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Combined effects of Cu and UVR on survival and melanin synthesis in *Chironomus riparius* larvae

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The tropical Andes encompass vast areas with altitudes above 4000 m, where the combination of high UV radiation and metal pollution creates environments that challenge the survival and persistence of aquatic life. Since the pigment melanin counteracts the negative effects of UVR, and has the additional ability to sequester metal cations, it has been hypothesized that melanin plays an important role in the ability of benthic invertebrates to persist under these harsh conditions. The aim of the present study was therefore to determine the combined effects of Cu and UVR on survival and melanin synthesis in *Chironomus riparius* larvae, by mimicking the extreme environment of high altitude Andean streams under laboratory conditions. It was observed that melanin production was induced by lethal Cu concentrations in the water as well as by exposure to UVR. The detrimental effects of Cu and UVR added up to multistress. Ninety-six hour pre-exposure to UVR decreased the sensitivity of the larvae to UV, but this was not due to an increase in melanin in the larvae. The observed responses to Cu and UVR may explain the persistence of chironomids under the most extreme metal and UV-B conditions in high altitude Andean streams.

Keywords: UV radiation, metal pollution, benthic invertebrates, Andes, high altitude, multistress, Chironomidae, Diptera, midge

The tropical Andes encompasses vast areas with altitudes above 4000 m, creating environments that challenge the survival and persistence of biota. The altitude, the close proximity to the equator, the low cloudiness and a thin ozone layer cause much higher UV irradiances compared to temperate latitudes (Kinzie *et al.* 1998). Elevated UV radiation (UVR) can be harmful to life, causing damage to DNA and proteins, and in transparent shallow waters it has been described as the major driver of the distribution and com-

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position of macroinvertebrate communities (Hurtubise *et al.* 1998, Loayza-Muro *et al.* submitted).

Mineral extraction has been the main economical activity in the Andean region for the last centuries and continues to grow. Metals from mining activities and natural leaching, due to weathering of metal-rich rocks, have deteriorated important freshwater bodies in the region, exceeding local and international quality standards (Loayza-Muro *et al.* 2010). Metals, especially in mixtures, can be toxic to many organisms (Hickey *et al.* 2002) and can accumulate in the food chain (Bervoets *et al.* 1997). Indeed, in high altitude Andean streams, metal pollution has been observed to induce strong shifts in benthic community composition with more sensitive taxa at reference sites and more tolerant taxa at polluted sites (Loayza-Muro *et al.* 2010). Hence, the combination of high UVR and metal pollution in the Andes may well represent extreme environmental conditions challenging the persistence of aquatic life. Despite these unique conditions little research has been performed in the Andean region or similar scenarios (Loayza-Muro *et al.* submitted).

In aquatic organisms, a relevant defence strategy against harsh UV conditions is the production of protective pigments like carotenoids, mycosporine-like amino acids (MAAs) and melanin. Melanin is the only pigment produced *de novo* by animals upon exposure to UVR. It counteracts the negative effects of UVR by converting the excess of harmful energy as heat (Krol *et al.* 1998, Herrling *et al.* 2008, Hansson *et al.* 2009), and it was recently discovered that it has the additional ability to sequester reactive metal cations (Szpoganicz *et al.* 2002, Hong *et al.* 2007). It has also been observed that, in vitro, the presence of some metals (*e.g.* Cu) induced the formation of melanin (Gallas *et al.* 1999). In the Andes, Loayza-Muro *et al.* (submitted) observed that Chironomidae persisted under the harshest UV and metal polluted conditions, and measured higher melanin levels in Chironomidae at both high UVR and at metal impacted sites (unpublished data). This suggested that melanin plays an important role in the ability of chironomids to persist under these conditions, by sequestering metals and counteracting the effects of UVR. Because there is a need for experimental verification of these field observations, the aim of the present study was to determine the combined effects of Cu and UVR on survival and melanin synthesis in *Chironomus riparius* (Chironomidae, Diptera) larvae, by mimicking the extreme environment of high altitude Andean streams under laboratory conditions.

