POLYTECHNIQUE MONTRÉAL

affiliée à l’Université de Montréal

et

l’Université du Québec en Abitibi -Témiscamingue

Évaluation de la toxicité des effluents miniers et métallurgiques : rôles de la spéciation, des interactions entre contaminants et des traitements avancés

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Évaluation de la toxicité des effluents miniers et métallurgiques : rôles de la spéciation, des interactions entre contaminants et des traitements avancés

Présentée par **Wijdane LIMOUNI**

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DÉDICACE

À l'âme de ma mère

À toute ma famille

.

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RÉSUMÉ

La plupart des études écotoxicologiques évaluent les risques environnementaux selon une approche substance par substance. Or, en milieu aquatique, les organismes sont exposés à un mélange complexe de contaminants susceptibles de générer des effets additifs, synergiques ou antagonistes. L'objectif de cette thèse est de complémenter cette approche substance par substance par l'intégration d'aspects liés à l'application de méthodes statistiques, à la spéciation chimique, aux interactions des contaminants au sein de mélanges et à l'évaluation de l'efficacité d'un processus de traitement par ozonation pour le contrôle de la toxicité d'effluents réels. Les approches méthodologiques déployées incluent l'analyse statistique de séries temporelles, la réalisation de bioessais sur des effluents synthétiques et réels et la réalisation d'essais de traitement par ozonation à microbulles.

Le premier volet de cette recherche doctorale a porté sur un jeu de données associé au suivi temporel (2016-2023) de la qualité et de la toxicité d'un effluent industriel. Malgré le respect des normes de rejet en matière de concentrations de contaminants, l'effluent montre des épisodes sporadiques de toxicité aiguë chez *Daphnia magna* (*D. magna*). L'hypothèse avancée pour expliquer ce phénomène est que la toxicité résulte de l'effet combiné de contaminants. Des analyses de corrélation (CA), des analyses de variance à sens unique (ANOVA), des analyses en composantes principales (ACP), des analyses en grappes hiérarchiques (HCA), des calculs de spéciation et des calculs de toxicité ont été utilisés pour tester cette hypothèse. Les résultats ont révélé que Cd et Cu influent significativement sur la toxicité, tandis que Fe pourrait l'inhiber dans certains mélanges. Les calculs de spéciation suggèrent que les formes biodisponibles Cd^{2+} et Cu^{2+}, même à des niveaux sublétaux, jouent un rôle central dans la toxicité observée.

En complément de l'analyse de la série temporelle de l'effluent industriel, des mélanges synthétiques ont été produits en laboratoire pour évaluer l'effet du mélange de contaminants (As, Cu, Zn, Se) sur la toxicité de l'eau envers *D. magna*. Les effets des mélanges binaires ont été analysés avec des méthodes mathématiques permettant d'identifier les interactions entre les contaminants. Les paires Cu-Zn et Zn-Se ont présenté des effets synergiques prononcés, avec des valeurs de EC_{50} inférieures de 1 unité toxique (UT). En revanche, les paires As-Cu, As-Se, Cu-Se et As-Zn ont montré des réponses additives avec des valeurs d'EC_{50} proches de 1 UT. Les mélanges ternaires ont été évalués avec le *MixModel* dans le logiciel R. Ce modèle a permis d'évaluer les effets principaux, les interactions binaires et les interactions ternaires dans l'espace de composition chimique des mélanges. Les résultats ont suggéré que l'effet du Cu est dominant lorsqu'il est présent à des concentrations élevées ou en combinaison avec Zn et Se. Des effets synergiques émergents et inattendus ont été observés pour certains mélanges ternaires (As−Cu−Se, As−Cu-Zn), montrant que les données binaires seules ne permettent pas de prédire les interactions de composants multiples.

Les travaux relatifs à l'analyse de séries temporelles et à l'évaluation de la toxicité de mélanges binaires et ternaires au sein de mélanges synthétiques ont permis de documenter des mécanismes associés à la toxicité de métaux et métalloïdes. Or, les effluents miniers et métallurgiques contiennent aussi d'autres catégories de contaminants, dont des substances oxydables et la salinité, deux composantes non considérées dans les analyses précitées. Des essais ont donc été réalisés pour évaluer l'influence de l'ozonation par microbulles sur la toxicité d'effluents miniers et métallurgiques. Les effluents examinés provenaient de deux mines d'or et d'une mine de Ni-Cu du nord du Canada, ainsi que d'une fonderie en Abitibi-Témiscamingue. Les compositions chimiques de ces effluents sont caractérisées par des différences marquées, notamment en ce qui concerne la salinité et les concentrations d'azote ammoniacal. Le traitement par ozonation à microbulles a été testé dans un réacteur de laboratoire pendant une période de 150 à 200 min, avec un pH ajusté à une valeur cible de 9. Des tests de toxicité sur *D. magna* ont été effectués avant et après le processus de traitement. Le traitement d'ozonation par microbulles a diminué la toxicité dans tous les effluents testés, révélant ainsi que les substances oxydables, en plus des métaux et métalloïdes, influencent significativement la toxicité des effluents.

Les travaux réalisés dans le cadre des trois principaux volets de cette thèse fournissent des connaissances essentielles à l'amélioration de l'évaluation et du contrôle de la toxicité des effluents métallurgiques et miniers. Ces connaissances permettent d'émettre des recommandations afin d'optimiser le couplage des approches d'évaluation des causes de la toxicité et de traitement des eaux impactées par des mélanges de contaminants.

ABSTRACT

Most ecotoxicological studies assess environmental risks using a substance-by-substance approach. However, in aquatic environments, organisms are exposed to complex mixtures of contaminants that can generate additive, synergistic or antagonistic effects. This thesis aims to complement the substance-by-substance approach by incorporating aspects such as the application of statistical methods, chemical speciation, interactions between contaminants in mixtures, and the evaluation of the effectiveness of ozonation treatment in controlling the toxicity of real effluents. The methodological approaches employed include time series analysis, bioassays on synthetic and real effluents, and microbubble ozonation treatment tests.

The first part of this doctoral research focused on analysing a dataset associated with the monitoring of the quality and toxicity of an industrial effluent over time (2016–2023). Despite complying with discharge standards for contaminant concentrations, the effluent exhibits sporadic episodes of acute toxicity in *Daphnia magna* (*D. magna*). The hypothesis put forward to explain this phenomenon is that the toxicity results from the combined effect of contaminants. To test this hypothesis, correlation analyses (CA), one-way analyses of variance (ANOVA), principal component analyses (PCA), hierarchical cluster analyses (HCA), speciation calculations and toxicity calculations were employed. The results revealed that Cd and Cu significantly influence toxicity, while Fe may inhibit it in certain mixtures. Speciation calculations concluded that the bioavailable forms Cd^{2+} and Cu^{2+}, even at sublethal levels, play a central role in the observed toxicity.

In addition to analysing industrial effluent time series, synthetic mixtures of contaminants (As, Cu, Zn, Se) were produced in the laboratory to evaluate their combined toxicity to *D. magna*. The effects of binary mixtures were analysed using mathematical methods to identify interactions between contaminants. The Cu-Zn and Zn-Se pairs exhibited pronounced synergistic effects, with EC_{50} values lower than 1 toxic unit (TU). By contrast, the As-Cu, As-Se, Cu-Se and As-Zn pairs exhibited additive responses, with EC_{50} values close to 1 TU. Ternary mixtures were evaluated using the MixModel in R. This model enabled the main effects, binary interactions and ternary interactions in the mixture composition space to be evaluated. The results suggested that Cu has a dominant effect when present at high concentrations or in combination with Zn and Se. Unexpected and emerging synergistic effects were observed for certain ternary mixtures (As-Cu-Se and As-Cu-

Zn), demonstrating that binary data alone cannot predict interactions between multiple components.

Research into time series analysis and the toxicity of binary and ternary synthetic mixtures has documented the mechanisms associated with the toxicity of metals and metalloids. However, mining and industrial effluents also contain other categories of contaminants, including oxidizable substances and salinity, two components not previously considered. Therefore, tests were conducted to evaluate the influence of microbubble ozonation on effluent toxicity. The effluents examined originated from two gold mines, one Ni-Cu mine and a smelter in northern Canada and Abitibi-Témiscamingue, respectively. These effluents have markedly different chemical compositions, particularly regarding salinity and ammoniacal nitrogen concentrations. Microbubble ozonation treatment was tested in a laboratory reactor for between 150 and 200 min, with the pH adjusted to reach a target value of 9. Toxicity tests on *D. magna* were performed both before and after treatment. Microbubble ozonation treatment reduced the toxicity of all the effluents tested, showing that, in addition to metals and metalloids, oxidizable substances significantly influence effluent toxicity.

The work carried out in the three main sections of this thesis provides essential knowledge for improving the assessment and control of industrial and mining effluent toxicity. This knowledge enables recommendations to be made for optimising the combination of approaches for identifying the causes of toxicity and treating contaminated water.

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ACP	Analyse en composantes principales
AOP	Advanced Oxidation Processes (procédés d'oxydation avancée)
BLM	Biotic Ligand Model (modèle du ligand biotique)
CA	Concentration Addition
CCME	Canadian Council of Ministers of the Environment
CEAEQ	Centre d'expertise en analyse environnementale du Québec
CEC	Contaminant of Emerging Concern (contaminant d'intérêt émergent)
CI	Chromatographie ionique
D. magna	*Daphnia magna*
DL	Detection Limit (limite de détection)
DMA	Drainage minier acide
DO	Dissolved Oxygen (oxygène dissous)
DOC	Dissolved Organic Carbon (carbone organique dissous)
DOM	Dissolved Organic Matter (matière organique dissoute)
EC	Electrical Conductivity (conductivité électrique)
EC_{50}	Concentration efficace causant un effet chez 50 % des organismes exposés
ECCC	Environnement et Changement climatique Canada
Eh / ORP	Oxidation–Reduction Potential (potentiel d'oxydo-réduction)

ETE	Le centre Eau Terre Environnement de l'INRS
HCA	Hierarchical Cluster Analysis (analyse de regroupement hiérarchique)
IA	Independent Action (action indépendante)
ICP-AES	Inductively Coupled Plasma – Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
LC_{25}	Concentration inhibitrice pour 25 % des organismes exposés
INRS-ETE	Institut national de la recherche scientifique- Eau Terre Environement
GRES	Groupe de recherche en eaux souterraines
IRME	Institut de recherche en mines et environnement
LC_{50}	Lethal Concentration for 50 % of the organisms
MDMER	Metal and Diamond Mining Effluent Regulations (Règlement sur les effluents des mines de métaux et des mines de diamants)
MELCC	Ministère de l'Environnement, de la Lutte contre les changements climatiques
MELCCFP	Ministère de l'Environnement, de la Lutte contre les changements climatiques, de la Faune et des Parcs
MES	Matières en suspension
MIXTOX	Modèle d'interaction pour mélanges toxiques
O_3-MB	Ozone à microbulles (Microbubble ozone)
OCDE	Organisation de coopération et de développement économiques (test 202)
PHREEQC	pH-Redox-Equilibrium programmé en C++

pH	Potentiel hydrogène
REMMMD	Règlement sur les effluents des mines de métaux et des mines de diamants
RSM	Response Surface Methodology (méthodologie des surfaces de réponse)
TDS	Total Dissolved Solids (solides dissous totaux)
TU	Toxic Units (unités de toxicité)
UV-LED	Ultraviolet Light-Emitting Diode (diode électroluminescente émettant des UV)

LISTE DES ANNEXES

AVANT PROPOS

Les recherches effectuées dans le cadre de cette thèse ont apporté de nouvelles connaissances quant à la toxicité des effluents miniers et métallurgiques. Ces résultats ont été valorisés par plusieurs contributions scientifiques, notamment sous forme des publications dans des revues avec comité de lecture, mais aussi par des communications à des conférences et des symposiums scientifiques nationaux et internationaux.

La thèse présente un format dit par articles. Chaque manuscrit se substitue à un chapitre et correspond à une étape clé du projet de recherche, de l'analyse historique des effluents industriels à l'évaluation expérimentale de la toxicité des mélanges en passant par les recherches de solutions innovantes de traitement. Les articles suivants ont été rédigés dans le cadre de ce travail de doctorat dont je suis l'auteure principale avec la coopération et la direction de mes directeurs et codirecteurs de recherche. L'état de soumission est précisé dans la suite.

Articles publiés, acceptés ou soumis dans des revues avec comité de lecture

- Limouni, W., Rosa, E., Couture, P., Cloutier, V., & Neculita, C. M. (2025). Assessing the relationship between trace metal co-occurrence, speciation, and toxicity in industrial effluents. *Environmental Toxicology and Chemistry*. Accepté avec corrections.
- Limouni, W., Dupraz, V., Couture, P., Neculita, C. M., Cloutier, V., & Rosa, E. (2025). Evaluating the acute toxicity of binary and ternary mixtures of Cu, Zn, As, and Se to *Daphnia magna*: A component-based approach using isobologram analyses and ternary diagrams. *Chemosphere*. Accepté avec corrections.
- Limouni, W., Rosa, E., Neculita, C. M., & Couture, P. (2025). Influence of ozone microbubbles on metallic elements speciation and acute toxicity of mining and metallurgical effluents to *Daphnia magna*. Manuscrit prêt à soumettre.

Présentations de conférences

Communications orales

- **Limouni, W.,** Rosa, E., Neculita, C.M., Cloutier, V., Couture, P. (9-11 juin 2025). *Toxicité aiguë des expositions binaires et ternaires de Cu, Zn, As et Se sur Daphnia magna : approche basée sur les composantes à l'aide d'analyses par isobologrammes et de* *diagrammes ternaires.* **Colloque conjoint en écotoxicologie du Chapitre Saint-Laurent et d'EcotoQ**, Montréal, Canada.

- **Limouni, W.,** Rosa, E., Neculita, C.M., Cloutier, V., Couture, P. (21-23 juin 2024). *Assessing the relationship between trace metal covariance, speciation and toxicity in industrial effluents.* **ICWPT**, Berlin, Allemagne.
- **Limouni, W.,** Rosa, E., Neculita, C.M., Cloutier, V., Couture, P. (15-18 septembre 2024). *Assessing the relationship between trace metal covariance, speciation and toxicity in industrial effluents.* **GeoMontréal**, Montréal, Canada.
- **Limouni, W.,** Rosa, E., Neculita, C.M., Cloutier, V. (26-29 septembre 2021). *Assessment of sources and mobility of selenium in mining context.* **GeoNiagara**, Niagara Falls, Canada.

Communications par affiche

- **Limouni, W.,** Dupraz, V., Couture, P., Rosa, E., Neculita, C.M., Cloutier, V. (11-15 mai 2025). *Assessment of acute toxicity in Daphnia magna exposed to binary and ternary metallic elements mixtures of As, Cu, Zn, and Se: Experimental investigations and modeling strategies.* **SETAC Europe**, Vienne, Autriche.
- **Limouni, W.,** Dupraz, V., Couture, P., Rosa, E., Neculita, C.M., Cloutier, V. (31 mai-2 juin 2023). *Évaluation de l'effet de mélange sur la toxicité des métaux et métalloïdes dans les effluents miniers et métallurgiques.* **Colloque conjoint EcotoQ-Ecobim**, Québec, Canada.
- **Limouni, W.,** Rosa, E., Neculita, C.M., Couture, P., Cloutier, V. (10-12 mars 2023). *Assessment of the association between the speciation of contaminants in mine effluents and their toxicity.* **GEOTOP**, Magog, Canada.

CHAPITRE 1 INTRODUCTION

1.1. Mise en contexte et problématique

Les activités d'extraction minière et de métallurgie jouent un rôle clé dans l'économie internationale notamment grâce à la production de métaux et métalloïdes indispensables au fonctionnement de nombreux secteurs industriels. Le Canada, et en particulier le Québec, fait partie des grands producteurs miniers à l'échelle mondiale (Dallaire-Fortier, 2024). Si ces industries représentent un moteur économique important, elles génèrent également des volumes considérables d'effluents issus notamment des eaux de ruissellement, du traitement du minerai, de l'utilisation d'explosifs et des procédés métallurgiques. Le rejet de ces effluents dans l'environnement engendre une pression sur les écosystèmes aquatiques (Campbell, 1995; Tiwary, 2001; Ryskie et al., 2023; Mykrä et al., 2024). Ces effluents doivent être considérés *a priori* comme complexes, en ce sens qu'ils constituent généralement des mélanges de contaminants dont la composition chimique et la toxicité dépendent notamment de l'environnement géologique, du climat, de la nature du gisement, des procédés mis en œuvre dans l'extraction des substances à valeur économique, de la gestion des rejets et de la gestion des eaux (Nordstrom, 2011). Les contaminants communs des mines de métaux de base et précieux incluent généralement des métaux traces (p. ex. : Cu, Zn, Ni, Pb), des métalloïdes (p. ex. : As, Se), l'azote ammoniacal (NH_3/NH_4^+), des matières en suspension (MES) et divers contaminants d'intérêt émergent tels que les produits de transformation des cyanures, les thiosels et les surfactants (Akcil & Mudder, 2003; Kossoff et al., 2014; Ryskie et al., 2021). Dans certains bassins miniers, les concentrations de contaminants peuvent atteindre des seuils préoccupants. À titre d'exemple, des études ont rapporté des concentrations en Cu pouvant dépasser 50 mg/L, des concentrations en As atteignant 5 mg/L et des teneurs en azote ammoniacal excédant 100 mg/L, largement au-dessus des limites protectrices pour la vie aquatique (Akcil & Mudder, 2003; Kossoff et al., 2014).

Les effets écotoxicologiques des métaux et métalloïdes sont essentiellement liés à leurs formes libres dissoutes, qui sont les plus biodisponibles que leurs formes complexées et donc les plus toxiques pour les organismes aquatiques (Campbell, 1995 ; Luoma & Rainbow, 2005). Les impacts de ces contaminants dans les écosystèmes aquatiques peuvent se manifester de différentes manières, comme par l'inhibition de fonctions biologiques essentielles et d'échanges ioniques, la production de stress oxydatif, la modification des activités enzymatiques et la création d'effets génotoxiques (Morris et al., 2021). Certains contaminants peuvent de surcroît s'accumuler dans les réseaux trophiques, nuire à la reproduction et engendrer des effets écologiques à long terme (Norwood et al., 2003a ; Lari et al., 2017). Même à faibles concentrations, les contaminants présents dans les effluents métallurgiques et miniers peuvent avoir des effets sublétaux sur la physiologie, le comportement et la reproduction des organismes aquatiques (Norwood et al., 2003a ; Lari et al., 2017).

Les milieux aquatiques récepteurs sont rarement exposés à une contamination unique, mais plutôt à des mélanges complexes de contaminants. Les expositions à des mélanges de contaminants impliquent des interactions pouvant générer des effets additifs, synergiques ou antagonistes compliquant la prédiction de la toxicité (Di Toro et al., 2001 ; Traudt et al., 2017). Certaines associations de contaminants peuvent inhiber la toxicité par compétition pour les sites biotiques (antagonisme) alors que d'autres conduisent à des effets additifs ou synergiques (Lari et al., 2017 ; Brix et al., 2017). Or, les études se concentrent souvent sur les effets de substances individuelles et non sur les effets écotoxicologiques résultant du mélange complexe dans lequel se trouvent les effluents émis (Norwood et al., 2003b; Vijver et al., 2011). De surcroît, l'évaluation de la spéciation et de la biodisponibilité des contaminants n'est pas intégrée de manière systématique dans l'évaluation des impacts potentiels du rejet d'effluents, alors qu'elles sont déterminantes dans l'interprétation des effets toxiques (Campbell, 1995; Slaveykova & Wilkinson, 2005).

Nonobstant les défis précités, différents dispositifs réglementaires visent à limiter les impacts associés au rejet d'effluents industriels et miniers sur l'environnement. À titre d'exemple, au Québec, la Directive 019 sur l'industrie minière fixe les seuils de rejet pour certains contaminants (MELCCFP, 2025). Au Canada, le Règlement sur les effluents des mines de métaux et des mines de diamants (REMMMD) prescrit de surcroît des méthodes de surveillance chimique et biologique des effluents (Gouvernement du Canada, 2025). Bien que ces règlements soient pertinents pour la protection des milieux aquatiques, ils se fondent uniquement sur les concentrations totales de contaminants considérés de façon individuelle et sur des paramètres tels que le pH et les MES, sans considérer de façon approfondie la spéciation chimique, la biodisponibilité et les interactions entre les contaminants (Yan & Saxena, 2022). Cela engendre un décalage manifeste entre conformité réglementaire de l'effluent et conséquences écotoxicologiques au sein du milieu récepteur. À titre d’exemple, en 2023, une analyse de données de suivi réalisée par Environnement et Changement climatique Canada a relevé près de 99 échecs à des bioessais normalisés (poissons et invertébrés) alors que les effluents considérés présentaient très peu de dépassements des normes de rejet (ECCC, 2023). Ainsi, le simple respect des seuils réglementaires définis principalement en fonction des concentrations totales de contaminants n’est pas en soi suffisant pour assurer la protection des écosystèmes aquatiques. Une évaluation plus détaillée des causes de la toxicité des effluents miniers et métallurgiques, prenant en considération la spéciation des contaminants et les effets antagonistes, additifs et synergétiques des contaminants au sein de mélanges est requise. C’est dans ce cadre que s’insère cette thèse qui vise à améliorer la compréhension intégrée des mécanismes responsables de la toxicité des effluents miniers et métallurgiques et, par le fait même, à apporter de nouvelles connaissances pertinentes à la résolution du paradoxe de la conformité réglementaire et de la protection effective de la qualité des milieux aquatiques soumis à la contrainte des effluents miniers et métallurgiques.

La démarche de cette recherche doctorale repose sur trois hypothèses mécanistiques clés, visant à élucider comment la spéciation et les interactions entre les contaminants contrôlent la toxicité et son atténuation dans les effluents miniers et métallurgiques.

H1 : Dans un ensemble de données combinant des échantillons toxiques et non toxiques, une relation statistique significative peut être établie entre les concentrations de métaux et métalloïdes dissous et le niveau de toxicité (exprimé en unités de toxicité, UT) et il existe des différences géochimiques statistiquement significatives entre les échantillons toxiques et non toxiques.

H2 : Les mélanges binaires et ternaires de Cu, Zn, As et Se engendrent des interactions toxicologiques qui ne sont pas additives. Dans les mélanges, le Se joue un rôle clé en tant que modulateur dans les effets d’atténuation ou d’amplification de la toxicité des métaux et métalloïdes selon les proportions de mélange et les conditions d’exposition.

H3 : L’ozonation microbulles à pH 9 modifiera considérablement la spéciation des métaux et métalloïdes et des espèces azotées (redox-sensibles) et peut ainsi entraîner une réduction mesurable de la toxicité aiguë des effluents métallurgiques et miniers.

1.2. Objectifs

1.2.1. Objectif général

L'objectif général de cette thèse est de contribuer à améliorer la compréhension intégrée des mécanismes responsables de la toxicité des effluents miniers et métallurgiques. Les démarches réalisées incluent l'analyse de la spéciation et des covariances des métaux et métalloïdes à partir de données de suivi d'un effluent industriel, l'évaluation expérimentale des interactions toxicologiques entre métaux et métalloïdes au sein d'effluents synthétiques et le test d'un procédé de traitement avancé (ozonation par microbulles) afin de proposer des solutions pour réduire efficacement la toxicité d'effluents métallurgiques et miniers.

1.2.2. Objectifs spécifiques

OS1 : Évaluer s'il existe des relations statistiques significatives entre les concentrations de métaux et métalloïdes dissous et le niveau de toxicité d'un effluent industriel et des différences géochimiques statistiquement significatives entre les échantillons toxiques et non toxiques de ce même effluent.

OS2 : Caractériser et prédire les effets aigus de Cu, Zn, As et Se, seuls et en mélanges binaires et ternaires, sur *D. magna* en caractérisant la nature des interactions par bioessais, isobologrammes et modèles prédictifs.

OS3 : Évaluer l'efficacité de l'ozonation à microbulles pour transformer la spéciation des contaminants et réduire la toxicité aiguë d'effluents miniers et métallurgiques.

1.3. Originalité

L'originalité de cette recherche repose sur une approche intégrée qui dépasse les analyses des effluents miniers et métallurgiques s'appuyant sur l'évaluation de la toxicité des contaminants considérés de façon individuelle. En effet, à ce jour, les connaissances relatives à la toxicité d'effluents miniers et métallurgiques dont la toxicité résulte de l'effet combiné de plusieurs contaminants mélangés demeurent lacunaires. Pour contribuer à l'avancement des connaissances de façon originale, cette thèse aborde des questions relatives (1) à la spéciation chimique des métaux et métalloïdes au sein d'effluents réels et synthétiques constituant des mélanges, (2) à l'application de méthodes statistiques visant à élucider des liens entre la toxicité et la composition chimique d'effluents, (3) à l'analyse mécanistique des interactions toxicologiques (additivité, synergie, antagonisme) via la réalisation de bioessais utilisant un organisme bioindicateur sensible (*D. magna*), et (4) à l'évaluation de l'influence d'un traitement par ozonation à microbulle pour la réduction de la toxicité d'effluents réels. En intégrant ces volets, ce projet de recherche doctorale fournira de nouvelles connaissances pertinentes à la résolution du paradoxe de la conformité réglementaire et de la protection effective de la qualité des milieux aquatiques soumis à la contrainte des effluents miniers et métallurgiques.

1.4. Contenu de la thèse

Cette thèse est structurée en huit chapitres, organisés de manière à progresser du cadrage théorique vers la validation expérimentale, puis vers l'exploration de solutions technologiques appliquées et l'émission de recommandations visant à assurer des retombées scientifiques et sociétales :

- **Chapitre 1 :** ce chapitre présente l'introduction générale de la thèse, le cadre scientifique et environnemental des effluents miniers et métallurgiques, la problématique générale et les objectifs et hypothèses de recherche qui encadrent l'ensemble de ce projet.
- **Chapitre 2 :** ce chapitre propose une analyse critique des travaux sur la toxicité des effluents miniers et métallurgiques, en abordant le rôle de la spéciation des éléments métalliques, les interactions entre contaminants et le potentiel des traitements avancés. Cette synthèse met en évidence les lacunes scientifiques et opérationnelles qui justifient le projet.
- **Chapitre 3 :** ce chapitre détaille la méthodologie retenue, les campagnes d'échantillonnage, les analyses physico-chimiques, les tests de toxicité aiguë sur *D. magna*, les outils de spéciation et de modélisation mis en œuvre.
- **Chapitre 4 :** ce chapitre, présenté sous la forme d'un article scientifique, se concentre sur l'évaluation des relations entre la co-occurrence de métaux et métalloïdes, leur spéciation et la toxicité d'un effluent industriel. L'approche repose en large partie sur l'application de calculs statistiques.
- **Chapitre 5 :** ce chapitre, présenté sous la forme d'un article scientifique, se concentre sur l'évaluation de la toxicité de mélanges binaires et ternaires de contaminants (Cu, Zn, As, Se). L'approche repose sur la réalisation de bioessais sur *D. magna*. Les interactions

toxicologiques (additivité, synergie, antagonisme) sont caractérisées au moyen de modèles théoriques (CA, IA), d'isobologrammes et d'approches prédictives (MixModel).

- **Chapitre 6 :** ce chapitre, présenté sous la forme d'un article scientifique, se concentre sur l'évaluation de l'efficacité d'un procédé d'ozonation à microbulles pour réduire la toxicité d'effluents métallurgiques et miniers. L'approche repose sur des essais de traitement couplés à des bioessais sur *D. magna*.
- **Chapitre 7 :** ce chapitre présente une discussion générale de synthèse au sujet des résultats expérimentaux et théoriques de l'ensemble de la thèse. Il propose une interprétation générale et discute les principaux résultats et identifie des enjeux écotoxicologiques et opérationnels relatifs au contrôle de la toxicité des effluents métallurgiques et miniers.
- **Chapitre 8 :** ce chapitre consiste en une conclusion générale mettant de l'avant les principales contributions scientifiques et sociétales susceptibles de découler de la thèse. Des pistes pour la gestion d'effluents et des priorités pour la recherche scientifique y sont identifiées.

1.5. Cohérence scientifique et articulation des axes de recherche

La structure de cette thèse suit une progression scientifique conçue pour répondre aux lacunes identifiées dans la littérature et à la complexité chimique des effluents miniers et métallurgiques. Le premier axe vise à déterminer les causes de la toxicité résiduelle en analysant simultanément les concentrations dissoutes, la spéciation modélisée et les covariances chimiques. Cette approche permet d'identifier les métaux et métalloïdes dominants, mais aussi les formes chimiques les plus susceptibles de contrôler la toxicité, fournissant ainsi la base pour les études mécanistiques des chapitres suivants. Le deuxième axe explore les interactions toxicologiques entre ces contaminants. Comme il n'est pas possible d'examiner tous les mélanges présents dans les effluents, des combinaisons originales de Cu, Zn, As et Se ont été retenues. Ces mélanges sont peu étudiés, particulièrement ceux impliquant le rôle modulateur du Se, alors que ce métalloïde peut atténuer ou amplifier la toxicité d'autres éléments selon les proportions et les conditions d'exposition. L'étude de mélanges binaires et ternaires permet ainsi de révéler des interactions additives, synergiques ou antagonistes impossibles à anticiper à partir des concentrations individuelles. Le troisième axe évalue une solution technologique directement liée aux mécanismes identifiés. Les premiers axes concernaient essentiellement des contaminants dont la toxicité est influencée par la spéciation et la possibilité de précipitation. Toutefois, les effluents contiennent également des espèces non précipitables mais oxydables, telles que NH_3 ou certaines formes d'As et de Se, qui contribuent à la toxicité résiduelle. Les procédés conventionnels ne ciblant pas efficacement ces espèces, l'ozonation à microbulles a été retenue pour sa capacité à les transformer. Les essais pilotes permettent d'évaluer si ces transformations se traduisent par une réduction mesurable de la toxicité dans un effluent réel.

Ainsi, l'enchaînement des chapitres suit une logique séquentielle : identifier d'abord les causes de la toxicité, puis comprendre les interactions et enfin tester une solution mécanistique. Cette cohérence renforce la portée des résultats et offre une vision intégrée de la gestion de la toxicité des effluents miniers et métallurgiques.

CHAPITRE 2 Revue de littérature

2.1. Contexte général et problématique

La littérature scientifique relative aux effluents miniers et métallurgiques met en évidence la grande complexité de leur composition et les défis associés à leur évaluation écotoxicologique (Nordstrom, 2011). Ces rejets comportent à la fois des métaux, des métalloïdes, des composés azotés, des teneurs en matières dissoutes totales parfois élevées (forte salinité) et des contaminants émergents présents en proportions variables (Ryskie et al., 2023). Plusieurs études portant sur les effluents miniers et industriels ont mis en évidence des concentrations en contaminants dépassant souvent les critères de protection de la vie aquatique et représentent donc un risque effectif pour les écosystèmes (Akcil & Mudder, 2003 ; Kossoff et al., 2014). Il existe un consensus scientifique pour affirmer que la toxicité des métaux et métalloïdes ne dépend pas uniquement de leur concentration totale mais également de leur spéciation chimique et de leur biodisponibilité (Campbell, 1995; Luoma & Rainbow, 2005). Les formes libres dissoutes des métaux et métalloïdes sont généralement plus toxiques parce qu'elles exercent des effets directs sur les sites biologiques d'interaction (p. ex. : branchies, transporteurs ioniques) alors que certains complexes inorganiques et organométalliques peuvent réduire l'exposition effective aux métaux et métalloïdes (Fortin, 2024).

Le problème des interactions entre contaminants constitue également un enjeu majeur (Norwood et al., 2003). Cela s'explique par le fait que les effets combinés des contaminants présents au sein de mélanges peuvent être additifs, synergiques ou antagonistes (Cedergreen, 2014). Il est donc difficile d'estimer la toxicité d'un mélange de contaminants en s'appuyant sur une approche « substance par substance », où les effets individuels sont considérés de façon exclusive (Cedergreen, 2014; Yan & Saxena, 2022; Dupraz et al., 2018). Or comme les effluents réels issus des mines et des fonderies contiennent souvent plusieurs dizaines de contaminants, l'évaluation de leurs effets écotoxicologiques revêt une complexité singulière.

Les cadres réglementaires relatifs aux effluents miniers, comme la Directive 019 (MELCCFP, 2025) au Québec (niveau provincial) et le REMMMD (Gouvernement du Canada, 2025) au Canada (niveau fédéral), reposent encore largement sur les paramètres généraux (moyennes mensuelles, pH, MES, seuils pour certains éléments métalliques). Ces paramètres sont certes pertinents et nécessaires, mais ils ne permettent pas de prédire la toxicité réelle des effluents. Les récents rapports d'Environnement et Changement climatique Canada (ECCC, 2025) confirment bien l'existence d'un décalage entre conformité réglementaire et impacts biologiques mesurés.

S'insérant dans le contexte préétabli, cette revue de littérature s'articule selon quatre principales composantes, soit (1) la composition chimique et la conformité réglementaire, (2) la spéciation et la biodisponibilité des métaux et métalloïdes, (3) les interactions entre contaminants au sein de mélanges et (4) les procédés de traitement avancés (précisément l'ozonation par microbulles) pour le contrôle de la toxicité. Ces composantes sont abordées afin de fournir une base de connaissances requise afin de mieux cerner les limites actuelles et d'améliorer l'évaluation du risque écotoxicologique associé aux effluents miniers et métallurgiques.

2.2. Conformité chimique et résultats des bioessais : un décalage persistant

Les données les plus récentes d'Environnement et Changement climatique Canada (tableau 2.1; ECCC, 2025) illustrent le paradoxe du décalage entre la conformité réglementaire (respect des concentrations maximales prescrites par le cadre légal) et les impacts biologiques mesurés en ce qui concerne les effluents miniers et métallurgiques. Ce tableau présente le nombre de dépassements déclarés par les mines canadiennes assujetties au REMMMD pour l'année 2023. La colonne A1 présente les non-conformités identifiées pour les substances considérées de façon individuelle (As, Cu, CN^-, Pb, Ni, Zn, MES, Ra-226, NH_3) et pour le pH. Les dépassements correspondent au nombre de concentrations moyennes mensuelles ou de mesures de pH qui sont hors de la plage réglementaire. La colonne A2 résume les résultats des essais de létalité aiguë réalisés sur des organismes aquatiques à partir des mêmes effluents. Les données révèlent que les mines assujetties au REMMMD ont montré un nombre limité de non-conformités chimiques. Ces non-conformités sont principalement associées aux teneurs en MES (41 cas), au pH (51 cas) au Zn (19 cas), au Ni (9 cas). Quelques dépassements sont aussi notés pour le Cu, l'azote ammoniacal (NH_3) et le ^{226}Ra. Pour ces mêmes effluents, les tests de létalité aiguë ont révélé 98 échecs biologiques, dont 77 avec *D. magna* et 21 avec la truite arc-en-ciel. Or, les données rapportées au tableau 2.1 illustrent bien le fait que les résultats de toxicité présentent un décalage marqué par rapport aux dépassements des critères chimiques.

Certains sites illustrent bien cette discordance, dont notamment l'effluent NO-12 de la Fonderie Horne (Glencore). Tel que rapporté au tableau 2.1, cet effluent n'a présenté aucun dépassement majeur en ce qui concerne les paramètres chimiques en 2023. Pourtant, ce même effluent a échoué 20 tests sur *D. magna* et à 2 tests sur la truite. Un autre cas témoignant de la discordance entre conformité chimique et toxicité est celui du site « Ancienne Croix » de Rio Tinto Fer et Titane. Cet effluent a présenté 6 inconformités liées à un pH < 6 mais présente 13 échecs pour les bioessais sur *D. magna* et 6 échecs pour les bioessais sur la truite.

Ces observations montrent que le respect des seuils chimiques exigés par la loi ne suffit pas pour garantir la protection des milieux aquatiques. Elles soulignent l'importance d'identifier de nouvelles méthodes permettant de complémenter les approches réglementaires afin de mieux protéger les écosystèmes récepteurs. Parmi les pistes de recherche scientifique d'intérêt, une meilleure prise en compte de la spéciation, de la biodisponibilité et des interactions entre les contaminants semble prioritaire (Niyogi & Wood, 2004).

Tableau 2.1 Conformité chimique et résultats des bioessais de létalité aiguë pour des effluents miniers au Canada (2023)

Mine / Site	**Dépassements chimiques (A1)**	**Échec aux bioessais (A2)**	**Commentaires**
Mine Caribou	8 dépassements pour les solides en suspension (SS), 2 pH > 9,5	Truite: 1 sur 14 *D. magna*: 0 sur 14	Effluent conforme pour les métaux, inconformité du pH → toxicité détectée
Mine d'or Elk (Puisard B)	1 dépassement Cu, 1 SS, 2 Ra-226, 1 NH_3	Truite: 1 sur 10 *D. magna*: 0 sur 11	Nombre limité de dépassements → mortalité observée pour un test sur truite
Glencore-Fonderie Horne	Conforme (pas de dépassements chimiques)	Truite: 2 sur 23 *D. magna*: 20 sur 35	Conformité chimique → nombreux bioessais échoués
HBMS-Flin Flon (North Weir)	2 dépassements pour Cu, 2 dépassements pour Zn	Truite: 2 sur 5 *D. magna*: 3 sur 6	Dépassements observés pour deux métaux → toxicité confirmée en bioessai
La Mine Niobec	Conforme (aucun dépassement chimique majeur)	*D. magna*: 6 sur 389	Conformité au niveau de la composition chimique → 6 bioessais échoués
Mine du Mont-Wright (HS-2)	6 inconformités pour le pH > 9,5	*D. magna*: 1 sur 12	Dépassements ponctuels de pH → un seul épisode de toxicité
Mine Raglan (DIR-Z3)	Conforme	D. magna: 1 sur 9	Effluent conforme → toxicité détectée sur un échantillon
Rio Tinto Fer & Titane-Havre-Saint-Pierre (Ancienne Croix)	12 inconformités pour le pH < 6	Truite: 6 sur 21 *D. magna*: 13 sur 22	Conformité en métaux, mais pH acide → forte toxicité biologique
SMC (Canada) Ltée	1 dépassement pour le Cu, 1 dépassement pour le Ni	*D. magna*: 3 sur 13	Deux dépassements chimiques → échec de 3 bioessais
Usine Beacon	1 dépassement pour les SS	Truite: 2 sur 2	Un seul dépassement en SS → deux échecs de bioessais sur la truite

2.3. Spéciation des métaux et biodisponibilité

La toxicité des éléments métalliques dans les milieux aquatiques ne dépend pas uniquement de leur concentration totale, mais avant tout de leur spéciation chimique, c'est-à-dire de la répartition des différentes formes chimiques présentes dans l'eau (Campbell, 1995). La spéciation conditionne directement la biodisponibilité des métaux et métalloïdes, soit la fraction du métal(oïde) susceptible d'interagir avec les organismes aquatiques en franchissant les membranes biologiques ou en se fixant aux ligands biologiques (ex. branchies, transporteurs ioniques) (Fortin, 2024). La biodisponibilité des métaux et métalloïdes est donc directement liée à la réponse toxique (Di Toro et al., 2001; USEPA, 2021). Les ions métalliques libres en phase dissoute constituent généralement la forme la plus biodisponible et la plus toxique (Adams et al., 2020). C'est notamment le cas pour Cu^{2+}, Zn^{2+}, Cd^{2+} et As(III), dont l'affinité élevée pour les sites de liaison biologiques s'avère déterminante des effets écotoxicologiques observés (Di Toro et al., 2001; Väänänen et al., 2018; Adams et al., 2020). À l'inverse, les métaux présents sous forme complexée, soit avec des anions inorganiques (p. ex. : $ZnCO_3$, $Cu(OH)_2$), soit avec la matière organique dissoute (p. ex. : complexes Cu-humate, Zn-fulvate), ou encore sous forme de précipités et de particules colloïdales, voient leur biodisponibilité et leur toxicité considérablement réduites (Tipping, 1994; Fortin, 2024).

Cette distinction entre la concentration totale et la spéciation est essentielle en écotoxicologie. En effet, deux échantillons d'eau ayant une concentration totale identique pour un élément métallique donné peuvent engendrer des impacts biologiques distincts en fonction de la proportion de formes libres biodisponibles (Neculita et al., 2008). Sous certaines conditions, même une faible concentration libre d'un contaminant métallique peut provoquer des effets notables (comme l'immobilisation ou la mortalité aiguë) chez *D. magna*, alors que des concentrations totales plus importantes du même contaminant peuvent rester inoffensives si l'élément métallique est complexé ou précipité (Adams et al., 2020; Brown & Markich, 2024). Il incombe de souligner que la spéciation est fortement influencée par le pH, le potentiel d'oxydoréduction (Eh), la dureté de l'eau (Ca^{2+}, Mg^{2+}) et la matière organique dissoute (DOM). Les paramètres précités exercent ainsi une influence indirecte (mais majeure) sur la toxicité de l'eau via leur impact sur la spéciation des métaux et métalloïdes (Di Toro et al., 2001; Fortin, 2024).

Afin d'illustrer concrètement l'influence de la spéciation métallique sur la biodisponibilité et les effets écotoxicologiques, le tableau 2.2 présente une synthèse d'exemples d'échantillons d'eau présentant des concentrations totales comparables pour différents éléments métalliques, ainsi que des informations sur leur spéciation, leur biodisponibilité relative et sur les réponses biologiques observées sur divers organismes aquatiques sensibles, notamment *D. magna.* Les données colligées à ce tableau illustrent l'influence déterminante de la spéciation chimique sur la toxicité effective des métaux, à concentration totale équivalente. Les résultats montrent que les formes ioniques libres (Cu^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}) sont celles qui présentent la biodisponibilité la plus élevée et induisent systématiquement des effets toxiques marqués (mortalité aiguë chez *D. magna*, inhibition de la croissance chez *Pseudokirchneriella subcapitata*, ou encore réduction de la reproduction lors d'expositions chroniques).

À l'inverse, les formes complexées avec la matière organique dissoute, les carbonates, ou les hydroxydes, ainsi que certaines formes organométalliques, modulent fortement la réponse biologique. Dans la majorité des cas (Cu, Cd, Zn, Ni, Pb), la complexation diminue la biodisponibilité et réduit la toxicité par rapport aux ions libres (Chen et al., 2018; Adams et al., 2020). Toutefois, certains complexes organiques comme le méthylmercure (CH_3Hg^+) constituent une exception : leur lipophilie élevée accroît la pénétration cellulaire et entraîne une toxicité supérieure à celle de Hg^{2+} (Beauvais-Flück et al., 2017).

Les valeurs d'EC présentées dans le tableau correspondent à des niveaux d'effet distincts : l'EC_{50} croissance traduit une réduction de 50 % du taux de croissance chez *P. subcapitata*, l'EC_{50} aiguë 48 h correspond à la mortalité de *D. magna* à court terme et les LC_{50} renvoient également à un effet létal mais selon des protocoles différents. La qualification de la biodisponibilité repose principalement sur la proportion d'ions métalliques libres, considérés comme les formes les plus réactives selon les principes du Biotic Ligand Model (BLM).

Ces observations montrent qu'une interprétation du risque environnemental s'appuyant seulement sur les concentrations totales peut être trompeuse et mener à une surestimation ou une sous-estimation de l'impact écotoxicologique réel. De telles observations justifient l'ajout de composantes liées à la spéciation chimique et de modèles mécanistes tels que le BLM dans les démarches scientifiques visant à bonifier l'évaluation des risques écotoxicologiques (De Schamphelaere & Janssen, 2004; Di Toro et al., 2001; Mebane et al., 2020; Tipping, 2002).

Tableau 2.2 Influence de la spéciation métallique sur la biodisponibilité et les effets écotoxicologiques chez les organismes aquatiques

	Formes chimiques	**Concentrations (EC_{50}/ LC_{50})**	**Organismes test**	**Effets observés**	**Biodisponibilité**	**Références**
Cu	Cu^{2+} (ion libre)	EC_{50} croissance = 48 µg/L	*P. subcapitata*	Inhibition de croissance	Très élevée	(Al-Hasawi et al., 2020)
	Cu^{2+} (ion libre)	EC_{50} aigu 48h = 185,2 µg/L	*D. magna*	Mortalité	Très élevée	(Xuan et al., 2024)
	Cu-DOM (complexes humiques)	EC_{50} > 200 µg/L (selon [DOM] mg/L)	*D. magna*	Mortalité réduite	Faible	(De Schamphelaere & Janssen, 2004)
Cd	Cd^{2+} (ion libre)	EC_{50} croissance = 18 µg/L	*P. subcapitata*	Inhibition de croissance	Très élevée	(Al-Hasawi et al., 2020)
	Cd-DOM (complexes acides humiques)	Toxicité réduite de 50-70% (par rapport au Cd^{2+})	*D. magna*	Réduction de mortalité	Faible	(De Schamphelaere & Janssen, 2004)
Zn	Zn^{2+} (ion libre)	EC_{50} croissance = 80 µg/L	*P. subcapitata*	Inhibition de croissance	Élevée	(Al-Hasawi et al., 2020)
	Zn–carbonate ($ZnCO_3$, $Zn(CO_3)_2^{2-}$)	EC_{50} > 500 µg/L	*D. magna*	Toxicité réduite	Modérée	(Tipping, 2002)
Pb	Pb^{2+} (ion libre)	EC_{50} croissance = 3,3 mg/L	*P. subcapitata*	Inhibition de croissance	Élevée	(Al-Hasawi et al., 2020)

Tableau 2.2 Influence de la spéciation métallique sur la biodisponibilité et les effets écotoxicologiques chez les organismes aquatiques (suite)

	Formes chimiques	**Concentrations (EC_{50}/ LC_{50})**	**Organismes test**	**Effets observés**	**Biodisponibilité**	**Références**
Hg	Hg^{2+} (ion libre)	EC_{50} croissance = 39 µg/L	*P. subcapitata*	Inhibition de croissance	Très élevée	(Al-Hasawi et al., 2020)
	CH_3Hg^+ (méthylmercure, organique)	$EC_{50} \approx 10$ µg/L	Rotifères (*Euchlanis dilatata*)	Forme la plus toxique	Très élevée	(Hernández-Flores et al., 2020)
Ni	Ni^{2+} (ion libre)	EC_{50} aigu 48h = 2707 µg/L	*D. magna*	Mortalité	Élevée	(Xuan et al., 2024)
	Ni-DOM (complexes humiques)	Réduction de toxicité de 40–60%	*D. magna*	Mortalité réduite	Faible	(CCME, 2021)

2.4. Toxicité des mélanges métalliques : interactions, modélisation et limites

Les effets combinés des contaminants présents au sein de mélanges complexes (dont font partie les effluents miniers et métallurgiques) diffèrent significativement de la simple additivité des effets individuels (Atakhanova, 2023). Cela met en relief les limites de l'approche d'évaluation de la toxicité appuyée sur une approche « contaminant par contaminant » et souligne l'intérêt scientifique lié au développement d'approches permettant d'élucider les causes de toxicité de solutions constituant des mélanges de contaminants (Norwood et al., 2003). Une prise en compte inadéquate des interactions entre les contaminants peut non seulement mener à une évaluation erronée des risques écotoxicologiques mais également compromettre l'efficacité de normes réglementaires (ECCC, 2025). Ces interactions influencent la biodisponibilité, la bioaccumulation et les mécanismes d'action physiologiques des contaminants, notamment via la compétition pour les sites de liaisons biologiques, l'altération du transport membranaire, l'induction ou l'inhibition de systèmes de détoxication et la formation de complexes dont les propriétés toxicologiques peuvent différer de celles des substances individuelles (De Schamphelaere & Janssen, 2004).

