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The Effects of Selected Metals and Rare Earth Elements On the Peroxidase Toxicity Assay

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Abstract

The search for quick and sensitive biosensors to detect changes in water quality and identify potentially toxic compounds, as recommendations to reduce fish testing increase. The peroxidase toxicity (Perotox) screen was recently proposed as a quick and sensitive biosensor for toxicity investigation. The purpose of this study was to examine the potential toxicity of environmentally relevant heavy metals cadmium (Cd) and copper (Cu), arsenic (As) and rare earth elements cerium (Ce), gadolinium (Gd), lanthanum (La) and samarium (Sm) using the Perotox screening test. Horseradish peroxidase (Per) without/with exogenously added DNA (DNA protection index) was exposed to increasing concentrations of the above elements for 5 min and its activity assessed. The data revealed that most elements were able to reduce Per activity at different potencies based on the calculated concentration that inhibit Per activity by 20 % (IC20): Cd~Cu>La~Gd>Sm>Ce>As. Inhibitions in Per activity led to oxidation of the monounsaturated detergent Tween 80 in the incubation media. The addition of exogenous DNA prevented Per inhibitions where the following elements show strongest interaction with DNA:Ce, Sm and As. However, the DNA protection index did not always lead to the formation of DNA strand breaks. The Perotox IC20 values were significantly correlated (r=0.6) with the reported 96 h acute lethality in Oncorhynchus mykiss juveniles making it a potential alternative for fish toxicity screening for compliance investigations. In conclusion, a simple, quick and inexpensive enzyme biosensor based on Per inhibitions is presented as a pre-screening methodology for the toxicity of various chemicals to fish.

Keywords: peroxidase, DNA protection, monounsaturated lipid oxidation, alternative methods, rainbow trout.

Introduction

Peroxidases (Per) are hemoproteins involved in the elimination of the highly toxic hydrogen peroxide (H_2O_2) by the co-oxidation of various endogenous and exogenous electron donor substrates such as ascorbic acid, phenols and reduced glutathione (O'Brien, 2000). Per catalyzes the following reaction: A-H + H_2O_2 → A-OH + H_2O and their main role is to keep H_2O_2 at safe levels in tissues and cells. It is estimated that 1-2 % of consumed oxygen during respiration leads to the production of H_2O_2 from superoxide dismutase (O2* → H_2O_2) (Cadenas & Davies, 2000). H_2O_2 is the main driver of oxidative damage in oxygen dwelling organism with an estimated toxicity of 0.1 µg/L (lethal concentration that kills 50% of fish or LC50) in rainbow trout at 17°C after 96h (Rach et al., 1997). Per are involved in the rapid elimination of H_2O_2 to prevent oxidative damage, such as unsaturated lipid peroxidation, protein carbonylation and 8-oxoguanosine damage in DNA. Exposure of fish to cadmium (Cd) leads to oxidative stress from inhibition of glutathione Per (George & Young, 1986). Cd also decreased glutathione S-transferase activity involved in the detoxication of non-polar compounds and displaced Zn from binding sites in cells (Gagné & Blaise, 1996). In another study with carp, exposure to Cd^{2+} lead to decreased antioxidant system involving superoxide dismutase, catalase and peroxidase (Wu et al., 2006). The addition of an electron donor (Ce^{3+}→ Ce^{4+} + 1e) alleviated the toxic effect of Cd^{2+} in carp kidneys. Numerous studies have shown that Per activity could be also induced by low levels of metals followed by (Javede et al., 2020) decreased activity as the concentration of the metals increases and overwhelms the antioxidant pathways. However, long-term Per activity could deplete endogenous antioxidants, which could lead to tissue damage.