To this purpose *C. riparius* larvae were exposed to Cu, to UVR and to both stressors combined. Cu was chosen because of its presence in Andean polluted streams (Loayza-Muro *et al.* 2010). To select a Cu concentration that guaranteed serious stress, but still assured a substantial number of surviving larvae for melanin analysis, first the 96-h LC50 for Cu was determined. In addition, the combined Cu and UVR experiment was repeated with larvae pre-exposed to UVR to simulate the natural situation of long-term exposure to high UVR in high altitude Andean streams.

MATERIALS AND METHODS

Outline of the study

In the first experiment *C. riparius* larvae were subjected to a range of Cu concentrations in the water in order to determine the 96-h LC50. Next, the combined influence of Cu and UVR on survival and melanin production in the larvae was investigated in a multifactorial experiment conducted in a Benthatron facility. The multifactorial experiment was repeated with larvae pre-exposed to UVR for 96 h. In all experiments, the Cu concentrations in the water and the melanin concentrations in the larvae were measured.

Test organism and culturing conditions

The nonbiting midge *Chironomus riparius* (Chironomidae, Diptera) has a wide distribution (Dornfeld *et al.* 2009). The short life cycle (3-4 weeks) consists of four larval stages, a pupal stage and an adult stage (Marinkovic *et al.* 2011). The larvae are mostly sediment-dwelling and are extensively used in toxicity experiments (León Paumen *et al.* 2008, Marinkovic *et al.* 2011).

In the present study, larvae were obtained from the University of Amsterdam's in-house *C. riparius* culture. The midges were cultured in aquaria containing 8 l of Dutch Standard Water (deionised water with 200 mg/l $CaCl_2 \cdot 2H_2O$, 180 mg/l $MgSO_4 \cdot H_2O$, 100 mg/l $NaHCO_3$ and 20 mg/l $KHCO_3$; pH = 8.2 ± 0.2) and 1.5 l of cleaned quartz sand (60-200 μm). The culture was maintained in a climate room at 20 °C, 65% humidity and a 16:8 h light:dark regime was applied. The cultures were fed a mixture of Trouvit (Trouw, Fontaine-les-Vervins, France) and Tetraphyll (Tetrawerke, Melle, Germany) in a 20:1 ratio.

Cu toxicity experiment

The 96-h LC50 for Cu was determined following OECD guideline 219 (OECD 2004) with slight modifications. First, a range finding experiment was performed followed by a definitive toxicity test. In the range finding experiment $CuCl_2$ (100 mg/l) was dissolved in DSW to a final volume of 100 ml to obtain the following nominal Cu concentrations in the water: 0 (control), 200, 400, 800, 1600 and 3200 μg/l. There were three replicates per concentration, each consisting of ten fourth instar larvae in a 100 ml beaker without sediment and food. The experiments were initiated by introducing the ten larvae into each test beaker using blunt tweezers. After 96 h the number of surviving larvae was counted. In the definitive toxicity experiment a narrower Cu concentration range was tested based on the results of the range finding experiment: 0 (control), 25, 50, 100, 200 and 400 μg/l.

Combined UVR and Cu experiments

The combined UVR and Cu experiments were conducted in 100-ml beakers in a benthatron facility during 96 h. Test beakers contained 100 ml of DSW without

sediment, food and aeration. Water temperature was kept at 18 °C and a 16:8 h light:dark regime was applied. Four treatments with three replicates each and ten larvae per replicate were assayed: control (without UV and without Cu), Cu (nominal concentration 100 μg/l ≈ nominal LC50 value), UV (1.75 W/m²) and the combination of Cu and UV. After 96 h of exposure the number of surviving larvae was counted and the melanin concentrations in the larvae were determined.

The experiment was repeated with larvae pre-exposed to UV. To this purpose, 96 h prior to the experiment, approximately 150 fourth instar larvae were transferred from the culture into a glass beaker containing 800 ml of DSW. Larvae were fed 0.5 mg of food/larva/day and water was aerated and kept at 18 °C. During these 96 h larvae were exposed to UVR (1.75 W/m²). To keep conditions similar between the two experiments, preceding the first experiment, larvae were also placed in a glass beaker for 96 h, but without UVR.

