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Shannon Weeks Santos

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THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE SCIENCES DU VIVANT, GÉOSCIENCES, SCIENCES DE L'ENVIRONNEMENT

SPÉCIALITÉ : Géochimie et Écotoxicologie

Par Shannon WEEKS SANTOS

Étude des réponses adaptatives et délétères des premiers stades du développement de truite arc-en-ciel, Oncorhynchus mykiss, exposés à des produits phytosanitaires utilisés en viticulture

Sous la direction de : Jérôme CACHOT
Co-Directeur : Patrice GONZALEZ

Soutenue le 11 janvier 2019

Membres du jury :

M. LAROCHE, Jean	Professeur, Université de Brest	Rapporteur
Mme ADAM, Christelle	Chercheur, IRSN	Rapportrice
M. DEVAUX, Alain	Ingénieur de Recherche, INRA	Rapporteur
Mme JEAN, Séverine	Maître de conférences, Université de Toulouse	Examinatrice
Mme BOLLIET, Valérie	Professeure, Université de Pau et des Pays de l'Adour	Examinatrice
Mme MORIN, Bénédicte	Maître de conférences, Université de Bordeaux	Invitée
M. PIERRON, Fabien	Chargé de recherche, CNRS, Université de Bordeaux	Invité

Titre : Étude des réponses adaptatives et délétères des premiers stades du développement de truite arc-en-ciel, Oncorhynchus mykiss, exposés à des produits phytosanitaires utilisés en viticulture

Résumé :

L'utilisation excessive de pesticides engendre des pollutions et des dégradations importantes de l'environnement y compris sur les écosystèmes aquatiques. L'activité viticole ne fait pas exception à cette règle, et c'est pour cette raison que ce travail de thèse s'est intéressé aux réponses adaptatives ou délétères aux premiers stades de vie de poissons exposés à trois produits phytosanitaires utilisés en viticulture ainsi qu'à leur mélange et à des échantillons environnementaux.

La thèse s'intéresse aux stades précoces de développement de la truite arc-en-ciel, mais également à la lignée cellulaire de foie de truite RTL-W1. Les embryons de truite ont été exposés au cuivre, au glyphosate et au chlorpyrifos seuls ou en mélange. Une autre partie de ce travail a consisté à étudier les effets toxiques des échantillons environnementaux d'eau et de sédiment provenant d'un cours d'eau, La Livenne, à proximité de parcelles viticoles. Toutes les expériences ont été faites en conditions contrôlées de laboratoire. Les réponses induites par ces expositions ont été mesurées à différents niveaux d'organisation biologique : au niveau moléculaire, phénotypique et comportemental pour l'étude in vivo (larves de la truite arc-en-ciel) ; et l'analyse des effets cytotoxiques de l'induction des espèces réactives de l'oxygène (ROS) et des dommages à l'ADN pour l'étude in vitro (lignée RTL-W1).

Les résultats de ce travail ont montré que l'exposition aux pesticides individuels, ou en mélange, chez les larves de truites arc-en-ciel n'a pas produit d'effets létaux aux concentrations testés. En revanche, ces substances ont provoqués différents effets sub-létaux, selon le composé et les concentrations étudiés, dont des effets tératogènes, des perturbations du comportement natatoire, des effets génotoxiques et l'expression différentielle de gènes cibles. Le cuivre s'est avéré le plus toxique provoquant un échec d'éclosion important. Des effets cytotoxiques et une production d'espèces réactives de l'oxygène (ERO) ont été observés sur cellules de truite exposées à des extraits d'eau de rivière. Par ailleurs, des effets comportementaux ont été observés sur les larves de truites exposées pendant 48 h à des sédiments et de l'eau de la Livenne. En conclusion, ces travaux de thèse ont mis en évidence des effets sub-létaux sur les cellules et les stades précoces de développement de truite arc-en-ciel exposés à des concentrations environnementales de pesticides utilisés en viticulture.

Mots clés : pesticides, cuivre, glyphosate, chlorpyrifos, eaux et sédiments de rivière, embryons et larves de truite arc-en-ciel, lignée cellulaire RTL-W1, embryotoxicité, génotoxicité, stress oxydant, comportement natatoire

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Environnements et Paléoenvironnements Océaniques et Continentaux

(EPOC-Ecotoxicologie Aquatique)

UMR CNRS 5805

Title: Study of the adaptive and deleterious responses of early stages of development of rainbow trout, Oncorhynchus mykiss, exposed to phytosanitary products used in viticulture

Abstract:

The excessive use of pesticides generates significant pollution and degradation of the environment, including aquatic ecosystems. The viticultural activity is not an exception for this rule, and this is why the aim of this work is to study the adaptive or deleterious responses in the early life stages of fish exposed to three phytosanitary products used in viticulture as well as to their mixture and environmental samples.

The thesis focuses on the early stages of development of rainbow trout, but also on the trout liver cell line RTL-W1. Trout embryos were exposed to copper, glyphosate and chlorpyrifos alone or as a mixture. Another part of this work consisted in studying the toxic effects of environmental samples of water and sediment coming from a river, La Livenne, close to vineyard plots. All experiments were done under controlled laboratory conditions. The responses induced by these exposures were measured at different levels of biological organization: at the molecular, phenotypic and behavioral level for the in vivo study (rainbow trout larvae); and the analysis of cytotoxic effects and induction of reactive oxygen species (ROS) and DNA damage for the in vitro study (RTL-W1 line).

The results of this work showed that exposure to individual or mixed pesticides in rainbow trout larvae did not produce lethal effects at the tested concentrations. In contrast, these substances caused different sub-lethal effects, depending on the compound and concentrations studied, including teratogenic effects, swim behavior disturbances, genotoxic effects, and differential expression of target genes. Copper was found to be the most toxic causing a major hatching failure. Cytotoxic effects and production of reactive oxygen species (ROS) were observed on trout cells exposed to river water extracts. In addition, behavioral effects were observed on trout larvae exposed for 48 h to sediments and Livenne water. In conclusion, these thesis studies revealed sub-lethal effects on cells and early stages of development of rainbow trout exposed to environmental concentrations of pesticides.

Keywords: pesticides, copper, glyphosate, chlorpyrifos, water and sediment from river, rainbow trout embryos and larvae, RTL-W1 cell line, embryotoxicity, genotoxicity, oxidative stress, swimming behavior

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Environnements et Paléoenvironnements Océaniques et Continentaux

(EPOC- Ecotoxicologie Aquatique)

UMR CNRS 5805

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Liste des abréviations et sigles

ACh	Acétylcholine
AChE	Acétylcholinestérase
ADN	Acide désoxyribonucléique
ADNc	Acide désoxyribonucléique complémentaire
AMPA	Acide aminométhylphosphonique
ANOVA	Analyse de variance (Analysis of variance)
ARN	Acide ribonucléique
ARNm	Acide ribonucléique messager
bax	Protéine X associé à Bcl2
BSA	Albumine sérique bovine
cat	Catalase
CPF	Chlorpyrifos
Cu	Cuivre
Cox1	Cytochrome-c-oxydase sous unité 1
DCE	Directive Cadre sur l'Eau
DD	Durée du développement (Jours x T°C)
DMSO	Dimethylsulfoxide
DNase	Désoxyribonucléase
DO	Densité optique
DT_{50}	Temps de demi-vie
EC_{50}	Concentration efficace 50 %
Ef1-α	Facteur d'élongation 1-α
ERO	Espèce réactive de l'oxygène
FAO	Organisation des nations unies pour l'alimentation et l'agriculture
Fpg	Formamidopyrimidine glycosylase
GLY	Glyphosate
gst	Glutathion –S-transférase
i.a.	Ingrédient actif
ICP	Inductively coupled plasma
ISO	Organisation internationale de normalisation (International Organization for Standardization)
IUPAC	International Union of Pure and Applied Chemistry
koc	Coefficient d'adsorption dans le sol

LC/MS	Chromatographie liquide couplée à la spectrométrie de masse
LD	Limite de détection
LMPA	Low melting point agarose
LC_{50}	Concentration létale 50 %
LOEC	Lowest observed effect concentration
LQ	Limite de quantification
MDA	malonedialdéhyde
mt1	Métallothionéine isoforme 1
mt2	Métallothionéine isoforme 2
NQE	Norme de qualité environnementale
NOEC	No observed effect concentration
OCDE	Organisation du Commerce et du Développement Économique
OP	Organophosphate
pH	Potentiel hydrogène
PCR	Réaction en chaîne par polymérase
PEIMA	Pisciculture Expérimentale INRA des Monts d'Arrée
qRT-PCR	Réaction de polymérisation en chaîne quantitative après rétrotranscription
RNase	Ribonucléase
REACh	Registration, Evaluation and Authorisation of Chemicals
ROCCH	Réseau d'observation de la contamination chimique
rpl7	Protéine ribosomique L7
RTL-W1	Rainbow Trout Liver-Waterloo 1
SAU	Surface agricole utile
sod	Superoxyde dismutase
TBA	Acide thiobarbiturique
TBARS	Thiobarbituric acid reactive substances
US-EPA	United States Environmental Protection Agency
WHO	World Health Organization

Introduction générale

Introduction

Depuis quelques années, la population mondiale est de plus en plus sensibilisée à l'utilisation des pesticides et à ses effets sur la santé humaine et l'environnement. Dans le cas de la France, l'un des plus gros producteurs agricoles européens, c'est aussi l'un des plus grands consommateurs de pesticides (Aubertot et al., 2005). Cependant, une grande disparité d'utilisation de ces produits phytosanitaires existe selon la nature de la culture. Ainsi, en métropole, 4 types de cultures (céréales, maïs, colza et vigne) ne représentent que 40 % de la surface agricole utile (SAU), mais consomment chaque année environ 80 % du total des pesticides du pays (Aubertot et al., 2005). Rien qu'en 2013, 60,000 tonnes de produits phytosanitaires ont été vendues en France, dont 14,000 pour le bassin Adour-Garonne (Agence de l'Eau Adour-Garonne, 2014). Dans le territoire de la Gironde, la culture dominante est la vigne qui correspondent à 50 % de la superficie totale cultivée du département. Cependant, la production viticole est très dépendante des traitements phytosanitaires. Environ 20 traitements phytosanitaires sont réalisés chaque année dans la vigne (Aubertot et al., 2005). L'utilisation intensive de ces produits phytosanitaires a entraîné la degradation des sols (Komárek et al., 2010; Ruyters et al., 2013).

Le terme pesticide englobe une grande diversité de substances différentes, qui se caractérisent par la spécificité de leur structure chimique, leur mode d'action et par l'organisme cible. Actuellement, certains types de pesticides, tels que les insecticides organochlorés, ont été interdits en raison de leur toxicité élevée et de leur persistance dans l'environnement. Cependant, même si ces pesticides ont été remplacés par des molécules moins toxiques ou plus biodégradables dans les pays développés, on estime qu'un demi-million de tonnes de pesticides « obsolètes » sont toujours dispersés dans des régions en développement telles que l'Asie, l'Amérique Latine et l'Afrique (Köhler and Triebskorn, 2013). Actuellement, les nouvelles formulations de pesticides ont évolué et sont conçues sur la base du mode d'action souhaité, avec le double objectif de traiter efficacement les organismes cibles, et de minimiser les effets indésirables sur les organismes non ciblés (Köhler and Triebskorn, 2013). Cependant, en raison des relations phylogénétiques et des habitats proches entre les espèces non cibles et les organismes nuisibles, il est devenu difficile d'atteindre ces objectifs (Köhler and Triebskorn, 2013).

Les pesticides peuvent générer différents effets indésirables, affectant non seulement la qualité de l'eau, de l'air et du sol, mais également les organismes qui les habitent. De ce fait, compte tenu de l'ampleur des traitements et des pratiques agricoles actuelles, ils ont entraîné une perte de la biodiversité dans de nombreuses régions du monde (Devine et al., 2008; Köhler and Triebskorn, 2013). Une fois appliqués sur les cultures, les pesticides peuvent atteindre les masses d'eau par le ruissellement et le lessivage des sols agricoles, par les masses d'air polluées, ainsi que par des applications directes pour lutter contre les plantes aquatiques nuisibles et d'autres organismes (comme les antifouling pour

protéger les bateaux contre les coques) (Babcsányi et al., 2014; Devier et al., 2005; Petit et al., 2013). Lorsqu'ils atteignent les écosystèmes aquatiques, les pesticides peuvent influencer la qualité de l'environnement en réduisant la diversité des espèces, en modifiant les chaînes alimentaires, et en modifient la stabilité et la résilience des écosystèmes (Pérez et al., 2011; Relyea and Hoverman, 2006). Certains pesticides sont présents de façon transitoire dans la colonne d'eau et de façon plus permanente dans les sédiments (Álvarez et al., 2013). L'accumulation de ces composés dans les sédiments aquatiques dépend essentiellement de leur hydrophobicité, de la nature du sédiment et de son pH, ainsi que la concentration de chaque polluant (Álvarez et al., 2013). Les sédiments qui sont pollués ont également un impact direct sur la biodiversité, notamment pour les espèces benthiques, le biofilm et la macrofaune (Chapman and Wang, 2001; Hyland et al., 2003).

Même si les pollutions aiguës par les pesticides ont diminué en milieu aquatique, les pollutions chroniques ou à long terme sont toujours d'actualité. La plupart des travaux d'écotoxicologie se concentrent principalement sur l'étude de pesticides individuels, alors que de nombreux rapports soulignent que les pesticides se trouvent généralement dans des mélanges complexes et à de faibles concentrations (Relyea, 2009; Stone et al., 2014). Bien que les concentrations individuelles soient souvent inférieures à la NOEC (Non Observed Effect Concentration), la présence de produits de dégradation et les interactions entre molécules en mélange peuvent entraîner des effets additifs, synergiques ou antagonistes (Beyer et al., 2014).

Dans le cadre de cette étude, les effets indésirables létaux et sub-létaux des pesticides isolés ou en mélange ont été caractérisés sur la truite arc-en-ciel, *Oncorhynchus mykiss*, en s'intéressant aux premiers stades de développement de ce poisson et à des cultures cellulaires. La sélection de molécules phytosanitaires a été faite par rapport à leur utilisation en viticulture en Gironde. Ainsi, pour représenter la diversité des pesticides utilisés en viticulture, un fongicide le sulfate de cuivre, un herbicide le glyphosate, et un insecticide le chlorpyrifos-éthyl ont été choisis. Afin de répondre à la problématique de l'usage des produits phytosanitaires et de leur toxicité sur les organismes aquatiques, une partie de cette travail s'est inscrit dans le cadre du projet PhytoCote du LabEx COTE visant à étudier le bassin versant de la Livenne, cours d'eau traversant un bassin viticole dans le Blayais.

Dans ce contexte, les différents objectifs de ces travaux de thèse ont été :

- Evaluer les effets indésirables de 3 molécules phytosanitaires utilisés notamment en viticulture avec des modes d'action différents : un fongicide (le cuivre), un herbicide (le glyphosate) et un insecticide (le chlorpyrifos) sur embryons et larves de la truite arc-en-ciel (**Articles 1**, **2** et **3**). Pour cela, un système d'exposition pour les embryons de truites a été développé avec un

système de renouvellement d'eau en flux continu. Pour avoir une plus grande pertinence environnementale, chaque pesticide a été étudié à des concentrations environnementales.

- Etudier les mécanismes des réponses de défense des organismes face à une exposition multiple à un mélange de pesticides (**Article 4**). Dans un contexte de contamination multiple, les 3 molécules en mélange ont été utilisées pour exposer des stades embryo-larvaires de la truite arc-en-ciel, en étudiant une nouvelle fois des concentrations environnementales.
- Evaluer les effets toxiques des échantillons d'eau et de sédiment collectés à proximité de parcelles viticoles sur la lignée cellulaire RTL-W1 et les larves de truite arc-en-ciel (**Article 5**). Cette partie de l'étude s'inscrit dans le cadre du Projet PhytoCote du LabEx COTE.
- Comprendre les effets de ces pesticides (le cuivre, le glyphosate et le chlorpyrifos) à différentes niveaux d'organisation biologique : au niveau moléculaire (expression de gènes, oxydation de protéines et lipides, génotoxicité, activité acétylcholinestérase) et sur la physiologie et les traits de vie des embryons et larves de poissons (survie, éclosion, morphométrie, comportement).

Le **chapitre I** aborde les généralités sur les pesticides ainsi que la consommation de produits phytosanitaires en France, principalement dans le département de la Gironde. Les modèles biologiques étudiés lors de ce travail de thèse, seront décrits dans cette partie.

La stratégie expérimentale et les analyses réalisées au cours de cette étude sont détaillés dans le **chapitre II**. Dans ce chapitre, les biomarqueurs, qui ont été étudiés sur les larves de truite, ont été présentés.

Les **chapitres III**, **IV** et **V** présentent les différents résultats acquis au cours de cette thèse rédigés sous forme d'articles.

Et finalement, le **chapitre VI** présent une discussion générale autour des principaux résultats obtenus ainsi que les perspectives de ce travail.

Chapitre I

Contexte de l’étude

1. Pesticides : Généralités

Les pesticides sont des produits chimiques, naturels ou synthétiques, employés par l'homme pour contrôler ou combattre des organismes, unicellulaires ou pluricellulaires, considérés comme ravageurs. Les principaux ravageurs agricoles comprennent les mauvaises herbes ; les insectes, arthropodes et vertébrés qui se nourrissent des cultures ; et les agents pathogènes qui causent des maladies dans les cultures, y compris les champignons, virus et bactéries. Le choix des pratiques de contrôle et des produits dépend de l'organisme à traiter (Abdollahi et al., 2004). Le mot « pesticide » englobe aussi les produits phytopharmaceutiques et les produits biocides. Parmi les produits biocides, il peut être mentionné les produits antiparasitaires (telles que les rodenticides, avicides, molluscicides, etc.), les produits de protection (pour les pellicules, du bois, des fibres, antimoisissures, etc.), les désinfectants (destinés à l'hygiène humaine, à l'hygiène vétérinaire, à la santé publique), et d'autres produits (de protection pour les denrées alimentaires ou les aliments pour animaux) (INSERM, 2012).

Le terme pesticide a été plutôt associé aux composés chimiques synthétiques ; pourtant, dès le début de l'activité agricole, les pesticides étaient fabriqués à partir de produits naturels. En effet, la lutte contre les ravageurs remonte à 4000 ans. Le premier pesticide connu était la poudre de souffre élémentaire utilisée dans l'ancienne Mésopotamie. Pour les peuples de Grèce et de l'Empire Romain, l'agriculture était d'une grande importance, et ils obtiendraient leurs pesticides de diverse sources végétales, ainsi que de certains minéraux. À partir du XVe siècle, des produits chimiques tels que l'arsenic, le mercure et le plomb étaient également appliqués dans les cultures pour éliminer les parasites. Plus tard, au XIXe siècle, d'autres types de traitement de pesticides apparaissent tels que les fongicides comme la bouillie bordelaise (à base de cuivre et chaux), qui est encore utilisée aujourd'hui. Avec le développement de la chimie organique, les pesticides de synthèse débutent depuis les années 1940 avec la commercialisation du DDT (dichlorodiphényltrichloroéthane), et les découvertes de nouveaux pesticides se font rapidement. L'utilisation des produits synthétiques a permis de lutter plus efficacement contre les parasites. Cependant, la présence de ces produits dans l'environnement a contribué à détériorer les écosystèmes et la santé humaine.

À différence des polluants provenant des industries, les déchets miniers, les résidus pharmaceutiques qui sont normalement rejetés involontairement, les pesticides représentent un des contaminants qui sont rejetés intentionnellement dans l'environnement, avec le but précis d'affecter négativement les systèmes biologiques de ravageurs (Abdollahi et al., 2004; Sánchez-Bayo, 2011). L'utilisation généralisée des pesticides, dans les programmes de santé publique et agricole, a provoqué une grave pollution de l'environnement et des risques pour la santé (Abdollahi et al., 2004). Ces produits font parfois partie des écosystèmes, mais un grand nombre d'entre eux sont toxiques pour les

organismes non-ciblés (Abdollahi et al., 2004). On estime que chaque année 4 millions de tonnes de pesticides sont appliquées aux cultures dans le monde, mais on estime que moins de 1 % du total de ces pesticides parvient aux ravageurs ciblés (Abdollahi et al., 2004; Gavrilescu, 2005; Sánchez-Bayo, 2011). D'après Sánchez-Bayo (2011), 25 à 50 % du pesticide pulvérisé par avion parvient aux cultures, et 62 à 90 % si les pesticides sont pulvérisés avec des machines au sol.

A l'échelle mondiale, les données montrent que, sans une lutte antiparasitaire efficace, les pertes de récoltes seraient en moyenne d'environ 40 % (Gavrilescu, 2005). Sans surprise, les populations d'animaux sauvages sont systématiquement affectées par l'exposition directe aux pulvérisations d'insecticides, en particulier les oiseaux présents dans les zones agricoles au moment de la pulvérisation des pesticides (Köhler and Triebskorn, 2013; Sánchez-Bayo, 2011). L'objectif de la chimie moderne des pesticides est de produire des pesticides qui sont efficaces en petites quantités, plus spécifique aux organismes cibles, et moins persistants dans l'environnement (Gavrilescu, 2005).

Les produits commerciaux n'ont pas de nomenclature bien précise, et leur nom technique fait référence à l'ingrédient actif (i.a.) dont le produit commercial est composé. Le nom chimique fait référence au nom de la molécule chimique de l'ingrédient actif du pesticide, selon les normes d'IUPAC (de l'anglais International Union of Pure and Applied Chemistry). La formulation d'un pesticide comprend l'ingrédient actif et d'autres substances inertes telles que les solvants, les diluants et les adjuvants qui contribuent à l'action de l'ingrédient actif (Anzalone, 2008).

Dans l'Union Européenne, la qualité des eaux de consommation a des limites de qualité fixées concernant les eaux brutes destinées à la consommation d'eau potable et à l'eau du robinet du consommateur (Parlement européen et Conseil de l'Union Européenne, 2008). La qualité limite dans les ressources en eau a été réglementé en 2 µg/L pour chaque pesticide et 5 µg/L pour l'ensemble des substances mesurés, tandis que la qualité limite pour le robinet du consommateur est de 0.10 µg/L pour chaque pesticide et 0.50 µg/L pour le total des substances mesurées. Par rapport à la qualité des masses d'eau, la Directive Cadre sur l'Eau (DCE, adoptée le 23 octobre 2000) a défini la liste des substances prioritaires, qui regroupe 45 molécules, dont 19 pesticides (tels que le diuron, l'atrazine et le chlorpyrifos), identifiés comme prioritaires vis-à-vis de leur toxicité et de leur permanence en milieu aquatique (DCE, 2013). Afin de définir l'état des cours d'eau, des normes de qualité environnementale (NQE) ont été fixés. L'état chimique est alors défini par 2 classes de qualité (bon ou mauvais), en comparant la concentration mesurée d'une molécule et la NQE.

1.1. Diversité des pesticides

Le terme pesticide englobe une collection très variée de substances différentes, souvent caractérisées par la spécificité de leur structure chimique et par l'organisme ciblé (Tableau 1). Cependant, ils peuvent être classés aussi selon leur mode d'utilisation, et selon leur interaction avec l'environnement (Gavrilescu, 2005). La classification en fonction de la structure chimique prend en compte une grande variété de groupes ou de familles de composés. Ces familles chimiques ont des caractéristiques chimiques et moléculaires communes. Par exemple, les insecticides organophosphorés comprennent environ 58 produits différents, tandis que la famille de la glycine ne compte qu'un seul membre, le glyphosate. L'avantage de ce type de classification est qu'elle permet l'identification de substances qui tendent à avoir des effets similaires sur les organismes nuisibles et l'environnement. Un autre classement des pesticides a été fait par l'Organisation Mondiale de la Santé (OMS) en fonction des dangers pour la santé humaine et du degré de toxicité du produit (Tableau 2).

Dans ce mémoire de thèse, j'ai choisi de classer les pesticides selon leur fonction et l'organisme nuisible à traiter. Nous verrons donc successivement les herbicides, les insecticides et les fongicides.

Tableau 1 : Produits chimiques agricoles y compris les pesticides et leur activité. Source : Boland et al. (2004)

Catégorie	**Activité/cible**
Algicide	Elimine les algues
Anorexigène	Prévient que les animaux se nourrissent de la culture ou du produit stocké
Appât	Attire les animaux provoquant des fléaux
Bactéricide	Elimine ou inhibe la croissance des bactéries
Fongicide	Désinfectant pour moisissures et champignons
Herbicide	Elimine ou inhibe la croissance des adventices
Régulateur de croissance d'insectes	Modifie les phases de développement ou de croissance des insectes
Insecticide	Elimine les insectes
Acaricide	Elimine les acariens
Molluscicide	Elimine les escargot et limaces
Nématicide	Elimine les nématodes
Rodenticides	Elimine les rats, les souris, les rongeurs
Stérilisant	Stérilise les insectes par voie chimique
Termicide	Elimine les termites

Tableau 2 : Classification des pesticides selon l'OMS.

Classe de danger OMS	Niveau de toxicité	LD_{50} pour le rat (mg/kg poids)		Exemples
		Oral	Dermique	
Classe Ia	Extrêmement dangereux	<5	<50	Parathion, diéldrin
Classe Ib	Très dangereux	5-50	50-200	Eldrin, dichlorvos
Classe II	Modérément dangereux	50-2000	200-2000	DDT, chlordane
Classe III	Peu dangereux	>2000	>2000	Malathion
Clase IV	Ne semble pas dangereux en cas d'usage normale	≥5000		Carbetamide, cycloprothrin

1.1.1. Herbicides

Les adventices ou les « mauvaises herbes » sont toutes des espèces de plantes qui pousse à l'état sauvage dans une zone cultivée ou contrôlée par l'homme, telles que les cultures agricoles et les jardins. Toute plante peut être considérée comme une adventice si elle pousse dans un endroit où n'est pas souhaitable. Les plantes qui poussent agressivement et qui empêchent le développement normal des autres espèces de plantes peuvent également être considérées comme des adventices. Généralement ces plantes peuvent être contrôlées par l'application d'un type ou d'une classe de pesticide spécifique.

Les herbicides sont des composés complexes utilisés pour éliminer ou contrôler la végétation et les mauvaises herbes dans les cultures. Les herbicides peuvent être classés en fonction de leur structure chimique, de leur utilisation et de leurs effets sur les plantes. Une définition commune des herbicides indique qu'il s'agit d'un « *produit chimique qui perturbe la physiologie ou le métabolisme d'une plante pendant un temps suffisant pour la tuer ou pour réduire la croissance* » (Anzalone, 2008).

Les herbicides d'un même groupe chimique ont des caractéristiques physico-chimiques communes qui leur permettent de prédire comment un nouvel herbicide du même groupe devrait être utilisé. Bien qu'ils soient généralement divisés en organiques ou inorganiques, la plupart des herbicides sont organiques et comprennent principalement les aliphatiques, les amides, les benzoïques, les pyridines, les triazines, etc.). Les herbicides organiques sont généralement composés de carbone, d'hydrogène, et d'oxygène et, en fonction de l'herbicide, et peuvent inclure aussi des atomes de fluor, de phosphore, de chlore, d'azote, etc. (Anzalone, 2008). La présence des adjuvants dans la formulation

de l'herbicide aura une influence sur les propriétés chimiques et structurelles de l'herbicide, telles que sa solubilité, sa volatilité et sa charge électrique (Anzalone, 2008; Varshney and Sondhia, n.d.).

En fonction de l'effet que cela peut avoir, les herbicides sont regroupés en substances sélectives ou non sélectives. La sélectivité est la capacité de certains herbicides à provoquer des perturbations physiologiques ou métaboliques entraînant la mort de certaines espèces végétales et pas d'autres (Anzalone, 2008). Les herbicides sélectifs sont des composés qui éliminent certaines mauvaises herbes sans endommager de manière significative une culture associée ou une autre espèce souhaitable. Les herbicides non sélectifs suppriment une large gamme de végétation. La classification en fonction du mode d'action prend en compte les différences dans les actions physiologiques et biochimiques des herbicides. Sous cet aspect, les herbicides peuvent être classés comme herbicides systémiques ou à effets localisés et systémiques ou comme herbicide de contact.

1.1.2. Insecticides

En dépit des progrès considérables accomplis par l'humanité dans les domaines de la science et de la technologie, très peu d'innovations ont été accomplis dans la lutte contre les insectes. Environ 10,000 espèces d'insectes se nourrissent des plantes près de 700 espèces dans le monde causent des dégâts aux cultures (Devine et al., 2008). De plus, de nombreux insectes parasites viennent attaquer les populations humaines et les animaux et propagent des maladies graves. Après la Seconde Guerre Mondiale, le développement et l'utilisation intensive d'insecticides tels que le DDT, la dieldrine, l'aldrine, le chlordane, entre autres, ont permis de lutter efficacement contre des centaines d'espèces d'insectes nuisibles. Cependant, les cycles de vie des insectes étant très courts, de nombreuses espèces ont développé une résistance aux insecticides (Devine et al., 2008).

Le DDT, le dieldrine, l'aldrine et d'autres insecticides organochlorés sont reconnues comme insecticides dangereux car ils persistent dans l'environnement sans se décomposer en substances inertes. Les insecticides organochlorés ont une structure formée par une chaîne d'atomes de carbone (C) et, en tant que groupes substituants, l'atome de chlore (Cl). Ces substances persistantes ont entraîné une diminution de la population d'oiseaux au cours des années 1960 aux Etats-Unis et en Europe (Becher and Hartig, 2005). Actuellement, l'utilisation d'insecticides organochlorés est interdite dans la plupart des pays du globe, et ils ont été remplacés par d'autres classes d'insecticides moins persistants tels que les organophosphorés, les carbamates (Figure 1) et les néonicotinoïdes. Par ailleurs, d'autres substances moins toxiques, telles que des insectifuges, des attractifs sexuels, des hormones d'insectes, des substances stérilisantes, ont été développé pour le contrôle physique et/ou biologique des insectes nuisibles (Yadav and Devi, 2017).

Les organophosphorés (OP) (Figure 1, 2) sont le terme générique qui comprend tous les insecticides contentant du phosphore. Les OP ont deux caractéristiques : ils présentent une toxicité élevée envers une grande variété d'organismes et la plupart sont chimiquement instables ou non persistants (Devine et al., 2008). En raison de cette dernière caractéristique, les OP ont été acceptés pour un usage agricole. Cependant, il est nécessaire de répéter plusieurs fois le traitement pour assurer une protection durable des cultures. Les OP doivent leur activité insecticide à leur capacité à phosphoryler l'enzyme cholinestérase, qui régule la transmission de l'influx nerveux dans les neurones cholinergiques. L'effet de l'inactivation de l'enzyme entraîne une accumulation d'acétylcholine au niveau de la synapse. Il en résulte une sur-stimulation des neurones moteurs, provoquant des tétanies musculaires, des convulsions, une paralysie et finalement la mort de l'organisme (EPA).

