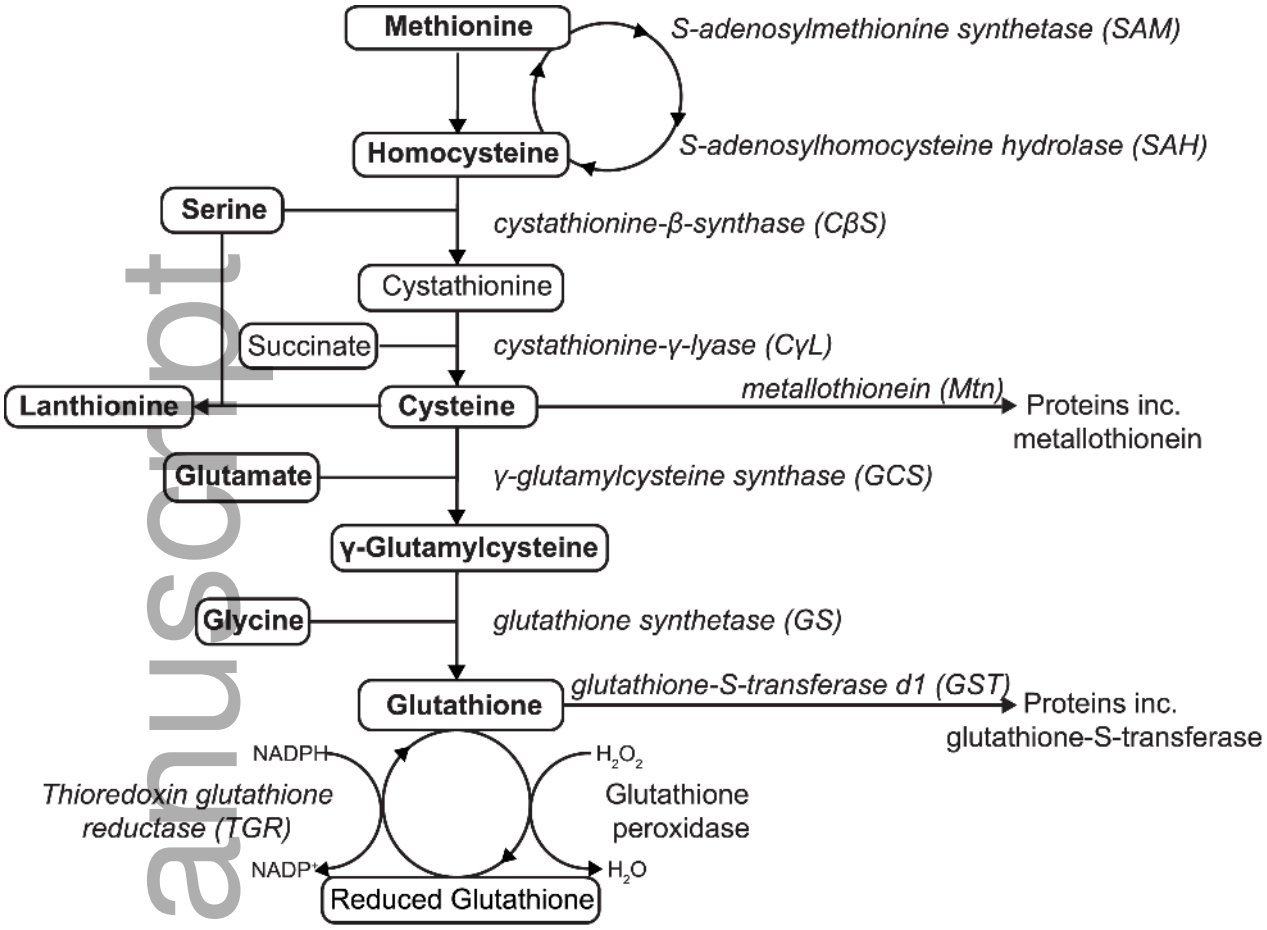

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