Analysis of Cu concentrations in water

To determine the actual Cu concentrations in the water, in all experiments 2 ml water samples per replicate were taken after 1h and 96 h of exposure. These were acidified with 20 μl 65% HNO$_3$. Copper concentrations in the samples were determined by flame atomic absorption spectrophotometry (Perkin-Elmer AAnalyst 100, Germany). The measured Cu concentrations in the water after 1 and 96 h of exposure were averaged for each replicate to obtain the actual Cu concentration.

Analysis of melanin concentrations in larvae

Melanin analysis was conducted following Hebert & Emery (1990). Eight fourth instar larvae per replicate were dried in a 2 ml Eppendorf tube at 40 °C for 24 h. The samples were then crushed, weighed and homogenized in 1 ml 5M NaOH for 10 min. in a bath sonicator. Next, 10 μl 3% H$_2$O$_2$ was added and the samples were placed in a 65 °C waterbath for another 24 h. Finally, the samples were centrifuged for 5 min at 6000 rpm and 600 μl of the supernatant was analysed in a Shimadzu UV-1601 spectrophotometer at 350 nm.

Statistical analyses

To determine the LC50 for Cu the logistic response model $y = c/(1+e^{b[log(x)-log(a)]})$, adopted from Haanstra *et al.* (1985), was fitted through the concentration-response data with y being survival, a the LC50, b the slope of the logistic curve, c the average survival in the control, and x the actual Cu concentration in the water. For the toxicity experiment, the effect of Cu on the melanin concentrations in the larvae was determined by one-way analysis of variance (ANOVA). A Bonferroni *post-hoc* test was conducted to determine significant differences between treatments. To determine the relationship between the melanin concentration in the larvae and the Cu concentration in the water a Pearson product-moment correlation test was run.

For the multifactorial experiment a two-way ANOVA was applied to determine whether there was an interaction between the effects of UVR and Cu. One-way ANOVA's were run to determine the effects of UVR, Cu and UVR+Cu on survial, and on the melanin concentrations in the larvae. A Bonferroni *post-hoc* test was conducted to determine significant differences between treatments. Independent samples T-tests were run to compare survival and melanin concentrations in the larvae between the multifactorial and pre-exposed multifactorial treatments. All tests were run in SPSS version 16.0 and a significance level of $P<0.05$ was applied.

RESULTS

Cu toxicity experiment

The actual Cu concentrations in the water for the range finding experiment were: 14 (control), 132, 259, 476, 890 and 1503 μg/l. For the definitive toxicity experiment the actual Cu concentrations were: 14 (control), 23, 36, 63, 132 and 259 μg/l. The actual Cu concentrations in the water corresponded to the nominal concentrations with a 93% recovery for the lowest test concentration, decreasing to 47% for the highest test concentration. Control survival in the range finding experiment was 73 and 77% in the definitive toxicity experiment, slightly below the 80% prescribed by OECD guideline 219 (OECD 2004). A clear dose-response relationship was observed for the effect of Cu on survival of *C. riparius* larvae after 96 h of exposure (Figure 1). From the fitted logistic response model the LC50 for Cu was calculated to be 80 μg/l (95% CL: 71-89).

There was a significant positive correlation between the melanin concentration in the larvae and the Cu concentration in the water ($r = 0.801$, $P = 0.009$; Figure 2). The NOEC for melanin production in the larvae induced by Cu in the water was 132 μg/l, the LOEC was 259 μg/l.
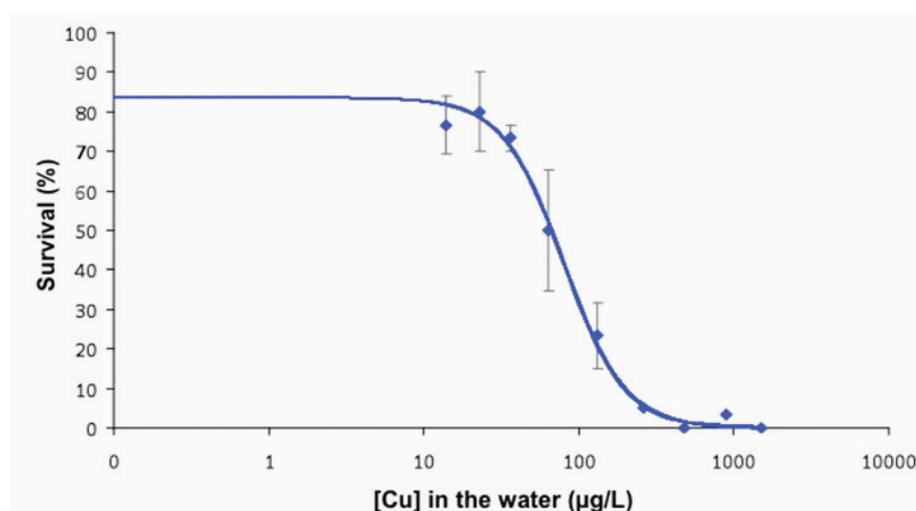