Les carbamates (Figure 1, 2) sont un groupe d'insecticides qui ont gagné en popularité. Ils possèdent des dérivés de l'acide carbamique dans leur structure. De même, comme les organophosphorés, les carbamates agissent en inhibant la cholinestérase (Devine et al., 2008). Cependant, les carbamates sont moins toxiques que les organophosphorés et one une durée de vie beaucoup plus courte.

Les néonicotinoïdes (Figure 2) sont une autre classe d'insecticides (systémique) introduits récemment (1991) (Kurwadkar and Evans, 2016). Les néonicotinoïdes sont des dérivés synthétiques de la nicotine, et agit également sur le système nerveux central (Watts, 2011). Comparé aux insecticides organophosphorés et carbamates, les néonicotinoïdes sont moins toxique pour les vertébrés (oiseaux et mammifères). L'imidaclopride, le premier néonicotinoïde, est rapidement devenu populaire en raison de son activité insecticide élevée, ainsi que de son faible taux d'application (0.3 mg/L) (Kurwadkar and Evans, 2016). Cependant, l'utilisation de l'imidaclopride, étant liée à la disparition des abeilles, a été restreint en France depuis 1999 pour le traitement des graines de tournesol, et finalement interdit dans les grandes cultures à partir de septembre 2018 par les états membre de l'Union Européenne (EU 2018/783).

Les insecticides à large spectre ont généralement des effets immédiats mais à court terme (Mondragon, 2002). Les insecticides sont l'un des outils agricoles ayant le plus d'impact sur l'environnement et en raison de leur utilisation abusive et au mauvais usage, ils entraînent des conséquences auxquelles il est difficile de remédier. La mer d'Aral en Asie Central, en est un exemple (Devine et al., 2008). De mauvaises pratiques agricoles mal planifiées et mal exécutées ont dévasté la région, et les résidus d'insecticides organiques et organophosphorés sont très abondants dans les sols de la région (Devine et al., 2008).

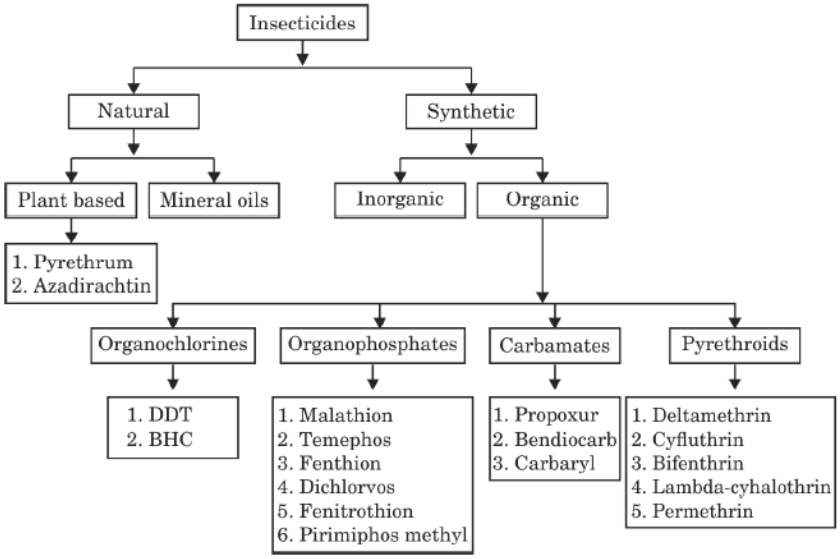


Figure 1 : Classification des insecticides (Yadav and Devi, 2017).

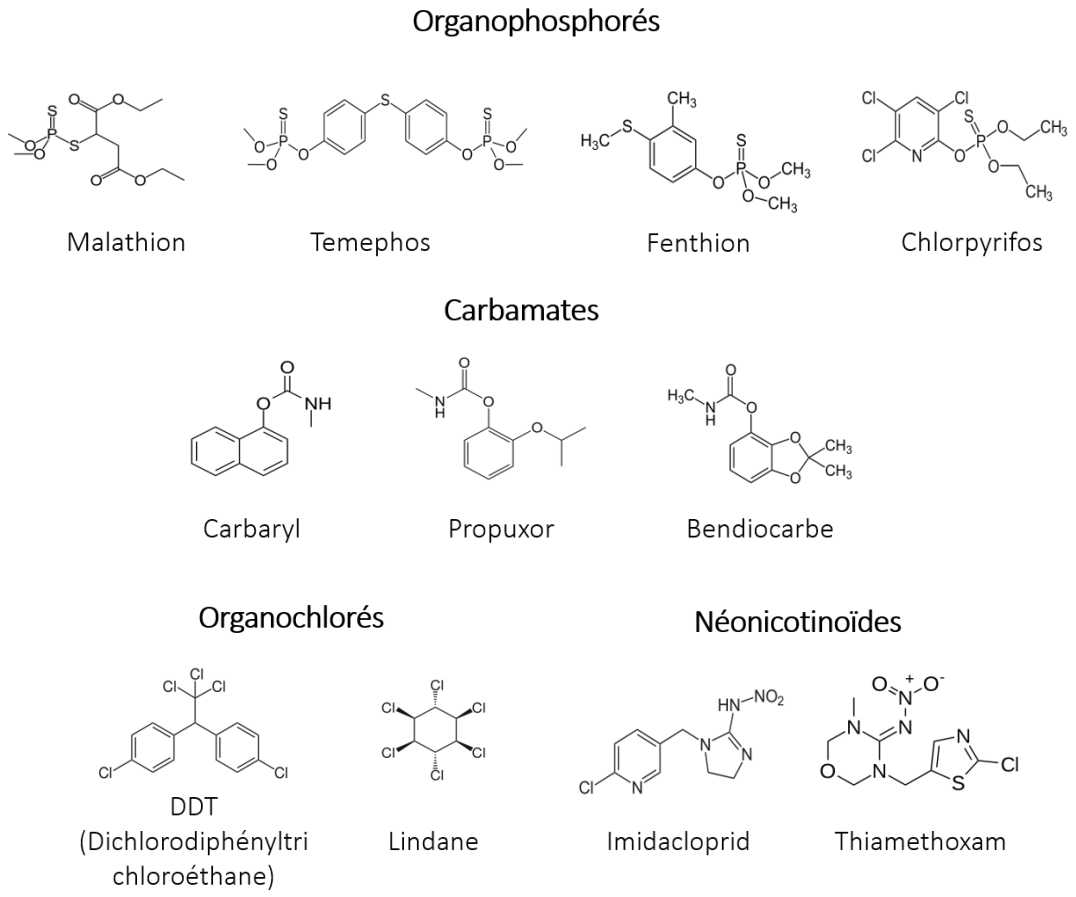


Figure 2 : Structure des quelques insecticides selon la classification.

1.1.3. Fongicides

Un autre type de dommages qui peuvent survenir dans les cultures et les plantes sont les maladies qui peuvent être causées par un certain nombre agents pathogènes comme les virus, les nématodes, les bactéries et les champignons. Les champignons sont la cause principale de pertes de récoltes dans le monde (Rouabhi, 2010). Le fongicide est une classe spécifique de pesticide utilisée pour éliminer ou empêcher le développement de champignons responsables des maladies dans les plantes. Les fongicides peuvent augmenter la productivité d'une culture et réduire les nécroses foliaires qui peuvent limiter la photosynthèse des plantes.

Il existe aussi des fongicides de contact ou systémiques (Rouabhi, 2010). Les fongicides de contact, appliqués sur le feuillage, ne sont généralement pas absorbés par la plante. Il reste donc à la surface des plantes, ne la protégeant que lorsque le fongicide a été déposé (Rouabhi, 2010). Ce type de fongicide empêche les spores de germer et de pénétrer dans les cellules. Les fongicides systémiques sont ceux qui sont absorbés par le feuillage ou les racines des plantes et sont distribués à travers les vaisseaux du xylème à la plante entière, en protégeant les feuilles de manière interne et externe.

Le mode d'action est très diversifié. Les fongicides peuvent endommager les membranes cellulaires en modifiant les structures des lipides, des stérols et d'autres composants membranaires (Yang et al., 2011). D'autres peuvent inactiver les enzymes ou les protéines essentielles des champignons, car certains fongicides interfèrent dans la biosynthèse des acides aminés et des protéines, en affectant les fonctions biologiques des organismes (Yang et al., 2011). Ils peuvent également interférer avec des processus clés tels que la production d'énergie ou la respiration mitochondriale, puisque, selon leur mode d'action, ils peuvent inhiber la NADH oxydoréductase (complexe I), la succinate déshydrogénase (complexe II), le cytochrome bc1 (complexe III) et les découpleurs de phosphorylation par oxydation (Yang et al., 2011).

1.2. Les produits commerciaux

La notion sur la toxicité des pesticides est principalement mise sur le composé actif. Cependant, les pesticides sont formulés pour augmenter leur efficacité contre les organismes cibles (Pérez et al., 2011). Chaque produit commercial contient une substance qui aura un effet pesticide ou toxique : il s'agit du principe ou ingrédient actif (i.a.). L'ingrédient actif est toujours identifié soit par son nom commun (atrazine, bentazone) ou soit par son nom chimique (1-chloro-3-éthylamino-5-isopropylamino-2,4,6-triazine ou 2,2-dioxyde de 3-isopropil-2,1,3-benzothiadiazine-4-one) (Nordby and Hager, 2004). Certains produits commerciaux incluent plusieurs ingrédients actifs pour combiner les effets de chacun.

Les autres ingrédients qui comptent pour une formulation sont les soi-disant inertes, qui normalement n'ont pas d'action biocide, mais comportent une série de fonctions différentes :

- Diluant : pouvant être l'eau, des huiles végétales ou certains solvants dérivés du pétrole, ou de l'argile ou du talc pour les solides.

- Adhérents : pour améliorer l'étalement du pesticide sur la surface traitée.

- Surfactant : fait référence aux produits chimiques qui ont une activité de surface prononcée dans les solutions aqueuses pouvant diminuer la tension de surface et perturber la perméabilité des membranes ou la fonction de transport des membranes.

- Agents d'alerte : pour prévenir des ingestions accidentelles par l'adjonction de colorants, odeurs importantes, de répulsif ou de vomitif.

La combinaison de ces substances dans un produit commercial, est ce que l'on appelle la composition. La même substance active peut être commercialisée sous différents "aspects ou formats" et avec des propriétés différentes les unes des autres. C'est ce que nous appelons des formulations. Certaines formulations de pesticides incluent un synergiste qui renforce l'activité toxique de l'ingrédient actif. Lors de l'examen des effets potentiels des pesticides sur la santé, il est important de tenir compte de la toxicité du principe actif ainsi que des autres ingrédients de la formulation.

Exemple : Commercial formulation of glyphosate

Glyphosate (nom commun) = N-(phosphonomethyl)glycine (nom chimique)

Les formulations commerciales de glyphosate sont des mélanges chimiques et doivent être considérées comme des mélanges dans les évaluations de toxicité (Pérez et al., 2011). La concentration en glyphosate dépendent des formulations commerciales ; bien que les informations disponibles sur les ingrédients inertes ne figurent généralement pas sur l'étiquette (Pérez et al., 2011). La formulation commerciale du glyphosate, Roundup®, est le nom de marque le plus populaire pour les herbicides à base de glyphosate. Roundup® contient du sel IPA de glyphosate (35-50%) plus des ingrédients inertes. La concentration de Roundup® est couramment exprimée en mg a.i. (active ingredient) /L ou en mg a.e. (acide equivalent) /L de glyphosate (Nordby and Hager, 2004). Le surfactant dans Roundup®, ainsi que dans certains autres produits à base de glyphosate, est le composé polyoxyéthylène amine (POEA) (Giesy et al., 2000).

1.3. Devenir des pesticides dans l'environnement : milieux aquatiques

La contamination des eaux de surface ou les eaux souterraines par les pesticides dépend de nombreux facteurs, notamment les propriétés du sol, les propriétés des pesticides, la charge hydraulique exercée sur le sol et la pratique de gestion des cultures (Gavrilescu, 2005). La principale source de pollution des pesticides provient des résidus produit lors de son application, sa dispersion, et dépend de ses propriétés chimiques de solubilité dans l'eau, la volatilité, la stabilité chimique et biologique (Figure 3).

Le devenir des pesticides dans l'environnement est influencé par de nombreux processus qui déterminent leur persistance et leur mobilité. En général, le mouvement d'un pesticide à partir du point d'application est contrôlé en premier lieu par des processus de transport du pesticide jusqu'au cours d'eau, et ensuite par des processus qui acheminent le pesticide de la colonne d'eau au sédiment. Les processus environnementaux qui régissent le devenir des pesticides dans les sols, les eaux de surface et les eaux souterraines peuvent être classés en trois types : (i) les processus de transport qui l'éloignent de son point initial d'introduction dans l'environnement et dans l'ensemble du système d'eau de surface, (ii) les processus de transfert, qui contrôlent ses mouvements parmi les compartiments environnementaux, tels que l'eau, le biote, les sédiments en suspension, les sédiments du lit des rivières, l'atmosphère, enfin (iii) les processus de transformation qui font référence à des processus biologiques et chimiques qui modifient ou dégradent complètement la structure d'un pesticide (Gavrilescu, 2005).

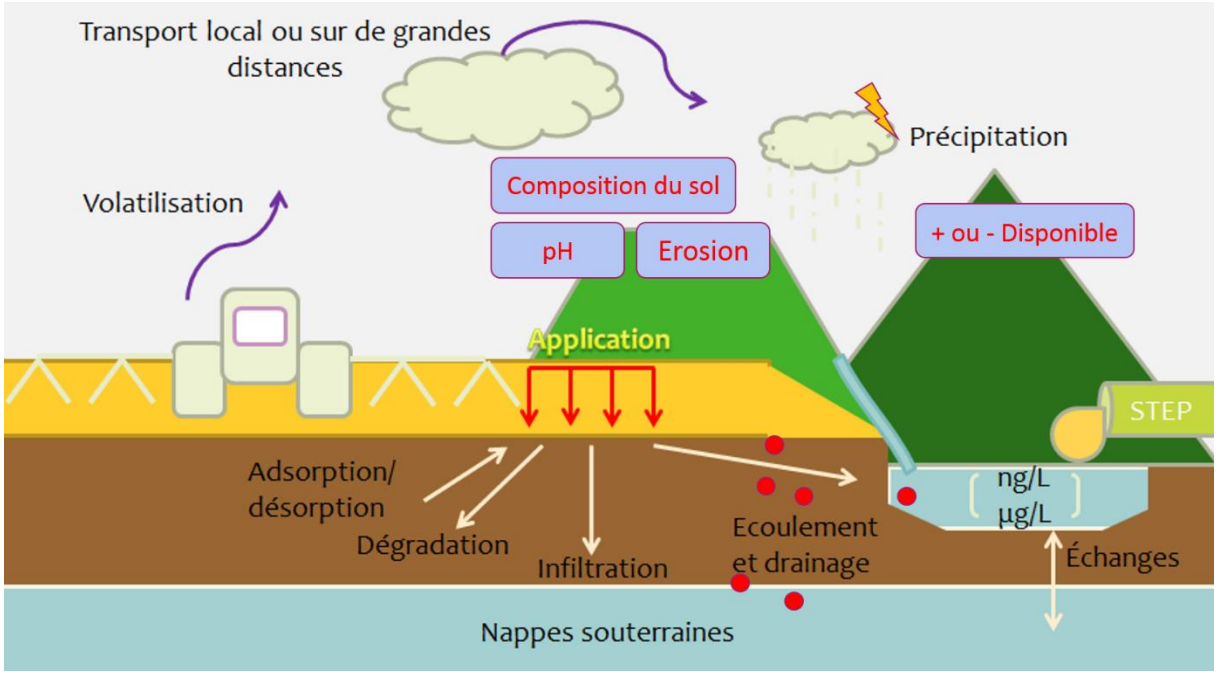


Figure 3 : Devenir des pesticides dans l'environnement.

La texture du sol et/ou la distribution de la taille des particules du sol influencent également la vitesse de déplacement de l'eau à travers le sol. Les sols à texture fine ont une plus grande surface et une perméabilité inférieure ; par conséquent, un temps de contact plus long et une plus grande surface de sorption pour l'atténuation de la pollution. Une teneur élevée en argile pourrait être avantageuse pour l'atténuation de la pollution en raison de la très petite taille des pores et de la grande surface disponible pour la sorption des cations. Une fois que les pesticides sont adsorbés par les colloïdes argileux, ils peuvent être inactivés ou dégradés (Gavrilescu, 2005).

Les autres caractéristiques des sols susceptibles d'influencer le transport ou la translocation des pesticides vers les milieux aquatiques sont (Figure 3) :

- La perméabilité des sols, qui correspond au taux de mouvement de l'eau à travers les sols.
- La profondeur du sol, qui influence la quantité et la durée du contact entre les polluants et les particules du sol.
- Le pH du sol, qui influence la solubilité des polluants et la vitesse du processus de biodégradation des polluants.
- La matière organique du sol, qui pourrait influencer le potentiel de sorption du polluant et le niveau d'activité biologique.
- La pente du sol, qui peut influer sur la quantité d'eau qui s'infiltrera dans le sol. Les pentes plus raides du sol conduisent à un plus fort ruissellement des eaux et donc des contaminants associés.

Les pesticides très solubles dans l'eau, ayant une faible tendance à s'adsorber sur les particules de sol et ayant une longue demi-vie ont le plus grand potentiel pour se déplacer vers les eaux de surface ou souterraines (Figure 4). Les grandeurs couramment utilisées pour évaluer les pesticides en fonction de leur potentiel de lessivage ou de déplacement avec le ruissellement de surface après application sont (Gavrilescu, 2005) :

- L'adsorption dans le sol : mesurée par le coefficient de partage carbone organique/eau : k_{oc}.
- L'hydrosolubilité : mesure la capacité de solubilisation et de transport du composé par l'eau.
- La persistance des pesticides : généralement mesurée par le temps de demi-vie, ou le temps en jours requis pour qu'un pesticide se dégrade à la moitié de sa concentration. Plus la demi-vie du pesticide est longue, plus le potentiel de déplacement du pesticide est grand.

Figure 4 : Photo du lessivage des sols aux abords d'une vigne après une journée de pluie.

1.4. La consommation de pesticides en France

Pendant quelques années, la France a été considérée comme le premier pays consommateur de pesticides en Europe. Il occupe actuellement la quatrième place, derrière l'Espagne, l'Italie et les Pays-Bas. Si l'on ne prend en compte que la consommation de pesticides par hectare de culture, la France occupe la 10ème place (Figure 5) (Eurostat, 2016). En effet, étant l'un des plus grands producteurs agricoles en Europe, avec une superficie de 29,423,031 hectares seulement dans la région métropolitaine (données AGRESTE pour 2016), la France est donc l'un des plus gros consommateurs de pesticides. Certains types de cultures pratiqués en France, tels que les céréales, le maïs, le colza et les vignes, sont très sensibles aux attaques d'insectes et aux maladies, de sorte que les traitements phytosanitaires sont pratiqués de manière intensive. Ces quatre cultures, représentent, à elles seules, 40 % de la SAU (surface agricole utile) et consomment chaque année environ 80% des pesticides dans tout le pays (Tableau 3) (Aubertot et al., 2005).

Un cas particulier est représenté par la vigne qui ne représente que 3 % de la SAU, mais consomme à elle seule 20 % de la consommation nationale en produits phytosanitaires (Tableau 3). Les céréales, qui occupent des surfaces beaucoup plus vastes, représentent elles 40 % de la consommation nationale (Aubertot et al., 2005).

Cependant, l'utilisation de ces pesticides (ainsi que des engrais) a permis d'accroître la productivité de l'agriculture par rapport aux années 1960. On estime que l'augmentation du rendement des terres agricoles a également permis de limiter la déforestation (Gatignol and Etienne, 2010). La Figure 6 montre l'évolution de l'usage agricole, entre les années 2009 et 2015. Les tendances, globalement à la hausse, montrent une augmentation des quantités de produits phytosanitaires vendues (DATALAB, 2018). Cette tendance peut s'expliquer par les aléas climatiques, avec des hivers doux et conditions climatiques humides au printemps qui sont favorables au développement des champignons causant des maladies sur les plantes (DATALAB, 2018; ECOPHYTO, 2018).

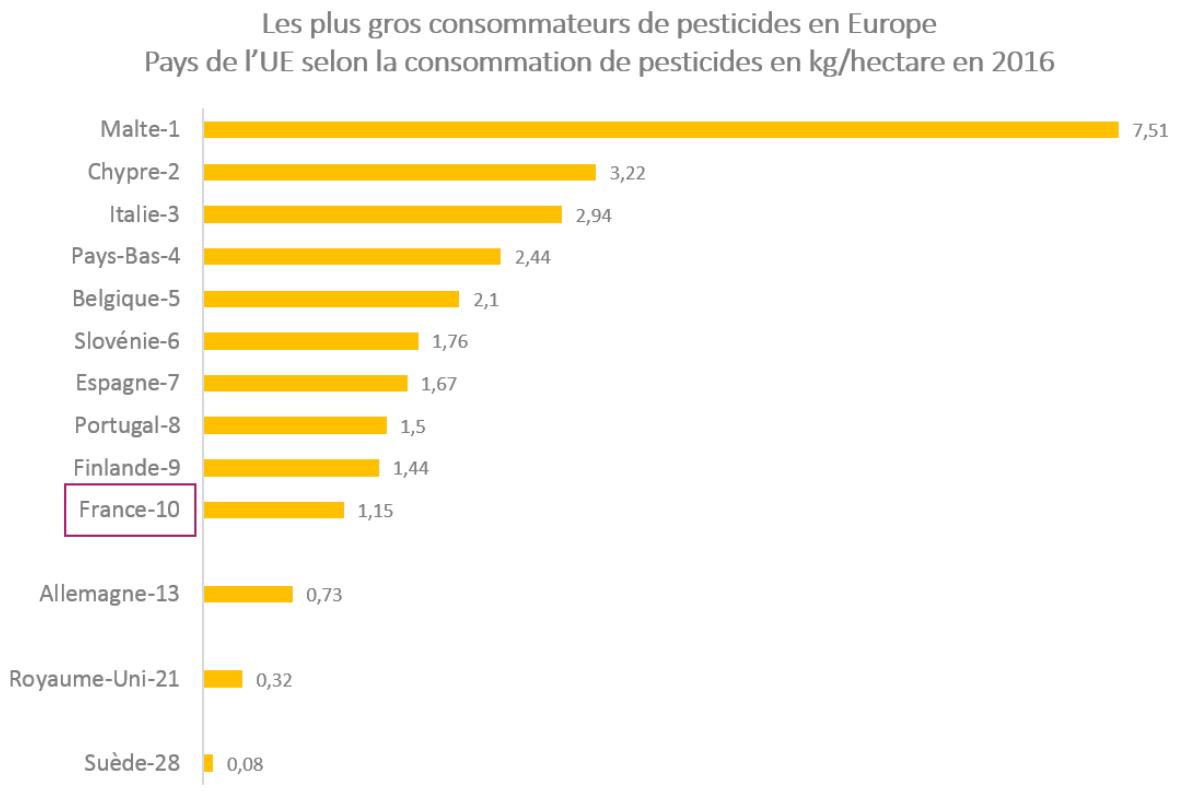


Figure 5 : Les pays les plus consommateurs de pesticides en Europe, année 2016. Source Eurostat https://fr.statista.com/infographie/15061/les-plus-gros-consommateurs-de-pesticides-en-europe

Tableau 3 : Consommation de pesticides en France pour quelques espèces de plantes agricoles (Gatignol and Etienne, 2010).

	Occupation du territoire et consommation de pesticides pour quelques espèces (données 2000, sources SCEES, UIPP)		
Cultures	%SAU (arrondi)	Consommation phytos (en % du total)	Remarques
Céréales à paille	24 %	40%	60% fongicides 35% herbicides
Maïs	7 %	10%	75 % herbicides
Colza	4 %	9%	
Vigne	3 %	20%	80 % fongicides
Ensemble	**38 %**	**79%**	

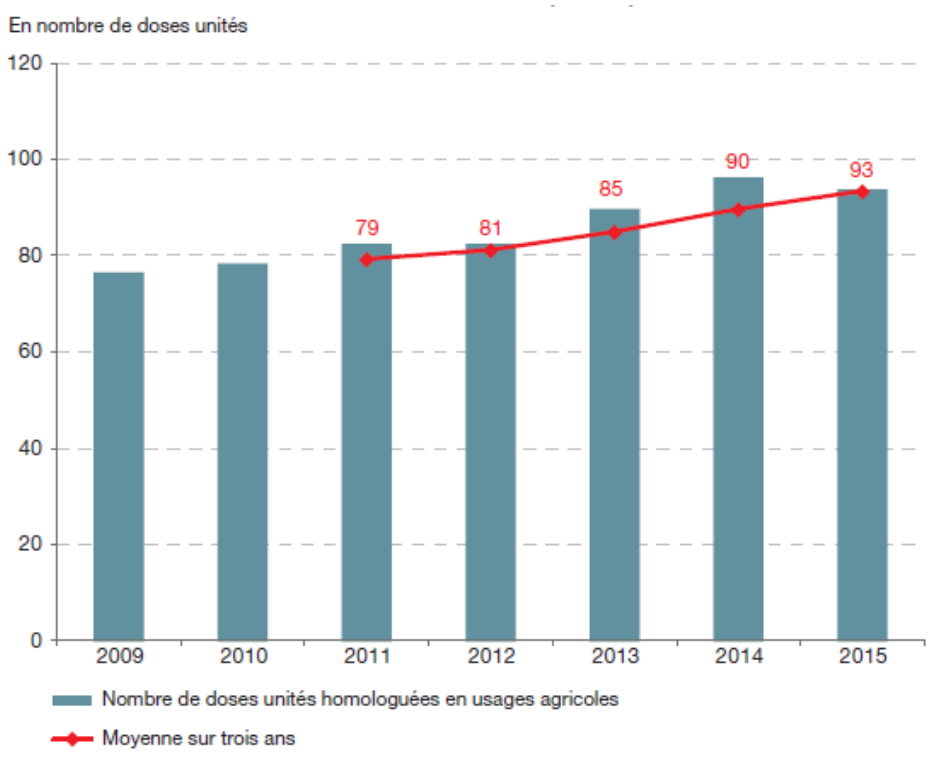


Figure 6 : Evolution du nombre de doses unités (NODU) à usage agricole (DATALAB, 2018).

1.4.1. Les traitements phytosanitaires en viticulture

La viticulture couvre une superficie de 86,000 hectares en France, ce qui ne représente que 3 % de la SAU. Cependant, la production viticole est très dépendante des traitements phytosanitaires, devenant une source importante de consommation de pesticides en France, notamment des fongicides (Monnier, 2014). A cause de l'introduction accidentelle des espèces invasives de bio-agresseurs, telles que le mildiou et l'oïdium, la vigne nécessite en moyenne 3 traitements fongicides par an (Monnier, 2014). Cela varie en fonction de la sensibilité des cépages à la maladie et des conditions climatiques (Monnier, 2014). Les traitements contre le mildiou et l'oïdium génèrent à elles seules 70 % des dépenses phytosanitaires (Aubertot et al., 2005). Le tableau 4 montre l'ensemble des principales substances utilisées dans les vignobles en France (Agreste, 2012). Parmi les produits fongicides, on trouve le folpel, le cuivre et le mancozèbe comme les plus utilisés en vigne. Les vignes reçoivent également au moins un traitement d'insecticide par an (Agreste, 2012). Ces traitements sont dirigés principalement contre les cicadelles (Agreste, 2012). L'indoxacarbe est l'une des substances les plus utilisées, suivi par les pyretrinoïdes (alphaméthrine et bifenthrine) et les organophosphorés (chlorpyrifos méthyl ou éthyl) (Tableau 5).

Selon Aubertot et al. (2005), les différents types de bioagresseurs pouvant avoir un impact agronomique sont regroupés en trois types d'agents :

- Agents de perte de récoltes : parasites qui se trouvent dans les parties aériennes de la plante et peuvent causer la destruction des grappes et des baies, ainsi que dans les feuilles. Pour traiter ce type de bioagresseur, l'utilisation de substances chimiques est considérée comme obligatoire par tous les viticulteurs.
- Agents responsables de la faible qualité des produits : les bioagresseurs qui affectent les propriétés organoleptiques ou les composants de typicité nécessaires à l'élaboration de vins répondant au standard d'appel (gamme d'alcool, composition phénologique, tanin, précurseurs d'arômes).
- Agents responsables de déchets ou de dégénérescence : les bioagresseurs attaquent généralement les organes ligneux, affectant la croissance ou le développement et donc la production de la plante, et pouvant provoquer à terme sa mort.

Tableau 4 : Principaux fongicides utilisés en vigne en 2010 (Agreste, 2012).

Matière active	Part des surfaces viticoles traitées (%) et nombre moyen de traitements				Maladies visées
	Bordelais		Bergeracois		
Folpel	83%	*3,0*	75%	*2,8*	*mildiou*
Cuivre (toutes formes)	76%	*1,8*	83%	*1,9*	*mildiou*
Soufre micronise	67%	*2,4*	74%	*2,9*	*oïdium*
Tébuconazole	50%	*2,0*	32%	*2,0*	*oïdium, black rot*
Cymoxanil	45%	*1,8*	27%	*1,7*	*mildiou*
Fosétyl-Aluminium	44%	*1,9*	28%	*1,9*	*mildiou*
Mancozèbe	35%	*1,8*	32%	*2,1*	*mildiou, excoriose, black-rot*
Quinoxyfène	33%	*1,3*	31%	*1,4*	*oïdium*
Spiroxamine	33%	*1,3*	30%	*1,4*	*oïdium, black-rot*
Metrafenone	28%	*1,3*	3%	*1,5*	*oïdium*
Trifloxystrobine	26%	*1,2*	2%	*1,0*	*mildiou, excoriose, black-rot*
Cyazofamid	25%	*1,4*	8%	*1,0*	*mildiou*
Metirame-zinc	22%	*2,0*	47%	*2,2*	*mildiou, oïdium, excoriose, black-rot*
Fluazinam	23%	*1,0*	2%	*1,0*	*pourriture grise*
Diméthomorphe	18%	*1,2*	34%	*1,4*	*oïdium, black rot*
Benalaxyl-M	20%	*1,6*	7%	*1,6*	*mildiou, black-rot*
Boscalid (510)	20%	*1,0*	1%	*1,0*	*oïdium, pourriture grise*

1.4.2. Les traitements phytosanitaires en Gironde

Une grande partie du territoire de la Gironde est occupée par des forêts et des cultures (Chambre d'agriculture de Gironde, 2014). La Figure 7 montre que la culture dominante en Gironde est le vignoble qui correspond à 50 % de la superficie totale cultivée, constituant l'un des secteurs les plus consommateurs de produits phytosanitaires. En 2013, 14,000 tonnes de produits phytosanitaires correspondant à 400 substances actives ont été vendues dans le bassin Adour-Garonne (Agence de l'eau Adour-Garonne, 2014). Parmi les substances les plus vendues au cours de cette période on trouve le glyphosate et le soufre par pulvérisation, chaque produit étant consommé à plus de 1 800 tonnes/an (Agence de l'eau Adour-Garonne, 2014) (Figure 8).

