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Immortal *Hydra* as a Model Organism for Metal Toxicity Studies

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Alisa Nykolayeva[1] and Aiman Shahid[2]

1. McMaster University, Honours Life Sciences, Co-op, Class of 2021
2. McMaster University, Honours Life Sciences, Class of 2020

ABSTRACT

Toxicology is an interdisciplinary scientific field that explores the impact, epidemiology, and treatment regimens for exposure to various toxic compounds and elements. Many toxicants such as metals have not yet been comprehensively examined, and a plethora of metal-related conditions are currently untreatable. *Hydra* is an immortal freshwater organism that serves as an excellent model for toxicity studies due to its natural availability, anatomical simplicity, yet comparatively complex physiology. This review will examine the significance of *Hydra* toxicity studies, outline current experimental designs, as well as summarize the most commonly tested metals. Altogether, comprehensive toxicity studies on *Hydra* might provide promising breakthroughs in the understanding of toxicity-related physiology, and can be applied to clinical research and practice to ultimately improve health and wellbeing of those affected by metal-related disorders.

Keywords: Model organism, *Hydra*, metal toxicity, morphology, experimental setup, regeneration

INTRODUCTION

Model Organisms

In research, model organisms are non-pathogenic living organisms that are carefully and extensively studied to contribute to the understanding of biological processes.[1] They are usually easily sustainable in laboratory conditions, bred in large quantities, have a short life-span, and are cost-effective to maintain.[1] Depending on the research incentive, these organisms are studied to understand genomic and genetic phenomena, disease origin and/or progression, anatomical and histological structures, developmental events, and pharmacologic outcomes, with the prospect that findings obtained from these studies will be applicable to more complex organisms such as humans.[1]

Hydra Anatomy and Regenerative Potential

Carnivorous cnidarian *Hydra* is a model organism used for genetic, developmental, pharmacological, and toxicity studies, and is simple enough to allow for efficient *in vivo* experimentation.[2] Biologically immortal, *Hydra* is a freshwater polyp that is said to be ageless due to the unlimited capacity to replace all cells in its body, and it is theorized to be able to survive for over 1400 years in ideal conditions.[3] The organism, thus, possesses the ability to regenerate a missing fragment or a wound upon injury anywhere along the body column, including the peduncle (foot), and the hydranth (head).[2] The hydranth consists of a hypostome, which is the oral tip made of epithelial cells that can temporarily open due to cell rearrangement to allow the entry of prey and excretion of regurgitated food.[4] The hypostome is surrounded by the tentacles that carry terminally differentiated cells called cnidocytes, that are involved in paralyzing and capturing prey.[5] The animal's body column is composed of the gastric section for food digestion, the budding section where a genetically identical polyp grows, and the peduncle.[4] At the very bottom of the peduncle, the basal disk, secretes various types of glycan-releasing granules that enable the *Hydra* to adhere to surfaces.[6] See Figure 1 for anatomical overview.

Hydra is a small diploblastic animal,[7] which makes it extremely sensitive to any environmental change.[4] It is

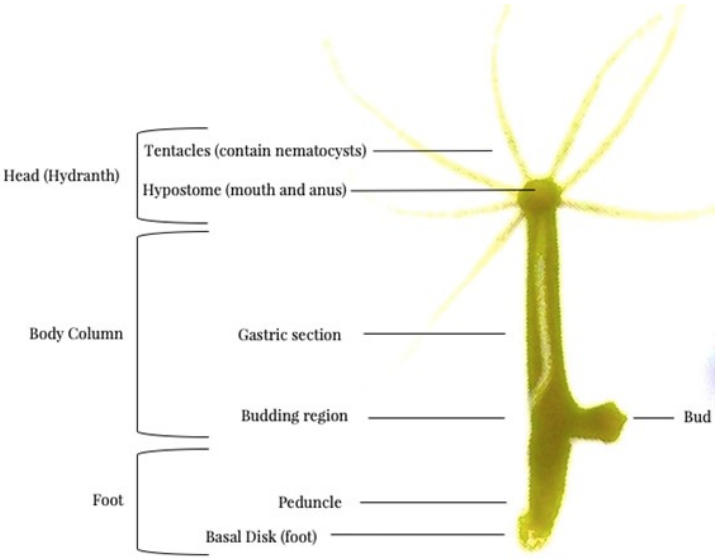

Figure 1. Overview of *Hydra* Anatomy. This figure identifies specific anatomical features of *Hydra* including the head, the body column, and the foot, as well as the developing bud.

