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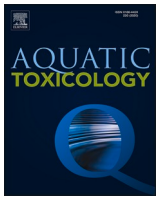

# Assessing the effects of single and binary exposures of copper and lead on *Mytilus galloprovincialis*: Physiological and genotoxic approaches

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ABSTRACT

It is becoming increasingly recognised that contaminants are not isolated in their threats to the aquatic environment, with recent shifts towards studying the effects of chemical mixtures. In this study, adult marine mussels (*Mytilus galloprovincialis*) were exposed to two aqueous concentrations of the essential trace metal, Cu (5 and 32 μg $L^{-1}$), and the non-essential metal, Pb (5 and 25 μg $L^{-1}$), both individually and in binary mixtures. After a 14-day exposure, metal accumulation was determined in the digestive gland, gill and mantle tissues by inductively coupled plasma-mass spectrometry following acid digestion, and a number of biochemical, neurotoxic and physiological markers were assessed. These included measurements of DNA damage using comet assay, total glutathione concentration, acetylcholinesterase (AChE) activity and clearance rate. Metal accumulation was greater in the digestive gland and gill than in the mantle, and based on computed free ion concentrations, was greater for Pb than for Cu. Copper exhibited an inhibitory effect on Pb accumulation but Pb did not appear to affect Cu accumulation. Comet assay results revealed DNA damage (i.e., genotoxic effects) in all treatments but differences between the exposures were not significant ($p > 0.05$), and there were no significant differences in AChE activities between treatments. The most distinctive impacts were a reduction in clearance rate resulting from the higher concentration of Cu, with and without Pb, and an increase in glutathione in the gill resulting from the higher concentration of Cu without Pb. Multivariate analysis facilitated the development of a conceptual model based on the current findings and previously published data on the toxicity and intracellular behaviour of Cu and Pb that will assist in the advancement of regulations and guidelines regarding multiple metal contaminants in the environment.

## 1. Introduction

Although there is a large body of literature examining the accumulation of and impacts of individual metals on marine invertebrates (e.g., Dallinger 1994; Rainbow 1997; Al-Subiai et al. 2011; Dallas et al. 2013; Cole et al. 2014; Chadwick and Bury 2023), environmental contaminants occur in combination forming complex mixtures (Jha, 2004). The type and concentration of these contaminants are unique to an ecosystem and are affected by human population, local geology and anthropogenic impacts (Ali et al., 2019). Interactions between two or more contaminants have been observed to induce different biological responses compared to single contaminant exposures and these responses can either be antagonistic, additive or synergistic. For instance, lower concentrations of arsenic (As) and cadmium (Cd) were found to express antagonistic effects on survival, accumulation, ion regulation and locomotion in the freshwater amphipod, *Gammarus pulex*, while higher concentrations resulted in additive effects (Vellinger et al., 2013). In a recent study employing the freshwater mussel (*Villosa iris*), growth appeared to respond additively to exposures of copper (Cu), nickel (Ni) and zinc (Zn) (Timpano et al., 2022).

The mechanisms of metal-metal combinations acting on marine invertebrates have not been fully characterised. Studies of mixtures of Cu and lead (Pb), for example, are often limited to their accumulation in target organisms (Debelius et al., 2009; García-Navarro et al., 2017; Marquez et al., 2018). Recently, however, Morais et al. (2023) showed that mercury (Hg), cobalt (Co) and nickel (Ni) enhanced biochemical

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alterations and induction of oxidative stress in the marine mussel, *Mytilus galloprovincialis*, when added in combination compared with individually. Such findings highlight the importance of studying the accumulation and sub-lethal biological effects of contaminants in combination and demonstrate the limitations of applying existing models to predict the toxicity of mixtures of metals.

Accordingly, the present study exposes *M. galloprovincialis*, as a species of ecological and economic importance, to high and low concentrations of the essential trace metal, Cu, and the non-essential metal, Pb, both alone and in binary mixtures. The aims were to explore the individual and combined impacts of these metals on specific accumulation (digestive gland, gill and mantle) and determine end-points indicative of different levels of biological organisation (i.e., DNA, enzymes, physiology). Copper and Pb are common contaminants of coastal marine ecosystems because of their multiple anthropogenic sources, such as marine paints, landfill leachate, fossil fuel combustion and mining waste (Wilson and Pyatt, 2007; Turner, 2010; Chiarelli and Roccheri, 2014; Richir and Gobert, 2016; Cruz et al., 2019). Moreover, both metals are accumulated in marine mussels in direct proportion to exposure concentrations (Sánchez-Marín et al., 2014; Cai and Wang, 2019). We also employ multivariate analysis (MVA), including cluster analysis and principal component analysis (PCA), to integrate multi-biomarker data into a mechanistic explanatory framework (Moore et al., 2021). Pathobiological modelling is an essential methodology for the derivation of explanatory frameworks that facilitate the development of a predictive capacity for estimating outcomes or risk associated with particular disease processes and stressful treatments (Moore, 2010; Moore et al., 2021; Sforzini et al., 2018). Previous studies on mussels and earthworms have shown that there is a strong relationship between lysosomal membrane stability (LMS), as an indicator of cellular health, and the responses of numerous cellular stress biomarkers (Moore et al., 2006, 2021; Sforzini et al., 2014, 2017, 2018).

## 2. Methods

### 2.1. Chemicals and animal collection

All chemicals and reagents used in the study were purchased from Merck Life Science UK Ltd, VWR International Ltd USA or Fisher Scientific UK Ltd. *M. galloprovincialis* were collected from Trebarwith Strand, North Cornwall (latitude 50 38′ 41″ N, longitude 4 45′ 43.5″ W), and in the laboratory mussels were acclimatised in 75 L plastic tanks with 50 L of filtered seawater (1 μM pore size; salinity ~ 34; pH ~ 8.0) for two weeks at 15 °C and over 12 h light:12 h dark cycles. During acclimation, *M. galloprovincialis* were fed three times a week with *Isochyrsis galbana* algae (~$1.0^5$ x $10^6$ cells $mL^{-1}$, Reed mariculture, Campbell, CA, USA), with a complete water change performed 2 h after each feed.

