Author/s:
Jeppe, KJ;Carew, ME;Pettigrove, V;Hoffmann, AA

Title:
Toxicant mixtures in sediment alter gene expression in the cysteine metabolism of Chironomus tepperi

Date:
2017-03-01

Citation:
Jeppe, K. J., Carew, M. E., Pettigrove, V. & Hoffmann, A. A. (2017). Toxicant mixtures in sediment alter gene expression in the cysteine metabolism of Chironomus tepperi. Environmental Toxicology and Chemistry, 36 (3), pp.691-698. https://doi.org/10.1002/etc.3570.

Persistent Link:
https://hdl.handle.net/11343/291716

15-00762

Environmental Toxicology

TOXICANT MIXTURES IN SEDIMENT ALTER GENE EXPRESSION IN THE CYSTEINE METABOLISM OF *CHIRONOMUS TEPPERI*

KATHERINE J. JEPPE,*† MELISSA E. CAREW,‡ VINCENT PETTIGROVE,† and ARY A. HOFFMANN‡

†Centre for Aquatic Pollution Identification and Management, School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

‡Bio21 Molecular Science and Biotechnology Institute, School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

(Submitted 3 September 2015; Returned for Revision 11 April 2016; Accepted 28 July 2016)

Abstract

Sediment contamination can pose risks to the environment, and sediment toxicity tests have been developed to isolate the impact of sediment from other factors. Mixtures of contaminants often occur in sediments, and traditional endpoints used in toxicity testing, such as growth, reproduction, and survival, cannot discern the cause of toxicity from chemical mixtures because of complex interactions. In urban waterways, the synthetic pyrethroid bifenthrin and the metal copper are commonly found in mixtures, so the present study was designed to investigate how these contaminants cause toxicity in mixtures. To investigate this, *Chironomus tepperi* was exposed to environmentally relevant concentrations of copper and bifenthrin-spiked sediments in a 2-way factorial mixture for 5 d. Growth and expression profiles of cysteine metabolism genes were measured after exposure. Growth increased at low copper concentrations, decreased at high copper concentrations, and was unaffected by

bifenthrin exposures. Copper exposures induced possible cellular repair by upregulating *S*-adenosylmethionine synthetase expression and downregulating expression of *S*-adenosylhomocysteine hydrolase and cystathionine-β-synthase. Metallothionein upregulation was also observed. Bifenthrin exposure altered cysteine metabolism to a lesser extent, downregulating cystathionine-β-synthase and γ-glutamylcysteine synthase. Synergistic, antagonistic, and dose-dependent interactions were observed, and there was evidence of conflicting modes of action and limited substrate production. These findings demonstrate how contextual gene expression changes can be sensitive and specific identifiers of toxicant exposure in mixtures.

Keywords: Cysteine metabolism, Gene expression, Sediment toxicity, Transsulfuration, Mixture exposure

* Address correspondence to kjeppe@unimelb.edu.au.

Published online XXX in Wiley Online Library (www.wileyonlinelibrary.com).

DOI: 10.1002/etc.3570

INTRODUCTION

Sediments are often highly contaminated because hydrophobic chemicals introduced to waterways bind to suspended particles, settle, and accumulate. In this way, sediments can reflect past and present contamination at concentrations that are orders of magnitude higher than concentrations in water in the same system [1]. Many aquatic organisms rely on sediment for protection and food, so sediment toxicity tests with benthic organisms are often included in routine aquatic risk assessment.

Sediment toxicity tests are useful because they standardize experimental conditions and isolate the impact of sediment from factors such as hydrology and habitat [2,3]. In southern Australia, a common sediment toxicity test organism is the chironomid *Chironomus tepperi*. Similar to *Chironomus riparius* and *Chironomus tentans* assessed in the northern

hemisphere [2,4], it breeds well in the laboratory and spends the majority of its life exposed to sediment [5]. Sediment toxicity tests with this organism traditionally assess mortality and sublethal whole-organism endpoints, such as reproduction or growth, and interpret these endpoints in light of sediment chemistry analyses (e.g., Kellar et al. [6]). However, toxicity from chemical mixtures in field sediments is often difficult to discern because of unpredictable interactions. Mixture interactions occur at various levels. In exposure media, interactions between contaminants can regulate their bioavailable fractions, whereas at the organism level, they may regulate their uptake and depuration processes and their reaction with biological targets [7]. Synergistic interactions of contaminants can cause toxicity at exposure concentrations lower than observed in single-contaminant exposures [8]. Chemical analysis of mixtures may not correlate with toxicity because often only part of the mixture associated with toxicity is identified [9,10]. Highly contaminated sites are particularly difficult to characterize because of the complexity of the mixtures and because of changes in chemical composition of sediments over time as deposition and degradation occur [9]. In these cases, it is essential to understand how toxicant-specific responses perform in mixtures in order to help identify contaminants and to improve rapid screening for chemical interactions [9].

Spiked mixture experiments are the first step toward improving our understanding of mixture toxicity in sediments. These experiments assess a few interacting factors in simplified mixtures, often focusing on chemical–chemical, chemical–nutrient, or chemical–geochemical interactions [11–13]. However, field sediments potentially comprise multitudes of interactions in a single mixture. Thus, after identifying responses to mixtures in simplified spiked combinations, these responses must be validated in field sediments, where geochemical, nutrient, and chemical interactions may occur simultaneously.

In aquatic environments, gene expression patterns are increasingly used as a tool to

examine how organisms respond to toxicants. They have the potential to identify exposure and sublethal stress that can lead to environmental degradation [14,15]. As a result, gene expression data are now accepted by the US Environmental Protection Agency as part of a weight-of-evidence approach for environmental assessment [16]. Several studies have investigated transcriptomic responses to pollution in freshwater biota [17,18]. However, whole-transcriptome techniques are still not cost-effective for routine toxicity testing. An alternative approach involves measuring transcript levels in a well-studied stress response pathway. Cysteine metabolism plays a central role in detoxification against environmental stress [19,20]. Previously, we reported the response of several genes regulating cysteine metabolism under the influence of waterborne copper (Cu) or cadmium [21]. The different transcriptional responses of genes observed implicated metal-specific detoxification pathways and suggested that gene expression profiles in this pathway could potentially provide informative biomarkers. However, contaminant mixtures can influence gene expression differently from individual contaminant exposures [7,18]. These mixtures can act additively, synergistically, or antagonistically or can influence expression patterns in idiosyncratic ways.