composed of only two cell lineages: the epithelial cell lineage which builds up the endodermal and epidermal tissue layers, as well as the interstitial cell lineage dispersed throughout the interstitial space.[8] Both cell lineages possess multipotent stem-cell properties allowing the cells to undergo continuous mitotic divisions, and upon the influence of various intracellular and extracellular factors — permits cell differentiation.[4] The tissue layer between the epidermal and endodermal tissue is called the mesoglea, which is a modified, fibrous extracellular matrix that is evolutionary similar to a basement membrane that is known to partake in molecular signalling.[4] *Hydra's* regenerative capacity is not yet comprehensively understood since there is a multitude of cellular and molecular events which contribute to the phenomenon.[4] However, the mesoglea might have crucial implications in understanding stemness since it regulates intracellular signalling while also acting as a substratum for cell migration.[9]

Phylogenetically, *Hydra* is a very divergent organism from higher animal forms; however, studying this organism provides a crucial knowledge base for development, stemness, differentiation, regeneration, and evolutionary mechanisms such as symbiosis.[10] Overall, roughly 80 different species of cnidarian *Hydra* were identified, and they were clustered into four species groups due to morphological similarity.[10] The genus *Hydra* originated an estimated 60 million years ago with *Hydra viridissima,* green *Hydra,* being the first diverging group, followed by three clades of brown *Hydra* that share a common ancestor.[10] The three clades are called *Braurie, Oligactis,* and *Vulgaris,* in the order of divergence.[10] Green *Hydra* carries photosynthetic endosymbiotic algae, *Chlorella,* which are known to modulate carbohydrate metabolism. However, other evolutionary advantages of the symbiont have been proposed and require further investigation.[11] Brown *Hydra* lack a symbiont, thus they are called aposymbiotic, and present with efficient lipid metabolism,[12] however, these species tend to exhibit higher protein loss during periods of starvation, which negatively affects their survival.[13]

Complete *Hydra* regeneration, either from a cut segment or a pellet containing aggregates of cells, occurs within a few days.[4] Numerous studies have proven that only the interstitial stem cells are required for complete regeneration of the animal since they are able to differentiate into any other cell type in *Hydra's* body.[14] Signalling to achieve differentiation and regeneration is accomplished by morphogens that are activated and/or upregulated during injury to ensure that the positional information is communicated to regenerate the missing segment.[9]

Since the animal is diploblastic, its epithelial layer is in continual contact with the external environment, and toxins only need to diffuse through two-cell layers to penetrate the organism.[10] This sensitivity and *Hydra's* regenerative capacity makes it suitable for acute and chronic toxicity studies, which are discussed further in this review.

Water Contamination by Heavy Metals

Although roughly 70% of Earth is covered in various types of water bodies, only 3% of it is freshwater, and only roughly 1% is usable, drinkable water. Canada is in a possession of 20% of the planet's freshwater supply, where only 7% is considered renewable due to industrial, medicinal, and nuclear wastes. The Canadian guidelines for drinking water quality have been established by the Federal-Provincial-Territorial Committee on Drinking Water (CDW), and are annually updated by Health Canada. The guide provides information on various types of water contaminants, accompanied by several parameters such as the maximal acceptable concentration (MAC), common sources of a parameter in water, health considerations, and a comments section.[15] Moreover, the guidelines in the document are systematically updated through extensive primary literature appraisal carrying relevant findings. Thus, the guide serves as a foundation to begin toxicity experimentation focusing on specific groups of compounds. Metal toxicity screening combined with clinical studies provides insight into chronic effects, acute toxicity, and birth defects associated with metal compounds in drinkable water. There are various metals, however, that are essential to human health in trace quantities. Such metals include copper, zinc, and chromium, and they are involved in mediating a multitude of biochemical processes. Non-essential metals include lead, mercury, arsenic, and cadmium, and exposure

