A. Comparison between the HC5-50 and essentiality and homeostasis levels

Copper has been proven to be an essential element for many biological processes. Copper is required for the functioning of a variety of enzymes such as superoxide dismutase (a scavenger of toxic oxy-radicals), cytochrome c-oxidase (part of the electron transport system in eukaryotic organisms), several oxidases (e.g. amine oxidase, ascorbate oxidase), mono-oxygenases and di-oxygenases (Cass and Hill, 1980). Copper is also essential for haemocyanin, which is a wide-spread oxygen-carrier in molluscs and arthropods and which is the second most widely distributed pigment in the animal kingdom (Brunori et al., 1979; Cass and Hill, 1980). According to Liebig's law of the minimum, each species has for each essential element an optimal concentration range in which it can satisfy its metabolic requirements and develop and perform in an optimal way (Hopkin, 1989). Van Assche et al. (1997) termed this range the optimal concentration range of essential elements (OCEE). The OCEE is linked with the natural concentration of the essential element in the species' natural habitat. It is further determined by the species' homeostatic capacity that allows it to regulate actively its metabolically required tissue concentrations and maintain optimal levels under varying external concentrations of the essential element. However, when the external concentration of the element becomes too low or too high, homeostatic regulation will not be sufficient and deficiency or toxicity can occur, respectively. Recently, studies have demonstrated the existence of an OCEE for invertebrates and algae for copper (Bossuyt and Janssen, 2003a, 2003b, 2003c). These studies have also indicated that the OCEE may shift to lower or higher concentrations upon acclimation to lower or higher concentrations. However, based on acute and chronic toxicity test data these authors only report a maximum of a factor of two to three decrease in sensitivity of *D. magna* and *P. subcapitata* in laboratory acclimation studies with these metals. A similar decrease in sensitivity by a factor of 2 was also demonstrated for O. mykiss, acclimated to increased waterborne copper levels (Taylor et al., 2004). Although the importance of this acclimation still needs to be addressed under field circumstances, acclimation effects only account for sensitivity differences up to factor two to three (within one species), whereas bioavailability has been demonstrated to account for differences well over 2 orders of magnitude within the same species (Erickson et al., 1996; Di Toro et al., 2001).

Within the ecotoxicity data base, a decreased growth of *O. mykiss* below 7.8 µg Cu/l and above 16 µg Cu/l indicated a copper OCEE range between 8 & 16 µg Cu /l (Seim et al, 1984).

Fort et al.,2000 further demonstrated developmental effects of copper deficiency and toxicity on *Xenopus laevis*. Three different copper containing diets (copper adequate diet, copper deficient diet and copper supplemented diet) were administered to adult *X. laevi* for 28 days. Egg production was assessed in the three groups. Larval developments were subsequently evaluated in a 4 days standard test, using varying copper concentrations. Reduced egg production as well as developmental effects were evidenced with the copper deficient diet (

Figure Error! No text of specified style in document.-1). Malformations related to copper deficiency included limb development as well as malformation of the eyes, brain, limbs and heart. Some of the observed copper deficiency symptioms were attributed to decreased lysil oxidase activities (a copper containing enzymze) and the resulting poor cross-linking of collagen fibers. **Figure Error! No text of specified style in document.**-2 compares mortality/malformations effects as a function of copper concentrations and demonstrates the U shaped copper doseeffects relationship. The curve indicates optimal concentrations range for copper between 1 and 10 µg /L. Interestingly, the development of larvae from parents fed copper supplemented diet did not show copper deficiency, indicating that with the copper supplemented diet the eggs had accumulated sufficient copper to sustain development.

