



## Short communication

# Predicting chronic copper and nickel reproductive toxicity to *Daphnia pulex-pulicaria* from whole-animal metabolic profiles<sup>☆</sup>



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## ABSTRACT

The emergence of omics approaches in environmental research has enhanced our understanding of the mechanisms underlying toxicity; however, extrapolation from molecular effects to whole-organism and population level outcomes remains a considerable challenge. Using environmentally relevant, sublethal, concentrations of two metals (Cu and Ni), both singly and in binary mixtures, we integrated data from traditional chronic, partial life-cycle toxicity testing and metabolomics to generate a statistical model that was predictive of reproductive impairment in a *Daphnia pulex-pulicaria* hybrid that was isolated from an historically metal-stressed lake. Furthermore, we determined that the metabolic profiles of organisms exposed in a separate acute assay were also predictive of impaired reproduction following metal exposure. Thus we were able to directly associate molecular profiles to a key population response – reproduction, a key step towards improving environmental risk assessment and management.

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## 1. Introduction

Three key objectives of the application of metabolomics to ecotoxicology are to quantify the health of organisms, to identify the stressors affecting health, and to build models to predict harm to individuals and populations (Williams et al., 2012). One strategy to draw associations across levels of biological organization is to connect molecular data obtained from omics investigations directly with standard toxicological endpoints such as survival, growth and reproduction (Vandenbrouck et al., 2010). If successful, these associations can form the basis of models that estimate potential organismal and/or population level effects (Garcia Reyero and Perkins, 2011), models that could be powerful tools for environmental risk assessments that might follow either environmental contamination or restorative interventions.

Historical mining and smelting operations in Sudbury, Ontario,

Canada led to the acidification and severe metal, especially copper (Cu) and nickel (Ni), contamination of thousands of lakes (Keller et al., 2007). Despite substantial recovery of water quality over recent decades, levels of Cu and Ni often still exceed Canadian water quality guidelines, and some key zooplankton, *Daphnia* in particular, have failed to recover, most likely because of persistent metal contamination (Labaj et al., 2015; Yan et al., 2016). *Daphnia* are integral players in healthy pelagic ecosystems (Flaherty and Dodson, 2005), and are a widely used test species in both regulatory toxicity testing and functional genomic studies, including metabolomics (Lampert, 2011; Taylor et al., 2009; Vandenbrouck et al., 2010). It is logistically difficult to design laboratory studies which incorporate multiple factors that modify toxicity, yet to successfully assess the risks of multiple stressors (Palmer and Yan, 2013) or the complex toxicant mixtures that typify natural ecosystems, this approach must be considered.

Here, we use metabolomics data to attempt to build multivariate statistical models that are predictive of chronic reproductive impairment, i.e. we evaluate the possibility of using whole-animal metabolic profiles from acute metal exposures to extrapolate to a key population level parameter. The experimental design consisted of both acute (48 h) and chronic exposures (21d) to sublethal,

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environmentally-relevant, Cu and Ni concentrations (Yan et al., 2016) using *Daphnia pulex-pulicaria*, a hybrid commonly found on the Canadian Shield. We had three objectives: i) to determine the chronic reproductive effects of single and binary metal mixtures of Cu + Ni to *D. pulex-pulicaria*; ii) to determine if metabolomics data generated after the chronic exposure was predictive of reproductive output; and iii) to determine if metabolomics data from acute exposures at identical concentration could predict chronic reproductive impairment – with implications for population effects – thereby providing a new tool for environmental risk assessment.