Selon le rapport de la Qualité des eaux des bassins Adour-Garonne, en 2014, 99 % des stations de surveillance ont détecté au moins un produit phytosanitaire dans les cours d'eau. Sur les 140 molécules recherchées, 94 ont été détectées, la plupart d'entre elles étant des herbicides. Parmi les molécules détectées dans plus de 40 % des échantillons d'eau de rivières, on trouve le glyphosate et son métabolite, l'AMPA, le S-métolachlore et l'atrazine, cette dernière étant pourtant interdite depuis

2003. L'herbicide S-métolachlore est également celui qui a été détecté avec des concentrations maximales supérieures à 20 µg/L (Agence de l'eau Adour-Garonne, 2014). Les autres pesticides détectés dans moins de 20 % des échantillons sont les herbicides bentazone, métaldéhyde, chlortoluron, nicosulfuron, diméthénamide, 2,4-D et el triclopyr, et le fongicide tébuconazole. Parmi les cours d'eau les plus touchés par la présence de produits phytosanitaires en 2014, on trouve Le Trec (avec 34 molécules trouvées), la Sausse et l'Ayroux (avec des concentrations accumulées allant jusqu'à 54 et 42 µg/L de pesticides) (Figure 8).

Dans le cadre de ce travail de thèse, nous nous sommes intéressés par l'étude de 3 molécules phytosanitaires différents : le sulfate du cuivre (comme fongicide), le glyphosate (comme herbicide) et le chlorpyrifos (comme insecticide). À continuation, leurs caractéristiques principales et ainsi que leurs effets toxiques dans les milieux aquatiques de ces molécules seront décrits rapidement.

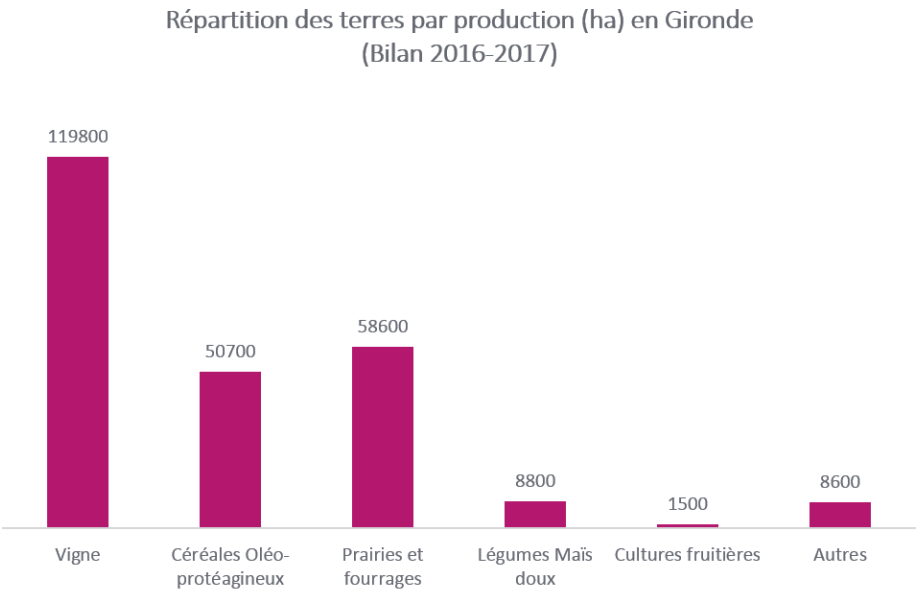


Figure 7 : Répartition des terres agricoles dans la région de la Gironde. Source : Chambre d'agriculture de la Gironde (2017).

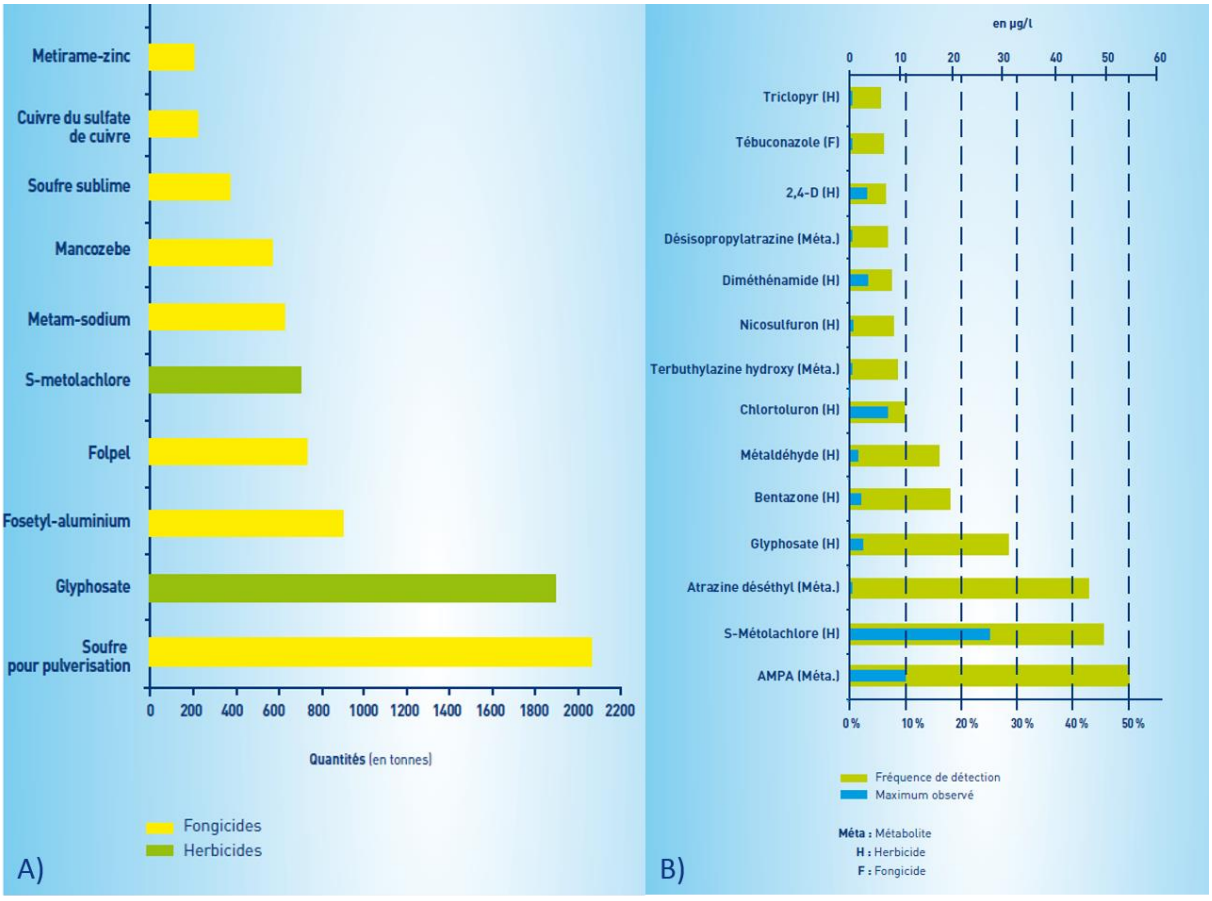


Figure 8 : Les 10 substances les plus vendues en 2013 (A). Les molécules les plus détectées en 2014 en rivières (B). Source : Agence de l'eau Adour-Garonne (2014).

Tableau 5 : Principaux insecticides utilisés en vigne en 2010 (Agreste 2012).

Matière active	Part des surfaces viticoles traitées (%) et nombre moyen de traitements				Ravageurs visés
	Bordelais		Bergeracois		
Indoxacarbe	25%	*1,23*	10%	*1,44*	*Tordeuses, cicadelle des grillures*
Flufénoxuron*	20%	*1,3*	33%	*1,4*	*Tordeuses, cicadelle des grillures*
Alphaméthrine	14%	*1,36*	26%	*1,8*	*Cicadelle de la flavescence dorée, Tordeuses*
Chlorpyriphos-méthyl	10%	*1,35*	18%	*1,38*	*Cicadelle de la flavescence dorée, cicadelle des grillures, tordeuses, cochenilles, pyrale*
Bifenthrine	9%	*1,21*	13%	*1,41*	*Tordeuses, cicadelle de la flavescence dorée, cicadelle des grillures, acariens*
Chlorpyriphos-éthyl	7%	*1,39*	23%	*1,44*	*Tordeuses, cicadelle de la flavescence dorée, cicadelle des grillures, cochenilles, pyrale*
Fénoxycarbe	7%	*1*	<1%	-	*Tordeuses, cochenilles, eulia*

2. Choix des molécules

2.1.Le cuivre

Actuellement, l'utilisation des fongicides organiques synthétiques est interdite en viticulture biologique en Europe. Cependant, l'utilisation de fongicides à base du cuivre est toujours autorisée (European Commision, 2002), et ils sont devenus indispensables pour protéger les vignobles contre les maladies fongiques telles que le mildiou. Le mildiou, causée par l'oomycète *Plasmopara viticola*, est une maladie cryptogamique pouvant attaquer tous les organes verts de la vigne tels que les feuilles, les jeunes branches et les grappes (Figure 9) (Burruano, 2000). Les plantes infectées ont des taches jaunes sur les feuilles, tandis que les spores du champignon apparaissent sur la face inférieure des feuilles avec une formation dense, blanche et cotonneuse (Figure 9). Cette maladie peut être grave pour la vigne car elle peut provoquer une défoliation prématurée et les raisins peuvent produire un vin de mauvaise qualité.

Des fongicides à base du cuivre ont été utilisés dans les vignobles comme traitement pour lutter contre ce type de maladies fongiques (Komárek et al., 2010; Ruyters et al., 2013). La bouillie bordelaise, depuis sa création en 1850, est toujours l'un des produits les plus commercialisés pour l'utilisation dans les vignobles. La bouillie bordelaise est un mélange de sulfate de cuivre hydraté ($CuSO_4 \cdot 5H_2O$) et de chaux ($Ca(OH)_2$) (Figure 10). A cause de l'acidité du sulfate de cuivre, il est nécessaire de le neutraliser avec la chaux pour éviter d'endommager les feuilles de la vigne (Monnier, 2014). Les bouillies bordelaises qui sont commercialisés en France peuvent contenir entre 17 à 50 % de cuivre (Monnier, 2014). Cependant, bien qu'il soit un excellent biocide, le cuivre peut affecter d'autres organismes bénéfiques et altérer la qualité des sols, les privant ainsi de leurs nutriments (Eijsackers et al., 2005). En raison de son application constante et du lessivage ultérieur au traitement, il en résulte une accumulation excessive du cuivre dans les sols du vignoble (Komárek et al., 2010).

Figure 9 : Vigne affecté par le mildiou. https://www.syngenta.es/mildiu-en-vina

Pour contrôler ou au moins limiter la pollution par le cuivre, la Commission européenne (CE 889/2008) a limité la dose annuelle maximale de cuivre pour le traitement des vignes de 8 à 6 kg Cu par hectare, ce qui correspond à une accumulation annuelle de 5 mg Cu kg^{-1} dans les premiers 10 cm du sol en supposant qu'il n'y ait pas de pertes (Ruyters et al., 2013). En fonction du pH du sol, le cuivre peut être solubilisé dans les sols acides (pH < 6) et contaminer les eaux souterraines (Komárek et al., 2010). Les apports des engrais azotés, tels que le nitrate d'ammonium, peuvent favoriser l'acidification du sol et augmenter la disponibilité du cuivre (Monnier, 2014). Le chaulage dans les sols peut monter le pH et stabiliser le cuivre (Monnier, 2014). Cependant, dans des sols dont le pH est d'environ 7.5 peut augmenter la solubilisation de la matière organique du sol et former des complexes avec le cuivre (Komárek et al., 2010; Monnier, 2014). Selon la composition des sols, le cuivre peut être associé à des hydroxydes de fer ou de manganèse et la matière organique en devenant moins mobile (Komárek et al., 2010). Dans les écosystèmes naturels, la teneur en cuivre dans l'eau est généralement comprise entre 0.2 et 5 µg/L (Willis and Bishop, 2016), tandis que dans les sédiments, le cuivre peut être compris entre 0.8 et 50 mg/kg (Willis and Bishop, 2016). Dans le cas de la Gironde, les concentration du cuivre dans le Bassin d'Arcachon se sont révélées entre 0.9 et 3.5 µg/L entre les années 2013 et 2014 (Gamain et al., 2017).

Figure 10 : Préparation de la bouillie bordelaise pour le traitement de la vigne
https://jardinage.ooreka.fr/tips/voir/285159/8-solutions-economiques-pour-proteger-son-jardin-en-hiver

2.1.1. Généralités

La présence du cuivre dans l'environnement est nécessaire, car le cuivre est un élément essentiel pour le bon développement et la croissance normale du poisson ainsi que des autres organismes vertébrés. L'accumulation et les effets du cuivre ne dépendent pas de la concentration totale dans l'eau, mais plutôt de la fraction biodisponible qui dépend des caractéristiques physico-

chimiques de l'eau (Kamunde and Wood, 2004). Généralement, l'ion Cu^{2+} prédomine dans les eaux à pH faibles (Kamunde and Wood, 2004). En augmentant le pH, la contribution du Cu^{2+} diminue avec l'augmentation simultanée du $CuCO_3$ et de $CuOH^+$ (Kamunde and Wood, 2004). Au pH typique d'eau douce naturel, l'espèce $CuCO_3$ est l'espèce dominante, tandis que les autres espèces telles que le Cu^{2+} et le $CuOH^+$ se trouve dans des proportions plus réduites (Kamunde and Wood, 2004). Cependant, ce sont les formes d'ion cuivrique (Cu^{2+}) et hydroxyde ($CuOH^+$) qui contribuent le plus à la toxicité chez les organismes aquatiques (Kamunde and Wood, 2004).

Tableau 6 : Information générale du sulfate de cuivre.

Sulfate de cuivre	
Nom chimique	
IUPAC	Sulfate de cuivre(II)
Synonymes	Sulfate cuivrique
CAS	
7758-99-8	
Formule moléculaire	
CuSO4	
CuSO4,5H2O (hydraté)	
Masse moléculaire	
159,609 ± 0,009 g/mol	
Solubilité dans l'eau à 20°C	
20.3g/100 mL	

2.1.2. Modes d'action

La toxicité du cuivre se produit lorsque les mécanismes homéostatiques échouent en raison d'une exposition aigüe ou chronique à des concentrations élevées de cuivre (Kamunde and Wood, 2004). Le cycle d'oxydoréduction du cuivre, qui est la base de sa fonction biologique, fait également partie de sa toxicité (Kamunde and Wood, 2004). Le cycle d'oxydoréduction du cuivre conduit à la formation d'espèces réactives de l'oxygène, pouvant causer des dommages cellulaires, notamment l'oxydation des protéines, la peroxydation de la membrane lipidique, et le clivage de l'ADN (Kamunde and Wood, 2004). De plus, le cuivre peut interférer avec l'homéostasie d'autres éléments essentiels,

tels que le Na et le Zn, ainsi qu'inactiver les protéines en se liant aux résidus de cystéine, d'histidine et de méthionine (Kamunde and Wood, 2004).

Le Cu, comme métal de transition, est capable de subir des réactions cycliques d'oxydoréduction et favoriser la formation d'espèces réactives de l'oxygène (ERO) (Gaetke et al., 2015). En présence de superoxydes ($O_2^{\cdot-}$) ou d'agent réducteurs, tels que l'acide ascorbique, le Cu^{2+} peut être réduit en Cu^{+}, lequel est capable de catalyser la formation de radicaux hydroxyles ($OH^{\cdot}$) à partir de peroxyde d'hydrogène via la réaction de Haber-Weiss (Bremner, 1998; Gaetke et al., 2015; Manzl et al., 2004) :

$$O_2^{\cdot-} + Cu^{2+} \rightarrow O_2 + Cu^{+}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$$

Comme un seul électron est nécessaire pour réduire Cu^{++}, l'autre électron peut être impliqué dans la production de radicaux hydroxyles (Gaetke et al., 2015). Le radical hydroxyle est capable d'initier des dommages oxydatifs en induisant des ruptures de brins de l'ADN et l'oxydation de bases (Gaetke et al., 2015).

2.1.3. Effets toxiques

Le cuivre est un oligo-élément essentiel pour la croissance et le bon fonctionnement du métabolisme de tous les êtres vivants. Chez les vertébrés, y compris les poissons, le cuivre est nécessaire au fonctionnement des enzymes dans les systèmes biologiques des organismes. Cependant, à des concentrations élevées, le cuivre peut être l'un des éléments les plus toxiques. Plusieurs facteurs peuvent déterminer la toxicité du cuivre chez les poissons et d'autres organismes aquatiques : la quantité du cuivre libre (Cu^{2+}) dans l'eau, la sensibilité des espèces exposées, le stade de développement, la présence des substrats formés de carbonate de calcium ou de magnésium pouvant diminuer le cuivre par adsorption, la présence d'autres substances dissoutes pouvant se lier avec le cuivre et réduire son activité (y compris les carbonates) et le pH de l'eau (Eisler, 1998; Kamunde and Wood, 2004).

Des concentrations élevées de cuivre dans les écosystèmes peuvent être nocives pour les organismes aquatiques. En effet, le Cu induit des effets toxiques lors d'une exposition chronique et une accumulation excessive dans les organismes vivants (Marr et al., 1996; Kamunde et al., 2002; Vardy et al., 2013). De nombreuses études ont mis en évidence les effets toxiques du cuivre chez les poissons et les mollusques (Barjhoux et al., 2012; Boukadida et al., 2017; Eaton et al., 1978; Gamain et al., 2017, 2016; Mai et al., 2012; Vardy et al., 2013). La sensibilité au cuivre peut varier considérablement d'une

espèce de poisson à l'autre. Par exemple, Vardy et al. (2013) ont déterminé les concentrations létales (CL50) après 96 h d'exposition au Cu pour deux espèces de poisson, respectivement à 10 et 21 µg/L de Cu pour les larves d'esturgeon blanc et de truite arc-en-ciel. McKim et al. (1978) ont examiné la mortalité embryonnaire de huit espèces de poissons différentes après exposition au Cu et ont observé une mortalité significative comprise entre 37 et 555 µg/L. En outre, cette sensibilité aux métaux peut également varier selon le stade de développement (McKim et al., 1978; Vardy et al., 2013) et les paramètres chimiques de l'eau tels que la dureté, le pH, le carbone organique et l'alcalinité (Brix et al., 2017; Crémazy et al., 2017; Eisler, 1998).

2.2.Le glyphosate

Actuellement, le glyphosate se distingue des herbicides modernes puisque, après 30 ans d'utilisation, il détient 50 % du marché des produits herbicides (Giesy et al., 2000). Le glyphosate est l'ingrédient actif de l'herbicide Roundup®, créé en 1970 par la société Monsanto (Giesy et al., 2000).

La consommation du glyphosate dans le monde a augmenté en raison de l'utilisation de plants et des semis de souches transgéniques résistantes. Par exemple, en 2014, aux Etats-Unis seulement, 94 et 96 % des cultures de soja et de maïs, respectivement, étaient des variétés génétiquement modifiées tolérantes au glyphosate. Cependant, l'utilisation constante d'un seul herbicide a favorisé l'émergence d'herbes résistantes (Mesnage and Antoniou, 2018), ainsi 14 espèces d'herbes ont développé une résistance au glyphosate dans 32 des 50 états (Mesnage and Antoniou, 2018)

En raison de certaines conditions qui combinent l'application directe sur les semis, les sols humides et les pluies immédiatement après son application, le glyphosate peut atteindre les eaux souterraines par percolation et/ou les eaux de surface par ruissellement (Bedmar, 2011).

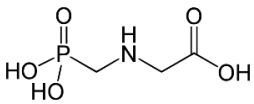
2.2.1. Généralités

Le glyphosate ($C_3H_8NO_5P$) est un sel d'isopropylamine de N- (phosphonométhyl) glycine (Tableau 7). Il a été développé pour l'élimination des herbes et des arbustes envahissants. C'est un herbicide à large spectre d'action systémique et non sélective, et à pénétration foliaire ; c'est-à-dire, qu'il est principalement absorbé par les feuilles et les parties vertes des plantes, puis va migrer vers d'autres parties du tissu végétal où il sera métabolisé. L'ingrédient actif du glyphosate est un dérivé de la glycine, où l'un des atomes d'hydrogène de la glycine est remplacé par un groupe phosphonométhyle.

Le glyphosate de qualité technique est un acide qui, pour augmenter sa solubilité dans l'eau, est formulé sous la forme du sel d'isopropylamine. En raison de son état ionique, il est hautement soluble et stable dans l'eau et ne devrait donc pas se volatiliser de l'eau ni du sol (Pérez et al., 2011). Les formulations commerciales du glyphosate sont composées du sel de glyphosate mélangé avec différents co-formulants, nécessaires pour permettre la pénétration du glyphosate dans les plantes (Mesnage et al., 2015). L'un des ingrédients connus est un surfactant le polyoxyéthylène amine (POEA), responsable de la toxicité de certaines formulations commerciales du glyphosate (Mesnage et al., 2013).

Le glyphosate a une forte capacité d'adsorption dans les sols, ce qui peut limiter ses déplacements dans l'environnement. La demi-vie du glyphosate dans les sols est d'environ deux mois (Giesy et al., 2000). Cependant, en fonction de la composition du sol, sa demi vie peut varier de quelques semaines à quelques années (Mercurio et al., 2014; Myers et al., 2016). Dans l'eau, la vitesse de dégradation peut être plus lente que dans les sols en raison de la quantité moindre de micro-organismes présents dans l'eau que dans les sols (Mensah et al., 2015). Les principaux produits de dégradation du glyphosate sont l'acide aminométhylphosphonique (AMPA) et le dioxyde de carbone.

Tableau 7 : Information générale du glyphosate.

Glyphosate	
ISO Nom commun	
Glyphosate (E-ISO, BSI, ANSI, WSSA, JMAF)	
Nom chimique	
IUPAC	N-(phosphonométhyl) glycine
Chemical Abstract	N-(phosphonométhyl) glycine
Formule structurelle	
CAS	
1071-83-6	
ECHA	
100.012.726	
Formule moléculaire	
$C_3H_8NO_5P$	
Masse moléculaire	
169,1 g/mol	
Solubilité	
12 g/L dans l'eau (25°C)	
Insoluble dans la plupart des solvants organiques	

1.1.1. Mode d'action

En tant qu'herbicide systémique, le glyphosate est facilement transporté à travers le phloème vers toutes les parties de la plante. Les molécules du glyphosate sont absorbées à partir de la surface des feuilles par les cellules végétales où elles sont transférées par le symplasme vers les méristèmes des plantes en croissance.

La voie shikimate est l'une des voies de biosynthèse importantes chez les plantes et les microorganismes, qui conduit à la formation des acides aminés aromatiques de type tryptophane, tyrosine et phénylalanine (Mensah et al., 2015). La biosynthèse de ces substances essentielles est favorisée par l'enzyme 5-énolpyruvylshikimate-3-phosphate synthase (EPSPS), l'enzyme cible du glyphosate. Cette enzyme est l'une des sept enzymes qui catalysent une série de réactions qui commencent par la réaction entre le shikimate-3-phosphate (S3P) et le phosphoénolpyruvate (PEP). Le glyphosate inhibe l'activité de l'EPSPS, empêchant ainsi la production de chorismate, dernier précurseur commun de la biosynthèse de nombreux composés aromatiques chez les bactéries, les champignons et les plantes. Ceci provoque un déficit dans la production des substances essentielles nécessaires aux organismes pour survivre et se propager. Cette voie est absente chez les animaux, ce qui peut expliquer la faible toxicité du glyphosate pour les animaux (Giesy et al., 2000; Pérez et al., 2011).

1.1.2. Effets toxiques sur les espèces non ciblées

De nombreuses études ont montré sa toxicité chez de nombreuses espèces de vertébrés et d'invertébrés (Morgan et al., 1991; Tsui and Chu, 2003; Vera et al., 2010). Cependant, les concentrations létales du glyphosate pour les organismes aquatiques, tels que les poissons, sont généralement plus élevées que les concentrations trouvées dans les cours d'eau après les traitements dans les cultures (Folmar et al., 1979). Néanmoins, il apparaît que les formulations commerciales de glyphosate sont plus toxiques que la molécule active pure en raison d'interférences avec les constituants du produit et notamment le surfactant polyoxyéthylène amine (POEA) (Folmar et al., 1979; Giesy et al., 2000; Navarro and Martinez, 2014; Tsui and Chu, 2003). Les poissons sont susceptibles d'être exposés au glyphosate par absorption directe au niveau des branchies à partir de l'eau ou par la voie alimentaire par ingestion de proies contaminés (Giesy et al., 2000). Çavaş and Könen, (2007) et Guilherme et al., (2012, 2010) ont étudié le potentiel génotoxique de Roundup et observé des niveaux importants de cassures de brins d'ADN sur les érythrocytes de poisson rouge (*Carassius auratus*) et de l'anguille européenne (*Anguilla Anguilla*). Une inhibition de l'acétylcholinestérase (AChE) a également été observée chez le poisson piava (*Leporinus obtusidens*) après exposition à 1 et 5 mg/L de Roundup pendant 90 jours (Salbego et al., 2010). L'activité natatoire a également été étudiée chez le poisson hybride surubim (Sinhorin et al.,

2014) et chez le poisson zèbre (*Danio rerio*) (Bridi et al., 2017), où des altérations du comportement de nage ont été observées à partir de 2,25 mg/L et 0,01 mg/L de glyphosate, respectivement. Par ailleurs, Uren Webster et al. (2014) ont remarqué que le glyphosate avait des effets néfastes sur la santé reproductive du poisson zèbre exposé à 10 mg/L. En outre, la formation d'espèces réactives de l'oxygène (ERO) a également été observée concernant les biomarqueurs tels que la peroxydation lipidique et les enzymes antioxydantes (de Moura et al., 2017; Glusczak et al., 2007; Harayashiki et al., 2013; Üner et al., 2006). Le tableau 8 présente les valeurs toxicologiques du glyphosate chez la truite arc-en-ciel.

Tableau 8 : Valeurs toxicologiques de référence pour le glyphosate.

Propriété	Valeur
Facteur de bioconcentration	Poisson BCF : 0.5 l/kg
Poissons	**Glyphosate acide :** LC_{50}-96h : 38 mg a.e./L (*O. mykiss*) NOEC-21 j : 25 mg a.e./L (*O. mykiss*) **Glyphosate sel :** LC_{50}-4j : 140-240 mg a.e./L (*O. mykiss*) **Roundup :** LC_{50}-4j : 2.5-16 mg a.e./L (*O. mykiss*) NOEC-21 j : 0.74 mg a.e./L (*O. mykiss*)

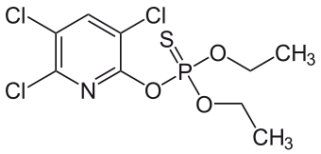
2.3.Le chlorpyrifos

Les organophosphates (OP) sont des inhibiteurs de l'activité cholinestérase utilisés principalement comme insecticides (Tableau 9). Ils sont également utilisés comme agents de guerre chimique (agents neurotoxiques). Les OP comprennent tous les insecticides contenant du phosphore dérivé de l'acide phosphorique et sont généralement les pesticides les plus toxiques pour les animaux vertébrés. Le chlorpyrifos fait partie de la liste

Dans certaines zones très cultivées, le chlorpyrifos (CPF) a été détecté principalement dans les sédiments et dans les cours d'eau, généralement transporté par les eaux de ruissellement (Jergentz et al., 2005; Phillips and Bode, 2004). Des concentrations maximales de CPF ont été mesurés à 0,06 µg/L dans le Kisco (sud-est de l'État de New York, États-Unis) et dans la Pampa Ondulada (Argentine), dont 42% des 26 échantillons testés présentaient des niveaux détectables de CPF où les concentrations variaient de < 0,2 à 10,8 µg/L dans les eaux de surface (Marino and Ronco, 2005). Jergentz et al. (2005) ont caractérisé 3 insecticides, dont le CPF, provenant de cours d'eau situés à proximité d'une région de

soja en Argentine et présentant des concentrations allant jusqu'à 0,45 µg/L dans la rivière Brown et de 0,3 µg/L dans l'Horqueta (ruissellement). Dans le département de la Gironde, le CPF a été retrouvé sur le bassin d'Arcachon dans des échantillons d'eau et sédiments (Auby et al., 2007). Les concentrations maximales de CPF dans l'eau qui ont été trouvé sont 15 ng/L provenant de Berle de Cassy et Ruisseau du Bétey (Tableau 10) (Auby et al., 2007). Egalement, 10 µg/Kg de CPF a été trouvé en Compriam. Le TCP (3,5,6-trichloro-2-pyrinidol), son produit de dégradation, a été retrouvé aussi en concentrations similaires au celui du CPF (table 10) (Auby et al., 2007).

Tableau 9 : Information générale du chlorpyrifos.

Chlorpyrifos		
ISO Nom commun		
	Chlorpyrifos (E-ISO, BSI, ANSI, ESA)	
Nom chimique		
	IUPAC	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
	Chemical Abstract	O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate
Formule structurelle		
		
CAS		
	2912-88-2	
ECHA		
	100.018.969	
Formule moléculaire		
	$C_9H_{11}Cl_3NO_3PS$	
Masse moléculaire		
	350,6 g/mol	
Solubilité		
	1.12 mg/L dans l'eau (24°C)	

2.3.1. Mode d'action

Comme la grande majorité des pesticides organophosphorés, le CPF agit en interférant avec la signalisation du neurotransmetteur acétylcholine (ACh). L'enzyme acétylcholinestérase joue un rôle important dans la transmission de l'influx nerveux. Le CPF inhibe la liaison de l'acétylcholine au site actif de l'enzyme, entraînant l'inactivation de l'enzyme (Canadian environmental Quality Guidelines, 2008). L'inhibition de l'activité acétylcholinestérase entraîne l'accumulation d'acétylcholine au niveau des récepteurs synaptiques des neurones cholinergiques, entraînant une transmission continue des impulsions nerveuses, une tétanie musculaire, puis une paralysie et finalement la mort de l'insecte. Le CPF a besoin d'une bioactivation métabolique pour pouvoir exercer son effet. L'enzyme CYP2B6 métabolise le CPF en chlorpyrifos-oxon (CPF-oxon) en remplaçant le groupe sulfure avec l'oxygène (Rathod and Garg, 2017). Le CPF-oxon est le principal responsable de la toxicité du CPF, car il peut être 1,000 fois plus puissant pour endommager les neurones (Faria et al., 2015; Rathod and Garg, 2017).

Tableau 10 : Occurrences (% des échantillons dans lequel les molécules présentent une concentration supérieure à la limite de quantification) et concentrations maximales de CPF et son produit de dégradation TCP dans les différents matrices entre mai 2005 et décembre 2006 (Auby et al., 2007).