often results in accumulation of the compound in organ tissues leading to metal overload. The main methods by which these metals are ingested by humans are through poorly filtered drinking water, as well as through the food chain.[16] Therefore, studying the implications of long-term, small-dose exposure is essential to uncover acute and chronic health effects of the aforementioned metals to human health.

Wet laboratories use model organisms to test the toxicity of metal compounds to obtain insight into the effects, mechanisms, and treatment implications of the research. The findings are also used by the government to develop regulations for drinking water quality and testing since optimal water filtration is limited in many areas of the world. This review discusses the common experimental designs used to perform toxicity screens on *Hydra,* specific metals tested in the screens, as well as the species of *Hydra* used for experimentation. Current limitations in any aspect are appraised by the review, and it includes a suggestion to explore a novel experimental design that will specifically target the regenerative potential of *Hydra's* stem cells. This design permits a more comprehensive approach to explore toxicity on a cellular level, and provides insight into the teratogenic and otherwise toxic potential of metal compounds.

DISCUSSION

Hydra Culturing and Experimental Design

Hydra exhibit a very dynamic body column, and some species extend up to 2 cm in length at the most relaxed state.[4] The tentacles of the animal, in a healthy state, are long and flowy, and are usually the first to provide a response to external stimuli.[5] At the state of morphology deterioration leading to the disintegration of the organism, the cnidarian exhibits notable physical features, allowing the observer to quantify the morphological, most commonly, by using the Morphology Scale, which was established by Wilby in 1988 (see Table 1).[17] The scale describes the organism's physical appearance from the stages of thriving to disintegration (death). Disintegrated *Hydra* presents as a cluster of cells attached to its substrates, such as rock or vegetation in the natural environment, or a glass dish, in the lab. The apparent morphological changes in *Hydra*, especially in response to toxicity, provide yet another reason the organism serves as an excellent model for toxicity experimentation.[18]

Naturally, *Hydra* is found in shallow, slow-moving freshwaters such as lakes and rivers, living in cultures of millions of *Hydra*.[8] As environmental conditions change, *Hydra* locomote using various methods that require muscle fibre contraction, tentacle movement, or floating.[8] To mimic natural habitat conditions of

Table 1. Adaptation of Wilby's (1998) Morphology Scale. The scale presents visual characteristics of the *Hydra* quantified with scores 10 (healthy state) to 0 (disintegrated state). The use of this morphology scale is useful in toxicity studies, as it contributes to consistency in data collection and analysis permitting accurate species response quantification and metal impact comparison.

Score	Morphology
10	Extended tentacles and reactive body column
9	Partially contracted tentacles; slow reactions to stimuli
8	Clubbed tentacles and slightly contracted body column
7	Shortened tentacles and a slightly contracted body column
6	Shortened tentacles and body column
5	Totally contracted body column, short and visible tentacles
4	Totally contracted body column, with no visible tentacles
3	Expanded body column, yet visible, short tentacles
2	Expanded body column, with no visible tentacles
1	Dead but intact
0	Disintegrated; appears as a cluster of cells at the bottom of the substrate

the animal in the lab and to prevent mass disintegration of *Hydra* culture, individual *Hydra* must be placed in glass dishes or bowls and filled with *Hydra* Medium (HM).[4] HM, similar in composition to freshwater, is a mixture of highly diluted salts in double distilled water. Namely, it consists of calcium chloride salt ($CaCl_2 \bullet 2H_2O$), TES buffer (N-tris [hydroxymethyl]methyl 1-2-aminoethanesulfonic acid buffer), and EDTA (Ethylenediaminetetraacetic acid) that are diluted in double distilled water.[18] The composition of the HM, however, might vary amongst the laboratories. The culture plates are usually kept in a temperature-controlled incubator, at around 20°C; however, the temperature can vary amongst the laboratories and species used.[18]