## 2. Materials and methods

### 2.1. *Daphnia* culture and exposures

The origin and culture maintenance of the daphniids was as previously reported (Taylor et al., 2015a). Animals were exposed following a  $3 \times 3$  matrix design to combinations Cu and Ni at 0, 1.0 and 1.8  $\mu\text{g/L}$  for Cu and 0, 1.8 and 18  $\mu\text{g/L}$  for Ni. In this design the 0:0 mixture served as an unexposed control and it was possible to compare the effects of single metal exposures to mixtures. Identical exposure concentrations were used for both chronic and acute tests. Acute (48 h) tests were conducted in a static system with no feeding, whereas chronic (21 d) tests were undertaken in semi-static conditions with twice weekly media changes and daily feeding as per standard culture conditions. Each treatment group had 10 replicates (separate exposure vessels) with *Daphnia* exposed in 50 mL of standard soft water media (FLAMES media without EDTA, pH  $6.4 \pm 1$ , hardness 9 mg  $\text{CaCO}_3/\text{L}$ ; Celis-Salgado et al., 2008). Acute tests were initiated with 5d old *Daphnia* (5 animals per replicate exposure vessel) to ensure sufficient biomass for metabolomics after 48 h of exposure, a duration that preceded primiparity. Chronic tests were initiated with individuals <24 h old and mortality and numbers of neonates produced per *Daphnia* were recorded daily throughout the 21 d test. Water chemistry analyses were performed throughout the test; see [supplementary information](#).

### 2.2. Metabolomics

Animals were immediately flash frozen in liquid nitrogen at the completion of each test and transferred to  $-80^\circ\text{C}$  for storage and shipping. Metabolites were extracted using a bi-phasic extraction method (Taylor et al., 2009, Wu et al., 2008). The polar fraction was analysed using a hybrid 7-T Fourier transform ion cyclotron resonance mass spectrometer (LTQ FT Ultra, Thermo Scientific). Samples were analysed in triplicate (negative ion mode,  $m/z$  70–590) and the data was SIM (selected ion monitoring)-stitched and filtered as described by Taylor et al. (2015b) using a 50% sample filter; see details in [supplementary information](#). We regressed (partial least squares regression; PLS-R) the measured reproductive output against the metabolic profiles of chronically exposed *Daphnia* seeking peaks predictive of reproduction (see [supplementary information](#)). Using the resulting regression model, the potential of utilising short-term exposure regimes to predict chronic reproductive impairment was investigated with the acute metabolic data; i.e. the acute metabolic profiles were input into the optimised statistical model, resulting in a predicted reproductive output for each *Daphnia* sample.

## 3. Results and discussion

### 3.1. Metal toxicity

The exposures to Cu, Ni, or Cu + Ni, by design, did not result in

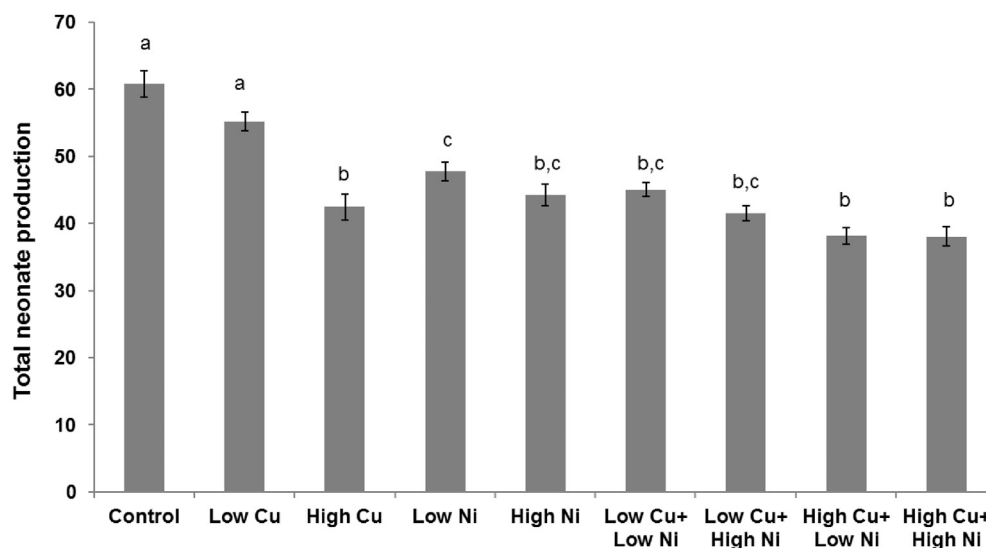
mortalities (in acute nor chronic exposures). Reproductive output (the total number of neonates produced by each individual *Daphnia*) was significantly impaired in all treatments except for the lowest concentration single Cu exposure (ANOVA;  $p < 0.01$ , Fig. 1). The number of broods was identical and time to primiparity was within 24 h for all treatment groups in the chronic assay. Similar to Regalado et al. (2014), we noted Cu reduced fecundity of daphniids but no delay in maturity. That we detected significant adverse effects on reproductive output at very low Cu concentrations is undoubtedly due to the very soft water and low pH of the FLAMES medium. The reproductive impairment observed in both single and binary Cu and Ni exposures may help to explain the lack of recovery of most daphniid species in many contaminated urban lakes in Sudbury where, despite substantial recovery in water quality (Palmer et al., 2013), Cu and Ni concentrations are still somewhat elevated.