The Per assay was used to screen in vitro for the potential toxicity of miscellaneous industrial effluents and led to Per inhibitions preventing the removal of H_2O_2 (Gagné & Blaise, 1997a). This study also showed that the strongest Per inhibitions by the effluents were associated with trout mortality (96h LC50). Moreover, the Per assay was more sensitive than the trout bioassay with the absence of false negatives (i.e., absence of Per inhibition with toxic effluents in fish). An interesting variation of the Per assay consists of adding exogenous DNA in the reaction mixture to determine

DNA interactions with the contaminants in the effluent. While the addition of DNA alone did not significantly influence Per activity, the concomitant addition of the effluent prevented inhibitions in Per activity (Gagné & Blaise, 1997a). This phenomenon was called the DNA protection index based on the Per activity ratio in the presence and absence of DNA. Interestingly, effluents displaying a DNA protection index were genotoxic in 70% of times as determined by the bacterial SOS chromotest. In another study, pre-incubation of Per to herbicides, detergents/surfactants, phenol and metals (Hg, Co, Ni) inhibited enzyme activity (Ilyina et al, 2000). Hence, the Perotox assay offers quick and inexpensive means to seek out potentially toxic substances found in industrial and municipal effluents. The Perotox assay was recently revisited to include a monounsaturated detergent (Tween-80) as a proxy for unsaturated lipid peroxidation (Gagné et al, 2025). The study revealed that inhibitions in Per activity led to increased oxidative damage by the formation of malonaldehyde. The reduction of Per activity was also associated with the collapse in periodic formation of Per intermediates (compounds I, II and III) leading to the accumulation of compound III, which is a non-catalytic intermediate of Per (Longu et al., 2004). Given the need to reduce the sacrifice of vertebrates (fish) in support of hazard assessment programs, new alternative methods are urgently needed, and the above studies suggest that Perotox assay is a potential alternative for the reduction of fish for toxicity screening. In addition, these enzyme biosensors could be easily amenable to machine learning (artificial intelligence) to forecast impacts of effluents and other environment stressors such as rain falls, domestic/industrial wastewaters and the performance of wastewater treatment plants (Baudhanwala et al., 2024; Gagné & Blaise, 1997b).

The purpose of this study was therefore to examine the effects of selected metals (Cd, Cu, As) and rare earth elements (Gd, Sm, Cs and La), known to be released in urban and industrial area, using the Perotox bioassay. In addition to the DNA protection assay with added DNA, the levels of malonaldehydes and DNA strand breaks were concurrently determined to understand the outcome of Per inhibitions by these elements. The Per inhibition potential was also compared with the reported toxicity of these elements in rainbow trout as a potential alternative to reduce fish testing and sacrifice.

Materials and methods

Sample preparation

Horseradish peroxidase (Per), salmon sperm DNA, Tween-80 (monounsaturated detergent), luminol, and bovine serum albumin were purchased from Sigma-Aldrich (Ontario, Canada). They were purchased as solids (except for Tween-80) and prepared at 0.1 mg/mL in phosphate-buffered saline (PBS: 140 mM NaCl, 1 mM KH2PO4 and 1 mM NaHCO3, pH 7.4). Hydrogen peroxide was diluted at 1 % in MilliQ water and stored in the dark at 4oC for no longer than 5 days. DNA was dissolved in PBS at 0.1 mg/mL, heated at 70oC for 20-30 min to ensure complete dissolution. The following elements were purchased at Sigma-Aldrich as dichloride salts ($CuCl_2$ and $CdCl_2$), trichloride salts ($LaCl_3$, $SmCl_3$, $GdCl_3$), tetrachloride salt ($CeCl_4$) and arsenic chloride ($AsCl_3$).