The present study was designed to investigate how expression profiles of cysteine metabolism genes in *C. tepperi* respond in a standard toxicity test involving a metal–pesticide mixture and to compare these expression profile responses to whole-organism endpoints traditionally used in these tests. A spiked sediment exposure was used to assess these responses, and 3 field sediments containing similar mixture concentrations were used to validate them.

The expression profiles of 9 genes involved in cysteine metabolism of *C. tepperi* were investigated. The genes encode enzymes involved in the remethylation cycle (*S*-adenosylmethionine synthetase [*SAM*] and *S*-adenosylhomocysteine hydrolase [*SAH*]), the

transsulfuration pathway (cystathionine-γ-lyase [*CγL*], cystathionine-β-synthase [*CβS*], γ-glutamylcysteine synthase [*GCS-CAT*], glutathione synthetase [*GS*], and thioredoxin glutathione reductase [*TGR*]), and 2 resulting stress response proteins (glutathione *S*-transferase delta 1 [*GSTd1*] and metallothionein [*Mtn*]). The expression of these genes had previously been characterized under metal exposure in water, which confirmed their potential for investigating mixture toxicity [21,22].

METHODS

Spiking of sediment

Sediment was collected from Glynns wetland, an uncontaminated wetland 30 km east of Melbourne (organic carbon, 2.2%). Sediment was sieved to 63 µm and allowed to settle for 24 h. It was then spiked using methods from Simpson et al. [23], with adjustments to be dilution pH 7 spiking, whereby a high-spike sediment stock was diluted to improve binding and equilibration of metal to sediment [24]. Sediment stock was spiked and pH adjusted in a nitrogen atmosphere. Spiking solutions were made up with deoxygenated water and added to sediment, and then sediment was mixed vigorously and rolled on a bottle roller for 2 h to 3 h. After 24 h, the pH was adjusted to 8 with 1 N sodium hydroxide, and sediments were equilibrated for 25 d, rolling for 2 h to 3 h 3 times a week. The Cu (as Cu[II] chloride) used to spike was analytical reagent grade; because it has been shown that pesticide formulation affects toxicity [25], we used a commercially available formulation of bifenthrin to be environmentally relevant (Hovex ant granules). After equilibration, the stock sediment was diluted and mixed in a 2-way factorial design to generate 3 exposure concentrations of each contaminant: low (Cu, 44 mg/kg; bifenthrin, 0.032 mg/kg), medium (Cu, 176 mg/kg; bifenthrin, 0.063 mg/kg), and high (Cu, 308 mg/kg; bifenthrin, 0.23 mg/kg). Based on preliminary data, these concentrations were sublethal and expected to induce stress. Hence, they were also expected to induce genetic profile changes linked to toxicity. Concentrations

of Cu were based on sediment quality guidelines [26,27]. The low Cu treatment concentration was above the threshold effect concentration guideline (31.6 mg/kg), the medium Cu concentration was above the probable effect concentration and interim sediment quality–low guidelines (149 mg/kg and 80 mg/kg, respectively), and the high Cu concentration was above the interim sediment quality–high guideline (270 mg/kg). There are currently no sediment quality guidelines for bifenthrin, so concentrations were based on observed field concentrations and preliminary laboratory exposures. Sediment from each treatment was taken for chemical analysis at the start of the exposure. Total Cu was analyzed through inductively coupled plasma atomic emission spectroscopy method EG005T (Australian Laboratory Services), and bifenthrin was analyzed using gas chromatography–tandem mass spectrometry method NR47 (National Measurement Institute). Measured concentrations are reported, with the exception of the unspiked control, which had a measured Cu concentration of 11 mg/kg and a bifenthrin concentration below the limit of range of 0.01 mg/kg but is reported as 0 mg/kg for both contaminants.

Site comparison

A comparison of sites was undertaken to investigate whether field-collected sediments caused similar responses to those observed in the spiked mixture experiment. Three sites known to have moderate synthetic pyrethroid and metal contamination were selected from within the greater Melbourne area. The top 2 cm of sediment was collected from each site, filtered to 63 µm, and allowed to settle for 24 h. Metal and synthetic pyrethroid concentrations were then determined as described and are displayed in Table 1. A metal contamination quotient (probable effect concentration quotient [PECq]) was calculated for the sites from MacDonald et al. [27] as

$$PECq = \sum \left(\frac{M/PECm}{n(M)} \right)$$

where M is the metal measured, PECm is the consensus-based probable effect concentration for M, and *n*(M) is the total number of metals measured. Synthetic pyrethroid contamination was determined as the sum of bifenthrin and permethrin concentrations (mg/kg; Table 1).

Organism exposures

Chironomus tepperi larvae for experiments were maintained at the Centre for Aquatic Pollution Identification and Management (University of Melbourne) and originally had been collected from Yanco, New South Wales, Australia, in March 2014 (courtesy of **<ZAQ;1>**M.M. Stevens). The culture was maintained under a 16:8-h light:dark cycle at 20 ± 1 °C in aerated culture medium (reverse osmosis water containing 0.07 mmol calcium chloride, 0.1 mmol sodium chloride, 0.08 mmol magnesium sulfate [$MgSO_4$], 0.12 mmol sodium bicarbonate, 0.01 mmol potassium dihydrogen phosphate, and 0.01 µmol iron[III] chloride), with ethanol-rinsed tissue paper as substrate. Larvae that hatched from eggs were reared to second instar (5 d old) under culture conditions and supplemented with 10 mg/egg mass ground commercial fish food 3 times per week (TetraMin).