Exposure to Cu alone reduced reproductive output by 9% (1.0  $\mu\text{g/L}$  Cu) and 30% (1.8  $\mu\text{g/L}$  Cu). Decreased reproduction of *Daphnia* following Cu exposure has been reported previously, although few studies have investigated metal toxicity in very soft waters. The acute and chronic toxicity of Cu to *Daphnia magna* is positively correlated to water hardness (Rodríguez and Arbildua, 2012). Natural Cladoceran populations obtained from soft water environments and subsequently exposed to Cu had an acute  $\text{EC}_{50}$  of 1.7  $\mu\text{g/L}$ , significantly lower than for animals obtained from harder waters (Bossuyt and Janssen, 2005; De Schamphelaere et al., 2007). In the single Ni exposures, reproduction was decreased by 21% (1.8  $\mu\text{g/L}$  Ni) and 27% (18  $\mu\text{g/L}$  Ni). Again, the soft water composition used in this study could explain these significant effects on reproduction at such low Ni concentrations. The chronic  $\text{EC}_{10}$  of animals exposed to Ni in soft water is significantly lower (2.95–6.86  $\mu\text{g/L}$ ) than those exposed in hard water (23.3–113  $\mu\text{g/L}$ ; Deleebeek et al., 2007). A similar study using *D. magna* found a 21d  $\text{EC}_{50}$  value of 23  $\mu\text{g/L}$  at low hardness, compared to 59–365  $\mu\text{g/L}$  in moderate to high hardness media (Deleebeek et al., 2008).

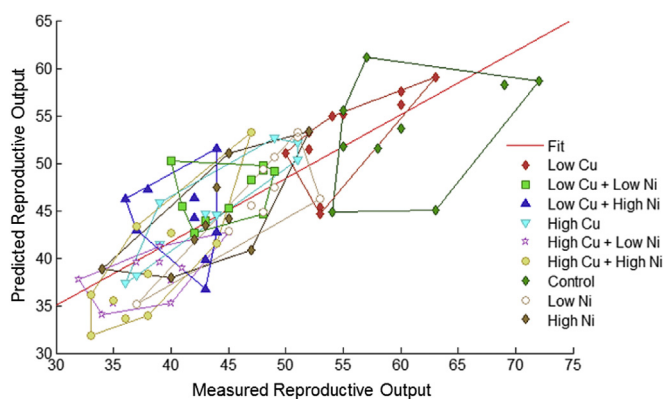
Reproductive output was further decreased with binary metal mixtures; 21–35% reductions in exposures containing low dose Cu with increasing Ni concentration and 37% at high dose Cu regardless of Ni concentration. This indicates there is an increased effect of sublethal Cu + Ni mixtures on the reproductive impairment in *D. pulex-pulicaria* when compared to the single metals. There have been no previous studies of the chronic effects of Cu + Ni mixtures on *Daphnia*. Charles et al. (2014) investigated the acute toxic effects of these metals on amphipods, determining that toxicity was synergistic when both metals were exposed at equal lethal concentrations ( $\text{LC}_{1-90}$ ), yet were antagonistic when using sub-lethal concentrations, highlighting the lack of mechanistic understanding of how metal mixtures affect aquatic organisms. The interaction between metals is variable and responses can vary from synergistic to antagonistic depending on the metals, the test species and the experimental design (Shuhaimi-Othman and Pascoe, 2007; Meyer et al., 2015).