Les effets combinés des contaminants peuvent se manifester selon trois grands types d'interactions :

- **Additivité** : Lorsque les éléments métalliques partagent des modes d'action similaires, l'effet combiné correspond à la somme des effets individuels (Backhaus & Faust, 2012).
- **Synergisme :** L'effet combiné dépasse celui correspondant à la somme des effets individuels. Ce phénomène est particulièrement préoccupant car il peut amplifier la toxicité des contaminants au-delà des valeurs établies par des évaluations s'appuyant sur les contaminants considérés de façon individuelle (Jonker et al., 2005 ; Cedergreen, 2014).
- **Antagonisme** : L'effet toxique du mélange de contaminants est inférieur à la somme attendue. Cela résulte généralement de phénomènes de compétition pour

les sites biotiques ou de l'activation de mécanismes de détoxication (Di Toro et al., 2001 ; Norwood et al., 2003).

En pratique, les interactions observées correspondent rarement à une stricte additivité (Cedergreen, 2014). Elles varient selon la combinaison métallique, les ratios de concentration, les conditions physico-chimiques (pH, dureté, matière organique dissoute) et l'espèce étudiée (Vijver et al., 2010; Traudt et al., 2016; Fettweis et al., 2021). Cette variabilité complique la prédiction de la toxicité des effluents complexes et souligne l'importance d'approches intégratives (Meyer et al., 2014).

La complexité des interactions métalliques a conduit au développement de plusieurs cadres de modélisation destinés à évaluer la toxicité des mélanges. Le tableau 2.3 en propose une synthèse comparative des principaux modèles, de leurs principes de fonctionnement, de leurs applications aux effluents miniers et métallurgiques, ainsi que de leurs atouts et limites. Les méthodes répertoriées mettent en évidence la complémentarité entre les modèles, chacun présentant des avantages et des limites pour l'évaluation de la toxicité des rejets métalliques. Les modèles additifs, l'addition des concentrations (CA) et l'action indépendante (IA), constituent des références de base permettant de prédire la toxicité attendue en absence d'interaction (Backhaus & Faust, 2012), alors que les représentations graphiques (isobologrammes) et les outils statistiques (RSM, MIXTOX) permettent de décrire plus précisément les interactions binaires et ternaires. Le BLM consiste pour sa part en une approche mécaniste qui prend en compte la spéciation et la biodisponibilité des métaux et métalloïdes selon les paramètres physico-chimiques. Il incombe de souligner qu'indépendamment de la robustesse des modèles, leur potentiel d'application dépend invariablement de la qualité et de l'exhaustivité des jeux de données expérimentales utilisées (Niyogi & Wood, 2004).

Outre les modèles théoriques de modélisation, de multiples recherches expérimentales ont examiné la toxicité de combinaisons d'éléments métalliques dans le but d'identifier les interactions entre les contaminants et les mécanismes associés à ces interactions. Le tableau 2.4 présente une synthèse d'informations issues de travaux récents couvrant différents contextes expérimentaux, organismes aquatiques et types de mélanges.

Tableau 2.3 Les principaux modèles de prédiction de la toxicité des mélanges métalliques en milieu aquatique

Méthode	**Principe**	**Application aux effluents métalliques**	**Forces**	**Limites**	**Références**
CA (Concentration Addition)	Somme des ratios concentration/ECx pour chaque métal (même mode d'action)	Utilisé pour estimer la toxicité d'effluents contenant plusieurs éléments métalliques	Approche simple, largement employée en évaluation des mélanges	S'appuie sur l'hypothèse forte de modes d'action identiques	(Backhaus & Faust, 2012)
IA (Independent Action)	Produit probabiliste des effets individuels (modes d'action différents)	Sert à comparer les prédictions dans les effluents où les éléments métalliques agissent par des mécanismes distincts	Plus réaliste que le CA pour les mélanges hétérogènes	Requiert des données dose-réponse précises pour chaque élément métallique	(Backhaus & Faust, 2012)
Isobologrammes	Représentation graphique de la déviation à l'additivité	Appliqués dans des bioessais binaires pour identifier la synergie ou l'antagonisme	Visualisation claire des interactions	Limité à deux composants, surtout qualitatif	(Traudt et al., 2017)
RSM (Response Surface Methodology)	Analyse statistique des surfaces dose-réponse	Déployée pour explorer les interactions ternaires dans des bioessais	Quantifie l'intensité et la direction des interactions	Plan expérimental lourd (non applicable à >3 composants)	(Meyer et al., 2015)

Tableau 2.3 Les principaux modèles de prédiction de la toxicité des mélanges métalliques en milieu aquatique (suite)

Méthode	Principe	Application aux effluents métalliques	Forces	Limites	Références
MIXTOX	Analyse statistique des écarts aux prédictions CA/IA	Utilisé sur les effluents binaires/ternaires pour tester si les réponses biologiques diffèrent de l'additivité prévue	Détection robuste de synergie et d'antagonisme	Ne décrit pas la forme de l'interaction	(Jonker et al., 2005)
BLM (Biotic Ligand Model)	Compétition des métaux pour les sites biotiques (intègre pH, dureté, DOM)	Développé pour prédire la toxicité dans des eaux naturelles, adapté à l'évaluation d'effluents miniers selon leur chimie	Intègre spéciation et biodisponibilité, reconnu dans la réglementation	Paramétrage complexe, validé seulement pour quelques éléments métalliques	(Di Toro et al., 2001; Santore et al., 2018)

Tableau 2.4 Études expérimentales sur les interactions toxicologiques entre métaux : synthèse des observations et mécanismes

Mélanges / Contexte	**Organismes**	**Interaction observée**	**Mécanismes identifiés**	**Réferences**
Ternaires Cd-Cu-Ni & **Cd-Ni-Zn**	*D. magna* (48 h)	Antagonisme ↔ additivité ↔ synergie selon ratio	Compétition pour sites biotiques et DOM, modulation de la biodisponibilité ionique	(Traudt et al., 2017)
Binaires & ternaires Cd, Cu, Zn (carbone organique dissous ~3 mg/L)	*D. magna* (48 h)	Réponses mixtes : antagonisme (Cd-Zn), additivité (Cu-Zn), synergie (Cu-Cd)	Compétition pour ligands DOM, formation de complexes métalliques affectant la spéciation	(Meyer et al., 2015)
Binaire Cu+Cd (*sublethal, ressource* & génotype)	*D. pulex* (21 j)	Additivité ↔ synergie selon contexte écologique	Stress oxydatif (Cu), compétition pour transporteurs	(Sadeq & Beckerman, 2020)
Cu+Cd (*sublethal, reproduction*)	*D. magna* (21 j)	Synergie	Perturbation énergétique, inhibition des défenses antioxydantes	(Lari et al., 2017)
Cu+Zn (branchies)	Poisson *O. mykiss*	Antagonisme partiel	Compétition Cu/Zn pour sites branchiaux, perturbation de la régulation ionique	(Niyogi & Wood, 2004)

Tableau 2.4 Études expérimentales sur les interactions toxicologiques entre métaux : synthèse des observations et mécanismes (suite)

Mélanges / Contexte	**Organismes**	**Interaction observée**	**Mécanismes identifiés**	**Réferences**
Cd+Zn (chronique)	*D. magna* (21 j)	Antagonisme (Zn protège contre Cd)	Zn réduit la toxicité du Cd par complexation/compétition aux sites cibles	(Pérez & Hoang, 2017)
Ternaires Ni-Cu-Zn (ratios 1:1:3 ; 5:1:13)	Algues (Espèces isolées & communautés)	Antagonisme chez des espèces isolées; synergie émergente chez des communautés	Redondance fonctionnelle, sélection d'espèces tolérantes, corrélations de sensibilité	(Fettweis et al., 2023)
Ni-Cu-Zn en microcosmes trophiques (algues et *D. magna*)	Algues + *D. magna* (communautés)	Additivité ↔ antagonisme selon la trajectoire trophique	Effets indirects via l'impact sur les algues sensibles, propagation dans la chaîne trophique	(Fettweis et al., 2024)
Cu (0,8 µM) + Cd (0,25 µM)	Embryons de *Danio rerio (poisson-zèbre)*	Effets combinés sur le développement embryonnaire	Perturbations morphologiques/physiologiques liées à des interactions toxiques	(Majid et al., 2024)
Cd, Cu, Zn, Ni (mélanges complexes)	*Artemia franciscana*	Tolérance élevée aux métaux multiples	Adaptation physiologique de la famille Artemiidae	(Pinto & Zanette, 2023)

Dans l’ensemble, les études identifiées au tableau 2.4 confirment que les mélanges métalliques produisent rarement des effets strictement additifs. Chez *D. magna* et *D. pulex*, les interactions entre Cu, Zn et Cd peuvent se caractériser par la synergie ou l’antagonisme selon les ratios, la spéciation et le contexte écologique, la synergie étant souvent associée au stress oxydatif, alors que la compétition pour les sites de transport membranaire tend à engendrer un effet antagoniste (Pérez & Hoang, 2017). Les travaux sur les poissons montrent l’importance de la compétition ionique aux branchies, modulée par les conditions physicochimiques du milieu (pH, dureté, concentrations en ions majeurs) (Niyogi & Wood, 2004). Les études récentes sur les groupes d'algues et les petits systèmes nourriciers montrent des synergies nouvelles et des effets indirects qui vont dans la chaîne alimentaire (Fettweis et al., 2023; Fettweis et al., 2024). À des niveaux supérieurs d’organisation, les recherches récentes sur les communautés des algues et les microcosmes trophiques révèlent des synergies émergentes et des effets indirects propagés dans la chaîne alimentaire (Fettweis et al., 2023; Fettweis et al., 2024). Finalement, des espèces résilientes comme *Artemia franciscana* démontrent des processus adaptatifs de séquestration et de détoxication (Pinto & Zanette, 2023). Ces conclusions mettent en évidence que l'additivité demeure une hypothèse de base utile, mais inappropriée pour saisir la complexité des effluents multi-composants. Il en résulte que le développement d'approches intégrées prenant en compte la spéciation chimique, les bioessais à plusieurs niveaux et la modélisation prédictive est essentiel (Norwood et al., 2003).

2.5. Traitement par ozone microbulles : mécanismes, efficacité et effets sur la toxicité

L’ozonation est l’une des technologies avancées d’oxydation (Advanced Oxidation Processes, AOPs) les plus exploitées pour le traitement des effluents miniers et métallurgiques (Ryskie et al., 2020). L’approche repose sur la capacité de l’ozone (O_3), seul ou en combinaison avec les radicaux hydroxyles (•OH) générés lors de sa décomposition, de transformer (oxyder) une grande variété de substrats organiques et inorganiques. L’utilisation du procédé d’ozonation avec microbulles d’ozone (O_3-MB), obtenues par différentes méthodes telles que la cavitation, le venturi et les membranes poreuses, présente un avantage marqué par rapport à l’injection de gaz des procédés conventionnels. En effet, les microbulles, d’un diamètre de moins de 200 µm, possèdent une surface interfaciale très grande, logent une quantité d’ozone plus importante qu’en phase liquide et présentent une durée de vie accrue dans l’eau plus (Xiao et al., 2022). Ces propriétés permettent d’optimiser la formation d’espèces réactives de l’oxygène (•OH, HO_2•, O_2•$^-$) et une meilleure diffusion du gaz dans l’eau (Völker et al., 2019).

Sur le plan mécaniste, l’ozonation à microbulle agit directement sur les espèces oxydables présentes dans les effluents (von Gunten, 2003). D’une part, l’azote ammoniacal (NH_3), très toxique pour les invertébrés et les poissons, est rapidement oxydé en NO_3^-, ce qui limite sa contribution à la toxicité chronique (Ryskie et al., 2023), bien que le risque d’eutrophisation puisse persister. D’autre part, Fe^{2+} et Mn^{2+} sont oxydés, menant à la formation d’oxydes ou hydroxydes peu solubles (Fe^{3+} et MnO_2), ce qui réduit fortement leur biodisponibilité (von Gunten, 2003). L’As(III) est, pour sa part, transformé en As(V), forme plus stable, moins toxique et susceptible de s’adsorber sur les phases solides. Le Se suit une dynamique similaire : le Se(IV), très biodisponible, est oxydé en Se(VI), généralement beaucoup moins assimilé par les organismes aquatiques (Karwowska & Sperczyńska, 2022). Il incombe de souligner que les réactions d’oxydation ayant lieu lors de l’ozonation peuvent aussi engendrer des risques, notamment en lien avec (1) l’oxydation du Cr(III) en Cr(VI), (2) la formation de BrO_3^- en présence de Br^-, (3) la formation de chlorate et (4) la formation de colloïdes toxiques (Khuntia et al., 2013). De tels mécanismes peuvent engendrer de nouvelles formes toxiques si le procédé n’est pas rigoureusement maîtrisé (Ryskie et al., 2023). Hormis ces limitations, des bioessais menés avec *D. magna* et d’autres organismes modèles confirment que l’ozonation à microbulles peut réduire significativement la toxicité des effluents miniers et métallurgiques. Plusieurs travaux ont évalué l’efficacité de l’ozonation, seule ou couplée à d’autres procédés, dans le traitement des effluents miniers et métallurgiques. Le tableau 2.5 présente une synthèse de ces études, en détaillant les matrices testées, les conditions opératoires, les effets sur la toxicité et les principales observations rapportées. Dans l’ensemble, les études révèlent que l’O_3-MB est une technologie prometteuse pour réduire la toxicité des effluents miniers et métallurgiques. Plusieurs études ont notamment montré l’enlèvement significatif de NH_3 avec des efficacités à plus de 90 % dans un cadre expérimental contrôlé (Ryskie et al., 2020, 2023). Le procédé agit également sur la spéciation de plusieurs métaux et métalloïdes, en réduisant ainsi leur fraction biodisponible. Cette diminution s’est traduite par une réduction mesurable de la toxicité aiguë lors des bioessais sur *D. magna* (Ryskie et al., 2023). Néanmoins, l’efficacité dépend principalement de la nature des effluents. La présence de cyanures, de métaux dissous ou de matière organique pourrait occasionner une perte d’efficacité et la formation de sous-produits non désirés, tels que les nanoparticules métalliques oxydées. Il existe aussi un manque d’informations sur la toxicité chronique et la pérennité du traitement en conditions réelles qui n’est pas bien connue. Enfin, le maintien de conditions optimales (pH alcalin, temps de contact, dosage en O_3) pose un défi technique et engendre une augmentation de salinité.

Tableau 2.5 Études sur l'efficacité de l'ozonation et procédés couplés dans le traitement des effluents contaminés

Matrice / Effluent	**Procédé O_3**	**Paramètres clés**	**Toxicité (aiguë / chronique)**	**Observations**	**Réference**
Mélanges d'effluents miniers réels (mine d'or, Québec)	Ozonation par microbulles, avec ou sans pré-traitement des métaux (chaux + floculant)	pH ≈ 9, NH_3-N initial de 1,1 à 58,4 mg/L, température 10 °C, Temps de résidence hydraulique 1-2,5 h, 22 g O_3/h	Aiguë (*D. magna*) : sans pré-traitement, toxicité anormale (à dilution 50%) malgré absence de toxicité sur un échantillon non dilué. Pas de toxicité aiguë détectée avec pré-traitement par ajustement de pH	L'absence de pré-traitement des métaux mène à apparition probable de nanoparticules oxydées responsables de la toxicité aiguë	(Ryskie et al., 2023)
Eaux simulées typiques de traitement d'or (CN, Ni, Cu, Zn)	Ozonation couplée à UV-LED (technique améliorée)	pH 10, O_3 ≈ 4 mg/s, électrodes SS-Fe/Al-Gr avec 15 mA/cm²	Enlèvement notable : 93 % Cu, 92 % Zn, 83 % Ni, 94 % CN. Toxicité non testée directement	Très haute efficacité pour élimination de cyanures et métaux dans matrices simulées	(Shahedi et al., 2023)
Effluents synthétiques et 5 effluents miniers réels, contenant NH_3 N (et autres contaminants)	Ozonation par microbulles en batch et flux continu	Flux continu : 1,1 L/min, 570 min ; enlèvement de 28-99 % pour NH_3-N	Toxicité non évaluée	Efficacité variable selon la matrice, la présence de cyanures/métaux diminue l'efficacité. Traitement faisable à l'échelle pilote	(Ryskie et al., 2020)

Tableau 2.5 Études sur l'efficacité de l'ozonation et procédés couplés dans le traitement des effluents contaminés (suite)

Matrice / Effluent	**Procédé O_3**	**Paramètres clés**	**Toxicité (aiguë / chronique)**	**Observations**	**Réference**
Eau industrielle (NH_3-N synthétique pilotée)	Ozonation microbulles	pH 6-9, débit d'O_3 variable, catalyse par Br^-	Toxicité non évaluée	Efficace à pH élevé par oxydation directe. Les radicaux $\cdot OH$ interviennent à pH faible. Br^- catalyse la réaction	(Khuntia et al., 2013)
Effluents miniers canadiens (Arctique/Abitibi)	Ozonation par microbulles (système pilote)	pH 9, temps de contact 15-90 min, O_3 10-20 g/h	Aiguë (*D. magna*, truite arc-en-ciel); réduction partielle mais persistance d'une toxicité résiduelle	L'ozonation réduit les concentrations en NH_3-N et métaux dissous, mais des effets osmotiques et des espèces oxydées stables engendrent une toxicité résiduelle	(Marcotte, 2021)

2.6. Synthèse

L'analyse de littérature souligne que l'évaluation précise de la toxicité des effluents miniers et métallurgiques ne peut se faire uniquement sur la base des concentrations totales des contaminants. La biodisponibilité des métaux et métalloïdes est influencée par la spéciation chimique, ce qui peut expliquer les variations des effets observés entre des solutions comportant des substances uniques et des mélanges. De plus, les interactions au sein des mélanges (synergie, antagonisme, additivité) compliquent les prévisions relatives à la toxicité. Les modèles conceptuels (CA, IA) demeurent des références précieuses, mais leur utilisation doit être enrichie par des méthodes mécanistes (BLM) et des techniques statistiques (MIXTOX, RSM) afin de mieux capturer la complexité des effluents réels. Techniquement, l'ozonation par microbulles semble être une alternative encourageante pour atténuer la toxicité en modifiant la spéciation et en facilitant l’enlèvement de contaminants oxydables, bien qu’elle nécessite d'être intégrée dans des processus combinés.

En synthèse, la protection des écosystèmes aquatiques soumis au rejet d’effluents miniers et métallurgiques nécessite une évaluation intégrée de la spéciation des métaux et métalloïdes, des espèces oxydables telles que NH_3, la réalisation de bioessais à plusieurs niveaux et la modélisation des mélanges.

CHAPITRE 3 Démarche méthodologique

La démarche méthodologique de cette thèse repose sur une approche intégrée combinant des analyses statistiques, échantillonnages saisonniers, tests biologiques de toxicité aiguë, essais de traitement en pilote et modélisation géochimique. La démarche est synthétisée dans la Figure 3.1, qui présente les différentes étapes et leur articulation, puis détaillée dans les sections suivantes. Il incombe de souligner que seules les grandes lignes des approches méthodologiques sont présentées dans le présent chapitre, les informations précises liées à chacun des trois chapitres constituant le cœur de cette thèse étant présentées aux **chapitres 4**, **5** et **6**. Cette approche est retenue afin de limiter les redondances imposées par la rédaction d'une thèse dont le contenu est en partie présenté sous forme d'articles scientifiques (**chapitres 4,5 et 6**).

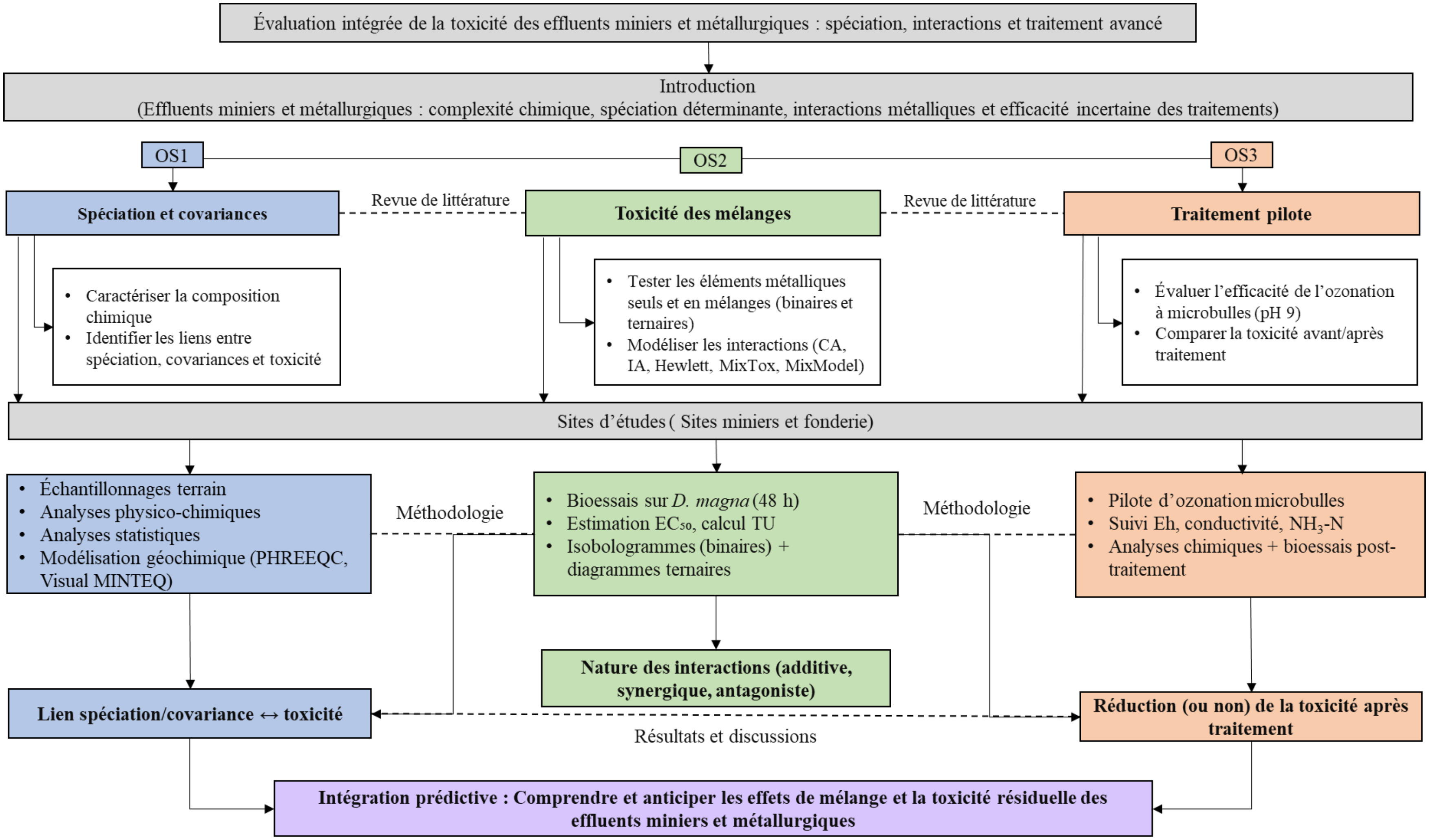

Figure 3.1 Schéma du déroulement du projet

3.1. Analyses statistiques des données historiques

Les données historiques du suivi de la qualité physico-chimique d'un effluent de la fonderie Horne (2016-2023) ont été compilées pour identifier des tendances temporelles et des liens éventuels entre la co-occurrence de métaux et métalloïdes et la toxicité. Les analyses statistiques ont été effectuées sous le logiciel R (v4.2.3) et ont comporté quatre volets principaux :

- Les corrélations de Pearson ont servi à explorer les associations entre les paramètres chimiques (métaux, métalloïdes, ions majeurs, paramètres physico-chimiques).
- Des régressions linéaires simples et multiples ont permis d'explorer le lien entre la toxicité (unités de toxicité, TU) et les concentrations de métaux et métalloïdes et d'identifier les variables explicatives dominantes.
- Une analyse en composantes principales (ACP) a été appliquée, après normalisation des données, afin de réduire la dimensionnalité et de détecter les gradients chimiques.
- Une classification hiérarchique ascendante (HCA) (distance euclidienne, méthode de Ward) a été utilisée pour regrouper les échantillons selon leurs signatures chimiques.

3.2. Échantillonnages saisonniers et analyses chimiques

Des campagnes d'échantillonnage saisonnières ont été menées sur les effluents de la fonderie Horne afin de documenter la variabilité temporelle dans les caractéristiques des effluents en lien avec les changements dans les conditions hydrologiques et climatiques (fonte printanière, basses eaux estivales, gel hivernal). Les prélèvements ont été réalisés sur le terrain, et le conditionnement ainsi que la préservation des aliquotes destinées aux différentes analyses chimiques ont été effectués conformément aux protocoles du MELCCFP et du CEAEQ (CEAEQ, 2021).

Les échantillons destinés aux analyses chimiques ont été conservés à 4 °C, tandis que ceux prévus pour les bioessais et les essais pilotes ont été congelés à –20 °C avant la réalisation des essais. Les analyses chimiques ont été effectuées à l'INRS-ETE par ICP-AES et ICP-MS pour les métaux et métalloïdes et par chromatographie ionique pour les anions majeurs (Cl^-, SO_4^{2-}, NO_3^-). Les paramètres de terrain (pH, température, conductivité, oxygène dissous) ont été mesurés *in situ* à l'aide de sondes multiparamètres calibrées le jour même. L'ensemble des méthodes incluait des contrôles qualité systématiques (blancs analytiques, standards certifiés, duplicatas).

Les données obtenues ont ensuite été intégrées dans les modèles de spéciation (PHREEQC avec la base de données thermodynamiques WATEQ4F et Visual MINTEQ avec les bases de données omp_2008.vdb, thermo.vdb, type6.vdb et gaussian.vdb) afin d'identifier les formes chimiques dominantes et d'évaluer leur rôle potentiel dans la toxicité observée.

3.3. Tests biologiques de toxicité aiguë

Les essais de toxicité ont été réalisés dans le laboratoire d'écotoxicologie de l'INRS-ETE à Québec (QC, Canada), selon le protocole normalisé du CEAEQ pour l'évaluation de la létalité aiguë chez *D. magna* (CEAEQ, 2021). Les organismes sont issus d'un élevage maintenu sur place, en conditions contrôlées (20 ± 2 °C, photopériode 16:8, alimentation régulière par *Chlamydomonas reinhardtii*), assurant une souche saine et stable. Seul les néonates âgés de moins de 24 h ont été utilisés pour garantir la sensibilité et la reproductibilité des essais. Chaque essai consiste à exposer des groupes de néonates à des dilutions croissantes d'effluents ou de mélanges synthétiques, préparés à partir des concentrations nominales en métaux et métalloïdes envisagés. La mortalité et l'immobilité sont évaluées après 48 h, selon les lignes directrices internationales (OECD, 2004) et les standards du CEAEQ (CEAEQ, 2021). Les résultats permettent de déterminer les concentrations efficaces médianes (EC_{50} et LC_{50}, exprimées en % d'effluent, 48 h) et d'exprimer la toxicité en unités de toxicité (TU) selon :

$$\mathrm{TU} = \frac{100}{\mathrm{EC_{50}\ ou\ LC_{50}}} \quad (1)$$

3.4. Analyse des interactions toxicologiques

L'évaluation des effets des mélanges métalliques a été réalisée via l'utilisation de plusieurs modèles de référence, intégrant des approches conceptuelles, statistiques et visuelles. Cette démarche visait à caractériser de façon intégrée la nature des interactions sous-jacentes à la toxicité (additivité, synergie, antagonisme). Les modèles de référence *Concentration Addition* (CA) et *Independent Action* (IA) ont d'abord été utilisés. Le modèle CA s'appuie sur l'hypothèse voulant que les substances d'un mélange présentent des mécanismes d'action similaires et contribuent de manière interchangeable à l'effet toxique total. La toxicité du mélange sera alors considérée comme égale à la somme des fractions des concentrations normalisées :

$$\mathrm{TU} = \frac{\mathrm{C_i}}{\mathrm{EC_{50i}}} \quad (2)$$

où C_i est la concentration du contaminant i dans le mélange et EC_{50i}, son effet médian individuel.

Le modèle IA considère pour sa part que les substances ont des mécanismes d'action distincts. L'effet combiné est donc calculé comme la probabilité que la toxicité soit affectée par au moins une des substances :

$$\mathrm{E_{mix}} = \sum_{i=1}^{n} (1 - \mathrm{E_i}) \tag{3}$$

où E_i est l'effet individuel de la substance i.

Ces deux modèles représentent des étalons permettant de comparer les déviations observées du comportement additif à la simple combinaison linéaire des réponses interactives. Plusieurs travaux récents confirment leur pertinence pour l'évaluation de la toxicité des mélanges métalliques (Cedergreen, 2014 ; Dupraz et al., 2018; Yan & Saxena, 2022). Le modèle statistique de Hewlett (1969) a été utilisé en complément aux deux approches précitées. Cette approche consiste en une mise en relation directe des réponses expérimentales observées et des réponses attendues selon les modèles CA ou IA. Si les écarts constatés sont significatifs, ils peuvent être considérés comme étant attribuables à de véritables interactions biologiques plutôt qu'à une simple variabilité aléatoire. Dans ce cadre, l'hypothèse nulle (H_0) correspond à l'absence de déviation entre les réponses observées et celles prévues par le modèle additif (CA ou IA), tandis que l'hypothèse alternative (H_1) correspond à une déviation significative indiquant une interaction synergique ou antagoniste (Jonker et al., 2005; Backhaus & Faust, 2012).

Le modèle MixTox est implémenté dans une macro Excel® et a été développé par Jonker et al. (2005). Cette approche complémente les modèles CA et IA et permet de considérer la synergie et l'antagonisme, la dépendance à la dose absolue (*dose-level dependence*) et la dépendance au ratio des composants (*dose-ratio dependence*). Le modèle MixTox a été ici utilisé pour caractériser la nature et l'intensité des interactions entre les différents éléments métalliques dans les mélanges binaires. Pour les mélanges ternaires, la fonction MixModel du paquet *mixexp* (Lawson & Willden, 2016) a été appliqué sous R. Cet outil développé spécifiquement pour des systèmes multi-composants permet d'estimer la contribution relative de chaque élément métallique, de tester les écarts à l'additivité relative et d'explorer la structure des interactions dans un espace tridimensionnel (Lawson & Willden, 2016).

L'analyse a été complétée par l'utilisation et l'élaboration d'outils graphiques. Des isobologrammes (Greco et al., 1995) obtenus grâce à un code R adapté de Dupraz et al. (2018) ont permis de représenter les combinaisons équitoxiques dans le cas des mélanges binaires. Pour le cas des mélanges ternaires, des diagrammes ternaires ont permis de représenter simultanément les proportions relatives des métaux ainsi que leurs effets respectifs sur la toxicité, ce qui a permis de d'illustrer les zones de forte toxicité et les combinaisons où une interaction particulière prévalait.

3.5. Essais de traitement pilote par ozonation microbulles

Les essais permettant d'évaluer l'efficacité de l'ozonation à microbulles ont été réalisés au laboratoire de l'IRME-UQAT, dans un réacteur de 18 L d'un pilote de traitement (Ryskie et al., 2021). Le réacteur d'ozonation était alimenté par un générateur d'ozone (débit de 11 g O_3/h) et un injecteur de microbulles (opérant entre 20 et 40 psi). Pour favoriser l'oxydation des espèces métalliques et azotées redox-sensibles, le pH a été ajusté à 9 durant tout le traitement par l'ajout de NaOH. Les effluents réels ont été traités pendant des temps de contact jusqu'à 210 min. Des mesures de suivi en continu ont été réalisées sur l'évolution du potentiel d'oxydoréduction (Eh), de la conductivité électrique (CE) et de la concentration en NH_3. Les analyses chimiques post-traitement ont été réalisées par ICP-AES et ICP-MS pour les métaux et métalloïdes et par CI pour les anions (Cl^-, SO_4^{2-}, NO_3^-) (section 3.3). Parallèlement, la toxicité des effluents a été déterminée par des tests de létalité aiguë sur *D. magna* (48 h) (section 3.4).

3.6. Intégration et modélisation géochimique

Les résultats analytiques et toxicologiques ont été intégrés dans une approche de modélisation géochimique afin de mieux comprendre les mécanismes responsables de la toxicité observée et d'identifier les espèces chimiques critiques. Deux logiciels complémentaires ont été utilisés, soit PHREEQC (v4.3.2, base de données WATEQ4F) et Visual MINTEQ (version 3.1, base de données comp_2008.vdb, thermo.vdb, type6.vdb and gaussian.vdb databases). Les calculs ont ciblé spécifiquement l'évaluation de la spéciation des métaux et métalloïdes, en tenant compte des conditions physico-chimiques mesurées (pH, alcalinité, Eh, CE, température) et en assumant l'équilibre thermodynamique.

CHAPITRE 4 Article 1: Assessing the relationship between trace metal co-occurrence, speciation, and toxicity in industrial effluents

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4.1. Abstract

This study focuses on evaluating the relationship between the co-occurrence and speciation of trace metallic elements with reference to the acute toxicity observed to *Daphnia magna*. Calculations were performed on data from the regular monitoring of an industrial effluent. The effluent generally met regulatory discharge criteria for metal(loid)s ($\mathrm{Fe} > \mathrm{Zn} > \mathrm{Al} > \mathrm{Cu} > \mathrm{Ni} > \mathrm{As} > \mathrm{Cd} > \mathrm{Pb}$) concentrations, but sporadic toxicity was observed, indicating that the interactions between trace metallic elements might affect toxicity. The methodological approaches include correlation analyses (CA), one-way analyses of variance (ANOVA), principal component analyses (PCA), hierarchical cluster analyses (HCA), and geochemical calculations performed for the purpose of assessing trace metallic elements speciation. The results suggest that Cd and Cu are the primary contributors to toxicity while Fe could inhibit toxicity. Moreover, speciation calculations suggest that the bioavailable forms of Cd^{2+} and Cu^{2+}, even at sublethal levels, could play a pivotal role in the observed toxicity. The analyses of changes in correlations between pairs of elements in non-toxic vs. toxic effluents further suggest synergistic $\mathrm{Cu} - \mathrm{Cd}$ and antagonistic Fe effects on toxicity. The approach developed in the present study has the potential for wider implementation. The identification of statistical links between the concentrations of different contaminants and toxicity could facilitate toxicants identification, particularly for effluents that meet regulatory standards in terms of contaminant concentrations.

Keywords: *Trace metals and metalloids, Effluent toxicity, Speciation, Multivariate analysis, interactions effects*

4.2. Introduction

The actual and potential impacts of industrial and mining effluent discharge on natural waters and ecosystems have been extensively documented in the scientific literature (Wright et al., 2017). Metal(loid)s found in such effluents, including Ni, Cr, Cu, Zn, Cd, Pb, and As, can have significant adverse environmental effects due to their inherent toxicity and potential for bioaccumulation (Dubé et al., 2005; Vendrell-Puigmitja et al., 2020; Thomas et al., 2021 Moldovan et al., 2022). Different legal frameworks have been established at the international, national, and regional levels with the objective of regulating the quality of effluents before they are discharged into the environment. These frameworks often provide criteria that may be expressed in the form of maximum allowable concentrations for a range of individual contaminants. The advantage of this approach is that it allows for establishing quantitative contaminant concentration thresholds that must not be exceeded in effluents. For example, in Canada, the *Metal and Diamond Mining Effluent Regulations (SOR/2002-222)* (MDMER) provide a framework for the oversight and release of mining effluents and provide quantitative criteria for the maximum concentrations of trace metallic elements before release to the environment (Minister of Justice, 2025). Nevertheless, in some cases, effluents in which all the concentrations of regulated contaminants are below the prescribed criteria can still entail aquatic toxicity (Munz et al., 2017). This phenomenon can be attributed to the presence of contaminants that are not associated with criteria, such as certain contaminants of emerging concern (CEC) that are not yet considered in the regulatory framework (Munz et al., 2017). Alternatively, it can be explained by the cumulative effects of different species being present in an effluent that represents a cocktail of contaminants, even though each contaminant in the cocktail is present at a concentration below the discharge criteria (Rzymski et al., 2017; Ahsan et al., 2019; Gui et al., 2023; Li et al., 2023). The toxicity of contaminant mixtures is contingent on the interplay between dissolved species, their speciation, and bioavailability (Birceanu et al., 2008; Ahn et al., 2020). The speciation of contaminants is also of great consequence regarding their bioavailability, and their interaction with living organisms. To illustrate, As(III) is regarded as more toxic than the more oxidised form, As(V) in aqueous environments (Smedley & Kinniburgh, 2002; Wang & Mulligan, 2006). Furthermore, the co-occurrence of certain dissolved species may give rise to additive effects, while others may result in synergistic or antagonistic effects (Rodea-Palomares et al., 2015; Lari et al., 2017; Hassold et al., 2021). A synergistic effect can be defined as a situation whereby the toxicity resulting from a mixture of contaminants is greater than the sum of the toxic effects associated with each contaminant taken individually. Conversely, an antagonistic effect can be defined as a situation whereby the toxicity of a mixture of contaminants is less than the sum of the toxic effects of each of the components considered individually. For example, the simultaneous presence of Cu and Zn can result in competition for binding sites in organism tissues, thereby altering the biological response to these metals in an antagonistic manner (Cooper et al., 2009; Meyer et al., 2014; Garman et al., 2020). The intricate interactions between diverse metals, such as Ni and Zn, which confer protection against Cd toxicity have also been identified (Lari et al., 2017; Nys et al., 2017; Traudt et al., 2017; Jamil Emon et al., 2023). An evaluation of toxicity based on an isolated focus on specific substances (each taken individually) may therefore result in an inaccurate assessment of the toxicity of mixtures. An oversight of this nature could result in environmental risks related to toxicity, given that receiving natural waters are exposed to complex mixtures of multiple contaminants, each present in different chemical forms (Backhaus and Faust, 2012; Traudt et al., 2017). Laboratory-based controlled tests can be conducted on binary (Perez and Hoang, 2017) and ternary (Traudt et al., 2017; Zeng et el., 2024) contaminant mixtures. However, assessing the interactions among multiple contaminants poses a significant challenge due to the sheer complexity involved. This complexity stems in part from the exponential increase in the number of possible combinations that must be evaluated in a laboratory setting when multiple contaminants are considered. Additionally, producing effluents in the laboratory that accurately represent field conditions can be challenging, thereby complicating the extrapolation of laboratory findings to real-world conditions. Significant gaps in the evaluation of toxicity risks associated with effluents comprising mixtures of contaminants still exist. In this context, statistical analysis of laboratory monitoring data (geochemistry and toxicity) from active industrial sites could help to better identify the causes of toxicant in contaminant cocktails. This is particularly salient in cases where effluents, despite adhering to current metal(loid)s concentration standards (e.g. the MDMER in Canada), exhibit toxicity. In such instances, the underlying cause of toxicity can be challenging to discern and difficult to address effectively.

Fitting in the pre-established context, this study seeks to identify the causes of toxicity in smelter effluents that generally meet the MDMER maximum concentration criteria. Two hypotheses (H1; H2) are specifically tested:

H1: In a dataset combining toxic and non-toxic samples, a significant statistical relationship can be established between the concentrations of dissolved metal(loid)s and the level of toxicity (expressed in toxicity units, TU).

H2: In a dataset separated into non-toxic (toxicity < 1 TU) and toxic (toxicity > 1 TU) subgroups, there will be statistically significant geochemical differences between subgroups.

Monitoring data from a smelter effluent comprising a mixture of contaminants were analyzed. H1 was tested using multiple linear regression analyses. H2 was tested by comparing toxic and non-toxic subgroups based on (1) dissolved metal(loid)s concentrations and speciation, (2) correlation analyses and, (3) principal component analyses (PCA) and hierarchical cluster analyses (HCA). The developed approach could be extrapolated to other locations, offering a framework for identifying toxicants in industrial and mining effluents that adhere to regulatory standards concerning dissolved metal(loid)s. This knowledge is critically required for better protecting ecosystems and adapt water management and treatment methods in active industrial sites.

4.3. Methodology and data sources

4.3.1. Data source and transformation

Historical data (2016-2023; n = 127 samples) from an industrial effluent were made available by the Glencore Horne Smelter, a company located in Abitibi-Témiscamingue (Quebec, Canada). The toxicity data used in this study originate from environmental monitoring programs previously conducted by the company to meet regulatory requirements under the supervision of the *Ministère de l'Environnement, de la Lutte contre les changements climatiques, de la Faune et des Parcs* (MELCCFP). Acute toxicity tests were carried out by the *International Organization for Standardization / International Electrotechnical Commission* ISO/IEC 17025-accredited laboratory using the MA. EAU. TOX. 1.0 method (CEAEQ, 2012). This method is equivalent to the procedure described in the *Organisation for Economic Co-operation and Development* (OECD) Test Guideline No. 202 (OECD, 2004), for the acute immobilisation test using *Daphnia magna* (*D. magna*). The tests were performed on *D. magna* for 48 hr, with immobilization, determined as the median effective concentration (EC50), as the final endpoint of measurement.

The results pertaining to acute toxicity on *D. magna* and to pH, alkalinity, temperature, Al, As, Ca, Cu, Fe, K, Mg, Mn, Na, Ni, N(5), P, Pb, Zn, and Cd, were made available. All concentration analyses and toxicity tests conducted to create the database were performed by a laboratory accredited by CEAEQ, thereby ensuring that the quality of the results meets the provincial requirements for environmental analyses. The pH and temperature data were measured onsite by the operator. The available database reports elemental concentrations in parts per million (ppm), as compositional data. This type of dataset is not ideal for performing statistical analyses because each concentration represents a part of the whole, which means that the different concentrations of single elements cannot vary independently (Blake et al., 2016; Bondu et al., 2020; Reimann et al., 2017). To address this challenge and enhance the robustness of the statistical analyses, a compositional log-ratio (CLR) transformation was applied to the data (Aitchison, 1986). This technique involves taking the logarithm of element ratios, thereby eliminating the constraints imposed by the closure of the composition space. This approach facilitates the statistical analyses and interpretation of the data, enhancing the reliability and precision of the results. The CLR transformation equation is written as:

$$\mathrm{clr}(x) = [\log(x1/\, g(x)), \ldots, \log(xD\, g(x))] \tag{1}$$

Where x represents the composition vector with D components and g(x) is the geometric mean of the components in vector x.

Toxicity was expressed in toxic units (TU), calculated as:

$$\mathrm{TU} = \frac{100}{\mathrm{EC50\ or\ LC50}} \tag{2}$$

where EC_{50} and LC_{50} represents the effluent concentration (% v/v) causing 50% immobilization or 50% mortality, respectively, of *D. magna* after 48 hr of exposure. This method provides a standardized approach for assessing effluent toxicity. An effluent is considered non-toxic (TU < 1) when, even at 100% (undiluted), it does not reach the EC50, implying EC_{50} > 100%, in accordance with criteria established by the OECD (2004) and USEPA (2002). A brief remark is added to include both EC_{50} and LC_{50} to remain consistent with immobilization-based protocols (OECD 202) and lethality-based endpoints used in other regulatory frameworks.

4.3.2. Statistical analyses

Multivariate analyses were conducted in *Rstudio v4.2.3* (R Core Team, 2023) to identify potential correlations between metallic trace elements and effluent toxicity. One-way analyses of variance (ANOVA) were conducted with a $p < 0.05$ significance threshold, and the least significant difference (LSD) test was employed to compare the concentrations of metallic trace elements in toxic and non-toxic effluent samples. Pearson correlation and principal component analyses (PCA) were performed with a varimax rotation to facilitate interpretation by reducing the number of factors for each component (Yadav et al., 2019). Hierarchical cluster analyses (HCA) were further conducted to cluster samples based on Euclidean distance for structured classification purposes.

4.3.3. Geochemical calculations

Geochemical calculations were performed using the PHREEQC software (Parkhurst and Appelo, 1999) with the *wateq4f.dat* database. In the calculations, the ionic balance error was adjusted to zero using sulfate ions. This was required as the available data did not contain all the anion species required to properly assess the ion balance. A fixed value of 6 was imposed for pe, consistent with onsite oxidation-reduction potential measurements conducted in 2021-2023. Scenario-based modelling with PHREEQC was also carried out to assess the influence of Cl^- on speciation calculations, by fixing Cl^- concentrations at 20, 38, 60, and 100 mg/L to reflect the range expected in mining and metallurgical effluents. In parallel, Visual MINTEQ was employed using the comp_2008.vdb, thermo.vdb, type6.vdb and gaussian.vdb databases and the NICA–Donnan model (Gustafsson, 2000) to estimate the role of dissolved organic matter (DOM) on metal speciation, using dissolved organic carbon (DOC) concentrations fixed at 1, 2, 5, 10, and 20 mg/L.