Peroxidase Toxicity Assay

For the Per toxicity assay (Perotox assay), the reaction mixture consisted of 0.1 µg/mL each of Per and albumin, 0.001% Tween-80 and 0.1 mM luminol. The reaction mixture was exposed to the following elements for 5 min: 25, 50 and 100 µg/L for Cu and Cd; 2.5 mg/L, 5 mg/L and 10 mg/L for Sm and Ce(IV); 0.1, 0.2 and 0.4 mg/L for La; 0.125, 0.25 and 0.5 mg/L for Gd and 25, 50 and 100 mg/L for As. These concentrations were selected based on previous experiments to determine the concentration range leading to gradual changes in Per activity and the reported toxicity in fish. The reaction was initiated by the addition of 0.1 % H_2O_2 and allowed to proceed for 15 min for luminescence readings each 30 sec in clear bottom white microplates (Synergy-IV, Biotek instruments, USA). In a separate duplicate well, DNA was added at 0.1 µg/mL for 5 min to the reaction mixture to determine the DNA protection index: Per activity with DNA/Per activity without DNA. Measurements at 418 nm was taken at each 30 sec to determine the relative levels of compound III (CIII) of Per (Longu et al., 2004). CIII is considered a non-catalytic intermediate of Per when the substrate (luminol or other compounds such as the contaminants in the samples) is depleted relative to H_2O_2. At the end of the incubation period (15 min), the reaction mixtures were analyzed for malonaldehyde formation (a precursor for lipid hydroperoxides) and DNA strand breaks. The acid-soluble DNA strands were obtained by adding 5 µL of ice-cold trichloroacetic acid to the reaction mixture (2.5% final concentration) and centrifuged at 10 000 x g for 5 min at 4°C. The supernatant (containing the small DNA strands in the order of < 100 oligonucleotides) was mixed with 10 µg/mL of Hoescht dye in 100 mM KH_2PO_4, pH 8.0, and fluorescence was determined at 350 nm excitation and 450 nm emission using a microplate reader as described above. Salmon sperm DNA was used as a positive control. The data was expressed as relative fluorescence units (RFU). For malonaldehyde (MDA), the levels were determined by the thiobarbituric acid reagent (Wills, 1987). A 10 µL sample was mixed with 90 µL of 1 mM $FeSO_4$ and 100 µL of 0.325% of thiobarbituric acid and incubated at 70oC for 10 min in a water bath. After cooling at room temperature, fluorescence readings were taken at 540 nm excitation and 590 nm emission. Solutions of tetramethoxypropane (stabilized form of malonaldehyde) was used for instrument calibration. The data was expressed as relative fluorescence units. Operational blanks were performed with MilliQ water only. The concentration that the decrease light emission rates by 20% (IC20) was calculated by visual analysis of the luminescence rates with the exposure concentrations.

Data Analysis

The exposure experiments to the selected elements were repeated three times. Per activity data were normalized to 1 (controls) and subjected to analysis of variance followed by the Least Square difference post hoc test. Relationships between the physical properties (atomic mass, ionic radius, electronegativity and DNA binding constants) and trout toxicity data (survival, LC50) were performed by the Pearson moment correlation as the distance metric during hierarchical tree analysis.

Results

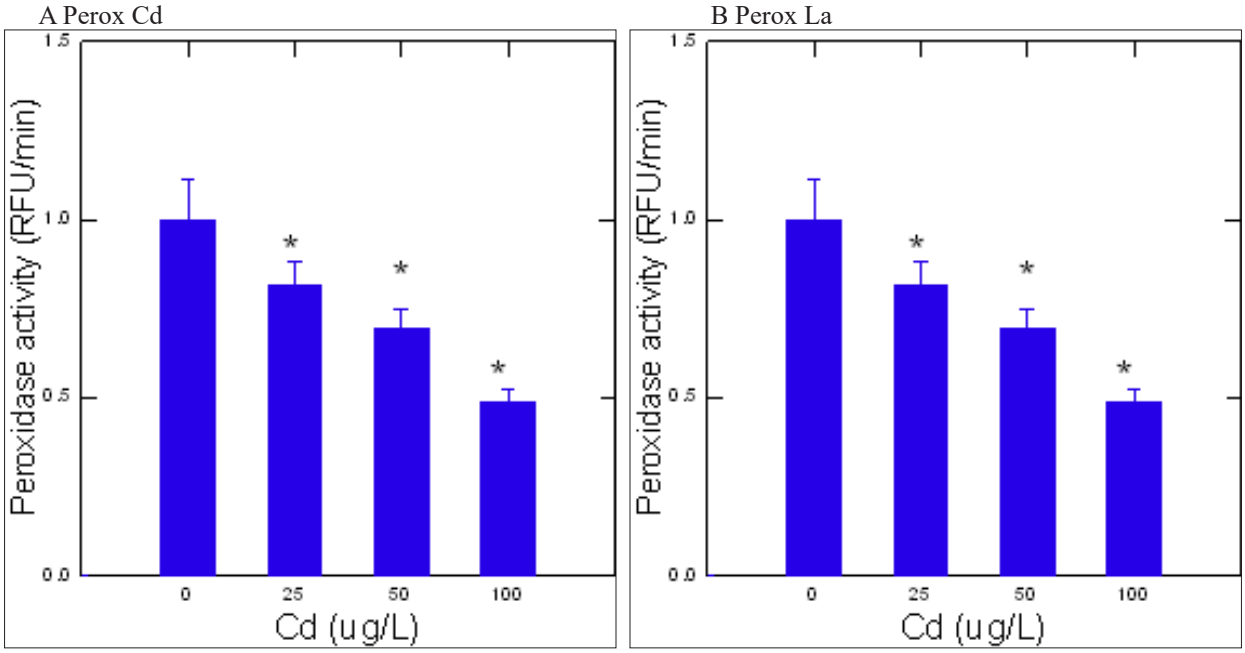
The basic physico-chemical characteristics of the selected elements were provided in Table 1. Cd and Cu are divalent metals of lower mass than rare earth elements (Sm, Ce, La and Gd) with higher electronegativity and lower redox potential. While the ionic radius was generally similar (69-122 pm), As and La had the lowest and highest radius in the group. The DNA binding constants were also included and revealed a log K range spanning 1 order of magnitude from 5.672 for Cd up to 7.079/6.895 for Gd/Ce. This indicates that rare earth elements (Sm, Ce, La and Gd) generally display more affinity towards DNA than for Cd and Cu.