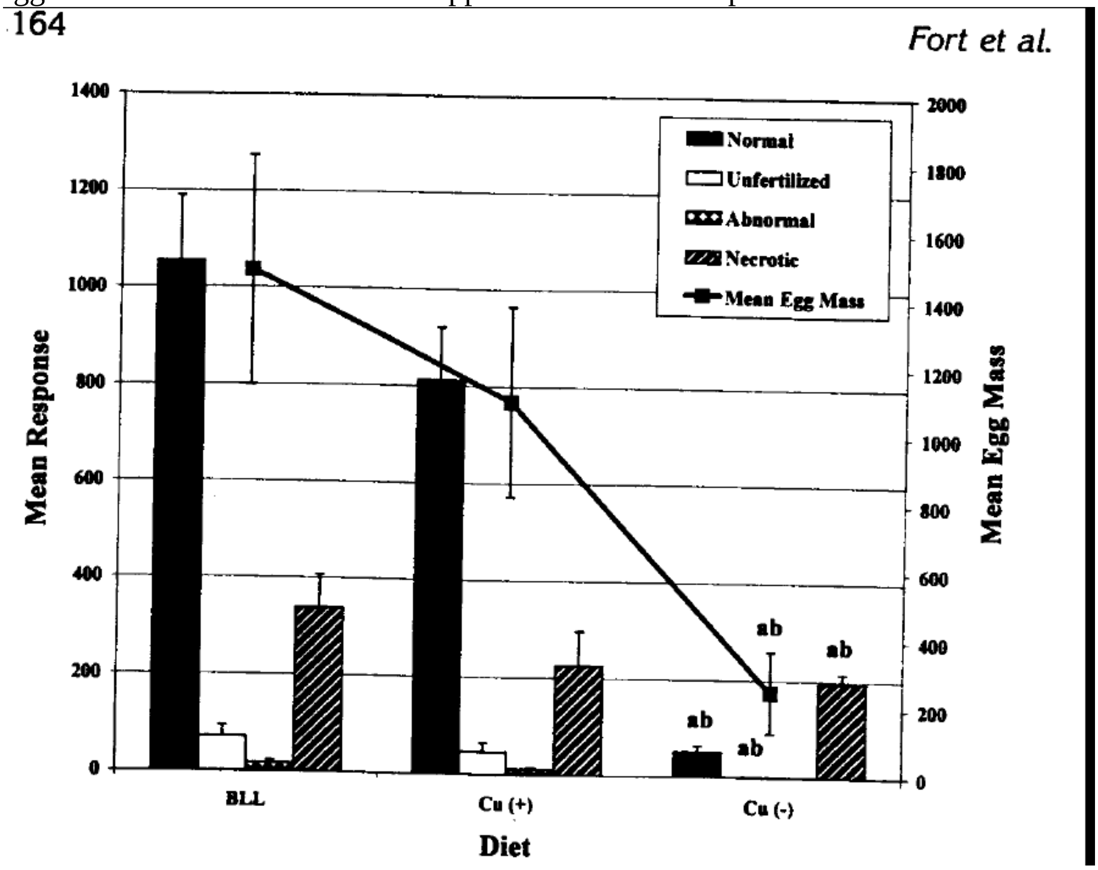


Figure Error! No text of specified style in document.**-1:** Effects of copper diet on egg development and larvae malformations of Xenopus larvae (BBL= normal diet; Cu(+)= copper supplemented diet, Cu(-)=copper defiecinet diet)

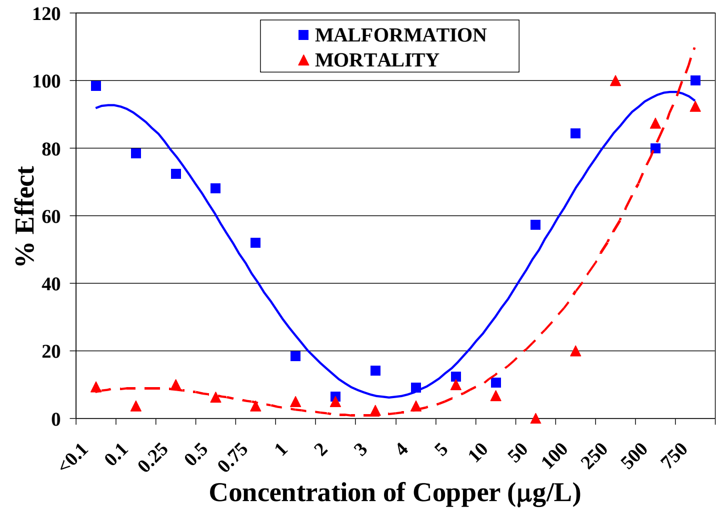


Figure Error! No text of specified style in document.**-2:** Effect of copper concentrations on 4-days development of *X. laevis*. Adult *X. laevis* were maintained on beef liver and lung diet for 28days.

Bossuyt et al, 2004 performed acclimation experiments with D. magna and showed that after three generation of acclimation, the optimal concentration ranges (from energy reserves and number of offsprings) remained constant between 1 and 35 µg Cu/L (**Figure Error! No text of specified style in document.**-3). Highest energy reserves were observed around 12 µg Cu/L. Below 1 µg Cu/L (a concentration often used as background copper concentration in the ecotoxicity media), copper defieciency was clearly observed.

Comparison between this homeostatic range with the chronic toxicity (expressed as 21 days EC_{50} values) of copper for the same species shows a clear overlap between the OCEE and the toxicity thresholds. In other words, chronic toxicity of copper for *D. magna* is expected to occur within the species' homeostatic capacity (**Figure Error! No text of specified style in document.**-4).

Such overlap could be explained because both the experiments in the toxicity and homeostatis studies were conducted in different test media, reflecting therefore different levels of bioavailability. Normalisation of both the ecotoxicity data and the homeostatic range to similar conditions (i.e. reasonable worst case EU conditions) using the chronic BLM resulted in a clear differentiation between the optimal copper levels and the toxicity levels for *D. magna* **Figure Error**! No text of specified style in document.**-5.** Deficiency and toxicity effects, normalised at the same level of bioavailability, seemed to occur when the external copper concentrations are < 1.1 µg/l and > 23 µg/l respectively.