The 1997 study by Trottier S. et al., is used here as a general example for the experimental setup of a typical *Hydra* toxicity study. The organisms are starved for 24 hours prior to experimentation, ensuring that the regurgitated waste does not contaminate the solution.[18] Feeding does not occur in the duration of the experiment,[19] likely to control for contamination as well as potential budding, since feeding stimulates an increase in the rate of asexual reproduction.[19] This was a cross-sectional study tracking three *Hydra* per treatment group, once daily.[18] A 12-well microplate was used, where a third of the wells were dedicated for the control variable which is the HM in this case.[18] The screen was carried on for 96 hours, where at every 24-hour time-points the *Hydra* were qualitatively assessed and imaged using a stereoscope.[18] Budding *Hydra* in the

experimental set-up were avoided, ensuring each individual well was occupied by three *Hydra* only.[18] Test solutions were diluted in HM using logarithmic dilution for pure substances and serial dilution for aqueous environmental samples.[18] Prior to placing the animal into the experimental microplate with its respective solutions, the animal was washed in a petri dish filled with the respective test compounds.[18] Different concentrations of the compound are utilized to observe the gradient of its effect on *Hydra* morphology.[18] Usually, replicate microplates are set up to increase the sample size for improved statistical power at the time of analysis. Two measures of drug potency are generally applied to determine the effect of a drug on an organism:[18] EC50 - half maximal effective concentration and LC50 - half maximal lethal concentration. EC50 is used to measure the induced response of the compound halfway between the baseline and maximal effect at some duration of exposure, as opposed to the LC50, which estimates the concentration of the compound that kills half of the population at some duration of exposure. In the case of *Hydra* toxicity screen in Trottier's study, EC50 is applied as the *Hydra* exhibit clubbed tentacles, whereas LC50 is applied as the *Hydra* disintegrate.[18] Thus, the study measured sublethal toxicity of the compound, as well as lethal effects, utilizing the same experimental setup.[18]

A 1998 study by Beach M. J. et al. utilized a 6 mL-well of a repli dish with a single *Hydra* populating the space. This was a longitudinal study, which tracked an individual *Hydra* over time, after having been exposed to a particular compound.[7] The overall sample size of the study was 20 individuals per each tested metal concentration, and the study randomly selected both, budding and non-budding subjects during the setup.[7] Beach's study measured toxicity similarly, but with the addition of a feeding protocol for the sub-lethality screen.[7] Similarly to Trottier's study, 24-hour time intervals were used to collect data on the animal's morphology and any associated conditions, and the experiment lasted for a total of 96 hours for the acute toxicity screen. For sub-lethal toxicity, at the 48-hour time point, the subjects were presented with five *Daphnia* neonates as their prey.[7] Feeding behaviour was recorded after each 20-minute interval for two hours and the response was assessed by the amount of shrimp ingested by each organism.[7] Differently from Trottier, however, *Hydra* cultures were kept at 18 hours light and six hours dark lighting regiment, at 20 +/- 1°C.[7]

A 2000 study by Karntanut W. and Pascoe D. conducted an experiment similar to Beach; however, testing was performed in 3 mL glass vials carrying 1 polyp per space, with a total of ten subject replicates.[17] As opposed to recording the LC50 and EC50, Wilby's scale was used to assess the trend of *Hydra* response, allowing for consistent numerical categorization of *Hydra*