For exposures, 5 replicate 600-mL beakers were used for mixture treatments, and 3 replicates were used for individual contaminant treatments. Each beaker contained 140 g of treatment sediment and 200 mL culture medium and was allowed to settle for 24 h prior to exposure [2,4]. At this stage, 70% of the culture medium was renewed and 20 larvae (5 d old) were added to each replicate. Larvae were exposed for 5 d. Water was renewed and larvae were fed 2.5 mg/larvae on days 1 and d 3. Dissolved oxygen, pH, ammonia, and electrical conductivity were measured at each water renewal. After exposures, larvae were removed from sediment using a 125-µm sieve and rinsed in deionized water. A subset of 10 larvae was snap frozen for gene expression analysis, and the remaining larvae were stored in 70% ethanol for dry weight analysis.

RNA extraction

The High Pure RNA Tissue Kit (catalog no. 12033674001; Roche) was used to isolate RNA according to the manufacturer's protocol, with some small adjustments. The larvae from each replicate were homogenized at 0 °C in 300 µL lysis buffer using a cryomill (Precellys 24; Bertin Technologies). The homogenate was transferred, and the beads were rinsed with a further 150 µL lysis buffer before extracting per the kit protocol. Extracted RNA was eluted in 50 µL ribonuclease-free water. The integrity of the total RNA was checked on a 1.5% agarose gel and quantified on a Take3 plate (BioTek). All RNA samples had a 260/280 ratio between 1.9 and 2.1 and were suitable for complementary DNAcid (cDNA) synthesis.

Reverse transcription

Total RNA (2 µg) was reverse-transcribed using an oligo-deoxythymidine primer and Moloney murine leukemia virus reverse transcriptase (catalog no. M1705; Promega), following the manufacturer's protocol. The cDNA was then checked for genomic DNA (gDNA) contamination using a pair of ribosomal protein L11 primers that flank a 345-bp intron. The expected cDNA amplicon was 466 bp, whereas amplification of the 811-bp product would indicate the presence of gDNA [28]. Template cDNA was diluted to 400 µL and stored at –20 °C.

Gene expression analysis

Primers used to amplify cysteine metabolism genes have been described previously [21]. Briefly, literature and online databases were searched, and cysteine metabolism gene sequences were recorded for species closely related to *C. tepperi.* Conserved regions were then identified using the ClustalW function in Molecular Evolutionary Genetics Analysis, Ver 5 [29], and degenerate primers were designed using Primer3Plus [30]. Directly sequenced amplicons from *C. tepperi* were then searched against the GenBank protein database [31] and FlyBase [32] to ensure that the correct gene sequences were amplified.

Primers suitable for quantitative polymerase chain reaction (qPCR; fragment length, 60–150 bp; optimal temperature, 65 °C; and primer length, 19–26 bp) were designed within the degenerate amplified sequence, and the qPCR amplicon was directly sequenced to confirm amplification of the target genes. Primers used for qPCR are displayed in Table 2, and further information including degenerate primers and amplicon sequences is available elsewhere [21]. Primers amplifying *TGR* were added for the present study, and the *TGR* amplicon showed strong homology to its target protein when subjected to a BLASTx search against the *Drosophila melanogaster* annotated protein database [31]. The top hit for *TGR* was a thioredoxin reductase-1 splice variant sharing 69% identity ($E = 2e^{-11}$), and the fragment included the **<ZAQ;2>***TGR* domain ($E = 1.56e^{-07}$).

Gene expression was measured using real-time qPCR, with gene-specific primers. Real-time qPCR was performed with the LightCycler® 480 (Roche Applied Science) in 10-µL reactions containing the following components: 1 µL of cDNA, 1 µL of ThermoPol 10× buffer (catalog no. B9004S; NEB), 0.18 µL of $MgSO_4$ (100 mM), 0.064 µL of deoxynucleotide triphosphate (25 mM), 0.25 µL of LightCycler 480 High Resolution Melting Master Mix (catalog no. 04909631001; Roche), 0.01 µL of Immolase DNA polymerase (catalog no. BIO- 21047; Bioline; 5 units/µL), and 6.696 µL of water and 0.4 µL of forward and reverse primer mix (10 µM each) [33]. The qPCR program was 10 min at 95 °C; followed by 50 cycles of 10 s at 95 °C, 15 s at 58 °C, and 15 s at 72 °C; followed by a melting curve analysis from 65 °C to 95 °C to ensure that a single product was formed. Resulting fluorescence data were normalized using the data-driven NORMAGene normalization method [34].

Statistical analysis

Differences in relative normalized expression of target genes between treatments were analyzed using R (Ver 3.2.1). All survival, growth, and gene expression data were found to

be normal with equal variance using the Kolmogorov-Smirnov (ks.test[stats]) and Levene (leveneTest[car]) tests with the exception of the site sediments. For the spiked mixture experiment, an overview of gene expression in the cysteine metabolism was obtained using principal component analysis (princomp[stats]) with a matrix of the 9 genes and 16 treatment groups. Gene expression differences between contaminants were tested with factorial analyses of variance (ANOVAs; aov[stats]) considering Cu, bifenthrin, and the interaction of Cu and bifenthrin. Treatments with significant interactions between contaminants were assessed for antagonism, synergism, or idiosyncratic trends by comparing mean values of each treatment. Because of multiple comparisons of treatments involving 9 genes, only differences at $p = 0.005$ were considered significant based on a Bonferroni correction. Parabolic regression lines were added where significant treatment effects were detected, to illustrate changes in expression with levels of Cu. For the preliminary site comparison, sediments from sites were compared to the control with a Kruskal-Wallis rank sum test.

RESULTS

Survival and growth

Survival of *C. tepperi* after Cu, bifenthrin, or mixture exposure did not differ significantly between treatments (ANOVA $F_{(15,53)} = 0.67$, $p = 0.803$; Figure 1A). Exposures were therefore sublethal, and mortality did not confound growth or gene expression results. Dry weight of larvae differed significantly with Cu exposure ($F_{(3,53)} = 25.70$, $p < 0.001$) but was unaffected by bifenthrin ($F_{(3,53)} = 0.62$, $p = 0.608$) with no interaction between these factors ($F_{(9,53)} = 1.37$, $p = 0.225$). Dunnett's *t* test revealed that low Cu led to a marginally nonsignificant increase in larval dry weight by 5 mg to 10 mg ($p = 0.072$), medium Cu was not different from controls ($p = 0.36$), whereas high Cu significantly reduced larval dry weight by 25 mg to 30 mg ($p < 0.001$) compared with control exposures and irrespective of bifenthrin concentration (Figure 1B). Furthermore, Tukey's contrasts indicated that high Cu

was different from all other Cu treatments ($p < 0.001$) and that medium Cu was different compared with low Cu ($p = 0.002$).