4.4. Results and discussion

4.4.1. Descriptive Statistics

The statistical summary of results pertaining to the distribution of acute toxicity tests on *D. magna* and chemical analyses conducted on the tested effluent are presented in Table 4.1. The results for pH, As, Cu, Fe, Ni, Pb, and Zn were compared to the standards and criteria set out in the MDMER (Minister of Justice, 2025), the Quebec directive D019 (MDDEP, 2012; MELCCFP, 2025), the intervention guide for soil protection and remediation from *the Ministère de l'Environnement et de la Lutte contre les changements climatiques* (MELCC) (Beaulieu, 2021). Overall, the measured parameters tend to comply with the threshold values set by both the D019 directive and the MDMER, particularly for Cu, Zn, Pb and Ni, which generally remain below the federal and provincial limits. However, when compared with the more stringent MELCC's intervention guide for soil protection and remediation reference values, several exceedances become apparent, notably for Cd, Ni. In addition, pH occasionally surpasses the upper limit of 9.5 allowed by both the D019 and the MDMER, while bioassays with D. magna indicate episodes of acute toxicity reaching 16

TU, far above the regulatory threshold of 1 TU set by D019. Therefore, although the effluent can be generally compliant with federal and provincial standards in terms of contaminant concentrations, it still presents a biological toxicity risk that is not captured by chemical compliance alone.

Table 4.1 Summary statistics for the chemical composition of the effluent ($n = 127$)

Parameters	LOD	Min	Q1	Med	Mean	Q3	Max	D019	MD MER	MELCC
pH	-	7.26	7.85	8.30	8.23	8.60	9.80	6 to 9.5		6 to 9
Alkalinity (mg/L $CaCO_3$)	2	24	42	48	47	52	79	-	-	-
Conductivity (µS/cm)	1	304	499	534	542	586	743	-	-	-
Hardness (mg/L $CaCO_3$)	1	125	179	194	198	216	287	-	-	-
Temperature (°C)	-	0.8	1.2	10.0	10.2	18.6	24.0	-	-	-
Toxicity (TU)	1	0.0	0.0	0.0	2.3	3.0	16.0	1	-	-
Al (mg/L)	5×10^{-4}	0.03	0.08	0.12	0.15	0.17	0.75	-	-	2
As (mg/L)	2×10^{-4}	9×10^{-4}	2.0×10^{-3}	2.5×10^{-3}	3.0×10^{-3}	3.3×10^{-3}	3.0×10^{-2}	0.4 (0.6)	0.6	0.34
Ca (mg/L)	0.5	32	57	62	63	69	98	-	-	-
Cd (mg/L)	2×10^{-4}	3×10^{-4}	1.3×10^{-3}	1.9×10^{-3}	2.2×10^{-3}	3.1×10^{-3}	8.2×10^{-3}	-	-	1.1×10^{-3}
Co (mg/L)	5×10^{-4}	<LOD	<LOD	2×10^{-3}	3×10^{-3}	4.7×10^{-3}	0.01	-	-	0.37
Cr (mg/L)	2×10^{-4}	0.00	2×10^{-4}	2×10^{-4}	3×10^{-4}	2×10^{-4}	0.02	-	-	Cr(III): 1 Cr(VI): 16×10^{-3}
Cu (mg/L)	5×10^{-4}	0.04	0.08	0.13	0.14	0.16	0.44	0.6 (0.2)	0.6	7.3×10^{-3}

Table 4.1 Summary statistics for the chemical composition of the effluent $(n = 127)$ (suite)

Parameters	**LOD**	**Min**	**Q1**	**Med**	**Mean**	**Q3**	**Max**	**D019**	**MDMER**	**MELCC**
K (mg/L)	0.02	1.2	2.2	2.4	2.4	2.6	3.4	-	-	-
Mg (mg/L)	0.02	4.3	8.4	9.4	9.4	10.3	15.1	-	-	
Mn (mg/L)	2×10^{-4}	0.01	0.04	0.06	0.09	0.11	0.57	-	-	2.3
Na (mg/L)	0.02	2.3	24.0	26.5	26.7	30.5	41.8	-	-	-
NH_3 (mg N/L)	0.01	<LOD	0.02	0.06	0.16	0.22	0.05	-	-	-
Ni (mg/L)	2×10^{-4}	5×10^{-3}	0.010	0.015	0.020	0.020	0.050	1(0.5)	1	0.26×10^{-3}
NO_3 (mg N/L)	0.01	<LOD	0.03	0.11	0.15	0.21	0.58	-	-	300
P (mg/L)	0.01	<LOD	0.01	0.03	0.04	0.05	0.48	-	-	1
Pb (mg/L)	2×10^{-4}	<LOD	8×10^{-4}	1.2×10^{-3}	1.5×10^{-3}	2.3×10^{-3}	0.038	0.4 (0.6)	0.2	34×10^{-3}
SO_4 (mg/L)	0.6	93	126	142	147	168	225	-	-	-
Zn (mg/L)	5×10^{-3}	0.03	0.08	0.12	0.15	0.18	0.78	1(0.8)	1	67×10^{-3}

LOD: Limits of detection, Min: Minimum, Q1: 1st Quartile, Med: Median, Q3: 3rd Quartile, Max: Maximum, [1]D019: Directive 019 sur l'industrie minière : the bold values are those from the 2012 version of the D019, which was applicable at the time of monitoring. The values in italics in parentheses are from the 2025 version of the D019 and were updated after the monitoring period referred to here. MDMER: Metal and Diamond Mining Effluent Regulations (2025), MELCC: Ministère de l'Environnement et de la Lutte contre les changements climatiques du Québec (Beaulieu, 2021).

Seasonal differences in effluent toxicity and dissolved metal concentration were found to be statistically significant. The data suggest increased toxicity during spring and fall and reduced toxicity during summer (Figures A1 and A2, Table A1). Seasonal variations in Fe, Zn, Al, Cu, Ni concentrations (which are statistically significative; $p < 0.05$) reveal higher values in the spring, winter and fall seasons, and lower values during the summer. These trends are consistent with previous findings reporting elevated metal concentrations during periods of enhanced runoff and erosion (Comber et al., 2019; Tokatlı et al., 2024; Karadeniz et al., 2024). In contrast, higher temperatures, reduced runoff, increased oxygenation, and generally higher pH during the summer may promote the precipitation and complexation of metals (e.g. as metal hydroxides or carbonates), thereby reducing their bioavailability and toxicity (Comber et al., 2020). Increased biological activity during the summer may also enhance the alteration or adsorption of certain contaminants (Milazzo et al., 2014). Such processes could explain the reduced toxicity observed in the summer.

4.4.2. Multiple linear regression analyses

The findings of the multiple linear regression analysis (see Table A2) indicated that the model fitted the data well ($F = 3.42$, $p > 0.001$), explaining 70% of the variation in toxicity ($R^2 = 0.70$). Of the measured variables, Cd, Cu and Fe were significant predictors ($p > 0.05$). Cd and Cu exhibited positive relationships with toxicity, whereas Fe showed a negative relationship, suggesting that higher iron concentrations may reduce the toxic effects. Other trace elements (Al, As, Ni, Pb and Zn) did not appear to contribute significantly ($p > 0.05$). These results support hypothesis H1, which confirmed a strong statistical relationship between dissolved metal(loid) concentrations and acute toxicity observations. The significant effects of Cd and Cu are consistent with their known toxicity to D. magna, which has been attributed to their affinity for biological ligands, disturbance of ionic regulation, and interference with enzyme activity (Grosell et al., 2007). Significant toxic effects were previously observed in *D. magna* exposed simultaneously to Cu and Cd, though individual concentrations were below their respective LC_{50} values (e.g., Cu: ~162 µg/L; Cd: ~166 µg/L; Lari et al., 2017). These mixtures often act through additive or synergistic interactions, enhancing toxicity beyond single-metal exposures. Reported mechanisms include increased oxidative stress, reduced antioxidant defences, and altered metal bioavailability, which together explain the stronger impacts of Cu-Cd mixtures (Traudt et al., 2017; Meyer et al., 2015).

The role of Fe in modulating effluent toxicity is multifactorial. Iron toxicity depends on its speciation and on the alkalinity of the effluent. Under anoxic conditions, Fe^{2+} was shown to entail toxicity in mining effluents as it remained in solution even at concentrations >500 mg/L (Neculita et al., 2008). The precipitation of Fe hydroxides may further cause a decrease in pH which can entail toxicity (Neculita et al., 2008). Nevertheless, when present in complex mixtures, Fe may attenuate the overall toxicity by altering the speciation and bioavailability of more toxic metals (Cardwell et al., 2023). This attenuation results from both direct chemical mechanisms and indirect physicochemical effects. On the one hand, dissolved Fe^{3+} and its precipitated forms as amorphous ferric hydroxides (often simplified as $Fe(OH)_3$) shows a strong affinity for metal cations such as Cu^{2+}, Cd^{2+} and Zn^{2+}. Iron hydroxides present as colloids or suspended solids can further adsorb free metal ions on their surface, thereby reducing the concentration of the free ionic forms which are the most bioavailable and toxic for aquatic organisms (Tipping et al., 2002; Cardwell et al., 2023; Gatcha-Bandjun et al., 2017). Fe can also indirectly modulate pH and redox conditions. For instance, redox reactions involving Fe^{2+} and Fe^{3+} may influence the redox potential, which in turn affects the speciation of redox-sensitive elements such as arsenic and selenium (Liu et al., 2013). Such observations suggest that Fe in industrial effluents may influence toxicity through its influence on the speciation and sorption of co-occurring metals. Based on the results discussed above, the regression equation for toxicity is as follows:

$$\mathrm{Toxicity} = -2.34 + 1627\ \mathrm{Cd} + 14\ \mathrm{Cu} - 5\ \mathrm{Fe} \tag{3}$$

4.4.3. Metal(loid)s concentration and speciation in toxic vs non-toxic effluents

4.4.3.1. Total concentrations

The distribution of metal(loid)s concentrations in the effluent is shown in Figure 4.1. The median concentrations reveal the following trend: $\mathrm{Fe} > \mathrm{Zn} > \mathrm{Al} > \mathrm{Cu} > \mathrm{Ni} > \mathrm{As} > \mathrm{Cd} > \mathrm{Pb}$. A comparison was made between the concentrations of these elements between two subgroups, one characterised by toxicity levels greater than 1 (toxic; $\mathrm{n} = 58$) and the other where toxicity levels are less than 1 (non-toxic; $\mathrm{n} = 67$). The data suggest that there were differences in trace metallic element concentrations between the two groups, with the interquartile range of Zn, Cu, Ni, and Cd concentrations being higher for the toxic subgroup, not overlapping with the interquartile range of concentrations in non-toxic samples.

In toxic samples, Cd concentrations ranged from 0.0003 to 0.0082 mg/L, with many values above the EC_{50} range of 0.002–0.008 mg/L commonly reported for *D. magna* (USEPA, 2001; OECD, 2004; De Schamphelaere & Janssen, 2004). Cu concentrations were found to be generally higher than acute EC_{50}, usually ranging from 0.03 to 0.08 mg/L under medium-hardness water conditions (80-100 mg/L $CaCO_3$) (USEPA 2007). Similarly, Zn was found to exceed the EC_{50} range of 0.07 to 0.3 mg/L in several samples, with 0.78 mg/L being the greatest concentration registered (USEPA, 2005).

This observation suggests a potential causal relationship between effluent toxicity and the concentrations of Zn, Cu, Ni, and Cd. This conclusion is consistent previously reported findings highlighting that some metals (e.g., Zn, Cu, Ni, Cd), although present at concentrations below acute toxicity thresholds, can generate significant ecotoxicological risks when present simultaneously in sediments (Okoro et al., 2024). The findings of this last study show that the cumulative risk, particularly for Ni, can exceed permissible ecotoxicological, despite apparent compliance with environmental standards. The same authors calculated cumulative risk indices (Hazard Index, HI) showed that the long-term risk exceeded reference values, particularly for Ni, due to chronic exposure or gradual bioaccumulation of these metals in aquatic organisms.

Unlike observations relating to Zn, Cu, Ni, and Cd, the interquartile ranges of Fe and Pb concentrations overlap between the toxic and non-toxic subgroups. In the case of As, the median concentration in the toxic subgroup was lower than that in the non-toxic subgroup. A comparison was made between the toxic and non-toxic groups to identify differences in the average concentrations of trace metallic elements. Statistical analyses involved F statistics and $p-$values with a significance level of 0.05. The findings suggest that the $p-$values for Fe, Cu, and Cd were $p < 0.05$, suggesting statistically significant differences between the average concentrations in the toxic vs non-toxic effluents (Table A3). This observation lends support to the validity of hypothesis 2 (H2), which postulated that significant statistical differences would be observed between the toxic and non-toxic subgroups.

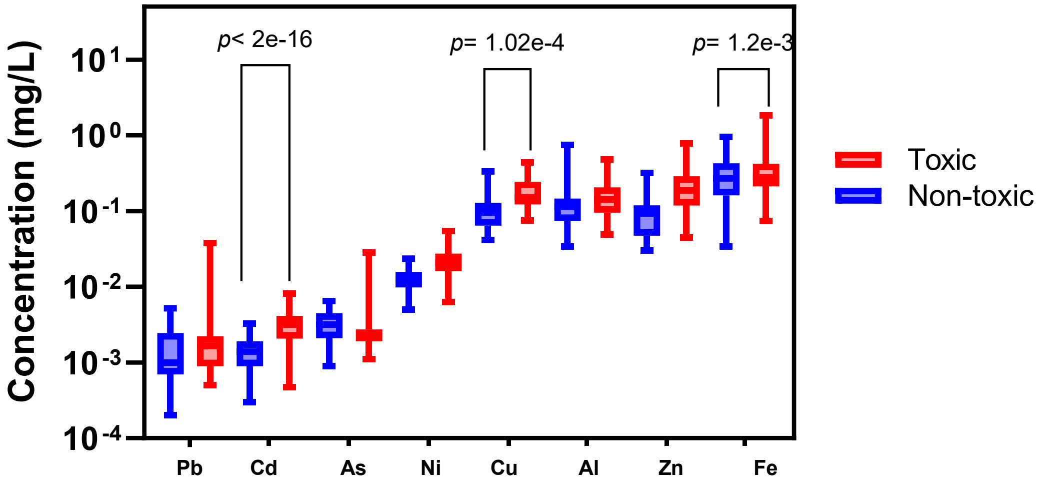


Figure 4.1 Comparison of metallic elements levels in final effluent: Toxic vs. Non-toxic group. The rectangle of the whisker boxes is representative of the interquartile range on either side of the median (horizontal line), with the vertical lines denoting the non-outlier range. The one-way ANOVA statistical results are shown for *p*-values < 0.05.

4.4.3.1. Speciation calculations

The speciation of Fe, Pb, Ni, Zn, Al, Cu, Cd and *As* in the non-toxic and toxic effluents was further estimated using calculations carried out in PHREEQC with the *minteq.v4* database. To complement the analysis of total metal concentrations presented earlier, a second ANOVA was performed focusing specifically on the free ionic forms of the trace metallic elements, which represent the bioavailable fractions most relevant to ecotoxicological effects. The statistical results (Figure 4.2; Table A4) reveal that Zn^{2+}, Cu^{2+}, Cd^{2+}, Fe^{2+}, and Al^{3+} exhibited highly significant differences in average concentrations between toxic and non-toxic groups ($p < 0.05$). These findings reinforce and refine the results from the total concentration analysis, highlighting that the observed toxicity could be associated with the bioavailable ionic species of these metals. The additional ANOVA on ionic forms provides further statistical support for hypothesis 2 (H2), confirming that significant differences exist between toxic and non-toxic subgroups not only in total concentrations, but also in the most ecotoxicologically relevant chemical forms.

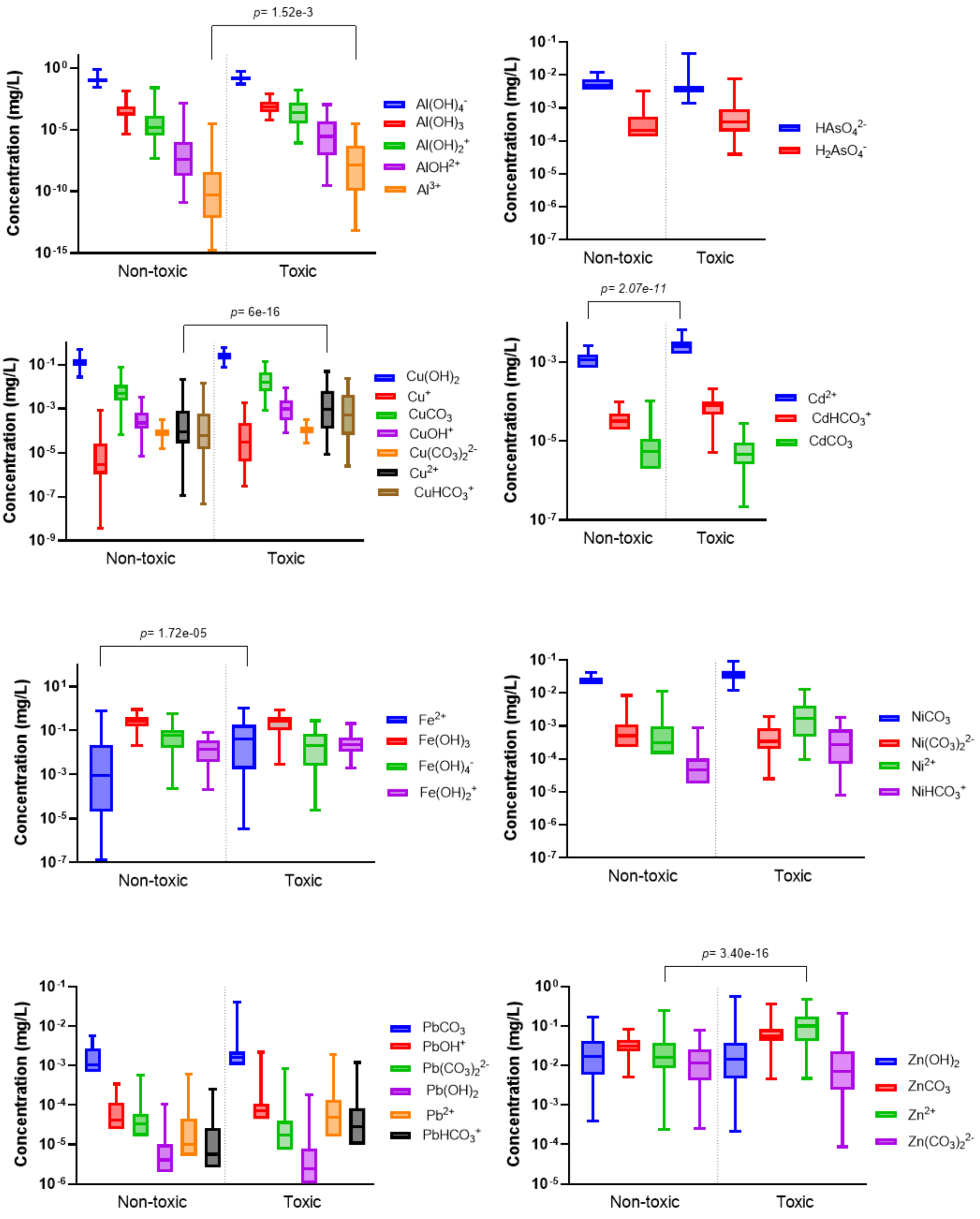


Figure 4.2 Speciation of metal(loid)s in the non-toxic (n=68) and toxic (n=59) groups of samples. The rectangles of the whisker boxes represent the interquartile range on either side of the median (horizontal line), and the whiskers represent the non-outlier range.

The principal outcome of the speciation calculations pertains to the disparities observed in the free cationic forms of trace metals between toxic and non-toxic samples. The calculated Fe^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} concentrations are generally higher in toxic samples that in non-toxic samples. This evidence lends support to the hypothesis that the free bivalent forms of these cations, which are more bioavailable, exert an influence on the toxicity of the effluents under examination (Dhaouadi et al., 2021). Lethal Fe concentrations (LC_{50}) for *D. magna* generally vary between 0.5 and 3 mg/L (Okamoto et al., 2015; van Anholt et al., 2002). In other contexts, Fe may induce sublethal effects such as reproduction inhibition and reduced growth (Okamoto et al., 2015). However, in the present study, Fe appears to play an inhibitory role in overall toxicity, highlighting the complexity of its interactions with other metals. Zn^{2+} concentrations, which varied between 0.01 mg/L and 0.1 mg/L in the effluent samples, are of particular concern because this ionic species is the most bioavailable of the dissolved Zn species, and potentially the most toxic to aquatic organisms. Previous studies showed that lethal concentrations (LC_{50}) for *D. magna* range between 0.1 and 0.7 mg/L (Li et al., 2019; Okamoto et al., 2015), suggesting that the observed concentrations can entail sublethal or even lethal effects. The concentrations of Cu^{2+} in toxic samples were found to range between 10^{-5} and 10^{-2} mg/L. Reported LC_{50}s for *D. magna* are as low as 0.02 mg/L (Okamoto et al., 2015), suggesting that Cu^{2+} is of concern in the effluent. The results of the calculations indicate that the discrepancies in the concentrations of the anionic forms of As are not particularly pronounced. The median values and interquartile ranges observed for the $HAsO_4^{2-}$ and $H_2AsO_4^-$ species were comparable between toxic and non-toxic effluents. In non-toxic samples, the concentrations of $HAsO_4^{2-}$ exhibited a high degree of consistency, clustering closely around a median value of 0.004 mg/L. In toxic samples, the non-outlier range of $HAsO_4^{2-}$ concentrations showed a greater dispersion around a median value of 0.003 mg/L. The non-outlier range of $H_2AsO_4^-$ concentrations also showed a greater dispersion in toxic samples (median value of 0.0003 mg/L) than in non-toxic samples (median value of 0.0002 mg/L). The hypothesis that arsenic may contribute to toxicity in some of the toxic samples cannot be ruled out, despite concentrations remaining below the reported lethal concentrations for *D. magna* (0.5-2 mg/L) (He et al., 2009). The lack of data on Cl^- concentrations could lead to an overestimation of the abundances metal(loid)s as free ions. Specifically, Cl^- has been observed to form complexes with Cu, Zn, and Cd, thereby reducing the free and bioavailable fraction of these elements (Di Toro et al., 2001). The exclusion of this variable from calculations performed using PHREEQC can lead to an overestimation of the abundances of free cationic forms (Cu^{2+}, Zn^{2+}, Cd^{2+}) that are known to be the most toxic to *D. magna* (Schamphelaere & Janssen, 2004). Scenario-based modelling, however, was conducted with PHREEQC to address this limitation by fixing Cl^{-} concentrations at 20, 38, 60, and 100 mg/L, thus covering the expected range occurring in the studied effluent. These simulations suggest that the omission of Cl^{-} does not significantly affect speciation calculations (Table A5, Table A6). The main species for Cu, Zn, and Cd remain free ions and their carbonate or hydroxide complexes, which are also the most toxic to *D. magna* (Schamphelaere & Janssen 2004). Furthermore, statistical analyses done on the speciation results across the various chloride concentration scenarios revealed no significant differences in metal species concentrations, further corroborating the robustness of the speciation conclusions even in the absence of measured Cl^{-} values.

The absence of DOM (and DOC) measurements also adds uncertainty to the speciation results. The only available data for the effluent under study pertain to 12 samples collected in 2024-2025 and suggest that DOC concentrations ranged between 2.2 and 6 mg/L, with a median of 3.2 mg/L. The free cationic forms (Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}), which are thought to be the most bioavailable and toxic to *D. magna*, could be overestimated if DOM is excluded from the calculations (Schamphelaere & Janssen, 2004). Visual MINTEQ was therefore used for scenario-based calculations. The DOC concentrations were set at 1, 2, 5, 10, and 20 mg/L. Speciation calculations were performed using the Visual MINTEQ 3.1 databases (comp_2008.vdb, thermo.vdb, type6.vdb, gaussian.vdb) and the NICA–Donnan model (Gustafsson, 2020), with an active DOM/DOC ratio of 1,65 and assuming the fulvic acid fraction represents 70% of DOM. Figure S3 presents the results of calculations across the different scenarios. The results suggest that Cu, Pb, Fe, and to a lesser extent Cd tend to bound to DOM. Zn and Ni were only marginally impacted, and As was unaffected. These observations are consistent with the previous studies that concluded that DOM strongly complexes Fe^{3+}, Cu^{2+}, and Pb^{2+}, while the binding of Zn^{2+}, Cd^{2+}, and Ni^{2+} is weaker and more sensitive to competition with major cations such as Ca^{2+} and Mg^{2+} (Perdue & Ritchie, 2003). Importantly, calculations suggest that metal complexation remains moderate (Figure A3) for DOC concentrations in the 2-6 mg/L range, suggesting that substantial fractions of Zn and Ni, and to a lesser extent Cu and Cd, still occur as free ions. Furthermore, although DOM was not included in the PHREEQC geochemical calculations, it was present in the raw effluents used for bioassays.

Thus, any protective effect of DOM on metal bioavailability is inherently reflected in the immobilization responses observed in *D. magna.*

4.4.3.2. Limitations associated with speciation calculations

The effluent studied in the present study is characterized by hardness values ranging from 180 to 280 mg/L (expressed as $CaCO_3$ equivalent), neutral to slightly alkaline pH values (between 7 and 9), and oxidizing conditions (pe ~6). Water hardness and $pe - pH$ conditions strongly influence the chemical speciation, bioavailability, and the toxicity of trace metals such as As, Fe, Cu, Zn, Pb, Ni, Cd, and Al. For instance, acidic pH values tend to increase the solubility and bioavailability of metals in their toxic ionic forms (Cu^{2+}, Zn^{2+}), whereas alkaline pH promotes their precipitation as less toxic complexes (Goulet et al., 2015). Similarly, higher pe values can favor the presence of less toxic oxidized species, such as Fe^{3+} or $As(V)$, while more reducing conditions can increase toxicity by promoting more bioavailable forms such as Fe^{2+} or $As(III)$ (Liu et al., 2020). Water hardness acts as a protective factor since Ca^{2+} and Mg^{2+} ions reduce competition from toxic metals for sensitive biological binding sites (Goulet et al., 2015). The scope of the interpretations proposed in the present study is limited to effluents that are comparable in terms of hardness, pe and pH. Water samples intended for dissolved metal concentration analyses are typically filtered and acidified in the field, thereby ensuring the preservation of the sample. Oxidation-reduction potential and pH measurements are generally conducted using a multiparameter field probe, enabling the estimation of *in situ* conditions. However, toxicity analyses are performed up to 48 h after sampling. The $pe - pH$ conditions of the samples, and the speciation of trace metals at the time of toxicity testing, are likely to be significantly different from the actual field conditions. This complicates the use of speciation calculations to elucidate the causes of toxicity. Moreover, DOM can form stable complexes with certain metals, reducing their bioavailability, although these complexes may sometimes decompose, releasing metals into more toxic dissolved forms (Li & Gong, 2021). The dataset employed in this study is constrained in its scope, as it does not encompass information pertaining to DOM concentrations. Some mechanisms that may influence toxicity are implicitly excluded.

4.4.4. Correlation analyses

Pearson correlation analyses were conducted on non-toxic and toxic samples to ascertain potential discrepancies in the co-occurrence of metal(loid)s in toxic versus non-toxic effluents. In the case of non-toxic samples (Figure 4.3A), positive correlations were observed between $\mathrm{Cd} - \mathrm{Ni}$ ($\mathrm{r} = 0.67$), and $\mathrm{Cd} - \mathrm{Zn}$ ($\mathrm{r} = 0.52$). It is likely that the concentrations of these elements did not reach a point where cumulative action caused toxicity. Previous studies showed that these metals can interact synergistically (Lari et al., 2017). Zn alone could cause severe malformations of embryonic development in sea urchins, and these effects were amplified in the presence of other metals, notably cadmium. These interactions resulted in morphological abnormalities, even at concentrations below those generally considered toxic (Kobayashi & Okamura, 2005). Furthermore, $\mathrm{Cd} - \mathrm{Ni}$ interactions observed in aquatic plants such as *Typha capensis*, a typical aquatic plant in contaminated environments, suggest co-absorption facilitated by shared physicochemical characteristics, potentially amplifying bioaccumulation and toxicity (Zaranyika & Nyati, 2017). Still in the non-toxic effluent group, negative correlations were observed between $\mathrm{As} - \mathrm{Zn}$ ($\mathrm{r} = -0.54$), $\mathrm{As} - \mathrm{Cd}$ ($\mathrm{r} = -0.55$), $\mathrm{Pb} - \mathrm{Zn}$ ($\mathrm{r} = -0.51$), $\mathrm{Pb} - \mathrm{Cd}$ ($\mathrm{r} = -0.41$) and $\mathrm{Pb} - \mathrm{Ni}$ ($\mathrm{r} = -0.60$). Such negative correlations between some of the metal(loid)s suggest that their toxic effects could not add up, and hence the effluent remained nontoxic. The calculations pertaining to toxic effluents (Figure 4.3B) showed significant discrepancies when compared to those of non-toxic effluents (Figure 4-3A). The toxic effluents show stronger correlations between $\mathrm{As} - \mathrm{Pb}$ ($\mathrm{r} = 0.40$), $\mathrm{Fe} - \mathrm{Al}$ ($\mathrm{r} = 0.48$), $\mathrm{Fe} - \mathrm{Pb}$ ($\mathrm{r} = 0.32$), and $\mathrm{Ni} - \mathrm{Zn}$ ($\mathrm{r} = 0.34$), suggesting the propensity for these trace metallic elements to be present together in toxic effluents. This co-occurrence may result from common geochemical sources, such as the co-release of As and Pb from sulfide ores, or from similar mobilization processes, such as acid dissolution or adsorption on metal oxides (Alarifi et al., 2023). From a toxicological perspective, these associations may amplify toxic effects through different mechanisms. The $\mathrm{As} - \mathrm{Pb}$ interaction, for example, is known to induce significant oxidative stress, as both metals are known to generate or facilitate the formation of reactive oxygen species (ROS), inhibiting cellular antioxidant systems. In addition, $\mathrm{Ni} - \mathrm{Zn}$ interactions may involve competition for membrane transporters, modulating their bioavailability and toxicity (Wu et al., 2016). Moreover, the $\mathrm{Fe} - \mathrm{Al}$ couple can influence the mobility of other metals via coprecipitation or adsorption phenomena, sometimes reducing their apparent toxicity while contributing to potentially reactive colloidal forms (Bao et al., 2021).

Changes in correlations for non-toxic vs toxic subgroups may allow for deciphering how the interactions between metal(loid)s influence toxicity (Figure 4.4A-F). One example is the change in correlation observed for $\mathrm{As}-\mathrm{Cu}$, with a positive correlation in the non-toxic subgroup turned negative for the toxic subgroup ($\mathrm{r}=0.094 \rightarrow \mathrm{r}=-0.25$). This may indicate that $\mathrm{As}-\mathrm{Cu}$ have antagonistic effects, and that toxicity is reduced when they co-vary and increased when only one of the elements shows higher concentrations. Such responses may involve competitive mechanisms between metals or changes in the metabolic pathways of living organisms (Juśkiewicz & Gierszewski, 2022; Niampradit et al., 2024). For instance, a positive $\mathrm{As}-\mathrm{Cu}$ correlation in non-toxic samples may be reversed in toxic environments due to competitive interactions, whereby the presence of one element inhibits the uptake or accumulation of the other. An alternative hypothesis is that living organisms can alter their metabolic activities to accommodate high levels of one element, thereby reducing the bioavailability or accumulation of other elements (Juśkiewicz & Gierszewski, 2022; Niampradit et al., 2024). All correlations involving Fe exhibited a marked divergence between non-toxic and toxic effluents (Table 4.2), suggesting that Fe entails a significant impact on toxicity in the monitored effluent. The presence of Fe in the effluent could contribute to the inhibition of the toxicity of elements such as Cd (Tipping et al., 2002). The data also suggest that several changes in the correlations between non-toxic and toxic effluents correspond to types C and E (Figure 4.4C; Table 4.2). The C-type changes involve a negative correlation between elements that is more pronounced in toxic than in non-toxic samples. The pairs of elements corresponding to C-type changes in Figure 4.4 ($\mathrm{Cd}-\mathrm{Fe}$, $\mathrm{As}-\mathrm{Ni}$, $\mathrm{Fe}-\mathrm{Ni}$, $\mathrm{Cu}-\mathrm{Pb}$, $\mathrm{Al}-\mathrm{Zn}$, $\mathrm{Fe}-\mathrm{Zn}$) are likely to have antagonistic effects on toxicity. E-type changes are also common ($\mathrm{Al}-\mathrm{Fe}$, $\mathrm{As}-\mathrm{Pb}$, $\mathrm{Fe}-\mathrm{Pb}$, $\mathrm{Ni}-\mathrm{Zn}$). These changes imply that the positive correlation between two elements is more pronounced in toxic than in non-toxic effluents. Pairs of elements showing E-type changes (Figure 4.4E) may have additive or synergistic effects on toxicity. The $\mathrm{Cu}-\mathrm{Cd}$ couple shows a D-type (Figure 4.4D) change between toxic vs non-toxic samples. Such a change suggests that Cd and Cu could have synergistic or additive effects on toxicity. Studies have clearly shown that Cu and Cd mixtures cause greater toxic effects in *D. magna.* Traudt et al. (2017) found that exposure to these metals together increases oxidative stress and lowers antioxidant defences, leading to higher mortality than exposures alone. Meyer et al. (2015) verified that Cu–Cd actions can be additive or synergistic depending on the environment and quantity ratios, leading to greater toxicity than predicted by simple models. Furthermore, Lari et al. (2017) highlighted that these mixtures can change the availability of metals and produce non-additive effects, with direct implications for assessing water quality. These observations confirm that this Cu–Cd group is an important point in toxicity, as seen with our statistical figures. Overall, consistent with the findings from section 4.4.4, the results tend to support the validity of hypothesis H2, which postulated that statistically significant geochemical differences would be observed between the non-toxic vs toxic subgroups.

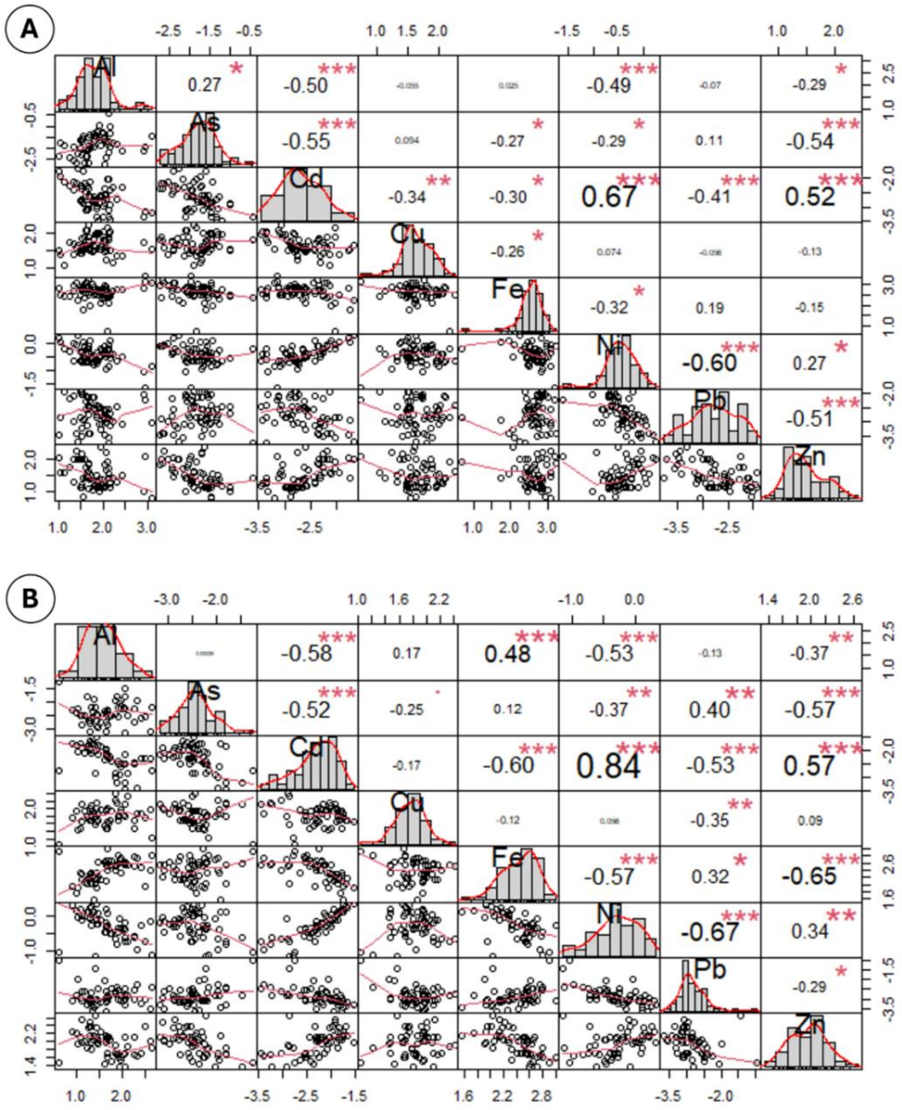
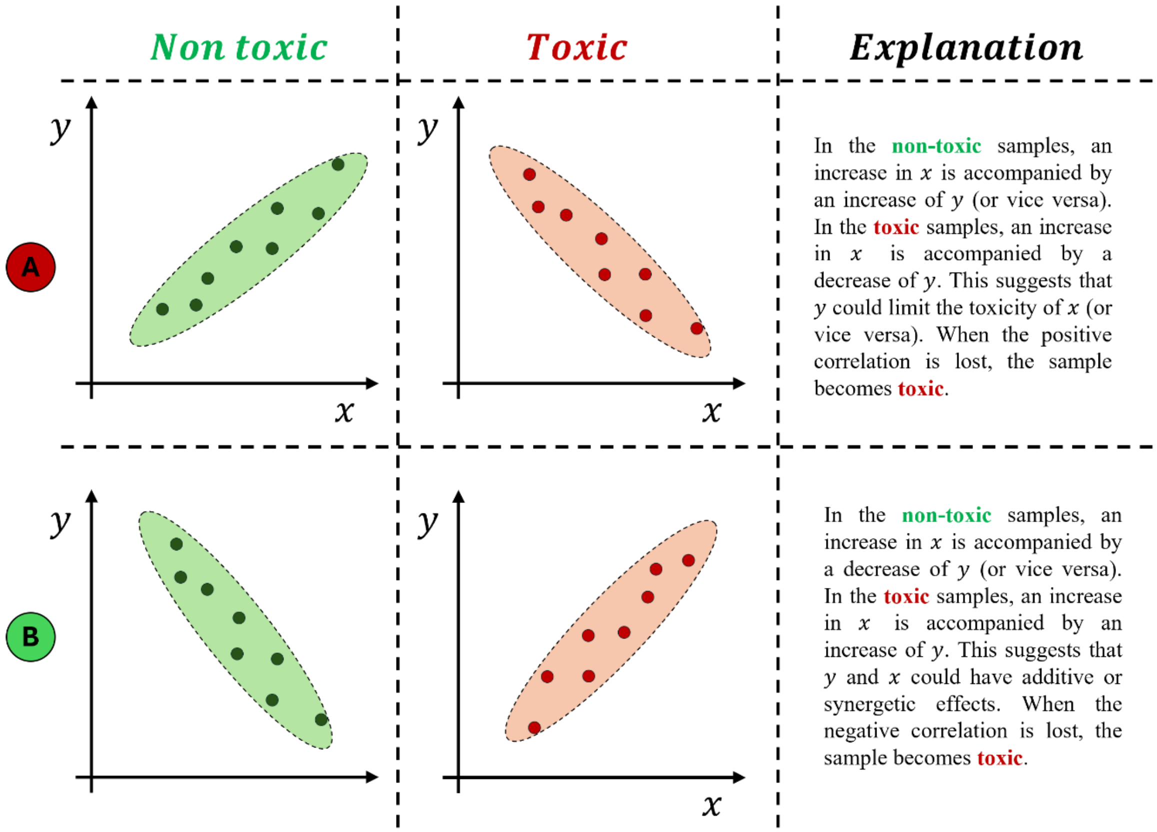


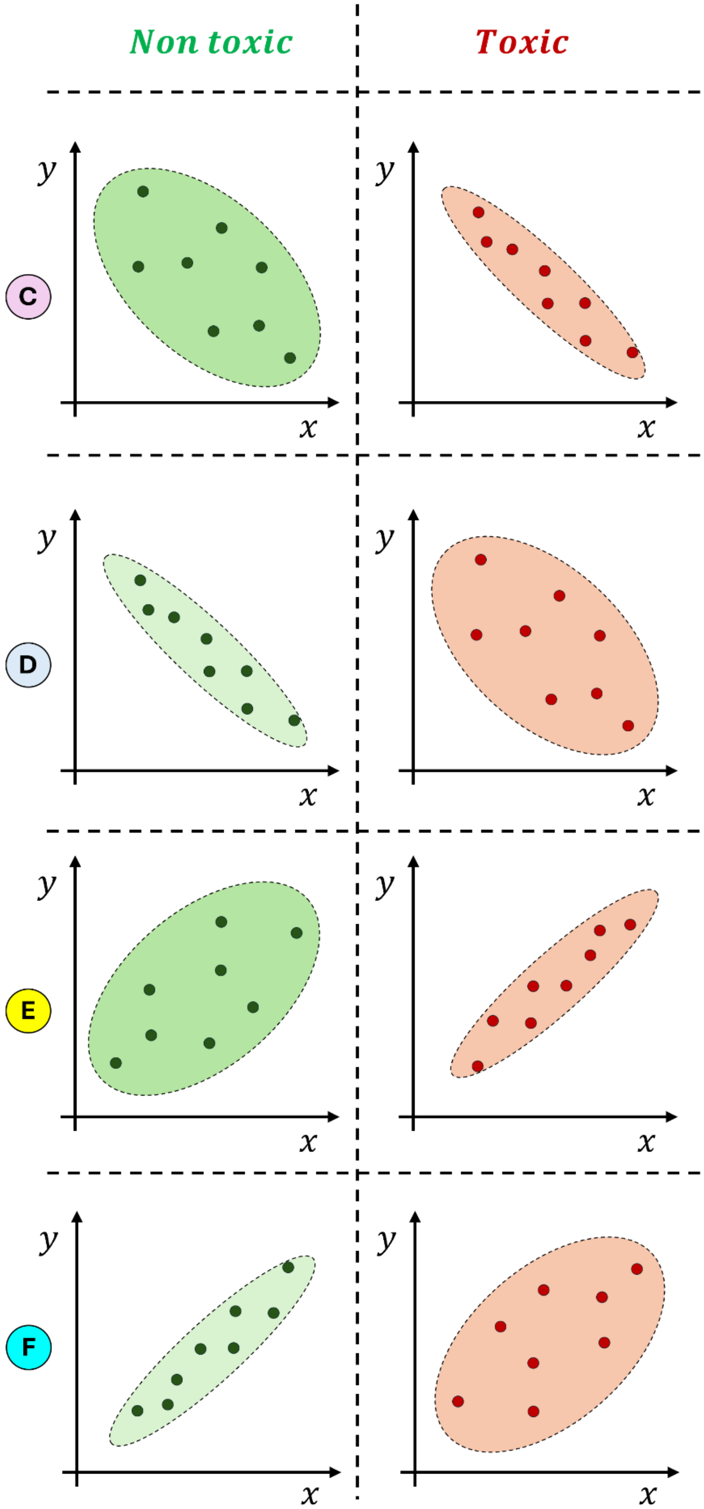
Figure 4.3 Correlation coefficient matrix for metallic elements in the non-toxic group (a) vs. toxic group (b). Histograms and red density curves on the diagonal show the distribution of each element.The upper panels display Pearson correlation coefficients with significance levels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The lower panels show scatterplots with red trend lines indicating pairwise relationships.



In the **non-toxic** samples, an increase in x is accompanied by an increase of y (or vice versa). In the **toxic** samples, an increase in x is accompanied by a decrease of y. This suggests that y could limit the toxicity of x (or vice versa). When the positive correlation is lost, the sample becomes **toxic**.

In the **non-toxic** samples, an increase in x is accompanied by a decrease of y (or vice versa). In the **toxic** samples, an increase in x is accompanied by an increase of y. This suggests that y and x could have additive or synergetic effects. When the negative correlation is lost, the sample becomes **toxic**.

Figure 4.4 A conceptual model for interpreting correlation inversions in non-toxic vs toxic effluents



Explanation

In the non-toxic samples, an increase in x is accompanied only by a weak decrease of y (or vice versa). In the toxic samples, an increase in x is accompanied by a significant decrease of y. This suggests that y could limit the toxicity of x (or vice versa). When $x - y$ become more negatively correlated, the sample becomes more toxic.

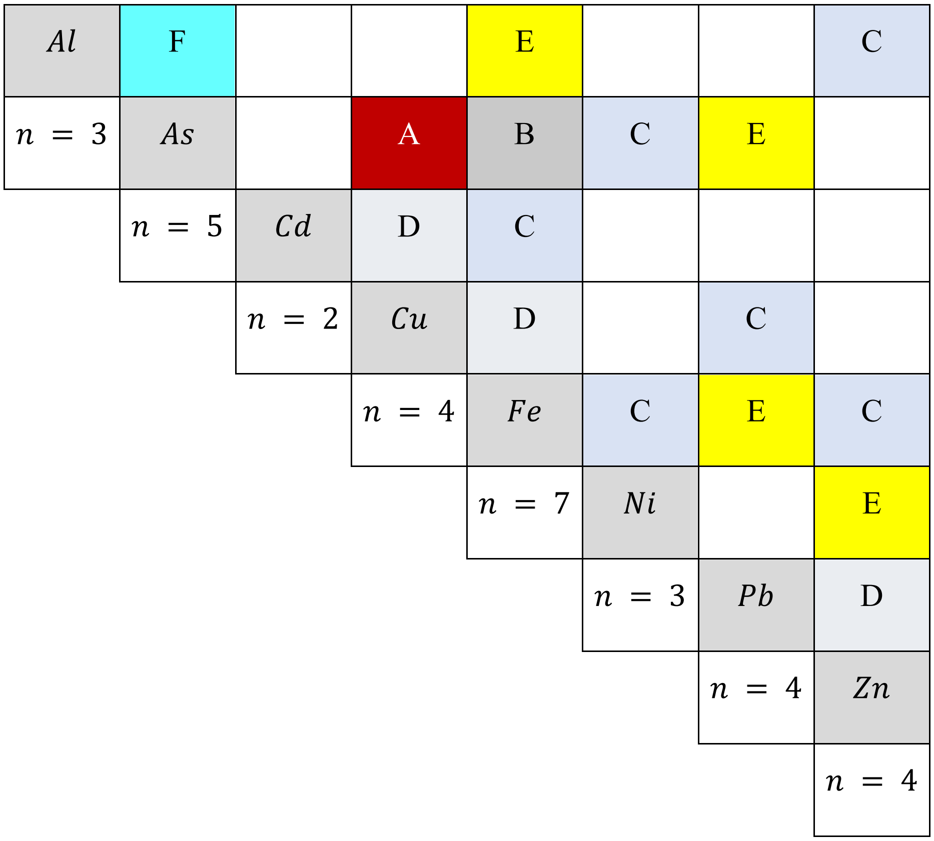
In the non-toxic samples, an increase in x is accompanied by a significant decrease of y (or vice versa). In the toxic samples, an increase in x is accompanied only by a weak decrease of y. This suggests that y and x could entail additive or synergetic effects on toxicity. When $x - y$ become less negatively correlated, the samples become more toxic.