Table 1: Physico-chemical characteristics of selected elements.

Elements	Mass	EN	Redox potential	Radius	DNA binding affinity (-log K)[1]
Cu	63.6	1.9	0.34	72	6.489 5.4
Cd	112	1.69	-0.40	103	5.672 5.5
Sm	150.4	1.17	-2.3	100	6.217 6.84
Ce	140	1.12	-2.34	107	6.895 6.55
La	139	1.1	-2.38	122	6.320 6.31
Gd	157	1.2	-2.28	97	7.079 7.12
As	74.9	2.18	-0.24	69	6.142 5.23

1. From (Sigel, 2003; Smith et al., 1991.; Wang et al., 2025).

The Perotox assay was then tested on each of the individual elements for changes in Per activity, DNA protection index and formation rates of the non-catalytic intermediate (CIII) of Per (Figure 1). The data are shown for the most (Cd) and least (La) toxic elements for rainbow trout. Per activity was significantly inhibited at a threshold concentration of 25 µg/L and 100 µg/L for Cd and La respectively. The DNA protection index also increased at these concentrations but showed a non-monotonic response (Figures 1C and 1D). For Cd, the DNA protection index was significantly increased at 25 µg/L and 100 µg/L for Cd and La respectively but gradually reduced as the exposure concentration of Cd and La increased. This suggested that higher concentrations of the elements overwhelmed the interaction capacity of DNA. Only Cd significantly decreased the DNA protection index compared to controls at 100 µg/L, while for La, the DNA index returned to control levels at 400 µg/L. In respect to the formation of complex III intermediate of Per (Figures 1D and 1E), a gradual increase in CIII formation was observed with increasing concentrations of Cd (50 µg/L) and La (100 µg/L) suggesting a gradual inactivation of enzyme activity from reduced availability of co-substrates and enzyme inactivation.