Cysteine metabolism

Principal component analysis of all treatments showed that individual bifenthrin treatments and low Cu treatments were not different from the control (Figure 2). In contrast, medium and high Cu treatments separated primarily along principal component 1 (74.1% variance explained) with variance increasing with Cu concentration. This analysis also suggested that the presence of medium and high bifenthrin in mixtures reduced Cu-driven variation.

Individual expression profiles of each gene are displayed in Figure 3, and results of the factorial ANOVA are displayed in Table 3. For *SAM* expression, Cu and bifenthrin concentrations had significant effects on expression and an interaction between treatments was evident ($p < 0.001$; Figure 3A and Table 3). A dose-dependent increase was present for Cu only and Cu+low bifenthrin mixture (2.0-fold and 1.5-fold increases for the high Cu treatment, respectively). This indicates that Cu was driving *SAM* expression. For higher bifenthrin concentration, there appeared to be antagonistic effects with Cu (showing only a 1.25-fold upregulation for high bifenthrin level and actually a 0.5-fold downregulation for the medium bifenthrin treatment). For the expression of *SAH*, Cu exposure had a significant effect ($p < 0.001$), whereas bifenthrin did not ($p = 0.11$). However, bifenthrin and Cu displayed a synergistic interaction ($p = 0.004$; Figure 3B and Table 3). Although medium Cu did not alter *SAH* expression, medium mixtures downregulated *SAH* expression to 0.6-fold, lower than either contaminant individually. Furthermore, *SAH* expression downregulated to 0.6-fold under high Cu exposure; when combined with increasing bifenthrin, it downregulated to as low as 0.3-fold, indicating synergistic downregulation (Figure 3B).

For the expression of *CβS*, Cu concentration had a significant effect ($p < 0.001$), and

bifenthrin did not ($p = 0.416$; Figure 3C). However, the interaction between Cu and bifenthrin was significant ($p < 0.001$) and antagonistic. Expression of *CβS* downregulated in medium and high individual exposures (Cu down to 0.5-fold, bifenthrin down to 0.4-fold) and low mixtures; however, higher-concentration mixtures of Cu and bifenthrin showed a return to control expression levels. These results make *CβS* expression difficult to predict in mixtures because of dose-dependent differences. For the expression of *CγL*, there was no significant difference below the Bonferroni-corrected p value of 0.005 (Cu $p = 0.032$, bifenthrin $p = 0.141$) and no interaction ($p = 0.756$; Figure 3D). The expression of *Mtn* differed between exposures (Cu $p < 0.001$, bifenthrin $p < 0.001$, and Cu:bifenthrin $p < 0.001$; Figure 3E and Table 3). Under individual medium and high Cu exposure, *Mtn* expression was upregulated over 10-fold compared with controls. For individual bifenthrin exposure, *Mtn* expression was downregulated between 0.6-fold and 0.8-fold. Upregulation of *Mtn* was still evident in the Cu and bifenthrin mixtures, but the magnitude of the upregulation was reduced (medium bifenthrin, 8-fold; high bifenthrin, 5-fold), indicating an antagonistic interaction (Figure 3E).

The expression of *GCS* differed under Cu ($p < 0.001$) and bifenthrin ($p < 0.001$) exposure (Figure 3F), with no significant interaction between the contaminants ($p = 0.052$). However, because of the low magnitude (±0.2-fold) of these changes, their implications are unclear.

For *GS* expression, there was no significant difference between treatments based on the Bonferroni-adjusted p value of 0.005 (Cu $p = 0.108$, bifenthrin $p = 0.141$, and interaction effect $p = 0.015$; Figure 3G and Table 3). The expression of *GST* was also not significantly different based on the Bonferroni-adjusted p value of 0.005 (Cu $p = 0.024$, bifenthrin $p = 0.144$, and interaction $p = 0.165$; Figure 3H). For the expression of *TGR*, only Cu treatments differed significantly (Cu $p = 0.001$, bifenthrin $p = 0.088$, and Cu:bifenthrin $p = 0.167$; Figure 3I). However, similar to *GCS*, the magnitude of these changes was small (0.25-fold).

Site comparison

The 3 sites with moderate metal and synthetic pyrethroid contamination were investigated for 4 genes showing responses in the spiked mixture experiment (Table 1 and Figure 4). Although the Cu concentration was not identical to that used in the spiked experiment, the combination of several metals indicated a moderate probable effect concentration quotient value similar to medium and high Cu exposures and a synthetic pyrethroid concentration between the medium and high spiked exposures (Table 1). For all sites, the expression of *SAM* did not differ from control levels (Kruskal-Wallis $\chi^2 = 0.39$, degrees of freedom [*df*] = 1, $p = 0.53$; Figure 4A). For the expression of *SAH* and *CβS*, all sites downregulated around 0.75-fold ($\chi^2 = 7.7$, $df = 1$, $p < 0.01$; Figure 4B) and 0.5-fold ($\chi^2 = 6.2$, $df = 1$, $p < 0.05$; Figure 4C), respectively. The expression of *Mtn* was significantly upregulated in all sites by about 5-fold ($\chi^2 = 8.0$, $df = 1$, $p < 0.005$; Figure 4D). These results align well with what would be expected for medium Cu and medium bifenthrin spiked exposures (triangle series in Figure 2).

DISCUSSION

The present study showed how mixtures influence stress-induced gene expression in *C. tepperi* after a 5-d sediment toxicity test and compared these gene expression changes to a commonly used sublethal whole-organism endpoint. The dry weight of *C. tepperi* larvae indicated a small degree of hormesis in low Cu exposures and toxicity in high Cu exposures based on guidelines for toxicity tests [2,4]. Using these endpoints, it would have been concluded that Cu was beneficial at low exposures and moderately toxic at high exposures and that the bifenthrin concentrations investigated in the present study were not toxic. However, changes in gene expression profiles altered at medium and high exposure concentrations. This suggests that gene expression responses occurred before a reduction in dry weight was observed and that gene expression could provide an early signal of pollution

exposure and associated stress.