### 2.2. Exposures

Acclimatised mussels were transferred to 2 L glass beakers (two individuals per beaker) with 1.8 L of filtered seawater 24 h prior to exposure. Ten mussels, in the size range 50–60 mm, were exposed to one of nine treatment groups for a period of 14 d at 15.1 ± 0.1 °C, with feeding (*Isochyrsis galbana,* cell density = 2 × $10^6$ $mL^{-1}$) and a water (and metal) change performed daily. Treatments consisted of a seawater control, and Cu added at a "low" concentration (5 μg $L^{-1}$) and a "high" concentration (32 μg $L^{-1}$) and prepared from a solution of $CuSO_4$, and Pb added at a "low" concentration (5 μg $L^{-1}$) and a "high" concentration (25 μg $L^{-1}$) and prepared from a solution of $Pb(NO_3)_2$. Metals were added both singularly and in binary mixtures and resulting concentrations in the exposures were designed to encompass the range of aqueous metal concentrations encountered in anthropogenically-impacted coastal waters. Thus, coastal concentrations of Cu range from 0.7 to 6.1 μg $L^{-1}$ (Van Veen et al., 2002; Pearson et al., 2017), and the Water Framework Directive (WFD) environmental quality standard (EQS) for Cu is 3.76 μg $L^{-1}$, with amendments based on the concentration of dissolved organic carbon (DOC) (DEFRA, 2014). However, higher concentrations can occur in regions directly affected by boating activities (Turner, 2010; Chadwick and Bury, 2023). Concentrations of Pb in coastal waters are generally below 1.25 μg $L^{-1}$ (Langston et al., 2003) and are not considered detrimental to the health of marine organisms (European Commission, 2013). However, concentrations higher than 12 μg $L^{-1}$ have been observed in highly polluted areas (Meng et al., 2008).

### 2.3. Analysis of Cu and Pb concentrations in water and tissue

In triplicate, water samples of 1 mL were taken on days 1, 3, 9 and 13. Samples were diluted five-fold with 2 % $HNO_3$ and spiked with 40 μg $L^{-1}$ of indium and iridium as internal standards. The digestive gland, gill and mantle were dissected from nine out of ten individuals at the end of the exposure and tissue of the same type was pooled together to make three replicates per treatment. Samples were freeze-dried before 20 to 50 mg were digested, along with a certified oyster reference material (NIST 1566b), in 70 % $HNO_3$ at 70 °C for ~ 4 h. Digests were diluted five-fold with distilled water and spiked with indium and iridium as above. Diluted water samples, tissue digests and procedural blanks were analysed in triplicate using a X-series II inductively-coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific Inc., Waltham, MA, USA) that had been calibrated with matrix-matched standards.

### 2.4. Determination of DNA strand breaks using the comet assay

Determination of DNA strand breaks using single cell gel electrophoresis (comet assay) was conducted as described in Jha et al. (2005). Briefly, haemolymph from ten mussels in each treatment was extracted and centrifuged at 3000 rpm for 2 min. The resulting pellet was resuspended in low melting point agarose (0.75 % in phosphate saline buffer) and pipetted in duplicate onto a pre-coated slide (1.5 % normal melting point agarose in tris-acetate-EDTA). After setting at 4 °C, slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1 % N-lauryl-sarcosine, 1 % Triton X-100, 10 % DMSO; pH adjusted to 10 with NaOH) for 1 h. Following lysis, cell were left in electrophoresis buffer (1 mM EDTA, 0.3 M NaOH; pH 13) to allow DNA to denature for 20 min. Electrophoresis was then conducted for 20 min at ~1 V $cm^{-1}$. Slides were placed in neutralisation buffer (0.4 Tris-HCL; pH 7) for 10 min, rinsed with distilled $H_2O$ and left to dry at room temperature. Cells were stained with ethidium bromide (20 μg $mL^{-1}$) and scored using an epifluorescent microscope (DMR; Leica Microsystems, Milton Keynes, UK). One hundred cells per slide were quantified using Comet IV imaging software (Perceptive Imaging, Bury St Edmunds, UK), with% tail DNA (tail intensity) considered the most reliable measure of DNA damage (Kumaravel and Jha, 2006).

### 2.5. Measurement of total glutathione

Total glutathione activity was based on the cyclic reduction assay of Owens and Belcher (1965) with alterations. Digestive gland and gill from three out of ten *M. galloprovincialis* per treatment group remaining after dissection and digestion (see above) were homogenised (x520D, Bennett Scientific Ltd, Devon, UK) with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1 % sodium dodecyl sulphate, 0.5 % sodium deoxycholate, 1 % Triton X100; pH 7.4) and resulting supernatants were stored at −80 °C until use. Thawed samples were diluted with assay buffer (100 mM potassium phosphate, 5 mM EDTA; pH 7.5) and mixed with an equal volume of buffered DTNB (100 mM potassium phosphate, 5 mM EDTA, 10 mM DTNB pH 7.5) and placed on ice. Sample aliquots of 40 μL were transferred to a 96 well plate, along with 210 μL aliquots of glutathione reductase solution (0.6 U, Sigma G-3664 from *Saccharomyces cerevisiae*, in assay buffer). After equilibration for 1 min, the reaction was started by adding 60 μL aliquots of NADPH (1 mM, Melford,

Ipswich, UK in assay buffer) and the absorbence recorded at 412 nm over 20 min using a plate reader (VersaMax Microplate reader USA, SoftMax Pro 5.4). Each run contained a blank buffer and a standard (20 μM reduced glutathione) and samples were measured in triplicate and results expressed as total glutathione in μmol $mg^{-1}$.

### 2.6. Determination of neurotoxicity using acetylcholinesterase activity

The acetylcholinesterase activity was assessed in the haemolymph of ten *M. galloprovincialis* per treatment, with protein content estimated using the Micro BCA™ Protein assay (ThermoFisher Scientific) according to the manufacturer's instructions. Twenty μL of haemolymph and 240 μL of buffered dithiobis (2-nitro-benzoic acid) (0.01 M DTNB in 0.1 M potassium phosphate; pH 8) were added into individual wells on a 96 well plate. After 5 min at room temperature, 40 μL of acetylthiocholine iodine (final concentration 7.47 μM) was added to each well and absorbence immediately read at 412 nm for 15 min (VersaMax Microplate Reader USA, SoftMax Pro 5.4).

### 2.7. Determination of clearance rate

Clearance rate of *M. galloprovincialis*, as a proxy for "scope for growth" (Liu et al., 2011), was conducted as described in Canty et al. (2009). Briefly, individuals were placed into 400 mL beakers with 300 mL of filtered seawater at 15 °C. Magnetic bars were added and beakers were placed on 15-point magnetic stirrers (RO 15 power, Kika-Werke GmbH & Co, Germany). After a 10 min acclimation period, 500 μL of *Isochyrsis* algal solution was added to each beaker to produce a concentration of ~12,000–15,000 cells $mL^{-1}$. Water samples were taken directly preceding the transfer of algae ($t_0$) and at $t = 20$ min, and algal concentrations were determined using a Beckman Z2 Coulter Particle Size and Count Analyser (Beakman Coulter, Brea, CA, USA). Clearance rate (*CR*; L $h^{-1}$) was calculated using the following equation:

$$CR = V(\log_e C_0 - \log_e C_t)/t$$

where $V$ is the volume of water (L), $t$ is time (min), and $C_0$ and $C_t$ are the algae concentrations at $t_0$ and $t$.