Station	Matrice	Molécule	Occurrence	Concentration maximale
Comprian	Eau	CPF TCP	2 % 14 %	9.8 ng/L 20 ng/L
	Sédiment	CPF	14 %	10 µg/kg PS
	Huîtres	CPF	Détecté 1 fois	< seuil
Jacquets	Eau	TCP	24 %	12 ng/L
	Sédiment	CPF	14 %	13 µg/kg PS
	Huîtres	CPF	Détecté 1 fois	< seuil
Ruisseau du Bourg	Eau	CPF TCP	11 % 11 %	8.2 ng/L 11 ng/L
Eyre	Eau	CPF TCP	9 % 5%	12 ng/L 5 ng/L
Berle de Cassy	Eau	CPF	5 %	15 ng/L
Ruisseau du Bétey	Eau	TCP	21 %	15 ng/L

2.3.2. Effets toxiques

Le chlorpyrifos peut avoir des effets létaux sur les espèces aquatiques. C'est le cas de l'étang de Bages-Sigean (Narbonne, France) où, en décembre 2004, une importante pollution par du CPF a été détectée (concentrations élevées de 6,200 mg/kg dans les sédiments) et une mortalité importante des poissons a été observée (CEDRE, 2005). Carr et al. (1997) ont décrit comment le CPF avait affecté plusieurs populations de poissons dans un petit étang proche d'un chantier de construction. En effet, une mortalité élevée et une inhibition importante de l'acétylcholinestérase cérébrale (AChE) ont été observées sur des écrevisses, des achigans à grande bouche, des méné jaune et des crapet arlequin. Cependant, d'autres populations comme la gambusie ne sont pas affectées, ce qui démontre la sensibilité différente de chaque espèce de poisson. Les valeurs de toxicité (96 h-CL50) pour les poissons d'eau douce vont de 9 µg/L pour la truite arc-en-ciel à 806 µg/L pour le barbue de rivière (Phipps and Holcombe, 1985). Dans l'article de Faria et al. (2015), l'exposition à des fortes concentrations de CPF-oxon (µM) a induit des malformations de la structure de la rétine des fibres musculaire et des nécroses dans le cerveau de poisson zèbre. Comme le CPF est très toxiques pour les organismes aquatiques (Tableau 11 et 12), les recommandations canadiennes pour la qualité des eaux ont établi une valeur de 0,02 µg/L de CPF pour la protection à court terme de la vie aquatique dans les eaux de surface, et une valeur 0,002 µg/L de CPF comme valeur à long terme pour la vie aquatique pour la protection de la vie en eau douce (Canadian environmental Quality Guidelines, 2008). La NQE (norme de qualité environnementales) a été établie en 0.03 µg/L de CPF (WFD, 2005).

Tableau 11 : Valeurs de LC_{50} pour différents espèces de poissons modèles.

Espèce	Test	Duration	Résultat	Références
Pimephales promelas	Toxicité aigüe	96 h	LC_{50} = 120 µg/L (Dursban)	Jarvinen and Tanner (1982)
Ictalurus punctatus	Toxicité aigüe	96 h	LC_{50} = 806 µg/L	Phipps and Holcombe (1985)
Lepomis macrochirus	Toxicité aigüe	96 h	LC_{50} = 10 µg/L	Phipps and Holcombe (1985)
Juvéniles O. mykiss	Toxicité aigüe	96 h	LC_{50} = 9 µg/L	Phipps and Holcombe (1985)
Juvéniles O. mykiss	Toxicité aigüe	96 h	LC_{50} = 7.1 µg/L (12.7°C) LC_{50} = 15 µg/L (7.2°C) LC_{50} = 51 µg/L (1.6°C) (Dursban)	Macek et al. (1969)

Tableau 12 : Effet sur l'activité AChE pour différents espèces de poissons.

Espèce	Stade de développement	Duration	Concentrations (µg/L)	Effet	Références
O. mykiss	Adulte	72 h et 96 h	6.75	Inhibition de l'activité AChE	Topal et al. (2016)
D. rerio	Larve	96 h	100 et 300	Inhibition de l'activité AChE	Jin et al. (2015)
D. rerio	Embryon	96 h	200–1000	Inhibition de l'activité AChE	Jeon et al. (2016)
D. rerio	Adulte	96 h	600	Augmentation de l'activité AChE	Jeon et al. (2016)
D. rerio	Adulte	96 h	1000	Inhibition de l'activité AChE	Jeon et al. (2016)
O. niloticus		14 jours	0.5-12	Inhibition de l'activité AChE	Chandrasekara and Pathiratne (2007)
G. affinis	Adulte	96 h	297	Inhibition de l'activité AChE	Kavitha and Venkateswara Rao (2008)

3. Les modèles biologiques

Puisque la contamination des milieux aquatiques par les substances chimiques ne cesse d'augmenter, il apparaît nécessaire de connaître l'impact potentiel de ces substances sur ces écosystèmes. Pour cela, des tests standardisés ou non sur les organismes aquatiques, et notamment sur les poissons, ont été largement employés, du fait que les milieux aquatiques sont le milieu récepteur final des pesticides. En plus les poissons peuvent être en contact direct avec le milieu contaminé. Cependant, à cause de nombreuses contraintes éthiques, économiques et scientifiques, le choix des méthodes d'analyse et de modèles biologiques tendent vers des méthodes alternatives qui ne sont pas couvertes par la réglementation européenne 2010/63/EU : les tests sur embryo-larvaire de poissons et sur culture cellulaire (Castaño et al., 2000; Garthoff, 2005).

L'entrée en vigueur de la réglementation européenne REACH (Registration, Evaluation, Autorisation and restriction of Chemicals), la demande de test de toxicité des produits chimique sur le

marché a augmenté. Cela implique l'utilisation des animaux afin de réaliser ces études. Afin de réduire le nombre d'animaux utilisés, le principe de 3 R (Réduire, Raffiner et Remplacer) est basé sur la minimisation du nombre d'individus, l'amélioration des expérimentations afin de réduire le stress ressentis par les animaux et le remplacement des animaux par des méthodes alternatives en utilisant des modèles in silico comme le QSAR (Quantitative Structure Activity Relationship) ou in vitro (tests sur stades précoces de développement ou sur culture cellulaire). Les stades précoces de développement des poissons permettent de remplacer l'utilisation d'individus à des stades protégés par des stades non protégés (Figure 13).

3.1. Modèle *in vivo* : Premiers stade de développement de truite arc-en-ciel

3.1.1. La truite arc-en-ciel

La truite arc-en-ciel (*Oncorhynchus mykiss*) (Figure 11) est une espèce de poisson de la famille des salmonidés, originaire d'Amérique du Nord dont la répartition à l'état sauvage va de l'Alaska jusqu'au nord du Mexique. Cependant, depuis 1874, la truite arc-en-ciel a été introduite dans les eaux de tous les continents sauf l'Antarctique (Figure 12) pour des motifs récréatifs pour l'aquaculture et la pêche sportive (FAO).

Les populations sauvages de truite arc-en-ciel ont décliné dans leurs propres aires de répartition en raison de la surexploitation, de la perte d'habitat, des maladies, de la pollution, etc. En conséquence, certaines populations de truites arc-en-ciel sont classées comme étant en voie de disparition ou préoccupantes. La truite arc-en-ciel est actuellement répertoriée comme indicateur des essais de toxicité en eau douce (OECD, 2013).

Figure 11 : Truite arc-en-ciel adulte

http://dnr2.maryland.gov/Fisheries/Pages/Fish-Facts.aspx?fishname=Rainbow%20Trout

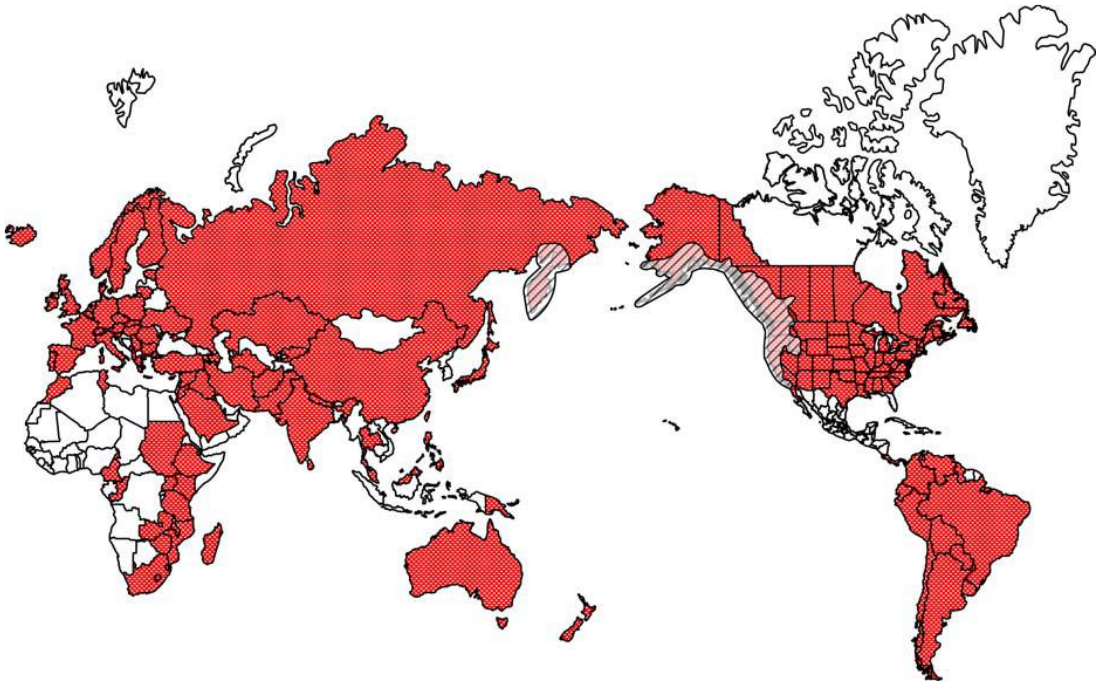


Figure 12 : Aire du répartition de la truite arc-en-ciel. Distribution initiale (diagonale, noire), et les endroits où elle a été introduite (rouge) (Crawford and Muir, 2007)

3.1.2. Généralités

La truite arc-en-ciel peut tolérer des températures qui vont de 0 à 25°C, bien que le frai et la croissance se déroulent dans une gamme plus étroite entre 9 et 12°C. Cette espèce de truite a de petites écailles et des couleurs chatoyantes. Le dos est d'une couleur bleue/verte olive, et présente des taches noires dans la tête, le corps, les nageoires dorsales et la queue. De plus, elle présente une bande ample irisée de ton rosacée dans la ligne latérale (Figure 8). Sa taille peut varier entre 20 et 40 cm et jusqu'à 80 cm de long pour un poids entre 500 g et 6 kg.

3.1.3. La reproduction

La truite arc-en-ciel fraie dans les cours d'eau et leurs affluents, ainsi que dans des cours d'eau provenant des lacs. Généralement, le frai à lieu du début à la fin de printemps (de janvier à juin dans l'hémisphère nord) avec une température d'eau comprise entre 6 et 7°C. Les affluents et les cours d'eau contenant des graviers d'une taille variant entre 1.27 et 7.6 cm sont les habitats les plus appropriés pour le frai de la truite. La femelle utilise sa queue et creuse une dépression dans le gravier. Ensuite, la femelle dépose une partie de ses œufs dans la dépression et le mâle les féconde. Les femelles de truite arc-en-ciel produisent généralement entre 2 000 et 3 000 œufs par kilogramme de poids. Chaque œuf peut

avoir un diamètre entre 4 et 5 mm. Les œufs fécondés sont recouverts de gravier. L'habitat idéal pour le frai est constitué de graviers bien aérés, dépourvus de sédiments et des eaux bien oxygénées. La truite pond ses œufs dans une gamme de profondeurs et de vitesse d'eau qui minimisent le risque de dessiccation lorsque le niveau d'eau diminue avec les saisons. Une profondeur d'eau suffisante et des graviers exempts de sédiments sont essentiels pour assurer la pénétration de l'eau dans les graviers, apportant de l'oxygène aux œufs et éliminant les déchets métaboliques associés à l'incubation et à l'éclosion. Après l'éclosion, les jeunes truites (appelées alevins) restent dans le gravier jusqu'à ce que l'absorption de la plupart des réserves du vitellus. Ils émergent du gravier (phase d'émergence) lorsque le tube digestif est totalement formé et fonctionnel et que les alevins s'alimentent sur des proies vivantes de type larves d'insectes et crustacés.

3.1.4. Description du développement embryo-larvaire

La période d'incubation peut varier entre 28 et 49 jours et est inversement proportionnelle à la température de l'eau (7-12°C) (Babiak and Dabrowski, 2003). Après l'éclosion, les larves disposent d'un grand sac vitellin et restent dans le fond, où ils vont demeurer jusqu'à ce qu'ils commencent à avoir un comportement pélagique. La vitesse de développement des œufs dépend de la température, et s'exprime, généralement, en degré jour (°J ou DD). Une fois que le sac vitellin est résorbé, la truite peut rester dans la colonne d'eau et commencer la recherche active d'aliment (Figure 13).

Au cours de l'embryogenèse et du développement larvaire de la truite, la croissance et la fourniture d'énergie dépendent des réserves endogènes du vitellus transférées par les géniteurs (Zengin and Akpinar, 2006). La croissance et la survie des larves dépendent notamment de la disponibilité d'aliments exogènes en quantité et en qualité suffisantes (Zengin and Akpinar, 2006). Avec les réserves en phospholipides et en acides gras polyinsaturés, le vitellus constitue un facteur important pour le développement de l'embryon et de la survie des larves (Zengin and Akpinar, 2006). Les acides gras, qui constituent le vitellus, ont un rôle important en tant que composants des phospholipides dans les biomembranes des poissons, associés à la fluidité de la membrane et à d'autres fonctions physiologiques (Zengin and Akpinar, 2006).

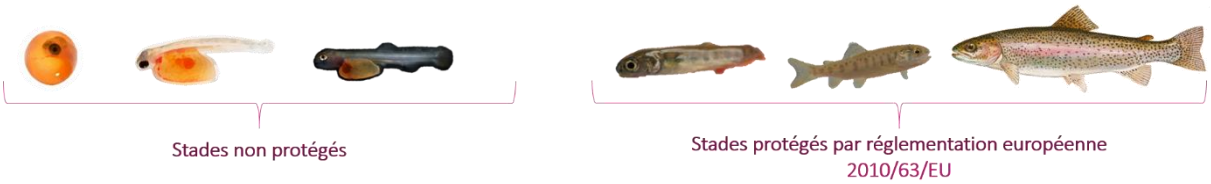


Figure 13 : Stades de développement de la truite arc-en-ciel (Oncorhynchus mykiss), de stade embryonnaire jusqu'au stade adulte.

3.1.5. Modèle en écotoxicologie

La truite arc-en-ciel peut être facilement élevée dans des conditions de laboratoire, et elle est considérée, avec d'autres espèces telles que le poisson zèbre ou le medaka japonais, comme un animal modèle en écotoxicologie (OECD, 2013). Cette espèce a été largement étudiée et caractérisée, et à cause de sa faible tolérance aux eaux de mauvaise qualité, est considérée comme une des espèces sentinelles les plus utilisées en écotoxicologie aquatique (Van Leeuwen et al., 1985). Smith et al. (2007) décrivent quelques avantages importants de cette espèce :

- Son application universelle permet la comparaison entre les différents groupes de recherche.
- Sensible aux changements dans l'environnement aquatique.
- Son génome a été caractérisé.

Dans cette étude, la truite arc-en-ciel au stade embryo-larvaire a été choisie pour étudier les possibles effets des pesticides. En effet, son lent développement embryo-larvaire permet de faire une exposition sub-chronique plus longue à ce stade qui est particulièrement sensible aux xénobiotiques.

3.2. Modèle *in vitro*

Afin de répondre aux restrictions éthiques, techniques et scientifiques des modèles *in vivo* (Castaño et al., 2003), y compris les tests sur les embryons et larves de poissons, des tests *in vitro* peuvent constituer une bonne alternative. Les cultures primaires et les lignées cellulaires constituent l'un des principaux composants des méthodes *in vitro*. Les cultures primaires sont des cellules obtenues à partir de tissus d'un organisme entier et qui sont conservées dans des conditions contrôlées de laboratoire pendant une courte période (quelques jours). Dans certains cas, les cultures primaires se développent pendant plusieurs générations et arrivent à former des lignées stables ou des cultures

cellulaires permanentes. Cependant, l'obtention d'une lignée stable dépend du type de cellule et des conditions de culture.

3.2.1. Lignée cellulaire

Comme décrit précédemment, une lignée cellulaire est une culture cellulaire établie de manière permanente. La lignée proliférera indéfiniment si elle dispose d'un milieu et d'un espace appropriés pour sa croissance (Ulrich, 2001, Encyclopedia of Genetics). Les lignées cellulaires diffèrent des cultures cellulaires par le fait qu'elles sont immortalisées. Les lignées cellulaires ont été développées à partir de cultures cellulaires (Bols et al., 2005), et elles diffèrent au niveau transcriptomique et morphologiquement des cellules dont elles sont originaires. Ce type de culture a la particularité d'être génétiquement, morphologiquement et fonctionnellement transformée (cellules aneuploïdes), de ne pas avoir d'inhibition de contact et donc de pouvoir se développer indéfiniment. Mais lorsqu'elles dépassent cette limite, les cellules peuvent entrer dans une phase de sénescence en perdant la capacité de proliférer en raison du raccourcissement des télomères, ce qui conduit progressivement à la mort cellulaire.

Les cultures cellulaires et les lignées cellulaires ont joué un rôle important dans l'étude des processus physiologiques, physiopathologiques et de différenciation cellulaire spécifique (Ulrich, 2001). Ils permettent d'étudier les altérations de la structure, de la biologie et de la composition génétique de la cellule dans des environnements contrôlés et définis (Bols et al., 2005). Les lignées cellulaires sont souvent préférées pour les études de toxicologie et devraient devenir plus pertinentes à l'avenir en remplacement des tests sur animaux pour la recherche et les essais en toxicologie (Thibaut et al., 2009).

3.2.2. Lignée RTL-W1

L'un des avantages de la truite arc-en-ciel est qu'elle possède une grande variété de lignées cellulaires établies à partir de différents tissus (Figura 14) (Bols et al., 2005). La lignée cellulaire RTL-W1 qui a été utilisée dans cette étude (Figure 15), a été développée à partir du foie d'une truite arc-en-ciel adulte de 4 ans (Lee et al., 1993). Cette lignée est considérée comme une lignée cellulaire immortelle mais non néoplasique (non tumorale) Lee et al. (1993). Ces cellules ont une morphologie polygonale et changent de forme en confluence. Elles contiennent un réticulum endoplasmique rugueux peu développé, des desmosomes, des lysosomes et des projections ressemblant à des microvillosités (Lee et al., 1993).

L'origine des cellules RTL-W1 est incertaine, mais elles peuvent provenir de cellules souches situées dans les structures canalaires biliaires (Lee et al., 1993). Les cellules épithéliales biliaires sont abondantes dans le foie de la truite arc-en-ciel (Lee et al., 1993). Ces cellules sont cultivées d'une manière très similaire aux lignées cellulaires de mammifères en utilisant le même milieu de culture complété par des sérums de mammifères tels que les sérums de veau fœtal ou de bovin (Bols et al., 2005; Fent, 2001). Les lignées RTL-W1 peuvent être cultivées sur une plage de températures étendue (entre 5 et 26 °C), les températures optimales étant comprises entre 18 et 22 °C (Bols et al., 2005).

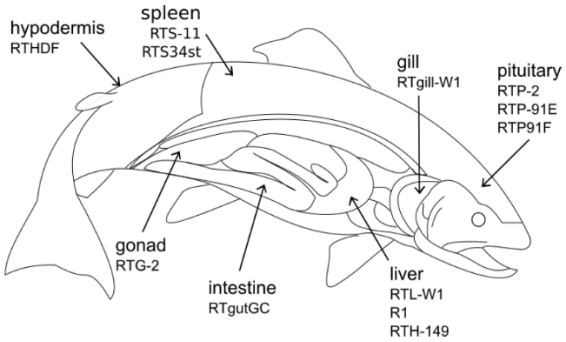


Figure 14 : Lignées cellulaires de différents tissues de la truite arc-en-ciel. Source : (Roux et al., 2015).

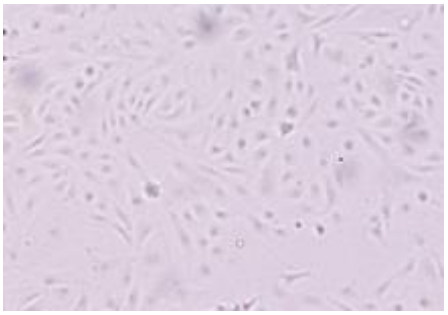


Figure 15 : Lignée cellulaire RTL-W1.

3.2.3. Utilisation en écotoxicologie

Dans les écosystèmes aquatiques, les interactions entre les substances chimiques et les organismes vivants se produisent d'abord au niveau cellulaire (Fent, 2001). Par conséquent, les réponses cellulaires peuvent être des manifestations précoces et directe de la toxicité de ces substances (Fent, 2001). Dans le domaine de l'écotoxicologie, non seulement les études in vitro devraient extrapoler les effets observés sur les effets toxiques in vivo, mais elles devraient également fournir des informations sur les réponses biologiques à des niveaux écologiques plus complexes (Castaño et al., 2003).

Les cultures cellulaires peuvent offrir certains avantages par rapport aux études sur des animaux entiers. Les tests in vitro sont plus reproductibles, utilisent moins de réactifs et d'échantillons et produisent moins de déchets. Contrairement à l'utilisation du poisson, les résultats sont obtenus plus rapidement et à moindre coût. Comme les cultures de cellules ne reproduisent pas toute la complexité des organismes pluricellulaires, l'interprétation des résultats mécanistes est généralement plus facile (Fent, 2001) mais l'extrapolation des résultats à l'organisme entier et plus hasardeux.

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Chapitre II

Bases Méthodologiques

Bases Méthodologiques

Ce travail de thèse a comme objectif d'étudier les effets sub-aigus de 3 pesticides, seuls ou en mélange, dans les premiers stades de vie de la truite arc-en-ciel. Ces pesticides, avec différents modes d'action, ont été choisi par rapport à leur usage dans la viticulture de la région de la Gironde :

- Fongicide : le sulfate de cuivre (composé principal de la bouillie bordelaise).
- Herbicide : le glyphosate (molécule active de la formulation commerciale Roundup®).
- Insecticide : le chlorpyrifos-éthyl

La dernière partie de ce travail de thèse s'inscrit dans le cadre du projet de PhytoCote du LabEx COTE. Le projet de PhytoCote s'intéresse à l'influence de l'application des pesticides dans les systèmes agricoles, leurs transferts vers les écosystèmes, leur bioaccumulation et leur potentiel toxique des parcelles agricoles aux écosystèmes terrestres et aquatiques. Au sein de ce projet, diverses questions scientifiques sont posées, et cette partie de la thèse a l'objectif de répondre à la problématique sur l'usage et la toxicité des produits phytosanitaires sur les organismes aquatiques. Pour cela, les premiers stades de développement de la truite arc-en-ciel ont été exposés aux échantillons environnementaux (d'eau et de sédiment) provenant d'un bassin versant viticole (La Livenne).

Lors de ces expériences, les effets de ces pesticides, et des matrices environnementales, ont été évalués à différents niveaux d'organisation biologique. Au niveau moléculaire, l'expression de gènes et l'oxydation de protéines et de lipides a été analysés sur les corps des larves entières ; l'activité de l'acétylcholinestérase a été mesuré dans les têtes des larves décapitées ; et la génotoxicité a été évaluée sur les cellules sanguines. La physiologie et les traits de vie des embryons et larves de poissons a été analysés par l'observation de la survie, l'éclosion, la morphométrie et le comportement.

Au cours des paragraphes qui vont suivre, nous ne décrirons que le principe général des approches que nous avons utilisées. Ceci afin d'éviter toute redondance avec les sections « matériels et méthodes » présentes dans nos articles et qui, elles, donnent plus de détails.

1. Préparation des solutions d'exposition

Les solutions mères du sulfate de cuivre (2 et 20 mg/L) et glyphosate (0.1 et 1 g/L) ont été préparées dans de l'eau Milli Q, tandis que la solution mère de chlorpyrifos (0.1 et 1 g/L) a été préparée dans du DMSO. Les solutions mères ont été conservées à l'obscurité à 4°C, pour les solutions du cuivre et glyphosate, et -20°C pour les solutions de chlorpyrifos, pendant tout la durée de chaque expérience.

Les solutions d'exposition de chaque pesticide ont été préparées dans des réservoirs de 5 L d'eau de source de Laqueuille (Tableau 13) qui permet d'alimenter 3 unités expérimentales, correspondant aux 3 réplicats d'une même condition. Ces solutions ont été renouvelées chaque 48 h et conservées à 4°C.

2. Modèle expérimental

Les embryons de la truite arc-en-ciel ont été fournis par la station d'expérimentation PEIMA (INRA de Sizun, France) au stade œillé à 250°J (°J : nombre de jours x température). A ce stade, les embryons sont assez robustes pour être manipulés. Lors de la réception, les embryons arrivent à une température de 6-7°C. Afin d'amener les embryons à la température d'exposition de 12°C, les œufs sont placés au bain marie à 12°C avant de les transférer dans le système d'exposition dans l'eau de source. Les œufs resteront en acclimatation 24 h avant d'être triés et répartis dans les aquariums d'exposition. Lors de cette acclimatation, il ne faut pas dépasser une amplitude thermique de 2°C par heure pour éviter un choc thermique dans les embryons.

Tableau 13 : Composition de l'eau de source de Laqueuille (63820, Laqueuille) en mg/L.

Analyse moyenne	mg/L
Calcium	4.7
Magnésium	1,8
Sodium	5,9
Potassium	2,8
Hydrogénocarbonates	40,3
Chlorures	1,2
Sulfates	0,2
Nitrates	0,5
Résidu sec à 180°C	74 mg/L
pH	7,5

3. Exposition embryonnaire

Pour chaque expérience, entre 3 et 4 conditions d'exposition ont été étudiées selon le cas. Les unités expérimentales (Figure 16), contenaient 1 L de solution d'exposition. Un système semi-dynamique de flux continu a été établi pour maintenir la circulation de l'eau dans les unités d'expérimentations (3 aquariums par réplicat) (Figure 17). Ce système est constitué par les réservoirs

d'eau d'exposition (avec les molécules étudier) qui sera pompée vers les aquariums avec une pompe péristaltique (Watson Marlow, USA). Le débit de l'eau est de 9 mL/minute. Les solutions d'exposition ont été changées tous les 2 jours pour limiter la dégradation du composé.

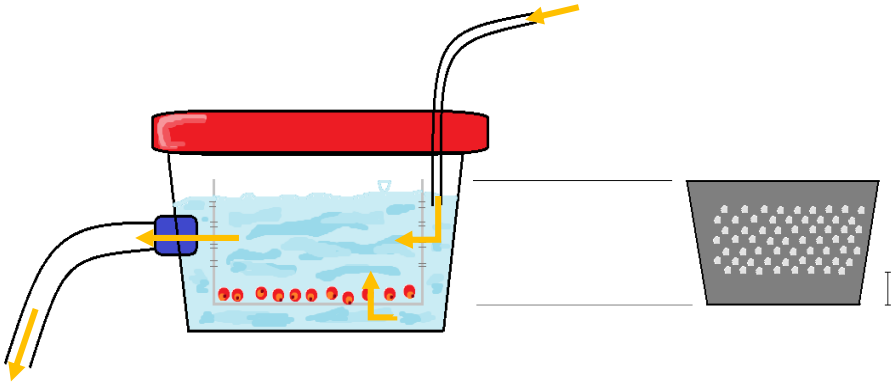

Figure 16 : Unités d'expérimentation pour les embryons et larves de truite arc-en-ciel exposés au pesticides.

Pour l'exposition, entre 50 et 100 embryons (selon l'expérience) au stade œillé (265°J) ont été comptés et placés dans chacune des unités d'expérimentation. Toutes les expériences se sont déroulées à l'obscurité dans une enceinte thermorégulée (Thorine) maintenue à 12°C (Figure 17). La température et l'oxygène dissous ont été mesurés quotidiennement (Sonde à O_2 FIBOX3 PreSens).

Les tests ont été arrêtés à environ 528°J, ce qui correspond à la fin de la résorption du sac vitellin et donc à la fin de l'alimentation endogène. A la fin du test, 6 larves par réplicat ont été décapitées afin de récupérer le sang pour l'étude de la génotoxicité (test de comète), 6 larves sont prélevées dans un tampon de conservation (RNA later) pour étudier l'expression génique, entre 10 et 12 larves sont sélectionnées pour réaliser les tests de comportement natatoire, entre 15 et 20 larves pour la mesure de la biométrie et les possibles malformations. Tous les individus restant sont alors congelés à -80°C pour les analyses biochimiques ultérieures (TBARS, protéines carbonylés, activité AChE). Les figures 18 et 19 montrent les différentes conditions étudiées (et leurs concentrations), pour les analyses des composés isolés (Figure 18) et les conditions du mélange de pesticides (Figure 19).

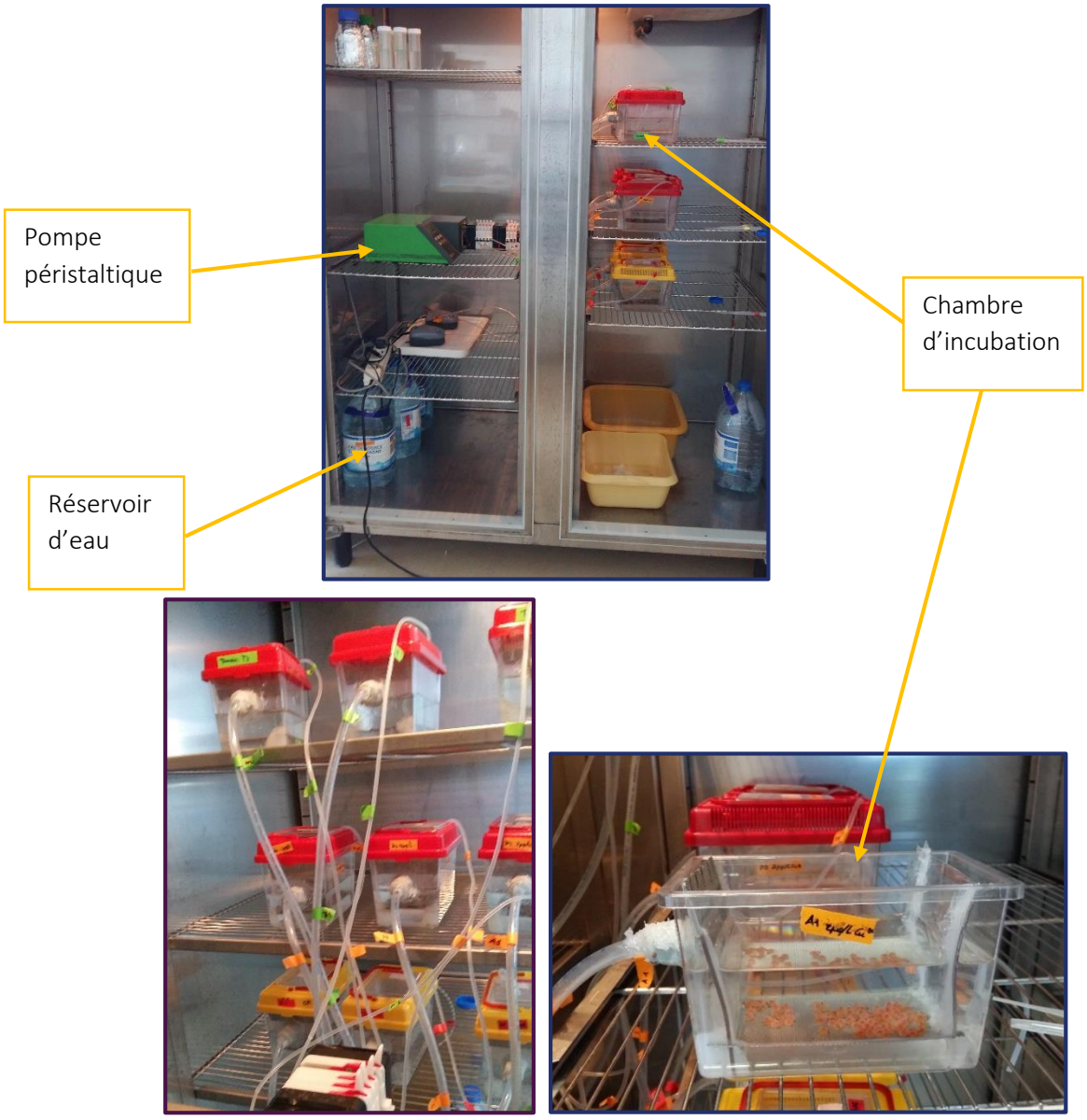

Figure 17 : Système d'exposition embryo-larvaire de la truite arc-en-ciel.