In the non-toxic samples, an increase in x is accompanied only by a weak increase of y (or vice versa). In the toxic samples, an increase in x is accompanied by a significant increase of y. This suggests that y and x could entail additive or synergetic effects on toxicity. When $x - y$ become more positively correlated, the samples become more toxic.

In the non-toxic samples, an increase in x is accompanied by a significant increase of y (or vice versa). In the toxic samples, an increase in x is accompanied only by a weak increase of y. This suggests that y and x could entail antagonistic effects on toxicity. When $x - y$ become less positively correlated, the samples become more toxic.

Figure 4.4 (continued) A conceptual model for interpreting the strength of correlations in non-toxic vs toxic effluents

Table 4.2 Summary of the changes observed in correlations between non-toxic and toxic effluents



Al	F			E			C
$n = 3$	*As*		A	B	C	E	
	$n = 5$	*Cd*	D	C			
		$n = 2$	*Cu*	D		C	
			$n = 4$	*Fe*	C	E	C
				$n = 7$	*Ni*		E
					$n = 3$	*Pb*	D
						$n = 4$	*Zn*
							$n = 4$

The letters in the correlation matrix represent the type of change observed in the correlations between non-toxic vs toxic effluents. The letters and colour codes correspond to those in Figure 4.4. The numbers (n) below the elements indicate how many correlation changes were observed for each element between non-toxic vs toxic samples.

4.4.5. Principal Component Analyses (PCA) and Hierarchical Cluster Analyses (HCA)

Principal component analyses (PCA) were conducted to ascertain the associations between metal(loid)s and elucidate their toxicity mechanisms. Figure 4.5A illustrates the biplot for the non-toxic samples, while Figure 4.5B depicts the biplot for the toxic samples. The first two axes of the PCA express 58% and 67% and of the total variance in the non-toxic and toxic datasets, respectively. The variability explained by the two axes is highly significant. No significant differences were observed between the non-toxic and toxic effluents with respect to axis 1. In both cases, there was an association between Ni − Cd − Zn with positive values, and between As − Al and $\mathrm{Pb}-\mathrm{Fe}$ with negative values. The position of Cu was close to zero in both cases. This lack of differences between the toxic and non-toxic subgroups suggests that component 1 of the PCA is an ineffective means of identifying the causes of toxicity. The addition of axis 2 enables the identification of differences in potential groupings. Specifically, $\mathrm{Al}-\mathrm{Fe}$ and $\mathrm{As}-\mathrm{Pb}$ appear to be more closely associated for toxic effluents than for non-toxic effluents. The positioning of the samples in the space defined by axes 1 and 2 indicates that the non-toxic effluents are distributed across the entire area, whereas the toxic effluents exhibit a tendency to cluster in the $\mathrm{Zn}-\mathrm{Ni}-\mathrm{Cd}$ and $\mathrm{Fe}-\mathrm{Al}$ poles. This observation may be indicative of the greater influence of these metal associations in toxic effluents.

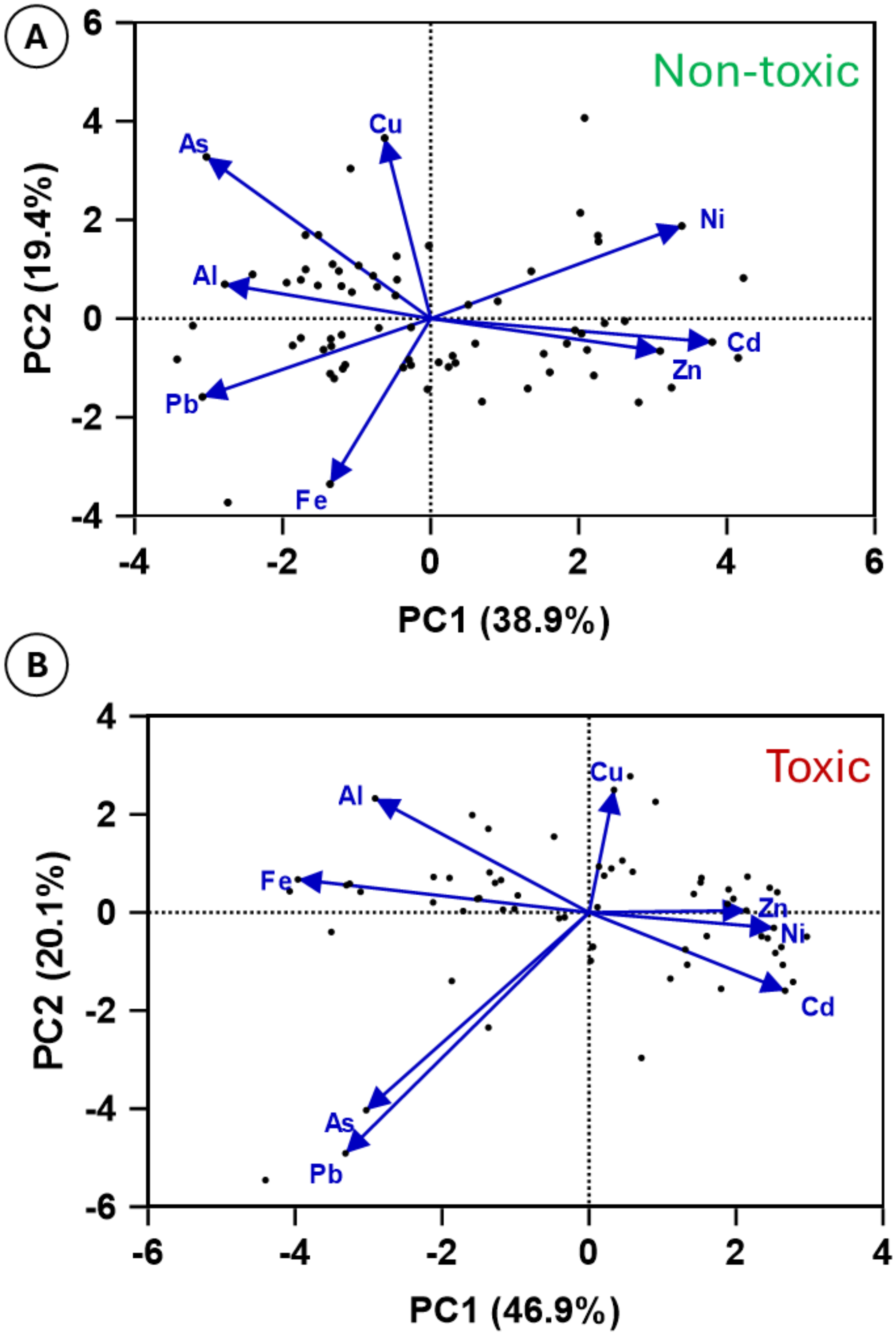


Figure 4.5 Varimax rotated principal component loadings of metallic elements in the final effluent for the non-toxic (a) and toxic (b) groups of samples

The HCA is used to cluster elements according to the Ward's cluster method and absolute correlation distance, utilising similarity as the criterion for grouping (Figure 4.6). The HCA results suggest that three main groups of elements are found in the non-toxic samples: (1) $\mathrm{Cd} - \mathrm{Ni} - \mathrm{Zn}$, (2) $\mathrm{Fe} - \mathrm{Pb}$, and (3) $\mathrm{Cu} - \mathrm{Al} - \mathrm{As}$. Three groups are also found in the toxic samples: (1) $\mathrm{Cd} - \mathrm{Ni} - \mathrm{Zn}$, (2) Cu, and (3) $\mathrm{Al} - \mathrm{Fe} - \mathrm{As} - \mathrm{Pb}$. The two dendrograms display a notable degree of similarity, particularly in their grouping of the $\mathrm{Cd} - \mathrm{Ni} - \mathrm{Zn}$ variables. However, there are also discrepancies in the configuration of the remaining clusters. These include the segregation of Cu and the formation of discrete groups for $\mathrm{Al} - \mathrm{Fe}$, and $\mathrm{As} - \mathrm{Pb}$ in the dendrogram for toxic samples.

The isolation of Cu in toxic samples suggests that it plays a significant role in the observed toxicity. Even at low concentrations, Cu can interfere with ionic regulation and cellular functions of aquatic organisms, thereby contributing to pronounced toxic effects (Mebane, 2023). The association of Cu with Al and As in non-toxic samples may be indicative of a moderating interaction. Indeed, some studies suggest that Al can form complexes with specific metal ions, thereby reducing their bioavailability and their potential toxicity (Zhang et al., 2022). This co-occurrence may explain why Cu and As appear to be less toxic in the presence of Al. In toxic samples, the association of As and Pb may indicate a synergistic interaction, whereby Pb increases the accessibility of As, resulting in an increase in toxicity. Recent research has demonstrated that Pb can influence cellular transportation pathways, thereby facilitating the entrance of other metals, such as As, into the *D. magna* cells, which increases their toxicity (Chen et al., 2023). Alterations in the correlation between Al and Fe in toxic and non-toxic samples may also influence toxicity. The association of Fe with other substances can indirectly affect the availability of toxic metals for bioaccumulation (Wu et al., 2016). The results presented here further suggest that Al could reduce the toxicity of Cu and As in non-toxic samples. Pb and As co-occurring in the toxic samples could be a factor contributing toward the observed toxicity. The findings of previous research conducted on zebra fish embryos suggest that the co-occurrence of As and Pb could result in a synergistic effect (Kiper & Freeman, 2022). While this has not yet been studied directly in *D. magna*, the possibility remains that Pb may potentiate As toxicity in other aquatic organisms through similar mechanisms.

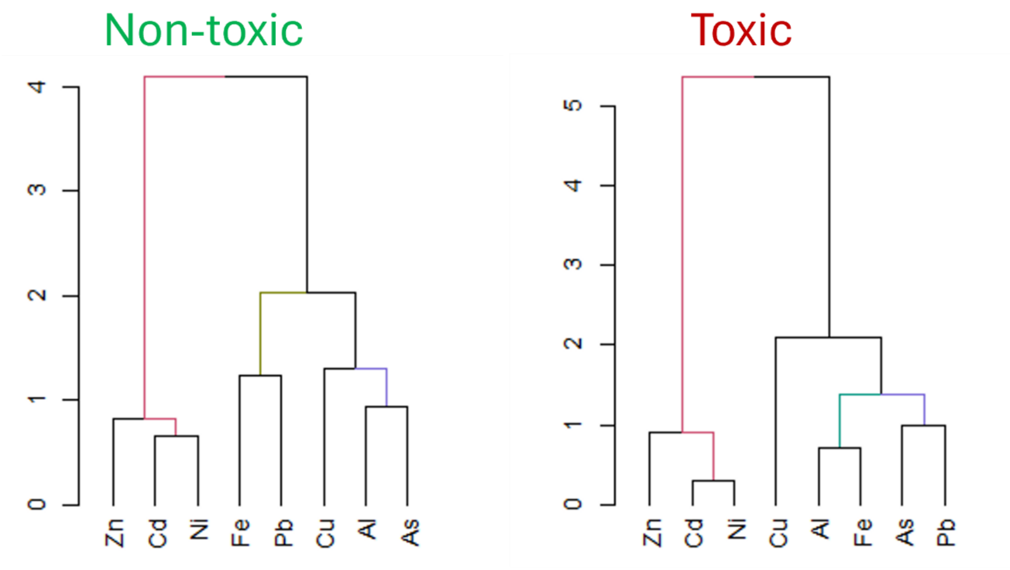


Figure 4.6 Dendrogram showing the relationships among metallic elements in non-toxic and toxic samples

4.4.6. A more holistic perspective on results

Table 4.3 presents a summary of the primary findings derived from the analyses conducted as part of this study for each of the targeted metal(loid)s. The results of the multiple regression analysis reveal a negative correlation between Fe and toxicity (Table 4.3 – column 2). Moreover, the concentration of Fe is higher in non-toxic samples than in toxic samples (Table 4.3 - column 3). As a result, all binary correlations involving Fe are distinct between the non-toxic and toxic samples (Table 4.3 - column 5). The elements that appear to be the most toxic are Cd and Cu. This is evidenced by the regression coefficients calculated in the multiple regression analyses (Table 4.3 - column 2). The calculations also suggest significant statistical differences in the concentrations of these elements in the toxic vs non-toxic subgroups (Table 4.3 - column 3). The correlation analyses revealed that these two elements show C-type changes in their correlations in non-toxic vs. toxic samples, suggesting an additive or synergistic effect (Table 4.3 - column 5). Geochemical calculations further suggest that the free ionic forms of these elements (Cd^{2+} and Cu^{2+}) are more concentrated in toxic than in non-toxic samples (Table 4.3 - column 4).

A conceptual model was developed to illustrate the most plausible causes of toxicity identified for the targeted effluent (Figure 4.7). The system is presented as a tilting platform with substances (squares and circles, representing dissolved species) that will balance on either side of the pivot point:

A. The more toxic a substance is, the more it is to the right of the pivot point. Substances that can reduce the toxicity of metal(loid)s, such as alkalinity, are to the left of the pivot point.
B. Changes in dissolved species concentrations or speciation induce alterations in the dimensions of the squares/circles that represent the substances, without causing a shift from left to right.
C. A substance that generates a synergistic or antagonistic effect acts like a ball that pushes a square to the left (antagonism) or to the right (synergy) on a curved part of the tilting platform.

In the system under consideration, Fe could be conceptualized as a stabilizing element, metaphorically represented as a rectangle positioned to the left of the pivot point (Figure 4.7). A higher concentration of iron appears to impart a degree of inertia to the system with respect to the toxicity imposed by other elements present in solution. This behavior has already been proven in previous studies, which reported that iron, especially ferric iron (Fe^{3+}), greatly decreases the trace metals' bioavailability and toxicity through various physicochemical mechanisms. Indeed, iron oxides and oxyhydroxides exhibit a strong affinity for metals such as Cu, Pb, Cd, or As, which they can immobilize via surface adsorption processes, simultaneous coprecipitation during their formation, or even by structural incorporation into the crystalline matrix (Shi et al., 2021; Zhang et al., 2023). The elements that primarily contribute to the weight on the right side of the pivot point, conceptualized as destabilizing forces, are Cu and Cd, two metals known for their high toxicity in aquatic organisms such as *D. magna*, particularly when present in a mixture. These elements appear to exert additive or synergistic effects, as revealed by previous studies (Norwood et al., 2003; Lari et al., 2017). These elements could be conceptualized as a pair occupying the right portion of the curved segment of the plateau, situated to the right of the pivot point (Figure 4.7). The synergy between Cu and Cd would mean that the circle is pushing the rectangle further downhill to the right, resulting in increased force on the plateau to the right of the pivot point, without any change in the total concentrations of the elements. When the forces to the right of the pivot point exceed those on the left, the plateau tilts and can reach the threshold corresponding to a toxicity of 1 TU (and beyond).

The methodological approach developed here, combining the use of statistical methods with a data set for monitoring a real effluent, represents a valuable complement to the approaches used in the laboratory to identify the causes of effluent toxicity. Laboratory approaches are characterized by enhanced precision, enabling the modulation of diverse contaminants' concentrations under controlled conditions. However, these approaches are limited in their ability to simultaneously assess many contaminants. A comprehensive assessment of all possible combinations of contaminant mixtures currently remains impractical due to the considerable combinatorial complexity involved (Kim et al., 2018). This limitation stems from the fact that, beyond binary and ternary mixtures, the number of solutions that must be tested in the laboratory for studying a cocktail of contaminants can become impractically high. Synthetic effluents studied in the laboratory do not always accurately represent the conditions encountered in the field, which complicates the transposition of laboratory observations to field conditions. Here, the analyses indicated the predominant influence of Fe, Cd, and Cu in the toxicity of the effluent under consideration. This finding is of relevance to the site operator, as it enables the optimization of efforts to reduce Cu and Cd concentrations, thereby mitigating the occurrence of toxicity episodes. In the context of an effluent consisting of a mixture of numerous contaminants, it is advantageous to target the specific contaminants that exert the greatest influence on toxicity. The approach proposed here facilitates the identification of priority targets. While the findings of this study cannot be readily extrapolated to other sites, the proposed approach has the potential for widespread implementation. The identification of statistical links between the concentrations of different contaminants and toxicity could facilitate a more precise determination of the causes of toxicity, particularly for effluents that meet current standards in terms of contaminants concentrations.

Table 4.3 Summary of the primary findings pertaining to the toxicity of the tested effluent

Trace metal(loid)s	**Regression coefficients (Section 4.4.2)**	**Data distribution (Section 4.4.1)**	**Speciation calculations (Section 4.4.3)**	**Changes in correlations between non-toxic and toxic subgroups (Section 4.4.4)**	**PCA & HCA (Section 4.4.5)**
Pb	194	$[\mathrm{Pb}]_{\mathrm{NT}} < [\mathrm{Pb}]_{\mathrm{T}}$	$[\mathrm{Pb}^{2+}]_{\mathrm{NT}} < [\mathrm{Pb}^{2+}]_{\mathrm{T}}$	$4\ (\mathrm{Fe_E} - \mathrm{Cu_C} - \mathrm{As_E} - \mathrm{Zn_D})$	Closer to As in toxic samples
Cd	1627	$[\mathrm{Cd}]_{\mathrm{NT}} < [\mathrm{Cd}]_{\mathrm{T}}$	$[\mathrm{Cd}^{2+}]_{\mathrm{NT}} < [\mathrm{Cd}^{2+}]_{\mathrm{T}}$	$2\ (\mathrm{Cu_D} - \mathrm{Fe_C})$	With Zn and Ni in all samples
As	218	$[\mathrm{As}]_{\mathrm{NT}} > [\mathrm{As}]_{\mathrm{T}}$	Greater variability in toxic samples	$5\ (\mathrm{Al_F} - \mathrm{Cu_A} - \mathrm{Fe_E} - \mathrm{Ni_C} - \mathrm{Pb_E})$	Closer to Pb in toxic samples
Ni	16.3	$[\mathrm{Ni}]_{\mathrm{NT}} < [\mathrm{Ni}]_{\mathrm{T}}$	$[\mathrm{Ni}^{2+}]_{\mathrm{NT}} < [\mathrm{Ni}^{2+}]_{\mathrm{T}}$	$4\ (\mathrm{Fe_C} - \mathrm{As_C} - \mathrm{Zn_E})$	With Cd and Zn in all samples
Cu	14.5	$[\mathrm{Cu}]_{\mathrm{NT}} < [\mathrm{Cu}]_{\mathrm{T}}$	$[\mathrm{Cu}^{2+}]_{\mathrm{NT}} < [\mathrm{Cu}^{2+}]_{\mathrm{T}}$	$4\ (\mathrm{Cd_D} - \mathrm{As_A} - \mathrm{Fe_D} - \mathrm{Pb_C})$	Isolated among toxic samples clusters
Al	− 0.495	$[\mathrm{Al}]_{\mathrm{NT}} < [\mathrm{Al}]_{\mathrm{T}}$	$[\mathrm{Al}^{3+}]_{\mathrm{NT}} < [\mathrm{Al}^{3+}]_{\mathrm{T}}$	$3\ (\mathrm{As_F} - \mathrm{Fe_E} - \mathrm{Zn_C})$	Closer to Fe in toxic samples
Zn	3.42	$[\mathrm{Zn}]_{\mathrm{NT}} < [\mathrm{Zn}]_{\mathrm{T}}$	$[\mathrm{Zn}^{2+}]_{\mathrm{NT}} < [\mathrm{Zn}^{2+}]_{\mathrm{T}}$	$4\ (\mathrm{Pb_D} - \mathrm{Ni_E} - \mathrm{Fe_C} - \mathrm{Al_C})$	With Cd and Ni in all samples
Fe	−5.01	$[\mathrm{Fe}]_{\mathrm{NT}} > [\mathrm{Fe}]_{\mathrm{T}}$	$[\mathrm{Fe}^{2+}]_{\mathrm{NT}} < [\mathrm{Fe}^{2+}]_{\mathrm{T}}$	$7\ (\mathrm{Cu_D} - \mathrm{Cd_C} - \mathrm{As_B} - \mathrm{Al_E} - \mathrm{Ni_C} - \mathrm{Pb_E} - \mathrm{Zn_C})$	Closer to Al in toxic samples

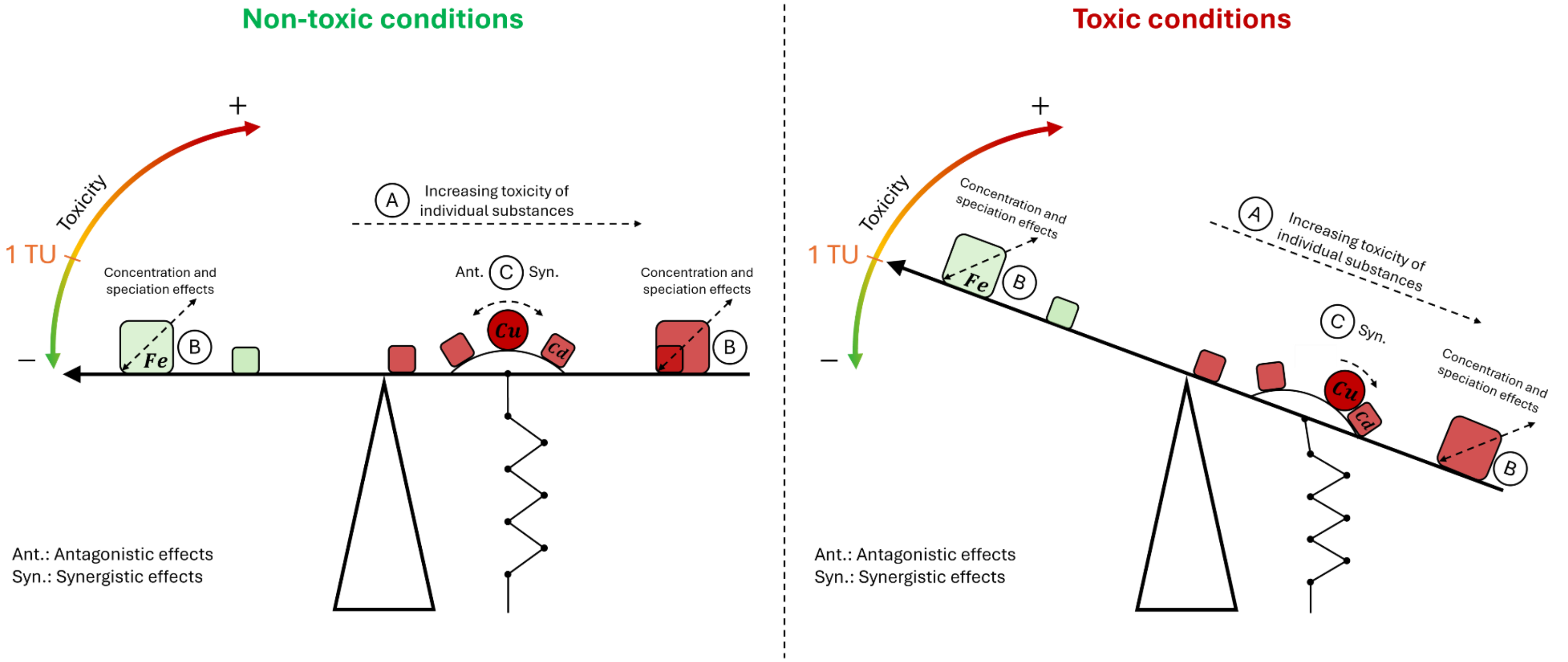


Figure 4.7 Conceptual model of toxicity causes for the studied effluents

4.5. Conclusion

This study attempted to characterize the complex interplay among the concurrent concentrations of metallic element, their speciation, and the toxicity of industrial effluents, with particular emphasis on the sporadic acute toxicity observed to *D. magna*. Several methods were used to identify the causes of effluent toxicity: multiple linear regression analyses, statistical comparisons, speciation calculations, correlation analyses, principal component analyses, and hierarchical cluster analyses. Plausible explanations for the toxicity episodes were identified by combining these methods. Concentrations of contaminants in the effluents under consideration were within the regulatory limits, but the findings show that physiochemical compliance does not guarantee the absence of toxicity. The results revealed that even at relatively low concentrations, certain metals can induce toxic effects, contingent on their speciation and interactions. The results lead to the conclusion that Cd and Cu are the main contaminants causing toxicity in the studied effluent, while Fe most likely indirectly limits the effluent toxicity. The analysis of changes in the correlations between metal(loid)s in toxic and non-toxic effluents is of particular interest. The results allowed to propose a conceptual model to support the joint interpretation of the results from different statistical methods. This conceptual model, which can be adapted to other contexts, could be a relevant tool for identifying additive, synergistic, or antagonistic effects between pairs of contaminants in an effluent. The results of speciation calculations also indicate significant differences in the concentrations of free metal ions in toxic and non-toxic effluents. This suggests that by taking speciation into account (rather than only considering the absolute concentrations of dissolved elements), a more comprehensive understanding of the causes of toxicity in effluents consisting of cocktails of contaminants may be possible. A better control of $pe - pH$ conditions during toxicity tests appears critical for better understanding the influence of trave metallic elements speciation on the toxicity of samples. This is because laboratory vs in situ $pe - pH$ conditions are likely to differ significantly, limiting the usefulness of speciation calculations. From a broader standpoint, the work conducted as part of this study has revealed that databases on chemical composition and toxicity of mining and industrial effluents are not readily accessible to the scientific community. Increased access to such databases would facilitate more detailed and robust statistical analyses. The production of such databases is a research priority.

4.6. References

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CHAPITRE 5 Article 2: Evaluating the acute toxicity of binary and ternary mixtures of Cu, Zn, As and Se to *Daphnia magna*: a component-based approach using isobologram analyses and ternary diagrams

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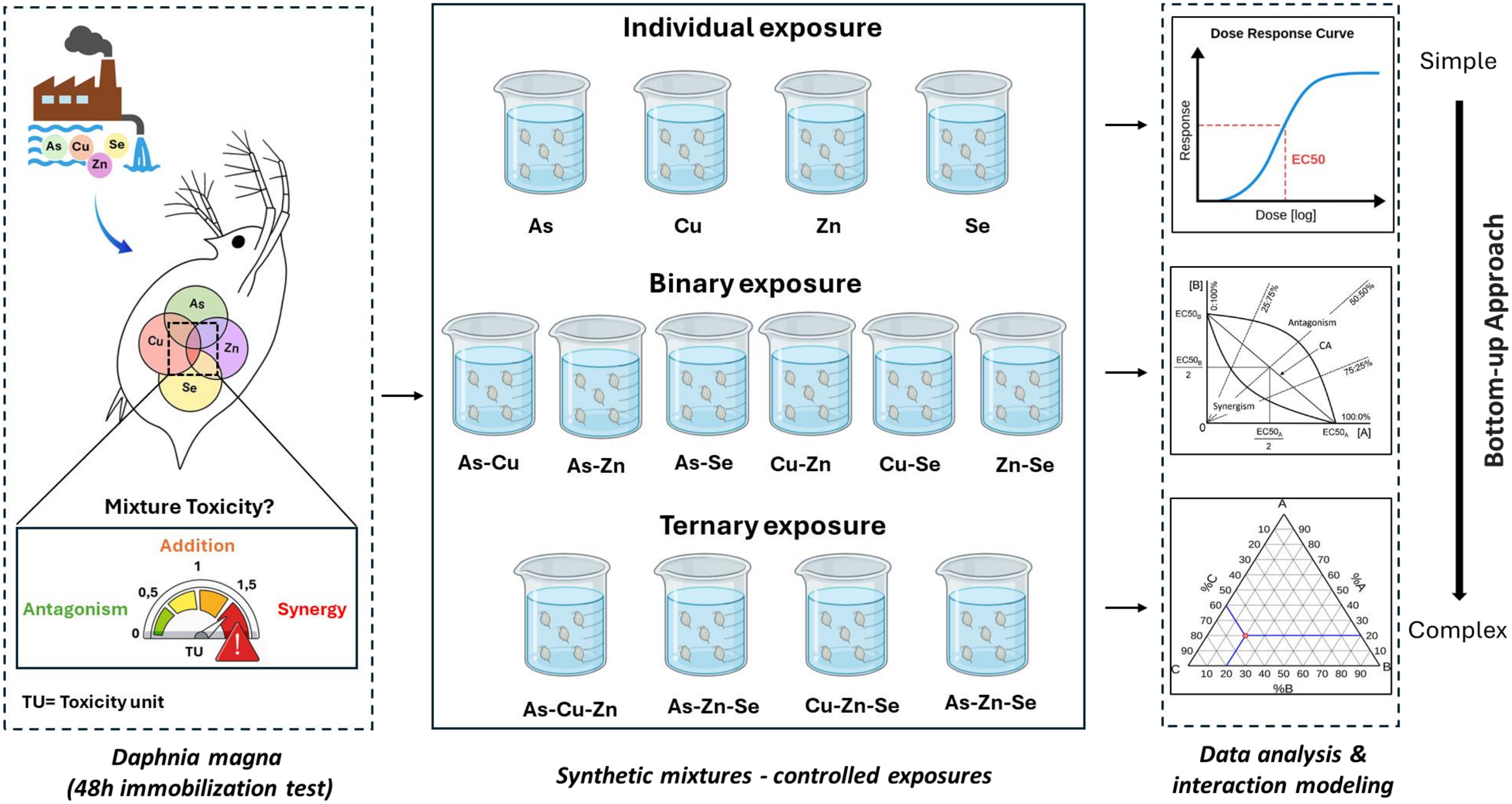
5.1. Abstract

The acute toxicity of binary and ternary combinations of Cu, Zn, As and Se to *Daphnia magna* was investigated. The aim was to provide a detailed characterization of the interactive effects of these elements in relation to aquatic toxicity. The binary and ternary combinations were evaluated using isobolograms, the concentration addition (CA) model, the independent action (IA) model and the Hewlett model. They were also implemented using the MixModel package in R. This approach facilitated the identification of potential synergism, additivity, and antagonism, in addition to the characterization of binary and ternary interactions within a mixture composition space. For the binary combinations, $\mathrm{Cu - Zn}$ and $\mathrm{Zn - Se}$ had the most robust evidence for synergism with EC_{50} estimates < 1 toxicity unit (TU), as low as 0.27 TU for $\mathrm{Cu - Zn}$, and 0.30 TU for $\mathrm{Zn - Se}$. Combinations of $\mathrm{As - Cu}$, $\mathrm{As - Se}$, $\mathrm{Cu - Se}$, and $\mathrm{As - Zn}$ were mostly additive, with EC_{50} estimates ~ 1 TU for all evaluated ratios. In all of the tested ternary combinations, Cu was identified the most toxic component. Emergent effects, which were defined as toxicity responses arising from combinations of toxicity which were not dependent on binary combinations, were observed in some ternary combinations. Synergism was observed in the $\mathrm{As - Cu - Se}$ and $\mathrm{As - Cu - Zn}$ mixtures, suggesting that binary combinations alone are insufficient for predicting interactions involving more than two components. Ternary diagrams also showed potential areas of high toxicity ($\mathrm{TU} > 3$), mostly in the Cu-dominated mixtures. These observations suggest that the toxicity assessment of trace metals and metalloids commonly found in mining and metallurgical effluents (in this case Cu, Zn, As, and Se) must take into account the effect of mixtures and not rely on substance-by-substance assessments.

Keywords: *Trace metal mixtures, Speciation, Interaction effects, Synergism, Antagonism*

Graphical Abstract



5.2. Introduction

Mining and metallurgical industries are widespread in many regions of the world, and the continuous and long-term release of effluents from these industries has the potential to detrimentally impact receiving waters (Atakhanova, 2023; Environment and Climate Change Canada, 2023). Contaminants such as Cu, Zn, As, and Se can be released during the processing of ores and the smelting of metals, and the latter have been observed to exhibit high toxicity towards aquatic biota (Chapman et al., 2010; USEPA, 2017; Ali et al., 2019). Although these elements occur naturally in the environment, industrial activities can cause levels to exceed the tolerance thresholds of aquatic organisms, thereby affecting their physiology, reproduction and survival (Grosell et al., 2006; Janz et al., 2010). In this context, it is imperative to develop a precise understanding of the mechanisms causing the toxicity of mining and metallurgical effluents.

Despite the large body of literature on toxicity thresholds for individual metals and metalloids, uncertainty remains regarding mixtures (Backhaus & Faust, 2012; Qiu et al., 2017). The overall toxicity of a mixture can differ substantially from that of its components due to interactions between contaminants. Depending on the nature of these interactions, the toxic effects may be synergistic (greater than expected from the combined effects of the individual metals), antagonistic (lower than expected) or additive (corresponding to the sum of individual effects). Therefore, the toxicity of a mixture should not be based solely on data relating to the toxicity of its individual components (Ferreira et al., 2008; Cedergreen, 2014; Dupraz et al., 2018). Synergistic toxicity has been reported for the Cu-Zn mixture at sub-lethal concentrations of *Daphnia magna* (*D. magna*) (Norwood et al., 2003; Lari et al., 2017). This resulted in mortality being almost 70% higher than expected based on the concentrations of both metals. Conversely, antagonistic interactions have been documented for As-Zn mixtures, in which reprieved mortality was considerably lower than expected for the euryhaline rotifer *Proales similis* (Arreguin-Rebolledo et al., 2024). The toxicity is further impacted by environmental factors such as chemical speciation, competition for biological binding sites, and complexation with organic or inorganic ligands (Santore et al., 2001; Niyogi & Wood, 2004; Fortin, 2024). For instance, Cu and Zn have been observed to compete for the same transporters at a cellular level (Grosell &

Wood, 2002; Li et al., 2024), while Se has been shown to inhibit As toxicity by either curtailing oxidative stress responses or forming insoluble complexes (Hughes, 2002; Sun et al., 2014).

Environmental regulations (e.g., USEPA, 2017; CCME, 2019) frequently depend on maximum allowable concentrations for contaminants considered in isolation, thus failing to reflect the actual risks involved when effluents contain complex mixtures of contaminants. This is a significant issue, as it is well-documented that aquatic organisms are frequently exposed to intricate amalgamations of contaminants (Traudt et al., 2017). In the field of ecotoxicology, two predominant models are frequently employed to predict the toxicity of mixtures: concentration addition (CA) and independent action (IA). The CA model posits the hypothesis that compounds which share the same molecular site of action will also have similar toxic effects, essentially acting as mutual dilutions (Loewe & Muischnek, 1926). The IA model posits the hypothesis that chemicals acting on independent targets may result in a binary response, whereby the target is either affected or unaffected. Hence, the probability of surviving a mixture following IA is equivalent to the product of the probability of surviving each of the chemicals individually (Hewlett, 1969; Jonker et al., 2005). Nevertheless, deviations from these models are frequently observed in real-world conditions, further complicating toxicity predictions (Dupraz et al., 2018). Some of these deviations are associated with processes such as transporter competition, changes in speciation or modulation by Se, which can either mitigate or exacerbate metal toxicity depending on the environmental conditions (Hamilton, 2004; Gong et al., 2020; Zhao et al., 2024). Despite the extensive documentation of binary mixtures, research on ternary and more complex mixtures remains limited. This lacuna is particularly concerning given the prevalence of industrial effluents containing multiple metals and metalloids, which have been observed to exhibit emergent toxicity in several cases (Kim et al., 2018; Zeng et al., 2024).

Due to the vast number of possible contaminant combinations, it is not feasible to assess every conceivable mixture in laboratory experiments involving bioassays. A viable strategy to determine patterns of interactions and mechanisms is to take a bottom-up approach, starting with binary mixtures and then moving towards ternary and multicomponent exposures (Zeng et al., 2024). For the specific context of mining and metallurgical effluents, *D. magna* is a relevant model organism for toxicity tests because it is a key component of freshwater food webs, it is highly sensitive to trace metals and metalloids such as Cu, Zn and As, and it is widely used in standardized toxicity tests (Norwood et al., 2003; Paylar et al., 2024). Its relevance is reinforced by its inclusion in the OECD guidelines for acute and chronic toxicity testing (OECD, 2012).

Fitting in the preestablished context, this study adopted an integrative approach combining (1) acute toxicity tests with *D. magna* for assessing effects of exposures to individual, binary, and ternary mixtures of Cu, Zn, As, and Se, (2) the analysis of contaminant interactions through the isobologram approach for identifying synergistic, antagonistic, or additive effects of binary mixtures, and (3) a MixModel approach for evaluating the effects of ternary mixtures. The hypothesis was that binary and ternary mixtures of Cu, Zn, As and Se would produce significant deviations from simple additive effects due to antagonistic and synergistic effects (Rusetskaya & Borodulin, 2015; Hasanuzzaman et al., 2022; Tian et al., 2024). By assessing the interactive effects of metallic elements commonly found in mine and metallurgical effluents, this study may contribute to a better understanding of the toxicity mechanisms in effluents containing multiple contaminants. This knowledge is vital for improving the characterization, management and treatment of effluents, as well as informing decision-making processes within environmental monitoring and management frameworks.

5.3. Materials and methods

5.3.1. *D. magna* culture

The culture of *D. magna* was carried under controlled conditions following the validated CEAEQ (2021) protocols. The water used for culture was supplied by a standalone aquatic housing system for zebrafish (*Danio rerio*) (Aquaneering). This system is equipped with mechanical, biological, and chemical filtration units as well as UV sterilization, ensuring continuous removal of particles, nitrogenous waste, and microbial contaminants. The design maintains stable water quality parameters (pH, hardness, electrical conductivity) suitable for aquatic organisms without the need for additional reagents or vitamins. The use of this closed-loop, validated system guarantees consistency and compliance with the CEAEQ (2021) guidelines for maintaining reliable *D. magna* cultures (Figure B1). The pH was adjusted between 7.0 and 8.0 using 0.1 M or 1 M sodium hydroxide (NaOH) or hydrochloric acid (HCl) solutions and total hardness was maintained between 160 and 180 mg $CaCO_3$/L. A stable temperature of 20.0 ± 2.0 °C was imposed under a photoperiod of 16 h of light (600 lux at the surface) and 8 h of darkness. Dissolved oxygen (DO) saturation was maintained between 80% and 100% with aeration provided by air stones. The aquariums had a volume of 18 L and could hold up to 100 organisms per tank from an adult stage on. The daily food supply included 100 mL of algae (*Chlamydomonas reinhardtiiat* with a density of approximately 10,000 cells/mL) per aquarium and a solution of dextrose and beef extract for adults older than 9 days. To ensure optimal conditions for the growth and reproduction of the organisms, 40% of the aquarium water was renewed every two days. Neonates less than 24 h old were obtained from the third brood of healthy females. Organisms were collected by sieving from culture aquaria, and only individuals of uniform size and appearance were selected for toxicity testing.

5.3.2. Acute toxicity tests

Acute toxicity tests were carried out using a static protocol with an exposure duration of 48 ± 1 h. The tests were performed at a constant temperature of 20.0 ± 2.0 °C, under a photoperiod of 16 h of light (600 lux intensity) and 8 h of darkness. Aeration ensured that the level of DO remained between 90% and 100%. Each experimental tube contained a solution of 10 mL, with 4 concentrations tested in 4 replicates and 5 neonates less than 24 h old per tube. No food was provided during the tests. The dilution water used for tests was the same as that used for culturing *D. magna*. The pH was maintained between 7.0 and 8.0 and total hardness was maintained between 160 and 180 mg $CaCO_3$/L. The measured effects included mortality and immobility of the exposed organisms. The results are expressed as 48 h LC_{50} and EC_{50} values. For a test to be considered acceptable, the control organisms had to remain below 5% for both death and unusual behavior.

Experiments were conducted with individual metallic elements, as well as with mixtures. Reference EC_{50} values were determined for individual metallic elements, while binary and ternary mixtures were prepared in parallel in distinct experiments. Control groups without added metallic elements were also tested to confirm conformity with the CEAEQ (2021) acceptance criteria. Each single-metal EC_{50} was set as 100%, and mixtures were tested at

75%, 50%, and 25% of these levels. For each binary and ternary mixture, a full dilution series was prepared starting from 100% of the defined combination down to lower proportions. All mixtures were prepared as equimolar combinations, which means that the expected EC_{50} by concentration addition corresponds to 1 toxic unit (1 TU). This design allowed for assessing interaction patterns both at and below EC_{50} levels.

5.3.3. Physicochemical analyses

Physicochemical analyses were performed for the characterization of test media and quantification of contaminants concentrations. Concentrations of dissolved As, Zn, Cu and Se were measured using inductively coupled plasma mass spectrometry (ICP-MS) Thermo X series. US EPA Method # 200.8 (USEPA, 1994) (detection limits: 0.00003 mg/L for As, 0.00006 mg/L for Cu, 0.0002 mg/L for Se, and 0.0006 mg/L for Zn) and inductively coupled plasma atomic emission spectroscopy (ICP-AES; Agilent 5110 Dual View; US EPA Method # 200.7 (USEPA, 1994); detection limits: 0.02 mg/L for As and Se, 0.004 mg/L for Cu, and 0.0008 mg/L for Zn). Quality control was assured using procedural blanks, calibration standards (multi-elements, 1000 mg/L), and certified reference materials (SCP Aquatic Quality Control). Duplicate analyses (10%) and instrument checks after every ten samples confirmed measurement accuracy and precision, with recoveries within 85-115%. Parameters such as temperature (maintained at 20.0 ± 2.0 °C), pH (7.0–8.0), DO (90–100% saturation), and electrical conductivity were monitored using standard probes.

5.3.4. Preparation of solution

5.3.4.1. Stock solutions and preparation

Individual, binary, and ternary solutions of As, Cu, Zn, and Se were prepared to test their acute toxicity. Stock solutions (PlasmaCal grade, purity >99 %) were prepared by dilutions of 1 g/L standard solutions for each element. Concentrations for the stock solution were analyzed by ICP-MS to ensure accuracy. The pH was adjusted between 7.0 and 8.0 to match the test medium conditions. Solutions were prepared immediately before the experiments to prevent changes in water chemical composition prior to tests.

5.3.4.2. Individual exposures

Individual exposures were used as toxicity evaluation benchmarks. Solutions of As, Cu, Zn, and Se were prepared at different concentrations to determine their respective toxicity. The concentrations tested for each metal were chosen based on preliminary data and existing literature to cover a significant toxicity range (Norwood et al., 2003; Lari et al., 2017). The ranges tested were 0.315 - 30 µg/L for As, 0.39 - 100 µg/L for Cu, 200 - 1000 µg/L for Zn, and 450 - 1000 µg/L for Se. This baseline data allowed for a better interpretation of the combined effects observed in binary and ternary mixtures.

5.3.4.3. Binary exposures

Binary mixtures were explored to assess potential interactions between pairs of contaminants. The six binary combinations evaluated were $\mathrm{As} - \mathrm{Cu}$, $\mathrm{As} - \mathrm{Zn}$, $\mathrm{As} - \mathrm{Se}$, $\mathrm{Cu} - \mathrm{Zn}$, $\mathrm{Cu} - \mathrm{Se}$, and $\mathrm{Zn} - \mathrm{Se}$. A fixed-ratio design was used in the experiment based on toxic units (TU), ensuring that the sum of the toxic contributions of both components remained equal to 1 TU for all mixtures. The ratios tested for each binary combination are presented in Table 5.1. The mixtures were prepared by combining stock solutions of the two contaminants in the specified proportions, followed by a final dilution to achieve the test concentrations. Additive, synergistic, or antagonistic interactions were possible under the ratios evaluated. The measured metal concentrations (µg/L) used for each binary mixture are provided in Table B1. Table B3 reports the physicochemical parameters (pH, dissolved oxygen, temperature, and electrical conductivity) measured at 0 h and 48 h during the exposure tests.

Table 5.1 Composition ratios, individual toxic units (TU), and corresponding nominal metal concentrations (µg/L) for binary mixtures of As, Cu, Zn, and Se used in acute toxicity tests with *D. magna*. TUa = Toxic unit of the first metal(oid) in the mixture; TUb = Toxic unit of the second metal(oid) in the mixture

Mixture Ratio	**TU_a**	**TU_b**	**As − Cu**		**As − Zn**		**As − Se**		**Cu − Zn**		**Cu − Se**		**Zn − Se**	
			[As]	**[Cu]**	**[As]**	**[Zn]**	**[As]**	**[Se]**	**[Cu]**	**[Zn]**	**[Cu]**	**[Se]**	**[Zn]**	**[Se]**
100:0%	1	0	16.0	0	16.0	0	16.0	0	13.0	0	13.0	0	620	0
75:25%	0.75	0.25	12.0	3.25	12.0	155	12.0	178	9.75	155	9.75	178	465	178
50:50%	0.5	0.5	8.00	6.50	8.00	310	8.00	355	6.50	310	6.50	355	310	355
25:75%	0.25	0.75	4.00	9.75	4.00	465	4.00	533	3.25	465	3.25	533	155	533
0:100%	0	1	0	13.0	0	620	0	710	0	620	0	710	0	710

5.3.4.4. Ternary exposures

Ternary mixtures were studied for four combinations: $\mathrm{As}-\mathrm{Cu}-\mathrm{Zn}$, $\mathrm{As}-\mathrm{Zn}-\mathrm{Se}$, $\mathrm{Cu}-\mathrm{Zn}-\mathrm{Se}$ and $\mathrm{As}-\mathrm{Cu}-\mathrm{Se}$. Sixteen ratios were designed for each ternary combination to represent balanced (equimolar) and unbalanced scenarios (single-contaminant dominance, two-contaminants dominance). Ternary mixtures were prepared by mixing stock solutions of the three contaminants in the proportions presented in Table 5.2, followed by dilutions to get the final concentrations. The measured metal concentrations (µg/L) used for each ternary mixture are provided in Table B2. Table B4 reports the physicochemical parameters (pH, dissolved oxygen, temperature, and electrical conductivity) measured at 0 h and 48 h during the exposure tests.

