Determining the Ideal Substrate in Exposure Experiments for Ecotoxicology – a Metabolomics Approach

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Abstract

It is crucial to understand the effects caused by experimental parameters, such as temperature; light availability; and food type that can interfere when determining a dose-response relationship in toxicology exposure experiments. Limiting variability is especially important when developing metabolite biomarkers of contaminant exposure since metabolomics is very sensitive to any internal and external factors that affect organism health and metabolic status. Ecotoxicologists are well aware of the importance of experimental design but the type of substrate used in exposure experiments is generally assumed to have no effect and is not assessed. This may not always be correct. Here we investigate the different metabolic changes in the freshwater crustacean, *Austrochiltonia subtenuis* to the heavy metal copper caused using three common substrate types, gauze; toilet paper; and cellulose. Each substrate type elicited a different metabolic response over the course of the experiment indicating substrate type affected the final results of the exposure test. Metabolites were assessed using Gas Chromatography-Mass Spectrometry. Several groups were shown to change including disaccharides, monosaccharides, fatty acids and their conjugates, and TCA cycle intermediates. The results illustrate that different metabolomic responses can occur from experimental design decisions previously thought not to have a significant effect. This may in turn affect policy decisions based on the results of the experiment. We propose that metabolomics can play an important part in the design of ecotoxicity tests and that all experimental parameters should be assumed to potentially have an effect until proved otherwise.

1. Introduction

Ecotoxicity studies aim to be consistent, reliable, and reproducible, to provide the scientific community with information that can lead to decisions concerning the toxicity of contaminants and development of ‘safe’ guideline values. At this purpose, many testing guidelines are in place, such as CRED (Criteria for Reporting and Evaluating Ecotoxicity Data Project) (Moermond et al. 2016), that aimed to produce a framework to follow for peer-reviewed papers for test set-up, and data reporting (Savić-Zdravković et al. 2020). It is crucial to follow such guidelines when designing an experiment but equally to understand how certain decisions may affect a causal response that might not be accounted for in the guidelines. For example, it is known that changes in experimental parameters such food or temperature or light regime may result in changes in sensitivity of an organism exposed to a contaminant. Similarly, the use of environmental sediment as a substrate for aquatic organisms in toxicology tests may be environmentally relevant but it can also increase the variability across results because it is almost impossible to obtain a sediment that is uniform in concentrations of nutrients and other trace contaminants. For this reason, alternatives to environmental sediments, such as cellulose or gauzes, are often used in ecotoxicology experiments. These substances are assumed to be uniform and not affect an organism’s sensitivity to contaminants, but this latter assumption is not often tested.

Currently, there is no preferred option for substrates used in exposure experiments. For example, cotton gauzes are commonly used for amphipods (Moore and Farrar 1996; Jeppe et al. 2017; Vu et al. 2017; Schlechtriem et al. 2019; Everitt et al. 2020). Alternatively, another substrate is ethanol rinsed supermarket

toilet paper, which has been typically used in exposures with *Chironomus*, (non-biting midges) (Townsend et al. 2012; Jeppe et al. 2014; Long et al. 2015; Mehler et al. 2017). A third alternative substrate is powdered cellulose (Marinković et al. 2011; Gagliardi et al. 2015).

Metabolomics is a highly innovative omics technology used to efficiently monitor environmental contaminants and their adverse outcome pathways, to clarify their mechanisms of actions and to observe and characterize various modes of organismal adaptation (Environmental Toxicology and Chemistry, 2016, 35, 20-35). Moreover, thanks to this technique, it is possible to understand how the concentrations of small molecule metabolites, such as amino acids, sugars and organic acids (the metabolome), change in response to external stimuli such as disease or diet. Environmental metabolomic studies investigate the changes in the metabolome of organisms following exposure to specific environmental stressors, such as toxicants (Viant 2009). Investigating changes in metabolites can provide information about the organism's physiological status and can help elucidate a stressor's mode of action. Environmental metabolomics has been applied to a wide range of organisms, such as invertebrates, crustaceans, fish and reptiles, following exposure to manmade and naturally occurring compounds (Matich et al. 2019). In the last decades, -omics techniques, in particular proteomics and metabolomics, have been coupled with separation techniques, such as chromatography and electrophoresis, nuclear magnetic resonance and mass spectrometry. The principle advantage of using those instrumentation and data processing has been the improvement in the detection sensitivity and interpretation of changes in the metabolome, assessing simultaneously thousands different molecular responses due to particular environmental exposures of an organism (Bahamonde et al. 2016; Pinu et al. 2019), leading to the application of metabolomics to identify biochemical biomarkers of contaminant exposure. Biomarkers can play a critical role in assessing environmental exposure to chemicals, the concentration of chemicals and length of that exposure (Beger et al. 2017). With the ever-increasing sensitivity of biomarkers and more detectible/traceable effects of pollutants, at very low exposure levels, it is essential that all possible experimental effects are accounted for.

In this project, the biochemical baseline responses of a common test organism, the amphipod *Austrochiltonia subtenuis*, exposed to copper, has been investigated and the variations in function of the substrate type have been assessed (gauze; toilet paper; and cellulose). Survival of post gravid adults and juveniles was recorded following a 14-day exposure for adults and a 28-day exposure for juveniles. The amphipods biochemistry was then assessed using Gas Chromatography-Mass Spectrometry (GC-MS), focusing on polar metabolites. A large number of metabolite groups, including disaccharides, monosaccharides, fatty acid and conjugates, and Tricarboxylic Acids (TCA), have been identified and they responded differently to each substrate. This highlights the importance of accounting for substrate type in ecotoxicology experiments and also demonstrates the utility of metabolomics for assessing subtle effects that may be caused by experimental factors previously thought to be stable.

This study is the first to use a metabolomics approach to validate experimental method design in ecotoxicology. We investigate the potential importance of using biochemical approaches to validate experimental design decisions in future applications in this area.

2. Method

2.1. Organism

The amphipods *Austrochiltonia subtenuis* were originally collected from Deep Creek, Bulla Rd, Bulla, Victoria, Australia (S 37° 37.919157' E 144° 47.995837') at varying life-stages and cultured in-house at RMIT University (Melbourne, Australia). The amphipods were maintained in aquaria using a standard artificial media (SAM) modified from Borgmann (1996). The SAM consisted of reverse osmosis water (18.2 mΩ) with 0.23 mM $NaHCO_3$, 0.061mM $CaCl_2$, 0.032 mM $MgSO_4$, 0.47 mM NaCl, 0.0087mM KCl, 0.17 mM $MgCl_2$ and 0.0009 mM NaBr. Cultures were maintained at 21 ± 1°C under a 16:8h light:dark photoperiod. The culture was fed with powdered fish food (Tetramin®, Tetra Werke, Melle, Germany) and yeast–cerophyll–trout chow (YCT) made-up inhouse every second day.

2.2. Substrates

Cellulose was purchased from Sigma-Aldrich (product no. C6429- Merck KGaA., St. Louis, Missouri, United States). Toilet paper was purchased from a local supermarket. It was rinsed thoroughly with 100% ethanol and left to dry under fume hood to ensure sterilization before use. Gauze was cut from sterilized bandage (Livingstone Triangular bandage – bleached calico), (Livingstone, Mascot, New South Wales, Australia) obtained from a local chemist.

2.3. Substrate exposure

Five replicate beakers (600 mL) were used for each treatment. Gauze was cut into 2x2 cm squares and two squares were added to each of the five replicates and topped up SAM. A one ply sheet of toilet paper was roughly torn into quarters for each of the five replicates and the beakers topped up with SAM. Approximately 1.5(± 0.05)g of powdered cellulose was weighed into each beaker, then topped up with SAM. A further five beakers with 400mL of SAM without a substrate, i.e. water only, were used as a negative control for the experiment. All beakers were placed in a light (16-8h cycle) and temperature-controlled room (19 ± 1^{o}C) over night with constant aeration to allow the substrates to settle. On day one of the test, gravid amphipods (n=200) were collected from culture tanks and 10 gravid amphipods were randomly added to each replicate.

2.4. Amphipod culture

During the exposure, amphipods were fed every second day with 1mL powdered fish food (Tetramin®, Tetra Werke, Melle, Germany) solution (90 mg of ground Tetramin/50 ml reversed osmosis water) and 1mL yeast–cerophyll–trout chow (YCT). The water was changed every week with freshly made up SAM and the quality of the water was checked (dissolved oxygen; conductivity; pH; temperature and ammonia). Dissolved oxygen was >80%; pH remained in range 7-8; conductivity and ammonia remained consistent (supplementary material, Table S1).

On Day 14, adult post-gravid amphipods were removed and counted, then quenched on dry ice and stored at -80^{o}C for further analysis. Juveniles were counted (total number for each replicate and survival in Supplementary Material, Table S2) and replicates were further separated to reduce space competition in beackers (i.e. cellulose replicates were further separated by 50/50 into cellulose 1A and 1B (Total 97, 1A=49 and 1B=48 individuals). Fresh substrates were added to each treatment and exposure continued for a further 28 days. Amphipods were fed every second day and water was changed each week and its quality checked.