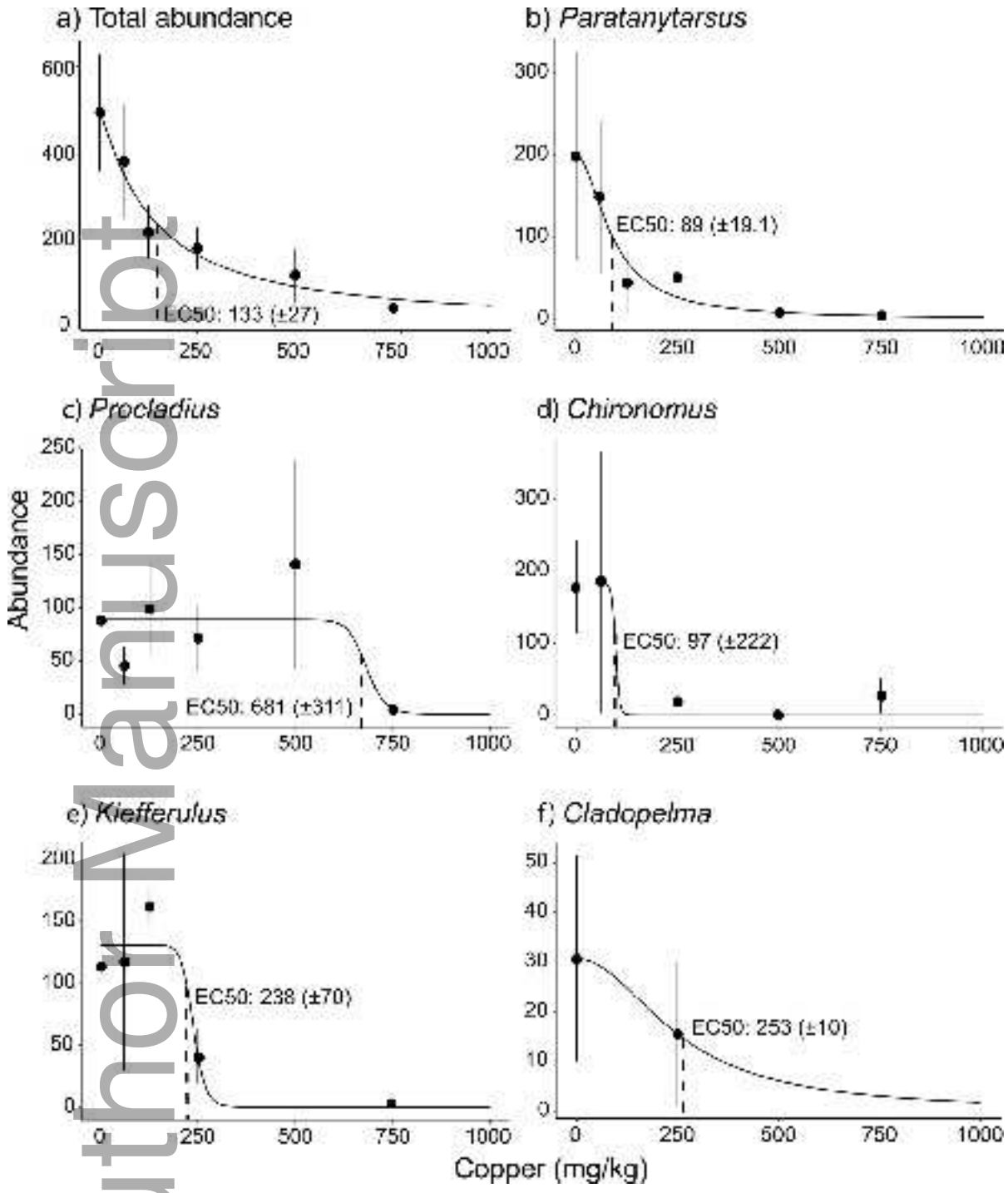

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