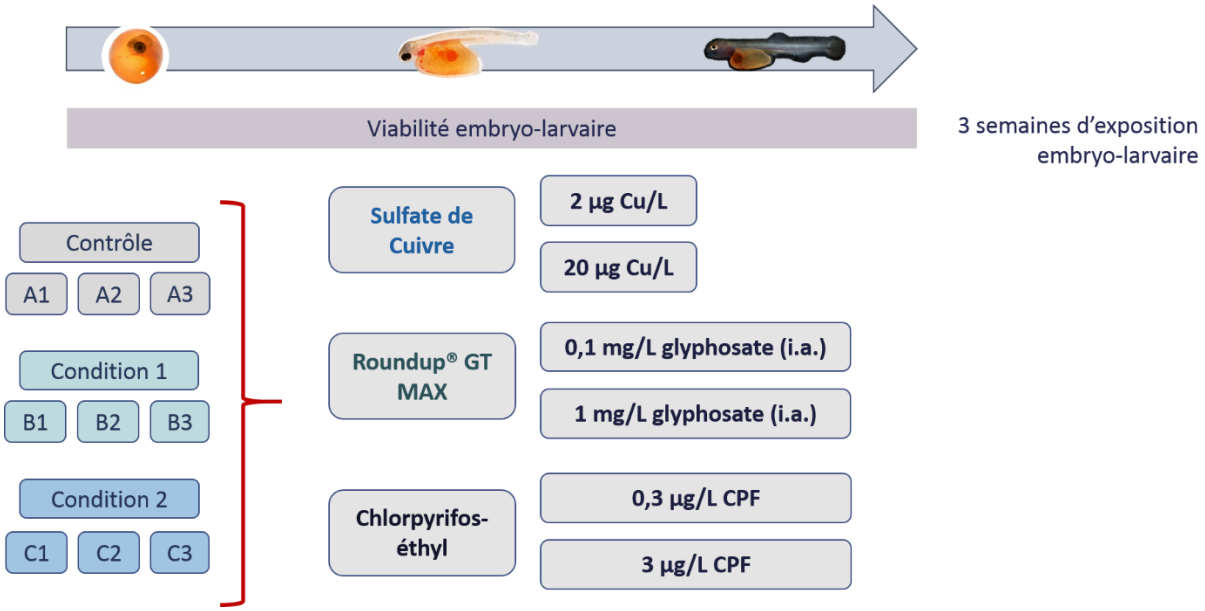

Figure 18 : Conception de l'expérimentation pour l'exposition embryo-larvaire de truite arc-en-ciel aux pesticides individuels. La température d'exposition a été mis à 12°C.

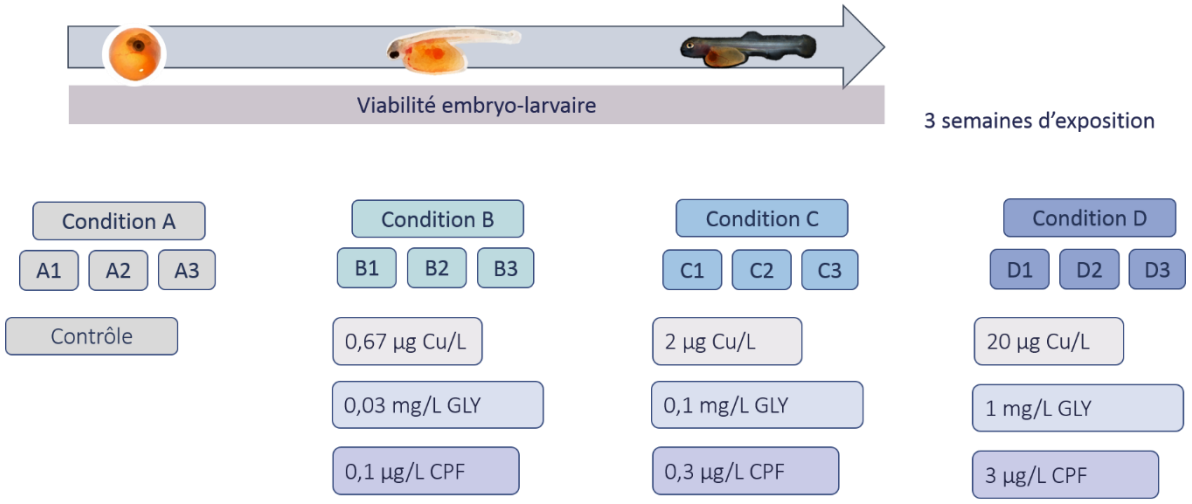

Figure 19 : Conception de l'expérimentation pour l'exposition des stades embryo-larvaire de truite arc-en-ciel au mélange de pesticides. La température d'exposition a été mis à 12°C.

4. Critère de toxicité à étudier

Pendant toute la durée de l'expérience, un suivi de mortalité est réalisé tous les jours sur les embryons, larves et les embryons demi-éclos (quand une partie du corps est à l'extérieur du chorion). Tous les individus morts sont retirés de l'aquarium afin d'éviter une prolifération bactérienne ou des champignons. Ce suivi permet d'observer des retards d'éclosion possibles ainsi que des mortalités dues à l'exposition aux polluants. Le pourcentage de mortalité correspond au nombre d'individus morts par rapport au nombre d'individus de départ.

A la fin de l'exposition, une série d'analyses et d'observations ont été réalisées afin d'étudier les possibles effets sub-létaux de ces pesticides sur les larves de truite arc-en-ciel. Les méthodes pour les analyses de biochimie, de génotoxicité, d'expression de gènes, de comportement sont décrites dans les articles inclus dans ce mémoire.

4.1. Biométrie et malformations

A la fin de l'expérience, entre 10 et 20 larves (selon l'étude) ont été choisies de manière aléatoire et observées avec une loupe binoculaire MZ7.5 Leica (x 0,63) équipée d'une caméra (DFP420C Leica) et d'un logiciel d'analyse d'images (Leica Microsystems software v3.8, Nanterre, France). L'analyse des photographies des individus permet d'appréhender la biométrie comme la longueur totale, la taille de la tête et ainsi que l'aire du sac vitellin des larves (Figure 20). Le rapport taille tête/longueur totale est calculé. Les malformations recherchées étaient les malformations squelettiques (scoliose, lordose), les œdèmes, les malformations craniofaciales (malformation ou absence de mâchoire, hypertrophie ou atrophie de l'œil), les anomalies vasculaires (hémorragies ou anémies) et du cœur (position, arythmie).

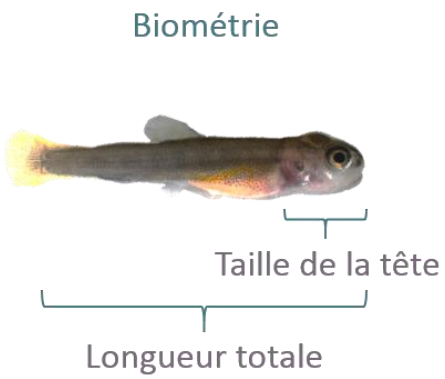

Figure 20 : Mesures biométriques sur les larves de la truite arc-en-ciel.

4.2. Description de biomarqueurs étudiés

Les biomarqueurs sont définis comme des changements dans la réponse biologique qui peuvent être liée à l'exposition à des substances chimiques. Walker (1995) le définit comme des changements biochimiques, physiologiques et histologiques dans les organismes susceptibles d'être déjà utilisés comme organismes sentinelles pour estimer soit l'exposition aux polluants soit les effets induits par la contamination. Par conséquent, les biomarqueurs doivent permettre de distinguer entre la variation naturelle et le stress induit par des polluants, et il doit exister une pertinence écotoxicologique dont la réponse doit être liée à l'impact de l'organisme. Les biomarqueurs étudiés lors de cette étude seront décrit dans les paragraphes suivants.

4.3. Comportement natatoire

Comme le comportement n'est pas un processus aléatoire, mais une séquence d'activités visibles et mesurables visant à assurer la survie de l'individu et des espèces, l'étude de comportement est un outil utile pour évaluer les effets de l'exposition aux xénobiotiques (Kane, 2005). Ces séquences d'actions mesurables, qui agissent depuis le système nerveux central jusqu'au sytème périphérique, intègrent des modifications au niveau génique, biochimique et physiologique (Kane, 2005). Les altérations dans le comportement représentent une réponse intégrative de l'organisme (Kane, 2005). Ces altérations peuvent être associées à une réduction de la survie avec des conséquences négatives pour la survie de l'individu ou de la population d'une espèce (Kane, 2005). L'étude des réponses natatoires des larves de la truite arc-en-ciel implique l'enregistrement de vidéo et l'analyse des trajectoires au niveau individuel avec un logiciel d'analyse d'images. L'étude du comportement inclut la distance totale, la vitesse, le temps de mobilité.

4.4. Analyses biochimiques

4.4.1. Activité de l'acétylcholinestérase

La mesure de l'activité de l'acétylcholinestérase (AChE) peut être considérée comme l'un des biomarqueurs les plus anciens, fréquemment utilisé pour diagnostiquer l'exposition à des composés anti-cholinestérasiques chez les poissons et d'autres organismes aquatiques (de Castilhos Ghisi, 2012). L'AChE est responsable de l'hydrolyse de l'acétylcholine (ACh) en choline et en acide acétique dans les terminaisons nerveuses cholinergiques, vital pour le fonctionnement normal des systèmes sensoriel et neuromusculaire (Van der Oost, 2003). L'inhibition de l'activité de l'AChE est liée au mécanisme d'action

de certains types d'insecticides tels que les organophosphates et les carbamates, elle est donc considérée comme un indicateur de l'exposition à ces composés (Cajaraville, 2000 ; Fulton, 2010). Ces pesticides se lient aux cholinestérases, entraînant leur inactivation et l'accumulation d'ACh dans la synapse nerveuse interférant avec le fonctionnement normal du système nerveux. Cela peut produire des spasmes rapides et une tétanie des muscles volontaires suivis d'une paralysie (Fulton, 2010).

La mesure de l'activité AChE a été réalisé sur les têtes des larves, des truites arc-en-ciel, préalablement décapitées à la fin de l'expérience (500°J). La méthode pour l'extraction de la fraction de S9 a développé selon le protocole de Lowry et al. (1951), et le dosage de l'AChE par la méthode de Ellman et al. (1961).

4.4.2. Peroxydation des lipides

L'une des conséquences du stress oxydatif induits par des composés xénobiotiques est la détérioration des cellules, des tissues et des organes provoqués par des dommages oxydatifs (Ayala et al., 2014). Des niveaux élevés de radicaux libres ou d'espèces réactives de l'oxygène (ERO) peuvent causer des dommages directs aux lipides (Ayala et al., 2014). Les deux espèces les plus répandues pouvant affecter les lipides sont principalement les radicaux hydroxyles ($HO^{\cdot}$) et hydroperoxyl ($HO^{\cdot}_2$) (Ayala et al., 2014). Le processus de la peroxydation lipidique peut être décrit comme un ensemble des réactions en chaîne durant lequel des oxydants attaquent les lipides contenant des doubles liaisons carbone-carbone, en particulier les acides gras polyinsaturés (AGPI) qui sont très sensibles aux réactions oxydatives (Ayala et al., 2014; Valavanidis et al., 2006). Cela implique l'extraction de l'hydrogène d'un carbone et l'insertion de l'oxygène donnant lieu à des radicaux lipides péroxyles et à des hydropéroxydes (Ayala et al., 2014). En réponse, les cellules peuvent favoriser la survie cellulaire avec l'activation de systèmes de défense anti-oxydants ou induire leur mort cellulaire par nécrose ou apoptose (Ayala et al., 2014). Sous un taux faible en lipides peroxydés, les cellules stimulent leur maintien et leur survie par le biais des systèmes de défense constitutifs des antioxydants (GST, Vitamine C et E, …) ou de l'activation de voies de signalisation qui régulent des enzymes antioxydantes (SOD, catalase, GPX, …) ce qui entraîne une réponse adaptative au stress (Ayala et al., 2014).

La dégradation des lipides induit la formation de composés comme des radicaux lipidiques alcoxyles, des cétones, des alcanes, des époxydes et des aldéhydes dont le malondialdéhyde (MDA) (Amiard et Amiard-Triquet, 2008). Dans ce travail, les effets de cette dégradation sont étudiés dans l'étape terminale qui sont des substances réactives à l'acide thiobarbiturique (TBARS) (Buege and Aust, 1978) (Amiard et Amiard-Triquet, 2008) dans des corps entiers des larves prélevés à la fin de chaque

expérience. Ce test met en évidence majoritairement la formation de MDA par méthodes colorimétrique selon le protocole développé par Buege and Aust (1978).

4.4.3. Protéines carbonylées

La réaction d'oxydation des protéines implique divers radicaux et des ERO, ce qui entraîne des modifications oxydatives telles que la formation de dérivés carbonylés dans les protéines (Valavanidis et al., 2006). Les groupes carbonyles (CO) (aldéhydes et cétones) sont produits sur les chaînes latérales des protéines quand ils sont oxydés (Dalle-Donne et al., 2003). Ces aldéhydes et cétones réactifs peuvent réagir avec le 2,4-dinitrophénylhydrazine (DNPH) pour former des hydrazones (Suzuki et al., 2010). Les dérivés des protéines carbonylés peuvent également être générés par le clivage oxydatif des proteines par la voie de l'α-amidation ou par l'oxydation des chaînes latérales du glutamyle (Dalle-Donne et al., 2003). Les groupes CO peuvent être introduits dans les protéines par une réaction secondaire produite lors de la peroxydation lipidique. La teneur en protéine carbonylées est l'un des indicateurs les plus généraux et le biomarqueur le plus couramment utilisé de l'oxydation des protéines (Dalle-Donne et al., 2003). L'accumulation de protéines carbonylées a été analysée dans de nombreuses études écotoxicologiques chez des poissons exposés à différents xénobiotiques (Dalle-Donne et al., 2003; Ferreira et al., 2010; Parvez and Raisuddin, 2005). Le dosage des protéines carbonylés ont été réalisé dans les larves du corps entier suivant le protocole de Augustyniak et al. (2015).

4.4.4. Dommage à l'ADN

Si l'impact du contaminant se produit pendant la phase de division cellulaire, il peut générer des mutations entraînant des effets héréditaires à long terme, tels que des modifications de la fréquence de gènes dans les populations exposées, des événements mutationnels, etc. (Amiard et Amiard-Triquet, 2008; de Castilhos Ghisi, 2012). Les biomarqueurs génétiques évaluent le premier niveau de réponse à l'échelle moléculaire (de Castilhos Ghisi, 2012). L'exposition d'un organisme à des substances génotoxiques peut entraîner une série d'événements pouvant affecter des niveaux de réponse plus élevés (de Castilhos Ghisi, 2012). Dès le premier contact d'une substance génotoxique avec la molécule d'ADN, il se produit une cascade d'événements commençant par des modifications structurelles comme des oxydations, des pertes de bases, des cassures de l'ADN ou la formation d'adduits à l'ADN (Amiard et Amiard-Triquet, 2008; Van der Oost, 2003). Si ce dommage primaire n'est pas réparé avant réplication de l'ADN, alors il sera définitivement fixé sous forme de mutations géniques ou chromosomiques. La présence de dommages primaires en grand nombre, peut également altérer la réplication et la

transcription de l'ADN. Par conséquent, le traitement et la fréquence de ces altérations peuvent entraîner des dommages irréversibles à l'ADN, induisant à des altérations structurelle et fonctionnelle de la cellule. La prolifération incontrôlée peut conduire à des mécanismes de cancérogenèse (Amiard et Amiard-Triquet, 2008). La détection et la quantification de ces événements peuvent être considérées comme des biomarqueurs d'exposition et d'effet chez des organismes exposés à des substances génotoxiques dans l'environnement (Van der Oost, 2003).

Plusieurs biomarqueurs sont utilisés comme outils de détection de l'exposition et des effets des génotoxiques (de Castilhos Ghisi, 2012). Ces biomarqueurs consistent en différents tests tels que l'évaluation des anomalies chromosomiques, des mutations géniques sur des gènes cibles, des adduits et des cassures dans l'ADN, des micronoyaux et des comètes. Au cours de ce travail de thèse, la génotoxicité des pesticides a été évaluée au moyen du test des comètes. Le test de comètes est l'un des tests les plus largement utilisés pour la détection des cassures de brins de l'ADN chez les organismes aquatiques lors d'une exposition *in vivo*, *in vitro* ou *in situ*. Cette technique repose sur la quantification des fragments d'ADN dénaturés qui migrent à partir du noyau cellulaire au cours de l'électrophorèse (Devaux et al., 1997). Le protocole de ce test a été fait selon Singh et al. (1988) avec des modifications (Le Bihanic et al., 2014; Weeks Santos et al., 2019) dans les cellules sanguines des larves qui ont été décapité. Egalement, ce test a été effectué sur la ligné cellulaire RTL-W1.

4.4.5. Expression de gènes cibles

Les biomarqueurs d'expression génétique sont limités aux polluants, dont le mode d'action implique (ou induit) des modifications de la transcription des gènes cibles. Des paramètres critiques de l'homéostasie de la cellule (comme l'intégrité de l'ADN, la pression osmotique, les niveaux de cations divalentes ou des dommages dans les composants de la cellule) sont continuellement surveillés par certaines protéines régulatrices qui fonctionnent comme des capteurs cellulaires. La cellule répond à ces stress en réajustant son métabolisme, en activant ou en synthétisant des protéines spécifiques liées au stress et destinées à compenser le dommage induit (Piña et al., 2007).

Le mécanisme de réponse cellulaire à la présence de xénobiotiques (ex. : une hormone, un métal ou une protéine endommagée) est décrit comme une succession d'évènements. Le récepteur détecte la présence de la molécule et s'active. Cette activation entraine la liaison du récepteur à une séquence spécifique de l'ADN dans le noyau cellulaire, en induisant la transcription de gènes cibles. Le produit de ce processus, l'ARN messager (ARNm), est exporté vers le cytoplasme cellulaire où l'information est traduite pour synthétiser la protéine correspondante, qui constitue la base moléculaire de la réponse biologique à la présence d'un xénobiotique (Piña et al., 2007). Les biomarqueurs géniques

mettent l'accent sur la mesure de la quantité d'ARN codant pour ces protéines (Piña et al., 2007). Avec l'introduction de la PCR, de 10 à 100 molécules d'ARNm peuvent être détectées et quantifiées. Cependant, l'apparition de la PCR quantitative en temps réel (qRT-PCR) a permis d'amplifier les séquences d'une manière plus précise en réussissant à quantifier les molécules d'ARNm. De plus, la haute sensibilité que la qRT-PCR permet son applicabilité à des plus petits organismes comme dans le cas de cette étude où les larves entières de la truite arc-en-ciel ont été analysés de manière individuelle (Piña et al., 2007). Pour ces analyses, des larves individuelles du corps entier ont été étudiées.

Chapitre III

Caractérisation des effets sub-létaux de 3 pesticides individuels sur les premiers stades de développement de la truite arc-en-ciel

Les écosystèmes aquatiques sont souvent considérés comme le réceptacle final des différents polluants tels que les pesticides, provenant principalement des rejets de l'agriculture. Plusieurs études ont montré que la présence de ces pesticides dans les milieux aquatiques, même à de faibles concentrations, peut impacter les organismes qui les habitent (Chapman and Wang, 2001; Hyland et al., 2003). Pour cette raison, il existe un intérêt d'étudier précisément les effets létaux, mais aussi sub-létaux que ces pesticides peuvent avoir sur les premiers stades de développement de poisson qui sont particulièrement sensibles à la qualité de leur milieu.

Ce chapitre est consacré à la présentation des résultats de 3 études différents, sous forme d'articles, avec l'objectif d'analyser l'impact de 3 molécules phytosanitaires sur les premiers stades de développement de la truite arc-en-ciel. Chaque molécule, possédant un mode d'action différent, représente une classe de pesticide : un fongicide (le cuivre), un herbicide (le glyphosate) et un insecticide (le chlorpyrifos). Le cuivre (**Article 1**) et le glyphosate (**Article 2**) ont été choisis à cause de leur large utilisation dans les vignes de Gironde, et de leur détection fréquente dans les écosystèmes aquatiques. Le chlorpyrifos (**Article 3**), moins utilisé, fait partie de la liste des 41 substances prioritaires de la DCE qui doivent être quantifiées pour déterminer le bon état chimique des eaux de surface.

Pour répondre à cet objectif, des embryons de truite arc-en-ciel ont été exposés jusqu'au stade larvaire à ces 3 molécules, individuellement, à des concentrations environnementalement pertinentes pendant 3 semaines. Dans l'**article 1**, les embryons ont été exposés à 2 et 20 µg/L Cu en utilisant le sulfate de cuivre. Dans l'**article 2**, la formulation commerciale du Roundup®GT Max a été utilisée pour exposer les embryons à 0,1 et 1 mg/L de glyphosate. Parallèlement, l'exposition au glyphosate a été menée également sur la lignée cellulaire RTL-W1 et la cytotoxicité et la génotoxicité de ce composé ont été analysées. Finalement, dans l'**article 3,** les embryons ont été exposés au chlorpyrifos pur avec des concentrations de 0,3 et 3 µg/L.

A la fin de l'exposition, plusieurs paramètres biologiques (survie, succès d'éclosion, malformation, croissance), comportementaux (activité de nage) et moléculaires (génotoxicité, peroxydation lipidique, carbonylation des protéines, expression de gènes) ont été étudiés sur les larves exposées.

Article 1

Sub-lethal effects of waterborne copper in early developmental stages of rainbow trout (*Oncorhynchus mykiss*)

Publié dans *Ecotoxicology and Environmental Safety*

Shannon Weeks Santos, Jérôme Cachot, Pierre-Yves Gourves, Christelle Clérandeau, Bénédicte Morin, Patrice Gonzalez

Principaux résultats de l'Article 1

- *Les résultats obtenus montrent des effets létaux du cuivre à 20 µg/L avec l'apparition d'embryons demi-éclos. En absence d'une forte mortalité embryonnaire (< 20 %), nos résultats suggèrent une protection du chorion chez les embryons exposés au cuivre. Cependant, après l'éclosion, les larves sont en contact direct avec le milieu contaminé par le cuivre ce qui entraîne une mortalité supérieure.*

- *Les deux concentrations de cuivre étudiées montraient une augmentation significative du nombre de larves malformées par rapport aux larves témoins. Parmi toutes les déformations observées, les anomalies vertébrales (cyphose, lordose, scoliose et les larves en forme de 'C') étaient majoritaires.*

- *Le comportement de nage, l'intégrité de l'ADN, et l'oxydation des lipides et des protéines n'ont pas été affectés par l'exposition au cuivre quelle que soit la concentration testée.*

- *Les gènes impliqués dans la détoxication des xénobiotiques (gst, mt1 et mt2), et l'arrêt du cycle cellulaire (p53), sont significativement réprimés dans les larves exposées à 20 µg/L de Cu. L'altération de l'expression de ces gènes pourrait compromettre la survie de cette espèce dans des conditions naturelles.*

Sub-lethal effects of waterborne copper in early developmental stages of rainbow trout (*Oncorhynchus mykiss*)

Shannon Weeks Santos, Jérôme Cachot, Pierre-Yves Gourves, Christelle Clérandeau, Bénédicte Morin, Patrice Gonzalez

Abstract

The aim of this work was to study the impact of copper during a sub-chronic exposure to environmental concentrations in the early life stages of rainbow trout (Oncorhynchus mykiss). Eyed-stage embryos of rainbow trout, at 265°D, were exposed in semi-static conditions to sub-lethal concentrations of CuSO4 up to the larval stage (528 °D) under laboratory-controlled conditions. During 3 weeks, they were exposed to the environmentally-realistic concentration of 2 µg/L Cu and to a 10-fold higher concentration, 20 µg/L Cu. Several biological (survival, hatching success, malformation, growth) and behavioral (swimming activity) and molecular endpoints (genotoxicity and gene transcription) were studied. Exposure to 20 µg/L Cu had an inhibitory effect on hatching and increased half-hatched embryos (25%). At the end of the exposure, no significant differences were observed in growth of the larvae exposed to the highest Cu concentration. However, larvae exposed to 2 µg/L Cu exhibited increased growth in comparison with non-exposed larvae. The percentage of malformed larvae was significantly higher for both copper conditions, with skeletal malformations being the most observed. Expression of several genes was evaluated in whole larvae using quantitative real-time PCR. Genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest (*p53*) were significantly repressed in both copper conditions when compared to control. In addition, potential genotoxic effects on larvae were investigated by the comet assay on blood cells, but this test did not demonstrate any significant DNA damage on larvae exposed to copper. This study confirms the adverse effects of copper on early life stages of rainbow trout even at the lowest environmentally relevant tested concentration.

Keywords: copper, *Oncorhynchus mykiss*, early life-stage, developmental toxicity, genotoxicity, gene transcription.

1. Introduction

Copper is one of the few metals that occurs naturally in ecosystems, as well as being one of the first used by humans since 8000 B.C. Over 95 % of all copper in circulation today has been extracted since 1900. It is an essential trace element involved in numerous biological processes, such as photosynthesis in plants. It also plays an essential role in certain enzyme functions in animals, including copper-zinc superoxide dismutase and cytochrome c-oxidase (Eisler, 1998). Copper is widely used in industrial, agricultural and urban activities, and consequently is one of the priority substances listed in the Water Framework Directive (WFD). With its significant anti-microbial and anti-fungal properties, copper has been used extensively in vineyards, resulting in high levels of transfer to soils and aquatic ecosystems (Devez et al., 2005; El Azzi et al., 2013; Komárek et al., 2010; Ruyters et al., 2013). Recent reports have shown that Cu-containing fungicides and boat antifouling paints appear to be the main anthropogenic sources of Cu in aquatic ecosystems (Babcsányi et al., 2014; Devier et al., 2005; Petit et al., 2013; Singh & Turner, 2009). Monitoring this element in the coastal marine environment by the Chemical Contamination Observation Network (ROCCH, IFREMER) using the Pacific oyster (*Magallana gigas*) as a sentinel species showed an increase in its concentration at certain points along the French coast, such as Arcachon Bay (Claisse and Alzieu, 1993; Gamain et al., 2016).

Pollution of surface water and groundwater is one of the biggest environmental issues. The background copper concentrations in unpolluted water bodies varied between 0.2 to 6 µg/L (Flemming and Trevors, 1989; Willis and Bishop, 2016). Levels of copper can increase from anthropogenic pollution as a result of urban and agricultural runoffs (Davis et al., 2001; Devez et al., 2005). Copper concentration in Beysehir lake (Turkey) was up to 100 µg/L in spring 2004 (Tekin-Özan, 2008). The river Lot (southern France), with a large history of metal pollution, concentrations of copper in water column was 17 µg/L in 1987; but, this concentration decreased in 2006 to 3.2 µg/L (Shinn et al, 2009). In several Asturians rivers (Spain), copper concentrations were between 2.5 and 4.9 µg/L (Linde et al., 1998), and in Boulder river (Montana, USA) was ranged from 1.4 to 23 µg/L (Farag et al., 2007).

High concentrations of copper in ecosystems may be harmful to aquatic organisms. Indeed, although essential, Cu induces toxic effects during chronic exposure and over-accumulates in living organisms (Marr et al., 1996; Kamunde et al., 2002; Vardy et al., 2013). Since copper can participate in Fenton and Haber-Weiss reactions, it can produce reactive oxygen species (ROS), which react with H_2O_2 and cause cellular damage to lipids, nucleic acids and proteins that can lead to cell death (Manzl et al., 2004; Prousek, 2007; Bopp et al., 2008). Many studies have highlighted the toxic effects of copper on fish and mollusks (Vardy et al., 2013; Gamain et al., 2016; Boukadida et al., 2017). Sensitivity to copper can vary greatly from one fish species to another. For example, Vardy et al. (2013) determined the lethal

concentrations (LC_{50}) at 96 h of Cu exposure at different life stages of fish, which for swim-up larvae of white sturgeon and rainbow trout was 10 and 21 µg/L of Cu, respectively. McKim et al. (1978) examined embryo mortality of eight different fish species after Cu exposure and observed significant mortality between 37 and 555 µg/L. Besides, this sensitivity to metals could also differ according to the development stage (McKim et al., 1978; Vardy et al., 2013) and water chemistry parameters like hardness, pH, organic carbon and alkalinity (Brix et al., 2017; Crémazy et al., 2017; Eisler, 1998).

Several reports have revealed that early life stages of fish are a great deal more vulnerable to pollutants than juveniles and adults (Eaton et al., 1978; Hutchinson et al., 1998; Van Leeuwen et al., 1985; Vardy et al., 2013). Embryos and larvae organs, such as liver, gills and kidney, are not entirely developed, which can result into a higher sensitivity to toxicants (Mohammed, 2013). Additionally, larvae have a permeable skin that could allow the uptake of pollutants (Mohammed, 2013). Because fish embryos and larvae have an important role in the trophic chain and, in the persistence of population as well, the use of these stages are relevant for the assessment of toxicity in the aquatic environment. Several reports have studied copper toxicity in early life stages of fishes (Barjhoux et al., 2012; El-Fiky, 2001; Kazlauskienë, 2002; Stasiūnaitė, 2005; Witeska et al., 2010).