Table 5.2 Composition ratios, individual toxic units (TU), and corresponding nominal metal concentrations (μg/L) for ternary mixtures of As, Cu, Zn, and Se used in acute toxicity tests with *D. magna*

Ratios	TU_a	TU_b	TU_c	**As – Cu – Zn**			**As – Cu – Se**			**Cu – Zn – Se**			**As – Zn – Se**		
				[As]	[Cu]	[Zn]	[As]	[Cu]	[Se]	[Cu]	[Zn]	[Se]	[As]	[Zn]	[Se]
33:33:33 %	0.33	0.33	0.33	5.28	4.29	205	5.28	4.29	234	4.29	205	234	5.28	205	234
60:20:20 %	0.60	0.20	0.20	9.60	2.60	124	9.60	2.60	142	7.80	124	142	9.60	124	142
20:60:20 %	0.20	0.60	0.20	3.20	7.80	124	3.20	7.80	142	2.60	372	142	3.20	372	142
20:20:60 %	0.20	0.20	0.60	3.20	2.60	372	3.20	2.60	426	2.60	124	426	3.20	124	426
40:40:20 %	0.40	0.40	0.20	6.40	5.20	124	6.40	5.20	142	5.20	248	142	6.40	248	142
40:20:40 %	0.40	0.20	0.40	6.40	2.60	248	6.40	2.60	284	5.20	124	284	6.40	124	284
20:40:40 %	0.20	0.40	0.40	3.20	5.20	248	3.20	5.20	284	2.60	248	284	3.20	248	284
85:7.5:7.5%	0.85	0.07	0.07	13.6	0.97	46.5	13.6	0.97	53,3	11.1	46.5	53.2	13.6	46.5	53.2
7.5:85:7.5%	0.07	0.85	0.07	1.20	11.1	46.5	1.20	11.1	53.3	0.97	527	53.2	1.20	527	53.2
7.5:7.5:85 %	0.07	0.07	0.85	1.20	0.97	527	1.20	0.97	603	0.97	46.5	603	1.20	46.5	603
50:25:25 %	0.50	0.25	0.25	8	3.25	155	8	3.25	178	6.50	155	178	8	155	178
25:50:25 %	0.25	0.50	0.25	4	6.50	155	4	6.50	178	3.25	310	178	4	310	178
25:25:50 %	0.25	0.25	0.50	4	3.25	310	4	3.25	355	3.25	155	355	4	155	355
70:15:15 %	0.70	0.15	0.15	11.2	1.95	93	11.2	1.95	106	9.10	93	106	11.2	93	106
15:70:15 %	0.15	0.70	0.15	2.40	9.10	93	2.40	9.10	106	1.95	434	106	2.40	434	106
15:15:70 %	0.15	0.15	0.70	2.40	1.95	434	2.40	1.95	497	1.95	93	497	2.40	93	497

5.3.5. Speciation calculations

The PHREEQC software was used with the WATEQ4F thermodynamic database to evaluate the speciation of contaminants in the test solutions. The WATEQ4F database was selected because it is commonly used for calculation speciation in mining effluents (Cravotta et al., 2022) and it contains the metals/metalloids that we considered here (Cu, Zn, As, Se). Input data for PHREEQC comprised measured dissolved concentrations of trace (e.g., Cu, Zn, As, Se) and major elements (e.g., Ca, Mg, Na, K) obtained by ICP-MS, and ICP-AES along with pH, and alkalinity. Fixed pe values of 4 and 12, corresponding to an Eh values of ~ 0.236 V and ~ 0.708 V at 25 °C, respectively. were applied to constrain redox conditions in the absence of direct Eh measurements. These values allow for covering a wide range of redox conditions, encompassing the conditions likely to occur during the experiments.

5.3.6. Data analysis

5.3.6.1. Concentration-Response

The concentration-response curves were fitted using a three-parameter log-logistic model (LL.3) with the *drc* package in R (Ritz & Streibig, 2014, 2005):

$$\mathrm{U} = \mathrm{d} \times (1 + \left(\frac{\mathrm{x}}{\mathrm{EC}_{50}}\right)^{\mathrm{b}})^{-1} \qquad (1)$$

where U represents the measured response (immobility after 48 h), x is the concentration of the metal element, EC_{50} is the concentration inducing 50% immobility, d is the upper asymptotic value corresponding to the immobility rate of unexposed controls, and b is the slope of the curve, reflecting the sensitivity to concentration variation. These curves allow for the precise determination of the median lethal concentrations of the individual contaminants on *D. magna*.

5.3.6.2. Binary mixture analyses

Interaction analyses between substances in mixtures were performed using the isobologram model based on the principles of CA and IA, and the MIXTOX tool which assesses synergistic, antagonistic, or additive effects at different doses. Isobolograms allow these interactions to be graphically represented by visualizing isoboles (Dupraz et al., 2018), corresponding to the concentration combinations producing a common toxic effect, here set at 50% immobility in *D. magna* (EC_{50}). The relative contributions of contaminants to the overall toxicity of the mixture are expressed in toxic units (TUs), determined by the following equation:

$$\mathrm{TU_i} = \frac{\mathrm{z_i}}{\mathrm{EC_{50i}}} \tag{2}$$

where zi is the concentration of the metal in the mixture and $\mathrm{EC_{50_i}}$ is its individual effective concentration. The sum of the TUs is theoretically equal to 1 for perfect additivity according to the CA model. Deviations from additivity are interpreted according to the following thresholds: $\mathrm{TU} < 0.5$ indicates synergy, $0.5 \leq \mathrm{TU} \leq 2$ indicates additivity, and $\mathrm{TU} > 2$ indicates antagonism (Belden et al., 2007; Cedergreen, 2014).

The extended Hewlett model (Hewlett, 1969), allowing symmetrical and asymmetrical representations of deviations from additivity, was applied when a significant deviation from the CA model was suspected. The Hewlett model is described by:

$$\mathrm{EC_{50_{mix}}} = ((\frac{\mathrm{pX}}{\mathrm{EC_{50_X}}})^{1/\lambda} + (\frac{\mathrm{pY}}{\mathrm{EC_{50_Y}}})^{1/\lambda})^{-\lambda} \tag{3}$$

where pX and pY represent the relative proportions of substances X and Y in the mixture

and $\mathrm{EC_{50_X}}$ and $\mathrm{EC_{50_Y}}$ correspond to their respective $\mathrm{EC_{50}}$, and λ is an interaction parameter describing the deviation from additivity, when $\lambda = 1$, the model reduces to classical concentration addition; $\lambda < 1$ indicates antagonism, while $\lambda > 1$ describes synergism. These models improve the statistical description of interactions when residue reductions are significant according to F tests (Dupraz, 2018; Hewlett, 1969).

The MIXTOX model (Jonker et al., 2005) was also used to explore contaminant interactions. Unlike isobole approaches, MIXTOX directly fits the entire concentration–response surface, allowing a more robust and detailed description of interactions. Two extensions are commonly used to detect deviations from reference models. The generalized synergism/antagonism (S/A) model uses a single interaction parameter, a, where $a < 0$ indicates synergism and $a > 0$ indicates antagonism. The dose-ratio (DR) and dose-level (DL) dependent extensions incorporate an additional parameter, b, which enables asymmetric deviations. Specifically, b modulates how the interaction shifts along either the relative dose proportion (DR) or total dose intensity (DL), revealing more complex interaction dynamics. These parameters are estimated by minimizing the residual sum of squares, and statistical significance is assessed using a χ^2 test (Dupraz et al., 2019; Jonker et al., 2005).

The models used to assess interactions rely on distinct conceptual frameworks and reference models, which limit their direct comparability. In the *drc* package (Ritz and Streibig, 2005; Ritz et al., 2015), interaction analysis is based on comparing observed data to the CA model. Deviations from this null model are evaluated using more flexible alternatives such as the Hewlett model, allowing the detection of significant synergistic or antagonistic interactions. Although the IA model is not implemented in *drc*, its predictions can be generated separately and overlaid on isobolograms, enabling a visual comparison, typically at the 50% effect level (e.g., EC_{50}) (Dupraz, 2018).

MIXTOX can be applied to binary data such as mortality or immobility, as well as to continuous responses. It can be applied using both IA and CA as reference models. Here, MIXTOX was applied with IA since the metals tested are assumed to act through dissimilar modes of action, which makes IA the most appropriate choice. The model accounts for synergism or antagonism, either as a general shift (S/A) or as a function of dose ratio or dose level (DR- or DL-dependent deviations).

As a result, when synergy is detected with *drc*, it means there's a deviation from the CA model, while synergy identified by MIXTOX reflects a deviation from IA. This difference in reference models is essential when interpreting or comparing results across the two approaches. Even if the results may look similar visually, such as in isobolograms, the underlying assumptions are not the same. Therefore, visual comparisons can be helpful, but they should always be interpreted considering the methodological framework used. Comparisons remain possible, especially at EC_{50}, but the methodological underpinnings differ and should guide the interpretation.

5.3.6.3. Modeling Ternary Mixtures (MixModel)

The assessment of interactions in ternary mixtures relies on a statistical approach known as MixModel, which allows modeling the combined effect of three elements on toxicity using the *mixexp* package in R (Lawson & Willden, 2016). MixModel performs a multiple regression fit that includes all individual component effects, binary interactions, and ternary interaction terms, allowing for the evaluation of the specific combined effects typically observed in such substances. The equation for this model is usually written as:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k + \varepsilon \qquad (4)$$

where Y represents the measured response (toxicity), X_i corresponds to the relative concentrations of the substances, β_i, β_{ij} and β_{ijk} are the coefficients estimated by the model, ε is the residual error of the model. Model fit is assessed using statistics such as the coefficient of determination (R^2), which measures the quality of the prediction, and the overall significance test, which indicates whether the interactions are relevant to explaining the response studied. Model predictions are represented as ternary diagrams, where each vertex of the triangle corresponds to 100% of an element in the mixture. The intermediate proportions of the three substances are arranged within the triangle. The toxicity is visualized via a color gradient, highlighting areas of greater or lesser intensity. Experimental points are integrated into visualization to verify the fit of the model to the real data.

5.4. Results and discussion

5.4.1. Single exposure toxicity results

The single exposure toxicity tests for Cu, Zn, As, and Se on *D. magna* showed clear dose-response relationships reflected in log-logistic curves characterized by initial low toxicity, a sharp increase in immobility as concentration rises, and a plateau at higher concentrations indicating maximum immobility (Figure 5.1). The EC_{50} values for individual contaminants are summarized in Table 5.3. Cu exhibited the highest acute toxicity to *D. magna,* ($EC_{50} = 13\ \mu g/L$), followed by As (16 µg/L), Zn (620 µg/L), and Se (710 µg/L). Cu and As showed similar EC_{50} values, while Zn and Se were much less toxic with EC_{50} values about 50 times higher.

Table 5.3 EC_{50} values for individual metallic elements in *D. magna.* EC_{50} ± standard error (SE) calculated from 3 independent experiments (each with 4 replicates)

Metallic element	**EC_{50} (µg/L)**	**Standard error (SE)**	**95% confidence interval (95% CI)**
Cu	13	±1.2	11.0 – 15.0
As	16	±1.8	13.0 – 19.0
Zn	620	±35	550 – 690
Se	710	±40	630 – 790

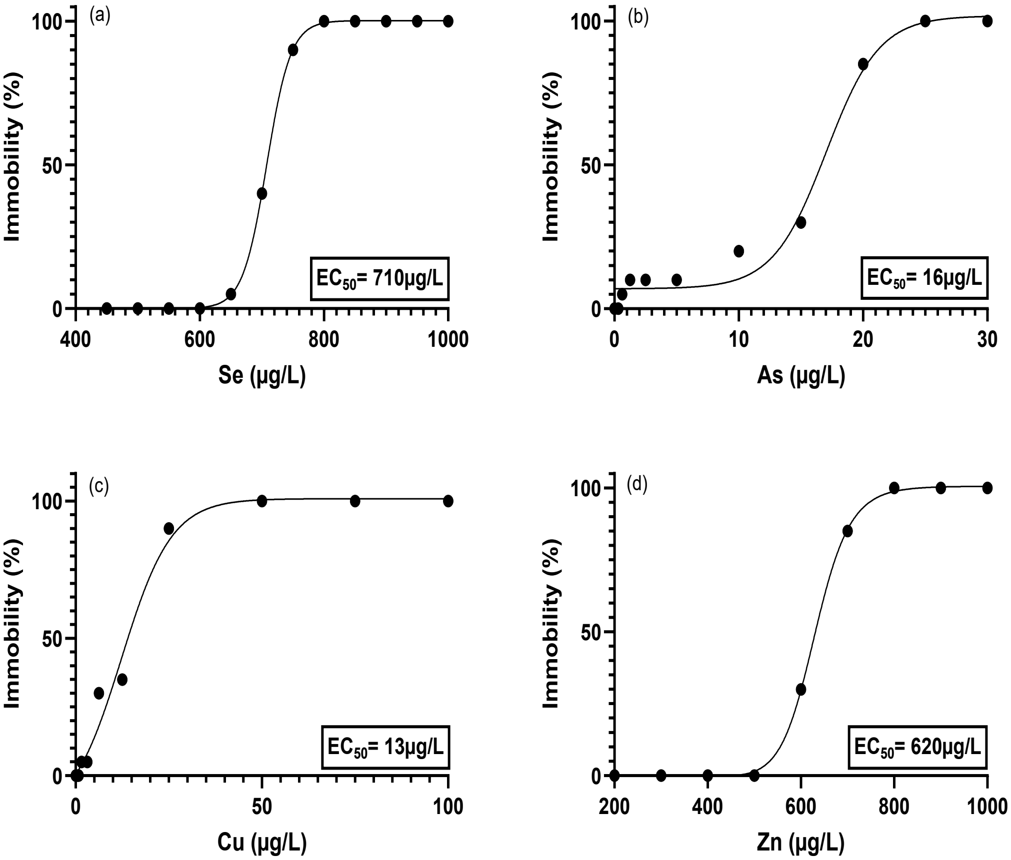


Figure 5.1 Concentration–response curves representing 48-h immobility in *D. magna* following individual exposure to each metal or metalloid. Dose–response curves were fitted using a four-parameter log-logistic model for: (a) Se, (b) As, (c) Cu, and (d) Zn. The EC_{50} values are shown for each element: 710 µg/L for Se, 16 µg/L for As, 13 µg/L for Cu, and 620 µg/L for Zn. Data represents mean immobility ± SE (n = 4).

These values are generally consistent with previous studies. Bossuyt & Janssen (2003) reported EC_{50} values for Cu ranging from 6 to 18 µg/L. *As* toxicity, however, varies widely depending on its chemical speciation. Sales et al. (2016) reported EC_{50} values of approximately 450 µg/L for As^{3+} and 500 µg/L for As^{5+}, whereas Czub et al. (2021) found a much lower value of 3.2 µg/L for an organoarsenic compound. The EC_{50} values obtained in the present study are significantly lower than those reported by Sales et al. (2016) but more comparable to those of Czub et al. (2021), suggesting the presence of more bioavailable or toxic arsenic species in the test solutions used in the present study. This discrepancy is discussed in greater detail in the section on arsenic speciation (see section 4.4.4).

The Zn toxicity results are consistent with those reported by Muyssen & Janssen (2002), who found EC_{50} values around 500 µg/L. Similarly, the EC_{50} value for Se (710 µg/L) aligns with Lemly (2002), who observed toxic effects near 700 µg/L.

Compared to other freshwater cladocerans and invertebrates, *D. magna* shows a noticeable sensitivity to trace metals. For instance, *Ceriodaphnia dubia* and *Moina macrocopa* can withstand higher concentrations, usually 20-30 µg/L for Cu and 800-1200 µg/L for Zn (Norwood et al., 2003; Muyssen & Janssen, 2005). In contrast, *D. magna* typically has EC_{50} values for Cu of 10-15 µg/L. For most metals, the ostracod *Cypris subglobosa* consistently exhibits higher EC_{50} values than *D. magna* (Khangarot & Das, 2009). The oligochaete worm *Tubifex tubifex* is even less sensitive, exhibiting an EC_{50} for Cu of 340 µg/L at 15 °C (Rathore & Khangarot, 2002). These differences confirm the findings of Okamoto et al. (2015), who ranked *D. magna* among the most vulnerable species to trace metals, supporting its use as a sentinel organism in ecotoxicological testing.

In the context of marine systems, a number of copepod species have been observed to demonstrate EC_{50} values for copper that range from approximately 5 to 50 µg/L (Heuschele et al., 2022). This finding suggests that they exhibit a degree of sensitivity to Cu comparable to that observed here in *D. magna*. Nevertheless, the EC_{50} values measured in the present study (median values of 13 µg/L and 620 µg/L, for Cu and Zn, respectively) were generally lower than those quoted in previous studies focusing on other organisms. This finding underscores the sensitivity of *D. magna* to trace metals.

5.4.2. Toxicity of binary mixtures

5.4.2.1. Synergistic interactions

The dose-response curves of the $\mathrm{Cu}-\mathrm{Zn}$ mixture showed toxicity values several times higher than that observed for the metals tested separately. An increase in toxicity was marked for mixtures containing a high proportion of Cu, particularly the $75:25$ and $50:50$ ratios, with EC_{50} values of 0.27 and 0.50 TU, respectively. Values below 0.5 TU indicate significant synergy (Belden et al., 2007). Experimental data points lie well below the reference curves for both CA and IA models (Figure 5.2a), indicating a clear deviation from additive behavior. The use of the Hewlett interaction model provides a better fit than the CA model ($p < 0.05$). The MIXTOX analysis (Table 5.4) further suggests synergy. The best-fit model was the DR (dose ratio)-dependent synergistic interaction, with parameters $\mathrm{a} = -3.06$ and $\mathrm{b} = 11.62$ ($p < 0.001$). These results corroborate previous studies that have identified Cu as an amplifying agent of Zn toxicity (Traudt et al., 2017). This amplification mechanism has been inferred to result from the disruption of ionic homeostasis or inhibition of detoxification systems (Paylar et al., 2024). Indeed, Cu disrupts ion regulatory pathways and induces oxidative stress in *D. magna*, thereby enhancing the toxic effects of other metals under co-exposure conditions (Paylar et al., 2024). Such observations, therefore call for consideration of synergy when assessing the risks of metal mixtures, especially in the presence of Cu.

A significant synergistic interaction was observed for the $\mathrm{Zn}-\mathrm{Se}$ mixture only for the equimolar mixture ($50:50$), which reached an EC_{50} of 0.37 TU, well below the additivity threshold ($\mathrm{TU} = 0.5$). The other mixtures ratios ($75:25$ and $25:75$) were within the additive range, with EC_{50} values of 0.64 and 0.96 TU, respectively. The experimental points deviate moderately from the CA curve (Figure 5.2b), suggesting a slight synergism. This is consistent with the Hewlett interaction model, which performed significantly better than the CA model ($p < 0.05$). The MIXTOX analysis (Table 5.4) confirms that the toxic effect resulting in the interaction of the two metals depends on the ratio of each metal in the mixture. The best-fit model was a DR-dependent synergism, yielding $\mathrm{a} = -10.95$ and $\mathrm{b} = 7.16$ ($p < 0.001$). This ratio-dependent synergistic effect means that some combinations of contaminants may become abnormally toxic even when the concentrations of the individual components are below lethal concentration.

Table 5.4 Summary of binary mixture interactions in *D. magna* based on isobologram and MIXTOX analyse

Mixture	**Isobole**	**Mixtox**	
	BFM(a)/EFF.(b) Int. param. ± s.e. p-value	**Reference Model (REF)**	**BFM/EFF. Int. param. *p*-value**
Cu – Zn	Hewlett/SYN $\lambda = 5.24$ $p < 10^{-3}$	IA	DR / SYN $a = -3.06$ $b = 11.62$ $p < 10^{-3}$
Cu – Se	ADD	IA	IA/ ADD
As – Zn	CA/ADD	IA	DR / ANT $a = -2.82$ $b = 0.45$ $p < 10^{-3}$
As – Cu	ADD	IA	DL / ANT $a = -4.38$ $b = 2.1$ $p < 10^{-3}$
As – Se	CA/ADD	IA	IA/ ADD
Zn – Se	Hewlett/DR/SYN $\lambda = 1.65$ $p < 10^{-3}$	IA	DR/SYN $a = -10{,}95$ $b = 7.16$ $p < 10^{-3}$

For isobolograms, the best-fitting model (BFM) and interaction type (EFF) are reported relative to the concentration addition (CA) model, with the Hewlett parameter λ shown when significant. For MIXTOX, the BFM and interaction type (synergism (SYN), antagonism (ANT), or additivity (ADD)) are presented relative to the reference model

(REF: CA or IA). Significant interaction parameters (a, or a and b for DR/DL models) are given when $p < 0.05$ (F-test). Negative values indicate synergism; positive values indicate antagonism. Parameter b reflects dose ratio (DR) or dose level (DL) dependence.

5.4.2.2. Additive interaction described by the CA model

Results obtained for the $\mathrm{As}-\mathrm{Se}$ mixture reveal that combined exposure leads to a slightly increased toxicity compared to individual effects. Two mixtures ratios, $50:50$ and $25:75$, showed slight decreases in EC_{50} (0.75 and 0.72 TU, respectively). However, these values remain above 0.5 TU, suggesting that combined effect does not differ from additive behavior to any significant extent. The isobologram analysis (Figure 5.2c) corroborates this observation. Experimental points lie close to (or just below) the predictions of the CA model. Fitting the data to the Hewlett interaction model does not yield a significant improvement over the CA model ($p > 0.05$), and the MIXTOX analysis (Table 5.4) confirms the absence of significant interaction: the best-fit model was IA/ADD, without evidence of deviation from additivity. The lack of a pronounced synergistic effect might be due to divergent or non-converging mechanisms of action for As and Se, and an explicit metabolic interference is lacking at intermediate concentrations. Indeed, previous studies have shown that Se can mitigate As toxicity in some aquatic organisms. Both inorganic (selenite) and organic (selenomethionine) forms of Se significantly alleviated arsenite-induced oxidative stress and cytotoxicity in cultured hepatocytes of *Oncorhynchus mykiss*, particularly at low to moderate exposure levels (Jamwal & Niyogi, 2017). These findings provide cellular-level evidence for antagonistic interactions between As and Se, depending on dose and speciation.

The tested $\mathrm{As}-\mathrm{Zn}$ mixtures showed EC_{50} values that are very similar or equal to 1 TU (from 1 to 1.1 TU). This suggests that there is no significant interaction between the two elements. The experimental data on the isobologram (Figure 5.2d) fit well with the CA model, which gives a better fit than the IA model. This observation suggests that the combined effects of $\mathrm{As}-\mathrm{Zn}$ in mixtures are additive, likely due to a common or similar mechanism of action. The Hewlett model does not perform significantly better than the CA model. These findings align with previous studies indicating that the combined effects of As and Zn are generally additive or exhibit slight variations depending on the tested concentrations and the indicator species used. The presence of Zn could reduce the bioavailability of As by promoting sorption and complexation, leading to antagonistic or near-additive combined effects, depending on the concentrations and environmental conditions such as temperature, pH and water hardness (Gong et al., 2020). Under the conditions tested here, the overall effects of the $\mathrm{As}-\mathrm{Zn}$ mixture are better described by a toxic addition, more precisely described by the CA model.

5.4.2.3. Additive interaction described by the IA model

The EC_{50} values for the three tested $\mathrm{As}-\mathrm{Cu}$ mixtures ranged between 1.4 and $1.6\ \mathrm{TU}$, indicating an absence of significant interaction according to the standard criteria employed for the examination of mixtures (Belden et al., 2007). The isobologram analysis (Figure 5.2e) shows that the experimental points align better with the IA model than with the CA model which tends to overestimate the combined effect. Fitting the Hewlett model does not improve the description of the data. In this last case, the observed additivity is well predicted by the IA model and that remains the most appropriate approach in this case. Although the CA model fits well at the 50% effect level (Figure 5.2d), the MIXTOX analysis (Table 5.4) identifies a DL dependent antagonism as the best fit ($\mathrm{a} = -4.38$, $\mathrm{b} = 2.10$, $p < 0.001$). This interaction model implies that the type and strength of the interaction vary with the overall dose of the mixture. Specifically, estimated parameters indicate that the $\mathrm{As}-\mathrm{Zn}$ mixture exhibits synergistic effects at low doses, which gradually shift toward antagonism as the dose increases, with the inflection occurring before the EC_{50} threshold (i.e., $\mathrm{TU} < 1$). This is also visible in the dose–response curves (Figure B2), where immobility at low exposure levels is slightly higher than predicted by CA, while the opposite trend is seen at higher doses. These results suggest that the interaction pattern may depend on the substance dose and thus the selected effect level (e.g., 10%, 50%, or 90% response), underscoring the importance of considering the full dose–response profile rather than relying solely on EC_{50} based models. This observation further supports the hypothesis that As and Cu exert their toxic effects through distinct mechanisms, As primarily disrupting enzyme functions related to oxidative stress, while Cu targets ion channels and cellular respiration, thereby reducing the likelihood of strong interactive effects at moderate or high doses (Fan et al., 2015; Paylar et al., 2024). Sublethal exposures of *D.*

magna to As and Cu revealed that the metabolites affected by As differed from those affected by Cu, indicating distinct toxicity pathways. This suggests that, when combined, their effects are independent rather than synergistic or antagonistic (Nagato et al., 2013). The overall effects of the $\mathrm{As}-\mathrm{Cu}$ mixtures can be considered to fall into a logic of additivity without clear interaction. The prediction from the IA model is superior, but still indicates potential synergism at low doses, as evidenced in Figure B2. Such observation could suggest a higher toxicity for this mixture at lower concentrations, potentially closer to those observed in the environment.

All tested $\mathrm{Cu}-\mathrm{Se}$ mixtures yielded a combined EC_{50} of 1.5 TU, hence showing the absence of significant toxicological interaction as per standard criteria (Belden et al., 2007). This conclusion is further supported by the isobologram analysis (Figure 5.2f). The experimental points plotted close to the IA model, while the CA curve slightly overestimated total toxicity. The Hewlett model (used to test for deviations from CA) and the S/A and DR-/DL-dependent deviation models implemented in the MIXTOX framework (used to test for deviations from IA) (Table 5.4) did not significantly improve fit ($p > 0.05$), confirming that the IA model provides the best description of this mixture's behavior. This is coherent because Cu and Se do not share common mechanistic pathways. Cu mostly disturbs ionic homeostasis and generates oxidative stress, while Se can bond with sulfur proteins and antioxidant enzymes for protective activities against oxidative damage (Schiavon et al., 2017; Paylar et al., 2024). In fact, additive effects are typically associated with shared modes of action when evaluated under the CA model (Altenburger, 2011). Nevertheless, in this case, the absence of significant deviation from the IA model suggests that this reference model, rather than CA, provides a more appropriate framework for capturing the joint effects of Cu and Se, whose modes of action are distinct.

In consideration of these findings and in accordance with existent literature, it is proposed that the pronounced synergistic effects of the Cu–Zn and Zn–Se mixtures can be attributed to oxidative stress. It has previously been demonstrated that exposure to Cu and Zn increased the production of reactive oxygen species and inhibited the antioxidant defence systems in *D. magna*, leading to an increased vulnerability under concurrent exposures (Barata et al., 2005). Another potential mechanism of action for the Cu–Zn mixed metals is competition for binding sites on thiol-rich proteins, as both metals will alter key enzymes and subsequently disrupt energy metabolism (Norwood et al., 2003). The additive responses reported for the mixtures including As suggest that the contaminants act independently, with each retaining its dominant mode of toxicity, as observed in reported mixture studies with *D. magna* (Heugens et al., 2001). Finally, antagonistic interactions that were reported occurred infrequently, but could likely be explained by intracellular complexation mechanisms that decrease ionic bioavailability, a response observed in other mixtures tested with this cladoceran (Blinova, 2004). These interpretations are consistent with the findings of Arreguin-Rebolledo et al. (2023), who demonstrated in *Proales similis* that oxidative stress, metabolic interference, and the formation of complexes that limit toxicity could influence the antagonistic and synergistic effects of binary metal mixtures.

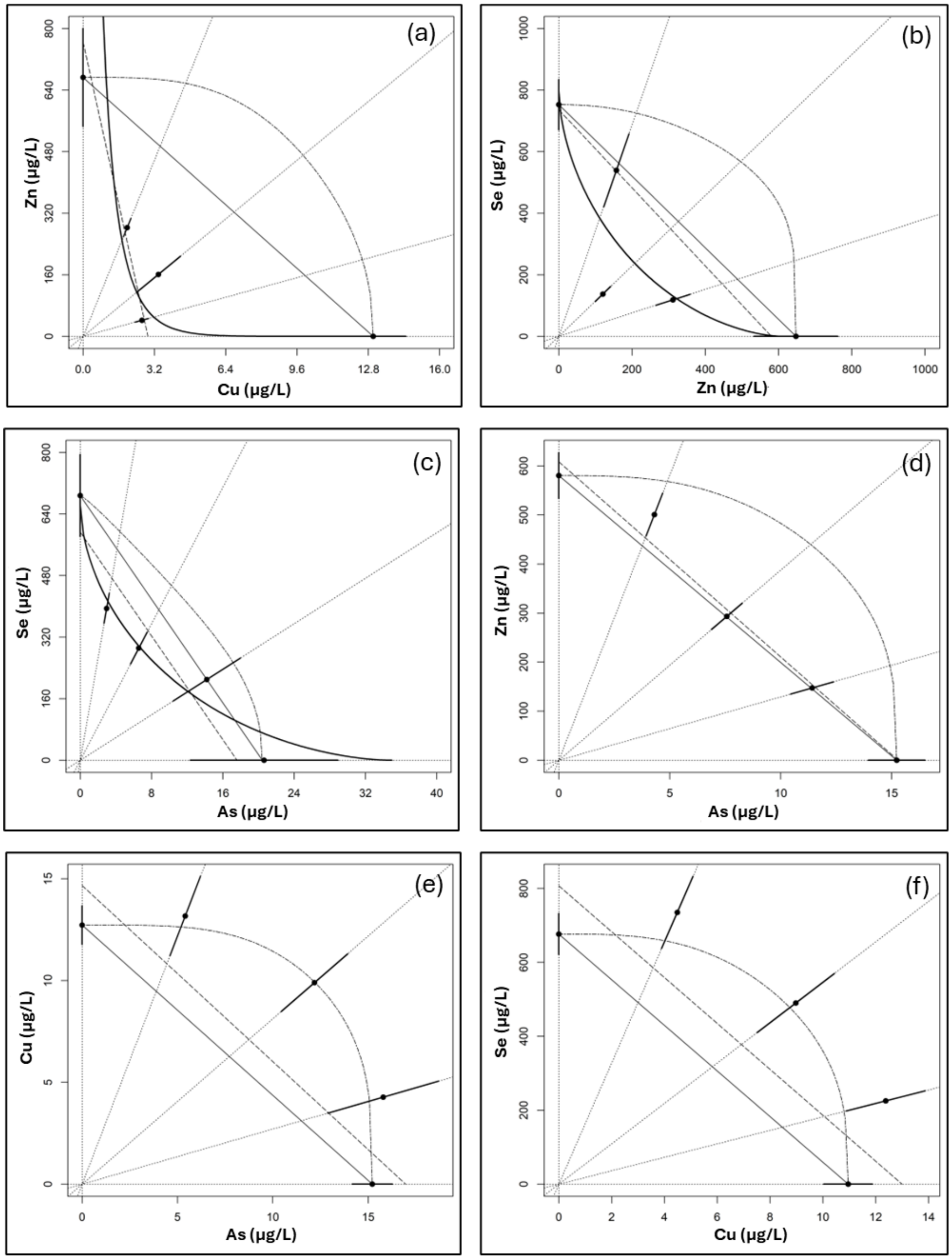


Figure 5.2 Isobolograms of binary mixtures of metallic elements. Points represent $EC_{50} \pm SE$. Solid straight line: CA isobole based solely on the two pure metallic elements; Dashed straight line: CA isobole fitted to the entire dataset (i.e., the two pure metallic elements plus the three mixture ratios); Curved dash-dotted line: IA isobole based solely on the two pure metallic elements; Bold curved line: Hewlett isobole plotted when the Hewlett model provides a significantly better fit than the CA model.

5.4.3. Toxicity of ternary mixtures

Ternary mixtures ($\mathrm{As}-\mathrm{Cu}-\mathrm{Zn}$, $\mathrm{As}-\mathrm{Zn}-\mathrm{Se}$, $\mathrm{Cu}-\mathrm{Zn}-\mathrm{Se}$, $\mathrm{As}-\mathrm{Cu}-\mathrm{Se}$) were evaluated using the MixModel framework (Lawson & Willden, 2016). This model employs polynomial regression based on the relative proportions of contaminants and allows capturing individual, binary and ternary interactions. Results reveal that toxicity responses in *D. magna* are dependent on elements combinations, interactions ranging from antagonistic to additive or synergistic

5.4.3.1. Non-significant ternary interactions

Results for the $\mathrm{As}-\mathrm{Cu}-\mathrm{Zn}$ mixtures (Table 5) showed that the individual effects of all three elements were highly significant ($p < 0.001$) and indicated that each individually contributed to toxicity. Among binary interactions, only $\mathrm{Cu}-\mathrm{Zn}$ was found to be statistically significant ($\mathrm{p} < 0.001$), which is consistent with the synergistic interaction observed between these two metals (section in 5.4.2.1). This has also been reported in earlier findings by Norwood et al. (2003) who described synergetic effects of $\mathrm{Cu}-\mathrm{Zn}$ mixtures on *D. magna*. In contrast, the binary interactions between $\mathrm{As}-\mathrm{Cu}$ and $\mathrm{As}-\mathrm{Zn}$, as well as the ternary $\mathrm{As}-\mathrm{Cu}-\mathrm{Zn}$ interaction, were not statistically significant ($p > 0.05$), suggesting that, within the tested concentration ranges, their effects could be considered additive or independent. The model used in this study provided an excellent fit (adjusted $\mathrm{R}^2 = 0.936$; $p < 2.2 \times 10^{-16}$) with low residual error, thus supporting the robustness of the MixModel for characterizing complex chemical interactions. The ternary plot (Figure 3a) shows the toxicity landscape based on the relative proportions $\mathrm{As}-\mathrm{Cu}-\mathrm{Zn}$. The zone of highest toxicity ($\mathrm{TU} > 3$) is seen to occur predominantly in the Cu-rich region, while mixtures dominated by Zn or As tend to exhibit lower toxicity ($\mathrm{TU} < 1$), thus reinforcing the central role of Cu in driving overall mixture toxicity. This pattern is consistent with the results from binary $\mathrm{Cu}-\mathrm{Zn}$ mixtures, for which the MIXTOX model identified a DR dependent synergistic interaction, particularly pronounced in the 75:25 ratio.

Results obtained from the $\mathrm{As}-\mathrm{Zn}-\mathrm{Se}$ mixtures (Table 5.5) suggest that all three elements entail significant individual effects on toxicity ($p < 0.001$). Zn and Se appeared to have slightly higher estimated effects than As. Among the binary mixtures, only $\mathrm{Zn}-\mathrm{Se}$ showed a highly significant interaction ($p < 0.001$), confirming a synergistic effect. This result is consistent with previous findings and aligns with earlier observations from this study, where a significant synergistic interaction was observed for the 50:50 ratio ($\mathrm{EC}_{50} = 0.37$ TU), while other ratios showed additive effects. Similar synergistic effects of $\mathrm{Zn}-\mathrm{Se}$ have also been reported in other aquatic organisms, reinforcing the relevance of this synergistic pattern (Altenburger, 2011). On the other hand, Se was found to exert modulatory effects on metal-induced oxidative stress. For example, Fasil et al. (2021) reported that combined supplementation of Se and Zn in zebrafish (*Danio rerio*) increased antioxidant defenses and decreased oxidative stress. The $\mathrm{As}-\mathrm{Zn}$ and $\mathrm{As}-\mathrm{Se}$ interactions were not statistically significant ($p > 0.05$), which tends to indicate additivity. The ternary $\mathrm{As}-\mathrm{Zn}-\mathrm{Se}$ interaction was not significant ($p > 0.05$), thus suggesting that any potential interaction may depend on specific concentration ratios, as observed for the $\mathrm{Zn}-\mathrm{Se}$ binary mixture, or on environmental conditions, such as temperature, pH and water hardness, and should therefore be investigated further. The ternary diagram (Figure 5.3b) reveals a high-toxicity zone (TU > 3) between the Zn and Se axes, consistent with the synergistic interaction between these two elements. On the other hand, mixtures dominated by As and Se tented to be less toxic (TU < 1.5), giving more evidence that $\mathrm{Zn}-\mathrm{Se}$ interactions are the main drivers of toxicity in this system.

5.4.3.2. Significant ternary interactions

Results for the $\mathrm{Cu}-\mathrm{Zn}-\mathrm{Se}$ mixtures (Table 5.5) indicated that all three elements had considerable and similar individual effects ($p < 0.01$). This suggests that Cu, Zn, and Se may have contributed comparably to the mixture’s toxicity. This observation agrees with previous studies that pointed out that although Cu, Zn, and Se have different modes of action, they can each induce oxidative stress, disturb ion balance, or interfere with enzymatic functions in aquatic organisms (Chen et al., 2022). Among the binary interactions, the $\mathrm{Cu}-\mathrm{Zn}$ interaction proved to be highly significant ($p < 0.001$), consistent with the synergistic effects identified between these metals in *D. magna* (Norwood et al., 2003). The $\mathrm{Zn}-\mathrm{Se}$ interaction was also significant ($p = 0.0027$), suggesting a secondary synergy, while $\mathrm{Cu}-\mathrm{Se}$ turned out not to be significant ($p = 0.20$)

implying an additive or independent effect. The ternary $\mathrm{Cu} - \mathrm{Zn} - \mathrm{Se}$ interaction proved to be significant ($p = 0.0026$), suggesting an overall non-additive response likely driven by the combined influence of the high individual toxicity of Cu, the role of Zn in enhancing Se bioavailability, and Se pro-oxidative effects at elevated concentrations (Norwood et al., 2003; Wang et al., 2007; Fasil et al., 2021). The ternary diagram (Figure 5.3c) corroborates these findings, showing two clear zones of high toxicity ($\mathrm{TU} > 3$), one close to the Cu apex and the other in between Zn and Se.

Mixtures dominated by Se (> 60%) showed lower toxicity. The model was well fitted (adjusted $\mathrm{R}^2 = 0.922$; $p = 1.13 \times 10^{-15}$) with a reasonable standard error (0.3967), though slightly higher than in other mixtures, possibly indicating greater biological variability upon exposure to $\mathrm{Cu} - \mathrm{Zn} - \mathrm{Se}$. Hence, effects of $\mathrm{Cu} - \mathrm{Zn} - \mathrm{Se}$ mixtures cannot be considered as reducible to the sum of binary combinations. Modeling of the $\mathrm{As} - \mathrm{Cu} - \mathrm{Se}$ mixtures (Table 5.5) showed that the individual effects of all three metallic elements were highly significant ($p < 0.001$). Unlike the other mixtures, all binary interactions were statistically significant, with negative coefficients for $\mathrm{As} - \mathrm{Cu}$, $\mathrm{As} - \mathrm{Se}$, and $\mathrm{Cu} - \mathrm{Se}$ ($p < 0.05$), indicating antagonistic effects between each pair of metals. The ternary $\mathrm{As} - \mathrm{Cu} - \mathrm{Se}$ interactions were nonetheless strongly significant ($p < 0.001$), with a positive coefficient, suggesting that despite binary antagonism, a synergistic effect may occur when the three metals are in balanced proportions. This phenomenon, binary antagonism offset by ternary synergy, has been documented in studies investigating mutual neutralization mechanisms between metal cations and anions (Altenburger et al., 2013). The ternary plot (Figure 5.3d) supports this interpretation. Generally, mixture effects were considered additive, with toxicity ranging from a 0.5 to 1.5 TU for most combinations and little spatial variation (Belden et al., 2007). However, a slightly lighter area near the center of the triangle, showing an increase of toxicity to about 1.8 TU. This elevation could be reflecting a localized synergistic interaction, particularly between Cu and Se , as previously observed in binary mixtures. In contrast, the $\mathrm{As} - \mathrm{Se}$ binary mixture exhibited an additive effect, which may explain the absence of highly toxic zones in As-rich regions. Altogether, these findings suggest a subtle balance between synergistic and antagonistic interactions, depending on the relative proportions of the individual components.

Table 5.5 Estimated parameters from the MixModel fitting for the toxic effects of ternary metal/metalloid mixtures (As, Cu, Zn, and Se) on *D. magna*

Mix	**Term**	**Estimate**	**Std. Error**	**t − value**	**p − value**	**Significance**
As − Cu − Zn	**As**	1.057e-0.2	2.239e-03	4.720	5.53e-05	***
	Cu	1.408e-02	2.239e-03	6.291	7.19e-07	***
	Zn	8.25 e-03	2.239e-03	3.685	9.33e-04	***
	As − Cu	-1.947e-04	1.270e-04	-1.533	0.136	
	As − Zn	4.850 e-05	1.270e-04	0.382	0.705	
	Cu − Zn	5.955e-04	1.270e-04	4.688	6.04 e-05	***
	As − Cu − Zn	-3.824e-06	6.919e-06	-0.553	0.585	
As − Zn − Se	**As**	9.929 e-03	1.380e-03	7.197	6.36e-08	***
	Zn	9.589 e-03	1.380e-03	6.950	1.22e-07	***
	Se	8.403e-03	1.380e-03	6.091	1.24e-06	***
	As − Zn	2.617e-05	7.828e-05	0.334	0.740	
	As − Se	1.307e-04	7.828e-05	1.669	0.105	
	Zn − Se	4.153e-04	7.828e-05	5.306	1.09e-05	***
	As − Zn − Se	-7.633e-06	4.264e-06	-1.790	0.0839	.
Cu − Zn − Se	**Cu**	1.454e-02	2.427e-03	5.989	1.64e-06	***
	Zn	7.989e-03	2.427e-03	3.292	2.62e-03	**
	Se	8.03e-03	2.427e-03	3.309	2.51 e-03	**
	Cu − Zn	6.318e-04	1.377e-04	4.588	7.96 e-05	***
	Cu − Se	-1.792e-04	1.377e-04	-1.301	0.203	
	Zn − Se	4.512e-04	1.377e-04	3.276	2.73 e-03	**
	Cu − Zn − Se	-2.474e-05	7.501e-06	-3.298	2.58 e-03	**
As − Cu − Se	**As**	1.046 e-02	8.736e-04	11.971	9.61e-13	***
	Cu	1.044 e-02	8.736e-04	11.954	9.95 e-13	***
	Se	9.494e-03	8.736e-04	10.868	9.66 e-12	***
	As − Cu	-1.272e-04	4.957e-05	-2.566	1.57e-02	*
	As − Se	-1.319e-04	4.957e-05	-2.661	1.26e-02	*
	Cu − Se	-1.473e-04	4.957e-05	-2.972	5.9e-03	**
	As − Cu − Se	1.415 e-05	2.700e-06	5.242	1.29 e-05	***

Signification codes : $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

The table reports the main effects of each metal as well as pairwise interaction terms within each mixture. For each term, the estimate, standard error $(\mathrm{Std.Error})$, $\mathrm{t-value}$, $p-$ value, and significance level are provided. Significant interaction terms help identify potential synergistic (positive coefficients) or antagonistic (negative coefficients) effects between metal components.

Overall, incorporating isobolograms and predictive models (e.g. IA, Hewlett, and MIXTOX) alongside the MixModel provides a more advanced visualisation of interactions within mixtures. These approaches go beyond the simple summation of toxic units that defines the CA model. Isobolograms provide a clear graphical representation of predicted and observed toxicities, allowing the identification of synergistic or antagonistic effects relative to the CA reference. MIXTOX, in turn, enables the quantitative assessment of deviations from the IA model based on toxicological data. In contrast, models such as the BLM describe the bioavailability of individual metals but do not account for interactive effects (Di Toro et al., 2001). The integrative approach implemented in this work represents a relevant methodological complement for evaluating the toxicity of complex mixtures.

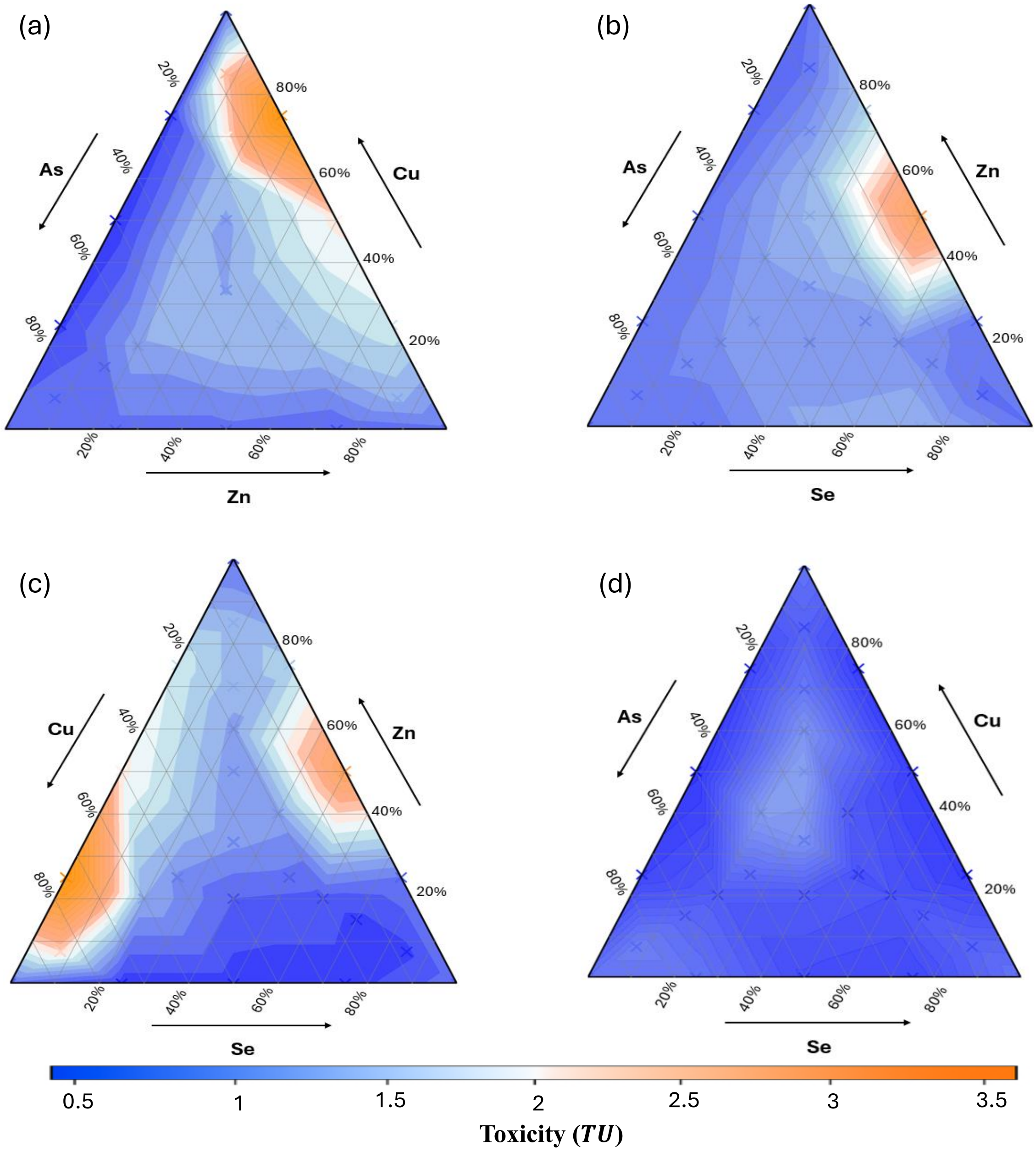


Figure 5.3 Acute toxicity (expressed in Toxic Units, TU) of ternary mixtures containing As, Cu, Zn and Se on *D. magna.* Each ternary plot represents a different metal combination: (a) As − Zn − Cu, (b) As − Se − Zn, (c) Cu − Se − Zn, and (d) As − Se − Cu. The relative proportions of each component are expressed as percentages along the triangle axes. Color gradients illustrate mixture toxicity, from low (0.5 TU, blue) to high (3.5 TU, orange). These plots highlight interaction patterns between metals, with regions of synergistic (higher toxicity) and antagonistic (lower toxicity) effects. Crosses represent the experimentally tested combinations.