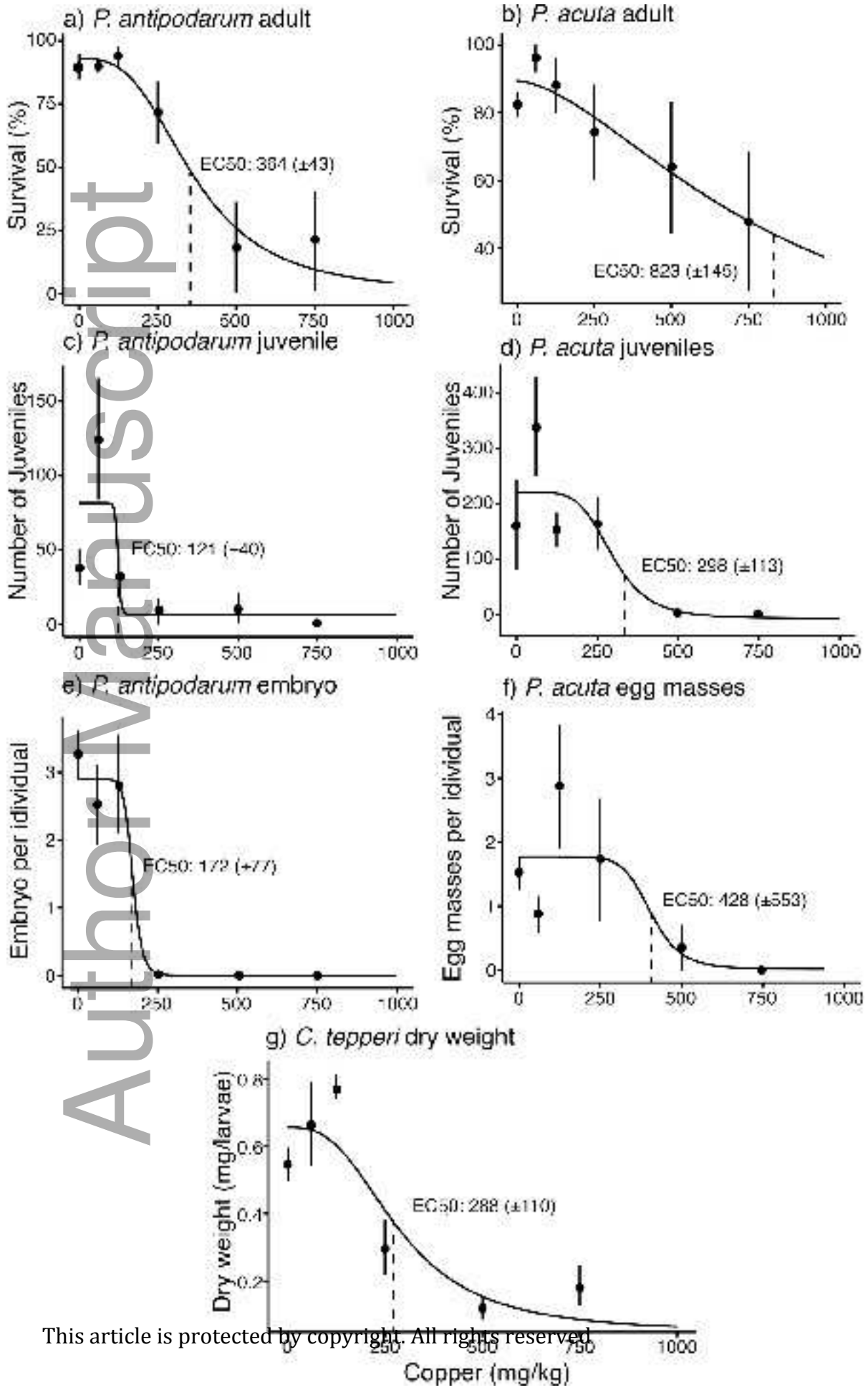

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