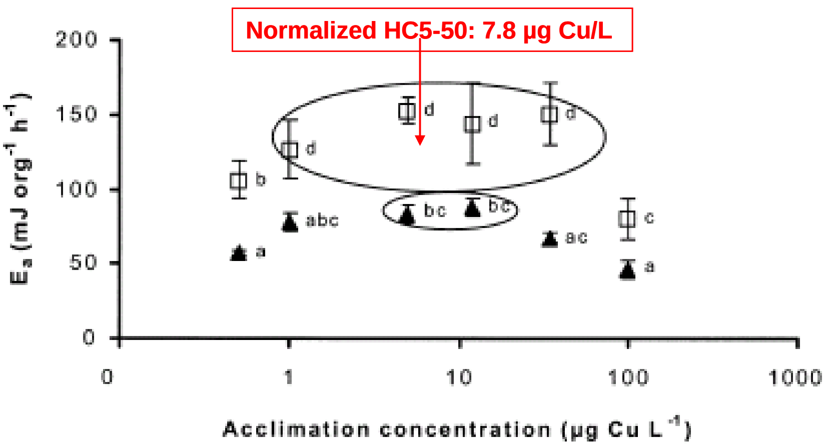
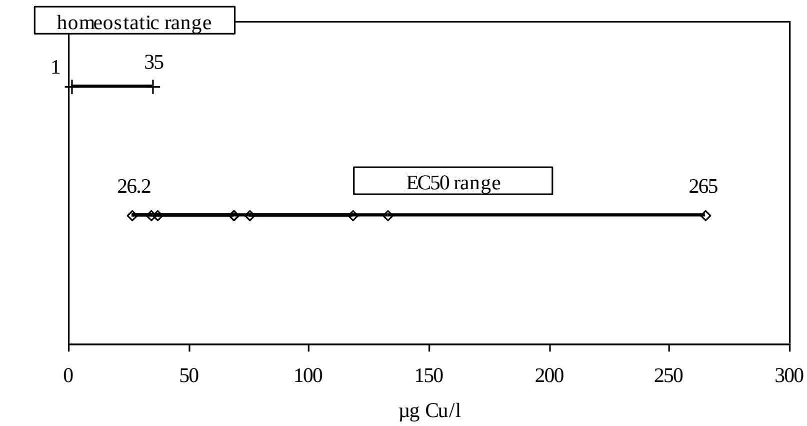


Figure **Error! No text of specified style in document.**-3 Energy reserves (Ea) of first (triangles) and 4th to 15th (squares) generation D. magna acclimated to different copper concentrations (error bars represent standard deviations. Mean levels for same letter are not significantly different at p<0.05). Ovals represent the optimal concentration range.

Figure Error! No text of specified style in document.**-4 :** Comparison between the chronic EC_{50} values and the homeostatic



range for *D. magna*

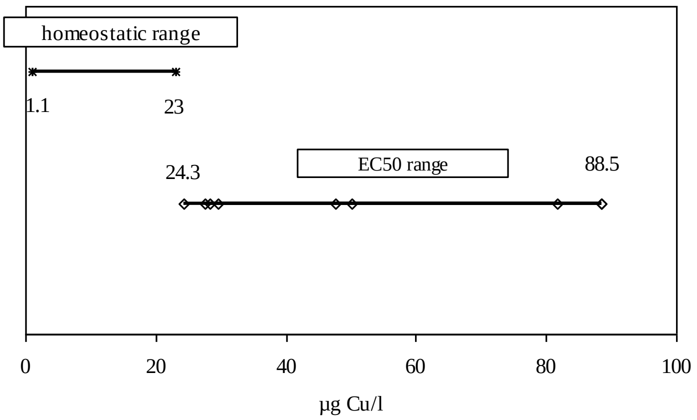


Figure Error! No text of specified style in document.**-5** :Comparison between the BLM-normalised chronic EC50 values and the homeostatic range for *D. magna*

BLM normalizations of the whole SSD, towards the water used for the acclimation experiments allows to calculate an HC5-50 of 7.8 µg Cu/L, being right at the centre of optimal concentration range **Figure Error! No text of specified style in document.**-3.

Some studies have evaluated the influence of dietary versus waterborne exposure on copper homeostasis and acclimation processes. Kanmundu et al. (2001 & 2005) demonstrated the importance of dietary uptake under normal copper exposure levels, and the importance of dietary uptake for copper homeostasic regulation of gill, and gut copper uptake as well as biliary excretion. Along the same reasoning, Bossuyt et al. (2004) performed acclimation studies with *D. magna* and demonstrated that acclimation is more related to the total copper concentration of the culture medium, than to the copper activity. Therefore, it is likely that although copper toxicity is related to bioavailable copper species and thus to water chemistry, higher organisms (fish and invertebrates) acquire the essential copper levels mainly through the diet and as a consequence copper toxicity is driven by bioavailability while copper deficiency and copper homeostasis seems to be driven by dietary and hence total copper levels.

Conclusion : Copper is an essential element and the above analysis confirms that the BLM derived HC5-50 will protect the organisms from copper toxicity as well as copper deficiency.