Figure 1. Average (± s.e.) survival (%) of *Chironomus riparius* fourth instar larvae after 96 h of exposure to Cu. Dots indicate the average data points (n = 3) and the line indicates the logistic response model of Haanstra *et al.* (1985).

Combined UVR and Cu experiment

The actual Cu concentrations in the water for the UVR and Cu experiment were: 14 (control), 76 (Cu), 3 (UVR) and 83 (Cu+UVR) µg/l. Control survival for the combined UVR and Cu experiment was 78%, slightly below the 80% prescribed by OECD guideline 219 (OECD 2004). Significant lower larval survival was observed in the Cu treatment ($P < 0.05$), the UVR treatment ($P = 0.001$) and in the Cu+UVR treatment ($P < 0.001$; Figure 3). According to the two-way ANOVA, lower survival was caused by significant main effects of both Cu ($P < 0.01$) and UVR ($P < 0.001$). Even though the Cu+UVR treatment showed the lowest survival, no interaction between Cu and UVR was observed ($P = 0.55$).
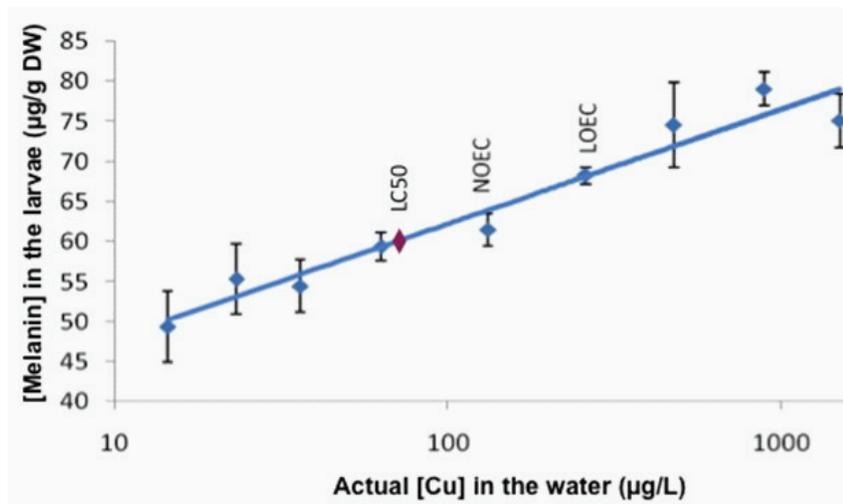

Figure 2. Positive correlation between the average (± s.e.) melanin concentration in the *Chironomus riparius* larvae (µg/g dw) and the Cu concentration in the water (µg/l) after 96 h of exposure to different Cu concentrations in the water (µg/l) ($r = 0.800$, $P = 0.010$). dw = dry weight, n = 3 per treatment. The LC50 was 80 µg/l, the NOEC was 132 µg/l, the LOEC was 259 µg/l.
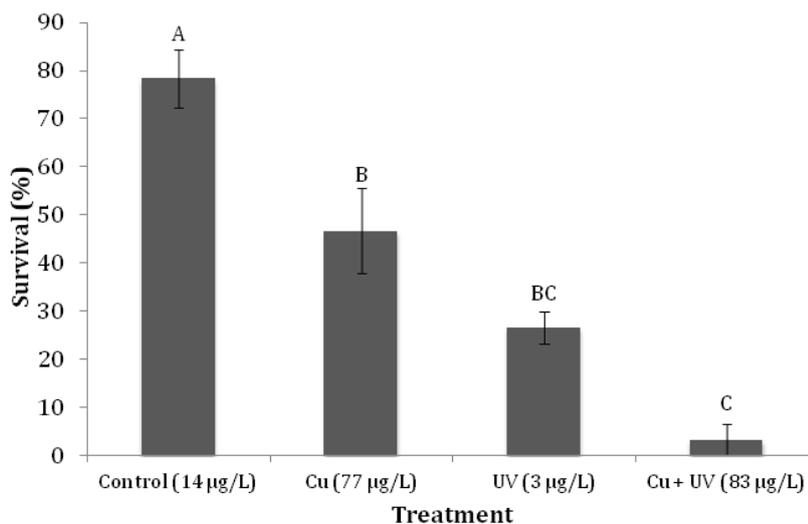