### 2.8. Metal speciation calculations

The equilibrium inorganic speciation of Cu and Pb in the different exposures was computed using Visual MINTEQ 3.1 and the default stability constants in its database and activity coefficients calculated with the Debye-Hückel equation. Temperature and pH were fixed at 15 °C and 8.0, respectively, and a salinity of 34 was made up of concentrations of the major ions ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$, $SO_4^{2-}$) in proportion to their relative abundance in average seawater.

### 2.9. Statistical analysis

All statistical analyses were performed using the software, R (RStudio, R 3.4.3 GUI 1.70 El Capitan build, https://www.rproject.org/), except for the MVA detailed below. All data were checked for normality (Shapiro-Wilk test, Q-Q plot) and Grubbs test was used to identify outliers. ANOVA was performed along with Tukey's post hoc test if normality assumptions were met, and the Kruskal-Wallis test was used if assumptions were not met. Post hoc pairwise comparison tests for nonparametric data were either Tukey and Kramar (Nemenyi) for complete equal data sets or a Tukey Kramar (Conover) for where $n$ was different between treatments. Level of significance for all tests was set at $p < 0.05$ (*) and data were presented as mean ± standard deviation, unless otherwise stated.

### 2.10. Multivariate analysis

Data collected from the comet assay, total glutathione, AChE activity and clearance rates were analysed using non-parametric MVA software, PRIMER v 6.1.5 (PRIMER-e Ltd., U. Auckland, New Zealand; Clarke, 1999). Data were log transformed [log$n$ $(1 + x)$] and standardised to the same scale. Principal component analysis (PCA), hierarchical cluster analysis and non-metric multi-dimensional scaling analysis, derived from Euclidean distance similarity matrices, were used to visualise dissimilarities between sample groups. The results were tested for significant differences between treatments using analysis of similarity (ANOSIM), a non-parametric equivalent of parametric analysis of variance. The biota and/or environmental (BIO-ENV) matching routine uses Spearman's rank correlations to compare a fixed matrix of similarities to a variable that tests all possible variable combinations. With this statistical test, patterns are connected between the effects of the biomarkers with the treatment groups, thus identifying potential "influential biomarkers".

## 3. Results

### 3.1. Aqueous metal concentrations

The measured (total) concentrations of Cu and Pb in the aqueous phase of the exposures are shown in Table 1, where L and H refer to the low and high concentrations of added metal, respectively. The control samples had concentrations of Cu and Pb that reflected concentrations in the coastal waters used in the exposures. In all treatments, measured concentrations of both Cu and Pb (alone and in binary mixtures) were within 20 % of nominal concentrations and as recommended by the OECD (1992).

Also shown in Table 1 are the percentages of free, ionic Cu ($Cu^{2+}$) and Pb ($Pb^{2+}$), in each exposure based on inorganic speciation computations (i.e., ignoring any organic ligands present). These percentages, coupled with measured concentrations, have also been used to estimate the concentrations of free, and therefore bioavailable, Cu and Pb in the exposures. The data reveal that the concentrations of free Cu or Pb are not simply a function of their total concentrations but that there is competition between the metals for certain inorganic ligands. Thus, while the percentage of free Cu is about 10 in the absence of added Pb, when the latter is added at the higher concentration, the percentage of

**Table 1**
Measured aqueous concentrations of Cu and Pb in the different treatment groups exposed to *M. galloprovincialis* for 14 d, along with calculated percentages and concentrations of free Cu and Pb (L = low, H = high). Errors are one standard deviation about the mean of three measurements.

| | Cu, μg $L^{-1}$ | % $Cu^{2+}$ | $Cu^{2+}$, μg $L^{-1}$ | Pb, μg $L^{-1}$ | % $Pb^{2+}$ | $Pb^{2+}$, μg $L^{-1}$ |
|---|---|---|---|---|---|---|
| Control | 3.26 ± 0.14 | 9.47 | 0.31±0.01 | 0.85 ± 0.15 | 0.52 | 0.004±0.001 |
| CuL | 7.37 ± 0.17 | 9.84 | 0.73±0.02 | 0.93 ± 0.16 | 0.27 | 0.002±0.000 |
| CuH | 30.67 ± 0.94 | 10.11 | 3.10±0.10 | 1.10 ± 0.21 | 0.08 | 0.001±0.000 |
| PbL | 4.00 ± 0.23 | 7.26 | 0.29±0.02 | 5.32 ± 0.56 | 2.07 | 0.11±0.01 |
| PbH | 4.14 ± 0.12 | 3.70 | 0.15±0.01 | 23.76 ± 0.26 | 4.55 | 1.08±0.01 |
| CuL+PbL | 7.61 ± 0.56 | 8.53 | 0.65±0.05 | 4.93 ± 0.40 | 1.18 | 0.06±0.00 |
| CuL+PbH | 7.89 ± 0.59 | 5.44 | 0.43±0.03 | 22.55 ± 0.55 | 3.33 | 0.75±0.02 |
| CuH+PbL | 31.19 ± 1.61 | 9.67 | 3.02±0.16 | 5.77 ± 0.51 | 0.38 | 0.02±0.00 |
| CuH+PbH | 29.61 ± 0.56 | 8.23 | 2.44±0.05 | 23.35 ± 0.31 | 1.39 | 0.32±0.00 |

free Cu is reduced to as low as 3.7. The percentage of free Pb is about 2 and 4.5 when lower and higher concentrations are introduced, but corresponding percentages are progressively reduced when increasing concentrations of Cu are added. Clearly, these effects and interactions must be factored into any observed biological responses below.

### 3.2. Copper and lead accumulation in tissue

Concentrations of Cu and Pb in the control and exposed mussels are shown in Fig. 1. The control mussels had mean concentrations of Cu in the digestive gland, gill and mantle of *M. galloprovincialis* of about 21.4 μg $g^{-1}$, 9.1 μg $g^{-1}$ and 4.2 μg $g^{-1}$, respectively. Concentrations in the lower exposure of Cu, with or without added Pb, were not statistically different to the corresponding controls. However, concentrations in the higher exposure of Cu, with and without added Pb, were significantly higher than the controls for each tissue type, with the gill and digestive gland subject to greatest accumulation.

The control mussels had mean concentrations of Pb in the digestive gland (2.3 μg $g^{-1}$), gill (5.9 μg $g^{-1}$) and mantle (2.8 μg $g^{-1}$) that were significantly lower than corresponding concentrations found in all treatments where Pb had been added. As with Cu, the digestive gland and gill accumulated greater concentrations of Pb than the mantle. In the absence of added Cu and when Cu was added at the lower concentration, Pb exhibited a concentratrion-dependant accumulation in each tissue type. The higher concentration of Cu, however, resulted in a reduction in accumulation of Pb when added at the higher concentration and compared with its accumulation in the absence of Cu. The latter observation is consistent with the free ion calculations shown in Table 1; namely, a reduction in the concentration of bioavailable Pb from 1.08 μg $L^{-1}$ (in the absence of added Cu) to 0.32 μg $L^{-1}$ (in the presence of the higher concentration of added Cu).