Early life stages of rainbow trout (*Oncorhynchus mykiss*) can be easily raised under laboratory conditions, and it is considered, along with other species, as a model animal in ecotoxicology (OECD, 2013). Due to its slow embryo-larval development, toxicity tests allow longer sub-chronic exposures to toxicants. This species has been extensively studied and, because of its low tolerance to poor water quality, is considered as one of the most commonly used sentinel species for aquatic ecotoxicology (De Boeck et al., 2004; Hansen et al., 2002; Kamunde et al., 2002; Olsson et al., 1990).

The purpose of the present work was to evaluate and to characterize the sub-lethal effects of environmental concentrations of copper to early developmental stage of rainbow trout. At the end of the 3-week Cu exposure, several end-points like viability, hatching rate, developmental abnormalities, larval swimming behavior, genotoxicity (DNA damage), lipid and protein peroxidation, and gene expression were analyzed.

2. Materials and methods

2.1. Chemicals

Copper sulfate ($CuSO_4 \cdot H_2O$, CAS 7758-99-8, 99.99%) and low melting point agarose (LMPA) were purchased from Sigma Aldrich (Lyon, FR). Phosphate-buffered saline (PBS), nitric acid 65% and normal melting point agarose (NMPA) were purchased from Fluka Chemie (Buchs, CH).

2.2. Exposure condition

Rainbow trout embryos (*Oncorhynchus mykiss*) at eyed stage were provided by INRA-PEIMA (Sizun, FFR). They were incubated in total darkness under a temperature of 12°C. Embryos were exposed to copper from 265 DD (degrees days) up to the larval stage of 528 DD for a total of 23 days of exposure. Copper solutions (2 and 20 µg/L Cu) were prepared using $CuSO_4 \cdot 5H_2O$ and the required concentrations were calculated according to the quantity of copper ions (Cu^{2+}). Each studied condition, and a control without Cu, involved 3 replicates with 100 embryos in 1 L aquaria. Exposure water was prepared in three 5 L tanks of Saint-Etienne spring water (63 mg/L Ca, 10.2 mg/L Mg, 1.4 mg/L Na, 0.4 mg/L K, 173.2 mg/L HCO_3^-, 51.3 mg/L SO_4^{2-}, 2 mg/L NO_3^-, pH 7.6, <1 mg/L Cl^-, <1.5 mg/L DOC). A peristaltic pump (Watson Marlow, USA) was used to maintain a continuous flow rate of water (9 mL/min) into the incubation chambers. To keep the require copper concentration in aquaria, water was renewed every 48 h. At the end of the experiment, larvae were sampled per replicate as follow: 10 larvae to record for biometrics and morphological anomalies, 6 larvae for the study of swimming behavior, 6 larvae for blood sampling for genotoxicity test. The remaining larvae were pooled and stored at -80°C for biochemical analysis and gene transcription.

2.3. Analysis of copper in water and larvae

Fish and water samples were analyzed to determine Cu concentrations. Fish samples (2 pools of 5 larvae from each replicate) were lyophilized and digested by nitric acid (0.3 mL of HNO_3 67 %). Four tubes containing the international certified reference material, Dolt-5 samples (Fish liver), were prepared in order to verify the accuracy of the method. Approximately 80 mg of thismaterial was digested by 3 mL of nitric acid in polypropylene tubes. Then, this mixture was heated by Hot Block for 3 h at 100°C, and after cooling 1.5 mL and 15 mL of ultrapure water (Milli-Q®) was respectively added to sample larvae and certified reference. Finally, 3 blank samples were also prepared in the same conditions (3 mL of HNO_3 before heating and 15 mL of Milli-Q water added after cooling). 40 mL of water sample of each condition were collected in 3 random days through the experiment, and acidified with 5 % of nitric acid (Nitric acid 65 %, Fluka). Copper concentrations in water of condition “20 µg/L” and fish samples were analyzed by inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES 720, Agilent Technologies), whereas copper concentrations in water of controls and “2 µg/L” conditions were analyzed by atomic absorption spectrophotometer (Varian SpectrAA 240Z, Agilent Technologies, Santa Clara, USA). Detection limits (DL) for ICP-OES was 2.26 µg/L Cu^{2+} and for atomic absorption was 0.5 µg/L Cu^{2+}.

2.4. Phenotypic effects

Dead embryos and larvae were recorded daily and removed immediately to prevent water alterations. Individuals were considered half-hatched when at least a part of the body was inside the chorion. Embryonic and larval mortality refer to the number of dead individual compared to the total number of embryos at the start of the experiment or total number of totally hatched larvae. Half-hatched mortality was only considered when at least a part of the body was inside the chorion. Hatching time refers to the number of degree days (DD: number of days x temperature) from fertilization to hatching. Hatching rate is calculated by dividing the number of half-hatched embryos by the total number of embryos at the beginning of the experiment. The appearance of anomalies was microscopically examined in 10 larvae per replicate randomly chosen. The larvae were placed in a Petri dish with carbonated water and ice to sedate them. Photos were taken for each larva with a stereomicroscope (MZ7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). From the photos, we measured total body and head length for each larva using Leica Microsystems software v3.8 (Nanterre, France). Larvae were also observed to locate possible malformations such as edemas, spinal and craniofacial deformities, cardiac anomalies, hemorrhages and yolks sac resorption.

2.5. Genotoxicity test

The comet assay was performed according to the Singh et al. (1988) protocol, adapted by Le Bihanic et al. (2014). Blood sampling were performed in 6 larvae per replicate by decapitation using a heparinized pipette. Samples were stored in a microtube with 200 µL of cryopreservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) and immediately frozen in liquid nitrogen until analysis. These storage conditions have already been described by Barillet et al. (2005) and Bony et al. (2008), this protocol has been validated for rainbow trout blood cells in our laboratory. 50 µL of cell suspension was added to 100 µL of LMPA (Low Melting Point Agarose). Two aliquots of 50 µL of the suspension were placed onto slides previously covered with NMPA (Normal Melting Point Agarose, 1 %, w/w) and a coverslip (18x18 mm) was added. The gels were left to harden for 15 min at 4°C and then the coverslips were removed from each slice and a second layer of LMPA was placed on the gels with new coverslips and hardened at 4°C for 15 minutes. The slices were put in a lysis solution (10 mM Tris; 2.5 M NaCl; 100 mM EDTA; 1% Triton x-100; 10% DMSO; pH adjusted to 10 with NaOH) at 4°C for 90 minutes. Slices were placed horizontally on a tray with electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH> 13) for 20 minutes to allow the DNA to unwind. Electrophoresis was performed with a voltage of 25 V and 290 mA for 20 minutes. After electrophoresis, slices were rinsed 3 times with a neutralizing solution (0.4 M Tris, pH 7.5) for 5 minutes at 4°C. Then, slices were soaked

with absolute ethanol for 20 minutes and dried at a room temperature for at least 24 hours. Slices were stained with 20 µg/mL of ethidium bromide solution. Comet analysis was performed using an epifluorescence microscope, and DNA damage was measured with Comet Assay IV software on 100 cells randomly selected. Results are expressed as percentage of degradation of DNA tail. Heavily degraded cells, or inexistent head with diffuse tail, were not counted in the tail DNA measurements as proposed by Kumaravel et al. (2009). However, they were counted as hedgehogs on a total of 100 cells per gel.

2.6. Behavioral analysis: swimming test

The swimming behavior of 528 DD larvae was studied using the Daniovision Image analysis system version 10.0 Noldus. Six larvae per replicate were placed in 6-well microplates containing 8 mL of exposure water. The microplates were placed in the recording chamber in the dark at 12 °C for about 30 min to acclimate the larvae. Then the analysis was started. An infrared camera in the chamber recorded the movements of each larvae focusing on their center of gravity. The analysis takes 30 min with dark/light/dark cycle of 10 min each. This cycle allows to analyze the behavior of larvae in response to light stimulation. Ethovision software allows to record the average speed of each larvae every 30 seconds. For each larva, the swimming performance was assessed by determining the total distance moved (cm) in 10-minute period, and time of mobility accumulated (s) on larvae. Mobility is defined as the period of time where the area of total larva is modified albeit central point of larvae had not changed. If more than 60 % of the area was changed, it was considered as highly mobile. Otherwise, less than 60 %, it was considered only as mobile.

2.7. Biochemical analysis

Preparation of supernatant

Pools of 5 larvae were frozen in liquid nitrogen and then stored at -80°C until analysis. At the moment of analysis, yolk sac from larvae were removed and then larvae (approximately 250 mg) were homogenized in a chilled phosphate buffer (0.1 M; pH 7.5) using an UltraTurrax® tissue homogenizer fitted with a potter at 3,000 rpm (4°C). The supernatant was centrifuged at 9,000 g for 25 min at 4°C to obtain the supernatant S9 fraction. This fraction contains soluble proteins from cytosol and microsomes (Parmentier et al., 2007). Total protein, TBARS levels and protein carbonyls content were analyzed using the S9 fraction.

Total protein

The total protein concentration was measured on S9 fraction by the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as a standard and Folin's reagent. Measurements were done using a spectrophotometer microplate reader (Synergy HT, BioTek).

Lipid peroxidation

Lipid peroxidation was determined using the adapted technique of Buege and Aust (1978) adapted to a microplate reader. 500 µL of a solution containing 20 % of trichloroacetic acid (TCA) and 20 % butylated hydroxytoluene (BHT) was added to 500 µL of S9 fraction and then mixture was centrifuged for 10 min at 9,000 g. Afterwards, 600 µL of supernatant with 480 µL of TRISbase (25 mM)-TBA (thiobarbituric acid-100 mM) and 120 µL of 0.6 N HCl heated at 80°C for 15 min. The tubes were then cooled and mixed. TBARS levels were read using a UV-spectrophotometer (Biotek Synergy HT) in a microplate at 530 nm. Results were expressed as nmoles of thiobarbituric acid reactive substance (TBARS) equivalents per milligram of protein.

Protein Carbonyl analysis

Protein carbonyl content was assessed using the spectrophotometry method described in Augustyniak et al., (2015). 50 µL of a solution of 11 % streptomycin sulfate – phosphate buffer (100 mM pH 7.4) were added to 500 µL of S9 fraction, then it was mixed and incubated for 15 min at room temperature. Afterwards, mixtures were centrifuged for 10 min at 6,000 g. Then 200 µL supernatant was added to 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 mM) used as a sample tube, and to 800 µL of HCl 2.5 M used as a control tube; then, incubated for 1 h at room temperature with vortexing every 15 min. Proteins were precipitated with 1 mL of 20 % TCA (trichloroacetic acid), vortexed and centrifuged for 10 min at 10,000 g. The pellets were rinsed with 1 mL of ethanol-ethyl acetate (v:v), vortexed and centrifuged three times. Then pellets were solubilized with 500 µL of 6 M guanidine HCl and centrifuged at 10,000 g for 10 min. The carbonyl content was measured using a UV-spectrophotometer (Biotek Synergy HT) at 370 nm. Results were expressed as nanomoles of DNPH incorporated/mg protein.

2.8. Gene expression

6 larvae per replicate were collected in a storage buffer (RNA later, Qiagen). Samples were frozen in liquid nitrogen and stored at -80°C until analysis.

RNA extraction

Total RNA extraction from whole larvae was performed with the kit "SV Total RNA Isolation system" (Promega) following the supplier's recommendations. Larvae were homogenized using the MP fastprep®-24 (Biorad, 6 m/s, 40s) with 14.4 mm diameter ceramics beads (MP Biomedicals, Lyssing Matrix D Bulk).

Retro-transcription of total RNA into cDNA

The retro-transcription of total purified RNA was carried out with the kit "GoScript Reverse Transcription System" (Promega). To execute the Reverse transcription, 1 µL of oligodT (1 µM) and 1 µL of hexanucleotides (1 µM) have been added to 10 µL of total purified RNA (1 µg). Then, the mix was heated at 70°C for 5 minutes with a thermocycler (Eppendorf Mastercycler) followed by 5 minutes at 4°C to allow primer annealing. Subsequently, 1 µL of dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of $MgCl_2$ (25 mM), 1 µL or reverse transcriptase (1 U/µL) and 0.5 µL or RNAsine were added. The reverse transcription reaction was performed for 1 h at 42°C in a thermocycler. The cDNA thus obtained were stored at -20°C pending analysis by quantitative real-time PCR reaction.

Quantitative real-time PCR

Thirteen genes were investigated and specific primer-pairs designed with primer3plus software (Table 1). All the primer-pairs used in this study have an efficiency upper than 95%. Real-time qPCR was carried out using GoTaq® qPCR Master Mix kit (Promega). PCR reactions contained 17 µL of a mixture of Nuclease-Free Water and GoTaq® qPCR Master Mix containing the SyberGreen fluorescent dye, 2 µL of specific primer pairs mix (200 µM each) and 1 µL of cDNA. Real-time quantitative PCR reactions were performed in a Mx3000P® qPCR System (Stratagene). The amplification program consisted in one cycle at 95°C for 10 min, then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity was determined for each reaction from the dissociation curve of the PCR products. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95°C.

Cycle thresholds (Ct) were obtained from MxPro™ qPCR software for each gene. Two different reference genes were investigated (Rpl7 and EF1α) and were found to be stable in our conditions. Consequently, relative quantification of each gene expression level was normalized according to the mean Ct value of these two reference genes according to the 2ΔCt methods described by Livak and Schmittgen (2001). The ratio of transcription level to exposed individuals with the control ones gives the induction factor (>2) or repression factor (<0.5) of studied genes.

Table 1: Accession number and specific primer pairs for the Oncorhynchus mykiss used in our study.

Gene	Accession number	Primer (5' – 3')
Rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
EF1α	NM_001124339.1	ATGGGCTGGTTCAAGGGATG[a] GATCATACCGGCCTTCAGGG[b]
Cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG[a] GTCCAGGATGGGAAGTTGC[b]
Sod	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
Gst	BT073173.1	ATTTTGGGACGGGCTGACA[a] CCTGGTGCTCTGCTCCAGT[b]
Mt1	M18104.1	GTGGATCCTGCAAGTGCTCA[a] GTAATGCACCAGGCCTCACT[b]
Mt2	XM_021597409.1	ATCTTGCAACTGCGGTGGAT[a] CCTCACTGACAACAGCTGGT[b]
Cox	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12S	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
Ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
Rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
Bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
p53	NM_001124692.1	CCTACCAATGAGTAAGACAT[a] CCCGAAACATCCCGCT[b]

[a]Forward primer

[b]Reverse primer

2.9. Statistics

Each studied condition was triplicated identically and each replicate was considered as an independent sample. All data are represented by the mean ± SE (Standard Error). Statistical analyzes were carried out using R (http://cran.r-projet.org/). The Normality of data distribution was verified on the residues by the Shapiro-Wilk test ($p < 0.01$) and the homogeneity of variances was evaluated by the Levene test ($p < 0.05$). In the case of normal distribution, a one-way ANOVA analysis was used ($p < 0.05$) followed by a Tukey post-hoc test. In the case that data was not normal, data was evaluated by non-parametric tests of Kruskal-Wallis ($p < 0.05$).

3. Results

3.1. Test condition

Dissolved oxygen in water varied between 81.4 and 95.7 % throughout embryonic and larval exposure. Table 2 shows Cu concentrations determined in the exposure water. Water samples were collected in 3 random days. In the control group, Cu concentrations were below the detection limit. Cu concentrations of the treatment at 20 µg/L were close to the nominal concentration. However, results from 2 samples of the treatment at 2 µg/L were below the detection limit (0.5 µg/L Cu).

Table 2: Mean copper concentrations measured in exposure chambers (µg/L).

	Nominal Cu concentration (µg/L)		
	0.0 (Control)	*2*	*20*
T1	<0.5	<0.5	20.34
T2	<0.5	<0.5	18.73
T3	<0.5	1.45	18.71

3.2. Embryonic and larval survival

No significant differences in embryonic and larval survival were observed between the control group and the condition at 2 µg Cu /L (Table 3). Regarding the condition of 20 µg/L Cu, a significant decrease of embryonic survival was observed compared to control. Mortality of half-hatched embryos was significantly higher for those exposed to 20 µg/L Cu compared to other conditions. In addition, Figure 1

and Table 3 show a significant lower hatchability for this condition in comparison to control and 2 µg/L Cu. On the other hand, the hatching time (DD) did not show any significant differences between the control and the two Cu exposure conditions (Table 3).

Table 3: Developmental endpoints in embryos and larvae of rainbow trout following exposure to waterborne Cu. Values are Means ± SE. Different letters in the same row indicate significant differences between conditions. N=3, ANOVA/Kruskall-Wallis, p<0.05.

	Control	2 µg/L Cu^{2+}	20 µg/L Cu^{2+}
Acute toxicity			
Embryo survival (%)	**97.3 ± 1.7[a]**	**97.3 ± 1.7[a]**	**88.3 ± 1.8[b]**
Larval survival (%)	92.9 ± 0.6	94.7 ± 1.1	90.3 ± 3.8
Half-hatched mortality (%)	**7.0 ± 1.5[a]**	**9.7 ± 5.7 [a, b]**	**25.0 ± 4.0[b]**
Sub-lethal toxicity			
Hatching rate (%)	**90.3 ± 3.2[a]**	**87.7 ± 7.3[a]**	**63.3 ± 3.2[b]**
Hatching time (DD)	332.8 ± 3.3	334.9 ± 2.1	336.8 ± 1.2
Total length (mm)	**17.6 ± 0.3[a]**	**18.36 ± 0.02[b]**	**17.9 ± 0.3[ab]**
Head length (mm)	3.8 ± 0.1	4.1 ± 0.02	4.0 ± 0.1
Ratio of head/body length (%)	21.8 ± 0.4	22.5 ± 0.1	22.2 ± 0.3
Whole body copper concentration (µg/g d.w.)	**5.66 ± 0.08[a]**	**5.71 ± 0.02[a]**	**6.03 ± 0.004[b]**
Abnormalities (%)			
Total deformed larvae	**23.3 ± 3.3[a]**	**58.4 ± 2.7[b]**	**67.8 ± 13.2[b]**
Edemas	6.7 ± 6.7	7.8 ± 4.2	17.8 ± 9.0
Yolk-sac malabsorption	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Spinal	**20.0 ± 5.8[a]**	**52.4 ± 3.5[b]**	**65.1 ± 11.8[b]**
Craniofacial	6.7 ± 3.3	10.8 ± 1.7	14.7 ± 10.8
Cardio-vascular	3.3 ± 3.3	10.8 ± 5.5	15.5 ± 7.8
Haemorrhages	0.0 ± 0.0	3.0 ± 3.0	15.5 ± 7.8

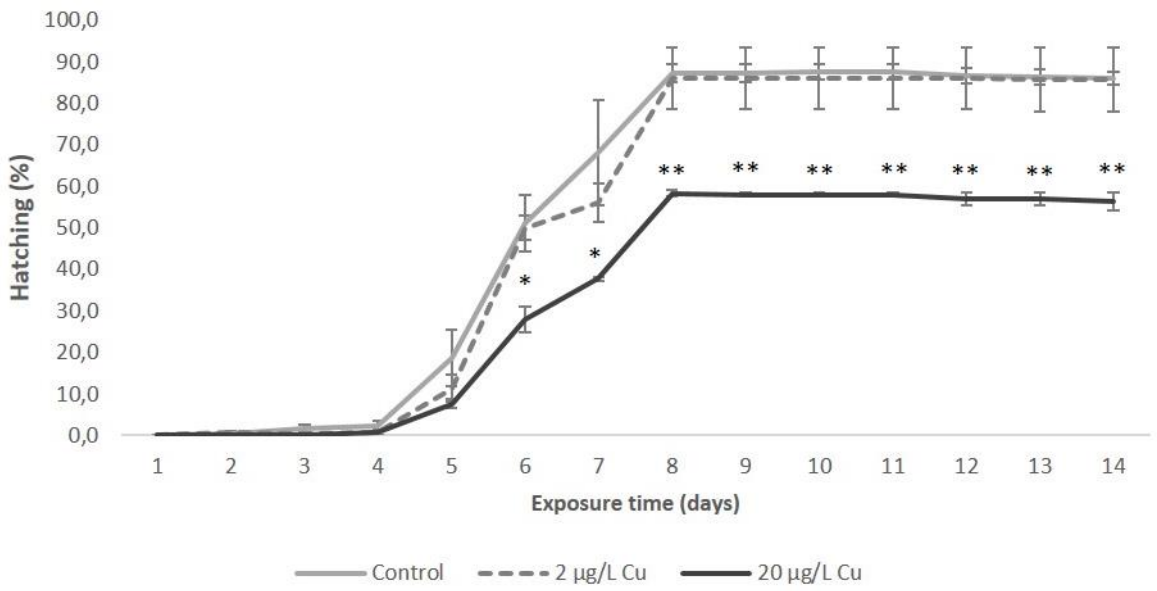

Figure 1: Hatching rate for each studied condition. Asterisks indicate significant differences compared to control condition. Values are Mean ± SE. N=3, ANOVA, *p<0.05, **p<0.01.

3.3. Larvae copper accumulation

After 3 weeks of exposure, larvae exposed to 20 µg/L Cu showed a significantly higher accumulation of Cu ($p < 0.01$) compared to control and larvae exposed to 2 µg/L Cu (Table 3). No significant difference in Cu accumulation was observed in larvae exposed to 2 µg/L Cu comparatively to control.

3.4. Biometrics

Exposure to the higher concentration of Cu (20 µg/L) did not promote significant changes on larvae biometrics compared to control (Table 3). However, larvae exposed to 2 µg/L Cu were significantly longer compared to control larvae. However, this difference is small representing only 4.14 % of increase between the two conditions. No significant differences were observed between the length of head and the ratio between the size of head and the total length for the two studied Cu conditions and the control (Table 3).

3.5. Malformations

Both copper conditions impaired normal development of larvae, where total malformations were significantly higher when compared to control condition (Table 3). Spinal anomalies (kyphosis, lordosis and c-shaped curvature) were the most frequently observed malformation, but its incidence was much

higher in the Cu exposed groups than in control. The incidence of all the other recorded abnormalities (edemas, yolk sac malabsorption and craniofacial malformation) tended to increase with increasing Cu concentration in the exposure water, although no significant differences were observed among groups (Table 3).

3.6. Behavior

Responses lo light stimulation of larvae are presented in Figure 2. The results represent the average distance traveled by larvae (Figure 2-A) and the cumulative time of high mobility (Figure 2-B) and mobility (Figure 2-C) of larvae. Over the light period, larvae from all conditions showed similar responses to light stimulation and all larvae increased their distance traveled and their mobility compared to the first dark period. However, no significant differences were observed between conditions on all studied parameters.

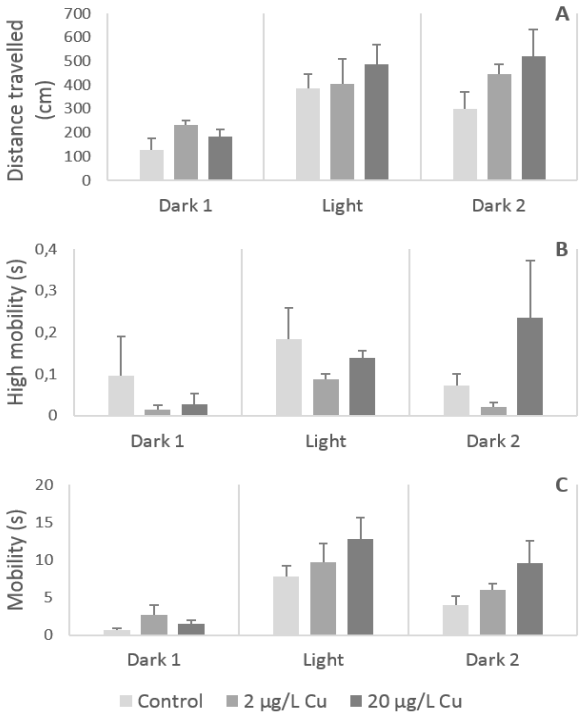

Figure 2: Total distance travelled by rainbow trout larvae (A), and cumulative time of high mobility (B) and mobility (C) of larvae. Locomotion was recorded in 30 minutes video analysis. Values represent average over 10 minutes interval. Values are Mean ± SD, N=3, ANOVA.

3.7. Genotoxicity

Results from the comet assay did not reveal any significant DNA damage in both Cu studied conditions when compared to control larvae (Figure 3-A). Nevertheless, numbers of severely damaged cells or ghost cells ("hedgehogs") were significantly increase in larvae exposed to 20 µg/L Cu in comparison to control (Figure 3-B).

3.8. Lipid and protein oxidation

Larvae exposed to 2 and 20 µg/L of Cu exhibited no significant changes on the content of thiobarbituric acid (Figure 3-D) and protein carbonylation (Figure 3-C) comparatively to non-exposed larvae.

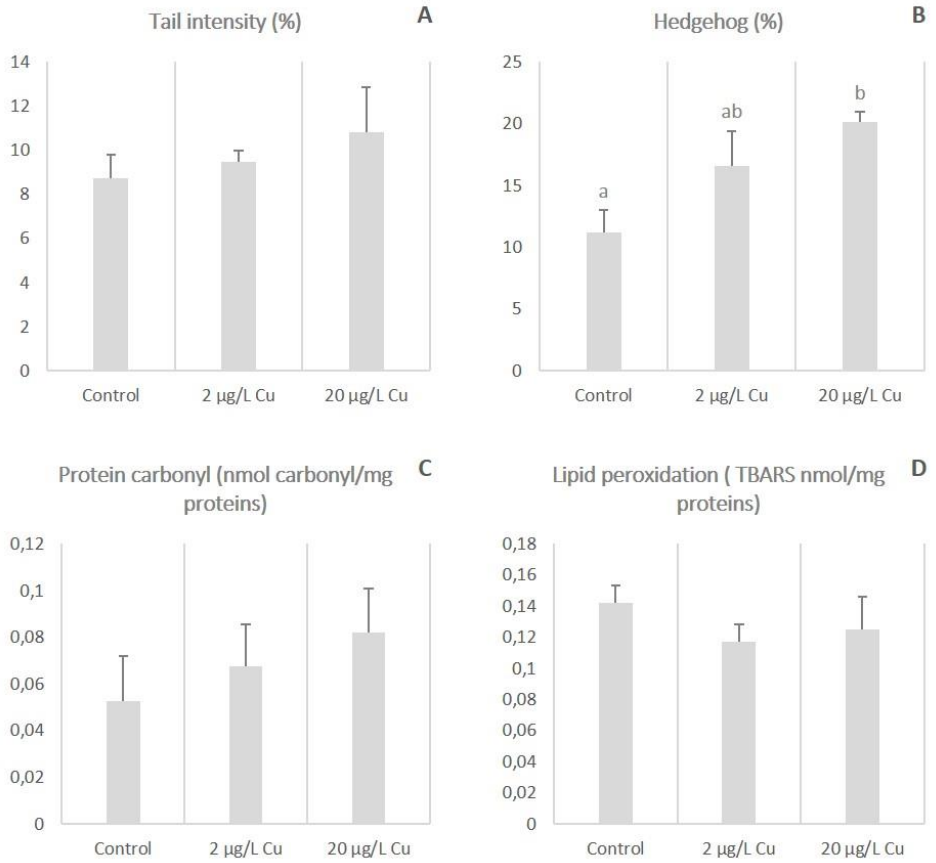

Figure 3: Percentage of DNA tail intensity (A) and hedgehog (B) from blood cells of rainbow trout larvae assessed with the comet assay. Protein carbonyl content (C) and lipid peroxidation expressed as TBARS (D) of whole-body rainbow trout larvae. Protein carbonyl is expressed as nanomoles of carbonyl/mg of protein, and lipid peroxidation is expressed as nanomoles of TBARS/mg of protein. All values are expressed as Mean ± SE, N=3, ANOVA.

3.9. Gene expression

Cu effects on gene transcription were pronounced on both larvae exposed to 2 and 20 µg/L Cu (Table 4). Indeed, genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest and apoptose (*p53*) were significantly repressed compared to control larvae. In contrast, gene transcription related to DNA repair system (*ogg1* and *rad51*) was not significantly altered at the end of 3-weeks Cu exposure. Also, no effects were observed on *cat* and *12s* genes for both studied conditions. In the case of larvae exposed to 2 µg/L Cu, the transcription of *sod* was significantly decreased when compared to control larvae.

Table 4: Transcription of genes involved in antioxidant defense (cat, sod), DNA repair (ogg1 and rad51), detoxification (gst, mt1 and mt2), mitochondria metabolism (cox, 12S), apoptosis (bax) and cell cycle arrest (p53) in whole-body rainbow trout larvae. Results are expressed as induction (> 2) and repression (< 0.5) factors relative to control condition. Asterisk indicate differences with the control condition (N = 3, ANOVA/Kruskal-Wallis, *p < 0.05; **p<0.01).

	cat	*sod*	*gst*	*mt1*	*mt2*	*cox*	*12s*	*ogg1*	*rad51*	*bax*	*p53*
2 µg/L Cu	/	0.18**	0.24*	0.28*	0.15*	/	/	/	/	/	0.16*
20 µg/L Cu	/	/	0.15**	0.14**	0.14*	0.39	/	/	/	0.32	0.12*

4. Discussion

Copper is a ubiquitous metal that is naturally present in the environment. Although copper is an important trace element for the proper functioning of the organism, strong concentrations may be harmful to aquatic organisms (Flemming and Trevors, 1989; McKim et al., 1978). In the present study, exposure to 2 µg/L Cu did not increase embryonic and larval survival and hatching rate of rainbow trout when compared to control. Regarding the exposure to 20 µg/L Cu, a significant decreased of embryonic viability was observed. However, the embryonic viability was superior to the survival criteria given by OECD (2013) at 75 % for control condition of rainbow trout. On the other hand, our results from this condition show a strong mortality during hatching of rainbow trout. Regarding the literature, our results are comparable to those found by other authors. McKim et al., (1978) demonstrated an almost complete embryo mortality on rainbow trout when exposed to 37 µg/L Cu. Compared to other species, rainbow trout showed greater sensitivity to Cu. For example, for a complete embryo mortality of brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*) and herring (*Coregonus artedi*) about

555 µg/L of Cu were needed (McKim et al., 1978). Vardy et al. (2013) determined that the LC_{50} at 96 h for yolk-sac stages of white sturgeon (*Acipenser transmontanus*), rainbow trout and fathead minnows (*Pimephales promelas*) was 22, 40 and 102 µg/L of Cu respectively. It was also observed that later larval stages of white sturgeon and rainbow trout were even more sensitive to the effects of Cu than yolk sac stages (9 and 22 µg/L of Cu respectively). Another study was performed on two different stages of rainbow trout, embryo and swim-up stages, where lethal responses to Cu were observed from 22 µg/L for both stages after a sub-chronic exposure (Besser et al., 2005). Cu lethality not only depends on the sensitivity of the fish species and stage, but also on the water chemistry parameters such as pH, concentration of DOM (dissolved organic manner), and hardness (Brix et al., 2017; Crémazy et al., 2017). Crémazy et al. (2017) observed that tolerance of juvenile rainbow trout to Cu was augmented when pH, hardness and concentration of DOM were increased.