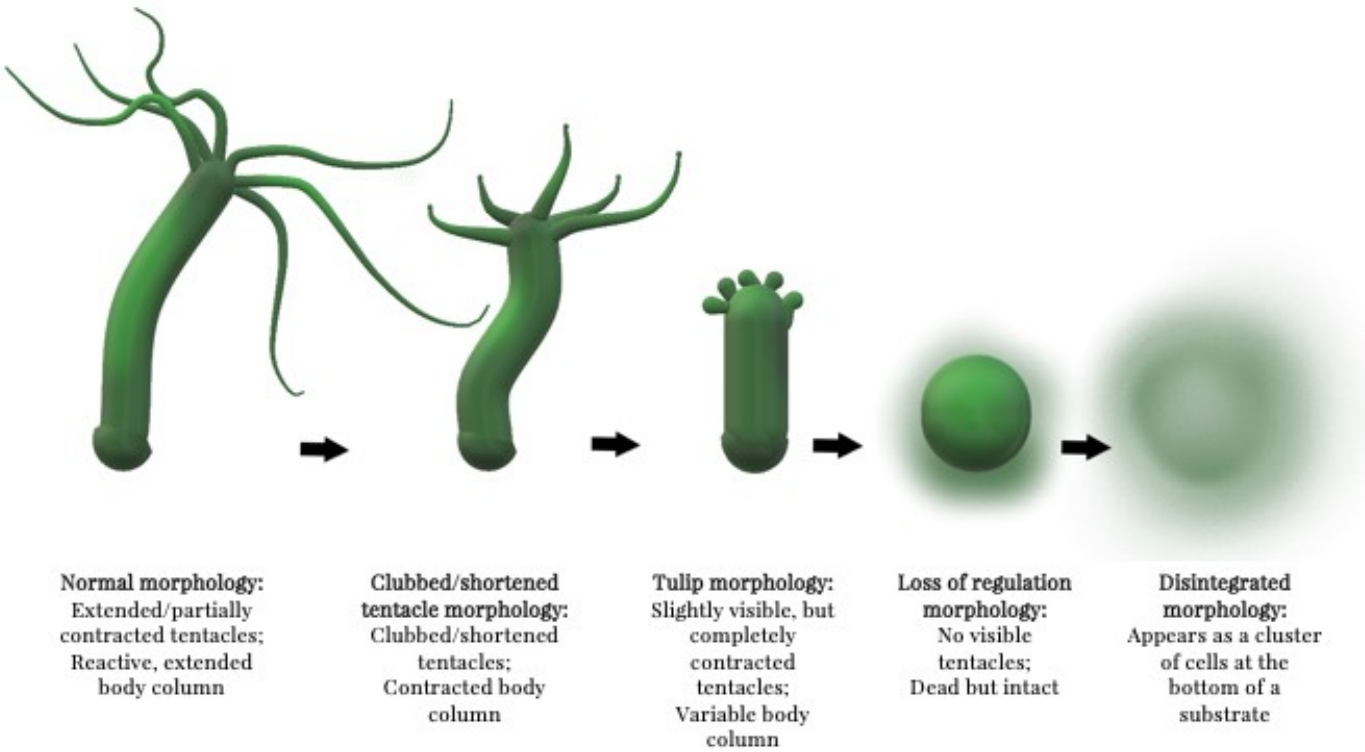

Figure 2. Condensed Overview of *Hydra* Morphology in Response to Toxicity. Stages of morphological deterioration were adapted from Wilby's (1988) Morphology Scale (Scores 10-0) are portrayed in a condensed manner. The overview features five stages: normal, clubbed/shortened tentacle, tulip, loss of regulation, and disintegration morphologies.

morphology per time-point. LT50 or median lethal time response was applied in addition to LC50 to further compute the response of the animal to the toxin.[17] See Figure 2 for the condensed overview of *Hydra* morphology in response to toxicity.