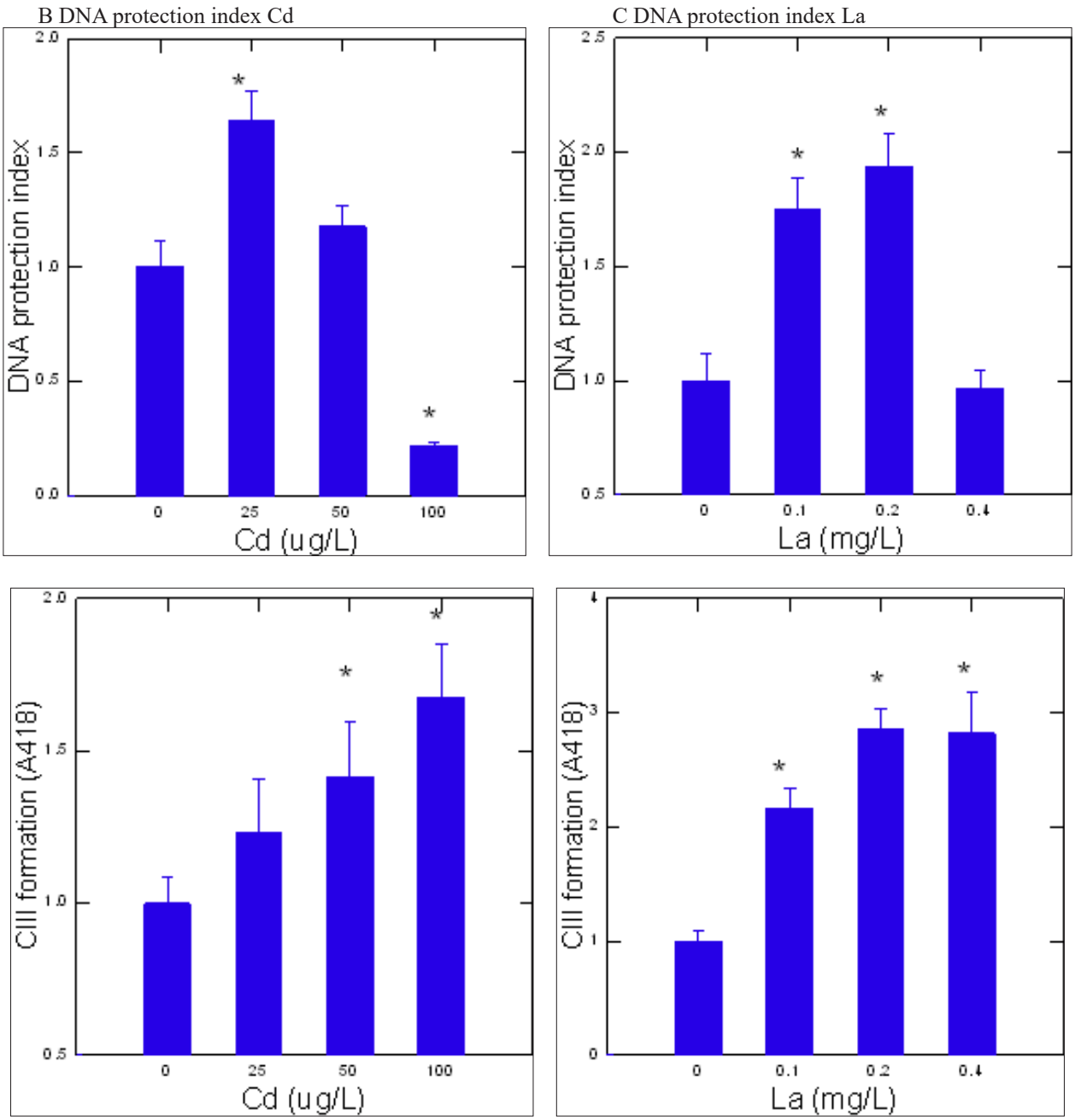


Figure 1: Representative responses of Perotox assay for Cd(II) and La(III).