On day 28, the experiment was ended. The juveniles were counted, dried and then pooled and placed on dry ice for instant quenching of metabolites, and stored at -80^{o}C until metabolomic analysis.

2.5. Copper exposure using different substrates

Fifteen beakers (600mL), were set up with 400mL of SAM spiked with 5% of the LC_{50} for copper (0.0605 mg/L), based on Australian and New Zealand marine and freshwater guidelines (ANZG, 2018). Here we used five replicates per treatment. Each beaker contained either gauze, toilet paper or cellulose and were run alongside 15 x 600mL substrate beakers with only 400ml of SAM, as controls. Forty 14-day old amphipods were collected from the amphipod culture from RMIT University (Melbourne, Australia) and quenched on dry ice at the commencement of the experiment for control. Another forty 14-day old amphipods were randomly added to each beaker and kept in a temperature-controlled incubator and fed every two days with 1mL powdered fish food (Tetramin®, Tetra Werke, Melle, Germany) solution (90 mg of ground Tetramin / 50 ml reversed osmosis water) and 1mL yeast–cerophyll–trout chow (YCT). At 7 days, water quality was recorded (supplementary material, Table S3) and 50% of water was changed. The exposure was kept for 14 days in a temperature-controlled incubator (19 ± 1 ^{o}C) with light (16-8h cycle with aeration. Cellulose, toilet paper and gauze were collected from each replicate (with and without copper) at the end of the exposure and stored in -20°C freezer prior to perform copper analysis.

At the end of the exposure, surviving amphipods were counted and placed in a microcentrifuge tube on dry ice for instant quenching of metabolites. Then stored in -80°C freezer until further analysis. Survival rates of amphipods from substrate and copper exposure was recorded.

2.6. Inductively coupled plasma mass spectrometry (ICP-MS) for water and substrate measurements

Water samples (50mL) were collected at the beginning and at the end of the exposures from each copper and control treatment to determine the copper concentration (n = 6). Copper concentration was measured using ICP-MS. 1mL of nitric acid has been added to each test tube to be then heated to 80^{o}C on a heating block in large test tubes (50mL) for over 2 hours. Once water samples had cooled, they were transferred to 50mL falcon tubes. Here, the water samples were filtered through a 45µm Syringe Filter, then made up to 50mL using Milli Q water.

Substrates collected at the conclusion of the experiment were placed in glass test tubes in 60^{o}C oven for 48 hours until completely dry. Dry substrates were then placed on a heating block and 200µL of nitric acid was added to each sample and heated for 5 hours. Once cool, 4mL of Milli Q water was added, and the supernatant was collected into falcon tubes. The substrate samples were filtered through a 45µm Syringe Filter, then made up to 5mL using Milli Q water in preparation for the ICP-MS.

All samples were analyzed for copper using an Agilent 7700×quadrupole-type ICP-MS (Agilent Technologies, Mulgrave, Australia), equipped with an Agilent ASX-520 autosampler following EPA Method 3051A (EPA and of Resource Conservation, 2007). The instrument was operated in He-mode. The integration time was 0.3s per mass, 1 point per mass, 3 replicates, and 100 sweeps per replicate. The Agilent Environmental standard for ICP-MS was used for quantification (Supplementary material, Table S4).

2.7. Sample preparation and metabolite extraction

Metabolites were extracted from each sample by first adding 600µL of methanol:chloroform (9:1 ratio), then homogenized individually by grinding the amphipods with a glass pipette and placed on dry ice. Once cooled, samples were sonicated for a 15 mins in an ultrasonic water bath with a timer and heater, to ensure total extraction, then they were placed back on dry ice. In order to collect the supernatant, all samples containing methanol:chloroform mixture were centrifuged at 5500 rpm (3382 RCF = 1.12 x 10 x $(5500/1,000)^{2}$) for 15 mins. The supernatant was placed into either a glass inserts or a clean microcentrifuge tube, depending on the biomass of the sample, and dried down overnight under an air pump in a fume hood. Substrate experiment had a greater tissue biomass, whereas copper exposed experiment didn't, so a glass insert was used to concentrate the sample. The dried extracts were derivatized using either 30µL (substrate) or 20µL (copper) of Methoxyamine in pyridine solution, (20 mg/mL). The samples were then vortexed for 30 seconds and left for 17 hours at room temperature in the fume hood. Next, 30µL (substrate) or 20µL (copper) of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were added to each sample, vortexed and left for one hour. Analytical grade hexane was then added to each sample (600µL for substrates; 300µL for copper) in order to perform GC-MS analysis. A pooled biological quality control (PBQC) was prepared by pooling 37.5µL of extract from each sample mixing thoroughly and aliquoted into 5 replicates. PBQCs were analysed along with the samples to assess repeatability, instrument drift and quality control.

2.8. Gas Chromatography-Mass spectrometry

The GC–MS analysis was performed using an Agilent 7890B gas chromatograph coupled to an Agilent 5977B mass spectrometer (Santa Clara, CA). The gas chromatography had a splitless mode with a purge flow to split vent at 2 mins. The separation was performed with an analytical column (30 m × 250 µm, 0.25 µm) using helium as carrier gas at a flow rate of 1mL per min. The injection volume was 1µL, the injector temperature was 250°C. The oven temperature was programmed from 35°C to 300°C at 25°C·min^{-1} and held at this temperature for 5 min, and finally rise to 310°C (at 5°C·min^{-1}) and kept for 5 min. Mass spectra were recorded at 1.5 scans/s over an m/z range of 35 –550.

Metabolites were putatively annotated as level 2 compounds according to the chemical reporting standards in Sumner et al. (2007) (based upon spectral similarity with public/commercial spectral libraries) where possible, using the NIST library for identification and using metabolite standards as part of an in-house database. The remaining compounds were either identified to level 3 or 4. Level 3 IDs were based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class. Level 4 IDs mean the compounds were unknown but although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data. Raw area retention times (RT) from each peak that occurred from each treatment was recorded and is listed in supplementary material (Table S5 and S6). Information regarding environmental metabolomic reporting standards to as described by Morrison et al. (2007) is provided in the supplementary information.

2.9. Statistics

A combination of multivariate and univariate data analysis was conducted as complete strategy for identification and selection of biomarkers using the total area of abundance for each metabolite, as described in Lacalle-Bergeron et al. (2020). Controls with adults and juveniles amphipods together with copper exposed amphipods were analysed separately. Metabolite values were normalized to the median value of each treatment to account for differences between samples. Median normalization was used to better account for unwanted system variation when dealing with metabolite extractions from whole organisms (Sinclair, et al., 2019).

Values were then transformed ($\log_{natural}$) to reduce variance between metabolite abundance. Multivariate statistical analysis was applied as a first step for interrogating the data in order to observe trends, grouping and/or outliers (Lacalle-Bergeron et al. 2020). Detecting differences across treatments using Principal Component Analysis (PCA) can be difficult due to the sensitive biological variables affecting metabolites between each individual amphipod (Lacalle-Bergeron et al. 2020). Orthogonal PLS-DA (with appropriate model overview) is a common method for determining separation in metabolomic data sets due to treatments when natural biological variation outweighs treatment variation (Blasco et al. 2015). Therefore, orthogonal partial least squares (OPLS-DA) analysis was carried out using online MetaboAnalyst software (Version 4.0, Xia Lab, McGill University, Anne de Bellevue, Quebec, Canada), using both adult, juvenile and copper data sets to visualize overall separation of treatments (Pang et al. 2021).

Analysis of variance (ANOVA) across metabolites and substrates for adults and juveniles as control and for amphipods exposed to copper, was used to determine significant differences between substrates ($p<0.05$). Following this, Tukeys *post hoc* test determined significance between treatments for each substrate. Metabolites were analysed using one-factor analysis of variance with treatment as the main effect with R Version 3.0.3 (R Development Core Team 2006). Each p-value was adjusted for false discovery rate using Benjamini-Hochberg (BH) method for 1% in order to determine which metabolites responded significantly to treatments. Pathway analysis was carried for significant metabolites using

online MetaboAnalyst software (Version 4.0, Xia Lab, McGill University, Anne de Bellevue, Quebec, Canada).

3. Results

3.1. Substrate Exposure to Adult and Juvenile Amphipods

3.1.1. Survival

There was a 36% reduction in adults survival after the water-only exposure. The survival of juveniles was too low for continuation of the juvenile 28-day exposure with water-only (> 5 surviving individuals per replicate from the post-gravid adult exposure). This result demonstrates that the amphipods need a substrate to survive and thus picking the correct material is essential to the experiment. There was a 5-10% decline in adults survival across all other

substrates (Fig. 1a). The percentage of juveniles survival varied more across the treatments, compared to adults (Fig. 1b). Toilet paper was characterized by a 36.4% juveniles survival, from initial count to day 28. Cellulose was characterized by a juveniles survival of 68% and gauze presented the highest average juveniles survival average of 81%.