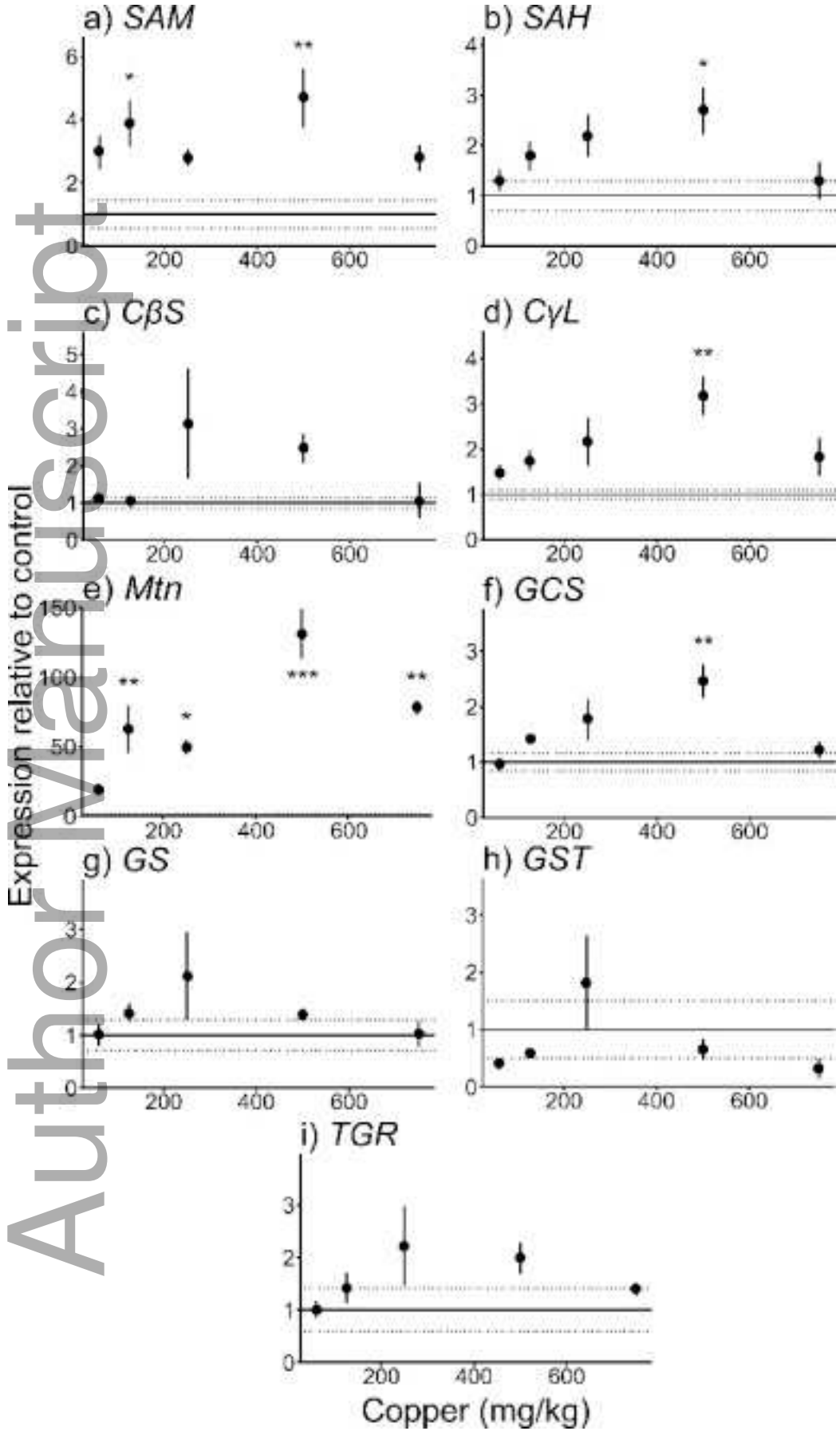

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