### 3.2. Metabolomics-based predictive models

Our second objective was to determine if data derived from metabolomics analyses could be used to predict chronic reproductive toxicity to *D. pulex-pulicaria*. The optimal PLS-R analysis employed 86 peaks from the metabolic profiles of all treatments from the chronic assay, yielding a statistical model that accurately predicted reproductive output (Fig. 2). The relationship between increasing metal concentration and decreasing reproductive output (Fig. 1) was predicted by the cross-validated model, with the metabolic profiles of the control samples predicted to have the highest reproductive output while *Daphnia* exposed to binary



**Fig. 1.** Total reproductive output after 21d of individual *D. pulex-pulicaria* exposed to single and binary mixtures of Cu and Ni. Data shown are mean  $\pm$  SEM, differing letters demarcate significantly different treatment means (ANOVA with Tukey's post-hoc test,  $p < 0.01$ ). Low and high concentrations are: Cu, 1.0  $\mu\text{g/L}$ , 1.8  $\mu\text{g/L}$ ; Ni, 1.8  $\mu\text{g/L}$ , 18.0  $\mu\text{g/L}$ , respectively.



**Fig. 2.** PLS-R model of metabolic data (86-peak cross-validated model: RMSE = 2.96, RMSECV = 4.78;  $p < 0.001$  against 1000 randomly permuted models) depicting predicted against measured reproductive output of *D. pulex-pulicaria* following exposure to single and binary metal mixtures of Cu and Ni.

mixtures predicted to have the lowest output. Hence, the metabolic profiles derived from our metabolomics approach were able to model an emergent property at a higher level of biological organization, i.e. reproduction (Garcia-Reyero and Perkins, 2011).

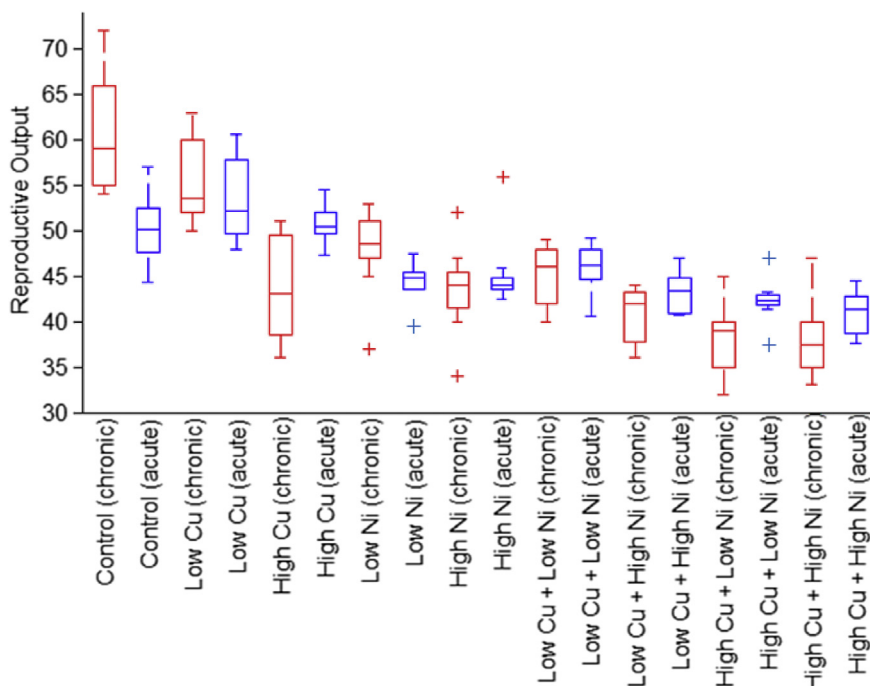
Our final objective was to determine if the *Daphnia* metabolic profiles from the acute metal assays could predict chronic reproductive impairment. Of the 86 peaks used to build the PLS-R model above, 79 of these were found in the acute metabolomics dataset with 35 of these being present in more than 50% of the acute samples. Therefore a second cross-validated PLS-R model was built from the original, chronic metabolomics and reproductive toxicity datasets, using just these 35 common peaks (RMSE = 5.69, RMSECV = 6.33;  $p < 0.001$ ). The metabolic profiles measured in the acutely exposed *Daphnia* were then applied to this new PLS-R model, as an independent dataset, to predict their chronic reproductive outputs (Fig. 3). The predicted reproductive outputs of the acutely exposed *Daphnia* correctly showed the trend of impaired reproduction with increasing metal concentrations.