3.1.2. Metabolites in adult and juvenile amphipods following to exposure to the different substrates

As large data matrices were obtained, statistical tools, such as multivariate analysis, were necessary to reduce the data complexity and reveal underlying trends in featured metabolites. Univariate analysis of significant metabolite features was then used to further elucidate the overall effects of the substrates on adult, juvenile and copper exposed amphipods. There was separation in the overall metabolite profile in adult and juvenile amphipods exposed to the different substrates (Fig. 2a and b). This was particularly marked in the juvenile amphipods (Fig. 2b)., where there was a clear separation between all three groups. In contrast there was less separation between the metabolic profiles of adult amphipods, particularly among the cellulose and gauze substrate groups.

Substrate had a significant effect on the abundance of several metabolites in both adult and juvenile amphipods. The adult amphipods had 20 significant metabolite features that changed in response to cellulose, toilet paper, gauze and water-only treatments (Table 1); whereas, the juveniles had 15 significantly different metabolite features, from three substrate treatments. (Table 2). Tetradecanoic acid was significantly different in the adult amphipods exposed to cellulose compared to the other treatments. Octadecanoic acid levels were significantly different in the juveniles while adult amphipods exposed to cellulose had a significant difference compared to the other substrates. The toilet paper and cellulose substrate groups showed 13 metabolites that significantly changed in the adults compared to the other

treatments. Whereas, in the juveniles, gauze and cellulose presented 8 significant metabolites that significantly changed compared to the other treatments.

Table 1

One-way analysis of variance for individual metabolites, measured following 14-day adult amphipods substrates exposure (gauze, cellulose, toilet paper and a water only negative control). Values listed are the significant differences of metabolite abundance between treatments (BH adjusted, P < 0.05). Using a 95% family-wise confidence level.

Metabolite	**Mean square residual**	**Treatment**	**Comparison between substrate**					
df	*13*	*3*	*Gz - Cell*	*TP-Cell*	*W-Cell*	*TP-Gz*	*W-Gz*	*W-TP*
Tetradecanoic acid	0.145	0.000	0.000	0.000	0.000	0.765	0.992	0.874
Gluconic acid	0.109	0.000	0.000	0.725	0.971	0.000	0.000	0.442
Glucose	0.188	0.000	0.014	0.644	0.000	0.119	0.002	0.000
Succinic acid	0.261	0.000	0.342	0.000	0.010	0.000	0.252	0.002
Glutaric acid	0.188	0.000	0.301	0.000	0.000	0.008	0.005	1.000
Octadecanoic acid	2.149	0.001	0.243	0.002	0.001	0.061	0.047	1.000
Glyceryl monostearate	0.477	0.001	0.833	0.006	0.002	0.029	0.011	0.984
Glycine	0.692	0.001	0.849	0.001	0.374	0.004	0.842	0.012
Trehalose	0.239	0.001	0.997	0.500	0.013	0.391	0.019	0.001
Palmitic acid	0.382	0.002	0.005	0.003	0.288	0.990	0.094	0.054
Unidentified 3	0.690	0.002	0.215	0.017	0.001	0.482	0.056	0.563
Unidentified 1	0.235	0.002	0.886	0.015	0.690	0.056	0.286	0.001
Androsterone	0.108	0.003	0.398	0.002	0.286	0.032	0.999	0.031
Unidentified 2	0.830	0.004	0.948	0.041	0.042	0.015	0.015	0.998
Propanoic acid	0.165	0.004	0.372	0.932	0.005	0.703	0.112	0.014
Valine	0.232	0.006	0.935	0.011	0.997	0.031	0.974	0.011
Turanose	2.218	0.007	0.854	0.026	0.017	0.106	0.077	1.000
Valeric acid	0.136	0.012	0.286	0.374	0.500	0.016	0.021	0.987
Myo inositol	0.215	0.013	0.037	0.930	0.970	0.012	0.060	0.710

1 Gz – Gauze; TP– Toilet paper; Cell– Cellulose; W – water.

2 List of unidentified metabolites, mass: charge ratio and retention time listed in supplementary material Table S1

Metabolite	Mean square residual	Treatment	Comparison between substrate					
Elaidic acid	0.137	0.017	0.080	0.085	1.000	1.000	0.064	0.069
1 Gz – Gauze; TP– Toilet paper; Cell– Cellulose; W – water.								
2 List of unidentified metabolites, mass: charge ratio and retention time listed in supplementary material Table S1								

Table 2
One-way analysis of variance for individual metabolites, measured following 28-day juvenile amphipods substrates exposure (gauze, cellulose, toilet paper). Values listed are the metabolites whose abundance differ significantly between treatments (BH adjusted, P < 0.05). Using a 95% family-wise confidence level.

Metabolite	Mean square residual	Treatment	Comparison between substrates		
df	*10*	*2*	*Gz - Cell*	*TP -Cell*	*TP –Gz*
Octadecanoic acid	9.61E+13	0.001	0.001	0.016	0.141
Glycerol	3.17E+12	0.002	0.002	0.019	0.446
Palmitic acid	9.64E+12	0.006	0.005	0.117	0.192
Proline	1.04E+14	0.011	0.802	0.039	0.011
Alanine	1.55E+14	0.012	0.910	0.033	0.013
Unidentified 3	1.06E+12	0.016	0.027	0.026	1.000
Unidentified 7	7.26E+11	0.018	0.014	0.277	0.225
Sucrose	4.64E+12	0.018	0.019	0.916	0.098
Unidentified 1	2.43E+10	0.018	0.029	0.030	0.988
Glutamic acid	3.27E+13	0.023	0.912	0.096	0.023
Glutamine	7.48E+12	0.024	0.064	0.026	0.754
Androst amine	1.43E+12	0.026	0.022	0.452	0.186
Glucose	1.15E+14	0.027	0.546	0.151	0.022
Talose	3.27E+13	0.031	0.187	0.509	0.027
Valine	1.46E+13	0.044	0.918	0.107	0.046
1 Gz – Gauze; TP– Toilet paper; Cell– Cellulose.					
2 List of unidentified metabolites, mass: charge ratio and retention time listed in supplementary material Table S2					

3.2. Copper exposure responses to alternative substrates

3.2.1. Survival

Survival of amphipods following the 14-days exposure to substrates with no copper, was between 84-91%. There was a slight decrease of 5% survival in the toilet paper substrate after copper exposure and a decrease of 3.5% in the cellulose after copper treatment. The use of gauze as a substrate induced a significant ($p<0.05$) decline in survival of amphipods exposed to copper (Fig. 3).

3.2.2. Change in metabolite abundance of amphipods following copper exposure

When the data from the amphipods exposed to copper have been combined with those from the controls, irrespective of substrate, an overlap was observed and only few treatments separated from the controls. However, potentially using that many groups in a model may make it unreliable (Fig. 4). When the substrates were considered separately, there was a separation in amphipod responses respect to copper, depending on the substrate (Fig. 5a-c). There was no clear separation between metabolites from day-0 control amphipods (collected at the commencement of the experiment), and amphipods exposed to copper spiked cellulose and the unexposed amphipods with cellulose as a substrate (Fig. 5a). Differently, there was a clear separation in the treatments with toilet paper as a substrate, between the control amphipods (sampled at day 0), copper spiked and unexposed amphipods (Fig. 5b). Additionally, there was an even more distinct separation between treatments when gauze was the substrate (Fig. 5c). It is initially clear from the OPLS-DA that gauze induced a larger metabolic response to copper then cellulose.

3.2.3. Univariate Analysis of Amphipods following Copper Exposure

The abundance of thirteen metabolites changed significantly in control groups compared to copper spiked substrates. Many of these metabolites showed a significant response between controls and substrate, not respect to copper treatments. For example, there were six significant metabolite features that differed significantly in the cellulose treatment. However only one metabolite, galactopyranoside, was altered in relation to exposed and unexposed amphipods to copper spiked cellulose (Table 3). Similarly, there were five metabolite features that significantly changed across in the toilet paper experiment, however four of these were between controls at the commencement of the exposure and the substrate unexposed and exposed to copper. Only one metabolite, glycine, responded significantly between copper-exposed and unexposed using toilet paper (Table 4). Furthermore, gauze had the greatest number of significant metabolites identified following copper exposure; 13 metabolites had a significant response in the gauze treatment (Table 5). Glycine increased an average of 50% in both copper exposed toilet paper and gauze; however it increased by 10% in toilet paper substrate group and decreased by 10% in the gauze substrate group. In both cellulose and gauze treatment groups, linolenic acid increased in abundance. Galactopyranoside increased in cellulose but remained similar abundance to the controls in

amphipods exposed to copper. In gauze, galactopyranoside levels increased when exposed to copper and decreased in the amphipods from the unexposed treatment.