In order to examine and compare accumulation of Cu and Pb by *M. galloprovincialis*, accumulation factors, AFs (L $kg^{-1}$), shown in Table 2, were calculated for each tissue type and for both total and free metal concentrations. Thus, $AF_{tot}$ represents the ratio of mean concentration in tissue (μg $kg^{-1}$, and derived from Fig. 1) relative to mean measured concentration in the seawater medium (μg $L^{-1}$, and given in Table 1), and $AF_{free}$ represents the ratio of mean concentration in tissue (as above) relative to mean calculated concentration of free metal in seawater (μg $L^{-1}$, and shown in Table 1). Values of $AF_{tot}$ range from a few hundred to about $10^5$ L $kg^{-1}$ and, overall, confirm that accumulation is greater in the digestive gland and gill than the mantle for both metals.

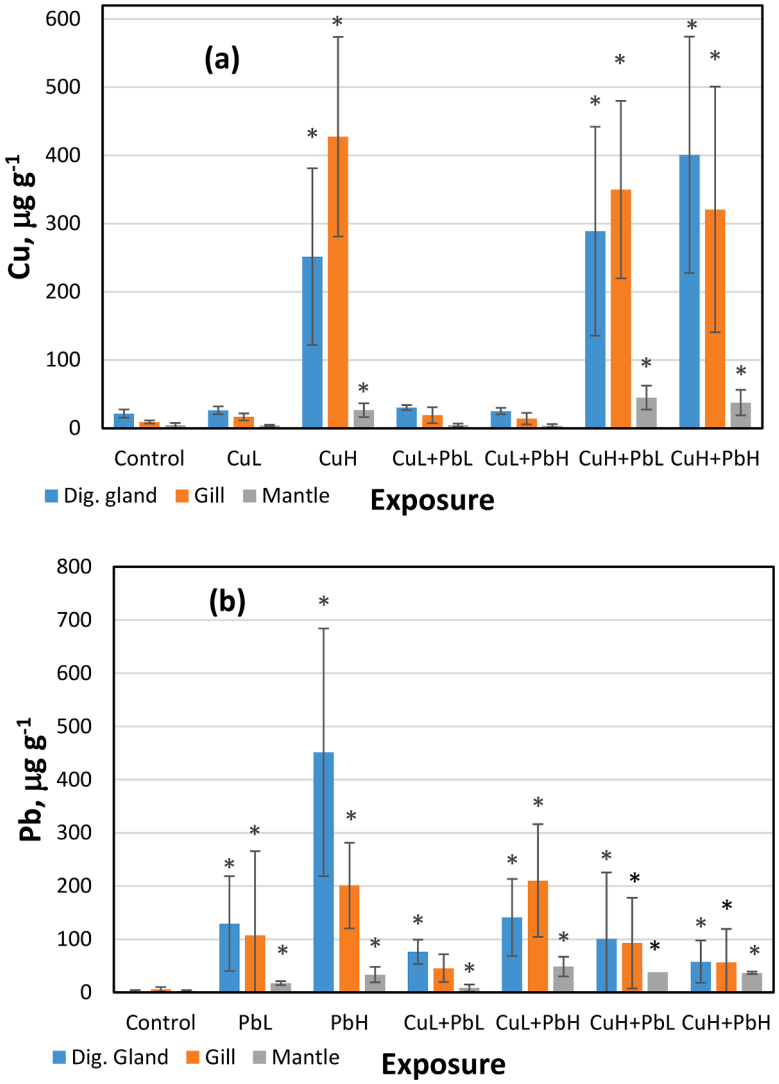


**Fig. 1.** The concentration of (a) Cu and (b) Pb in the digestive gland, gill and mantle of *M. galloprovincialis* after the 14-d exposures to different Cu and Pb concentrations (L = low, H = high). Error bars are one standard deviation about the mean of three measurements and asterisks denote significant differences to the corresponding controls.

**Table 2**
Mean accumulation factors for total Cu and Pb and free Cu and Pb in tissue of *M. galloprovincialis* in the different treatments.

| | $AF_{tot}$, L $kg^{-1}$ (1000s) | | | $AF_{free}$, L $kg^{-1}$ (1000s) | | |
|---|---|---|---|---|---|---|
| metal, exposure | dig. gland | gill | mantle | dig. gland | gill | mantle |
| Cu | | | | | | |
| Control | 6.6 | 2.8 | 1.3 | 69.4 | 29.4 | 13.6 |
| CuL | 3.6 | 2.2 | 0.5 | 36.1 | 22.8 | 5.3 |
| CuH | 8.2 | 13.9 | 0.9 | 81.1 | 137.9 | 8.6 |
| CuL+PbL | 4.0 | 2.5 | 0.6 | 46.7 | 29.4 | 7.0 |
| CuL+PbH | 3.2 | 1.8 | 0.5 | 58.4 | 32.8 | 8.4 |
| CuH+PbL | 9.3 | 11.2 | 1.4 | 95.7 | 115.9 | 14.9 |
| CuH+PbH | 13.5 | 10.8 | 1.3 | 164.4 | 131.6 | 15.4 |
| Pb | | | | | | |
| Control | 2.8 | 7.0 | 3.3 | 527.2 | 1339 | 622.7 |
| PbL | 24.3 | 20.1 | 3.3 | 1176 | 974.1 | 158.0 |
| PbH | 84.8 | 37.8 | 6.3 | 417.5 | 185.9 | 31.1 |
| CuL+PbL | 3.2 | 1.9 | 0.4 | 1310.0 | 782.8 | 144.5 |
| CuL+PbH | 28.6 | 42.6 | 9.9 | 187.5 | 279.6 | 64.7 |
| CuH+PbL | 4.5 | 4.1 | 1.7 | 4573 | 4196 | 1728 |
| CuH+PbH | 10.0 | 9.8 | 6.4 | 178.1 | 174.0 | 113.1 |

### 3.3. DNA damage, glutathione concentration, neurotoxic effects and clearance rate

The percentage comet tail intensity (DNA damage) in the exposures is shown in Fig. 2a. DNA damage was lower in the control than in all treatment groups exposed to added Cu and Pb and either singularly or in combination. *M. galloprovincialis* exposed to the combination of Cu and Pb at the highest concentrations exhibited DNA damage that was significantly greater than that for the higher Pb concentrations when added singularly but not the higher Cu concentration added singularly. In contrast, neurotoxic effects, determined using the AChE activity in haemolymph, exhibited no significant differences between the control and any treatment in which Cu and/or Pb had been added (Fig. 2b).

The GSH activity of the digestive gland and gill in *M. galloprovincialis* exposed to different concentrations and combinations of Cu and Pb is shown in Fig. 2c and d, respectively. In the digestive glands, activities were variable across the treatments, and concentrations were significantly greater than the control only when Cu and Pb had been added together and at the lower and higher concentrations, respectively. In the gills, GSH concentrations were significantly higher than the control only when Cu had been added at its higher concentration.