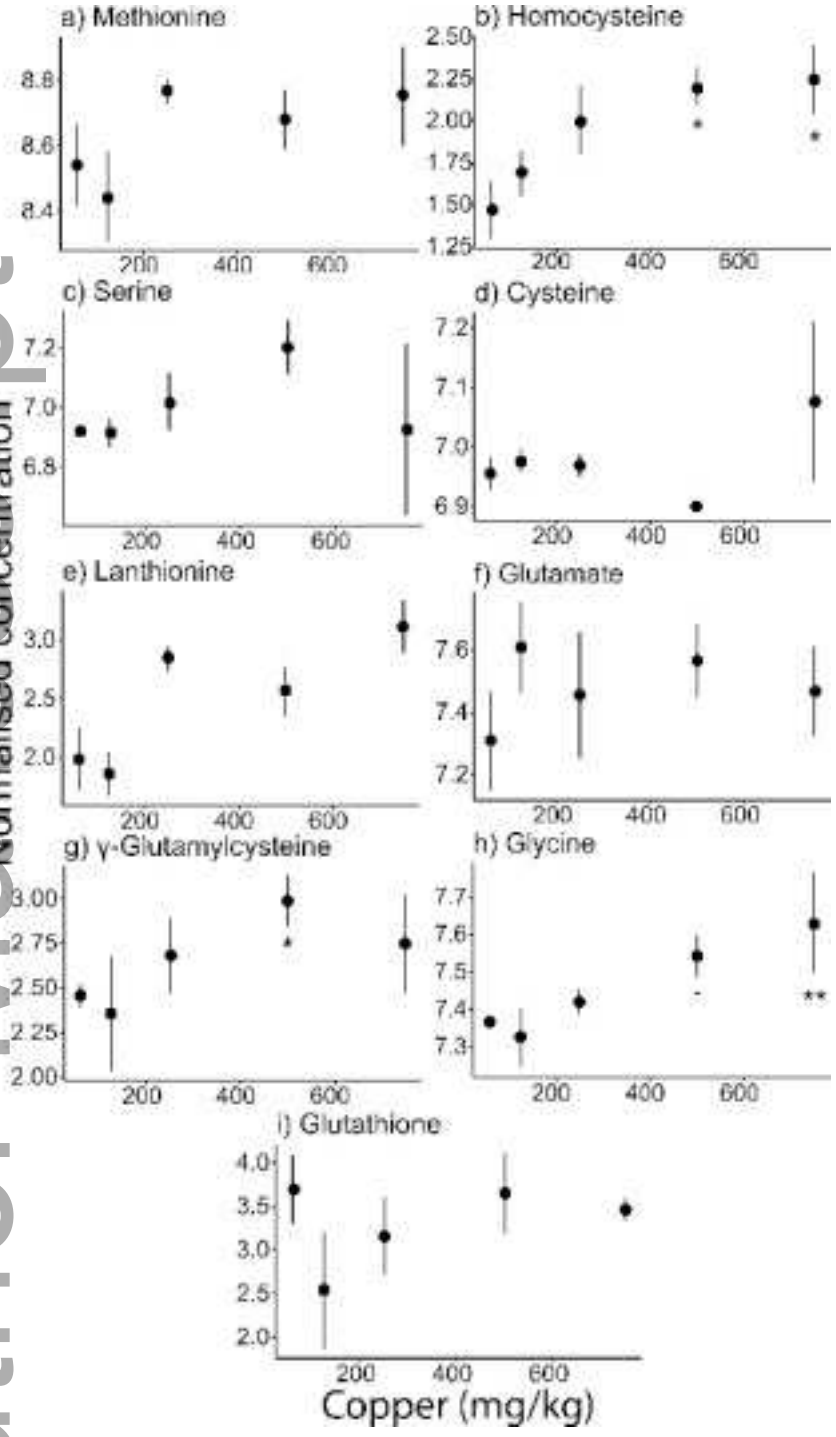

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