Table 3

One-way analysis of variance for individual metabolites, measured following 14-day amphipod copper spiked cellulose substrate. Values listed are the significant differences of metabolite for treatments (P < 0.05) and between treatments, including day 0 control amphipods (BH adjusted, p < 0.05). Using a 95% family-wise confidence level.

Metabolite	**Mean square residual**	**Treatment**	**Comparison between Cellulose and copper spiked cellulose amphipods**		
df	*11*	*2*	*Cell – Ctrl*	*Cell – Cu Cell*	*Cu Cell – Ctrl*
Glycerol	0.123	0.000	▼ 0.000	0.747	▼ 0.000
Trehalose	0.079	0.001	▼ 0.001	0.352	▼ 0.005
Galactopyranoside	0.150	0.006	▼ 0.009	0.012	0.989
Lactic acid	0.672	0.012	▼ 0.159	0.332	▼ 0.009
Linolenic acid	0.107	0.018	▼ 0.019	0.052	▼ 0.806
Eicosenoic acid	0.718	0.032	▼ 0.135	0.760	▼ 0.031
▼ Decreased in abundance compared to control amphipods.					
▲ Increased in abundance compared to control amphipods.					

Table 4

One-way analysis of variance for individual metabolites, measured following 14-day amphipod copper spiked Toilet Paper substrate. Values listed are the significant differences of metabolite for treatments (P < 0.05) and between treatments, including day 0 control amphipods (BH adjusted, p < 0.05). Using a ninety-five percent family-wise confidence level.

Metabolite	**Mean square residual**	**Treatment**	**Comparison between Toilet Paper and copper spiked Toilet Paper amphipods**		
df	*12*	*2*	*TP – Ctrl*	*TP – Cu TP*	*Cu TP – Ctrl*
Glycerol	0.243	0.000	▼ 0.000	0.811	▼ 0.000
Lactic acid	0.368	0.000	▼ 0.000	0.857	▼ 0.000
Palmitic acid	0.218	0.000	▼ 0.000	0.069	▼ 0.001
Glycine	0.211	0.001	▼ 0.647	0.004	▼ 0.001
Eicosenoic acid	0.701	0.026	▼ 0.044	1.000	▼ 0.043
▼ Decreased in abundance compared to control amphipods.					
▲ Increased in abundance compared to control amphipods.					

Table 5
One-way analysis of variance for individual metabolites, measured following 14-day amphipod copper spiked Gauze substrate. Values listed are the significant differences of metabolite for treatments ($P < 0.05$) and between treatments, including day 0 control amphipods (BH adjusted, $p < 0.05$). Using a ninety-five percent family-wise confidence level.

Metabolite	**Mean square residual**	**Treatment**	**Comparison between Gauze and copper spiked Gauze amphipods**		
df	*12*	*2*	*Gz – Ctrl*	*Gz – Cu Gz*	*Cu Gz – Ctrl*
Glycerol	0.142	0.000	▼ 0.000	0.773	▼ 0.000
Glucose	0.490	0.000	▼ 0.000	0.020	▼ 0.000
Glycine	0.178	0.000	▼ 0.444	0.000	▼ 0.000
Propanoic acid	0.245	0.000	0.883	0.000	▼ 0.000
Succinate acid	0.636	0.000	▼ 0.196	0.000	▼ 0.000
Palmitic acid	0.936	0.000	▼ 0.000	0.003	▼ 0.142
Tetradecanoic acid	0.324	0.001	1.000	0.002	▼ 0.002
Galactopyranoside	0.998	0.001	▼ 0.352	0.001	▼ 0.012
Isopropanol	0.538	0.002	▼ 0.333	0.002	▼ 0.022
Linolenic acid	0.103	0.003	▼ 0.667	0.016	▼ 0.003
Lactic acid	1.803	0.005	▼ 0.360	0.053	▼ 0.004
Trehalose	0.063	0.007	▼ 0.092	0.303	▼ 0.006
Eicosenoic acid	1.533	0.034	▼ 0.027	0.338	▼ 0.309
▼ Decreased in abundance compared to control amphipods.					
▲ Increased in abundance compared to control amphipods.					

3.3. Metabolite Pathway analysis

3.3.1. Pathway analysis of adult & juvenile amphipods following substrate exposure

The experimental observations can be placed into relevant biological context using pathway analysis tools (Karnovsky and Li 2020). At this purpose, a pathway analysis was determined taking in consideration significant metabolites identified to respond at different life stages, and it has been observed which pathways are affected in the organism. Seven metabolite pathways were identified to

respond across all three substrates (cellulose, toilet paper and gauze) in amphipods at both life stages. Whereas nine pathways were uniquely found to change in the juveniles and seven in the adults (Fig. 5).

3.3.2. Pathway analysis of amphipod from different substrates exposed to copper

In this work, it has been observed that four pathways changed significantly in response to copper, irrespective of substrate: galactose metabolism; glycerolipid metabolism; glycolysis metabolism and pyruvate metabolism (Fig. 6). Interestingly, when gauze was used as a substrate, an additional six metabolite pathways were affected by copper exposure, including alanine, aspartate and glutamate metabolism, butanoate metabolism, glutathione metabolism, propanoate metabolism and the tricarboxylic acid (TCA) cycle. These metabolite pathways have not been observed when toilet paper or cellulose were used as substrates. Additionally, toilet paper and cellulose had affected pathways that were different between the two substrates, while all the pathways were detected in the gauze treatment. For example, alpha-linolenic acid metabolism and starch and sucrose metabolism were shown to be affected by both cellulose and gauze treatments whereas toilet paper and gauze affected aminoacyl-tRNA biosynthesis, fatty acid regulation, glycine, serine and threonine metabolism and glyoxylate and dicarboxylate metabolism.

4. Discussion

Clear separation across substrates in the juvenile amphipods following 28-days exposure was observed, especially when compared to adult amphipods response following 14-days exposure. This might be due to the extended length of exposure for the juveniles and due to their higher sensitivity to external conditions respect to adults, being under major developmental stages of their life cycle (Vu et al., 2017).

4.1. Substrate life stages exposure

The use of a negative control (i.e. no substrate) emphasized the rationale behind carrying out exposures with artificial substrates, as these experiments resulted in low survival in adults after 14-days (Fig. 1A). This indicates that the organisms were likely under stress from not having substrates (which they would usually burrow into). Specifically, glucose reduced in abundance in the water-only controls compared to all other substrates in both post-gravid adults and juveniles (Table 1 and 2). Glucose contributes to glycolysis, gluconeogenesis, and glycogenolysis and is the predominant fuel for cells that depends largely on anaerobic metabolism, cells that lack mitochondria, and tissues such as the brain that normally cannot use other metabolic fuels (Bhagavan and Ha 2011). Other monosaccharides and disaccharides, e.g. talose, sucrose and turanose, responded differently to the different substrates in adults and juveniles, indicating a high likelihood of impact on biological functioning that relies on these metabolites. For example, gluconic acid decreased in abundance from the gauzes treatment but increased in the other two substrates. Gluconic acid is a carboxylic acid formed by the oxidation of glucose, which then acts as the fundamental building blocks for polysaccharides (Fuessl et al. 2012). In

adult amphipods, trehalose and myo inositol responded to the different substrates (Table 1). Trehalose had the highest abundance in toilet paper exposed groups, followed by gauze and then cellulose. It is a widespread disaccharide whose function appears to protect cells against desiccation, which can have irreversible impacts on an organism's overall function if disrupted (Hengherr et al. 2008). Myo inositol had an increased abundance in toilet paper and cellulose groups but decreased in the gauze exposed group. Myo inositol is also an important sugar that mediates cell signal transduction in response to a variety of hormones and growth factors (López-Gambero et al. 2020). The effect on growth factors, varying in the different substrates, can lead to misidentification of effect from a contaminant of interest depending on the substrates used. The change in abundance of these metabolites between treatments show that the amphipods are responding differently to the different substrates which is an important aspect in order to assess exposures using metabolomics endpoints.

Several amino acids were identified in the adult and juvenile amphipods whose levels changed in response to the different substrates, such as valine, proline, alanine, glutamic acid and glutamine. These contribute to the biosynthesis of proteins, can act as a source of energy, and aid in the utilisation of sugars (Cruzat et al. 2018). These changes in amino acids can lead to effects on growth and fecundity overtime in the amphipods, potentially impacting links to causal-effect investigations.