Clearance rate results, shown in Fig. 3, reveal a highly significant reduction compared with the control whenever Cu was added at the higher concentration, and both in the absence and presence of added Pb. Added Pb by itself, however, did not result in a significant reduction of *CR* compared with the control.

### 3.4. Multivariate analysis

The MVA used only the comet and clearance data as the acetylcholinesterase activity was not affected by any of the treatments (Fig. 2b) and samples for glutathione analysis were limited to three animals per treatment. Investigation of the biomarker data using ANOSIM gave a global significance of $R = 0.352$ ($p < 0.001$, $n = 90$) for all treatments, while pairwise analysis showed significant differences between the controls and low Cu ($p < 0.006$, $n = 10$), as well as all high Cu treatments ($p < 0.001$, $n = 10$). High Cu treatment (both singularly and in

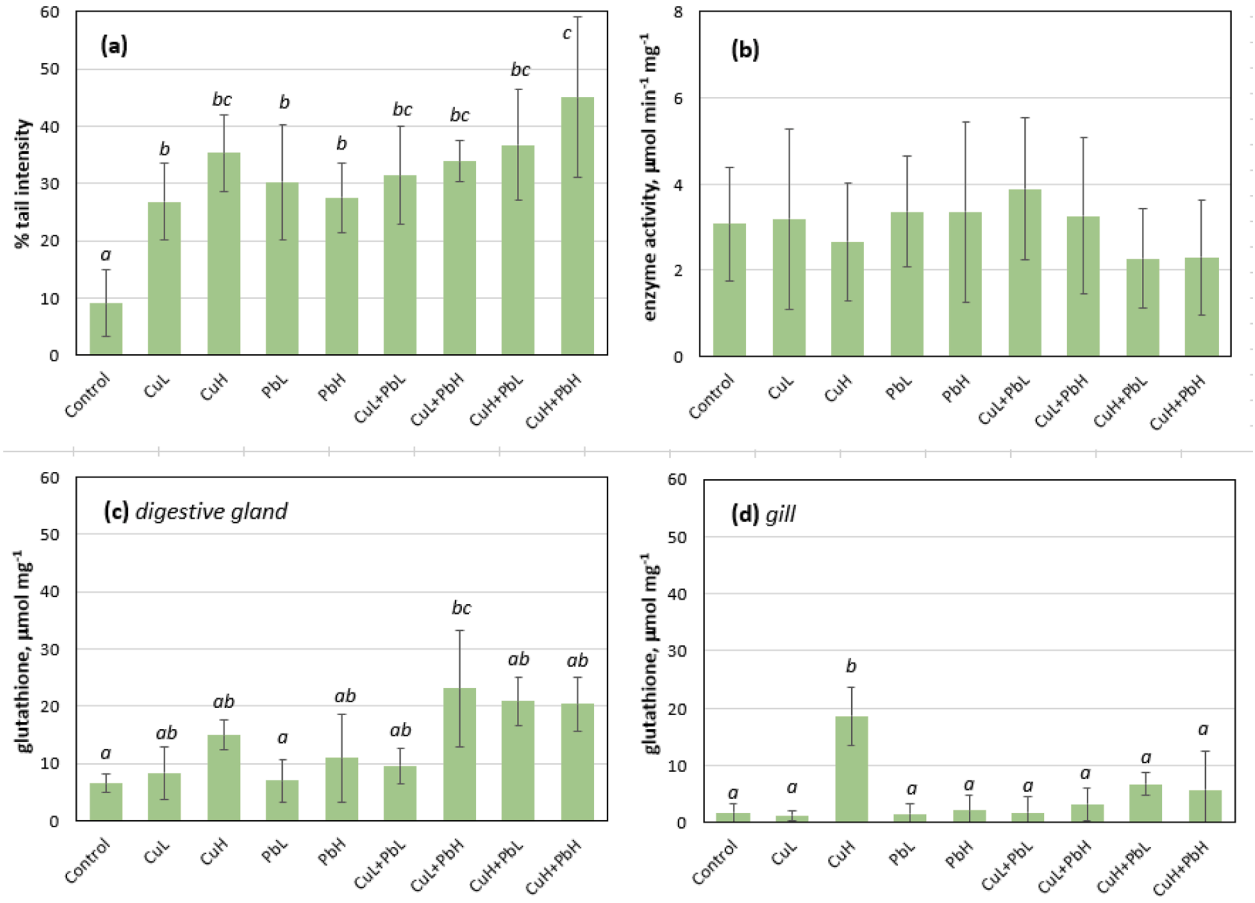


**Fig. 2.** The effect of 14-d exposure of Cu and Pb to *M. galloprovincialis* on (a) DNA damage, represented as % tail DNA, (b) enzyme activity of acetylcholinesterase per mg of protein, and glutathione activity per mg of protein of (c) the digestive gland and (d) gill. Different letters indicate significance differences between treatments ($p < 0.05$). Error bars are one standard deviation about the mean of ten ((a) and (b)) or three ((c) and (d)) individuals.

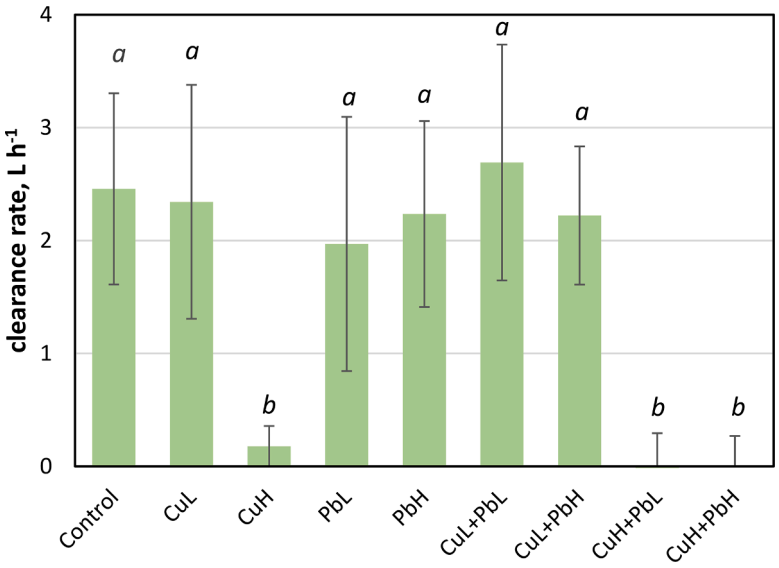


**Fig. 3.** The effect of 14-d exposure of Cu and Pb to *M. galloprovincialis* on clearance rate. Different letters indicate significance differences between treatments ($p < 0.05$). Error bars are one standard deviation about the mean of ten measurements.

combination) were significantly different from all Pb and low Cu treatments and their combined treatments ($p < 0.001, n = 10$). However, low and high Pb in combination with low Cu were not significantly different from the controls, perhaps indicating an antagonistic effect that is probably due to the DNA damage component (as indicated in Fig. 2a). The high Cu and low Pb and high Cu and high Pb treatments were not significantly different (ANOSIM; Fig. 4a) while clearance rate was found to be significantly inversely correlated with DNA damage ($r = -0.211, p < 0.05, = 90$, two-tailed).