Although low Cu-only exposure caused an increase in dry weight, there were no significant changes in gene expression. However, 4 genes displayed consistent and near-linear dose–response expression patterns in medium and high Cu-only exposures. The upregulation of *SAM* expression and downregulation of *SAH* expression at medium and high Cu exposures, respectively, suggested increased cellular methylation, which is important for cell function and repair [35]. The downregulation of *CβS* expression at medium and high Cu exposures is likely to inhibit CβS substrate production from homocysteine, which in turn increases the homocysteine available for *SAM* (and cellular methylation) but also slows the downstream production of cystathionine [36,37]. Furthermore, the upregulation of *Mtn* observed during medium and high Cu exposures suggested protective metal sequestration through metallothionein production, which is likely to further diminish the cysteine pool in the cell [38]. A marginal downregulation of *GCS* and *TGR* was evident in low and medium Cu treatments, and these responses require further investigation to establish if they are informative of Cu metabolism.

Exposure to bifenthrin-only treatments caused fewer overall changes in cysteine metabolism gene expression in *C. tepperi*. Only downregulation of *SAM*, *Mtn*, and *GCS* expression was observed following bifenthrin exposures. The downregulation of these genes could potentially prevent protective responses and increase sensitivity by limiting substrate production along the cysteine metabolism pathway [39].

Interactions between the effects of Cu and bifenthrin on the expression of cysteine metabolism genes were common and varied depending on which gene was considered and to some extent on the severity of the stresses, in some cases being reduced at relatively higher concentrations. Strong antagonism was observed in *SAM* expression. The presence of bifenthrin in the mixture limited *SAM*, and hence SAM substrate production, which could in

turn limit the cellular methylation required for Cu protective responses. Furthermore, the synergistic downregulation of *SAH* expression is indicative of the pathway being driven toward cellular methylation as SAM availability is limited. There was strong antagonism between Cu and bifenthrin observed in *CβS* expression; whereas both contaminants individually downregulated *CβS* expression, *CβS* expression returned to control levels in medium and high mixtures. This result could imply chemical neutralization or an increased demand for downstream products under high exposures. An investigation of related pathways could shed more light on this interaction. The antagonism between Cu and bifenthrin observed in *Mtn* expression indicates possible limitation of MT production under bifenthrin exposure, which could reduce the protective metal response of the cell and increase sensitivity to Cu in mixtures [40]. The reason for a reduction in the effects of mixtures at high compared with medium concentrations is unclear but might relate to organisms starting to shut down gene expression at particularly high levels of pollutants.

Of the 4 genes that displayed consistent and near linear dose–response expression patterns under Cu-only exposures, only 2 (*Mtn* and *SAH*) retained this pattern in the presence of bifenthrin. This highlights the need to understand gene expression under mixture toxicity if it is to be used as an indicator of exposure field sediments. Results from the site comparison were relatively consistent with expectations based on the spiked mixture experiment. The expression profiles of the sites investigated aligned with the medium Cu and medium bifenthrin mixture treatments. The probable effect concentration quotient of several of these sites was higher than the medium spiked treatment, with this index assuming an additive interaction between all metals. However, this is not always the case, with Cu and nickel showing synergistic reduction in *Daphnia* reproduction [41] and mouthpart deformities being reduced when zinc and lead were combined in contrast to individual metal treatments [13]. Furthermore, long-term weathering, organic matter content, and hardness of sediments can all

alter the bioavailability of contaminants in field sediments compared to spiked sediments [24,27]. Thus, although the present study cannot indicate specific interactions that will occur in particular sediments at a site, the results nevertheless suggest that the metal and synthetic pyrethroid effects in the sediments tested alter cysteine metabolism gene expression, consistent with observations in the medium spiked mixture.

The present study has demonstrated that gene expression can identify exposure in mixtures at sublethal concentrations. Gene expression analysis could be a valuable tool for environmental monitoring because it can detect exposure before traditional whole-organism endpoints. Gene expression of cysteine metabolism was generally more responsive to metal exposure than to synthetic pyrethroid exposure. However, the presence of synthetic pyrethroids in a mixture altered the response of genes in this pathway. This provides useful information contributing to the development of an adverse outcome pathway, where gene expression data on cysteine metabolism are incorporated into environmental risk assessment [42].

CONCLUSION

In the present study, we have confirmed the potential of several *C. tepperi* genes as molecular biomarkers of cysteine metabolism responses to Cu and bifenthrin exposure in a mixture. The present study showed that gene expression is altered at lower concentrations than apical endpoints observed in whole organisms. Furthermore, the results identified several expression interactions that should be considered when looking at contaminants in mixtures.

Acknowledgment—The authors thank R. Reid for technical assistance and S. Long and R. Lee for advice and guidance. The present study was funded primarily by the Australian Research Council through their linkage and fellowship schemes, with additional support from

The Victorian Centre for Pollution Identification and Management.

Data Availability—Gene sequence data are available via GenBank. Organism response data are available from the author on request (kjeppe@unimelb.edu.au).

References

1. Burton GA. 1991. Assessing the toxicity of fresh-water sediments. *Environ Toxicol Chem* 10:1585–1627.
2. Organisation for Economic Co-operation and Development. 2004. Test No. 218: Sediment–water chironomid toxicity test using spiked sediment. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
3. US Environmental Protection Agency. 1996. Chironomid sediment toxicity test: Ecological effect test guidelines. OPPTS 850.1790. Washington, DC.
4. ASTM International. 1997. Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates. E 1706-05. In *Annual Book of ASTM Standards*, Vol 11.06. Philadelphia, PA, USA.
5. Stevens MM. 1993. Larval development in *Chironomus tepperi* (Diptera, Chironomidae) under laboratory conditions. *Environ Entomol* 22:776–780.
6. Kellar CR, Hassell KL, Long SM, Myers JH, Golding L, Rose G, Kumar A, Hoffmann AA, Pettigrove V. 2014. Ecological evidence links adverse biological effects to pesticide and metal contamination in an urban Australian watershed. *J Appl Ecol* 51:426–439.
7. Spurgeon DJ, Jones OAH, Dorne J, Svendsen C, Swain S, Sturzenbaum SR. 2010. Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. *Sci Total Environ* 408:3725–3734.
8. Yang RSH, El-Masri HA, Thomas RS, Dobrev ID, Dennison JE, Bae DS, Campain JA, Liao KH, Reisfeld B, Andersen ME, Mumtaz M. 2004. Chemical mixture toxicology: From descriptive to mechanistic, and going on to in silico toxicology. *Environ Toxicol*

Pharmacol 18:65–81.