A number of fatty acid metabolites identified are involved in a range of chemical processes that contribute to the functioning of organisms such as breakdown of fats by pancreatic lipase (glyceryl monostearate); lipid peroxidation (tetradecanoic acid); metabolism of amino acids, including lysine and tryptophan (pentanedioic acid); precursors for a variety of important metabolites such as glutathione, porphyrins, purines, haem, and creatine, that are antioxidant and/or anti-inflammatory (glycine). Other metabolites affected by the substrate treatments include glycerol, which is the backbone found in lipids, known as glycerides (palmitic acid. Glycerol has also the ability to increase levels of cholesterol and to promote fat deposition that is stored as glycogen or used for lipids that can be used as temporary protection during chemical synthesis or other chemical reactions (elaidic acid). There are a number of metabolites involved in a variety of cellular functions, which has been shown to be affected by the choice of substrate alone. This can then lead to potentially affecting the development and response to pollutants in juveniles more so then adult amphipods. The extent of functions and pathways that have been altered due to the exposure of different substrates has shown to be paramount when investigating the baseline responses in organisms.

Substrate Pathways

There were six specific pathways in adults-only that responded to the substrates (Fig. 5), such as glycine, serine and threonine metabolism, which feeds into the tricarboxylic acid (TCA) cycle, via pyruvate. The TCA cycle has also been highlighted in the adults to be affected by the substrates (Fig. 5). The TCA cycle is used by organisms to generate energy for survival. It also provides precursors of certain amino acids that are used in numerous other reactions (Ryan et al. 2021). Its central importance to many biochemical pathways suggests that, once the overall health of the organism is disrupted, it can have much larger

follow on impacts (Cavalcanti et al. 2014). Propanoate metabolism is another intermediate of the TCA cycle that has undergone changes with the different substrates, clearly indicating that already after 14-days adult amphipods had a significant response to the various substrates. On the other hand, nine pathways responded to the different substrates in juveniles, including but not limited to, arginine and proline metabolism, which is one of the central pathways for the biosynthesis of the amino acids arginine and proline from glutamate (Patin et al. 2017). This is further demonstrated with glutamine and glutamate metabolism that responded to the different substrates as well (Fig. 5). Glutamine and glutamate metabolism can also have an effect on ammonia homeostasis (Kelly and Stanley 2001). Additionally, there were ten pathways in adults and juveniles that responded to the different substrates, such as alanine, aspartate and glutamate metabolism; galactose metabolism; glutathione metabolism and glycolysis and gluconeogenesis. It is important to note that the total number of pathways affected by the choice of alternative substrates could have follow on effects for the health and functioning of the amphipods, no matter where the stage of its life cycle.

This is an explorative study demonstrating a shift in metabolites caused by substrates in a typical ecotoxicology assessment. A well-known challenge in metabolomic studies is the ability to identify and link a metabolite to a biological process. Unknown metabolites deemed significant can often still be valuable to other researchers. Therefore, minimal requirements of reporting for unknown metabolites (retention time, prominent ion and fragment ion) has still been fulfilled in this current study (Supplementary Material, Table S4 and S5) (Sumner et al. 2007; Lacalle-Bergeron et al. 2020). Regarding the identified metabolites, certain pathways indicated activation and inhibition occurring in many sugar and lipid functions. These all are likely to feed into the overall fitness of the organism, potentially reducing its tolerance to other contaminants by weakening their energy reserves, as the overall survival indicated. Baseline environmental metabolomics can play a pivotal role in identifying biomarkers for environmental exposure. Therefore, these methodological decisions need to be assessed prior to running an exposure to understand what may be occurring before a contaminant is added. The data also show that metabolomics has a part to play in testing experimental design.

Copper exposure

Exposures to the heavy metal copper were used to determine whether there was increased tolerance/sensitivity in the amphipods as a result of the choice of substrate. Survival remained relatively consistent across the three substrate treatment groups, suggesting this may not be a sensitive assessment tool. However, metabolomics analyses provided a more in-depth insight into the varying effects of the substrates on the amphipods, when exposed to copper. Cellulose had more of an overlap of response, compared to the amphipod's response to copper in toilet paper and gauze. The separation between control and treatment groups (substrate and copper spiked), was particularly clear in the gauze treatment. This was further shown in the univariate analysis with gauze treatment, having 13 significantly responding metabolites identified, compared to cellulose that had six metabolites and toilet paper, that had five metabolites. However, between the metabolites that significantly responded, cellulose treatment had only two metabolites that differed between unexposed and copper-spiked amphipods:

galactopyranoside (p-value = 0.012), a contributor to the galactose metabolism and linolenic acid (p-value = 0.052), an essential fatty acid. Toilet paper was characterized by only glycine (p-value = 0.004) as a significantly responding metabolite between the substrate and copper spiked. Furthermore, when gauze was used as a substrate, an additional seven metabolites responded significantly between unexposed and copper spiked amphipods: glucose, propanoic acid, succinate acid, palmitic acid, tetradecanoic acid, isopropanol and lactic acid (all with a p-value <0.05) (Table 5).

Copper Pathways

Similar pathways have been identified in the experiment involving adults and juveniles substrates exposure, as to be expected as the only change was the addition of spiking the heavy metal copper to the treatment groups. Interestingly, four pathways were highlighted across each of the treatments groups: galactose metabolism, an important component of glycolipids and glycoproteins (Cohn and Segal 1973); glycolysis/gluconeogenesis metabolism that converts glucose to pyruvate prior to entering the TCA cycle; glycerolipid metabolism, necessary for membrane formation, caloric storage and crucial for intracellular signaling processes (Voelker 2013); pyruvate metabolism, contributing to the TCA cycle (Fig. 6). The two pathways that have been highlighted in both cellulose and gauze are linolenic acid metabolism, essential metabolism of fatty acids and starch and sucrose metabolism, responsible for the conversion of disaccharides to monosaccharides. The six pathways highlighted in both toilet paper and gauze are fatty acid biosynthesis, degradation and elongation; glycine, serine and threonine metabolism, that aids in protein balance and immune reactions in organisms; glyoxylate and dicarboxylate metabolism, characterized by a variety of reactions involving the two metabolites that contribute to the TCA cycle (Li et al. 2020); and amino acyl-tRNA biosynthesis that is pivotal to determine how the genetic code is interpreted as amino acids (Ling et al. 2009). Gauze presented an additional five pathways, including the TCA cycle, mentioned earlier to have a number of metabolites that feed into this cycle but only being detected in the gauze treatment; alanine, aspartate and glutamate metabolism is responsible for glutamine turning into glutamate, which is used by the TCA cycle (Sinclair et al. 2019). Additionally, butanoate glutathione and propanoate metabolisms has been detected in the gauze treatment.

Amphipods exposed to copper tended to show a more sensitive response when using gauze as a substrate, as suggested by the number of significantly responding metabolites to affected pathways. Additionally, gauze substrate resulted in better differentiating between copper-exposed and unexposed amphipods. Alternatively, cellulose made the amphipods more tolerant to the same concentration of copper. The alternative substrate for aquatic ecotoxicology assessments needs to not only be optimal for the organisms but also to provide repeatability and ease of use. The difficulties associated with toilet paper, cellulose and gauze can lead to indirect effects on the set up and pack down of exposures. Gauze provided repeatability from the sterilized bandaged being used, as it did not degrade over the period of exposure and most importantly had the highest survival. Therefore, gauze was the most user friendly and optimal substrate for experimental repeatability.

Future work can determine the range of parameters that may increase or decrease an organism's stress response to contaminant exposure, such as determining the effect of sampling from an established culture, the pack down methodology, feeding regimes, and light and temperature exposure (Rosenblum et al. 2006; Shi et al. 2012). With the sensitivity of biomarkers ever increasing, it is paramount to understand the impact of laboratory practices on an organism both in terms of reproducibility and repeatability and most importantly for accurate and trustable results.

5. Conclusion

Ecotoxicology experiments are useful tools to deeply understand the effects of exposure to contaminants on terrestrial and aquatic organisms and they generally provide data to derive guideline values for the protection of species in the environment. On this research work, it has been found that gauze was the most appropriate substrate to use for ecotoxicology experiments using amphipods as target organisms and it should be used going forward, especially when looking at metabolomic endpoints and other biochemical sublethal endpoints. This information can add value to our overall understanding of causal relationships between exposure and contaminants in the ecosystems or potentially linked to human health. Results from such experiments can carry weight in the way experiments are conducted or how environments are remediated, therefore it is essential to have a valid and trustable connection between the identified effects and the exposure. The current study demonstrates the need to ensure the methodology that has been widely adopted and used for many years, such as the choice of substrates in ecotoxicological experiments, is appropriate for the sensitive markers of exposure we are in search of today.

Declarations

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Data Availability: Data is available in Supplementary martial. Raw data/ additional data available upon request from the corresponding author.

Animal Research (Ethics): Not Applicable (N/A) for this research.

Consent to Participate (Ethics): All authors consent to participate.

Consent to Publish (Ethics): All authors consent to publication.

Plant Reproducibility: Not Applicable (N/A) for this research.

Clinical Trials Registration: Not Applicable (N/A) for this research.