PCA, combined with cluster analysis, showed that all experimental treatments with the high concentration of Cu (with and without added Pb) were both visually distinct from (red clusters) and statistically different to the control sample (70 % of control values within the green cluster; Fig. 4a), and that high Cu in combination was distinct from higher Cu added singly (ANOSIM, $p < 0.05$, $n = 10$). Overall, the first principal component (PC1) captured 60.5 % variation in the data and the second principal component (PC2) explained an additional 39.5 % (Fig. 4). The clearance rate and comet assay were both correlated with the first and second principal components ($p < 0.001, n = 90$, two-tailed; Fig. 4a). PCA bubble plots for clearance rate and comet assay values

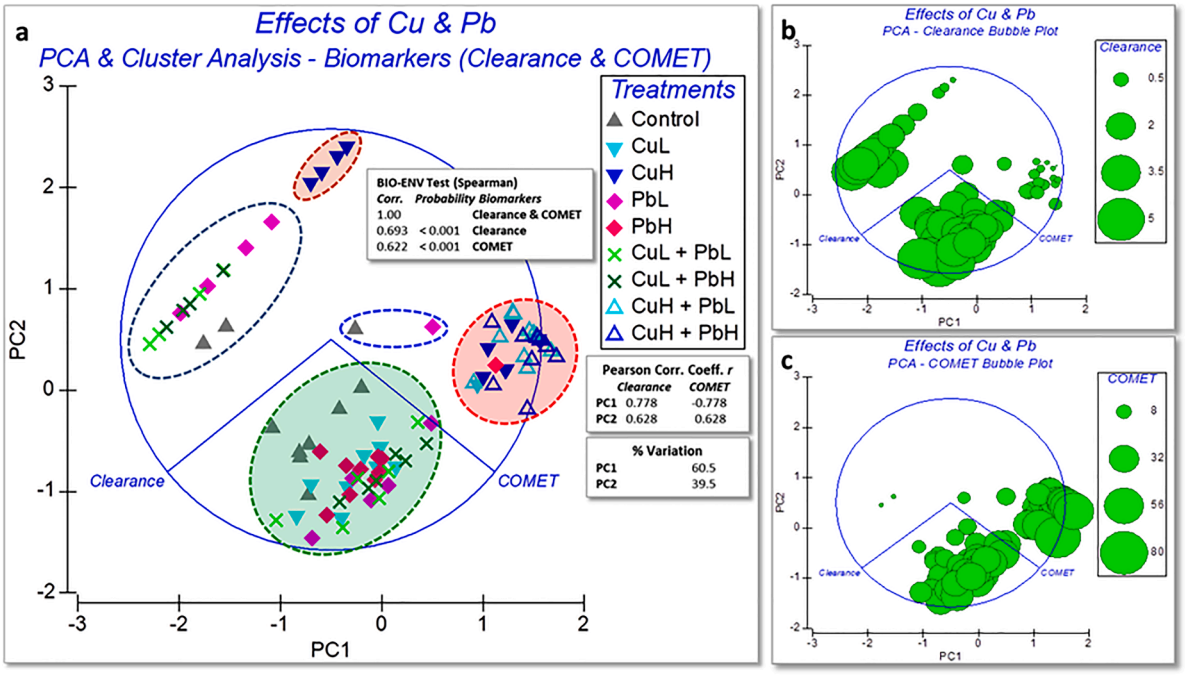


**Fig. 4.** (a) PCA and superimposed cluster analysis of comet assay in haemocytes, acetylcholinesterase activity in the haemolymph and clearance rate, with superimposed hierarchical cluster analysis. The Euclidean distance of the cluster boundaries (dark blue dotted ovoids) is 1.6, and the % variation captured by the first and second principal components (PC1 and PC2) is shown on the plot. The vector for each of the biomarkers is also indicated within the solid-line blue circle. Correlations with PC1 and PC2 are shown together with the BIO-ENV Spearman rank correlations for the combined and single biomarkers. (b) PCA bubble plot for clearance rate (as an indicator of physiological status) showing decreasing values with increased stress (towards the right-hand side). (c) PCA bubble plot for DNA damage (comet assay) showing increasing values with increasing stress (towards the right-hand side).

show decreasing values and increasing DNA damage with increasing stress, respectively (Fig. 4b and c).

The BIO-ENV routine for various combinations of biomarkers indicated that clearance rate and comet were both significant biomarkers ($r_s = 0.622$ for comet, $p < 0.001$; and $r_s = 0.693$ for clearance, $p < 0.001$; $n = 90$) for determining the outcome of the MVAs when mussels were exposed to Cu and Pb, singularly and in combination (Fig. 4a).

## 4. Discussion

Qualitatively, our results regarding the accumulation of Cu and Pb by *M. galloprovincialis* are in agreement with those of García-Navarro et al. (2017) who found that Cu and Pb added in binary mixtures or ternary mixtures (with Cd, and albeit at higher concentrations) accumulated to the greatest extent in the digestive gland amongst the soft tissues studied. Our AFs also confirm that the uptake of Cu appears to be largely unaffected by the presence of Pb while the uptake of Pb appears to be inhibited by the presence of Cu, with both effects most evident when higher concentrations of the metals are added (Cu = 32 μg $L^{-1}$, Pb = 25 μg $L^{-1}$). Because the calculated fractions of free Pb are smaller than the corresponding calculated fractions of free Cu, values of $AF_{free}$ are always greater for Pb than for Cu; in many cases, the discrepancies are between one and two orders of magnitude. Although we ignored any complexation of Cu and Pb by organic ligands in natural seawater, meaning that the fractions of computed free Cu and Pb may have been underestimated, the results of Table 2 suggest that, overall, $Pb^{2+}$ has a greater propensity for accumulation by *M. galloprovincialis* than $Cu^{2+}$. The speciation calculations in Table 1 also suggest that any inhibitory effects of Cu on Pb accumulation are at least partly the result of shifts in speciation as the metals compete for available inorganic ligands in seawater. These observations are qualitatively consistent with the findings reported by Chen et al. (2010) regarding the competitive uptake of Cu and Pb by the freshwater, unicellular green alga, *Chlamydomonas reinhardtii*. Here, binding constants for biological uptake sites were greater for Pb than Cu and, over part (but not the entirety) of the concentration range studied, increasing Cu concentrations resulted in decreasing Pb uptake but Pb appeared to have little competitive effect on Cu uptake.