Figure 3. Average (± s.e.) survival (%) of *Chironomus riparius* larvae after 96 h of exposure to the different treatments in the combined UVR and Cu experiment, with actual Cu concentrations in the water between parentheses. Different characters indicate significant differences between treatments at the $P < 0.05$ level. n = 3 per treatment.

Pre-exposed combined UVR and Cu experiment

The actual Cu concentrations in the water for the pre-exposed UVR and Cu experiment were: 1 (control), 72 (Cu), 3 (UVR) and 76 (Cu+UVR) µg/l. Control survival for the pre-exposed combined UVR and Cu experiment was 87%, which is in accordance with the 80% prescribed by OECD guideline 219 (OECD 2004). Pre-exposure to UVR caused a significantly higher survival of the larvae exposed to UVR (P = 0.001) and Cu+UVR (P<0.001; Figure 4) compared to not pre-exposed larvae, but not of those exposed to Cu only. Pre-exposure to UVR did, however, not result in higher melanin concentrations in the larvae in any of the treatments (Figure 5).
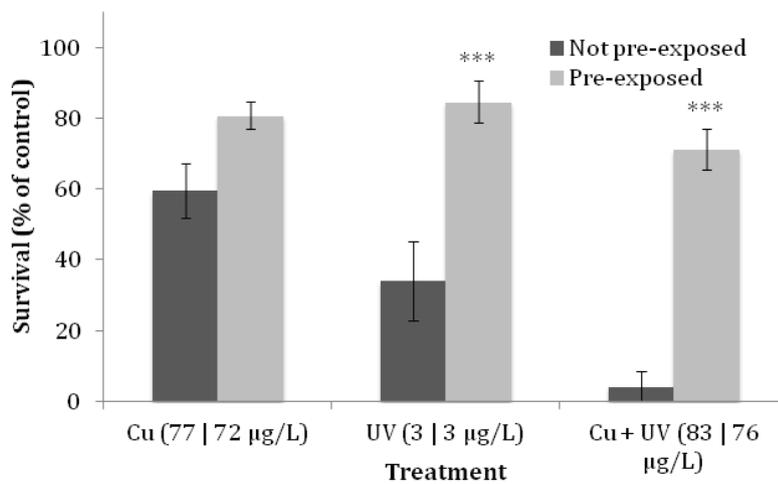

Figure 4. Average (± s.e.) survival (% of control) of *Chironomus riparius* larvae in the combined UVR and Cu experiment and in the pre-exposed combined UVR and Cu experiment after 96 h of exposure to the different treatments, with actual Cu concentrations in the water between parentheses. n = 3 per treatment; *** = P<0.001.
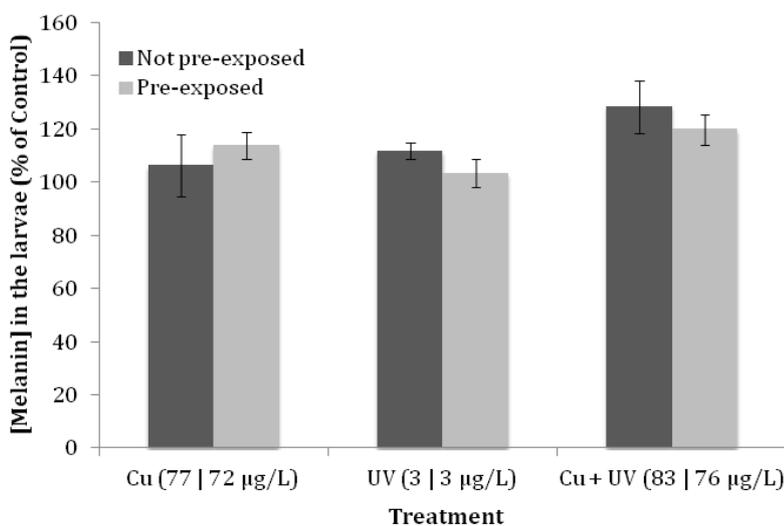