Author Contribution: G.M.S.: Conceived and designed the research, conducted experiments, analyzed data, and wrote the manuscript. M.D.G.: Conceived and designed the research, conducted experiments, and editing of the manuscript. O.A.H.J.: Designed the research, data interpretation, presentation and edited the manuscript. S.M.L.: Conceived and designed the research, data interpretation, presentation and edited the manuscript. All authors have read and approved the manuscript.

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Figures

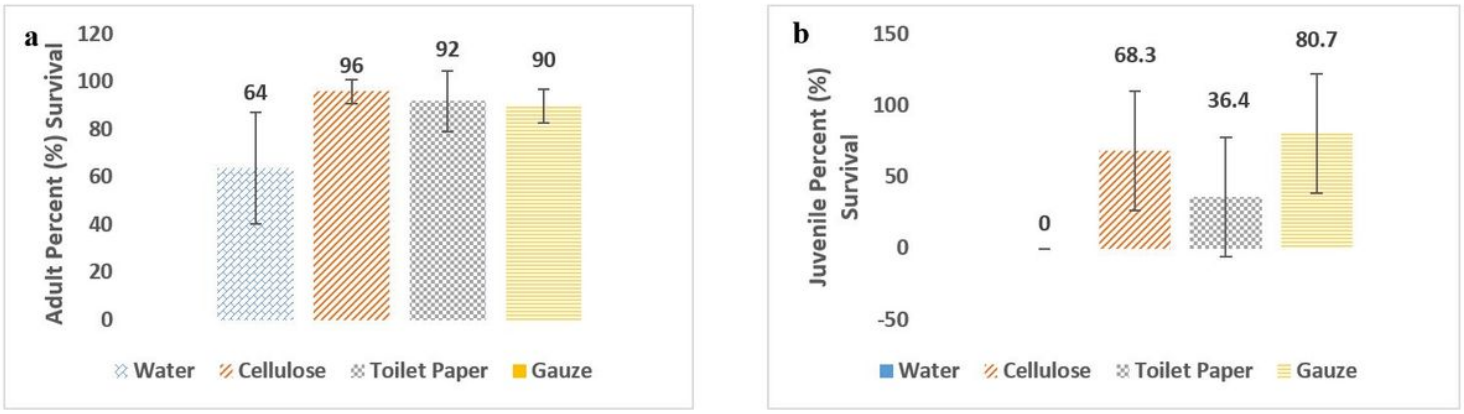

Figure 1

a) Mean (+/- SEM) survival of adult amphipods in three substrates (cellulose, toilet paper and gauze) and a negative control of water only. b) Survival of juvenile amphipods in substrates (gauze, toilet paper and cellulose). There was no survival in the water treatments. N = 5 replicates per treatment.

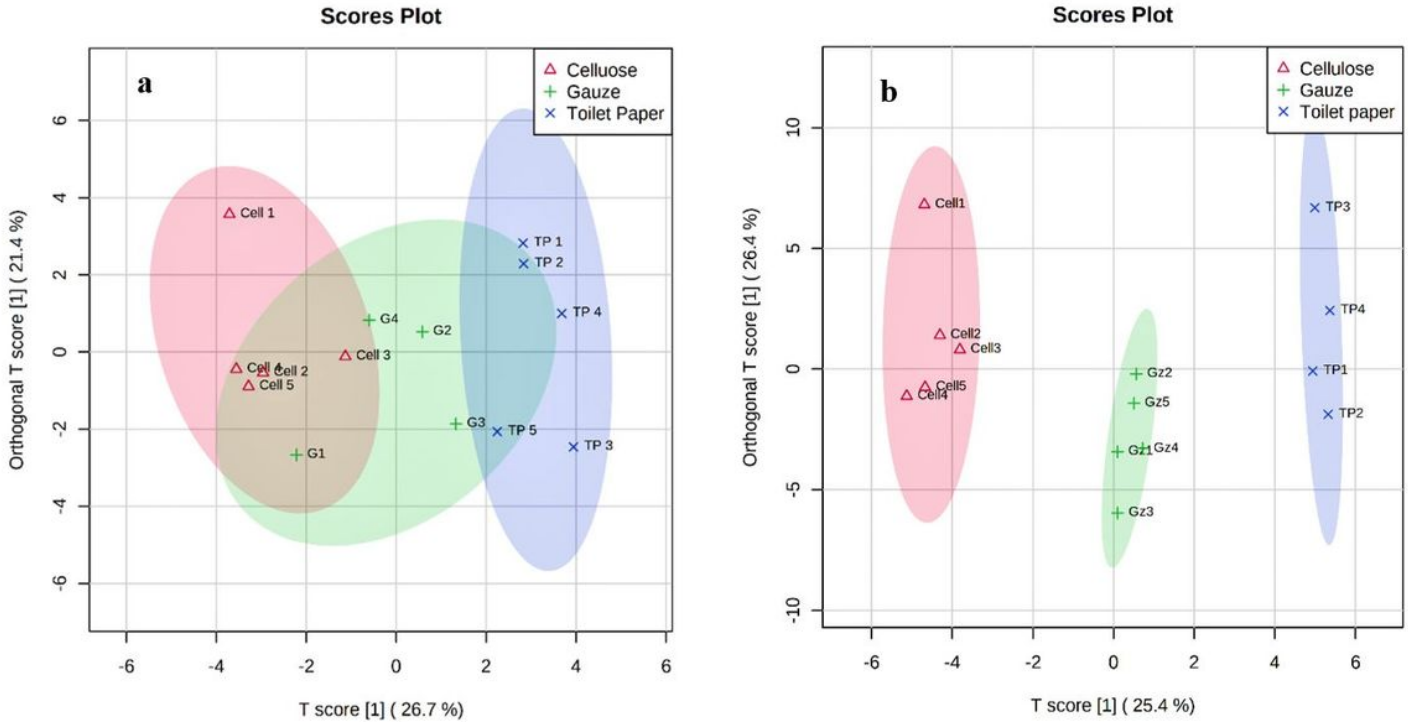

Figure 2

Orthogonal PLS-DA: separation of metabolites from adult (a) and juvenile (b) amphipods exposed to different substrates. Orthogonal PLS-DA Model overview can be found in the supplementary material

(Figure S1 and S2).