There was no clear effect of Cu or Pb on AChE activity levels in *M. galloprovincialis* (Fig. 2b), although gill tissue may have been a more suitable tissue in this respect as it has higher levels of AChE (Perić and Petrović, 2011). We note that Cu has been reported to decrease in activity in the crab, *Carcinus maenas*, but only at concentrations that were orders of magnitude greater than those employed in the present study (Elumalai et al., 2002). The combined highest treatment (32 μg $L^{-1}$ Cu + 25 μg $L^{-1}$ Pb) resulted in the greatest DNA damage, but the variability of the results do not allow us to establish whether the effects of Cu and Pb were additive or non-additive. We are unaware of any studies that indicate the induction of DNA damage by a low concentration combination of contaminants in adult *M. galloprovincialis*. However, in *M. galloprovincialis* embryos exposed to a mixture of Cu and Ag increased, additively and through oxidative stress, DNA damage compared to a control and singularly exposed embryos (Boukadida et al., 2019). In contrast, Cd and benzo[*a*]pyrene in combination decreased the DNA damage to the freshwater mussel, *Dreissena polymorpha,* compared with singular exposures (Vincent-Hubert et al., 2011). These discrepancies and uncertainties further highlight the requirement for more studies on the mechanisms involved in and induced by contaminant mixtures in the natural environment.

Glutathione has a number of known protective roles in organisms, including acting as an antioxidant defence and a Cu chelator, which may be impacted by exposure to individual metals (Richetti et al., 2011; Abarikwu et al., 2017). Moreover, it has been found that at many locations in the natural environment, antioxidant defences increased at polluted sites (Cole et al., 2014; Chen et al., 2015; Abarikwu et al., 2017; Defo et al., 2018). The effects of Cu and Pb on glutathione in the digestive gland of *M. galloprovincialis* were inconclusive, but the higher concentration of Cu alone resulted in a clear increase in glutathione in the gill (Fig. 3) and this was inversely correlated with clearance rate.

Perić and Burić (2019) also observed an increase in glutathione in *M. galloprovincialis* gills exposed to similar levels of Cu that were not apparent when 5 μg $L^{-1}$ of the organophosphorus pesticide, chlorpyrifos, was introduced with the metal. An increase of glutathione in response to Cu could be an indication that there is a need for an oxidative response, with addition of an extra contaminant resulting in glutathione being used for detoxification (Figueroa et al., 2020). There is clearly a need for further detailed investigation of anti-oxidant protection processes in both gills and the digestive gland. This would necessitate assessing the overall role of the antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase to counteract the formation of reactive oxygen species responsible for oxidative stress (Power and Sheehan, 1996). More generally, these observations support suggestions that the gills of mussels are more sensitive organs than the digestive gland to metal contaminants, at least following initial exposure (Butrimavičienė et al., 2019).

The feeding rate of many aquatic organisms is affected by metabolic changes induced by exposure to contaminants, including metals (Sopinka et al., 2010; Moghimi et al., 2018; Vasconcelos et al., 2022), with such behavioural effects often providing an indicator of the harmful effects of contaminants in the environment (Sardo and Soares, 2010). All of the exposures to *M. galloprovincialis* containing the highest Cu concentration (32 μg $L^{-1}$), and including those in the presence of added Pb, exhibited a significant reduction in clearance rate compared with the control (Fig. 2c). Similar results were found when *M. edulis* were exposed to 18, 32 and 50 μg $L^{-1}$ of Cu (Al-Subiai et al., 2011). By contrast, Pb itself had no measurable impact on clearance rate in our exposures (Fig. 2c). These observations suggest that *M. galloprovincialis* could be reallocating energy to detoxification processes, and specifically those involved in detoxifying Cu but not Pb.

MVA is the first step for developing predictive models for the health of mussels (Moore, 2010; Moore et al., 2021; Sforzini et al., 2014) and can be used as a tool for integrating biomarker data into a "health status space". The observations in this study support previous assertions that PCA can assist interpretation of multiple biomarker responses to environmental stressors (Sforzini et al., 2017; 2018; Moore et al., 2021). The only biomarker, however, that can be used directly as an indicator of physiological health status is clearance rate, as it can be used as a proxy for scope for growth (van der Veer et al., 2006; Barillé et al., 2011). In the present study, clearance rate and comet assay for DNA damage were both highly significantly correlated with PC1 and PC2, and clearance rate and DNA damage, as inputs into the MVA, were both found to be influential biomarkers using the BIO-ENV routine (Fig. 4). The inference is that PC1 and PC2 for the two selected biomarkers (clearance rate and DNA damage) can be used as indicators of adverse effects status, as has been shown in previous investigations (Moore et al., 2021). Furthermore, DNA damage measured with the comet assay is likely indicative of oxidative cellular damage, and oxidative stress induced by the high Cu concentration is the most probable factor contributing to overall physiological dysfunction (Moore et al., 2020, 2021).

It must be emphasised that the use of PCA in this way only indicates associations and not mechanistic or causal links. In order to effectively model causative processes, PCA needs to be combined with directed mechanistic networks, comprising the cellular physiological and pathophysiological interactions within a complex dynamic system (Moore, 2010; Moore et al., 2021).

Given the strong correlation between PC1 and clearance rate and the known mechanistic links between feeding, physiological scope for growth and lysosomal function (i.e., endocytosis and intracellular digestion) as effective measures of health status, a preliminary conceptual model has been developed based on the current findings and previously published data on the cellular behaviour of copper and lead (Fig. 5; Moore et al., 2006, 2007; Gu et al., 2019). Copper will largely be