9. Donnelly KC, Lingenfelter R, Cizmas L, Falahatpisheh MH, Qian YC, Tang Y, Garcia S, Ramos K, Tiffany-Castiglioni E, Mumtaz MM. 2004. Toxicity assessment of complex mixtures remains a goal. *Environ Toxicol Pharmacol* 18:135–141.
10. Eide I, Neverdal G, Thorvaldsen B, Arneberg R, Grung B, Kvalheim OM. 2004. Toxicological evaluation of complex mixtures: Fingerprinting and multivariate analysis. *Environ Toxicol Pharmacol* 18:127–133.
11. Mehler WT, Du J, Lydy MJ, You J. 2011. Joint toxicity of a pyrethroid insecticide, cypermethrin, and a heavy metal, lead, to the benthic invertebrate *Chironomus dilutus*. *Environ Toxicol Chem* 30:2838–2845.
12. Xia XH, Chen X, Zhao XL, Chen HT, Shen MH. 2012. Effects of carbon nanotubes, chars, and ash on bioaccumulation of perfluorochemicals by *Chironomus plumosus* larvae in sediment. *Environ Sci Technol* 46:12467–12475.
13. Martinez EA, Moore BC, Schaumloffel J, Dasgupta N. 2004. Effects of exposure to a combination of zinc- and lead-spiked sediments on mouthpart development and growth in *Chironomus tentans*. *Environ Toxicol Chem* 23:662–667.
14. Galay-Burgos M, Spurgeon DJ, Weeks JM, Sturzenbaum SR, Morgan AJ, Kille P. 2003. Developing a new method for soil pollution monitoring using molecular genetic biomarkers. *Biomarkers* 8:229–239.
15. Adams SM, Shepard KL, Greeley MS, Jimenez BD, Ryon MG, Shugart LR, McCarthy JF, Hinton DE. 1989. The use of biomarkers for assessing the effects of pollutant stress on fish. *Mar Environ Res* 28:459–464.
16. Van Aggelen G, Ankley GT, Baldwin WS, Bearden DW, Benson WH, Chipman JK, Collette TW, Craft JA, Denslow ND, Embry MR, Falciani F, George SG, Helbing CC, Hoekstra PF, Iguchi T, Kagami Y, Katsiadaki I, Kille P, Liu L, Lord PG, McIntyre T,

O'Neill A, Osachoff H, Perkins EJ, Santos EM, Skirrow RC, Snape JR, Tyler CR, Versteeg D, Viant MR, Volz DC, Williams TD, Yu L. 2010. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: Hurdles, achievements, and future outlook. *Environ Health Perspect* 118:1–5.

17. Marinkovic M, de Leeuw WC, de Jong M, Kraak MHS, Admiraal W, Breit TM, Jonker MJ. 2012. Combining next-generation sequencing and microarray technology into a transcriptomics approach for the non-model organism *Chironomus riparius*. *PLoS One* 7:e48096.
18. Hook SE, Skillman AD, Gopalan B, Small JA, Schultz IR. 2008. Gene expression profiles in rainbow trout, *Onchorynchus mykiss*, exposed to a simple chemical mixture. *Toxicol Sci* 102:42–60.
19. Sugiura Y, Kashiba M, Maruyama K, Hoshikawa K, Sasaki R, Saito K, Kimura H, Goda N, Suematsu M. 2005. Cadmium exposure alters metabolomics of sulfur-containing amino acids in rat testes. *Antioxid Redox Signal* 7:781–787.
20. Jones DP, Go YM, Anderson CL, Ziegler TR, Kinkade JM, Kirlin WG. 2004. Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *FASEB J* 18:1246–1248.
21. Jeppe KJ, Carew ME, Long SM, Lee SF, Pettigrove V, Hoffmann AA. 2014. Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium. *Comp Biochem Physiol C Toxicol Pharmacol* 162:1–6.
22. Long SM, Tull DL, Jeppe KJ, De Souza DP, Dayalan S, Pettigrove VJ, McConville MJ, Hoffmann AA. 2015. A multi-platform metabolomics approach demonstrates changes in energy metabolism and the transsulfuration pathway in *Chironomus tepperi* following exposure to zinc. *Aquat Toxicol* 162:54–65.
23. Simpson SL, Batley GE, Charlton AA, Stauber JL, King CK, Chapman JC, Hyne RV,

Gale SA, Roach AC, Maher WA. 2005. *Handbook for Sediment Quality Assessment*. CSIRO, Bangor, NSW, Australia.

24. Hutchins CM, Teasdale PR, Lee SY, Simpson SL. 2008. Cu and Zn concentration gradients created by dilution of pH neutral metal-spiked marine sediment: A comparison of sediment geochemistry with direct methods of metal addition. *Environ Sci Technol* 42:2912–2918.
25. Schlenk D, Lavado R, Loyo-Rosales JE, Jones W, Maryoung L, Riar N, Werner I, Sedlak D. 2012. Reconstitution studies of pesticides and surfactants exploring the cause of estrogenic activity observed in surface waters of the San Francisco Bay delta. *Environ Sci Technol* 46:9106–9111.
26. Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand. 2000. Australian and New Zealand guidelines for freshwater and marine water quality. Canberra, ACT, Australia.
27. MacDonald DD, Ingersoll CG, Berger TA. 2000. Development and evaluation of consensus-based sediment quality guidelines for freshwater ecosystems. *Arch Environ Contam Toxicol* 39:20–31.
28. Martinez-Guitarte JL, Planello R, Morcillo G. 2007. Characterization and expression during development and under environmental stress of the genes encoding ribosomal proteins L11 and L13 in *Chironomus riparius*. *Comp Biochem Physiol B Biochem Mol Biol* 147:590–596.
29. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739.
30. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—New capabilities and interfaces. *Nucleic Acids Res* 40:e115.

31. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. 2013. GenBank. *Nucleic Acids Res* 41:D36–D42.

32. Attrill H, Falls K, Goodman JL, Millburn GH, Antonazzo G, Rey AJ, Marygold SJ; FlyBase Consortium. 2016. FlyBase: Establishing a Gene Group resource for *Drosophila melanogaster*. *Nucleic Acids Res* 44:D786–D792.

33. Takahashi KH, Rako L, Takano-Shimizu T, Hoffmann AA, Lee SF. 2010. Effects of small Hsp genes on developmental stability and microenvironmental canalization. *BMC Evol Biol* 10:11.

34. Heckmann LH, Sorensen PB, Krogh PH, Sorensen JG. 2011. NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. *BMC Bioinformatics* 12:250.

35. James SJ, Melnyk S, Pogribna M, Pogribny IP, Caudill MA. 2002. Elevation in *S*-adenosylhomocysteine and DNA hypomethylation: Potential epigenetic mechanism for homocysteine-related pathology. *J Nutr* 132:2361S-2366S.

36. Hughes SL, Bundy JG, Want EJ, Kille P, Sturzenbaum SR. 2009. The metabolomic responses of *Caenorhabditis elegans* to cadmium are largely independent of metallothionein status, but dominated by changes in cystathionine and phytochelatins. *J Proteome Res* 8:3512–3519.

37. Rao AM, Drake MR, Stipanuk MH. 1990. Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes. *J Nutr* 120:837–845.

38. Banni M, Dondero F, Jebali J, Guerbej H, Boussetta H, Viarengo A. 2007. Assessment of heavy metal contamination using real-time PCR analysis of mussel metallothionein mt10 and mt20 expression: A validation along the Tunisian coast. *Biomarkers* 12:369–383.

39. Pin-Lan L, Fan Y, Ningjun L. 2007. Hyperhomocysteinemia—Association with renal

transsulfuration and redox signaling in rats. *Clin Chem Lab Med* 45:1688–1693.

40. Hoffman AE, Miles L, Greenfield TJ, Shoen C, DeStefano M, Cynamon M, Doyle RP. 2015. Clinical isolates of *Candida albicans*, *Candida tropicalis*, and *Candida krusei* have different susceptibilities to Co(II) and Cu(II) complexes of 1,10-phenanthroline. *Biometals* 28:415–423.
41. Taylor NS, Kirwan JA, Johnson C, Yan ND, Viant MR, Gunn JM, McGeer JC. 2016. Predicting chronic copper and nickel reproductive toxicity to *Daphnia pulex-pulicaria* from whole-animal metabolic profiles. *Environ Pollut* 212:325–329.
42. Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29:730–741.

Figure 1. (**A**) Survival and (**B**) dry weight of *Chironomus tepperi* larvae after exposure to different concentrations of copper and bifenthrin ($n = 3$) and mixtures of both ($n = 5$). Treatment effects were tested with analysis of variance (***$p < 0.001$; ° = marginal nonsignificance). Error bars indicate standard error of the mean.

Figure 2. Principal component analysis scores plots of 9 *Chironomus tepperi* cysteine metabolism genes after 5-d exposure to 16 treatments of copper and bifenthrin in 2-way factorial mixture of low, medium, and high concentrations (single treatments, $n = 3$; mixture treatments, $n = 5$). BH = bifenthrin high concentration; BL = bifenthrin low concentration; BM = bifenthrin medium concentration; CH = copper high concentration; CL = copper low concentration; CM = copper medium concentration; *CβS* = cystathionine-β-synthase; *CγL* = cystathionine-γ-lyase; Ctl = control; *GCS* = γ-glutamylcysteine synthase; *GS* = glutathione synthetase; *GST* = glutathione *S*-transferase delta 1; *Mtn* = metallothionein; PC = principal component; *SAH* = *S*-adenosylhomocysteine hydrolase; *SAM* = *S*-adenosylmethionine

synthetase; *TPx* = thioredoxin glutathione <**ZAQ;3**>reductase.

Figure 3. Expression profile of *Chironomus tepperi* cysteine metabolism genes after 5-d sediment exposure to copper and bifenthrin (n = 3) and mixtures of both (n = 5): (**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-CAT*); (**G**) glutathione synthetase (*GS*); (**H**) glutathione *S*-transferase delta 1 (*GST*); and (**I**) thioredoxin glutathione reductase (*TGR*). Expression was measured using real-time quantitative polymerase chain reaction and normalized using the data-driven algorithm NORMAgene. Data are displayed relative to control exposures (black line) ± standard error of the mean (black dashed lines). Parabolic trend lines are displayed for genes with significant responses to either contaminant or an interaction between the contaminants. Significance was determined with a factorial analysis of variance. Error bars indicate standard error of the mean.

Figure 4. Expression of (**A**) *S*-adenosylmethionine synthetase (*SAM*), (**B**) *S*-adenosylhomocysteine hydrolase (*SAH*), (**C**) cystathionine-β-synthase (*CβS*), and (**D**) metallothionein (*Mtn*) in 3 field sediments. Data are displayed relative to control exposures (black line) ± standard error of the mean (black dashed lines). Sites were contaminated with moderate levels of metal and synthetic pyrethroids as indicated in Table 1. Site differences were tested with Kruskal-Wallis tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Error bars indicate standard error of the mean.

<<ENOTE>> AQ1: Please add the affiliation, city, state, and country for M.M. Stevens.

<<ENOTE>> AQ2: OK as changed from "TRG"?

<<ENOTE>> AQ3: Figure 2: Correct definition for "TPx"?

<<ENOTE>> AQ4: Table 3: This note previously said significant differences were indicated

in bold. However, none of the values appeared to be bold. ET&C does not use bold font within tables, so this has been converted to a table footnote. Please indicate which values should have an “a” note. If none should, please delete the note.