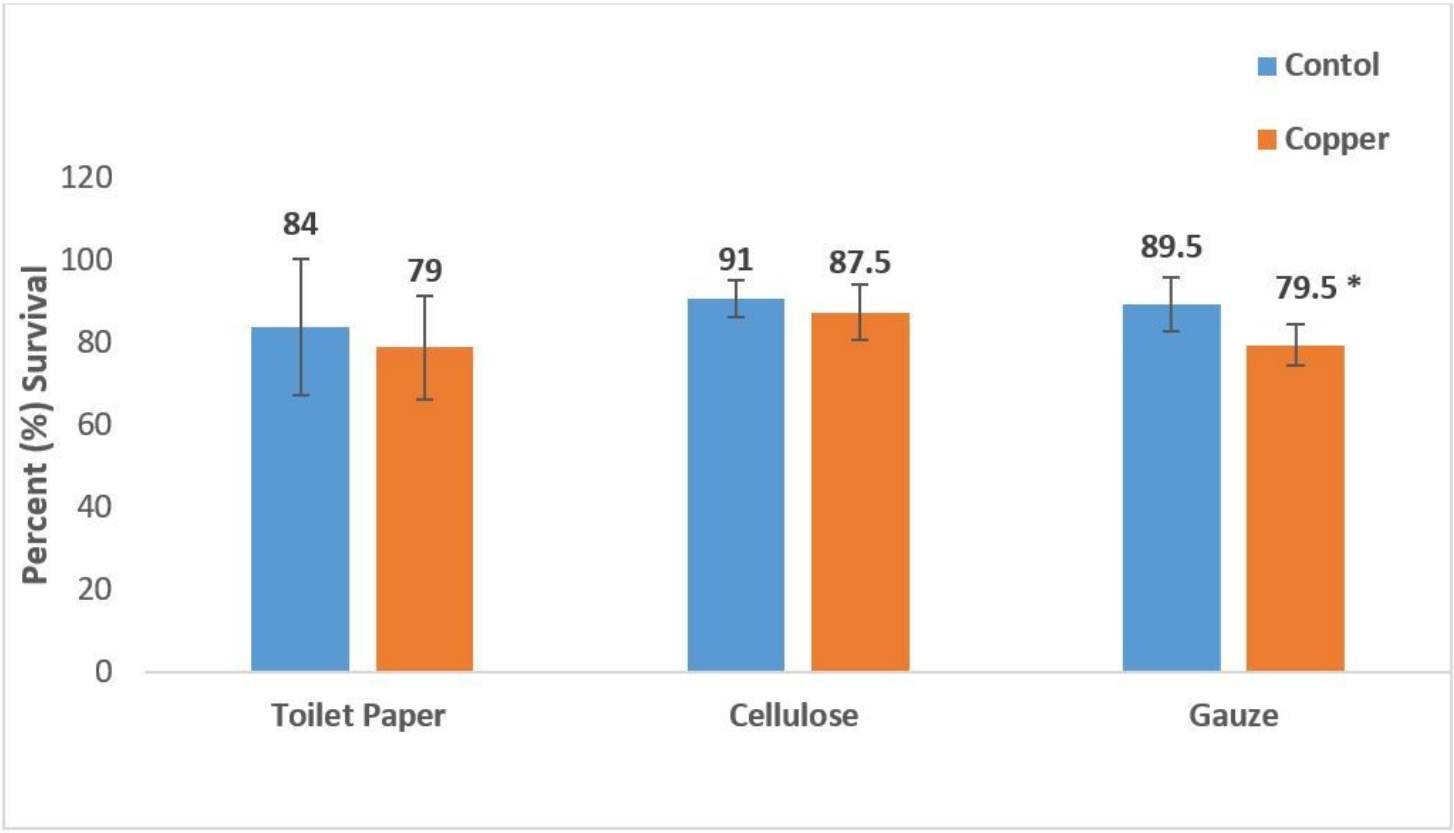

Figure 3

Mean (+/- SEM) Survival of amphipods following 14-day exposure to substrates (gauze, toilet paper and cellulose) spiked with copper. N = 5 replicates per treatment. Two-tailed t-test across substrates revealed. survival of amphipods significantly reduced (p= 0.002)* in copper when compared to gauze substrate.

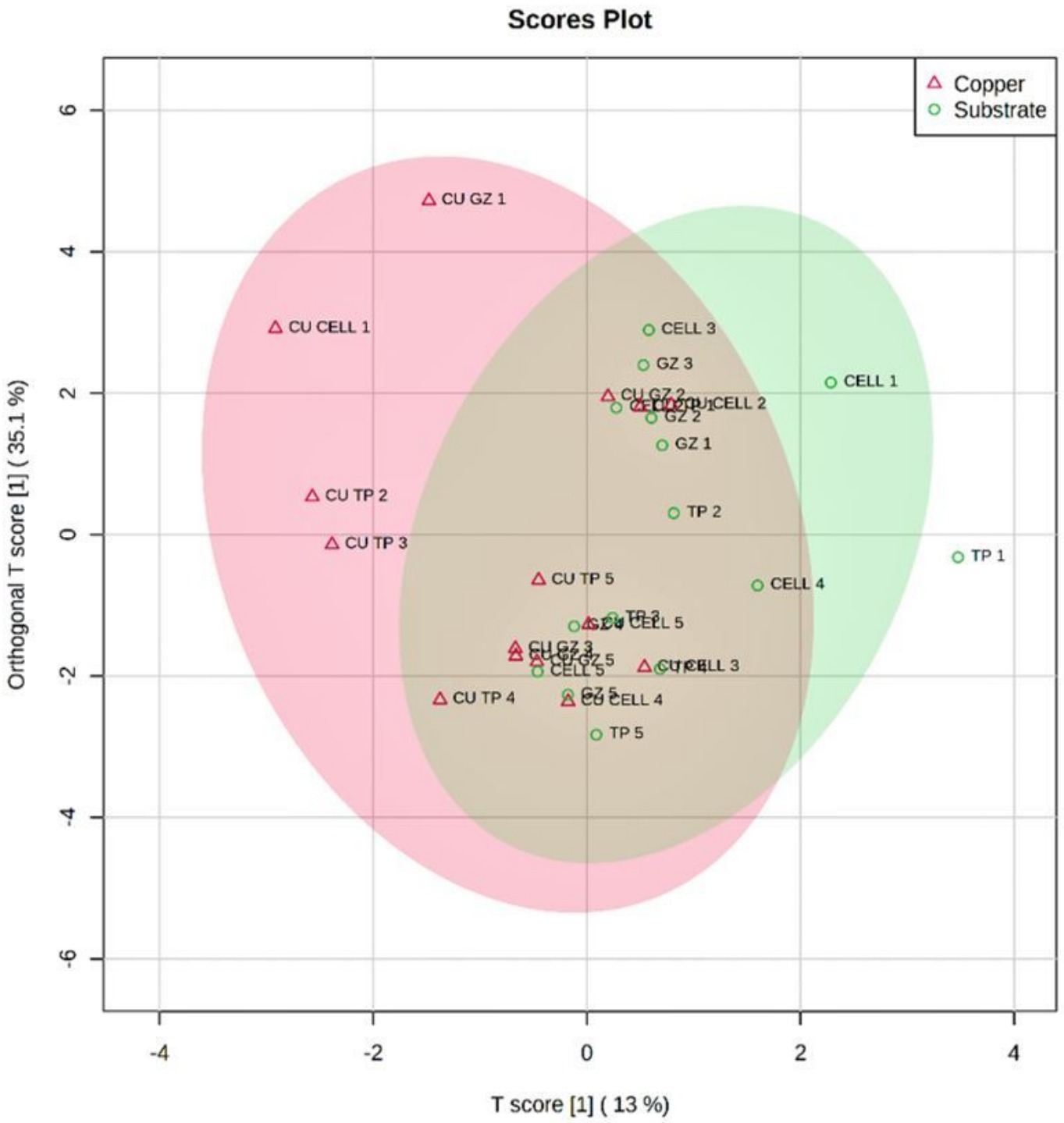

Figure 4

Orthogonal PLS-DA: separation of metabolites from adult amphipods across substrate treatment groups, following GC-MS. Model overview can be found in the supplementary material (Fig. S3).

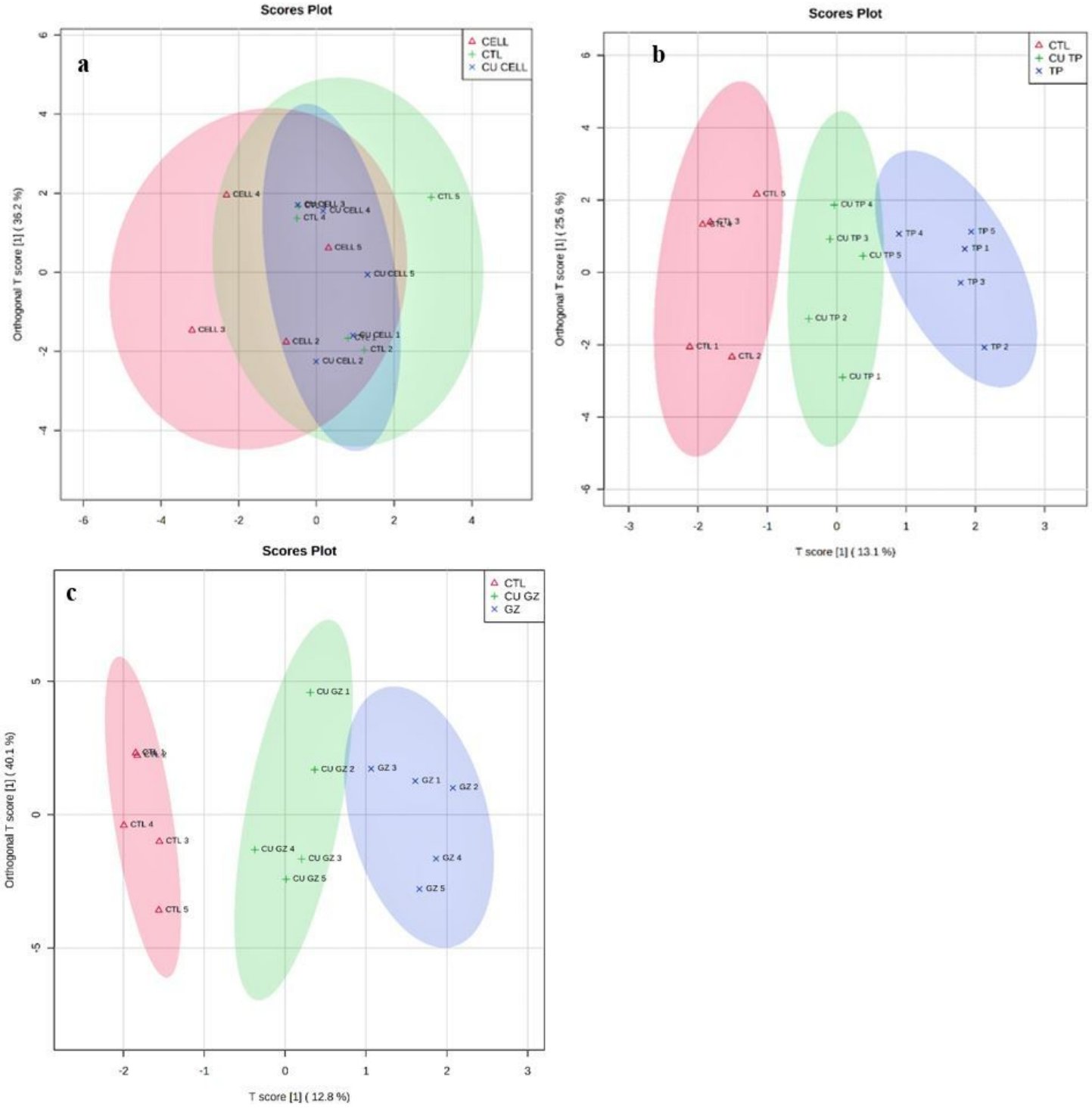

Figure 5

Orthogonal PLS-DA of metabolites extracted from amphipods exposed to copper for 14 days using Cellulose (a) Toilet Paper (b) or Gauze (c) as substrate following GC-MS. Controls in each figure were collected at the commencement of the exposure period (day 0).