This both improves our confidence in the predictive capabilities of the statistical model, and, of practical significance, indicates that

short-term, more cost-effective exposure studies can produce metabolic profiles that appear predictive of organismal responses, with implications for population outcomes. This is the first reported study of combined acute and chronic metabolomics data with simultaneously recorded reproductive data from a keystone freshwater organism being used in this manner. A previous study attempted to link acute metabolic data with chronic reproduction in separate studies of two species of earthworm (Whitfield Åslund et al., 2013). This approach to modelling whole organism reproductive changes using acute data has the potential to greatly benefit environmental risk assessment and management. Although currently only applicable to the parameters of this specific study of two metals, the value of utilising predictive models based on metabolic profiles to aid in environmental risk assessment is further enhanced by the very low, sublethal concentrations used in this investigation, and the concordance of our laboratory results with patterns of daphniid recovery in Sudbury lakes. However, there are still knowledge gaps that urgently need to be addressed to further validate this approach. First, more extensive studies of a wider range of pollutants are required to construct and test predictive models that are chemical class-specific and others that may be applied more broadly across many stressors. Secondly, the metabolites with predictive capability should be identified to strengthen the mechanistic understanding of the toxicity, and not rely solely on statistical models of unidentified peaks. This latter point is particularly relevant for the creation of Adverse Outcome Pathways (AOP), a knowledge management framework directed towards linking molecular initiating events with adverse outcomes at the population level, via a series of identifiable key events at immediate hierarchies (Ankley et al., 2010).

#### 4. Conclusion

One of the greatest challenges in ecotoxicology is to link the early warning signals that can be detected by rapid molecular screening techniques, such as metabolomics, to whole organism and population level endpoints. Here we have established that the chronic metabolic profiles of individual adult *D. pulex-pulicaria* can be used to build a multivariate statistical model that is predictive of reproductive impairment following exposure to both single and



**Fig. 3.** Box plots depicting the measured reproductive output from the chronic exposure (red boxes) and the predicted reproductive output from the acute metabolic data (blue boxes, derived from a 35-peak PLS-R model built with chronic metabolic data) of *D. pulex-pulicaria* following exposure to single and binary mixtures of Cu and Ni. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binary metal mixtures of Cu and Ni. Furthermore, this reduced fecundity can also be predicted using the metabolic data measured in juvenile daphniids subject to acute (48 h) exposures. Since chronic studies are both time and resource expensive, the ability to use acute study data holds great promise for future utilisation of cost-effective high-throughput molecular assays to predict higher level biological responses related to population dynamics.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2016.01.074>.

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