The threshold concentration (EC20) of the Perotox assay with the DNA protection index (maximum fold change) and trout toxicity data were calculated with the selected elements (Table 2). The IC20 values for the Perotox assay ranged between 30 to 5000 µg/L with Cd and Cu being the most potent elements as follows: Cd~Cu>La~Gd>Sm>Ce>As. In respect to DNA protection index, the highest values, indicative of strong interaction to DNA, were obtained for Ce, Sm and As. In respect to the reported 96h trout mortality (LC50), Cd and Cu were also the most toxic elements in the following order: Cd>Cu> Sm >Gd> As> Ce >La. The influence of these elements towards the formation of lipid hydroperoxides (malonaldehyde) and DNA strand breaks were also examined (Table 3). In respect to malonaldehyde levels involved in the oxidation of the unsaturated detegent Tween-80, all elements were able to induce lipid peroxidation during Per activity inhibitions (Table 3). Cu displayed a stronger oxidizing property than Cd with 2-fold increase in malonaldehyde compared to 1.2- fold increase for Cd at 25 µg/L. The oxidant Ce(IV) produced the highest increase in malonaldehyde levels reaching 5 fold increases at 5 mg/L. Interestingly, the increase in lipid hydro peroxides were often followed by a decrease as the concentration of the element increased suggesting that malonaldehyde levels were further oxidized to hyperoxides but this was not measured. The levels of acid-soluble DNA strand breaks were also examined to determine whether the DNA protection responses involved not only interaction with DNA but lead to DNA strand breaks in these oxidative conditions (Per, H_2O_2 and metal/element) (Table 3). The analysis revealed only few cases of DNA strand break formation for these elements with the exception of Cu, which increased DNA breaks at one concentration.

Table 2: Toxicological properties of selected elements

Elements	Trout mortality LC50 (mg/L)[1]	Perotox IC20 (mg/L)	DNA protection index
Cu	0.2	0.03	2.3
Cd	0.03	0.03	2
Sm	1.6	1.3	3
Ce	95[1]	2	6
La	120[1]	0.11	2
Gd	8	0.19	2
As	18	5	3

1. From (Dubé et al., 2019; Hanana et al., 2021; McGeachy & Dixon, 1989).

Table 3: Formation of lipid peroxides and DNA strand breaks

Exposure Concentration	Cu LPO DNAs	Cd LPO DNAs	Sm LPO DNAs	Ce(IV) LPO DNAs	La LPO DNAs	Gd LPO DNAs	As LPO DNAs
Control	1±0.05 1± 0.08	1±0.05 1±0.03	1±0.05 1±0.05	1±0.05 1±0.02	1±0.05 1±0.01	1±0.04 1±0.05	1±0.01 1±0.05
C1[1]	**2.1±0.2[2]** **1.5±0.1**	**1.2±0.1** 0.99±0.01	**1.9±0.1** 1±0.01	**2.5±0.1** 1.1±0.01	**1.9±0.07** 1.1±0.01	**1.6±0.01** 1±0.01	**1.4±0.08** 1±0.03
C[2]	1.1±0.1 0.09±0.05	0.9±0.1 0.91±0.01	2±0.07 1±0.01	**5±0.3** 1±0.01	**2±0.05** 1.1±0.02	**1.2±0.1** 0.9±0.02	**1.7±0.1** 1±0.04
C[3]	**0.35±0.02** 1±0.05	**0.5±0.07** 0.9±0.05	**1.9±0.1** 1±0.1	**1.5±0.07** 0.9±0.03	1.1±0.05 0.9±0.02	**0.5±0.1** 0.9±0.02	**2.2±0.1** 1±0.06

1. Cd and Cu: 25 µg/L (C1), 50 µg/L (C2), 100 µg/L (C3); Sm and Ce(IV): 2 mg/L (C1), 5 mg/L (C2), 10 mg/L (C3); La: 0.1 mg/L (C1), 0.2 mg/L (C2), 0.4 mg/L (C3); Gd: 0.125 mg/L (C1), 0.25 mg/L (C2), 0.5 mg/L (C3); As: 10 mg/L (C1), 50 mg/L (C2), 100 mg/L (C3).
2. Boldface indicates significant difference from controls ($p<0.05$).

In the attempt to have a global understanding between the physico-chemical properties and the toxicity of the selected elements as determined by the Perotox and rainbow trout toxicity tests, a hierarchical tree analysis was performed (Figure 2). The analysis revealed that Perotox assay was closely associated with DNA protection assay and trout lethality data. The Perotox EC20 values were significantly correlated with the reported trout toxicity data (LC50; r=0.60) and the DNA protection index (r=0.51). The DNA binding constants were generally closely associated with the redox potential and ionic radius of the selected elements.