Mortality of half-hatched embryos exposed to 20 µg/L of Cu was significant during hatching time; hence, the hatchability was significantly reduced compared to the control group. Hatching can be a very critical step for fishes. Throughout the embryonic stage, the chorion offers protection to embryos in a contaminated environment. However, during hatching this protection is no longer present and the larvae are in direct contact with the contaminated medium (Arukwe et al., 1997). One hypothesis is that copper may have an inhibitory effect on the activity of the chorionase enzyme, which degrades the inner area of the chorion to facilitate the hatching by mechanical and osmotic process (Johnson et al., 2007; Jezierska et al., 2009; Barjhoux *et al.*, 2012).

At the end of exposure, control larvae had accumulated 5.66 ± 0.13 µg/g Cu (d.w.). According to Shearer (1984), Cu accumulation in unexposed larvae of rainbow trout can vary between 2 and 7 µg/g Cu d.w. Results of Cu accumulation on unexposed larvae from Shearer (1984) and Hansen et al. (2002) are comparable to our results. Marr et al. (1996) revealed that swim-up fry rainbow trout exposed to 9 µg/L Cu accumulated 9.89 µg/g Cu after 20 days exposure and continue to accumulate up to 17.91 µg/g after 60 days exposure. This accumulation is higher than that obtained in our study after 3-weeks of exposure at 20 µg/L Cu. Several authors have suggested that copper accumulation is regulated by the depuration and the slow exchange capacity of metals with the surrounding medium (Carbonell and Tarazona, 1994; Marr et al., 1996). Despite the lethal effects observed, no significant changes on larvae biometry was reported at the end of exposure to 20 µg/L Cu. A decrease on total size of larvae was expected, since the process of metabolism and homeostasis consume energy and the growing rate of fish may be affected as explained by Marr et al. (1996). Several studies have shown a relationship between the metal accumulation and the total size of larvae when they are exposed to metals (Guo et al., 2016; Marr et al., 1996). However, our results seem to be consistent with the low Cu accumulation in larvae exposed to this condition. Regarding Vardy et al (2013) results about the differences in

tolerance according to the larval stage, we might presume that the lack of Cu accumulation in our larvae is probably link to the fact that, at the end of the 3-weeks Cu exposure, our larvae were still absorbing their yolk-sac and probably just starting to change to exogenous feeding. Swim-up rainbow trout larvae used in Marr et al. (1996) were fed and were more active leading to a larger exposure to Cu since more water is pumped through the gills. Since our larvae were not fed, there was no contamination or Cu accumulation by trophic way.

For larvae in control conditions, the most commonly observed malformations were edema, vertebral abnormalities and craniofacial anomalies. These kinds of malformations in control condition have already been noticed by several authors (Barjhoux et al., 2012; Jezierska et al., 2009; Witeska et al., 2010), and can be attributed to parental factors or laboratory culture conditions. In this study, exposure to 2 and 20 µg/L Cu showed a significant increase on malformed larvae compared to control larvae. Vertebral abnormalities (kyphosis, lordosis, scoliosis and C-shape form) were the most recorded among all observed deformities. In the literature, vertebral deformations are also the most observed malformations (Barjhoux et al., 2012; Jezierska et al., 2009; Witeska et al., 2010). For instance, 80% of malformations recorded on barbel larvae (*Barbus barbus*), after exposure to different concentrations of copper and lead were vertebral abnormalities (Ługowska and Kubik, 2011). Japanese medaka larvae (*Oryzias latipes*) showed 35 and 59 % of spinal deformities following an embryonic exposure to copper and cadmium spiked-sediment (Barjhoux et al., 2012). These defects can be caused by disturbances induced by metal exposure that may occur at early stages of development or difficulties encountered during a laborious hatching (Jezierska et al., 2009).

One aim of this work was to study the impact of copper on swimming behavior of larvae. Exposed larvae covered similar distances as control larvae and no significant difference was observed on travelled distance. The study of Haverroth et al. (2015) showed significant reduction on total distance traveled and restricted mobility on zebrafish (*Danio rerio*) exposed to 6 µg/L Cu after 24 hours. This can suggest that locomotor patterns were weakened (Haverroth et al., 2015). Atchinson et al. (1980) described the different ways by which metals can alter the locomotion on fish, either by the influence on the attraction or the evasion to a contaminated area, the impaired sensory perception on hyper or hypoactivity, or reducing the swimming performance.

The comet assay did not reveal any significant induction of DNA damage promoted by Cu exposure. However, several studies have reported DNA damage upon exposure to Cu or other metals (Barjhoux et al., 2012; Bopp et al., 2008; Canalejo et al., 2016; Oss et al., 2013; Santos et al., 2010). Barjhoux et al. (2012) demonstrated the genotoxic potential of Cu and Cd on *Japanese medaka* larvae after embryonic exposure to environmental sediments from contaminated sites of the Lot-Garonne-

Gironde continuum. In one study, a significant increase of micronuclei levels was also observed on European seabass (*Dicentrarchus labrax*) exposed to Cu (0.01-1 mg/L) for 96 h (Canalejo et al., 2016). However, at 24 h exposure, differences were only observed on fish exposed to the strongest Cu concentration (1 mg/L). Canalejo et al. (2016) also observed that micronuclei frequency was even more important for fish exposed to Cu than those exposed to As or Pb at the same concentrations. Environmental concentrations of Cu (3.2 to 128 µg/L) induced DNA strand breaks in a concentration-dependent manner in blood cells of mature male stickleback after exposure for 4-days (Santos et al., 2010). Oss et al. (2013) observed an increase of micronuclei levels after 30-days exposure to environmental concentrations of Cu (0, 25, 50 µg/L) on juvenile fat snook (*Centropomus parallelus*), and these differences did not change after 30-days of recovery. In our study comet assays were performed after 3-weeks exposure which could allow DNA repair system activation and efficient DNA damage repair. However, this is not in accordance to Oss et al. (2013) which did not observed recovery of micronuclei. On the other hand, we found an increase of very damaged cells counted as “hedgehog” in the group of 20 µg/L Cu. An increase of these cells may be explained by the generation of DNA strand breaks through the production of reactive oxygen species by Cu. These “hedgehog cells” were currently considered as apoptotic or necrotic cells (Barjhoux et al., 2012). However, some studies have evidenced that these hedgehog cells could be repaired and should not be considered as apoptotic cells (Lorenzo et al., 2013; Meintières et al., 2003). To determine the real nature of these cells, other analytical techniques would be required such as flow cytometry.

One way Cu exerts its toxicity is by the formation of reactive oxygen species (ROS), which at important levels can damage the cell through lipid peroxidation, protein denaturation and DNA damage (Romeó et al., 2000; Sevcikova et al., 2011; Vutukuru et al., 2006). Catalase (cat) and superoxide dismutase (sod) are two of the enzymes that protect cells from the oxidative damage caused by ROS. Sod catalyzes the dismutation of the superoxide anion radical into oxygen and hydrogen peroxide, which could be further decomposed by the *cat* into water and oxygen. In our study, after a sub-chronic exposure to Cu, RT-PCR results for *cat* expression did not reveal any significant modulation on rainbow trout larvae. Woo et al. (2009) observed an important induction of *cat* gene in liver when Javanese medaka (*Oryzias javanicus*) was exposed to different concentrations of Cu, Cd, and Zn for 48 h. In the study of Casanova et al. (2013) *cat* expression on tambaqui fish liver increased after 1h of exposure to copper and decreased to basal levels at 3 h of exposure. The expression level of cytoplasmic *sod* was decreased on larvae exposed to 2 µg/L of Cu when it was compared to control group. No significant induction of *sod* expression was observed on liver of Javanese medaka (Woo et al., 2009) and zebrafish (Craig et al., 2007) after exposure to Cu. However, Craig et al. (2007) stated an important induction on *sod* and *cat* genes expression on liver when zebrafish was under protective treatment of 10 mM Na^+

plus Cu. Activation and deactivation of the antioxidant defense system are determined by the duration and the intensity of the stress applied to the organism (Casanova et al., 2013).

Larvae exposed to copper conditions exhibited a significant repression of genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest and apoptosis (*p53* and *bax*). Regarding detoxification genes, it was surprising since, after 21 days of Cu exposure, it was expected an induction, in particular of those genes encoding the metallothioneins (*mt1* and *mt2*). Metallothionein (MTs) proteins have the capacity to bind xenobiotic heavy metals through their thiol groups providing protection against metal toxicity and oxidative stress. Indeed, previous studies have demonstrated an induction of *mt1* and *mt2* genes when exposing fish to metals including Cu (Gonzalez et al., 2006; Olsson et al., 1989; Sheader et al., 2006; Woo et al., 2006). The study of Doering et al., (2015) also observed a down-regulation response of *mt* on liver explants from white sturgeon exposed for 24 h to Cu. Glutathione-s-transferase (gst) has an important role in detoxification of xenobiotic compounds, catalyzing the conjugation of reduced glutathione (GSH) to a variety of endogenous and exogenous electrophilic functional groups. Induction of *gst* gene expression has been reported in Javanese medaka liver after being exposed to different heavy metals (Cu, Ag, Cd, Ni, and Cr) and in European flounder (*Platichthys flesus*) liver exposed to Cd after 1 and 3 days of exposure (Sheader et al., 2006; Woo et al., 2009). However, we studied gene expression in whole trout larvae and not in a specific fish organ like mostly previous studies refer. Gene repressions of *mt1*, *mt2* and *gst* could be explained by different hypotheses. The first one, it is the high sensitivity that rainbow trout larvae have to Cu could lead to a significant repression of these genes after 3-weeks exposure at these concentrations of Cu. Certain studies had already observed the differential regulation of *mt* gene expression in fish exposed to metals like Cu and Cd (Minghetti et al., 2008; Tiwari et al., 2010). Differential expression of *mt* gene was observed in intestine, gill, kidney and liver of sea bream (*Sparus aurata*) after exposure to dietary (0.3-130 mg/kg) or waterborne (300 µg/L) copper for 30 days (Minghetti et al., 2008). Tiwari et al. (2010) studied the kinetics of *mt* expression on freshwater murrel (*Channa punctata*) after exposure to 3.74 mg/L of Cd indicating that the expression varied according to the time of exposure. At this study, Tiwari et al. (2010) observed induction of *mt* expression on liver and repressions on kidney and gill within the first 8 h of exposure, and induction on kidney and repressions of *mt* expression on liver and gill after 14 days of exposure. Both studies revealed that *mt* expression was tissue specific. Besides, rainbow trout larvae could also be more sensitive during the transition from endogenous yolk-sac reserve to the exogenous feeding as hypothesized by Vardy et al. (2013). Indeed, Vardy et al. (2013) found that later larval life stages of white sturgeon and rainbow trout were more sensitive to Cu than at yolk-sac stage. In our study, at the end of these 3-weeks of Cu exposure, rainbow trout larvae were probably starting to turn to exogenous feeding, and since their biological activity was increased more water passed

through the gills increasing their exposure to Cu (Vardy et al., 2013). Another hypothesis could be that larvae may have implemented other mechanisms of detoxification. Indeed, other proteins like ABC transporters have been previously described to be efficient for trace metal detoxification (Gonzalez et al, 2006).

Cell cycle arrest is one of the principal functions of p53 when it recognizes DNA damage (Verhaegh et al., 1997). Having a defective *p53* gene could lead to tumorigenesis and cancer. In our study, transcription of *p53* was repressed in both Cu conditions. According to several studies (Verhaegh et al., 1997; Sandrini et al., 2009) *p53* gene expression could be regulated by the activation of p53 protein. Therefore, a possible inactivation of p53 protein, after Cu exposure, could lead to the inactivation of *p53* gene. Additionally, the study of Verhaegh et al. (1997) reported the inactivation of p53 protein after direct interaction with copper on human cell line. Regarding the expression of *bax* gene, only a slight non-significant repression (p= 0.0656) was observed in larvae exposed to 20 µg/L of Cu. Interestingly, Sandrini et al. (2009) observed that *bax* gene was induced and *p53* gene was repressed on zebrafish liver cell line after 24 h of exposure to Cu. Concerning the DNA repair-related genes *ogg1* and *rad51*, we did not see any significant modulation. But since we did not observe genotoxicity on blood cells with the comet assay, a possible explanation is that DNA strand breaks may have been repaired prior the comet analysis at 21 days of Cu exposure.

Malondialdehyde (MDA), measured as TBARS (thiobarbituic acid reactive substances), is one of the end-products of lipid peroxidation and it is a biomarker of oxidative stress. ROS degrade polyunsaturated lipids forming MDA, which appears to be potentially mutagenic since it reacts with deoxyadenosine and deoxyguanosine, arising DNA adducts (Janero, 1990). In our study, both Cu concentrations did not increase MDA levels in larvae. However, several authors have reported the effects of high concentrations of metals and other xenobiotics on lipid peroxidation in aquatic organisms (Baker et al., 1998; Doyotte et al., 1997; Radi and Matkovics, 1988; Romeó et al., 2000; Thomas and Wofford, 1993; Vutukuru et al., 2006). For example, Vutukuru et al. (2006) showed an increase of lipid peroxidation in the viscera of *Esomus danricus* exposed to 5.5 mg/L of Cu. Baker et al. (1998) fed juvenile grey mullet (*Chelon labrosus*) with a high-Cu diet for 4 weeks and found a rise of 3-fold in hepatic MDA. Pandey et al., (2001) observed that *Channa punctatus* exposed to 10 mg/L of Cu for 4 weeks did not induced lipid peroxidation in liver, kidney and gill. Interestingly, Cu showed to have a protective role on lipid peroxidation in liver when fish were exposed to endosulfan for 24 h (Pandey et al., 2001).

Regarding the carbonyl protein content, non-significant increase was observed on larvae after 3 weeks of copper exposure. Protein carbonyl content is one of the most used biomarker of protein oxidation (Stadtman and Berlett, 1998) because of its irreversible alteration. Craig et al. (2007) observed

an induction of protein carbonyl levels in liver and gills after 48 h of exposure to 15 µg/L of Cu on zebrafish. Besides, Craig et al. (2007) also remarked the protective actions of Ca^{2+} and Na^{2+} ions in generation of protein carbonyl levels in gills and liver when they were added during Cu-treatment. Ransberry et al., (2015) alluded tissue-specific responses, since exposure of 50 and 200 µg/L of Cu on zebrafish induced protein carbonyls in intestines, but not in the gills and livers.

Cu is known to cause oxidative damage in lipids and proteins, with a potential to induce genotoxic effects. However, in our study no significant alterations were observed on lipid peroxidation, protein contents and DNA damage. We could hypothesize that the concentrations of Cu used in this study were not enough to cause oxidative alteration in lipid, protein and DNA of rainbow trout.

5. Conclusion

Copper is an essential trace element important for the metabolism of the body. However, at high concentrations it can interfere with certain processes such as protein synthesis and cellular damage. This study provides an extensive evaluation of Cu impact or early developmental stages of rainbow trout that includes phenotypic, behavioral, genetics and biochemical endpoints. Results revealed sub-lethal effects of copper on development mainly on embryos exposed to the higher copper concentration. Defects included increase of half-hatched embryos, spinal deformities, genotoxicity and deregulation of genes involved in detoxification and cell cycle arrest. Mortality was higher at hatching but larvae appeared to adapt to the contaminated condition. However, alteration on gene expression, primarily those involved in detoxification (gst, mt1 and mt2) could compromise the survival of this species under natural conditions, since environmentally realistic concentration of copper were used in this study.

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Article 2

A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, *Oncorhynchus mykiss*

Soumis dans Aquatic Toxicology

Shannon Weeks Santos, Patrice Gonzalez, Bettie Cormier, Nicolas Mazzella, Christelle Clérandeau, Bertille Bonnaud, Soizic Morin, Bénédicte Morin, Jérôme Cachot

Principaux résultats de l'Article 2

- *L'exposition au Roundup® n'a pas entraîné de mortalité significative chez les embryons et les larves de truite arc-en-ciel pendant toute la durée de l'exposition. Il n'a pas été observé d'augmentation significative des malformations chez les larves exposées à ce composé. Chez la lignée RTL-W1, les données de cytotoxicité obtenues ont permis de déterminer des CE50 de 730 et 710 mg/L pour le glyphosate et sa formulation commerciale le Roundup®.*

- *La taille de la tête des larves était significativement plus petite que celles exposées à 1 mg/L de glyphosate. Également, le rapport entre la taille de la tête et la longueur totale des larves montrait une diminution significative en fonction de la concentration d'exposition. Ces résultats démontrent qu'une exposition chronique au glyphosate à des stades précoces de développement peut induire une diminution de la croissance.*

- *Les larves exposées à 0,1 mg/L de glyphosate étaient plus actives sous stimulation lumineuse. Cela pourrait démontrer qu'une exposition à de faibles concentrations de glyphosate peut induire une hyperactivité chez les larves de truite arc-en-ciel.*

- *Dans nos conditions d'exposition, le glyphosate n'a induit aucun dommage à l'ADN sur les cellules sanguines des larves de truite. Cependant, l'exposition de cellules de truite RTL-W1 au glyphosate a induit des dommages oxydatifs à l'ADN détectées par le test des comètes après traitement enzymatique au Fpg. Cette contradiction pour s'expliquer par l'intervention du système antioxydant et de réparation de l'ADN dans les cellules sanguines des larves. La réduction des niveaux de TBARS et la répression de l'expression de gènes de cat et cox1 chez les larves exposées au glyphosate pourraient confirmer cette hypothèse.*

A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, *Oncorhynchus mykiss*

Shannon Weeks Santos, Patrice Gonzalez, Bettie Cormier, Nicolas Mazzella, Christelle Clérandeau, Bertille Bonnaud, Soizic Morin, Bénédicte Morin, Jérôme Cachot

Abstract

Most pesticides use in agriculture are finally found in the aquatic environment by the action of runoff and leaching of treated crops. One of the main use herbicide is glyphosate, and this compound or its metabolites are frequently detected in surface water in Europe. In the present study, *in vivo* and *in vitro* studies were carried out using the early life stages of rainbow trout (*Oncorhynchus mykiss*) and the cell line RTL-W1 (a liver cell line from rainbow trout) to characterize toxic effects of glyphosate at environmentally-realistic concentrations. Both studies were performed using the commercial formulation Roundup® GT Max, and technical-grade glyphosate for the *in vitro* study. Eyed-stage embryos were exposed for 3-weeks to sub-lethal concentrations (0.1 and 1 mg/L) of glyphosate using Roundup. Numerous toxicity endpoints were recorded such as survival, hatching success, larval biometry, developmental abnormalities, swimming activity, genotoxicity (formamidopyrimidine DNA-glycosylase Fpg-modified comet assay), lipid peroxidation (TBARS), protein carbonyls and gene expression. Both concentrations did not affect embryonic nor larval survival, and there was no significant increase of morphological abnormalities. However, a significant decrease was observed on head size of larvae exposed to 1 mg/L of glyphosate. A significant increase of mobility was observed for larvae exposed to the weakest concentration compared to control larvae. Remarkably, TBARS levels were significantly decreased on larvae exposed to 1 mg/L (a.i.), and *cat* and *cox1* gene were differently expressed from controls. DNA damage was detected by the Fpg-modified comet assay in RTL-W1 cell line exposed to the technical-grade glyphosate and Roundup formulation. The results suggest that sub-chronic exposure of glyphosate, at relatively environmental concentrations, represent a potential risk for aquatic organisms.

Keywords: glyphosate, rainbow trout embryo, cell line RTL-W1, biomarkers, cytotoxicity, genotoxicity, embryotoxicity.

1. Introduction

The increased use of herbicides has caused serious problems to the environment, including aquatic ecosystems, affecting the physiological balance of the organisms that are exposed. One of the most commonly used herbicide is the glyphosate-based herbicide, whose molecules, usually transported by agricultural runoff, are frequently detected in surface water at elevated concentrations (Peruzzo et al., 2008). Glyphosate is the active ingredient of Roundup® herbicide; and is commonly used in the form of salt of isopropylamine glyphosate. Glyphosate is a broad-spectrum, non-selective and systemic herbicide for the control of weeds and grasses, used in both agricultural and non-agricultural areas. Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), the shikimic acid pathway. This enzyme is responsible for the synthesis of aromatic amino-acids, and is common to all plants (Giesy et al., 2000; Myers et al., 2016). The main degradation products of glyphosate are aminomethyl phosphonic acid (AMPA) and CO_2 (Grandcoin et al., 2017).

Half-life of glyphosate has been determined in several studies, ranging from few days to 2 weeks in freshwater (Giesy et al., 2000). However, its dissipation depends on the local conditions regulated by chemical, physical and biological factors (Giesy et al., 2000). In a more recent study (Myers et al., 2016), it was stated that half-life of glyphosate in water could be longer than expected and it could last more than 60 days. In another study (Mercurio et al., 2014), half-life time of glyphosate in the marine water was determined to 47 days at 25°C in low-light, 267 days at 25°C in dark, and 315 days at 31°C in dark, demonstrating its persistence under low light conditions.

The environmental exposure to glyphosate is extensive, due to the vast quantities used annually all over the world (Van Bruggen et al., 2018). The increase use of glyphosate is closely related to the endorsement of genetically modified glyphosate-resistant crops (Van Bruggen et al., 2018), cultivated at about 100 million hectares in 22 countries (mostly soybean, maize, canola and cotton) (http://www.fao.org/docrep/015/i2490e/i2490e04d.pdf). This situation is particularly true in North and South America, where elevated glyphosate concentrations were found in different streams and lakes near agricultural basins. For instance, in the Pampa region (Argentina) glyphosate residues were detected up to 4.52 µg/L in surface water (Castro Berman et al., 2018). Similar levels of concentration were found in surface water of the Southeast of Buenos Aires, with values ranging from 0.5 to 7.6 µg/L (Aparicio et al., 2013). However, higher concentrations were detected in streams near transgenic soybean cultivation in Pergamino-Arrecifes (north of Buenos Aires), where levels of glyphosate in water varied from 100 to 700 µg/L (Peruzzo et al., 2008). Coupe et al., (2012) studied the fate of glyphosate in different agricultural basins in North America and France, and maximal concentrations of glyphosate and his main metabolite AMPA (value between brackets) were detected in Bogue Phalia basin

(Mississippi) up to 73 µg/L (28 µg/L), SFIR New Province (Iowa) with 290 µg/L (400 µg/L), Sugar Creek (Indiana) with 430 µg/L (29 µg/L), and Rouffach (France) with 86 µg/L (44 µg/L).

Numerous studies show its toxicity in several organisms (Guilherme et al., 2010; Lushchak et al., 2009; Tsui and Chu, 2003). However, lethal concentrations of glyphosate in aquatic organisms, such as fish, are generally higher than the concentrations found in streams following applications of crops (Folmar et al., 1979). Notwithstanding, commercial formulations of glyphosate are more toxic than the pure molecule due to interference such as polyethoxilene amine surfactant (POEA) (Folmar et al., 1979; Giesy et al., 2000; Navarro and Martinez, 2014; Tsui and Chu, 2003) which helps the active ingredient penetrate the plant surface. Since fish are susceptible to glyphosate exposure by direct uptake from waterborne by the gills and through their diet (Giesy et al., 2000), several works have studied the sub-lethal effects of glyphosate on different stages of fish. For example, Çavaş and Könen (2007) and Guilherme et al. (2012, 2010) studied the genotoxic potential of Roundup remarking significant levels of DNA strand breaks on erythrocytes of goldfish (*Carassius auratus*) and European eel (*Anguilla anguilla*) respectively. Acetylcholinesterase (AChE) inhibition has also been observed in piava (*Leporinus obtusidens*) after exposure to 1 and 5 mg/L of Roundup for 90 days (Salbego et al., 2010). Behavior has also been studied in the hybrid fish surubim (hybrid fish) (Sinhorin et al., 2014) and in zebrafish (*Danio rerio*) (Bridi et al., 2017) where swimming alterations were observed from 2.25mg/L and 0.01mg/L of glyphosate respectively. Moreover, Uren Webster et al., (2014) remarked that glyphosate had detrimental effects on reproductive health of zebrafish exposed to 10mg/L. In addition, formation of reactive oxygen species (ROS) has also been observed regarding biomarkers as lipid peroxidation and the antioxidant enzymes in different species of fish (de Moura et al., 2017; Glusczak et al., 2007; Harayashiki et al., 2013; Üner et al., 2006).

The use of rainbow trout fish (*Oncorhynchus mykiss*) in ecotoxicology is very well documented; and a few works have been done studying the toxicity of glyphosate in this species (Hildebrand et al., 1982; Morgan and Kiceniuk, 1992). However, no studies have been done examining the sub-lethal effects of glyphosate on early life stages (ELS) of rainbow trout. ELS of rainbow trout can be easily raised under laboratory conditions, and because of its slow embryo-larval development, toxicity tests allow longer sub-chronic exposure to toxicants.

In the other hand, the use of cell lines allows the screening of molecules, the study of the mode of action of chemicals and the toxicity assessment of complex environmental samples (Bols et al., 2005; Castaño et al., 2003). It is considered as an alternative to the use of whole fish assays (Pannetier et al., 2018) to determine the toxicity of pollutants and for the monitoring of environmental quality. For this work, a reference cell line of rainbow trout, RTL-W1 (Rainbow Trout Liver-Waterloo 1), was selected.

This cell line, developed by Lee et al. (1993), is derived from untransformed liver tissue of rainbow trout. The RTL-W1 line consists of adherent fibroblastic cells and has the ability to metabolize xenobiotics.

The aim of this work was to study effects and mechanisms of toxicity of glyphosate, using a commercial formulation Roundup, on rainbow trout, focusing on cell cultures and ELS and a wide range of endpoints. An embryo-larval exposure of rainbow trout was conducted using Roundup® for 3 weeks and several endpoints were studied such as viability, hatching success, biometric changes, locomotion, genotoxicity, lipid and protein oxidation, and gene expression. In addition, a reference cell line, RTL-W1, was implemented to screen the cytotoxicity using technical grade glyphosate and Roundup®.

2. Materials and methods

2.1. *In vivo* study: rainbow trout

2.1.1. Test chemicals

Preparation of glyphosate solutions was made using the commercial formulation of Roundup® GT Max. The active substance is 480 g/L of glyphosate acid, which is equivalent to 588 g/L of potassium salt of glyphosate. Two stock solutions were prepared at 0.1 and 1 g/L of glyphosate (active ingredient a.i.) with osmosis water. From these stock solutions, exposure solutions at 0.1 and 1 mg/L of glyphosate (a.i.) were prepared.

2.1.2. Exposure system

Eyed stage embryos from rainbow trout (*Oncorhynchus mykiss*) were obtained from INRA-PEIMA (Sizun, FR). Rainbow trouts were exposed to 0 (control), 0.1 and 1 mg/L of glyphosate (a.i.) in total darkness and with a temperature of 12°C in a climate chamber for 3 weeks. Each studied condition consisted in 3 replicates with 100 embryos in 1 L aquaria. Exposure solutions was prepared in three 5 L tanks of spring water from Laqueuille (4.7 mg/L Ca, 1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, pH 7.5, <1.2 mg/L Cl^-) and was renewed every two days. A peristaltic pump (Watson Marlow, USA) was used to maintain a continuous flow rate of water (9 mL/min) into the incubation aquaria. Dissolved oxygen concentration was measured each day with a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburf, Germany) and data was recorded with OxyView v6.02 software (PreSens Precision Sensor).

2.1.3. Chemical analysis in water

Water samples were analyzed to determine glyphosate concentration and its main metabolite aminomethylphosphonic acid (AMPA). Water samples were collected at T_0 and T_{48} (48 h after exposure and before water was changed). Water samples were analyzed to determine glyphosate concentration and its main metabolite aminomethylphosphonic acid (AMPA) using the method described by Fauvelle et al. (2015). Briefly, 5 mL of each samples were spiked with 150 µL of glyphosate and AMPA 13C 15N at 20 ng mL^{-1}. Then, 325 µL of 50 mM borate-Na solution and 200 µL EDTA-Na_2 200 mM were added. After homogenization, solutions were left for 5 minutes, 4.5 mL of acetonitrile and 600 µL of FMOC-Cl (50 mg mL^{-1}) were added and samples were left in dark for 30 minutes in order to form FMOC derivates. Acetonitrile was evaporated under nitrogen flow until the volume was below 5 mL. Then, a liquid-liquid extraction with 1.5 mL of ethyl acetate was performed three times. Ethyl acetate was evaporated under nitrogen flow for 15 minutes. One hundred µL of formic acid were added and the samples volume was adjusted to 5 mL. Then, a solid phase extraction was performed on OASIS HLB cartridges (3 mL, 60 mg, 30 µ m particle size, Waters) conditioned with 1 mL of MeOH and 1 mL of formic acid 0.1 %. Samples were loaded on cartridges and the cartridges were rinsed with 1 mL of formic acid 0.1 % and 1 mL deionized water. The cartridges were dried under nitrogen flow before elution with 2 mL of ammonium hydroxide/deionized water/MeOH 2/30/68. Extracts were evaporated under nitrogen flow until stabilization volume (0.5 mL). The volume was adjusted to 1 mL with deionized water. Analyses was performed by HPLC-ESI MSMS.

2.1.4. Embryo-toxicity assay

Viability of embryos and larvae was recorded daily and dead specimens were removed immediately. Half-hatched embryos were considered when part of the body was inside the corium. Embryonic or larval mortality was the number of dead individuals compared to the total number of embryos at the start of the experiment or total number of hatching larvae. Half-hatched embryo rate was calculated by dividing the number of half-hatched embryos by the total number of embryos at the beginning of the experiment. Hatching time express in degree days (DD) was the duration of embryonic development from fertilization to hatching. At the end of the experiment, larvae were placed in Petri dish with carbonated water and ice to sedate them and photos were taken for each larva with a stereomicroscope (MZ 7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). From the photos, total body length and head length were measured for each larva. The presence of developmental anomalies (including edemas, yolk-sac absorption, spinal

malformations, craniofacial anomalies, presence of hemorrhages) was recorded in 15 larvae per replicate randomly chosen.

2.1.5. Swimming behavior analysis

The swimming behavior analysis was carried out on 12 larvae per replicate at 528 DD. The larvae were acclimated individually 30 minutes in the dark at 12°C in 6-well microplates containing 8 mL of exposure solution. The microplates were placed in the recording chamber (Daniovision Image Analysis System with Ethovision software version 12.0 Noldus) connected to a thermoregulation system set at 12 ± 0.5°C (Pilot one®, Huber). Larvae were subjected to a light/dark cycle of 30 minutes divided in 10 minutes dark, 10 minutes light, and 10 minutes dark. This cycle is designed to analyze the photomotor response of larvae in response to light stimulation. An infrared camera in the recording chamber records the movement of each larva focusing on their center of gravity. The average velocity of each larva was calculated over 30 seconds. The total distance traveled, time of mobility and the time spent in the peripheral area of the wells were determined for each larva.