Figure 5. Average (± s.e.) melanin concentrations (% of control) in *Chironomus riparius* larvae from the combined UVR and Cu experiment and the pre-exposed UVR and Cu experiment after 96 h of exposure to the different treatments, with actual Cu concentrations in the water between parentheses. n = 3 per treatment.

DISCUSSION

In high altitude Andean streams metal pollution and high UVR were shown to shape macroinvertebrate communities and it was observed that Chironomidae persisted under the harshest UV and metal conditions (Loayza-Muro *et al.* submitted). This raised the question how chironomids manage to persist under these extreme environmental conditions. In aquatic organisms, a relevant defence strategy against harsh UV conditions is the production of melanin (Krol *et al.* 1998, Herrling *et al.* 2008, Hansson *et al.* 2009). High up in the Andes, Loayza-Muro *et al.* indeed measured higher melanin levels in Chironomidae (unpublished data).

Recently it was discovered that melanin also has the ability to sequester reactive metal cations (Szpoganicz *et al.* 2002, Hong *et al.* 2007) and that, in vitro, the presence of some metals (*e.g.* Cu) induced the formation of melanin (Gallas *et al.* 1999). Likewise, Loayza-Muro *et al.* (submitted) observed that chironomids from metal polluted high altitude Andean streams contained more melanin than those from reference sites at the same altitude. Also in the present laboratory study, melanin was produced by chironomid larvae upon exposure to Cu, confirming the field observations of Loayza-Muro *et al.* (unpublished data). Melanin induction by Cu was observed above 132 μg/l Cu, explaining why no effect of Cu on the melanin concentrations in the larvae was observed in the combined Cu and UVR experiment, in which the animals were exposed to the 96 h LC50 of 80 μg/l. The LC50 was chosen as test concentration in the combined Cu and UVR experiment in order to guarantee serious stress, but still assure a sufficient number of larvae for melanin analysis. Yet, this concentration was too low to induce melanin production.

Pre-exposure to UVR for 96 h resulted in lower mortality of *C. riparius* larvae in the UV and Cu+UV treatments. However, this lower sensitivity was not caused by an increase in the melanin concentration in the larvae. This suggests that a different protective mechanism was induced by pre-exposure to UVR, like the synthesis of other protective pigments or enhanced free radical scavenging and anti oxidant capacity (Meng *et al.* 2009). Pigments like carotenoids and mycosporine-like amino acids (MAAs) are known adaptations to harsh UVR conditions and thus possible candidates for causing the observed lower sensitivity to UVR of pre-exposed larvae. However, carotenoids and MAAs are not produced *de novo* by animals, implying that the larvae would have acquired them from their food during pre-exposure.

It is concluded that by mimicking the extreme environment of high altitude Andean streams under laboratory conditions, the results obtained in the present study confirmed several observations made in the field. The effects of Cu and UVR added up, causing multistress conditions, suggesting that in the Andes, the metal mixtures in the water and the much higher UV irradiances may explain the impoverished communities under the most extreme metal and UV-B condi-

tions (Loayza-Muro *et al.* submitted). We also confirmed the production of melanin by chironomids upon exposure to Cu and showed that pre-exposure to UVR decreased the sensitivity to UV and Cu+UV, which may explain the persistence of chironomids under the most extreme metal and UVR conditions in high altitude Andean streams.

REFERENCES

Bervoets, L., Blust, R., de Wit, M. & Verheyen, R. 1997. Relationships between river sediment characteristics and trace metal concentrations in tubificid worms and chironomid larvae. Environmental Pollution 95: 345-356.