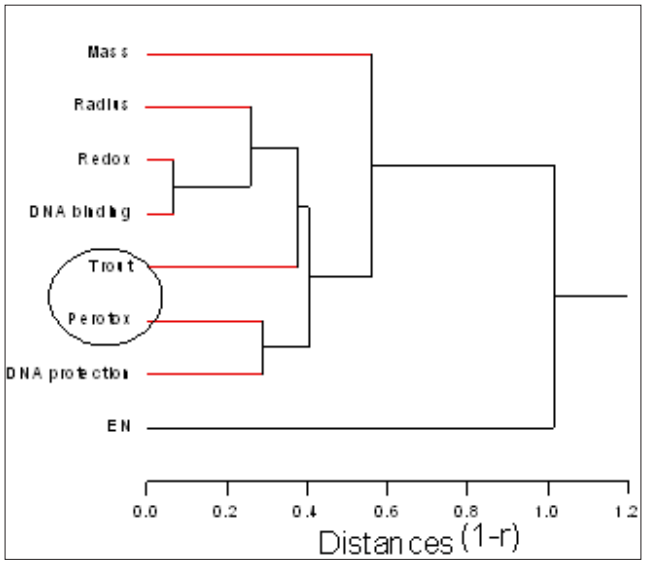


Figure 2: Hierarchical tree analysis

The Perox and rainbow trout toxicity data (LC50 and EC_20) and DNA protection index were examined in respect to atomic mass, ionic radius, redox potential, electronegativity (EN) and DNA binding affinity constant. The relative distance was measured using the Pearson moment correlation (1-r). Significant correlations were for r>0.53 or 1-r<0.47.

Discussion

In this study, the toxic properties of selected trace elements were examined using the Perotox and compared to the reported 96-h trout acute lethality assays. The Perotox assay was previously used as a biosensor to determine the water quality of municipal and industrial effluents (Gagné & Blaise, 1997). Based on industrial effluents, the enzyme sensor was more sensitive than the rainbow trout lethality test and was able to predict toxic effluents towards rainbow trout, making it a potential alternative to the 96h trout acute lethality bioassay. The Perotox assay was recently revisited to include monounsaturated lipid (Tween-80) and determine enzyme CIII intermediate to better understand the toxicological consequences of inhibitions in Per activity (Gagné et al., 2025). The toxicological meaning of the Perotox assay is that sustained levels of H_2O_2 from Per inhibitions initiate oxidative stress such as malonaldehyde formation leading to lipid hydro peroxide formation. The reported LC50 data for rainbow trout was significantly correlated with Perotox assay, suggesting that Perotox assay could be used as a fish alternative for the present elements. The observed inhibitions in Per activity in the present study were corroborated by the following studies. In vitro inhibition in Per activity was shown by Cd at a concentration of 1 µg/L (Attaallah and Amine, 2022) representing circa a concentration a 30 time less than the LC50 reported for trout. Cu was shown to denature Per protein at a concentration of 23 mg/L using gel electrophoresis (Mahmoudi et al., 2003) but its activity was inhibited at a concentration of 1.3 µg/L (Xianyu et al., 2013), which was circa 150 times lower than the LC50 for rainbow trout. The minimum As concentration to inhibit Per activity was 1 mg/L, representing a concentration 18 times less than the reported LC50 value for trout. In respect to Ce (IV), Per activity inhibition was observed at 2.8 mg/L (Xu and Chen, 2011), which was 34 times lower than the estimated LC50 in rainbow trout. High concentrations of La (40 mg/L), Ce (40 mg/L), and Nd (43 mg/L) inhibited Per activity in Tetrastigma hemsleyanum plant cell suspension (Xin et al., 2013). Taken together, these elements were shown to inhibit Per activity at concentration 100 times lower than the LC50 for rainbow trout suggesting that Per enzymes are sensitive target to the selected elements. It was previously shown that a small (20 %) decrease in fish survival exposed to miscellaneous pollutants, Per activity was strongly inhibited making it a sensitive and predicable biomarker for fish toxicity responses (Mukherjee, Bhattacharya 1975). In respect to municipal effluent, inhibitions of Per activity occurred at a mean concentration 38-fold lower than the trout LC50 indicating that this trend holds true for complex mixtures as well (Gagné & Blaise, 1997b). Per enzymes are hemoproteins containing heme prosthetic group for its active center. Heme is generally recognized to bind other divalent metals than the natural Fe2+/Fe3+ couple (Scheuhammer & Cherian, 1986; Anderson et al., 1984). Ag, Cu and Hg binds more strongly to hemoglobin compared to cadmium, zinc, nickel and tin highlighting the capacity of hemoproteins to interact with metals. Most lanthanides and actinides were shown to bind carbonyl and amine group of hemoglobin leading to loss of a-helix conformation (Kumar et al., 2016). For instance, at Ce concentrations > 75 µM (corresponding to 10 mg/L), interaction with heme moiety was observed leading to lower oxygen binding to the hemoprotein. This study also showed that Ce (IV) was required to be reduced to Ce (III) before binding to heme with a concomitant decrease in toxicity (from 10 % to 100% death Chironomids larvae for Ce (IV) to Ce (III) respectively).