5.4.4. Speciation calculations

The thermodynamic equilibrium calculations performed in PHREEQC (with the WATEQ4f database) were intended to identify the dominant dissolved species for the contaminants (As, Cu, Se, Zn) present in the mixtures. Table 5.6 illustrates the distribution of the predominant species in the different mixtures, while the speciation patterns for binary and ternary metal mixtures are presented in Figures B3 and B4. The calculations indicate that the arsenate species ($\mathrm{HAsO_4^{2-}}$) predominates within all mixtures, notwithstanding the imposed pe value (4 and 12). This suggests that $\mathrm{As(V)}$, the more oxidised form of As, is predominant over $\mathrm{As(III)}$. Arsenate has been demonstrated to induce toxicity through its interference with phosphate in metabolism (Smedley & Kinniburgh, 2002). Furthermore, calculations indicate that $\mathrm{Cu(OH)_2}$ predominates in all mixtures, although the interquartile range of concentrations for the free form ($\mathrm{Cu^{2+}}$) intersects with the interquartile range of $\mathrm{Cu(OH)_2}$ concentrations in the $\mathrm{Cu-Zn}$, $\mathrm{As-Cu}$, $\mathrm{Cu-Se}$, and $\mathrm{As-Cu-Zn}$ mixtures. $\mathrm{Cu^{2+}}$ has been demonstrated to possess ecotoxic properties, even at trace levels, attributable to ionic disruption and to its enzyme inhibition (Paylar et al., 2022). Selenite ($\mathrm{HSeO_3^-}$) was the predominant selenium species in all mixtures when a pe value of 4 is imposed. Selenite has been demonstrated to induce toxicity through a range of mechanisms, including oxidative stress, the generation of free radicals, and the disruption of membrane enzymes (Klaczek et al., 2024). Nevertheless, selenate ($\mathrm{SeO_3^{2-}}$) appears to be the dominant species when a pe of 12 is imposed (under more oxidizing conditions). This observation emphasises the need for more detailed analyses of selenium speciation when evaluating the toxicity of mixtures to Daphnia magna. The free form of Zn ($\mathrm{Zn^{2+}}$) was found to be predominant in all mixtures. This bioavailable form of Zn has been demonstrated to induce oxidative stress, ion exchange, and affect membrane integrity in *D. magna* (Muyssen & Janssen, 2002; Paylar et al., 2022). Further details on potential interactions between dissolved species are provided in Table 5.6.

Table 5.6 Dominant dissolved species in mixtures and potential interactions influencing toxicity

Mixtures	$HAsO_4^{2-}$	Cu^{2+}	$Cu(OH)_2$	$HSeO_3^-$	SeO_4^{2-}	Zn^{2+}	Potential interactions and causes of toxicity
As − Zn	✓	-	-	-	-	✓	The relative abundances of $HAsO_4^{2-}$($\sim$100%) and Zn^{2+} (~83%) suggest that their combination might generate additive or synergistic effects, mainly through oxidative stress or disruption of enzymes (Gong et al., 2020). Nevertheless, the tests conducted in the present study did not reveal additive or synergistic effects (sect. 3.2.2).
Zn − Se	-	-	-	✓ at pe = 4	✓ at pe = 12	✓	The relative abundance of Zn^{2+} is ~84% while the dominant Se species depend on redox conditions. Their combination could entail synergistic effects through free radical generation and membrane enzyme disruption (Klaczek et al., 2024). Se − Zn mixtures showed synergistic effects (sect. 3.2.1).
Cu − Zn	-	×	✓	-	-	✓	The simultaneous presence of two highly bioactive cations (Cu^{2+}($\sim$20%); Zn^{2+}($\sim$100%)) suggests possible synergistic interaction (Traudt et al., 2017; Paylar et al., 2024). Cu − Zn mixtures showed synergistic effects (sect. 3.2.1).

Table 5.6 Dominant dissolved species in mixtures and potential interactions influencing toxicity (suite)

Mixtures	$HAsO_4^{2-}$	Cu^{2+}	$Cu(OH)_2$	$HSeO_3^-$	SeO_4^{2-}	Zn^{2+}	Potential interactions and causes of toxicity
As − Se	✓	-	-	✓ at pe = 4	✓ at pe = 12	-	The relative abundance of $HAsO_4^{2-}$is ~100% while the dominant Se species depend on redox conditions. Because the modes of action are physiologically different (one causes disruption of energy, the other causes oxidative stress), they are unlikely to entail direct potentiation (Jamwal and Niyogi, 2017). The As − Se mixtures studied in the present study showed a slightly increased toxicity with respect to individual effects (sect. 3.2.2).
As − Cu	✓	×	✓	-	-	-	The co-presence of $HAsO_4^{2-}$(~83%) and Cu^{2+} (~20%) can lead to additive or even synergistic toxicity (Fan et al., 2015; Paylar et al., 2024). The results obtained in the present study suggest additivity (sect. 3.2.3).
Cu − Se	-	×	✓	✓ at pe = 4	✓ at pe = 12	-	The relative abundance of Cu^{2+} is ~20% while the dominant Se species depend on redox conditions. Both species are present in bioavailable forms but act through different biological mechanisms. The lack of direct functional interaction between their modes of action results in an additive, non-synergistic response consistent with independent action (Paylar et al., 2022). Tests suggested the absence of significant toxicological interaction (sect. 3.2.3).

Table 5.6 Dominant dissolved species in mixtures and potential interactions influencing toxicity (suite)

Mixtures	$HAsO_4^{2-}$	Cu^{2+}	$Cu(OH)_2$	$HSeO_3^-$	SeO_4^{2-}	Zn^{2+}	Potential interactions and causes of toxicity
As − Cu − Zn	✓	×	✓	-	-	✓	The presence of $HAsO_4^{2-}$(~100%), Cu^{2+} (~20%) and Zn^{2+}(~80%) could entail additive or synergistic toxic effects mainly due to the simultaneous hindrance of enzymatic, ionic, and metabolic systems (Smedley & Kinniburgh, 2002; Paylar et al., 2022). No significant interaction was observed for the ternary mixture; effects were considered additive or independent (sect. 3.3.1).
As − Cu − Se	✓	×	✓	✓ at pe = 4	✓ at pe = 12	-	The relative abundance of $HAsO_4^{2-}$ is (~100%), that of Cu^{2+} is ~20% while the dominant Se species depend on redox conditions. The combination of Cu^{2+} and $HSeO_3^-$ implies a possible synergetic effect, while Se could also entail an antagonistic effect on As toxicity (Paylar et al., 2022). In the present study, significant ternary interactions were observed, with overall synergy when the three metallic elements were present in balanced proportions (sect. 3.3.2).

Table 5.6 Dominant dissolved species in mixtures and potential interactions influencing toxicity (suite)

Mixtures	$HAsO_4^{2-}$	Cu^{2+}	$Cu(OH)_2$	$HSeO_3^-$	SeO_4^{2-}	Zn^{2+}	Potential interactions and causes of toxicity
Cu − Zn − Se	-	×	✓	✓ at pe = 4	✓ at pe = 12	✓	The relative abundance of $HAsO_4^{2-}$ is ~100%, that of Cu^{2+} is ~20%, while the dominant Se species depend on redox conditions. A synergistic effect is possible between Zn^{2+}which disrupts ion regulation, and $HSeO_3^-$ which impairs antioxidant defenses (Paylar et al., 2022; Muyssen et al., 2003; Klaczek et al., 2024). However, Se could also modulate the bioavailability of Cu and Zn by either forming complexes or interfering with their transport pathways. In the present study, significant ternary interactions that are overall non-additive responses were observed (sect. 3.3.2).
As − Zn − Se	✓	-	-	✓ at pe = 4	✓ at pe = 12	✓	The relative abundance of $HAsO_4^{2-}$ is ~100%, that of Zn^{2+} is ~80%, while the dominant Se species depend on redox conditions. The combination of Zn^{2+} and $HSeO_3^-$ could entail a synergetic effect, while Se may also attenuate As-related toxicity through antagonistic modulation (Fasil et al., 2021). No significant ternary interaction was detected, suggesting that the overall effects can be considered additive or independent (sect. 3.3.1).

Symbols: "✓" = dominant species; "×" = minor or absent species; "-" = not applicable or not detected

5.4.5. Environmental relevance and ecological risk

In the present study, synthetic effluents (solutions) were employed in the form of controlled experimental designs, with the objective of isolating mixtures effects on *D. magna*. This approach was undertaken in accordance with the guidelines stipulated for acute tests on *D. magna* (OECD TG 202) and the Canadian Reference Methods Framework for acute effluent lethality (EPS 1/RM/14). The nominal concentration ranges were calibrated to represent the ranges typically observed/reported at the point of discharge of mining and metallurgical effluents in Canada, according to the MDMER regulations and its annual reports (Environment and Climate Change Canada, 2023). The acute responses obtained suggest that mixtures can induce marked effects on *D. magna* at concentrations comparable to regulatory or reported ranges for Cu, Zn, As, and Se, indicating a potential ecological risk. These findings are consistent with the risk framework for mixtures (CA and IA) and with experimental observations where certain metal binaries entail synergistic toxic effects towards *D. magna* (Belden et al., 2007; Lari et al., 2017), supporting the need for an integrated reading of mixture effects when evaluating the toxicity of trace metals and metalloids. It is acknowledged that the chemical composition of the synthetic effluents utilized in this study (i.e. created under laboratory conditions with controlled hardness and salinity, absence of dissolved organic matter, absence of suspended solids, limited number of contaminants) does not permit direct extrapolation to real-world (field) conditions. In subsequent studies, the validation of these findings using real effluents collected under various field conditions will be imperative.

5.4.6. Study limitations and prospects

The present study yielded novel insights into the toxicity of binary and ternary Cu, Zn, As, and Se mixtures to *D. magna*. Several limitations to achieve a more comprehensive contextualization and interpretation of the findings are highlighted in the following. The synthetic effluent used here are laboratory-prepared solutions containing only four contaminants while real industrial effluents contain a wider array of contaminants. Ecological representativeness and the direct application of the results to more complex environmental matrices could be limited. The study also only examined acute toxicity. However, as demonstrated by Qiu et al. (2017), prolonged exposures have been shown to result in sublethal, cumulative or delayed effects, which are not detectable in acute assays. Improved ecological relevance would derive from including chronic toxicity tests targeting functional endpoints such as growth, reproduction, or survival, which are also the basis of most regulatory frameworks. Consistently, although the EC_{50}values, isobolograms and interactions models such as those proposed by MIXTOX or Hewlett can provide valuable indications about binary interactions, it is also necessary to take their limitations into account. The ability of a polynomial model to detect threshold effects, or strongly non-linear interactions, is inherently limited (Altenburger et al., 2013). More advanced approaches exist (Qiu et al., 2017; Zeng, 2024), but they are often complex or not easily applicable to standard ecotoxicological datasets. Therefore, the models used here offer a pragmatic and relevant approach for detecting deviations from additivity under realistic experimental conditions. Finally, the impact of fluctuations in pivotal water chemistry parameters, including pH, hardness, DOC, and major ions (Ca^{2+}, Mg^{2+}, SO_4^{2-}), as well as environmental variables such as pH, redox potential, salinity and temperature, were not investigated. These parameters directly influence speciation and bioavailability. Not accounting for this reduces the possibility of extrapolating results to other aquatic systems. For example, higher water hardness may reduce Cu and Zn toxicity as it favors metal ion complexation, while acidic conditions may increase the fraction of bioavailable metal ions. Similarly, increased salinity alters ion competition and osmoregulation, while higher temperatures can accelerate metabolic rates, leading to increased sensitivity to contaminants (Heugens et al., 2001).

The study poses questions regarding the current regulatory frameworks, which are often based on individual contaminant thresholds. The observed significant toxicity at concentrations lower than environmental guidelines suggests that mixture effects should be more explicitly integrated into risk assessment protocols. It is proposed here that the ecological validity of mixtures toxicity assessments could be enhanced by approaches based on effect summation, isobolograms, or predictive models that account for key environmental parameters such as pH, water hardness, temperature, and major ion composition, which influence metal speciation and bioavailability.

It is recommended that future research endeavors to expand the scope of the contaminants under scrutiny, with a particular focus on those commonly encountered in mining and metallurgical effluents. Furthermore, there is a pressing need to comprehensively assess the interactions of contaminants with nutrients and organic contaminants, but also through multistressor approaches that account for environmental variables such as temperature, pH, DOC and DO, factors that are increasingly affected by human activities and global climate change. The presence of multiple bioindicator species, including algae, fish embryos and benthic invertebrates, would also facilitate a more comprehensive evaluation of ecosystem-level effects. Finally, the integration of empirical data with advanced modelling tools, such as response surface models (RSM), mechanistic models and Bayesian approaches, in conjunction with machine learning strategies, has the potential to enhance our ability to predict mixture toxicity in a broader array of environmental scenarios.

5.5. Conclusion

The acute toxicity of binary and ternary mixtures of Cu, Zn, As, and Se towards *D. magna* revealed different interaction patterns among mixtures. Synergistic effects were observed in $\mathrm{Cu}-\mathrm{Zn}$ and $\mathrm{Zn}-\mathrm{Se}$ mixtures, especially at equimolar or Cu-dominant ratios. In these cases, the MIXTOX and Hewlett models better captured non-additive effects than the CA or IA models. Other combinations, such as $\mathrm{As}-\mathrm{Zn}$ and $\mathrm{Cu}-\mathrm{Se}$, mainly followed additive patterns consistent with CA or IA predictions.

Some ternary mixtures showed responses that differed from those expected based on binary tests or simple additivity. The $\mathrm{Cu}-\mathrm{Zn}-\mathrm{Se}$ and $\mathrm{As}-\mathrm{Zn}-\mathrm{Se}$ mixtures indicated that the synergistic effects found in $\mathrm{Cu}-\mathrm{Zn}$ and $\mathrm{Zn}-\mathrm{Se}$ pairs persisted in the ternary systems. In the $\mathrm{As}-\mathrm{Zn}-\mathrm{Se}$ mixture, binary interactions were additive, but the overall ternary response was stronger, showing a conditional synergy that appeared only when the three elements were present together.

Cu was consistently the most toxic element in mixtures, while Se showed the lowest toxicity. Se also acted as a modulator in some mixtures, particularly $\mathrm{Zn}-\mathrm{Se}$ and $\mathrm{Cu}-\mathrm{Zn}-\mathrm{Se}$.

Overall, this work demonstrates the need to bring together bioassays and advanced statistical tools to identify and evaluate the interactions between contaminants in mixtures. The findings indicate that environmental impact assessments involving effluent discharge containing trace metals and metalloids such as Cu, Zn, As, and Se should consider the interactions between contaminants, as opposed to solely focusing on the individual effects of each contaminant. Ultimately, a better incorporation of synergism and antagonism concepts in legal frameworks focusing on the maximum allowable concentrations of trace metals and metalloids in effluents discharged to the environment could allow for an enhanced protection of aquatic ecosystems.

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CHAPITRE 6 Article 3 Influence of ozone microbubbles on metallic elements speciation and acute toxicity of mining and metallurgical effluents to *Daphnia magna*

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6.1. Abstract

The present study focuses on the evaluation of the influence of microbubble ozonation on the toxicity of industrial and mining effluents. The eight effluents tested were sampled from two gold mines and one Ni-Cu mine in northern Canada, as well as a smelter situated in Abitibi-Témiscamingue. The chemical compositions of these effluents are characterised by marked differences, particularly regarding salinity (Cl^-ranging from < 0.05 mg/L to > 50 mg/L) and ammoniacal nitrogen concentrations ($\mathrm{NH_3-N}$ ranging from < 0.1 mg/L to > 450 mg/L). Tests were conducted using the microbubble ozonation treatment in a 16 L laboratory reactor over a period of 150 to 200 min, with the pH adjusted to a target value of 9. The physicochemical characteristics of the effluents and the $\mathrm{NH_3-N}$ concentrations were measured before, during, and after treatment. The concentrations of major ions and trace elements were measured in both the pre- and post-treatment samples. Toxicity tests on *Daphnia magna* were also conducted before and after the treatment process. The results showed initial $\mathrm{NH_3-N}$ concentrations ranging from 0.06 to 482 mg/L and a decrease to values below the detection limits (0.003 mg/L) after treatment. The concentrations of Fe and Mn decreased in most of the treated effluents, suggesting the precipitation of oxyhydroxides. Conversely, Zn concentrations slightly increased in certain effluents, suggesting the potential mobilisation of Zn from particulate matter, most likely ZnS particles. The microbubble ozonation treatment was found to have a significant effect on the reduction of toxicity on *D. magna* in all tested effluents. Following treatment, only one effluent sample, characterised by the highest salinity (Cl^-> 50 g/L), exhibited toxic properties. The results confirm the efficacy of microbubble ozonation in reducing toxicity through the removal of oxidizable species in mining and industrial effluents.

Keywords**:** *Microbubble ozonation, Mining and metallurgical effluents, Acute toxicity, Trace metals, Ammoniacal nitrogen*

6.2. Introduction

Mining and metallurgical activities generate high volumes of drainage and wastewater, which frequently contain intricate contaminant mixtures. Studies conducted in North America, Europe and Asia have shown that mine effluents are a primary source of ecological stress (Tiwary, 2001; Méndez & Maier, 2008). Trace metals (Cu, Zn, Fe, Mn), metalloids (As, Se), nitrogen compounds (ammoniacal nitrogen ($\mathrm{NH_3-N}$), consisting of both ammonia (NH_3) and ammonium (NH_4^+), nitrate, and nitrite), and cyanide species ($\mathrm{CN^-}$, $\mathrm{SCN^-}$) have been identified as contaminants frequently associated with base and precious metal mines (Johnson & Hallberg, 2005; Nordstrom, 2011; Gould et al., 2012). The presence of these contaminants is attributed to various sources, including mine drainage, process waters, surface runoff, and groundwater inflows. The composition of mine waters is subject to considerable variation both over time and space (Younger, 2001), which renders the treatment and control of aquatic toxicity particularly challenging. This is especially problematic in climates characterised by marked seasonality, such as is the case in Canada.

Legislation facilitates the establishment of standards with the aim of controlling the impact of mining water on ecosystems. In Canada, the federal Metal and Diamonds Mining Effluent Regulations (MDMER) are designed to regulate the quality and toxicity of mine waters discharged to receiving natural waters (Canadian Minister of Justice, 2025). These regulations are complemented by mandatory Environmental Effects Monitoring programs, which are implemented to assess the environmental impact of mining activities. Compliance with provincial legislation is also required, including the Directive 019 on the mining industry which sets out consistent criteria and monitoring requirements for the mining industry in Quebec (MELCCFP, 2025). However, compliance with threshold pertaining to maximum allowable contaminant concentrations as identified in regulations does not guarantee the protection of ecosystems; several studies have demonstrated that effluents meeting these regulatory criteria can still be toxic to aquatic organisms (Lowell et al., 2000; Atakhanova, 2023). A relatively recent federal performance report has recorded fish toxicity tests where chemical thresholds were met in mines regulated under Canadian MDMER (ECCC, 2023). The report states that 21 fish toxicity tests alongside 78 invertebrate toxicity tests that have failed. This brings forth evidence that ecological safety is not guaranteed by regulatory compliance. Among the potential explanations for why an effluent that complies with the maximum concentrations prescribed by regulation is still toxic, mixtures effects along with bioavailability and speciation of contaminants (e.g., salinity, species sensitive to redox or pH conditions) and their potential additive, synergistic or antagonistic are of particular interest (Ryskie et al., 2021). The free ionic forms of trace metals and metalloids (e.g., Cu^{2+}, Zn^{2+}) are generally considered more bioavailable and toxic than carbonate- or organic-bound complexes (Adam et al., 2019). The necessity to progress beyond the utilisation of "total concentrations" thresholds in ecological risk assessments, and to instead consider contaminant speciation is also acknowledged (De Schamphelaere & Janssen, 2004). This further highlights the critical importance of developing increasingly effective water treatment methods. Indeed, conventional treatment methods such as neutralisation, chemical precipitation, coagulation-flocculation and reverse osmosis significantly reduce the contaminant load. Nevertheless, they do not systematically guarantee the absence of aquatic toxicity. As an example, reverse osmosis generates brines that can entail toxicity due to the osmotic stress caused by high salinity (Pervov et al., 2023; Baghbanzadeh et al., 2023), while chemical precipitation faces challenges pertaining to the removal of stable dissolved metal complexes and creation of residual salinity (Pohl, 2020; Meng et al., 2022). Toxicity has been found to remain in treated effluents due to the stability of dissolved toxic species or complex interactions between contaminants and their transformation products (Neculita et al., 2020).

In consideration of the aforementioned background, advanced oxidation processes (AOPs) are considered as interesting options for performant treatment of effluents with complex chemistries (Glaze et al., 1987; Ryskie et al., 2021, Gervais et al., 2020). Among the available technologies, the ozone microbubble ($O_3 - MB$) process has been demonstrated to exhibit certain advantages over conventional ozonation and other AOPs (Ryskie et al., 2021). These advantages include superior gas-liquid transfer, an extended bubble residence time, and an enhanced generation of hydroxyl radicals ($\bullet OH$), thus accelerating the oxidation of nitrogen compounds and metals (Xiao et al., 2022). The $O_3 - MB$ was also shown to be efficient at low temperatures due to increased O_3 solubility (Marcotte et al., 2021). Hence, $O_3 - MB$ can be very useful in cold climates where biological processes face major challenges related to cold water conditions (Khuntia et al., 2013; Xiao et al., 2022). Recent studies report that $O_3 - MB$ can remove over 90% of $NH_3 - N$ from mining effluents, while also reducing metals and metalloids (Marcotte et al., 2021; Ryskie et al., 2023). Nevertheless, toxicity toward *Daphnia magna* (*D. magna*) can be observed in treated effluents due to the formation of metal oxide nanoparticles during the $O_3 - MB$ process (Ryskie et al., 2023). The toxicity of such nanoparticles on aquatic organisms is well documented (Singh, 2023). Therefore, alkaline precipitation as pretreatment step before $O_3 - MB$ is required to limit the formation of nanoparticles and reduce residual toxicity (Ryskie et al., 2023).

In line with the pre-established context, this study aims at evaluating the performance of alkaline $O_3 - MB$ as an advanced treatment technology to reduce the toxicity of mining and metallurgical effluents. The focus is set on waters and effluents from three mines and one smelter presenting a wide range of contaminants concentrations, including metallic elements, sensitive to redox conditions species, and salinity. Building on previous studies that mainly focused on $O_3 - MB$ contaminant removal efficiencies (Ryskie et al., 2023), this research develops new knowledge by integrating acute toxicity bioassays conducted with *D. magna* with effluents showing contrasted chemical properties. This integration provides a new understanding of the links between chemical transformation and biological responses at a mechanistic level, which could lead to the development of ecologically meaningful, predictive endpoints for quantifying the residual toxicity of effluents.

6.3. Materials and methods

6.3.1. Effluent sources and sampling methods

The present study focuses on effluents from a smelter and three mining sites in Canada. The sites were sampled with the objective of testing ozonation treatment on samples with different salinities, nitrogen and sulfur compounds, and trace metals and metalloids concentrations. An overview of the origin and characteristics of the effluents studied is provided in Table 6.1.

Samples from an active copper smelter in Rouyn-Noranda, Quebec, Canada, were collected by the UQAT research team. Sampling was conducted at three representative locations along the mine water circuit: (i) a settling pond, (ii) a runoff area water from an acid-generating tailings site and (iii) the final discharge point. Samples were collected within a restricted timeframe, from 2024-07 to 2024-08. In the field, samples were collected using a peristaltic pump. The feed tube was placed a few centimetres below the water surface, and pumping was carried out at a low flow rate to avoid increased sediment mobilisation and turbidity. Samples intended for toxicity tests were stored in 19 L containers and frozen at -9°C until the tests were performed. A first group of samples intended for metal and metalloid analyses were filtered on site at 0.45 μm, stored in 100 mL tubes, and acidified to $\mathrm{pH} < 2$ with $\mathrm{HNO_3}$. A second group of samples intended for metal and metalloid analyses were stored in 100 mL tubes and acidified to $\mathrm{pH} < 2$ with $\mathrm{HNO_3}$ without prior filtration. Samples intended for $\mathrm{NH_3-N}$ analyses were stored in 1 L bottles and then stabilised with NaOH without filtration. All samples for chemical analyses were stored at 4 °C until analysis. *In situ* physicochemical parameters (temperature, pH, oxidation-reduction potential (ORP) and electrical conductivity (EC)) were measured directly in the field, downgradient from the sampling points, using a YSI Pro Plus multiparameter probe.

The effluents from the three mining sites were sampled and provided by industrial partners of RIME-UQAT. These sites comprise gold and nickel mines located in arctic and subarctic climates. The samples collected by industrial partners were stored in 19 L containers and sent to the RIME-UQAT laboratories for physicochemical characterization and treatment testing. Upon arrival at the laboratory, the effluents were separated into different aliquots according to the procedure described above. The physicochemical parameters (temperature, pH, ORP and EC) were measured in the laboratory using the YSI Pro Plus probe. Samples collected at mining sites might have undergone geochemical changes during transport to the laboratory. Their geochemical characterization is, therefore, representative of their condition upon arrival at the laboratory.

Table 6.1 Summary of tested effluents main characteristics

Site	Location	Facility type	Known oxidizable species	Water conductivity
M1	North of Canada	Gold mine effluent	$\mathbf{NH_3 - N}$ (from explosives)	≈ 100 mS/cm
M2	North of Canada	Gold mine effluent (processing plant, cyanidation)	$\mathbf{NH_3 - N}$, (from explosives and cyanidation); trace $\mathbf{CN^-}$, $\mathbf{SCN^-}$ (from cyanidation), particulate and dissolved organic matter	≈ 7 mS/cm
M3-1	Nunavik	Ni–Cu underground mine (treated contact water)	Thiosalts ($\mathbf{S_2O_3^{2-}}$, $\mathbf{S_4O_6^{2-}}$), small amounts (<6 mg/L) of $\mathbf{NH_3}$, (from explosives)	≈ 1 mS/cm
M3-2	Nunavik	Ni–Cu concentrator (effluent)	Thiosalts ($\mathbf{S_2O_3^{2-}}$, $\mathbf{S_4O_6^{2-}}$)	≈ 0.7 mS/cm
M3-3	Nunavik	Ni–Cu satellite mine (effluent)	Thiosalts ($\mathbf{S_2O_3^{2-}}$, $\mathbf{S_4O_6^{2-}}$)	≈ 0.3 mS/cm
GFH1	Abitibi	Copper smelter (final effluent)	Particulate and dissolved organic matter	≈ 0.5 mS/cm
GFH2	Abitibi	Copper smelter (intermediate control point)	Particulate and dissolved organic matter	≈ 0.5 mS/cm
GFH3	Abitibi	Copper smelter (intermediate control point)	Particulate and dissolved organic matter	≈ 0.5 mS/cm

6.3.2. Logical organization of the analytical procedures

The sequence of procedures involved in analysing the chemical composition and toxicity of effluents alongside the application of ozonation treatment is showed in Figure 6.1. The untreated effluents are used to perform chemical analyses and toxicity tests (Figure 6.1A). Following the initial characterisation, the pH of the effluents is adjusted to a value close to 9 (Figure 6.1B), prior to starting up of the ozonation treatment (Figure 6.1C). During ozonation, the pH of the effluent is controlled by adding NaOH to reach a target value of 9. The effluents treated by ozonation are then subjected to chemical analysis and toxicity testing (Figure 1D). This enables the chemical composition and toxicity of the effluents to be compared before and after treatment. The subsequent subsections provide a more detailed exposition of the methodological approaches employed in this analytical procedure.

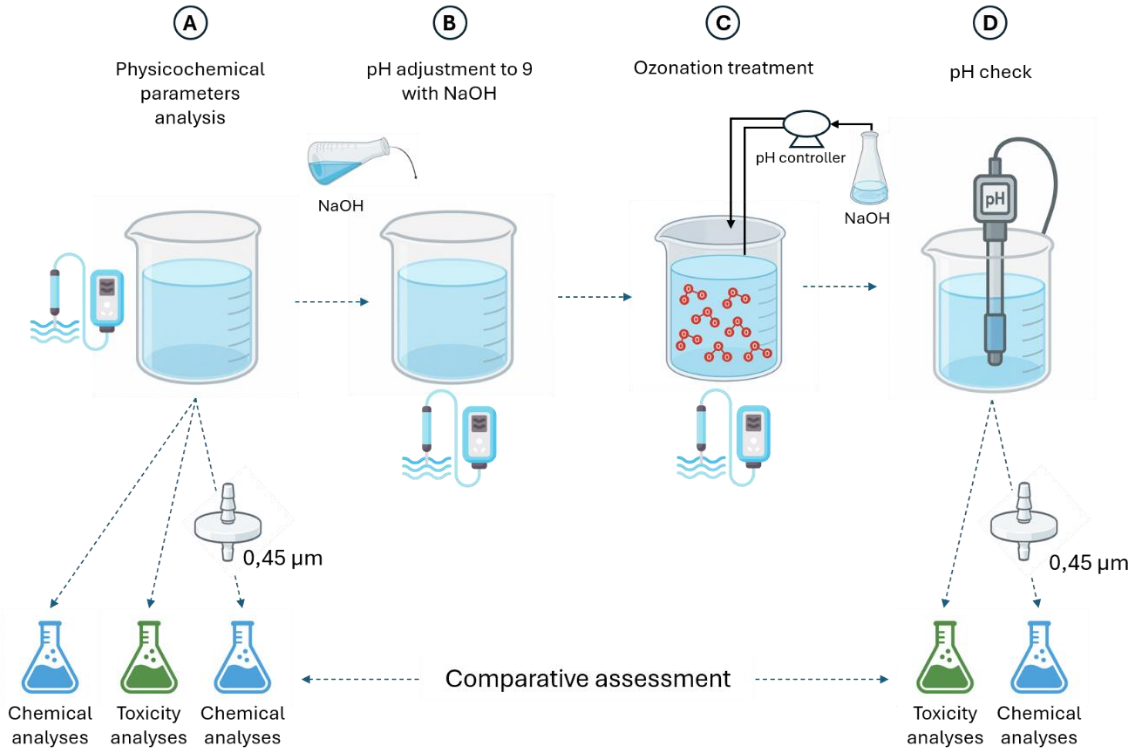

Figure 6.1 Analytical procedures and effluent treatment processes. When pH adjustment resulted in the formation of visible precipitates, a filtration step was added between steps B and C

6.3.3. Chemical analyses

Chemical analyses (major ions, both cations and anions, and trace elements) were conducted on raw (Figure 6.1A) and treated effluents (Figure 6.1D). These analyses were performed at the INRS laboratory in Quebec City, Canada. Anions (Cl^-, NO_3^-, NO_2^-, SO_4^{2-}) were quantified by ion chromatography (IC; PAC AS11-HC 4µm; Integrion HPIC from Thermo). Total and dissolved metals and metalloids were analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Agilent 5110 Dual View; US EPA Method # 200.7) and inductively coupled plasma mass spectrometry (ICP-MS; Thermo X series. US EPA Method # 200.8).

6.3.4. Acute toxicity tests

Acute toxicity tests were conducted on *D. magna* both on the raw (Figure 6.1A) and treated effluents (Figure 6.1D). The 48 h bioassays were conducted in accordance with the standard method of the *Centre d'expertise en analyse environnementale du Québec* (CEAEQ MA.500 – D.mag.1.1). The test organisms were less than 24 h old at the time of experiments. These were obtained from laboratory cultures that were maintained at 20 ± 2 °C, with a photoperiod of 16h:8h (light:dark) to simulate normal conditions, with water renewal every two days, and feeding with *Chlamydomonas reinhardtii*. Effluent dilutions (0%, 0.9%, 1.6%, 3%, 4.2%, 5.6%, 7.5%, 10%, 18%, 24%, 32%, 56%, 75%, 100%) were prepared using the raw and treated effluents and dilution water reconstituted to an alkalinity of 160-180 mg $\mathrm{CaCO_3}$/L. The tests were conducted in a static, non-renewal environment, utilising 10-mL tubes that each contained five daphnids. Four replicates were completed for each concentration ($\mathrm{n} = 20$ per condition). The tests involved the implementation of both negative control, which comprised the utilisation of only reconstituted water, and positive control, which incorporated the use of potassium dichromate solution ($\mathrm{K_2Cr_2O_7}$). Prior to exposure, the pH of treated samples (frequently > 9) was adjusted to 7.2 ± 0.2 with 0.1 N $\mathrm{H_2SO_4}$ to mitigate the potential for pH-induced toxicity. Ozonated water, akin to any other sample that has undergone treatment, was utilised for the purpose of further comparative analyses of biological effects on two endpoints related to mobility (impaired and complete loss), which were observed at 24 h and 48 h. This adjustment was made to mitigate response-related artefacts associated with the toxicity of extreme pH.

6.3.5. pH adjustment and ozone microbubble treatment

Treatment tests (Figure 6.1B-C) were conducted at the UQAT laboratory, in Rouyn-Noranda, Canada. The $\mathrm{NH_3 - N}$ was monitored using a selective electrode (Orion Thermo Fisher Scientific Orion for Ammonia) according to a standard method (APHA, 2017; Method 4500-$\mathrm{NH_3}$ D) (APHA, 2017), with a precision of precision of ~14 % RSD (corresponding to standard deviations of ±0.003–0.038 mg/L, depending on concentrations). Physicochemical parameters were monitored using a YSI Pro Plus multiparameter probe. The pH of the water was first adjusted by adding NaOH before ozone treatment (Figure 6.1B). This adjustment is intended to achieve an optimal pH close to 9, which is necessary for the ozone treatment process (Ryskie et al., 2023; Shekarian et al., 2025). Certain metals and metalloids may also precipitate at this stage

(Shekarian et al., 2025). The effluents were then treated in an 18 L continuous recirculation pilot system equipped with a Primozone® GM1 ozone generator producing up to 11 g O_3/h and a pump producing < 50 µm microbubbles (Figure 6.1C; Figure 6.2). During the ozonation process, sampling was conducted at 15 min intervals to monitor pH, ORP, EC, and $NH_3 - N$. Treatment continued until the residual $NH_3 - N$ concentrations decreased to < 0.1 mg N/L.

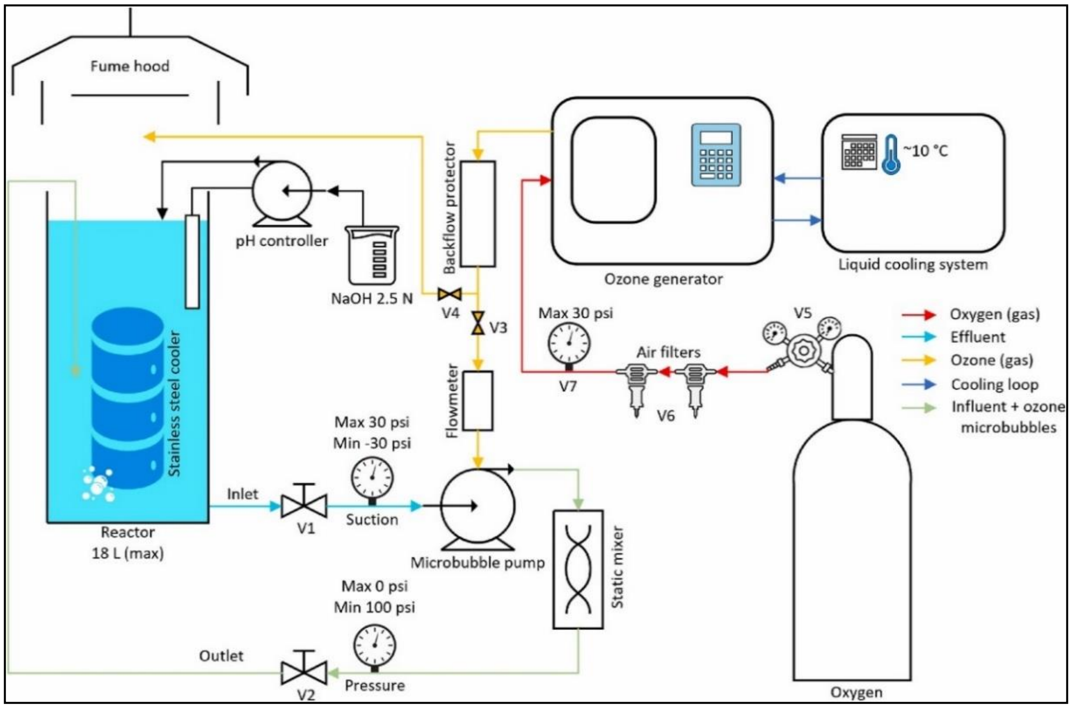

Figure 6.2 Schematic pilot-scale ozonation system under recirculated flow

(from ©Le Bourre, 2020)

6.4. Results and discussion

6.4.1. Physicochemical parameters (pH, EC)

The ozonation process requires pH adjustment to compensate for the decrease in pH caused by the oxidation of oxidizable species, including NH_3-N, sulfur compounds, and organic matter. The adjustment of pH also buffers the mineral acidity (H^+ release entailed by the precipitation of acidogenic Fe and Mn oxyhydroxides). The pH of the untreated, adjusted (before ozonation) and ozonated effluents is presented in Table 6.2. Despite the pH being adjusted every 15 min to a target value of ≈ 9 during ozonation, the M3 and GFH effluents exhibited pH below 9 at the end of the ozonation treatment. The decrease in final pH is most likely explained by the precipitation of Fe and Mn oxyhydroxides (Córdova-Udaeta et al., 2025), suggesting that the treated effluents did not reach an equilibrium state immediately after ozonation. The addition of NaOH for pH adjustment also explains the observed increase in effluents EC during treatment (Figure 6.3). The oxidation of $\mathrm{NH_3 - N}$ to $\mathrm{NO_3^-}$ is another process that can entail an increase in water EC. The precipitation of Fe and Mn oxyhydroxides (e.g.: $\mathrm{Fe(OH)_3(s)}$, $\mathrm{MnO_2(s)}$) likely acted as a competing mechanism that can decrease EC during ozonation through the removal of dissolved ions (Córdova-Udaeta et al., 2025). The EC of the M2 and GFH effluents gradually and unevenly increased during the ozonation process, as an indication that the removal of dissolved species by precipitation did not offset the increase in EC resulting from the addition of NaOH. The EC of the highly saline M1 effluent showed a marked increase at the onset of the ozonation process, followed by a slight decrease. This pattern likely reflects the simultaneous ion release from oxidation reactions and removal of dissolved species by adsorption and coprecipitation with newly formed minerals, including Fe and Mn oxides (Neculita et al., 2008).

Table 6.2 Evolution of pH values measured before, during and after microbubbles ozonation

	Before ozonation		**After ozonation**
	Initial pH	**pH after adjustment**	**Final pH**
M1	7.02	9.14	9.28
M2	7.63	9.2	9.02
M3-1	7	9.1	7.01
M3-2	6.72	9.5	8.71
M3-3	7.21	9.2	7.62
GFH1	6.2	9.08	7.82
GFH2	9.25	NA	7.46
GFH3	8.4	7.8	7.82

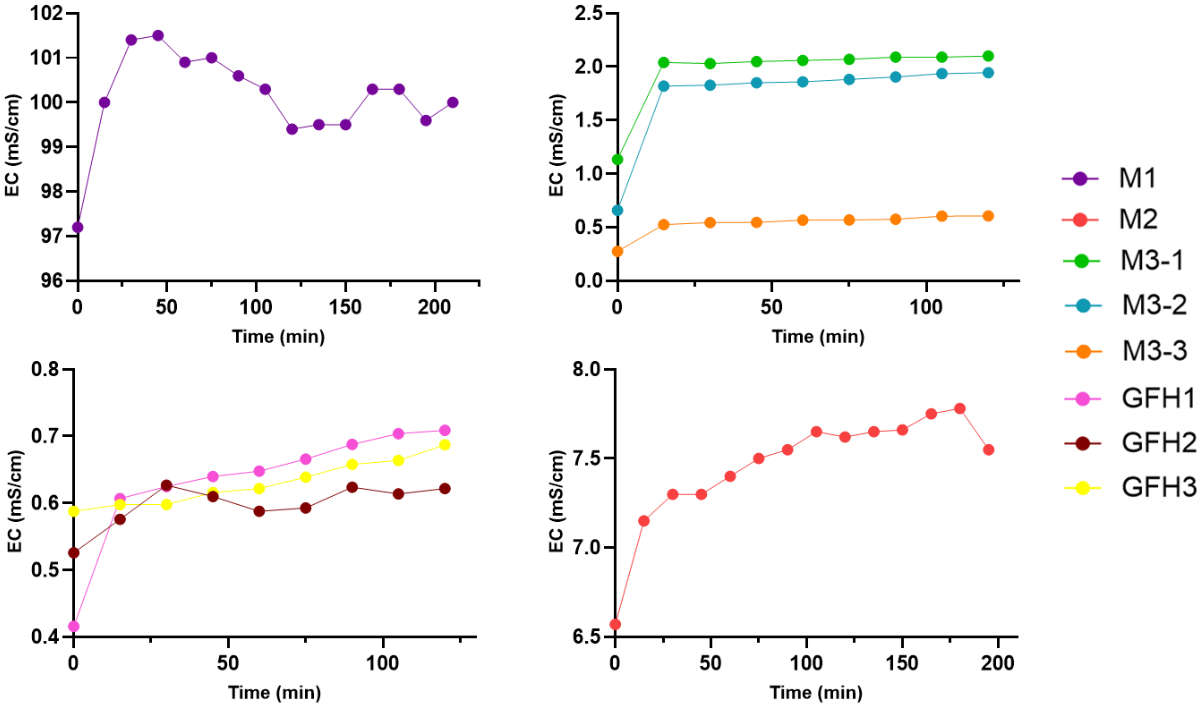

Figure 6.3 Evolution of water electrical conductivity (EC) during microbubble ozonation

6.4.2. Oxidizable species (N, S and organic matter)

The microbubbles ozonation process decreased $NH_3 - N$ concentrations to below the detection limit for all tested effluents. The change in $NH_3 - N$ concentrations over time varied depending on the effluents tested (Figure 6.4). The effluent from M1 is the only one that showed an increase in NH_3 concentration upon the initiation of the ozonation process. This phenomenon is most plausibly attributable to a measurement error, as the presence of S and N compounds that could be precursors to $NH_3 - N$ is not suspected in this effluent. The data further suggest that this increase in $NH_3 - N$ is quickly reversed during the ozonation process, as demonstrated by the linear decrease in concentrations after the peak concentration is reached. For this effluent, the removal of $NH_3 - N$ to a value < DL required approximately 200 min. The results on the M1 effluent show that the decrease in $NH_3 - N$ concentration was slower at the beginning of the ozonation process, reaching a maximum value after around 75 min. This observation suggests that other oxidizable species, including $NH_3 - N$ precursors, were competing with $NH_3 - N$ oxidation in the early stages of ozonation. In the case of M3 and GFH effluents, the initial NH_3 concentrations were already below 10 mg/L and 0.20 mg/L, respectively. Nonetheless, in both cases, the $NH_3 - N$ concentration fell below the detection limit in less than 50 min. Overall, the results demonstrate the effectiveness of the ozonation system in removing $NH_3 - N$ over a wide range of concentrations and salinity levels.

Here, the only N-compounds that were analyzed are $NH_3 - N$, NO_2^- and NO_3^-. However, certain effluents may contain other N-containing species, such as thiosalts, cyanates, thiocyanates, and organic matter. Nevertheless, a nitrogen mass balance (Table 6.3) can be used to estimate the proportion of nitrogen oxidised to NO_3^- that originates from unmeasured sources (other than $NH_3 - N$ and NO_2^-). The results suggest that the difference between the increase in NO_3^- concentration and the decrease in $NH_3 - N$ and NO_2^- concentrations is negligible for the M3 and GFH effluents. This indicates that the primary N-based compounds in these effluents are $NH_3 - N$, NO_2^- and NO_3^-, and that the contribution of other N compounds is minimal. The data associated with the M1 and M2 effluents suggest excess NO_3^- production relative to the loss of $NH_3 - N$ and NO_2^- during ozonation. This suggests that other oxidisable nitrogen compounds (not measured here) might have been present in the effluents prior to ozonation. It should be noted that this balance is only approximate, given that the accuracy of $NH_3 - N$ measurements made with the selective probe.