Dornfeld, C.B., Moreira-Santos, M., Espindola, E.L.G. & Ribeiro, R. 2009. Do larvae and ovipositing *Chironomus riparius* (Diptera: Chironomidae) females avoid copper-contaminated environments? *Human and Ecological Risk Assessment* 15: 63-75.

Gallas, J.M., Littrell, K.C., Seifert, S., Zajac, G.W. & Thiyagarajan, P. 1999. Solution structure of copper ion-induced molecular aggregates of tyrosine melanin. Biophysical Journal 77: 1135-1142.

Haanstra, L., Doelman, P. & Voshaar, J.H.O. 1985. The use of sigmoidal dose-response curves in soil ecotoxicological research. Plant and Soil 84: 293-297.

Hansson, L. & Hylander, S. 2009. Effects of ultraviolet radiation on pigmentation, photoenzymatic repair, behavior, and community ecology of zooplankton. Photochemical & Photobiological Sciences 8: 1266-1275.

Hebert, P.D.N. & Emery, C.J. 1990. The adaptive significance of cuticular pigmentation in *Daphnia*. Functional Ecology 4: 703-710.

Herrling, T., Jung, K. & Fuchs, J. 2008. The role of melanin as protector against free radicals in skin and its role as free radical indicator in hair. Spectrochimica Acta Part A-Molecular and Biomolecular Spectroscopy 69: 1429-1435.

Hickey, C.W. & Golding, L.A. 2002. Response of macroinvertebrates to copper and zinc in a stream mesocosm. Environmental Toxicology and Chemistry 21: 1854-1863.

Hong, L. & Simon, J.D. 2007. Current understanding of the binding sites, capacity, affinity, and biological significance of metals in melanin. Journal of Physical Chemistry B 111: 7938-7947.

Hurtubise, R.D., Havel, J.E. & Little, E.E. 1998. The effects of ultraviolet-B radiation on freshwater invertebrates: Experiments with a solar simulator. Limnology and Oceanography 43: 1082-1088.

Kinzie, R.A., Banaszak, A.T. & Lesser, M.P. 1998. Effects of ultraviolet radiation on primary productivity in a high altitude tropical lake. Hydrobiologia 385: 23-32.

Krol, E.S. & Liebler, D.C. 1998. Photoprotective actions of natural and synthetic melanins. Chemical Research in Toxicology 11: 1434-1440.

León Paumen, M., Borgman, E., Kraak, M.H.S., van Gestel, C.A.M. & Admiraal, W. 2008. Life cycle responses of the midge *Chironomus riparius* to polycyclic aromatic compound exposure. Environmental Pollution 152: 225-232.

Loayza-Muro, R.A., Elias-Letts, R., Marticorena-Ruiz, J.K., Palomino, E.J., Duivenvoorden, J.F., Kraak, M.H.S. & Admiraal, W. 2010. Metal-induced shifts in benthic macroinvertebrate community composition in Andean high altitude streams. Environmental Toxicology and Chemistry 29: 2761-2768.

Loayza-Muro, R.A., Elias-Letts, R., Marticorena-Ruiz, J.K., Palomino, E.J., Duivenvoorden, J.F., Kraak, M.H.S. & Admiraal, W. Benthic community responses to metal leaching and ultraviolet radiation in high altitude Andean streams. Submitted.

Marinkovic, M., Verweij, R.A., Nummerdor, G.A., Jonker, M.J., Kraak, M.H.S. & Admiraal, W. 2011. Life cycle responses of the midge *Chironomus riparius* to compounds with different modes of action. Environmental Science & Technology 45: 1645-1651.

Meng, J., Zhang, C., Zhu, F., Wang, X. & Lei, C. 2009. Ultraviolet light-induced oxidative stress: Effects on antioxidant response of *Helicoverpa armigera* adults. Journal of Insect Physiology 55: 588-592.

OECD, 2004. Guideline 219: Sediment-Water Chironomid Toxicity Using Spiked Water. Organization for Economic Cooperation and Development, Paris, France.

Szpoganicz, B., Gidanian, S., Kong, P. & Farmer, P. 2002. Metal binding by melanins: studies of colloidal dihydroxyindole-melanin, and its complexation by Cu(II) and Zn(H) ions. Journal of Inorganic Biochemistry 89: 45-53.