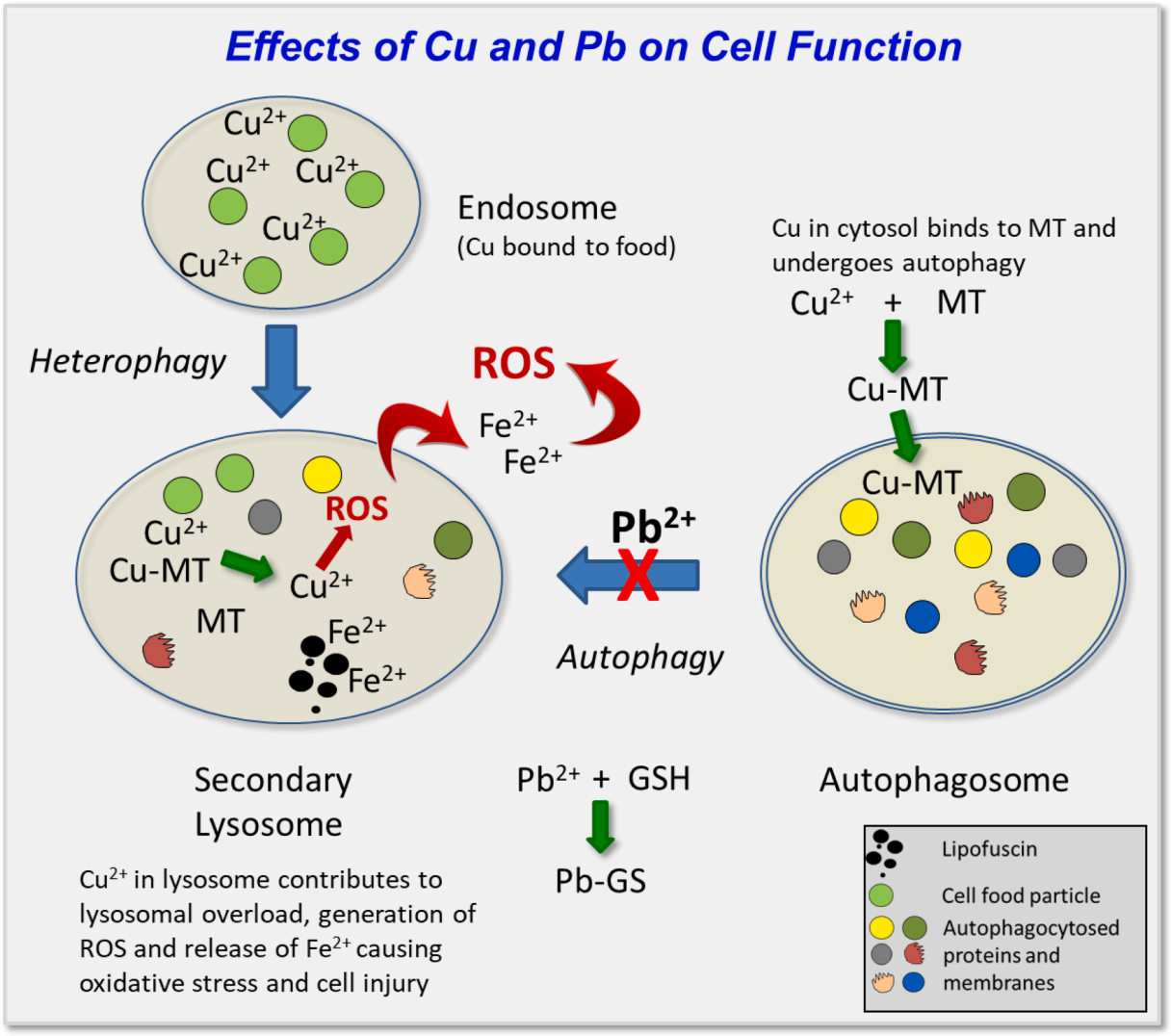


**Fig. 5.** Conceptual model for the intracellular behaviour of Cu and Pb in mussel digestive gland columnar epithelial cells (i.e., digestive cells) based on current and previously published data (Moore et al., 2006, 2007; Gu et al., 2019). Copper, and perhaps Pb, will be taken up bound to food particles. Lead may also be taken up by diffusion across the cell membrane. Copper binds to metallothionein and is transferred to the lysosomal compartment, where accumulation will result in lysosomal overload, lysosomal membrane damage, release of lipofuscin bound iron and resultant generation of reactive oxygen species and oxidative stress. MT – metallothionein; GSH – reduced glutathione; ROS – reactive oxygen species.

taken up by endocytosis of Cu bound to food particles in the digestive gland and accumulates in the lysosomal compartment where it is associated with cytosolic and lysosomal metallothionein (Moore et al., 2007). High Cu exposure will result in lysosomal overload and membrane damage with resultant release of intra-lysosomal iron that generates reactive oxygen species (ROS) and leads to oxidative stress (Moore et al., 2007, 2021; Myers et al., 1993; Rizzollo et al., 2021). Lead is largely bound to glutathione in the cytosol but may also block the fusion of autophagosomes with secondary lysosomes (Gu et al., 2019; Fig. 5). By blocking this fusion of the vacuolar lysosomal system, Pb may potentially disrupt the normal autophagic recycling of redundant and damaged cellular components (e.g., oxidatively damaged membranes and proteins). There is, however, no evidence in this investigation that Pb is exacerbating copper toxicity, as might be expected from enhanced dysfunction of the autophagic process (Fig. 4).

An increase in DNA damage may have a link to the decrease of clearance rate in mussels exposed to Cu. Such a connection has previously been demonstrated in mussels exposed to the pharmaceutical compound, cyclophosphamide (Canty et al., 2009). The mechanisms underpinning this link are currently unknown, but oxidative stress is probably a significant factor, as clearance rate and DNA damage were both shown to be influential biomarkers by the BIO-ENV routine. However, given that behavioural effects on ciliary action for feeding are probably directly controlled by the nervous system, it is now emerging that neurotoxicity is a frequent effect of genotoxic and chemotherapeutic agents (Canty et al., 2009; Kisby et al., 2006; Rzeski et al., 2004).

Although there are no known studies that have investigated a possible link between Pb and the behavioural and genotoxic parameters in aquatic invertebrates, previous singular exposures of Cu to mussels (*M. edulis*) have shown a correlation between clearance rate and DNA damage (Canty et al., 2009; Al-Subiai et al., 2011). Furthermore, Cu exposed to mussels (*M. edulis*) also indicated that there was a correlation between DNA damage (comet assay) and the glutathione concentration in the adductor muscle (Al-Subiai et al., 2011). These biomarkers indicate a potential for a predictive approach to the exposure of Cu and Pb, either alone or in combination. However, because of the sparse number of biomarkers in the present study, and the very limited sample size for glutathione ($n = 3$), more patho-biological endpoints are needed to form a robust network for the impact of binary mixtures on mussels.

## 5. Conclusions

Mixtures of contaminants have the potential to exert antagonistic, synergistic and additive effects on exposed organisms. With respect to the marine mussel, *M. galloprovincialis*, Cu and Pb, added singularly and in combination, were accumulated to greater extents in the gill and digestive gland than in the mantle, but, based on free ionic concentrations, the accumulation of Pb was greater than that of Cu. However, while Cu was observed to act antagonistically towards Pb uptake, the presence of Pb did not appear to inhibit the uptake of Cu, effects we attribute to the greater range of membrane transporters available to the essential metal. Despite these interactions, however, the most significant impacts on *M. galloprovincialis* (a reduction in clearance rate and increase in glutathione activity) were observed when Cu was added without Pb. Clearance rate, as an indicator of physiological status, was also found to be significantly and inversely correlated with DNA damage using data from all experimental treatments, suggesting that an increase in DNA damage may have either a direct or indirect impact on physiological status. Although the precise mechanisms behind these links are currently unknown, MVA and PCA have facilitated the development of a preliminary conceptual model for behaviour and toxicity.

This study highlights that single contaminant exposures at environmentally relevant concentrations cannot always predict outcomes when contaminants co-exist. Further studies are required to elucidate the chemical and biological mechanisms involved in multiple contaminant exposures and to modify and inform guidelines and regulations.

## CRediT authorship contribution statement

**Charlotte Crowther:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Andrew Turner:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Michael N. Moore:** Investigation, Formal analysis, Writing – review & editing. **Awadhesh N. Jha:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Project administration.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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