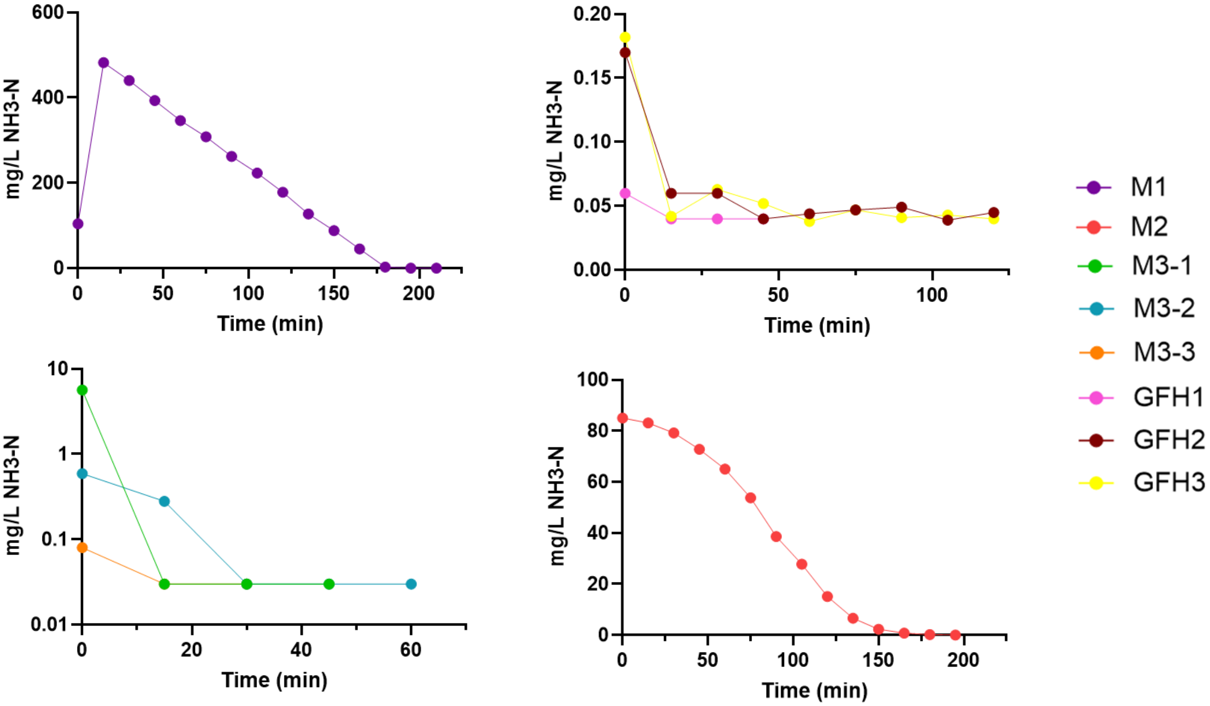

Figure 6.4 Evolution of ammoniacal nitrogen ($NH_3 - N$) during microbubble ozonation

Table 6.3 Nitrogen mass balance for the effluents that have undergone ozone oxidation. All concentrations are in mg-N/L

		$\mathbf{NH_3-N}$	$\mathbf{NO_2^- - N}$	$\mathbf{NO_3^- - N}$	$\mathbf{\Delta NH_3 - N}$	$\mathbf{\Delta NO_2^- - N}$	$\mathbf{\Delta NO_3^- - N}$	**Balance**
M1	**Before O_3**	104.00	90.83	608.17	-103.99	-90.83	221.23	26.41
	After O_3	0.01	<DL	829.40				
M2	**Before O_3**	85.00	1.04	18.89	-85.00	-0.93	128.18	42.25
	After O_3	<DL	0.11	147.07				
M3-1	**Before O_3**	5.64	0.47	29.15	-5.61	-0.47	4.29	-1.78
	After O_3	0.03	0.01	33.45				
M3-2	**Before O_3**	0.59	<DL	3.19	-0.56	0.00	2.12	1.56
	After O_3	0.03	<DL	5.31				
M3-3	**Before O_3**	0.08	0.01	1.66	-0.05	-0.01	-0.03	-0.08
	After O_3	0.03	0.01	1.63				
GFH1	**Before O_3**	0.06	0.01	0.11	-0.03	-0.01	0.20	0.16
	After O_3	0.03	<DL	0.31				
GFH2	**Before O_3**	0.17	0.01	0.21	-0.12	-0.01	0.32	0.19
	After O_3	0.05	<DL	0.52				
GFH3	**Before O_3**	0.18	0.01	0.24	-0.14	-0.01	0.39	0.23
	After O_3	0.04	<DL	0.63				

6.4.3. Major ions, fluoride and bromide

The results of the major ion analyses are presented in Table 6.4. Results show increases in Na concentrations after ozonation, consistent with the addition of NaOH during treatment for pH adjustment. Fluoride concentrations also increased during ozonation in all effluents. This may be due to the release of F^- from mineral or colloidal phases under high pH and oxidative conditions, consistent with similar observations reported in studies focusing on alkaline waters and AOPs (Fuge, 2019; Reimann et al., 2003). This increase in fluoride concentrations may pose an ecological concern since concentrations as low as 1 mg/L may entail sublethal effects on aquatic invertebrates (Camargo, 2003). Bromide, when present, was consistently reduced to < 0.3 mg/L after ozone treatment. Bromide has a very high reaction rate with ozone and hydroxyl radical, subsequently oxidizing to bromate (Morrison et al., 2023).

Table 6.4 Summary of results for the main cations and anions concentrations in the effluents before and after ozonation

		Alkalinity (mg/L $CaCO_3$)	**F^- (mg/L)**	**Cl^- (mg/L)**	**SO_4^{2-} (mg/L)**	**Br^- (mg/L)**	**Na^+ (mg/L)**	**K^+ (mg/L)**	**Ca^{2+} (mg/L)**	**Mg^{2+} (mg/L)**
M1	**Before O_3**	96	< DL	52769	1279	< DL	18430	875	6378	2417
	After O_3	64	0.57	55151	1298	< DL	18200	930	6036	2327
M2	**Before O_3**	131	0.28	411	2867	< DL	887	316	292	47,8
	After O_3	76	0,521	407	3481	< DL	1635	312	75.2	47.7
M3-1	**Before O_3**	22.9	0.057	594	152	7.31	19	4.32	325	36.9
	After O_3	59	0.383	526	125	< DL	75.1	3.88	263	31.6
M3-2	**Before O_3**	71	0.188	176	812	4.43	125	9.68	245	40
	After O_3	61.3	0.45	177	914	< DL	178	9.47	188	38
M3-3	**Before O_3**	61.8	0.057	47.5	112	0.53	62	0.986	35.1	10.1
	After O_3	119.8	0.377	40.9	100	< DL	88.6	0.92	20.9	8.71
GFH1	**Before O_3**	39.8	0.205	38.3	152	1.13	33.8	2.72	61.8	9.74
	After O_3	100.5	0.328	49.5	156	< DL	90.9	3.19	34.5	9.42
GFH2	**Before O_3**	33.2	0.129	66.3	166	< DL	38.3	1.52	63.8	6.76
	After O_3	58	0.439	66.4	140	< DL	67.3	1.86	43.4	6.78
GFH3	**Before O_3**	20.8	0.115	57.7	177	< DL	36.8	1.73	63.4	8.4
	After O_3	70.8	0.318	55.6	142	< DL	77.4	1.69	45.4	8.04

6.4.4. Trace element concentrations

The combined treatment of alkaline pH adjustment (NaOH, pH 9) and microbubble ozonation induced measurable changes in the concentrations of most dissolved metals and metalloids across all effluents (Table 6.5). The effects were site- and element-dependent, reflecting the interplay of oxidation, precipitation, and colloidal redistribution processes. Similar patterns of metal removal and mobilization have been reported during pilot-scale trials of microbubble ozonation in mining effluents (Ryskie et al., 2023).

Fe and Mn exhibited consistent decreases after treatment in most effluents. For instance, Fe decreased from 0.067 to 0.003 mg/L for the GFH1 effluent (−95%), while Mn decreased from 0.427 to 0.002 mg/L for the M1 effluent (−99.5%). Such results are consistent with the oxidation of Fe^{2+} and Mn^{2+} to oxides and hydroxides under alkaline and oxidizing conditions (von Gunten, 2018). Aluminum concentrations decreased in most effluents (e.g., GFH1: 0.071 → 0.013 mg/L), an observation that could indicate the sorption of Al^{3+} onto $Fe - Mn$ oxides and hydroxides or the precipitation of Al hydroxides (Bazilevskaya, 2011).

Cu and Ni showed somehow contrasting behaviors. For the M2 effluent, both metals decreased sharply (Cu: 0.385 → 0.009 mg/L; Ni: 1.18 → 0.020 mg/L), consistent with adsorption/coprecipitation onto freshly formed $Fe - Mn$ oxides and hydroxides (Seridou et al., 2021). Similar decreases were also recorded at GFH1 and GFH3. In contrast, some other effluents displayed marked increases (e.g., Cu at M3-1: 0.006 → 0.055 mg/L; Ni at GFH2: 0.013 → 0.027 mg/L). Such increases may reflect colloidal redistribution processes triggered by ozonation, as suggested in previous studies focusing on microbubble ozonation tests on gold mine effluents in Québec (Ryskie et al., 2023).

Zn concentrations increased significantly in most effluents, sometimes by more than one order of magnitude. For example, Zn concentrations rose from 0.006 to 0.376 mg/L, in the M3-1 effluent, and from 0.019 to 0.299 mg/L, in the GFH2 effluent. Increases in Zn concentrations were also observed in the M3-2, M3-3, M2, and GFH3 effluents. Decreases in Zn concentrations were only observed at M1 (0.012 → <DL) and GFH1 (0.050 → 0.036 mg/L). The increases in Zn concentrations may be linked to the oxidative destabilization of sulfide phases (ZnS) and subsequent release of Zn^{2+}, despite the tendency of alkaline conditions to promote the precipitation of Ni and Zn bearing carbonates or hydroxides (Liang, 2024). A comparable

release of Zn under ozonation was highlighted before and the phenomenon was attributed to the oxidation of fine sulfide-rich particles (Ryskie et al., 2023).

The removal of As was site-specific. In the M2 effluent, concentrations decreased substantially from 0.45 to 0.069 mg/L (−85%), suggesting the oxidation of As(III) to As(V) and subsequent potential sorption onto Fe oxides and hydroxides (Dixit & Hering, 2003). Conversely, small increases in As concentrations were detected in the GFH1, GFH3, and GFH2 effluents, where As concentrations rose from below detection limits to ~0.006–0.007 mg/L. This could result from competition with carbonate, phosphate, or silicate anions, which can reduce As(V) retention on $Fe(OH)_3$ surfaces (Smedley & Kinniburgh, 2002).

Cr concentrations systematically increased after treatment, from below detection limits to measurable values (e.g.: GFH1: <0.0004 → 0.0174 mg/L). This observation suggests the oxidation of Cr(III) to Cr(VI), a more soluble and toxic form that is stable under alkaline and oxidizing conditions (von Gunten, 2018). Specific speciation analyses would be required to confirm the presence of Cr(VI) after ozonation.

Cd generally remained close to detection limits, with small decreases in the GFH1, GFH2, and GFH3 effluents, and a minor increase in the M3-1 effluent. Lead dropped below detection in the GFH1 effluent and remained unchanged in the other effluents. Selenium remained stable in all effluents.

From a toxicological perspective, the removal of Mn^{2+}, Cu^{2+}, and Ni^{2+} is a substantial benefit as these metals are bioavailable and toxic to *D. magna* (Norwood et al., 2003). Fe^{2+} is usually less toxic at environmental concentrations but removing it as well could be advantageous since it decreases interactions that alter the mobility of the other metals. However, two side effects raise concern: (i) the generalized release of Zn^{2+}, a metal with well-documented chronic and acute toxicity to cladocerans (De Schamphelaere & Janssen, 2004), and (ii) the likely formation of Cr(VI), whose toxicity and mobility are considerably higher than those of Cr(III). These results are in line with previous findings, which also reported that microbubble ozonation reduced acute toxicity in gold mine effluents only when combined with metal pretreatment steps (Ryskie et al., 2023). The findings further highlight the need for process optimization and coupling with polishing treatments (e.g., clarification, adsorption onto Fe oxides or activated carbon) to stabilize residual metals and prevent residual toxicity after treatment.

Table 6.5 Summary of results for the main metals and metalloids concentrations in the effluents before and after ozonation

	#	**Al**	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Se**	**Zn**
M1	**Before O_3**	0.44	<DL	<DL	<DL	<DL	<DL	<DL	0,427	<DL	<DL	0.012
	After O_3	0.58	<DL	<DL	<DL	0.018	<DL	<DL	0.002	<DL	<DL	<DL
M2	**Before O_3**	0.037	0.45	<DL	1.19	<DL	0.385	0.004	0.021	1.18	0.34	0.006
	After O_3	0.02	0.069	<DL	0.77	0.02	0.009	0.035	0.011	0.02	0.33	0.017
M3-1	**Before O_3**	0.033	<DL	0.0003	0.003	<DL	0.006	0.008	0.143	0.194	<DL	0.006
	After O_3	0.028	<DL	0.0006	<DL	0.0099	0.055	0.001	0.038	0.22	<DL	0.376
M3-2	**Before O_3**	0.03	<DL	<DL	<DL	<DL	0.003	0.006	0.051	0.109	<DL	0.01
	After O_3	0.026	<DL	<DL	<DL	0.008	0.016	0.001	0.033	0.051	0.005	0.106
M3-3	**Before O_3**	0.211	<DL	<DL	<DL	<DL	<DL	0.009	0.03	0.22	<DL	0.005
	After O_3	0.0123	<DL	<DL	<DL	0.0057	0.016	0.002	0.01	0.02	<DL	0.073
GFH1	**Before O_3**	0.071	<DL	0.002	<DL	<DL	0.061	0.067	0.03	0.011	<DL	0.05
	After O_3	0.0134	0.007	0.0004	<DL	0.0174	0.026	0.003	0.003	0.009	<DL	0.036
GFH2	**Before O_3**	0.057	<DL	0.0011	<DL	<DL	0.027	0.026	0.013	0.013	<DL	0.0186
	After O_3	0.0091	0.006	0.0007	<DL	0.0062	0.045	0.008	0.01	0.027	0.005	0.299
GFH3	**Before O_3**	0.089	<DL	0.0011	<DL	<DL	0.036	0.012	0.048	0.02	<DL	0.014
	After O_3	0.0136	0.007	0.0007	<DL	0.0083	0.02	0.004	0.029	<DL	<DL	0.053

6.4.5. Acute toxicity to *D. magna*

The alkaline microbubble ozonation treatment consistently reduced acute toxicity across all effluents tested with *D. magna* (Figure 6.5). Before treatment, the toxicity expressed in toxic units (TU) spanned nearly two orders of magnitude, from relatively low values at M3-1 (0.46 TU) and M3-3 (0.72 TU), to highly toxic conditions at M1 (16.2 TU) and M2 (14.1 TU). After ozonation, toxicity decreased at all sites, ranging from 0.14 TU to 10.9 TU. The most substantial decreases were observed at M2 (-73%) and M3-1 (-70%), while moderate reductions occurred at M1 (-33%), GFH3 (-58%), and GFH2 (-66%).

From an ecotoxicological perspective, the conversion of $\mathrm{NH_3}$ to $\mathrm{NO_3^-}$ represents a significant detoxification process. Un-ionized ammonia (NH_3) is highly toxic to *D. magna* and fish, with LC_{50} values typically in the sub-mg/L range (CCME, 2010), whereas $\mathrm{NO_3^-}$ is much less toxic at environmentally relevant concentrations (Camargo et al., 2005). Thus, even though EC increased due to nitrate accumulation, the replacement of $\mathrm{NH_3}$ by $\mathrm{NO_3^-}$ is advantageous for reducing acute toxicity. However, attention must be paid to the transient formation of $\mathrm{NO_2^-}$, which is also toxic to aquatic organisms when present at elevated concentrations (Zhang et al., 2021; Ryskie et al., 2023). Here, the absence of $\mathrm{NO_2^-}$ buildup in the treated effluents indicates that ozonation favored its rapid conversion to the more stable $\mathrm{NO_3^-}$ under the applied conditions ($pH \approx 9$, high oxidant availability) (Ryskie et al., 2023; Shahedi et al., 2024). The toxicity reductions could also be related to the removal of redox-sensitive metals $(\mathrm{Fe}, \mathrm{Mn})$ and bioavailable cations such as $\mathrm{Cu^{2+}}$ and $\mathrm{Ni^{2+}}$, whose concentrations dropped by >90% at several sites. The precipitation of $\mathrm{Fe(OH)_3}$ and $\mathrm{MnO_2}$ and the subsequent adsorption/coprecipitation of trace metals are well-established mechanisms that may potentially explain these detoxification effects (Lee et al., 2002).

However, although the microbubble ozonation process shows promising results in terms of toxicity reduction, certain key questions should be investigated in greater depth to better understand the key processes occurring during the treatment process:

1. Zn mobilization: Post-treatment increases in $\mathbf{Zn}$ concentrations were observed in most M3 effluents and in effluent GFH2 (e.g., 0.019 → 0.299 mg/L). This observation is consistent with the oxidative destabilization of $\mathbf{ZnS}$. The $\mathbf{Zn^{2+}}$ is highly toxic to cladocerans, and, if present, its release may partially offset the benefits ozonation (De

Schamphelaere & Janssen, 2004). Further studies on Zn mobilization during ozonation would be required to clearly identify the mechanisms involved.

2. Speciation calculations: It would be relevant to evaluate the speciation o redox-sensitive species before and after ozonation treatment. For example, in the present study, chromium concentrations increased in all effluents, likely reflecting oxidation of $\mathbf{Cr(III)}$ to $\mathbf{Cr(VI)}$, a soluble and highly toxic species under alkaline and oxidizing conditions (von Gunten, 2018). Even small concentrations can contribute disproportionately to toxicity. A better understanding of redox-sensitive elements (e.g., Cr, As, Se) speciation before and after ozonation seems much needed.
3. F mobilization: A generalized increase in $\mathbf{F^-}$ was detected (e.g., M1: <0.1 → 0.57 mg/L), potentially reaching levels of ecotoxicological concern, since concentrations approaching 1 mg/L have been shown to impair aquatic invertebrates (Camargo, 2003). A better understanding of the sources of fluorine-containing compounds and their transformation pathways during ozonation is required.
4. Ionic strength effects: Increases in $\mathbf{Na^+}$ and alkalinity at several sites (e.g., GFH1, GFH3) may have influenced stress responses in *D. magna* by altering nonregulation, as reported in studies on cladocerans exposed to high EC waters (Morris et al., 2021). The results revealed that the post-treatment toxicity (10.9 TU) remains critical for the M1 effluent. This residual toxicity is most likely induced by the high salinity of the effluent (van Dam et al., 2014). A better understanding of the causes of residual toxicity, including the identification of the potential toxicant(s), would be needed.

The findings from the present study are consistent with previous pilot-scale studies of microbubble ozonation applied to mining effluents. A lime-based pretreatment (without filtration of produced TSS) followed by ozonation reduced the acute toxicity of effluents to *D. magna* but also noted an increase in toxicity due to the mobilization of Zn and colloidal metal oxides (Ryskie et al., 2023). It is possible that a similar process affected the effluents tested here. From an ecotoxicological point of view, the removal of Fe^{2+}, Mn^{2+}, Cu^{2+}, and Ni^{2+} might play a major role in detoxification since such species are among the most bioavailable and those which show acute toxicity to *D. magna*. However, the high toxicity of the M1 and GFH3 effluents, coupled with the associated risk factors of Zn, Cr(VI) and F^- mobilisation, suggests that ozonation alone may not be sufficient for all effluents. This further supports that ozonation at alkaline pH is a powerful yet incomplete detoxification process. For the achievement of toxicity reduction and stability, a complementary post-treatment process such as clarification, adsorption onto Fe oxides or granular activated carbon is also necessary (Ryskie et al., 2023).

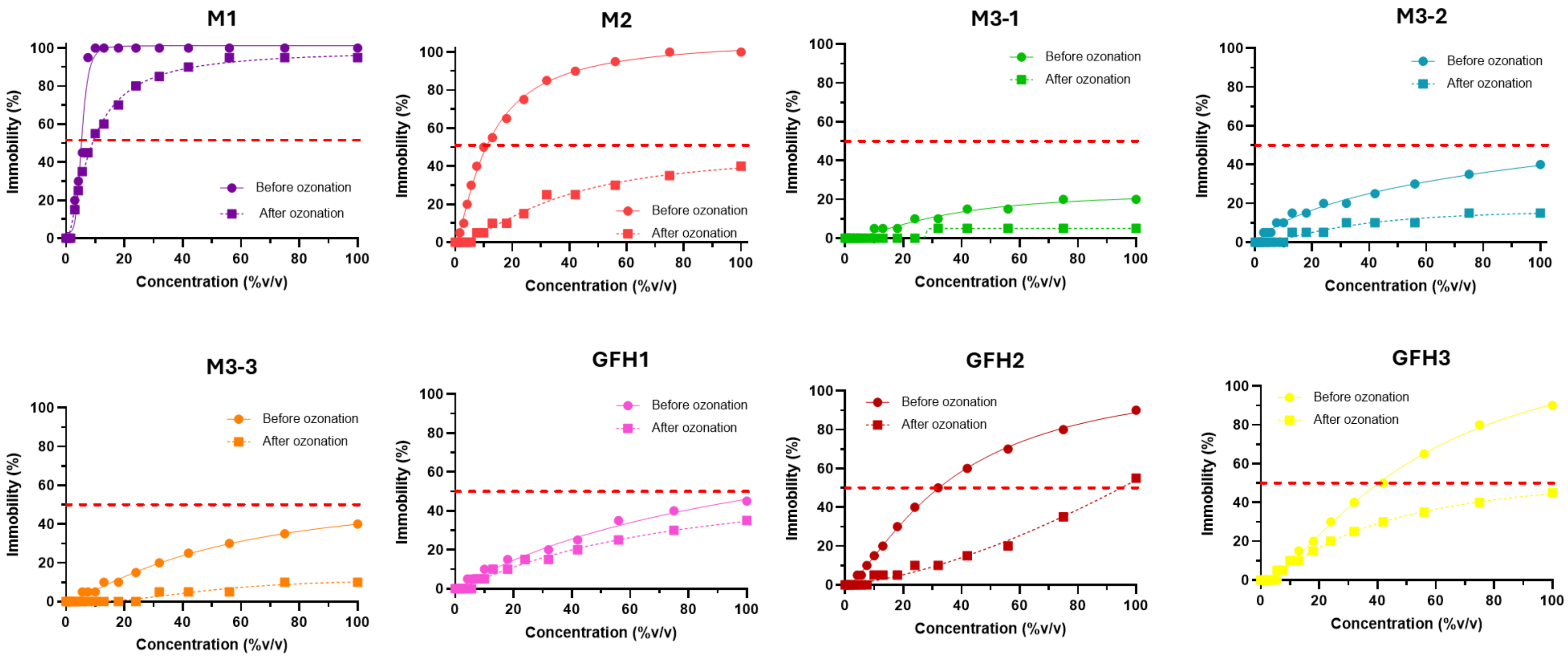

Figure 6.5 Dose-response toxicity relationships for the tested effluents before and after ozonation

6.5. Conclusion

The objective of this study was to develop new knowledge regarding the potential use of microbubble ozonation with pH control (target value of 9) to reduce the toxicity of mining and metallurgical effluents. The effluents were found to contain three primary categories of contaminants: (1) metals and metalloids, (2) salinity, and (3) oxidizable species, such as $NH_3 - N$. The results revealed a quantifiable decline in the toxicity of all effluents following ozonation. These findings suggest that the removal of oxidizable contaminants allows for reducing the toxicity of the effluents under investigation. In addition to the removal of oxidizable substances (including $NH_3 - N$), microbubble ozonation treatment resulted in a decrease in Fe and Mn concentrations, suggesting the precipitation of oxyhydroxides. Numerous inquiries persist regarding the precise origins of toxicity prior to and following treatment. The following research areas have been identified as priorities: (1) the evaluation of the processes involved in Zn release during ozonation; (2) the evaluation of the processes involved in F mobilisation during ozonation; and (3) the evaluation of the influence of treatment on the speciation of dissolved species sensitive to Eh-pH conditions (e.g.: Cr, As, Se). A more precise assessment of the threshold at which the salinity of treated effluents can cause toxicity would be required, particularly because ozonation requires pH adjustment, which leads to a measurable increase in effluent salinity. The results of this study demonstrate the value of developing and optimizing microbubble ozonation processes for controlling the toxicity of industrial and mining effluents.

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CHAPITRE 7 Discussion générale

7.1. Intégration des résultats et apports scientifiques

Les résultats de ce travail de recherche apportent des éléments de compréhension sur les mécanismes responsables de la toxicité des effluents miniers et métallurgiques et des pistes d'action pour leur réduction.

Le premier volet (**Chapitre 4**) a fourni de nouvelles connaissances en lien avec la toxicité d'un effluent de la Fonderie Horne en Abitibi-Témiscamingue. Il s'agit d'un effluent au sein duquel des concentrations des contaminants sont généralement sous les critères réglementaires mais qui engendre la toxicité envers *D. magna* de façon épisodique. Les analyses statistiques réalisées sur le jeu de données historique ont permis de mettre en évidence le rôle prépondérant du Cu et du Cd parmi les causes de la toxicité. Les calculs de spéciation suggèrent pour leur part que les formes ioniques libres, Cu^{2+} et Cd^{2+}, pourraient fortement influencer la toxicité de l'effluent. Les données tendent également à indiquer que sous certaines conditions, le Fe pourrait intervenir indirectement comme élément protecteur inhibant la toxicité. En effet, sa concentration généralement plus élevée dans les échantillons non toxiques par rapport aux échantillons toxiques et sa corrélation inverse avec la toxicité supportent l'hypothèse voulant qu'il puisse limiter la biodisponibilité de contaminants tels que Cu et le Cd et ainsi inhiber la toxicité. L'ensemble de ces résultats montre que la biodisponibilité, et non la concentration totale, détermine la toxicité aiguë. Ces travaux permettent à la fois d'appuyer certains constats déjà identifiés dans la littérature scientifique et de fournir de nouvelles connaissances en matière d'évaluation des causes de la toxicité. D'une part, les constats relatifs à la toxicité du Cu et du Cd et à leurs formes biodisponibles (Cu^{2+} et Cd^{2+}) renforcent une imposante littérature portant sur ce sujet (Di Toro et al., 2001). D'autre part, le développement et l'application de calculs statistiques appliqués à un jeu de données historiques impliquant des données géochimiques (données compositionnelles) et des données relatives à des essais de toxicité (considérée ici comme une valeur binaire, toxique ou non toxique) s'avère scientifiquement original. En effet, l'intégration couplée de données géochimiques compositionnelles et de données relatives à la toxicité au sein de calculs statistiques complémente des développements récents liés à l'analyse de données compositionnelles (Filzmoser et al., 2018) et à l'analyse de données écotoxicologiques par approches statistiques (Norwood et al., 2003; Jonker et al., 2005; Backhaus & Faust, 2012). Sur le plan sociétal, ces résultats fournissent des connaissances nouvelles susceptibles de permettre une meilleure gestion de l'effluent analysé. Le Fe, inversement corrélé à la toxicité, apparaît comme un facteur pouvant limiter la biodisponibilité du Cu^{2+} et du Cd^{2+}. Cette compréhension peut appuyer les décisions de gestion et l'ajustement des stratégies de traitement.

Ces constats ont trouvé un prolongement direct dans le second volet de la thèse (**Chapitre 5**) consacré à l'expérimentation sur des mélanges synthétiques. Des tests ont été menés sur des solutions avec un contaminant unique (As, Cu, Zn, Se) puis sur des mélanges binaires et ternaires de ces contaminants. Les bioessais sur *D. magna* tendent à supporter les constats issus du **chapitre 4** et montrent que les mélanges binaires et ternaires entraînent des effets toxiques qui ne se limitent pas à la simple additivité. Des effets synergiques marqués ont été observés pour les mélanges binaires Cu–Zn et Zn–Se, lesquels se sont avérés plus toxiques que ce qui pouvait être estimé par l'addition des effets induits par les substances prises séparément. Les autres combinaisons ont principalement montré des effets additifs, révélant que l'occurence de conditions de synergie ne peut être généralisée à toutes les paires de contaminants dans le groupe de substances testées (As, Cu, Zn, Se). Les mélanges ternaires se caractérisent par une complexité plus marquée que les mélanges binaires. Par exemple, le mélange Cu-Zn-Se semble présenter des niches de toxicité circonscrites et liées aux synergies fortes sur les couples binaires dominants. Les mélanges As-Cu-Se et As-Cu-Zn ont pour leur part montré des effets de synergie qui ne pouvaient être anticipés des résultats issus des mélanges binaires. Les résultats du travail ont également mis en évidence le rôle du Cu comme contaminant toxique et les fonctions modulatrices du Se, lequel peut exacerber ou inhiber la toxicité selon les caractéristiques des mélanges. L'application des modèles analytiques (isobologrammes, MIXTOX, MixModel) a été centrale à l'identification des comportements non additifs entre les contaminants. Les diagrammes ternaires issus de MixModel ont pour leur part permis de préciser les zones critiques de toxicité, notamment pour les mélanges riches en Cu, en révélant le lien entre spéciation, biodisponibilité et effets combinés. Ces résultats soulignent que les approches réglementaires actuelles, fondées principalement sur les concentrations des substances considérées de façon individuelle, ne rendent pas compte des risques engendrés par des effluents complexes. Ces travaux sont originaux sur le plan scientifique principalement en raison de l'évaluation de la toxicité de mélanges ternaires. En effet, la part la plus imposante de la littérature scientifique relative à la toxicité des mélanges porte sur des combinaisons binaires de contaminants (Cedergreen, 2014; Arreguín-Rebolledo et al., 2024). Cela s’explique notamment par la complexité des modèles requis pour évaluer les mécanismes de toxicité au sein des mélanges ternaires (Backhaus & Faust, 2012). Les graphiques produits (isobologrammes et diagrammes ternaires) permettent aussi de compléter les travaux récents (Dupraz et al., 2019) en étendant l’analyse des interactions du cadre binaire vers un espace ternaire. Cette approche offre une représentation quantitative de la surface de réponse des mélanges à trois composantes et permet de tester empiriquement si les effets ternaires peuvent être prédits à partir des résultats binaires.

Enfin, le troisième volet de la thèse (**Chapitre 6**) a permis de complémenter les connaissances issues des chapitres 5 et 6 par l’évaluation de l’influence des composés oxydables, jusque-là non considérés spécifiquement dans l’évaluation de la toxicité des mélanges, sur la toxicité d’effluents réels, dont celui ayant fait l’objet du **chapitre 5**. Des essais d’ozonation à microbulles en conditions alcalines ont révélé une grande efficience de la méthode, notamment pour l’enlèvement de l’azote ammoniacal (>90% selon les cas) et la réduction de la toxicité pour tous les effluents testés. Le traitement a aussi engendré une diminution mesurable des concentrations en Fe et Mn, lesquels ont vraisemblablement précipité sous forme d’oxydes ou hydroxydes insolubles. Le rendement de la méthode en matière de réduction de la toxicité dépend des caractéristiques des effluents. La présence de précurseurs de l’azote ammoniacal, comme les cyanates, semble générer la production de NH_3 lors de l’initiation de l’ozonation, un processus en compétition avec l’oxydation du NH_3. L’ozonation, qui a impliqué ici l’ajustement du pH par ajout de NaOH, engendre une augmentation mesurable de la salinité des effluents. Cette salinité peut engendrer des effets toxiques, ce qui limite l’applicabilité de la méthode à des effluents dont la salinité post-traitement est en deçà du seuil de toxicité induit par la force ionique de la solution. Ces travaux représentent une contribution scientifique originale notamment parce que l’application du traitement par ozonation à microbulle pour les effluents miniers et métallurgiques demeure très peu explorée dans la littérature scientifique. Les nouvelles connaissances produites dans le cadre de cette thèse complémentent directement les travaux de Marcotte et al. (2022) et Ryskie et al. (2023). Les retombées sociétales pourraient se manifester à plus long terme, lorsque la méthode pourra être appliquée à l’échelle du pilote de terrain et éventuellement, à l’échelle de la réalité d’un site industriel.

Les nouvelles connaissances véhiculées dans cette thèse excèdent la somme des nouvelles connaissances véhiculées dans les **chapitres 4**, **5** et **6** en raison des liens qui peuvent être directement établis entre ces contributions (Tableau 7.1). Dans son ensemble, cette thèse de doctorat constitue une contribution en lien avec l’évaluation et le contrôle de la toxicité d’effluents miniers et métallurgiques. Sur le plan méthodologique, un cadre analytique regroupant la chimie, l’écotoxicologie et un procédé de traitement avancés a été proposé. La contribution expérimentale de cette recherche repose sur les nouvelles connaissances concernant les interactions des éléments métalliques d'un mélange et leur influence sur la toxicité. Les résultats illustrent l'existence d'effets additifs et synergiques et soulignent la prépondérance des cations libres dans le déclenchement de la toxicité. Ces observations soulignent l'importance d'étudier les contaminants dans leur complexité et non dans une approche fragmentée. Enfin, sur le plan appliqué, cette thèse montre que l'ozonation par microbulles peut être une technologie prometteuse et novatrice pour réduire la toxicité des effluents.

Tableau 7.1 Sommaire des principales contributions scientifiques novatrices (individuelles et combinées) des chapitres constituant le cœur de la thèse

	Chapitre 4	**Chapitre 5**	**Chapitre 6**
Chapitre 4	Développement d’approches statistiques novatrices visant à élucider les liens entre la toxicité des effluents et leur composition chimique	**Le chapitre 5** complémente directement le chapitre 4 en permettant une évaluation plus précise des effets d’antagonisme, de synergie et d’additivité entre les contaminants. **Le chapitre 5**, qui s’appuie sur des solutions synthétiques plus simples que les effluents réels étudiés au **chapitre 4**, permet le déploiement de modèles mathématiques plus poussés pour l’identification des mécanismes responsables de la toxicité.	**Le chapitre 6** complémente directement **les chapitres 4 et 5** en ce sens qu’il permet d’aborder le rôle des substances oxydables, peu considérées dans **les chapitres 4 et 5**, sur la toxicité d’un effluent industriel réel. Le couplage des résultats issus du **chapitre 6** à ceux des **chapitres 4 et 5** illustre l’importance d’évaluer la toxicité résultant non seulement des métaux et métalloïdes, mais également des espèces oxydables et de la salinité résiduelle. Cela illustre la nécessité de réaliser des essais de toxicité sur des solutions synthétiques (mélanges binaires et ternaires) contenant des substances oxydables (p. ex.: NH_3 ou matière organique) et d’intégrer les substances oxydables à l’analyse statistique de séries temporelles.
Chapitre 5	Pris ensemble, **les chapitres 4, 5 et 6** fournissent des connaissances nouvelles sur les causes de toxicité de l’effluent de la Fonderie Horne.	Développement et application d’approches graphiques permettant d’évaluer les effets toxiques non additifs au sein de mélanges ternaires de contaminants	
Chapitre 6	De surcroît, la combinaison des trois chapitres fournit un éventail de méthodes novatrices pour l’analyse de la toxicité des effluents miniers et métallurgiques (méthodes statistiques, isobologrammes et diagrammes ternaires) ainsi que des développements en matière de traitement des effluents par ozonation à microbulles.		Évaluation quantitative de la réduction de toxicité résultant de l’enlèvement des contaminants oxydables d’effluents réels

7.2. Réponse aux objectifs et tests d’hypothèses

L’objectif principal de cette thèse était de contribuer à améliorer la compréhension intégrée des mécanismes responsables de la toxicité des effluents miniers et métallurgiques. La démarche de recherche s’est articulée autour de trois objectifs spécifiques (OS) associés à des hypothèses (H) de recherche :

OS1 : Évaluer s’il existe des relations statistiques significatives entre les concentrations de métaux et métalloïdes dissous et le niveau de toxicité d’un effluent industriel et des différences géochimiques statistiquement significatives entre les échantillons toxiques et non toxiques de ce même effluent;

> **H1** : Dans un ensemble de données combinant des échantillons toxiques et non toxiques, une relation statistique significative peut être établie entre les concentrations de métaux et métalloïdes dissous et le niveau de toxicité (exprimé en unités de toxicité, UT) et il existe des différences géochimiques statistiquement significatives entre les échantillons toxiques et non toxiques.

OS2: Caractériser et prédire les effets aigus de Cu, Zn, As et Se, seuls et en mélanges binaires et ternaires, sur *D.magna* en caractérisant la nature des interactions par bioessais, isobologrammes et modèles prédictifs;

> **H2** : Les mélanges binaires et ternaires de Cu, Zn, As et Se engendrent des interactions toxicologiques qui ne sont pas additives. Dans les mélanges, le Se joue un rôle clé en tant que modulateur dans les effets d’atténuation ou d’amplification de la toxicité des métaux et métalloïdes selon les proportions de mélange et les conditions d’exposition.

OS3 : Évaluer l’efficacité de l’ozonation à microbulles pour transformer la spéciation des contaminants et réduire la toxicité aiguë d’effluents miniers et métallurgiques.

> **H3** : L’ozonation microbulles à pH 9 modifiera considérablement la spéciation des métaux(loïdes) et des espèces azotées (redox-sensibles) et peut ainsi entraîner une réduction mesurable de la toxicité aiguë des effluents métallurgiques et miniers.

L’**OS1** est atteint. Les analyses statistiques et de spéciation ont montré que Cu et Cd, sous leurs formes libres, sont les principaux contributeurs à la toxicité aiguë, tandis que sous certaines conditions, le Fe joue un rôle d’inhibiteur de toxicité indirect. Ces résultats tendent à valider l’hypothèse **H1** en ce sens que des relations statistiques peuvent en effet être établies entre la composition chimique des effluents, la co-occurence de contaminants et la toxicité.

L’O**S2** est atteint également. Les bioessais sur *D. magna* montrent que les mélanges métalliques, en induisant des effets non additifs, sont sous-tendus par des synergies marquées pour Cu–Zn et Zn–Se et des comportements additifs pour d’autres combinaisons. Le Cu est l’élément toxique dominant, tandis que le Se module la réponse toxique selon les autres contaminants présents. Les résultats tendent à supporter l’hypothèse **H2**.

Enfin, l’**OS3** est également atteint. L’ozonation en microbulles ayant permis de réduire de manière significative le NH_3 et engendré une diminution peu contestable de la toxicité des effluents. Ces résultats supportent en grande partie la validité de l’**H3**, bien que l’efficacité de l’ozonation pour la réduction de la toxicité reste conditionnée par les caractéristiques des effluents à traiter, dont la salinité.

Dans l’ensemble, les résultats tendent à valider les trois hypothèses formulées et confirment la pertinence d’une approche intégrée, combinant analyses chimiques, écotoxicologiques et technologiques, pour comprendre et réduire la toxicité des effluents complexes.

7.3. Limites et considérations critiques

Bien que les résultats soient significatifs, il convient d’en reconnaître plusieurs limites afin de replacer cette recherche dans son contexte, dans ses enjeux, et dans ses limites.

7.3.1. Limites scientifiques et expérimentales

- **Espèces utilisées pour les bioessais** : Les expérimentations écotoxicologiques ont été réalisées exclusivement sur *D. magna*, une espèce modèle sensible couramment utilisée. Bien que *D. magna* convienne aux essais de toxicité aiguë, elle ne permet pas de représenter tous les niveaux trophiques en lien avec la diversité des sensibilités écologiques. D’autres espèces de poissons, d’algues ou d’invertébrés benthiques auraient pu être ajoutées à cette caractérisation.

- **Nombre restreint des contaminants étudiés** : Les travaux n'ont porté que sur un nombre limité de métaux et métalloïdes (Cu, Zn, As, Se, Cd, Fe) et sur l'évaluation de l'effet de l'élimination des espèces oxydables sur la toxicité. Bien qu'il soit impossible, à l'heure actuelle, d'évaluer de manière exhaustive toutes les combinaisons de mélanges de contaminants en raison de la complexité combinatoire considérable que cela implique, d'autres contaminants présents dans les effluents, notamment le Hg, Cr, Co, Sb, ainsi que certains anions comme les sulfates, les chlorures, les CNO^- et les SCN^- ainsi que la salinité et certains contaminants d'intérêt émergent (p. ex.: thiosels) pourraient également jouer un rôle dans la toxicité, mais n'ont pas été étudiées ici.
- **Systèmes interactionnels complexes** : Les effets étudiés portent sur des systèmes de types binaires ou ternaires, alors que les effluents réels sont des véritables cocktails de dizaines de substances. La transposition directe des résultats issus de la recherche, pour les systèmes étudiés en laboratoire à des systèmes naturels au milieu des écosystèmes, doit être considérée avec prudence.

7.3.2. Limites méthodologiques

- **Spéciation calculée et non mesurée** : Les résultats de spéciation dépendent d'outils et de modèles (PHREEQC, Visual MINTEQ) s'appuyant sur des bases de données thermodynamiques. Malgré leur robustesse, ces outils donnent une vision simplifiée de la réalité complexe des matrices avec lesquelles ils doivent fonctionner et sont sensibles aux paramètres d'entrée (pH, Eh, matière organique). Ici, l'absence de mesures directes de la matière organique dissoute et de quelques ions majeurs, notamment le Cl^-, a pu diminuer la robustesse des modélisations. Différents scénarios avec différentes concentrations en matière organique dissoute et en Cl^- ont été simulés pour évaluer l'influence des variabilités sur la spéciation pour pallier cette limite. Ce choix a permis de saisir la sensibilité des résultats mais ne peut se substituer à des mesures. De surcroît, les calculs de spéciation ont été réalisés en assumant l'équilibre thermodynamique, négligeant ainsi les déséquilibres induits par la cinétique des réactions.
- **Procédure de conservation** : La congélation des échantillons, bien qu'elle fasse partie des procédures normalisés du CEAEQ pour assurer leur stabilité, peut induire des modifications de spéciation (transformations redox, précipitations, pertes partielles de composés volatils), constituant ainsi une source d'incertitude dans la représentativité des essais réalisés.

- **Conditions pH-Eh** : Les différences entre les conditions *in situ* (naturelles) et celles reproduites en laboratoire constituent une source d'incertitudes. La spéciation des métaux dépend du potentiel d'oxydoréduction et du pH qui exercent une forte influence et peuvent modifier fondamentalement la biodisponibilité et la toxicité des effluents.
- **Statistiques et modélisation** : Bien que des outils comme MIXTOX et MixModel aient permis d'améliorer la caractérisation des phénomènes d'interactions entre les contaminants étudiés, ils n'ont été calibrés que sur des conditions expérimentales précises et ne saisissent pas la variabilité temporelle et spatiale des effluents naturels.

7.3.3. Limites appliquées et technologiques

- **Variabilité des effluents** : Le traitement est dépendant des caractéristiques initiales des effluents (pH, salinité, CE, composés azotés). Cette variabilité n'augure pas d'une performance généralisable. Ainsi, les résultats obtenus en laboratoire devront être confrontés à des mesures sur des pilotes de terrain et sur des applications à plus grande échelle.
- **Durabilité et intégration** : L'ozonation est efficace sur le NH_3 et certaines espèces métalliques, mais ne peut traiter tous les contaminants. L'intégration nécessite d'être mise en commun avec d'autres procédés physico-chimiques ou biologiques au niveau de la gestion opérationnelle des effluents pour garantir le traitement complet de la toxicité.

CHAPITRE 8 Conclusions et recommandations

8.1. Conclusion

La question de la gestion des effluents miniers et métallurgiques reste un problème important pour la protection des écosystèmes aquatiques. Ces effluents contiennent des métaux, métalloïdes et d'autres contaminants présents en proportions variables. Les règlements encadrant le rejet de ces effluents, comme le REMMMD au Canada, s'appuient largement sur des valeurs limites de concentrations totales pour différents contaminants. Cependant, les suivis écotoxicologiques ont montré à plusieurs reprises que des effluents conformes aux critères de rejet en matière de concentration des contaminants pouvaient néanmoins induire une toxicité biologique significative, révélant un décalage entre conformité chimique et protection écologique réelle. Pour contribuer à répondre à cette problématique, cette thèse a adopté une démarche intégrée en combinant des analyses chimiques et de spéciation, des bioessais standardisés utilisant *D. magna*, des outils statistiques et de modélisation et des expériences pilotes de traitement. Cette méthodologie a permis une meilleure compréhension des mécanismes de toxicité associés aux effluents complexes et l'exploration de solutions technologiques pour leur atténuation. Les analyses chimiques couplées à des bioessais ont montré que la toxicité aiguë observée ne peut être attribuée uniquement aux concentrations totales des métaux et métalloïdes mais qu'elle est plutôt influencée par les formes biodisponibles des contaminants et les interactions entre ces derniers. Les calculs de spéciation tendent à indiquer que les ions métalliques libres influencent significativement la toxicité. Des tests conduits sur des mélanges métalliques binaires et ternaires ont démontré que les interactions entre contaminants peuvent entraîner des effets additifs ou synergiques, conduisant à des niveaux de toxicité dépassant parfois les prévisions basées sur des substances individuelles. De plus, des essais au moyen d'un pilote d'ozonation par microbulles, en conditions alcalines, ont démontré l'efficacité du procédé pour l'enlèvement du NH_3 et la diminution quantifiable de la toxicité. En synthétisant ces différentes composantes (mélanges, spéciation, bioessais et traitement), cette recherche offre une perspective pertinente sur les mécanismes responsables de la toxicité des effluents miniers et métallurgiques (Figure 8.1).