Numerous other studies have been performed using similar, but not identical experimental setup. This, although inconsistent, allows for further understanding of *Hydra's* physiology in relation to metal toxicity, as every study provides for a unique set of variables. Some designs are cross-sectional, but longitudinal studies are commonly performed to avoid various co-variables such as the state of deterioration, which may affect neighbouring *Hydra* to disintegrate as well. If the subject occupies the space alone, this variable would be eliminated. Irrespective of the experimental design, physiological toxicity can be concluded based on the experimental data. The main compelling advancement to the studies would be to perform toxicity screens where the *Hydra* is dissected and allowed to heal and regenerate over a period of time. This will provide a more comprehensive insight into metal toxicity since it will target regeneration mechanisms and its susceptibility to metal toxins.

Compounds Tested and Species Response

Hydra have been shown to accumulate metals through waterborne and foodborne routes,[20] evident through sampled nematocysts contents that led to reduced prey capture.[4] Traces of metals were also found in the algae of the symbiotic *Hydra*.[4] Thus, *Hydra* is an excellent bioindicator for metal toxicity studies, as it lacks metallothionein, a binding protein, that aids with sequestration of metals.[4]

The 2002 study by Karntanut W. and Pascoe D. used *Hydra vulgaris* (pink *Hydra*) and copper sulfate ($CuSO_4 \bullet 5H_2O$), cadmium chloride ($CdCl_2 \bullet 21/2\ H_2O$), and zinc chloride ($ZnSO_4 \bullet 7H_2O$) as test compounds to conduct their study. Ranges of concentrations for the study were 0.018–0.32 mg l^{-1} for copper, 0.10–3.20 mg l^{-1} for cadmium, and 18.0–56.0 mg l^{-1} for zinc. LC50 values at the 96hr time point were 0.025–0.084 mg l^{-1}, 0.16–0.52 mg l^{-1} and 11–14 mg, respectively, and this was the study's terminal point.[21] Data analysis identified that copper exhibits more toxicity than cadmium or zinc, however, cadmium was comparatively more toxic than zinc. Qualitative observations identified that *Hydra* morphology changed in response to the toxic compound, beginning from progressive tentacle clubbing, shortening, and detachment.[21] The body of the organism seemed to be progressively contracted.[21] These descriptions have been quantified by morphology scores, adapted from Wilby (1998). The regular HM mimics true living conditions of the organism and promotes proper conditions for osmoregulation.[4] However, during exposure to the compound, the body of the organism was swollen,[21] indicating an osmoregulatory impairment.[4]

Hydra viridissima has its distinctive green colour due to symbiote photosynthetic algae, which might have been affected by the targeting metal, since copper is a potent algaecide.[22] The pink *Hydra*, which does not carry the symbiont, however, was not affected until subjected to higher copper concentration. Pollino C. and Holdway D. A. (1999), have also reported that green *Hydra* have been more sensitive,[22] perhaps, due to the inhibition of photosynthesis performed by the algae.[23] This finding was also confirmed by Karntanut W. and Pascoe D. (2005) study, where amongst four species of *Hydra: Hydra oligactis,* two strains of *Hydra vulgaris,* and *Hydra viridissima,* the later exhibited higher sensitivity to both copper and cadmium.[22] Karntanut W. and Pascoe D. study has also discovered that bleached *H. viridissima* exhibited higher sensitivity to low concentrations of copper without its symbiont, in opposition to *H. viridissima* with intact symbiont that was more affected.[24] Karntanut hypothesized that *Chlorella* sequesters the copper, which results in symbiotic green *Hydra* being more resilient to the metal at low concentrations.[24] This benefit however, is indifferent to higher copper concentrations, at which green *Hydra* appear to be more sensitive than other species.[24]