Table 1. Metal and synthetic pyrethroid concentrations of site and spiked sediment treatments selected for cysteine metabolism gene expression[a]

Treatment	Ar	Cd	Cr	Cu	Pb	Hg	Ni	Ag	Zn	PECq	Bif	Per
Field collected												
Site 1	5	1	25	33	33	0.1	12	2	315	0.31	0.089	0.08
Site 2	<5	<1	47	67	94	0.1	36	<2	913	0.54	0.095	0.0
Site 3	5	<1	56	88	64	<0.1	40	<2	794	0.64	0.11	0.0
Lab spiked												
Unspiked	<5	<1	22	11	12	<0.1	10	<2	49	0.19	<0.001	<0.0
Low				44						0.22	0.032	
Medium				176						0.33	0.063	
High				308						0.44	0.23	

[a] Chemical concentrations were summarized as probable effect concentration quotient for metals and sum of synthetic pyrethroid concentrations.

Ar = arsenic; Cd = cadmium; Cr = chromium; Cu = copper; Pb = lead; Hg = mercury; Ni = nickel; Ag = silver; Zn = zinc; PECq = probable effect concentration quotient; Bif = bifenthrin; Perm = permethrin; SP = synthetic pyrethroid summed.

Table 2. Primers used to amplify target genes for quantitative polymerase chain reaction, including primer sequence, amplified fragment size, and GenBank accession number (if available)

Gene	Primer 5'–3'	Fragment size (bp)	GenBank accession no.
SAM	F – CCAAAGTTGCATGTGAAACTGTAAGC	66	Too short to be submitted,
	R – TTGATGTGATTTCACCACACAATAGG		see Jeppe et al. [21]
SAH	F – GCATTGGGTGCATCTGTTCG	107	KF740474
	R – CCGCGCCATGCAAATACAC		
CβS	F – GCAATGGCATGTGCTGTTCG	100	KF740475
	R – TTTCAGCACCAAGAGCTTTCAAGAC		
CγL	F – GGGAATGGAAGTGATTTTTGTCG	98	KF740476
	R – TTGTTGGCGTTTCCAACCAC		
Mtn	F – ACCAATCTTGCGGCCAAGG	137	Too short to be submitted.
	R – TGCAACAGTTCGTTGCAGCAG		see Jeppe et al. [21]
GS	F – CAAGCATTAGCAAAGCCTGGAATC	61	Too short to be submitted,
	R – TTGTGATTTCATCCTCATCAGTCAAG		see Jeppe et al. [21]
GCS	F – TGCGACGTGGAGAGAAGGTG	130	KF740473
	R – CCCATTCCAAAACCCATTGC		
GST	F – GCTATGGAAACTGCAATGGGATTC	83	Too short to be submitted,
	R – TCAGCAACAGTGAGGCTATCACC		see Jeppe et al. [21]
TGR	F – ACGGATGGGAACTCGAGAAGCC	110	KJ996072
	R – TCACGCAAATCAACTCTTGTCACCC		

SAM = *S*-adenosylmethionine synthetase; *SAH* = *S*-adenosylhomocysteine hydrolase; *CβS* = cystathionine-β-synthase; *CγL* = cystathionine-γ-lyase; *Mtn* = metallothionein; *GS* = glutathione synthetase; *GCS* = γ-glutamylcysteine synthase; *GST* = glutathione *S*-transferase delta 1; *TGR* = thioredoxin glutathione reductase.

Table 3. Significance of copper and bifenthrin on *Chironomus tepperi* cysteine metabolism genes after 5-d sediment exposure to copper and bifenthrin (n = 3) and mixtures of both (n = 5) as assessed by a factorial analysis of variance

Gene	Contaminant	*df*	Mean square	*F*	*p*
SAM	Bif	3	0.54	12.81	<0.001
	Cu	3	0.94	22.38	<0.001
	Cu:Bif	9	0.39	9.26	<0.001
	Residual	51	0.04		
SAH	Bif	3	0.09	2.11	0.111
	Cu	3	1.31	29.98	<0.001
	Cu:Bif	9	0.14	3.21	0.004
	Residual	51	0.04		
CβS	Bif	3	0.03	0.97	0.416
	Cu	3	0.31	10.56	<0.001
	Cu:Bif	9	0.21	7.28	<0.001
	Residual	51	0.03		
CγL	Bif	3	0.11	1.88	0.144
	Cu	3	0.18	3.18	0.032
	Cu:Bif	9	0.04	0.63	0.762
	Residual	51	0.06		
Mtn	Bif	3	26.96	8.15	<0.001
	Cu	3	327.22	98.96	<0.001
	Cu:Bif	9	24.26	7.34	<0.001
	Residual	52	3.31		

GS	Bif	3	0.16	1.90	0.141
	Cu	3	0.38	2.13	0.108
	Cu:Bif	9	0.02	2.58	0.015
	Residual	52	0.01		
GCS	Bif	3	0.08	14.37	<0.001
	Cu	3	0.09	34.90	<0.001
	Cu:Bif	9	0.11	2.05	0.052
	Residual	51	0.04		
GST	Bif	3	0.14	1.89	0.143
	Cu	3	0.26	3.43	0.024
	Cu:Bif	9	0.12	1.52	0.165
	Residual	52	0.08		
TGR	Bif	3	0.04	2.30	0.088
	Cu	3	0.11	6.01	0.001
	Cu:Bif	9	0.03	1.52	0.167
	Residual	51	0.02		

[a] **<ZAQ;4>**Significant difference after Bonferroni adjustment ($p < 0.005$).

df = degrees of freedom; SAM = *S*-adenosylmethionine synthetase; SAH = *S*-adenosylhomocysteine hydrolase; CβS = cystathionine-β-synthase; CγL = cystathionine-γ-

lyase; Mtn = metallothionein; GS = glutathione synthetase; GCS = γ-glutamylcysteine synthase; GST = glutathione *S*-transferase delta 1; TGR = thioredoxin glutathione reductase; Bif = bifenthrin; Cu = copper.