An interesting add-on of the Per assay consists of the addition of DNA, which can prevent inhibitions of Per activity. So-called the DNA protection index, it successfully identified genotoxic effluents in 70% of the cases, as determined by DNA repair activity in bacteria (Gagné & Blaise, 1997). In the present study, the following elements had the highest DNA protection index suggesting interaction with DNA and potentially genotoxic: Ce, As and Sm (Table 2). Ce had the highest DNA protection index, suggesting that this element could be genotoxic. It was previously shown that single stranded sites were more susceptible to hydrolysis by Ce (IV)/EDTA treatment (Kitamura & Komiyama, 2002). In rainbow trout exposed to Ce (III), the expression of growth arrested DNA repair gene was significantly induced at a threshold concentration of 3.9 mg/L (Dubé et al. 2019). This a concentration corresponds to concentration 29 times lower than the reported LC50 in trout. Some indications of DNA strand breaks were observed in mouse lung epithelial cells exposed uncoated nCe_2O_2 but not with Ce (III) (Soloria-Rodriguez et al., 2024). DNA damage was observed at concentration range between 10-100 mg/L, which is close to the reported LC50 for rainbow trout. In another study, Ce (IV) nanoparticles (CeO_2) were genotoxic based on micronuclei formation and chromosome aberration test in human peripheral blood cultures at concentrations as low as 0.78 mg/L (Arslan & Akbaba, 2020). Finally, the long-term exposure to Ce (IV) resulted in heat shock expression and apoptosis in Drosophila melanogaster (Wu et al., 2012). Moreover, DNA damage and p53 antitumor protein expression were observed in flies at tissue concentrations reaching 100 µg/g. In respect to Sm, reduced DNA strand breaks were observed in freshwater mussels Dreissena polymorpha exposed to 250 µg/L Sm (Hanana et al., 2018). In rainbow trout juveniles, the expression of growth arrest DNA repair gene was induced at a threshold concentration of 0.12 mg/L corresponding to a concentration 13 times lower than the LC50 (Dubé et al., 2019). For As, a 96h exposure As (III) induced cyto-genotoxicity in Labea rohita fish (Khalid & Azmat, 2025). The 96 h LC50 for this fish species was 20 mg/L, in the same range of rainbow trout (McGeachy & Dixon, 1989).

In conclusion, a simple biosensor named the Perotox assay is presented to evaluate the potential toxicity of selected elements. The assay also determined DNA interactions during the Per assay. The selected elements were all inhibitors of the Per reaction leading to reduced elimination of H_2O_2, hence to the oxidation of the monounsaturated detergent Tween 80. Although DNA interactions were found for most elements but more strongly so with Ce, As and Sm, these interactions did not always lead to DNA breakage during the Per reaction. The Perotox assay inhibition data (IC20) was generally more sensitive and significantly correlated with the reported trout mortality data (r=0.6) making it a potential alternative to rainbow trout. However, this should be validated with other types of environmental contaminants (aromatics, heterocyclic compounds, pesticides etc) and matrices such as surface waters and effluents/wastewaters.

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