The 2001 study conducted by Holdway A. D. et al., screened cadmium and zinc on two *Hydra* species: *Hydra vulgaris* and *Hydra viridissima* (green *Hydra*). Mortality of green *Hydra* occurred at cadmium concentration of 1.6 µg/L, whereas the pink *Hydra* stayed resistant to the compound until the concentration reached 100 µg/L.[25] At t-96h, the terminal time-point of the study, the LC50 values for 3 µg/L of cadmium chloride were 27 times higher for green *Hydra* than pink *Hydra*.[25] Zinc toxicity testing has also exhibited higher resistance in *Hydra vulgaris,* with total mortality occurring at 8,000 µg/L, whereas the green *Hydra* appeared disintegrated at 2,000 µg/L.[25] Such drastic differences in impact of metals on species morphology and survival likely occur due to differences in species-specific physiology. Further research by Holdway concluded that elimination of light in experimental design had no significant impact on *Hydra* regeneration, concluding that photosynthesis is not being affected by cadmium.[25] The impact is speculated to have targeted the differences in metabolism as the green *Hydra* primarily utilizes carbohydrates for energy, rather than lipid-centered metabolism demonstrated by the pink *Hydra*.[25] Further studies are needed to determine how cadmium interferes with carbohydrate metabolism. Cadmium is a transition metal with a very lengthy half life, varying by the isotope.[26] This feature allows cadmium to accumulate over time, and there are no proven effective interventions to reverse cadmi-

um over load.[26] Cadmium and zinc have similar bioligands, thus, upon exposure, cadmium interferes with many zinc-mediated metabolic processes.[26] The differential zinc impact has occurred due to defective osmoregulation that occurs in *Chlorella* as zinc triggers altered cell membrane permeability.[4] Nitrogen fixation and photosynthesis are also known to be affected, thus, reduced rates of mitosis are observed leading to disintegration of the organism.[4] Comparing the metals' impact, cadmium is noticeably more potent at lower concentrations than zinc. This phenomenon occurs on the basis of the fact that cadmium is not an essential metal, as opposed to zinc. Zinc is used for numerous cellular functions, such as signalling, cell membrane structure, and is a common biochemical cofactor. Thus, its effect might be sequestered by the organism, until the concentration crosses a certain threshold.

CONCLUSION

The freshwater polyp *Hydra* serves as excellent model organism for metal toxicity studies due to its ease of culture, sensitivity to toxic compounds, and timely response to exposures. Experimental setup of toxicity screens is very efficient allowing for both, cross-sectional and longitudinal studies to be performed. The review has appraised multiple studies that tested the impact of different ranges of copper, zinc, and cadmium on *Hydra* morphology. Copper and zinc are essential metals and partake in a multitude of biochemical pathways. Specifically, copper is known to participate in biochemical cascades and reactions involving transcriptional regulators, chaperones and storage proteins, cell surface/secretory compartment transporters and receptors, oxidoreductases, oxidases, monooxygenases, and electron transfer-involving proteins.[27] This heavy metal has been found in both eukaryotes and prokaryotes, and specifically in *Hydra*, copper is known to impact reactive oxygen species (ROS)-mediated reactions that result in DNA damage, activation of programmed cell death called apoptosis, and modulation of antioxidant biomarker genes, all evident through morphological changes in response to exposure.[28] Limited information is available regarding the role of zinc in modulating toxic responses in *Hydra*. However, zinc is an essential trace metal and it is involved in a large multitude of biochemical processes including cell proliferation and differentiation, prevention of free radical formation, and binding of metallothioneins, that are absent in *Hydra*.[29] More research is suggested to determine the molecular mechanisms of zinc toxicity on *Hydra*. Cadmium, on the other hand, is not an essential metal, and tends to accumulate in cells due to its long half life, resulting in a toxic response at low concentrations. Thus, findings from *Hydra* toxicity screens are relevant and applicable for use during the compilation and revisions of the Guidelines for Canadian Drinking Water Quality. This will be used to develop policies and regulations ultimately improving human health. Additionally, *Hydra* is a small animal found attached to rocks or vegetation, as well as it may be found floating in water. Naturally, *Hydra* may be ingested by larger freshwater organisms, thus contributing to the food chain and further bioamplification of metals. Further research needs to be conducted to investigate how metals impact *Hydra's* regenerative potential, therefore, regeneration assays are recommended to help investigate current understanding of metal-related pathologies.

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