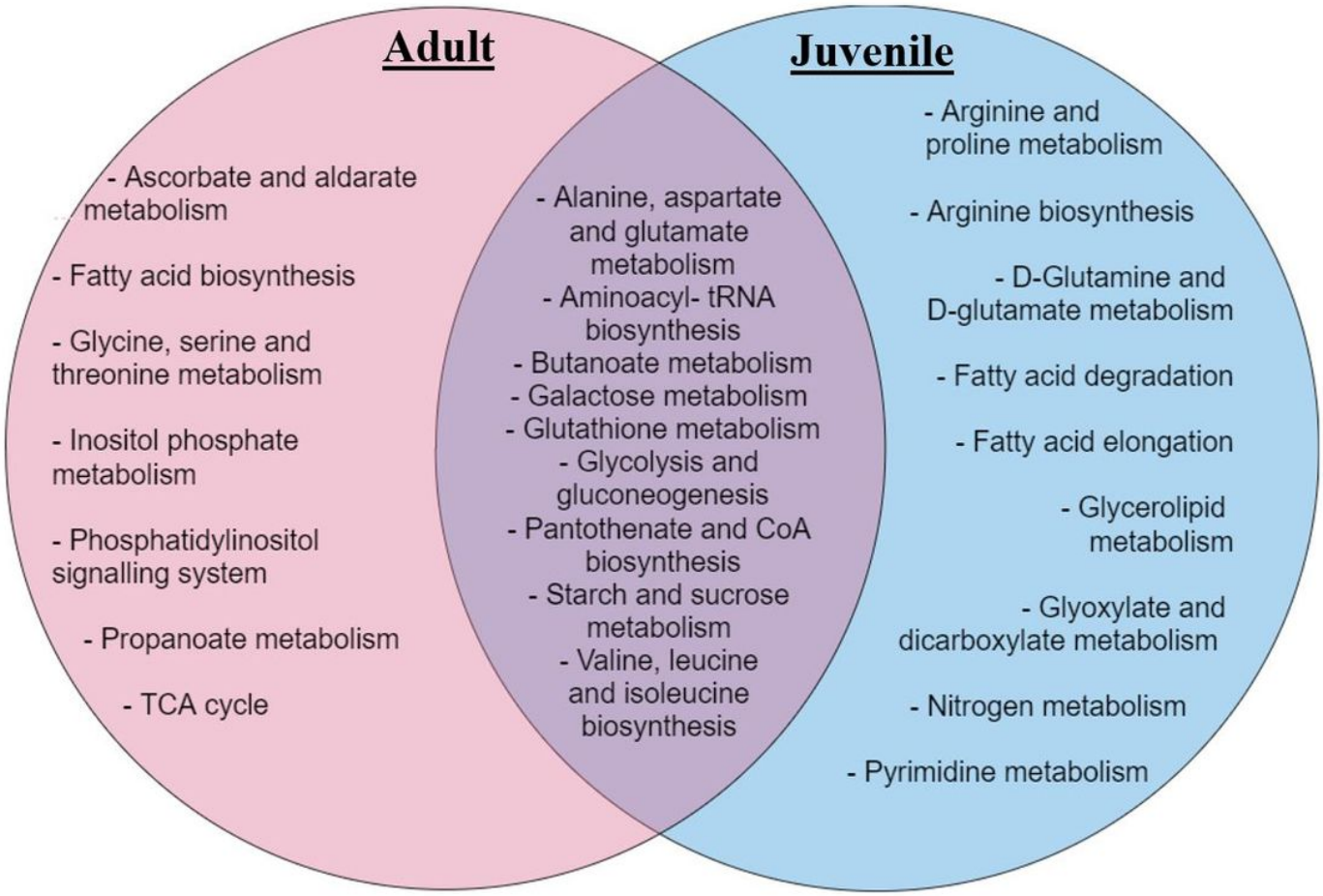

Figure 6

Venn diagram showing significant metabolite pathways that responded to the different substrate groups (cellulose, toilet paper and gauze), uniquely in adult amphipods and juvenile amphipods, as well as the pathways identified in both.

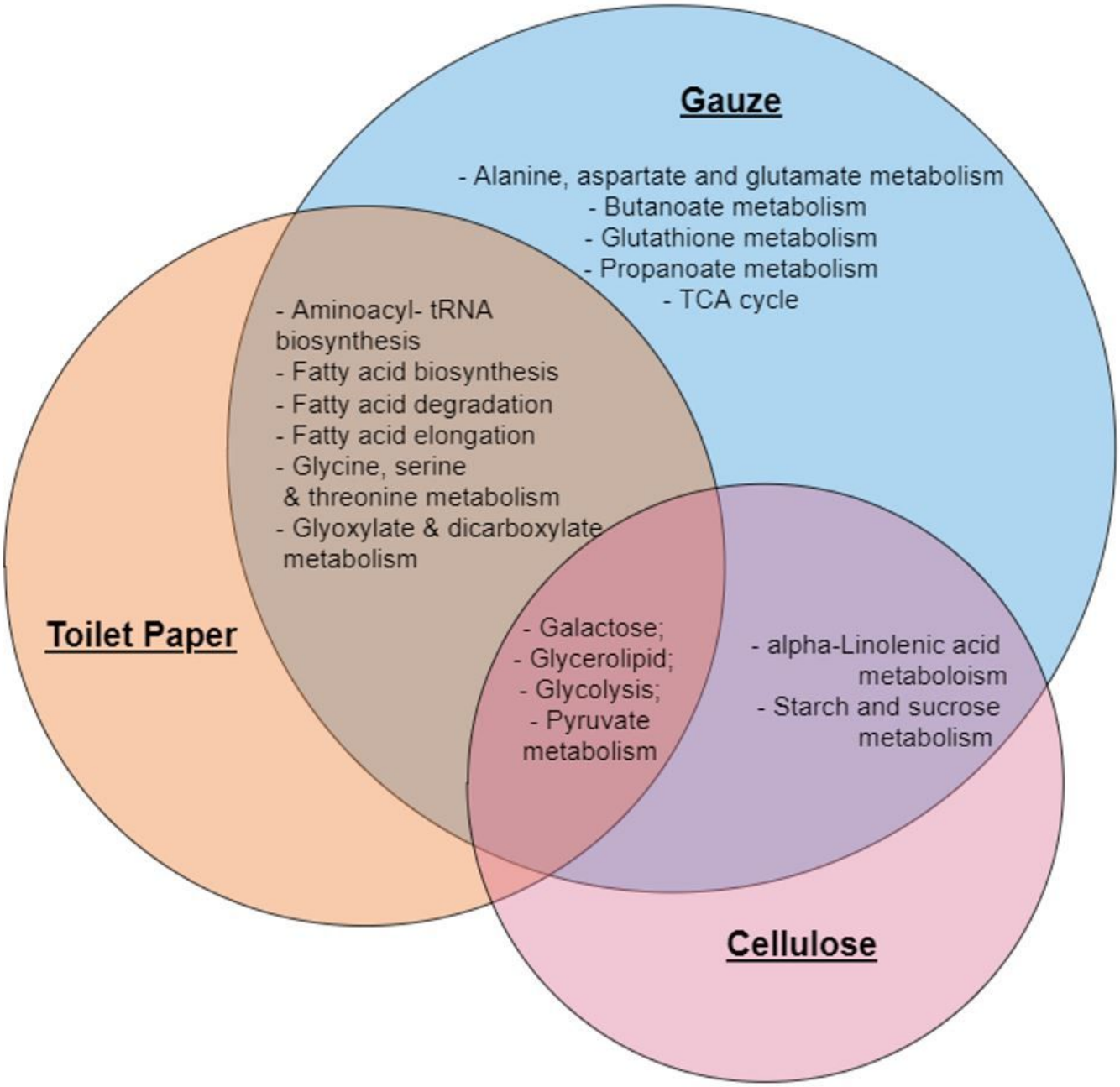

Figure 7

Venn diagram showing significant metabolite pathways that changed in response to copper in each of the substrate treatments (cellulose, toilet paper and gauze).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementarymaterialSinclairetal2021.docx