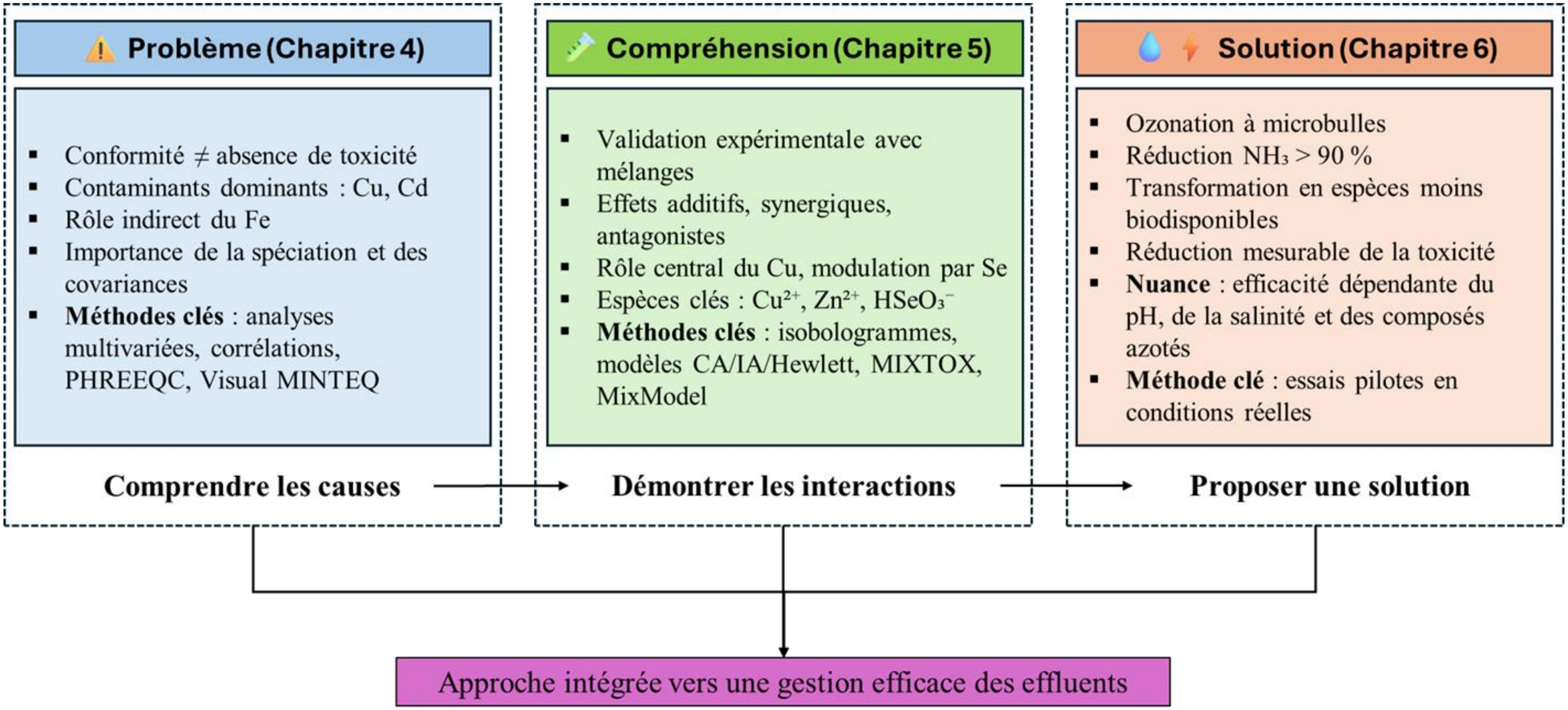

Figure 8.1 Approche intégrée pour la compréhension et la gestion des effluents

8.2. Recommandations

Pour la recherche

- **Compléter les bioessais** : associer à *D. magna* d'autres organismes faciles à maintenir en laboratoire (ex. algues vertes, poissons zèbres, amphipodes). Cela permettrait de couvrir différents niveaux trophiques sans multiplier les contraintes logistiques.
- **Élargir la gamme de contaminants étudiés** : inclure dans les prochains tests le Cd, les cyanates, les thiocyanates, les thiosels et la salinité, souvent retrouvés dans les effluents miniers et connus pour avoir des effets écotoxicologiques, mais absents de cette étude.
- **Mesurer systématiquement la matière organique dissoute** : intégrer ce paramètre dans les campagnes analytiques pour renforcer la robustesse des calculs de spéciation. L'ajout d'une substance organique de référence dans des essais réalisés en laboratoire, sur des mélanges synthétiques, serait également pertinent.
- **Améliorer la vérification expérimentale des calculs de spéciation** : combiner les calculs de modèles avec quelques mesures directes dans les prochains travaux. Par exemple, des mesures en lien avec l'abondance des principales espèces d'As (As(III) et As(V)) et de Cr (Cr(III) et Cr(VI)) seraient pertinentes.
- **Poursuivre le développement des modèles de mélanges** : tester l'application de MIXTOX et MixModel sur des cocktails plus représentatifs des effluents réels (≥4-5 contaminants), afin de se rapprocher des conditions industrielles.
- **Améliorer l'accès aux données**: Créer une base de données internationale ouverte sur composition–spéciation–toxicité des effluents.

Pour l'industrie

- **Un suivi régulier de paramètres clés** : complémenter les suivis réguliers, où applicable et pertinent, par l'ajout d'analyses des cyanates, thiocyanates, thiosels et autres contaminants d'intérêt émergent. Augmenter la fréquence de suivi de la matière organique dissoute dans les programmes existants. Ces paramètres influencent directement la spéciation et la formation de sous-produits durant l'ozonation.

- **Vérifier la transférabilité des essais réalisés en laboratoire sur l'effluent industriel aux conditions réelles**, en tenant compte des paramètres hydrauliques du site, dont le débit et le temps de contact.
- **Poursuivre par des essais pilotes en continu sur site afin d'optimiser l'ozonation à microbulles en situation opérationnelle,** notamment en ajustant le pH, la dose d'ozone et la durée de traitement, et en évaluant l'influence du débit et des variations saisonnières sur la performance.
- **Évaluer l'association des traitements classiques et avancés** : intégrer l'ozonation avec la neutralisation/chaux ou les biofiltres afin d'assurer une élimination des espèces oxydables et des métaux. À ce stade, les données disponibles ne permettent pas d'en confirmer la faisabilité ni la performance en conditions réelles. Il est donc recommandé de considérer cette combinaison comme une orientation de recherche prioritaire avant toute application opérationnelle.
- **Surveiller les sous-produits du traitement** : inclure dans les suivis les bromates et chlorates générés par ozonation pour s'assurer que le procédé ne crée pas de nouvelles sources de toxicité.
- **Sensibiliser le personnel** : renforcer les compétences des équipes industrielles sur l'interprétation de la spéciation et des bioessais, afin de passer d'un suivi « chimique » à un suivi « chimique-biologique ».

Pour les politiques publiques

- À moyen terme, il serait pertinent de complémenter le cadre réglementaire pour inclure des considérations relatives à la spéciation et à la biodisponibilité des contaminants dans les évaluations de risque.
- À plus long terme, des lignes directrices dédiées à la toxicité des mélanges complexes devraient être élaborées, afin d'offrir aux décideurs et aux industriels un cadre clair et opérationnel.

8.3. Perspectives à long terme

Les résultats de cette thèse montrent que la gestion durable des effluents miniers et métallurgiques doit nécessairement reposer sur une approche intégrée. Celle-ci s'appuie sur trois axes essentiels :

1. **Un axe chimique** : des analyses complètes dont au moins la spéciation et les covariances entre contaminants doivent être analysées.
2. **Un axe biologique** : via des bioessais en utilisant diverses espèces, dans un souci de diversification des sensibilités écologiques recherchées.
3. **Un axe technologique** : agissant sur des procédés innovants et adaptatifs tels que l'ozonation en microbulles combinée avec la précipitation post-traitement, où applicable.

Cette voie tripartite est indispensable pour surmonter des obstacles actuels, sécuriser la protection des écosystèmes aquatiques et appréhender durablement l'activité minière et métallurgique sur le long terme.

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ANNEXE A MATÉRIEL SUPPLÉMENTAIRE DU CHAPITRE 4

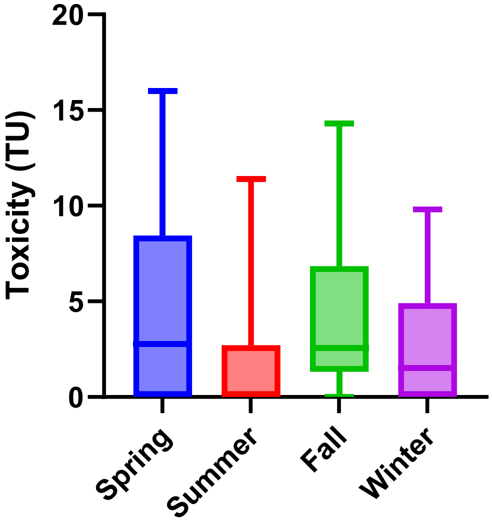


Figure A1 Variations in effluent toxicity (expressed in TU) between the spring (n = 22 samples), summer (n = 24 samples), fall (n = 22 samples), and winter (n = 20 samples) seasons. The rectangles of the whisker boxes represent the interquartile range on either side of the median (horizontal line), and the whiskers illustrating the non-outlier range

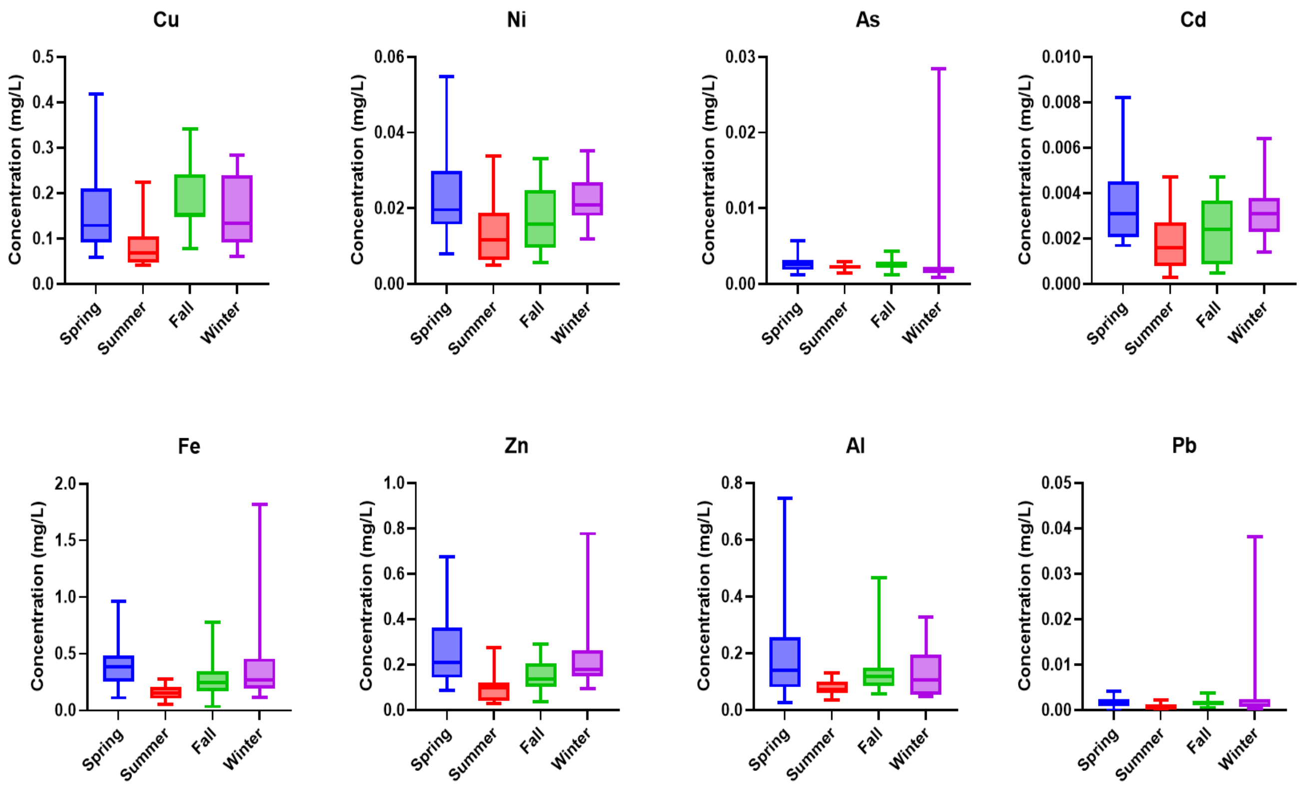


Figure A2 Variations in total concentrations of trace metal(loid)s (expressed in mg/L) between the spring (n = 21 samples), summer (n = 22 samples), fall (n = 20 samples), and winter (n = 18 samples) seasons. The rectangles of the whisker boxes represent the interquartile range on either side of the median (horizontal line), and the whiskers illustrating the non-outlier range

Table A1 Seasonal Influence on Effluent Toxicity: Evidence from ANOVA and Kruskal-Wallis Tests

Element	**p (ANOVA)**	**p (Kruskal)**	**Max season**	**Min season**
Fe	0.0029	0	Winter	Summer
Zn	0.0003	0.0001	Spring	Summer
Al	0.0057	0.0048	Spring	Summer
Cu	0.0006	0	Fall	Summer
Ni	0.0039	0.002	Spring	Summer
As	0.4999	0.0258	Winter	Summer
Cd	0.0009	0.0013	Spring	Summer
Pb	0.0864	0.0025	Winter	Summer
Toxicity (TU)	0.0539	0.0282	Spring	Summer

Table A2 Multiple linear regression results

	Estimate	**Std. Error**	**t value**	**Pr (>|t|)**	
(Intercept)	-2.34	0.74	-3.165	0.002	**
Cd	1627.12	507.31	3.207	1.7e-3	**
Cu	14.60	4.01	3.638	4.1e-4	***
Fe	-5.015	2.04	-2.455	0.02	*

Signifcance of codes: *$p < 0.1$, **$p < 0.05$, **$p < 0.01$, ***$p<0.001$

Table A3 Summary table of one-way ANOVA results based on total concentrations of trace metal(loid)s. Df: degrees of freedom; Sum Sq: sum of squares; Mean Sq: mean of squares; F value: Fisher statistic; Pr(>F): probability value (*p*-value)

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**	**Signification**
Al	1	10.0	10.0	2.21	0.14	
As	1	14.6	14.6	3.22	0.08	.
Cd	1	1094.0	1094.0	240.90	< 2e-16	***
Cu	1	73.6	73.6	16.21	1.02e-4	***
Fe	1	49.9	49.9	10.98	0.0012	**
Ni	1	0.2	0.2	0.05	0.83	
Pb	1	2.8	2.8	0.611	0.44	
Zn	1	4.1	4.1	0.911	0.34	
Residuals	116	526.8	4.5			

Signification of codes: *$p < 0.1$, **$p < 0.05$, **$p < 0.01$, ***$p<0.001$

Table A4 Summary table of one-way ANOVA results based on free ionic forms of trace metal(loid)s. Df: degrees of freedom; Sum Sq: sum of squares; Mean Sq: mean of squares; F value: Fisher statistic; Pr(>F): probability value (*p*-value)

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**	**Signification**
Al^{3+}	1	49.1	49.1	10.54	0.00152	**
Cd^{2+}	1	256.1	256.1	55.00	2.07e-11	***
Cu^{2+}	1	410.5	410.5	88.15	6.00e-16	***
Fe^{2+}	1	93.6	93.6	20.11	1.72e-05	***
Ni^{2+}	1	2.1	2.1	0.45	0.50358	
Pb^{2+}	1	0.1	0.1	0.02	0.88678	
Zn^{2+}	1	419.7	419.7	90.13	3.40e-16	***
Residuals	117	544.8	4.7			

Signification of codes: *$p < 0.1$, **$p < 0.05$, **$p < 0.01$, ***$p<0.001$

Table A5 Analysis of variance (ANOVA) of metal species concentrations (mg/L) in toxic effluents at chloride concentrations of 20, 38, 60, and 100 mg/L

Variable	Df	Sum Sq	Mean Sq	F value	Pr (>F)
$ZnCO_3$	3	6.81E-05	2.27E-05	6.71E-03	9.99E-01
Pb_2OH^{3+}	3	3.04E-06	1.01E-06	3.75E-03	1.00E+00
$ZnHCO_3^+$	3	2.22E-13	7.39E-14	1.97E-03	1.00E+00
$Pb_3(OH)_4^{2+}$	3	2.21E-08	7.35E-09	1.07E-03	1.00E+00
H_3AsO_3	3	1.61E-23	5.37E-24	5.91E-04	1.00E+00
$Zn(OH)_2$	3	1.05E-23	3.51E-24	5.79E-04	1.00E+00
$AlOH^{2+}$	3	1.60E-10	5.34E-11	5.36E-04	1.00E+00
Al^{3+}	3	1.92E-05	6.41E-06	5.18E-04	1.00E+00
$H_2AsO_3^-$	3	3.18E-28	1.06E-28	3.73E-04	1.00E+00
$H_4AsO_3^+$	3	1.41E-18	4.69E-19	3.51E-04	1.00E+00
Zn^{2+}	3	6.38E-06	2.13E-06	3.27E-04	1.00E+00
$ZnOH^+$	3	1.78E-08	5.95E-09	2.89E-04	1.00E+00
$Al(OH)_3$	3	4.69E-31	1.56E-31	2.83E-04	1.00E+00
$Zn(OH)_4^{2-}$	3	2.31E-39	7.70E-40	1.91E-04	1.00E+00
$Zn(OH)_3^-$	3	3.78E-44	1.26E-44	7.56E-05	1.00E+00
$HAsO_3^{2-}$	3	1.68E-07	5.59E-08	6.32E-05	1.00E+00
$Zn(CO_3)_2^{2-}$	3	2.22E-12	7.41E-13	1.66E-05	1.00E+00
$Al(OH)_2^+$	3	2.24E-07	7.45E-08	5.84E-07	1.00E+00
$Al(OH)_4^-$	3	2.57E-11	8.56E-12	2.02E-07	1.00E+00

Table A6 Analysis of variance (ANOVA) of metal species concentrations (mg/L) in non-toxic effluents at chloride levels of 20, 38, 60, and 100 mg/L

Variable	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**
$ZnCO_3$	3	1.57E-05	5.23E-06	1.02E-02	9.99E-01
Pb_2OH^{3+}	3	2.85E-25	9.51E-26	6.25E-03	9.99E-01
$ZnHCO_3^+$	3	2.31E-07	7.70E-08	2.12E-03	1.00E+00
$Pb_3(OH)_4^{2+}$	3	4.79E-32	1.60E-32	1.44E-03	1.00E+00
H_3AsO_3	3	3.66E-14	1.22E-14	1.16E-03	1.00E+00
$Zn(OH)_2$	3	8.53E-07	2.84E-07	9.47E-04	1.00E+00
$AlOH^{2+}$	3	2.93E-06	9.78E-07	6.94E-04	1.00E+00
Al^{3+}	3	2.30E-24	7.67E-25	6.06E-04	1.00E+00
$H_2AsO_3^-$	3	2.89E-09	9.62E-10	4.24E-04	1.00E+00
$H_4AsO_3^+$	3	2.03E-06	6.76E-07	3.91E-04	1.00E+00
Zn^{2+}	3	1.05E-28	3.51E-29	3.45E-04	1.00E+00
$ZnOH^+$	3	5.11E-11	1.70E-11	2.93E-04	1.00E+00
$Al(OH)_3$	3	6.59E-40	2.20E-40	1.75E-04	1.00E+00
$Zn(OH)_4^{2-}$	3	1.43E-16	4.77E-17	1.34E-04	1.00E+00
$Zn(OH)_3^-$	3	1.95E-08	6.49E-09	1.28E-04	1.00E+00
$HAsO_3^{2-}$	3	2.43E-44	8.12E-45	1.03E-04	1.00E+00
$Zn(CO_3)_2^{2-}$	3	1.02E-12	3.39E-13	1.95E-06	1.00E+00
$Al(OH)_2^+$	3	1.77E-07	5.91E-08	3.34E-07	1.00E+00
$Al(OH)_4^-$	3	3.02E-13	1.01E-13	1.97E-09	1.00E+00

ANNEXE B MATRÉRIEL SUPPLÉMENTAIRE DU CHAPITRE 5

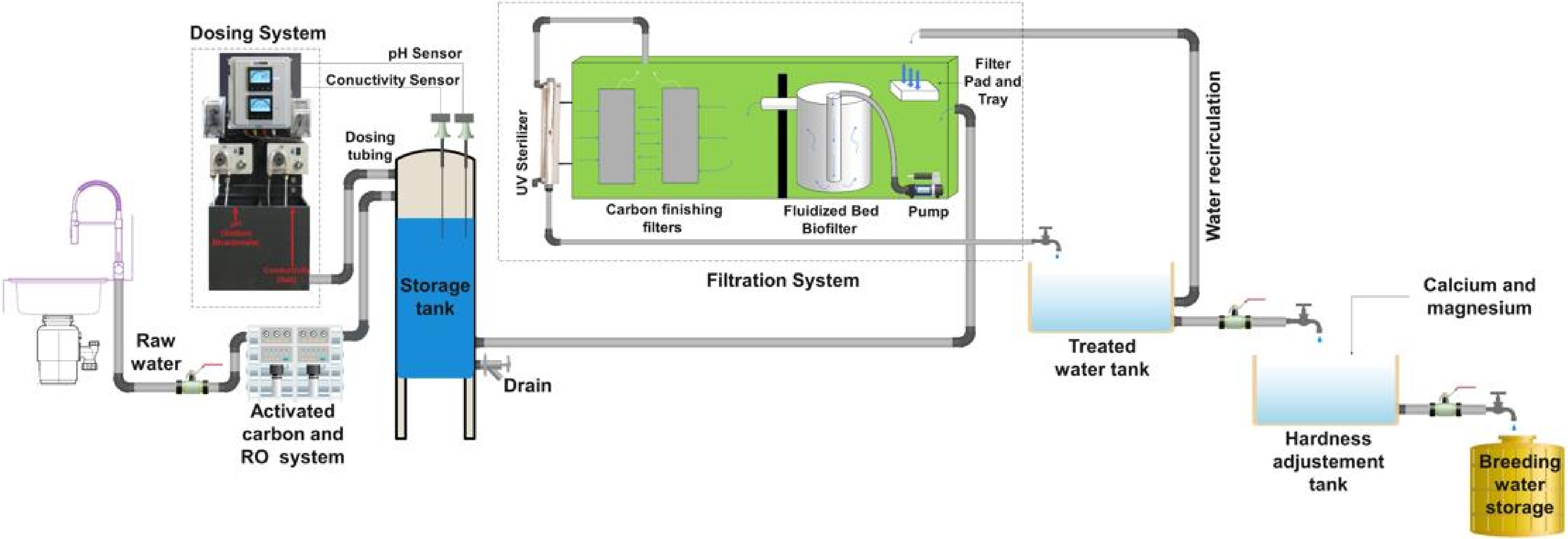


Figure B1 Water treatment system for the preparation of breeding water (diagram created using EdrawMax)

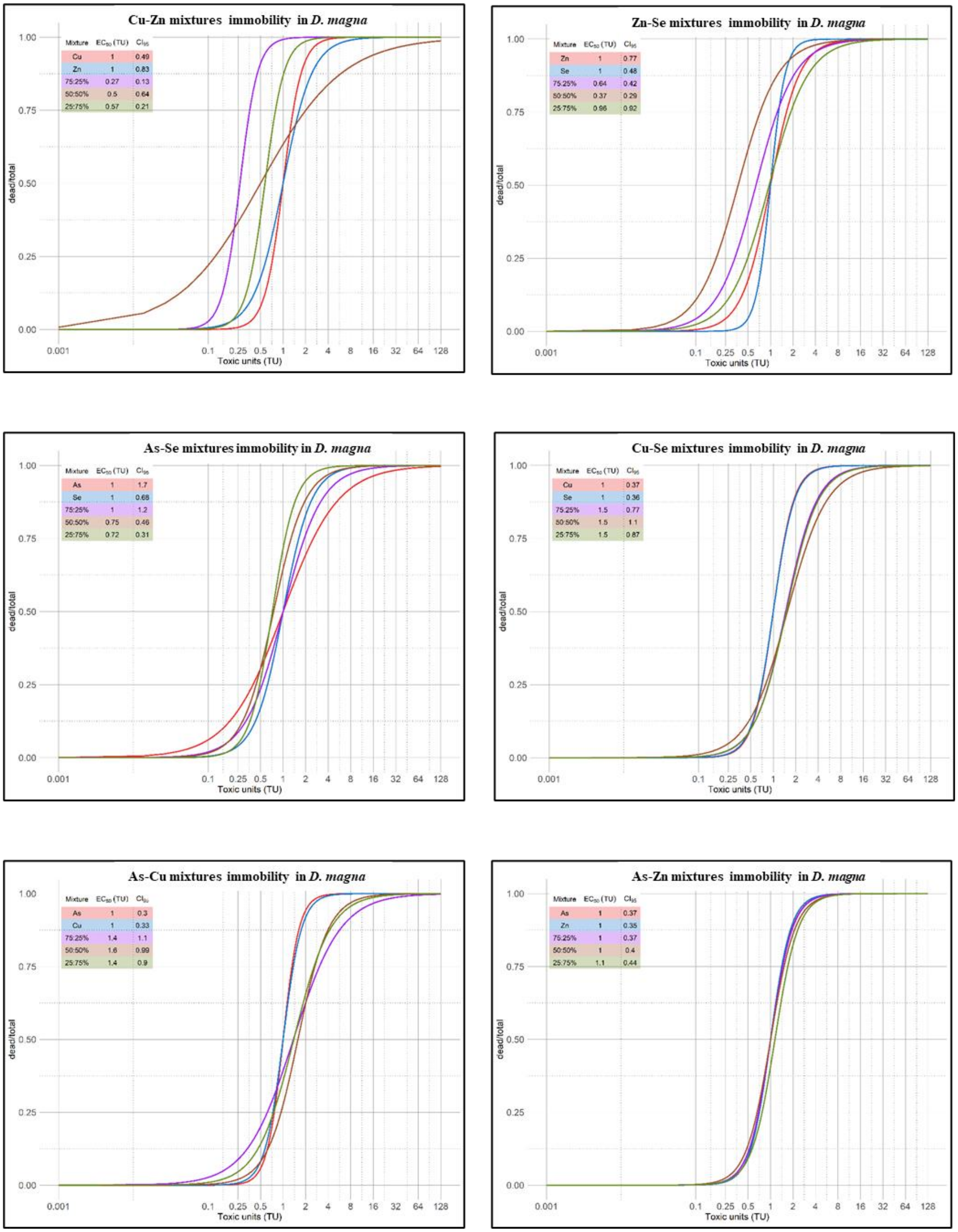
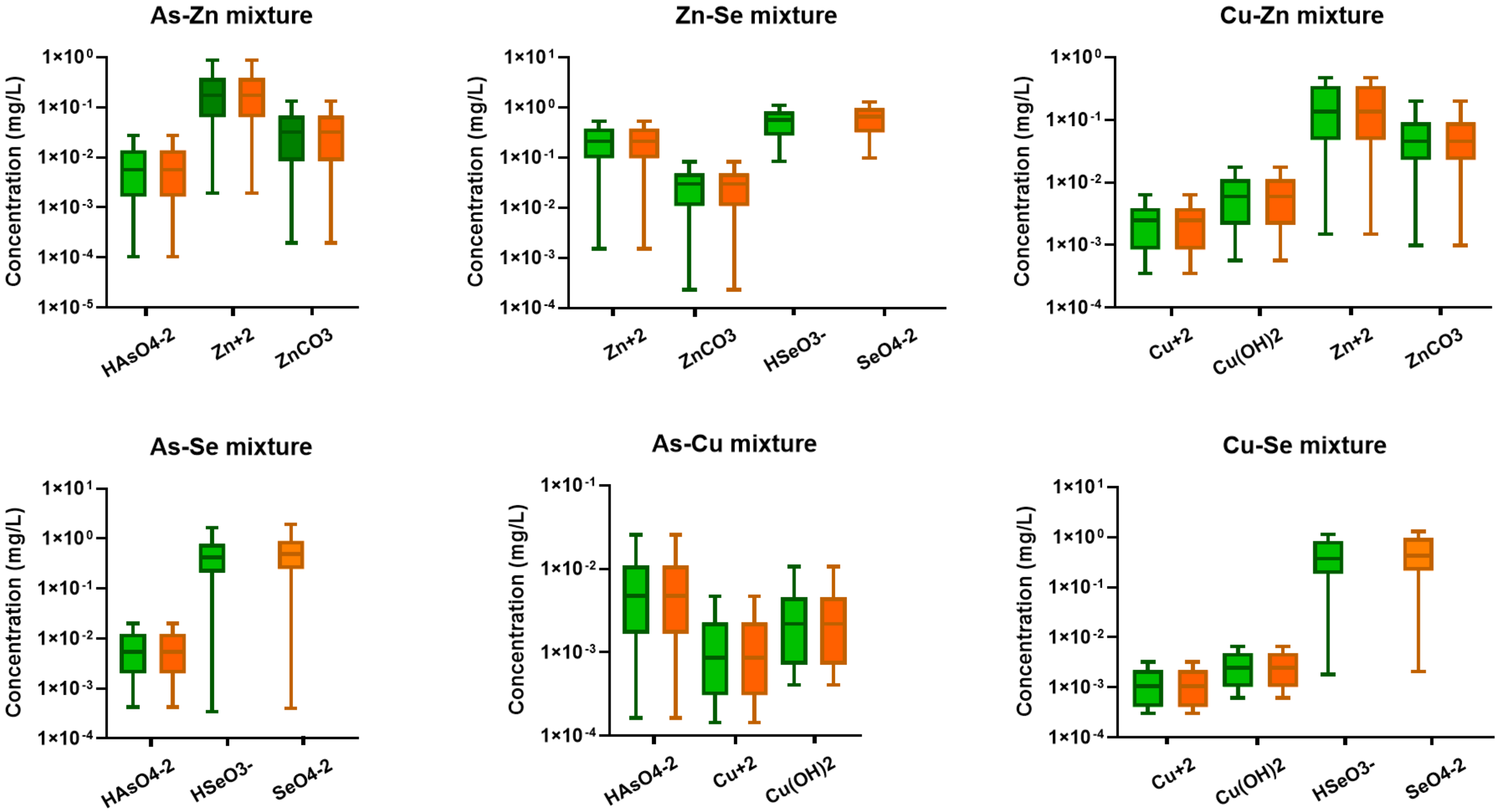


Figure B2 Dose-response analysis of binary metal mixtures in *D. magna*


As-Zn mixture

Zn-Se mixture

Cu-Zn mixture

As-Se mixture

As-Cu mixture

Cu-Se mixture

Figure B3 Speciation patterns of metallic elements in binary mixtures. Speciation was calculated using PHREEQC (with the WATEQ4F database) based on the measured concentrations of metals in all tested mixtures. The boxplots represent the distribution of calculated concentrations of the dominant species within each binary mixture. Results obtained under moderately reducing conditions (pe = 4, shown in green) and under oxidizing conditions (pe = 12, shown in orange) are presented for comparison

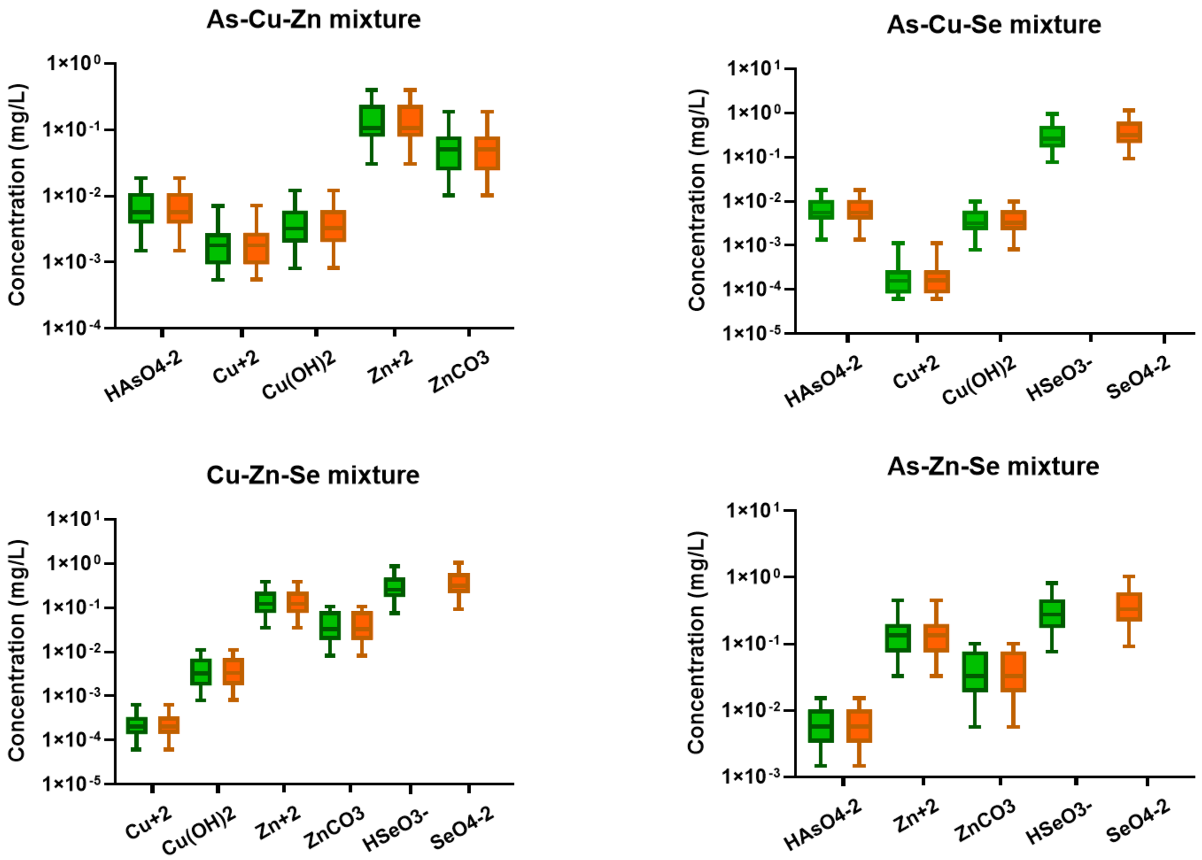


Figure B4 Speciation patterns of metallic elements in binary mixtures. Speciation was calculated using PHREEQC (with the WATEQ4F database) based on the measured concentrations of metals in all tested mixtures. The boxplots represent the distribution of calculated concentrations of the dominant species within each binary mixture. Results obtained under moderately reducing conditions (pe = 4, shown in green) and under oxidizing conditions (pe = 12, shown in orange) are presented for comparison

Table B1 Composition ratios, individual toxic units (TUs), and corresponding measured metal concentrations (µg/L) for binary mixtures of As, Cu, Zn and Se used in acute toxicity tests with *D. magna.* TU_a = Toxic unit of the first metal(oid) in the mixture; TU_b = Toxic unit of the second metal(oid) in the mixture

Mixture Ratio	**TU_a**	**TU_b**	**As-Cu**		**As-Zn**		**As-Se**		**Cu-Zn**		**Cu-Se**		**Zn-Se**	
			[As]	**[Cu]**	**[As]**	**[Zn]**	**[As]**	**[Se]**	**[Cu]**	**[Zn]**	**[Cu]**	**[Se]**	**[Zn]**	**[Se]**
100:0%	1	0	16.7	0.89	16.4	2.23	16.7	0	14.2	2.54	12.3	0	619	2.97
75:25%	0.75	0.25	12.9	3.49	12.4	161	12.9	166	10.7	163	10.3	178	440	178
50:50%	0.5	0.5	8.53	6.93	8.27	320	8.53	331	5.84	326	6.97	355	319	363
25:75%	0.25	0.75	4.02	9.8	4.21	466	4.02	567	3.17	442	3.54	532	161	538
0:100%	0	1	0.7	12.5	0.525	607	0.962	694	1.33	618	1.5	710	1.87	718

Table B2 Composition ratios, individual toxic units (TU), and corresponding measured metal concentrations (µg/L) for ternary mixtures of As, Cu, Zn, and Se used in acute toxicity tests with *D. magna.* TU_a = Toxic unit of the first metal(loid) in the mixture; TU_b = Toxic unit of the second metal(loid) in the mixture; TU_c = Toxic unit of the third metal(loid) in the mixture

Ratios	**TU_a**	**TU_b**	**TU_c**	**As-Cu-Zn**			**As-Cu-Se**			**Cu-Zn-Se**			**As-Zn-Se**		
				[As]	**[Cu]**	**[Zn]**	**[As]**	**[Cu]**	**[Se]**	**[Cu]**	**[Zn]**	**[Se]**	**[As]**	**[Zn]**	**[Se]**
33:33:33 %	0.33	0.33	0.33	5.45	4.27	201	5.62	4.24	224	4.37	209	243	4.87	201	252
60:20:20 %	0.6	0.2	0.2	9.86	2.61	115	9.74	2.57	144	2.48	115	139	9.31	129	153
20:60:20 %	0.2	0.6	0.2	3.23	7.54	122	3.01	8.32	146	7.26	122	151	3.07	123	147
20:20:60 %	0.2	0.2	0.6	3.04	2.43	350	3.3	2.67	457	2.43	402	448	2.98	357	445
40:40:20 %	0.4	0.4	0.2	6.22	5.01	116	6.36	5.27	136	4.91	127	152	6.04	122	135
40:20:40 %	0.4	0.2	0.4	6.82	2.48	252	6.34	2.52	264	2.42	237	291	6.49	232	291
20:40:40 %	0.2	0.4	0.4	3.03	5.45	249	3.14	5.4	304	5.29	239	291	3.14	251	290
85:7.5:7.5 %	0.85	0.07	0.07	13.9	0.98	42.8	14	0.95	53.8	0.93	44	50.6	12.7	48.7	50.5
7.5:85:7.5 %	0.07	0.85	0.07	1.29	10.6	49.2	1.13	10.7	50.6	11.8	48.9	54.8	1.22	43.5	54.7
7.5:7.5:85 %	0.07	0.07	0.85	1.18	1.04	540	1.12	1.01	631	1.05	488	573	1.16	549	570
50:25:25 %	0.5	0.25	0.25	7.99	3.18	155	8.54	3.04	177	3.29	150	173	7.41	163	181
25:50:25 %	0.25	0.5	0.25	4.29	6.82	149	4.3	6.43	172	6.95	151	171	3.79	161	187
25:25:50 %	0.25	0.25	0.5	3.88	3.28	293	4.29	3.2	364	3.46	333	349	3.87	287	343
70:15:15 %	0.7	0.15	0.15	11.8	1.93	90.9	12.1	1.81	105	1.97	88.7	110	11.3	91.4	114
15:70:15 %	0.15	0.7	0.15	2.32	8.56	90.9	2.5	9.22	108	8.42	99.8	110	2.45	86.3	98.6
15:15:70 %	0.15	0.15	0.7	2.45	1.88	419	2.21	1.94	490	1.9	444	533	2.29	466	489

Table B3 Physicochemical parameters (pH, dissolved oxygen, temperature, and conductivity) measured at 0 h and 48 h during binary metal mixture exposure tests on *D. magna*

Mixture	**Mixture Ratio**	**pH**		**O₂%**		**T(°C)**		**Conductivity (µS/cm)**	
		0h	**48h**	**0h**	**48h**	**0h**	**48h**	**0h**	**48h**
As-Cu	**100:0%**	7.7	7.76	91	90	20.7	20.8	761	767
	75:25%	7.21	7.26	93	91	20.8	21	772	782
	50:50%	7.3	7.32	92	89	20.8	20.9	779	795
	25:75%	7.37	7.36	90	88	20.8	21	778	790
	0:100%	7.36	7.35	91	89	20.4	20.6	760	765
As-Zn	**100:0%**	7.7	7.77	92	90	20.7	20.8	761	770
	75:25%	7.75	7.78	90	89	20.8	20.9	788	801
	50:50%	7.72	7.76	93	90	20.8	21	799	815
	25:75%	7.58	7.59	92	90	21	21.2	792	807
	0:100%	7.6	7.59	94	93	20.8	20.9	800	809
As-Se	**100:0%**	7.7	7.76	93	91	20.7	20.8	761	768
	75:25%	7.6	7.65	91	89	20.6	20.7	789	800
	50:50%	7.41	7.44	91	87	20.6	20.8	738	756
	25:75%	7.3	7.3	90	86	20.7	20.9	799	812
	0:100%	7.33	7.34	91	89	20.6	20.8	793	802
Cu-Zn	**100:0%**	7.23	7.28	90	88	20.3	20.5	786	799
	75:25%	7.03	7.06	93	90	20.3	20.5	799	814
	50:50%	7.54	7.54	91	87	20.6	20.7	801	821
	25:75%	7.4	7.43	91	85	20.4	20.5	806	820
	0:100%	7.36	7.38	92	87	20.4	20.6	760	773
Cu-Se	**100:0%**	7.36	7.41	92	87	20.4	20.6	760	770
	75:25%	7.61	7.64	93	90	21	21.1	777	787
	50:50%	7.52	7.52	94	91	20.9	21	788	808
	25:75%	7.5	7.51	91	86	20.8	21	789	801
	0:100%	7.33	7.33	89	83	20.6	20.7	793	800
Zn-Se	**100:0%**	7.33	7.35	94	93	20.6	20.8	793	803
	75:25%	7.41	7.43	93	91	20.9	21	747	761
	50:50%	7.26	7.27	92	90	20.9	21.1	811	828
	25:75%	7.31	7.31	93	90	20.9	21.1	801	817
	0:100%	7.6	7.58	92	88	20.8	20.9	800	808

Table B4 Physicochemical parameters (pH, dissolved oxygen, temperature, and conductivity) measured at 0 h and 48 h during ternary metal mixture exposure tests on *D. magna*

Mixture	**Mixture Ratio**	**pH**		**O2%**		**T(°C)**		**Conductivity (µS/cm)**	
		0h	**48h**	**0h**	**48h**	**0h**	**48h**	**0h**	**48h**
As-Cu-Zn	**33:33:33 %**	7.7	7.76	91	90	20.7	20.8	761	767
	60:20:20 %	7.21	7.26	93	91	20.8	21	772	782
	20:60:20 %	7.3	7.32	92	89	20.8	20.9	779	795
	20:20:60 %	7.37	7.36	90	88	20.8	21	778	790
	40:40:20 %	7.36	7.35	91	89	20.4	20.6	760	765
	40:20:40 %	7.7	7.77	92	90	20.7	20.8	761	770
	20:40:40 %	7.75	7.78	90	89	20.8	20.9	788	801
	85:7.5:7.5 %	7.72	7.76	93	90	20.8	21	799	815
	7.5:85:7.5 %	7.58	7.59	92	90	21	21.2	792	807
	7.5:7.5:85 %	7.6	7.59	94	93	20.8	20.9	800	809
	50:25:25 %	7.7	7.76	93	91	20.7	20.8	761	768
	25:50:25 %	7.6	7.65	91	89	20.6	20.7	789	800
	25:25:50 %	7.41	7.44	91	87	20.6	20.8	738	756
	70:15:15 %	7.3	7.3	90	86	20.7	20.9	799	812
	15:15:70 %	7.23	7.28	90	88	20.3	20.5	786	799
As-Cu-Se	**33:33:33 %**	7.03	7.06	93	90	20.3	20.5	799	814
	60:20:20 %	7.54	7.54	91	87	20.6	20.7	801	821
	20:60:20 %	7.4	7.43	91	85	20.4	20.5	806	820
	20:20:60 %	7.36	7.38	92	87	20.4	20.6	760	773
	40:40:20 %	7.36	7.41	92	87	20.4	20.6	760	770
	40:20:40 %	7.61	7.64	93	90	21	21.1	777	787
	20:40:40 %	7.52	7.52	94	91	20.9	21	788	808
	85:7.5:7.5 %	7.5	7.51	91	86	20.8	21	789	801
	7.5:85:7.5 %	7.33	7.33	89	83	20.6	20.7	793	800
	7.5:7.5:85 %	7.33	7.35	94	93	20.6	20.8	793	803
	50:25:25 %	7.41	7.43	93	91	20.9	21	747	761
	25:50:25 %	7.26	7.27	92	90	20.9	21.1	811	828
	25:25:50 %	7.31	7.31	93	90	20.9	21.1	801	817
	70:15:15 %	7.6	7.58	92	88	20.8	20.9	800	808
	15:70:15 %	7.7	7.76	91	90	20.7	20.8	761	767

Table B4 Physicochemical parameters (pH, dissolved oxygen, temperature, and conductivity) measured at 0 h and 48 h during ternary metal mixture exposure tests on *D. magna* (suite)

Mixture	**Mixture Ratio**	**pH**		**O2%**		**T(°C)**		**Conductivity (µS/cm)**	
		0h	**48h**	**0h**	**48h**	**0h**	**48h**	**0h**	**48h**
Cu-Zn-Se	**33:33:33 %**	7.3	7.32	92	89	20.8	20.9	779	795
	60:20:20 %	7.37	7.36	90	88	20.8	21	778	790
	20:60:20 %	7.36	7.35	91	89	20.4	20.6	760	765
	20:20:60 %	7.7	7.77	92	90	20.7	20.8	761	770
	40:40:20 %	7.75	7.78	90	89	20.8	20.9	788	801
	40:20:40 %	7.72	7.76	93	90	20.8	21	799	815
	20:40:40 %	7.58	7.59	92	90	21	21.2	792	807
	85:7.5:7.5 %	7.6	7.59	94	93	20.8	20.9	800	809
	7.5:85:7.5 %	7.7	7.76	93	91	20.7	20.8	761	768
	7.5:7.5:85 %	7.6	7.65	91	89	20.6	20.7	789	800
	50:25:25 %	7.41	7.44	91	87	20.6	20.8	738	756
	25:50:25 %	7.3	7.3	90	86	20.7	20.9	799	812
	25:25:50 %	7.33	7.34	91	89	20.6	20.8	793	802
	70:15:15 %	7.23	7.28	90	88	20.3	20.5	786	799
	15:15:70 %	7.54	7.54	91	87	20.6	20.7	801	821
As-Zn-Se	**33:33:33 %**	7.4	7.43	91	85	20.4	20.5	806	820
	60:20:20 %	7.36	7.38	92	87	20.4	20.6	760	773
	20:60:20 %	7.36	7.41	92	87	20.4	20.6	760	770
	20:20:60 %	7.61	7.64	93	90	21	21.1	777	787
	40:40:20 %	7.52	7.52	94	91	20.9	21	788	808
	40:20:40 %	7.5	7.51	91	86	20.8	21	789	801
	20:40:40 %	7.33	7.33	89	83	20.6	20.7	793	800
	85:7.5:7.5 %	7.33	7.35	94	93	20.6	20.8	793	803
	7.5:85:7.5 %	7.41	7.43	93	91	20.9	21	747	761
	7.5:7.5:85 %	7.26	7.27	92	90	20.9	21.1	811	828
	50:25:25 %	7.31	7.31	93	90	20.9	21.1	801	817
	25:50:25 %	7.6	7.58	92	88	20.8	20.9	800	808
	25:25:50 %	7.7	7.76	91	90	20.7	20.8	761	767
	70:15:15 %	7.21	7.26	93	91	20.8	21	772	782
	15:15:70 %	7.37	7.36	90	88	20.8	21	778	790