2.1.6. Biochemical analyses

Preparation of supernatant

At the end of the exposure, 5 pools of 2 larvae were frozen in liquid nitrogen and stored at -80°C until analysis. Larvae (approximately 250 mg) were homogenized in a phosphate buffer (0.1 M; pH 7.5; 4°C) using an UltraTurrax® tissue homogenizer fitted with a potter at 3,000 rpm (4°C). Then, samples were centrifuged at 9,000 g for 25 min at 4°C. The supernatant S9 fraction obtained were placed in different tubes for total protein, TBARS and protein carbonyl measurements.

Total protein

The total protein concentration was determined following the method of Lowry et al. (1951) on S9 fraction. Bovine Serum Albumin (BSA) was used as a standard. Measurements were performed using a spectrophotometer microplate reader (Synergy HT, BioTek).

Lipid peroxidation (TBARS)

Lipid peroxidation was assessed following the method of Buege and Aust (1978) adapted to a microplate reader. Five hundred µL of S9 fraction was added to 500 µL of a solution containing 20 % of butylated hydroxytoluene (BHT) and 20 % of trichloroacetic acid (TCA). Then, the mixture was centrifuged for

10 min at 9,000 g. Afterwards, 600 µL of supernatant was added to 480 µL of TRISbase (25 mM) - TBA (thiobarbituric acid – 100 mM) and 120 µL of 0.6N HCl and heated at 80°C for 15 min. Then, the mixtures were cooled and mixed. TBARS levels were read using a UV-spectrophotometer (Synergy HT, BioTek) in a microplate at 530 nm. Results were expressed as nmoles of thiobarbituric acid reactive substance (TBARS) equivalents/ mg of protein.

Carbonylated protein analysis

Carbonylated protein content was measured using the method described in Augustyniak et al. (2015). 50 µL of 11 % streptomycin sulfate – phosphate buffer (100 mM pH 7.4) was added to 500 µL of S9 fraction, mixed and incubated for 15 min at room temperature. Then, the mixture was centrifuged for 10 min at 6,000 g. Afterwards, supernatant was divided in two tubes (200 µl each) where 200 µL of supernatant was added to 800 µL of HCl 2.5 M used as a control tube, and 200 µL of supernatant was added to 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 Mm) used as a sample tube. Subsequently, the mixture was incubated for 1 h at room temperature with vortexing every 15 min. Proteins were precipitated with 1 mL of 20 % TCA (trichloroacetic acid), vortexed and centrifuged for 10 min at 10,000 g. The pellets were rinsed with 1 mL of ethanol-ethyl acetate (v:v), vortexed and centrifuged three times. Then pellets were solubilized with 500 µL of 6 M guanidine HCl and centrifuged at 10,000 g for 10 min. The carbonyl content was measured using a UV-spectrophotometer (Biotek Synergy HT) at 370 nm. Results were expressed as nmoles of DNPH incorporated/mg protein.

2.1.7. Gene expression

Six larvae per replicate were collected in a storage buffer (RNA later, Qiagen). Samples were frozen in liquid nitrogen and stored at -80°C until analysis.

RNA extraction

Total RNA extraction from whole larvae was performed with the kit “SV Total RNA Isolation system” (Promega) following the supplier’s recommendations. Larvae were homogenized using the MP fastprep®-24 (Biorad, 6 m/s, 40s) with 14.4 mm diameter ceramics beads (MP Biomedicals, Lysing Matrix D Bulk).

Retro-transcription of total RNA into cDNA

The retro-transcription of total purified RNA was realized with the kit “GoScript Reverse Transcription System” (Promega). The reverse transcription was performed using 1 µL of oligodT (1 µM), 1 µL of hexanucleotides (1 µM) and 10 µL of total purified RNA (1 µg). Then, the mixture was heated at 70°C for

5 min with a thermocycler (Eppendorf Mastercycler) followed by 5 min at 4°C to allow primer annealing. Subsequently, 1 µL of dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of $MgCl_2$ (25 mM), 1 µL or reverse transcriptase (1 U/µL) and 0.5 µL or RNAsine were added. The reverse transcription reaction was performed for 1 h at 42°C in a thermocycler. The cDNA thus obtained were stored at -20°C pending analysis by quantitative real-time PCR reaction.

Quantitative real-time PCR

Twelve genes were selected and specific primer-pairs were designed with primer3plus software (Table 1). Real-time qPCR was carried out using GoTaq® qPCR Master Mix kit (Promega). PCR reaction mix contained 17 µL of a mixture of Nuclease-Free Water and GoTaq® qPCR Master Mix (2X) plus 2 µL of specific primer pairs mix (200 µM each) and 1 µL of cDNA. Real-time quantitative PCR reactions were performed in a Mx3000P® qPCR System (Stratagene). The amplification program consisted in one cycle at 95°C for 10 min and then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity of amplifications was determined for each reaction from the dissociation curve of the PCR products. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C.

Cycle thresholds (Ct) were obtained from MxPro™ qPCR software for each gene. Two different housekeeping genes were used for standardization (*rpl7* and *ef1α*) and were found to be stable in our conditions. Consequently, relative quantification of each gene expression level was normalized according to the mean Ct value of these two reference genes and using the 2ΔCt methods (Livak and Schmittgen, 2001). The expression factor (induction if >2 and repression if <0.5) of each gene was calculated for each condition by dividing the transcription level of exposed individuals by control ones.

Table 1: Accession number and specific primer pairs for the Oncorhynchus mykiss used in our study.

Gene	Accession number	Primer (5' – 3')
rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
ef1α	NM_001124339.1	ATGGGCTGGTTCAAGGGATG[a] GATCATACCGGCCTTCAGGG[b]
cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG[a] GTCCAGGATGGGAAGTTGC[b]
sod	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
gst	BT073173.1	ATTTTGGGACGGGCTGACA[a] CCTGGTGCTCTGCTCCAGT[b]
er-b	AJ242741	AGCCCTCTCCTCCACCCTACCA[a] ACAGCTGGCTGAGGAGGAGTT[b]
cox1	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12s	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
cyp19a1	XM_021598638	CTCTCCTCTCATACCTCAGGTT[a] AGAGGAACTGCTGAGTATGAAT[b]

[a]Forward primer

[b]Reverse primer

2.2. *In vitro* study: RTL-W1

2.2.1. Cell exposure

The RTL-W1 cell line was obtained from rainbow trout liver (Lee et al., 1993). For cell culture, L15 Leibovitz medium supplemented with 5 % FBS (Fetal Bovine Serum) and 1 % Penicillin/Streptomycin (100 IU/mL) was used. The cells were kept in polypropylene flasks of 75 cm^2 (Cell start® cell culture Flask Greiner) at 20 °C. The analysis was carried out with cells aged from passage from 65-72.

The cytotoxicity and genotoxicity test were carried out in 96- and 24-well polypropylene microplates, respectively. For both MTT and comet assay, cell lines were seeded 24 h prior glyphosate exposure in triplicate. Cell density was 200 000 cells/mL. For MTT assay, cell lines were contaminated to concentrations from 0.05 to 1000 mg/L of glyphosate for 24 h, using both technical and commercial formulation Roundup®. For comet assay, the concentrations tested were the same studied in our *in vivo* study (0.1 and 1mg/L of glyphosate) using technical and commercial formulation of glyphosate.

2.2.2. MTT assay for cytotoxicity evaluation

Cytotoxicity test was performed using serum free L15 medium containing 10 % of 3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). 24 h after chemical exposure, the medium was removed and cells were rinsed with PBS. 100 µL of the MTT solution was added to the wells. After incubation of 1 h in the dark (time to allow cells to reduce tetrazolium to formazan), the MTT solution was removed and 100 µL of isopropanol solution (4% 1N HCl) was added. Then the microplate was shaken horizontally in the dark for 15 min to dissolve the formazan crystals. Following this step, the formazan coloration was quantified in a Bio-Tek Synergy HT spectrophotometer at 570 nm.

2.3. Genotoxicity test

The alkaline comet assay was performed following Le Bihanic et al. (2014) and Weeks et al. (2019) in blood cells of larvae, and RTL-W1 cell line following Pannetier et al., (2018). Blood sampling was performed in 6 larvae per replicate (previously anesthetized with ice water and few drops of carbonated water) by decapitation using a heparinized pipette. Samples were stored in microtube with 200 µL of cryopreservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) and immediately frozen in liquid nitrogen until analysis. The comet assay for the RTL-W1 cell line (1.5 to 2 x 10^5 cells/mL) was performed 24 h after cell exposure to glyphosate. For each condition, 4 replicates were prepared. The cells were rinsed, trypsinized and transferred into

microtubes. The cells were then centrifuged (5 min, 20°C at 1000 rpm) and suspended in 100 µL of L15 medium (without FBS) before being mixed with 200 µL of low melting point agarose (0.75% LMPA). 50 µL of cell suspension (blood and cell line) were deposited on slides previously coated with NMPA (Normal Melting Point Agarose, 0.8% w/w) and covered with an 18x18 mm coverslip. The slides were then immersed in a lysis solution (10 mM Tris; 2.5 M NaCl; 100 mM EDTA; 1% Triton X-100; 10% DMSO; pH adjusted to 10 with NaOH) at 4°C for 90 min in the dark. At the end of the lysis, the slides were then rinsed 3 times for 5 min in an enzyme buffer at pH 8 (Biolabs, Evry, France). Then the slides were immersed for 30 minutes in hellendahls, one containing 60 mL of buffer with 12 µL of the enzyme Fpg (Biolabs, Evry, France) diluted in 1/5000, and the other with only buffer. Following the exposure to enzyme, the slides were incubated in an alkaline solution (0.3 M NaOH, 1 mM EDTA, pH> 13) at 4°C for 40 minutes for the cell line and 20 minutes for blood cells to allow the DNA to unwind. Electrophoresis was then performed in the same solution with a voltage of 25 V and 300 mA for 20 min. The slides were rinsed 3 times with neutralizing solution (0.4 M Tris, pH 7.5) for 5 min at 4°C. Afterwards, the slides were dehydrated in absolute ethanol for 20 min and then allowed to dry at a room temperature for at least 12 h. Slides were stained with 20 µg/mL of ethidium bromide solution and covered with a 22x22 mm coverslip. Comet lecture was realized using an epifluorescence microscope (Olympus BX51) (zoom x20) equipped with an Olympus U-RFL-T reflected fluorescence system lamp. The comets were quantified using the Comet Assay IV software (Instrument Perspective LtD). Results are expressed as percentage of degradation of DNA tail for 100 randomly selected nuclei per slide.

2.4. Statistics

Each studied condition was performed in triplicate and each replicate was considered as an independent sample. All data are expressed by the mean ± SE (Standard Error). For the MTT test, the EC_{50} was calculated by PRISM 5 software (GraphPad software, California, USA). Statistical analyzes were carried out using R (http://cran.r-projet.org/). The Normality of data distribution was verified on the residues by the Shapiro-Wilk test ($p < 0.01$) and the homogeneity of variances was evaluated by the Levene test ($p < 0.05$). In the case of normal distribution, a one-way ANOVA analysis was used ($p < 0.05$) followed by a Tukey post-hoc test. In the case that data was not normal, comparisons were carried out by non-parametric tests of Kruskal-Wallis ($p < 0.05$).

3. Results

3.1. Exposure conditions

Table 2 shows the concentrations of glyphosate in water for each experimental conditions. The analyses were carried out at 0 and 48 hours after exposure to estimate the possible losses of the molecule. The measured concentrations of glyphosate were comparable (± 20 %) to the nominal concentrations. No concentration variation was noted during 48h. The glyphosate's metabolites, aminomethylphosphonic acid (AMPA) were also analyzed but not detected at T0 and T48.

Table 2: Measured concentration of glyphosate in the exposure water for each studied condition.

Nominal concentration of glyphosate (mg/L)	Measured concentration (mg/L)	
		Glyphosate
0.0[1]	T0	0.0 ± 0.0
	T48	0.0 ± 0,0
0.1[2]	T0	0.12 ± 0.0
	T48	0.12 ± 0.01
1.0[3]	T0	1.22 ± 0.01
	T48	1.22 ± 0.01

[1]Limit of quantification: 30 ng/L

[2]Limit of quantification: 600 ng/L

[3]Limit of quantification: 6000 ng/L

3.2. Embryonic and larval survival

Dissolved oxygen in the exposure water varied between 83.8 and 93% throughout the duration of this study. Exposure to 0.1 and 1 mg/L of glyphosate (a.i.) did not induce significant mortality in trout embryos and larvae throughout the duration of exposure (table 3). Both, embryonic and larval survival, were greater than 90 % in all studied conditions. All embryos succeeded to hatch. The duration of development was slightly longer for both groups exposed to glyphosate compared to control, however no significant differences were observed.

Table 3: Effects on viability and development of rainbow trout during glyphosate exposure. Values represent Mean ± SD (N = 3). The results show no significant difference.

	Control	0.1 mg glyphosate/L	1 mg glyphosate/L
Embryonic viability (%)	96.3 ± 2.1	95.3 ± 3.8	95.3 ± 3.5
Larval viability (%)	91.9 ± 3.4	93.2 ± 3.6	92.2 ± 6.2
Cumulative viability (%)	88.6 ± 5.2	88.8 ± 3.6	88.0 ± 8.6
Hatching rate (%)	99.0 ± 0.02	97.6 ± 2.4	95.8 ± 1.8
Duration of development (DD)	307.9 ± 4.4	311.4± 3.1	314.0± 6.8

3.3. Biometry

No significant differences were observed in total larval length between studied conditions and control (Figure 1-A). Nevertheless, measurement of head size of larvae showed significant decreases on larvae exposed to 1 mg/L of glyphosate compared to control (Figure 1-B). Head size in unexposed larvae was 4.76 ± 0.04 mm against 4.55 ± 0.11 mm for larvae exposed to 0.1 mg/L of glyphosate, and 4.43 ± 0.14 mm on larvae exposed to 1 mg/L of glyphosate. The ratio between total length and head size (Figure 1- C) showed a significant dose-dependant decrease from control (24.79 ± 0.14 %) and larvae exposed to 0.1 mg/L of glyphosate (24.14 ± 0.27 %) and 1mg/L of glyphosate (23.37 ± 0.3%).

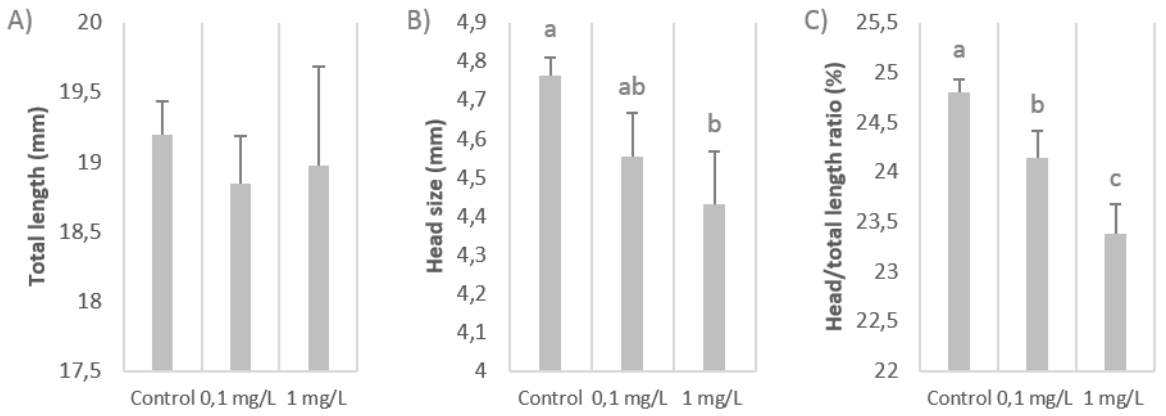

Figure 1: Biometric analyzes of larvae after exposure to 0.1 and 1 mg/L of glyphosate. (A) total body length (mm), (B) head length of larvae (mm) and (C) ratio of head size to total length of larvae (%) are showed. Different letters indicate significant differences between conditions (Mean ± SD N = 3, ANOVA, $p < 0.05$).

3.4. Malformations

Embryo–larval exposure to glyphosate did not result in significant induction of malformation when compared to non-exposed larvae. Control condition presented 13.3 ± 6.7 % of malformed larvae. However, larvae exposed to 1 mg/L of glyphosate (a.i.) showed a significant increase of developmental anomalies over larvae exposed to 0.1 mg/L of glyphosate (a.i.) with 8.9 ± 3.8 % and 26.7 ± 6.7 % respectively (Figure 2).

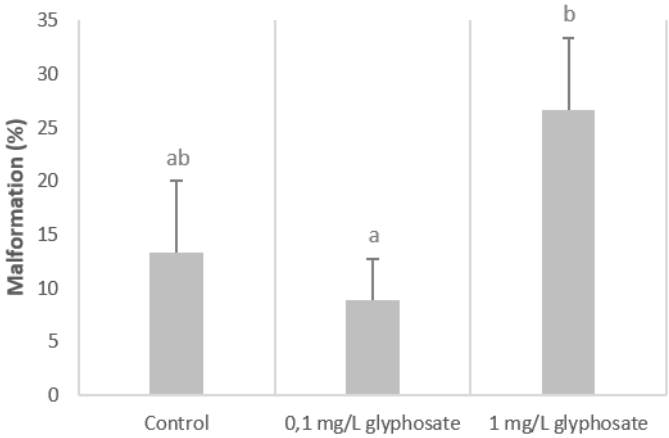

Figure 2: Percentage of malformed rainbow trout larvae after 21 days of exposure to glyphosate. Different letters indicate significant differences (Mean ± SD, N = 3, ANOVA, $p<0.05$).

3.5. Swimming behavior

Figure 3 shows the responses of larvae to light stimulation. Results represent the average speed of larvae exposed to glyphosate with alternating periods of luminosity. Under each condition, the same tendency was observed with an increase in larval velocity during the light period. No significant differences were observed for both periods of darkness when comparing the different treatments. However, when the light was turn on, the stress caused an increase in the average speed of the larvae exposed to 0.1 mg/L of glyphosate (120.7 ± 10.3 mm/s) when compared to control and larvae exposed to 1 mg/L of glyphosate (86.9 ± 7.6 and 82.6 ± 4.1 mm/s respectively).

Figure 4 shows the average cumulative time of immobility, mobility and high mobility for larvae exposed to both glyphosate conditions and control. Larvae exposed to 0.1 mg/L of glyphosate were significantly highly mobile (8.04 ± 1.25 s) in the light period when it was compared to control (4.72 ± 0.63 s) and larvae exposed to 1 mg/L of glyphosate (4.19 ± 0.38 s).

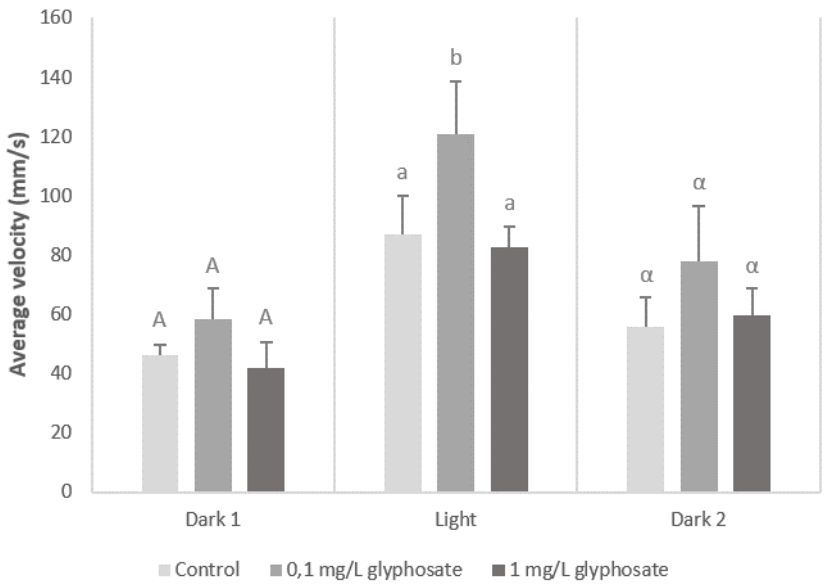

Figure 3: Mean velocity (mm/s) of larvae exposed to glyphosate after a light stimulation. Different letters indicate significant differences for each period of illumination (Mean ± SD N = 3, ANOVA, $p < 0.05$).

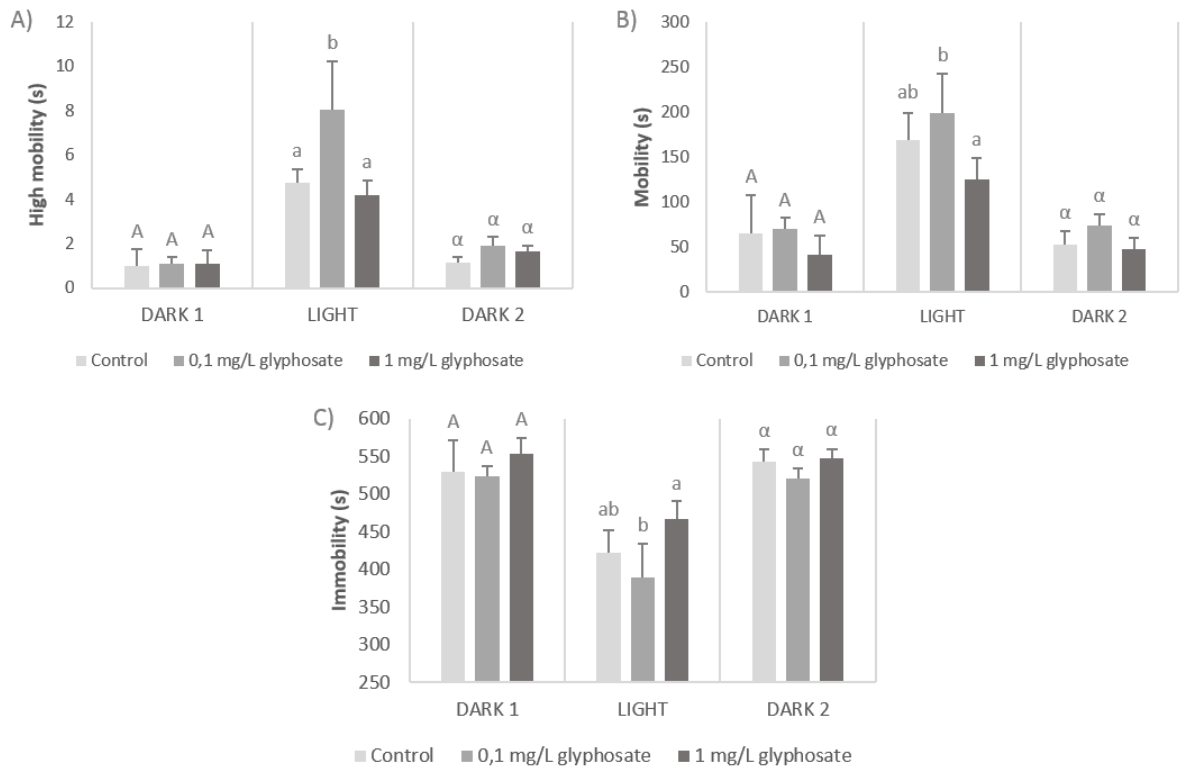

Figure 4: Cumulative time of high mobility (a); mobility (b); and immobility (c) on larvae exposed to glyphosate. Different letters indicate significant differences between each period of time (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.6. Genotoxicity

The average damage of DNA for each studied condition, with- and without treatment by Fpg is presented in Figure 5. No significant differences were observed in DNA damage in all conditions when cells were not treated with Fpg enzyme (6.85 ± 2.11 % for control, 8.52 ± 2.33 % for 0.1 mg/L of glyphosate condition, and 7.28 ± 1.69 % for 1 mg/L of glyphosate condition). A global increase of DNA damage was observed after Fpg treatment but no significant differences were observed between conditions (20.86 ± 3.73 % for control condition, 22.37 ± 2.12 % for larvae exposed to 0.1 mg/L of glyphosate and 19.88 ± 1.02 % for larvae exposed to 1 mg/L of glyphosate).

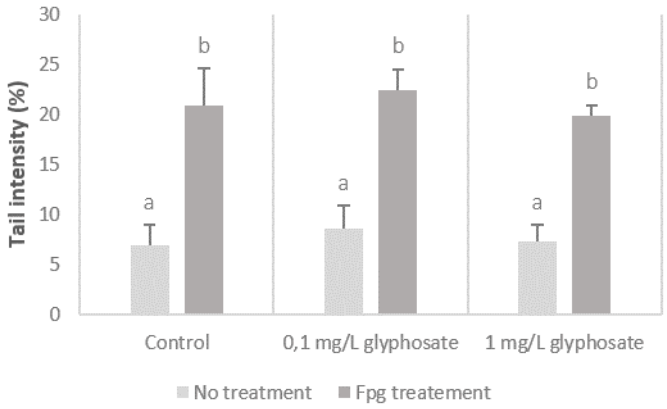

Figure 5: DNA damage in blood cells from rainbow trout larvae after exposure to 0.1 and 1 mg/L of glyphosate, with- and without addition of enzymatic Fpg treatment. Different letters indicate significant differences between treatments (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.7. Lipid peroxidation (TBARS) and protein carbonyls

TBARS levels showed a significant reduction in larvae exposed to 0.1mg/L of glyphosate when compared to control (figure 6-A). In the other hand, larvae exposed to glyphosate did not show any significant changes in protein carbonyls (figure 6-B).

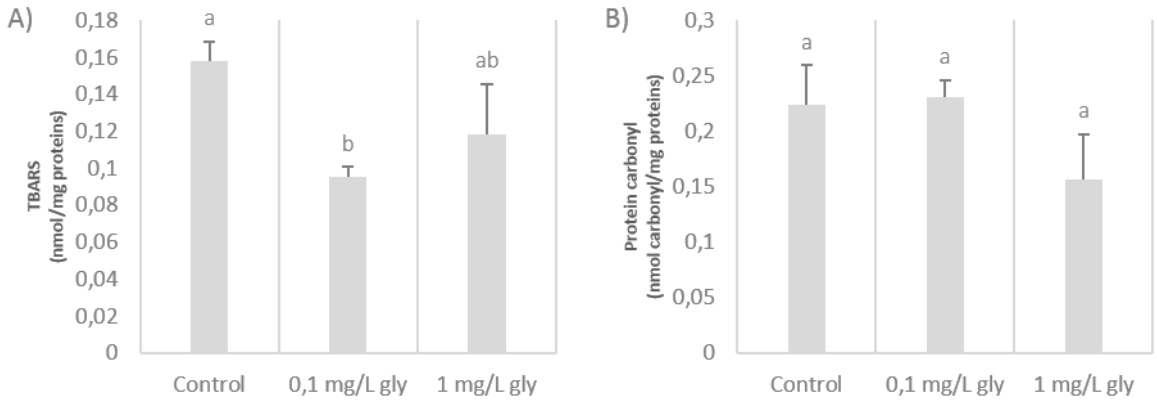

Figure 6: Lipid peroxidation (A) expressed as nanomoles of TBARS/mg of protein and protein carbonyls (B) expressed as nanomoles of carbonyl/mg of protein in rainbow trout exposed to glyphosate. Different letters represent significant differences. All values are expressed as Mean ± SE, N=3, ANOVA.

1.1. Gene expression

After 3-week exposure of rainbow trout to glyphosate, only few significant changes were observed on gene expression on larvae exposed to 1 mg/L. *Cox1* gene was significantly down-regulated when *cat* gene level was increased. The expression of *sod*, *gst*, *ERb*, *12s*, *ogg1*, *rad51*, *bax* and *Arom* were not significantly differentially regulated following glyphosate exposure (table 5).

Table 4: Transcriptional expression levels in rainbow trout larvae exposed to 0.1 and 1 mg/L of glyphosate. Gene expression values were normalized to housekeeping gene Ribosomal Protein L7 (rpL7) and Elongation Factor 1α (ef1α). Data was expressed in comparison to the control treatment as an induction (above 2) or repression (below 0.5) factors. Asterisks refer to significant difference with the control treatment (n=3, ANOVA, $p<0.05$).

Condition	cat	sod	gst	erb	cox1	12S	ogg1	rad51	bax	arom
0.1 mg/L glyphosate	/	/	/	/	/	/	/	/	/	/
1 mg/L glyphosate	0.22*	/	/	/	2.13*	/	/	/	/	/

3.8. Cytotoxicity

The cytotoxicity data for glyphosate and Roundup® (a.i.) was obtained using the MTT assay on RTL-W1 (Figure 7). Cytotoxicity was observed only at concentrations above 250 mg/L of glyphosate, and 200 mg/L of Roundup® (a.i.). The EC_{50} calculated at 24 h was 730 and 710mg/L for glyphosate and Roundup® (a.i.), respectively.

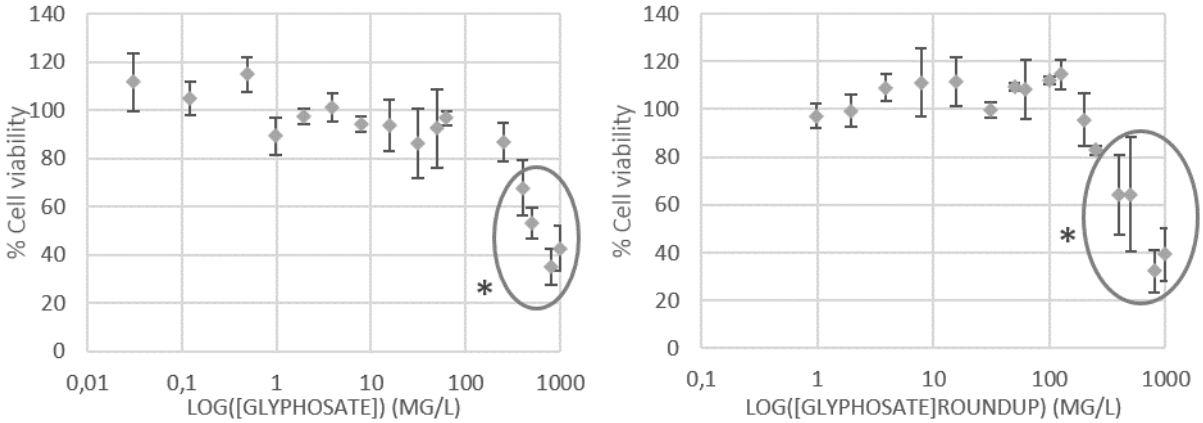

Figure 7: Comparative cytotoxicity of glyphosate (A) and Roundup (B) on the RTL-W1 cell line after 24 h of exposure. Asterisks represent significant differences compared to control. Values represent Mean ± SE. (N=3, Kruskal-Wallis, $p<0.05$).

3.9. Genotoxicity

With the standard comet assay, no genotoxic effect was detected after exposure to both glyphosate and Roundup® whatever the tested concentrations. However, with the modified Fpg assay, significant genotoxic effects compared to the control (18.07 ± 2.87 % damage) were observed as early as the lowest concentration of glyphosate tested (33.64 ± 3.11 % DNA damage) and Roundup (a.i.) (26.17 ± 2.05 %). No significant differences between both concentrations tested were observed (Figure 8).

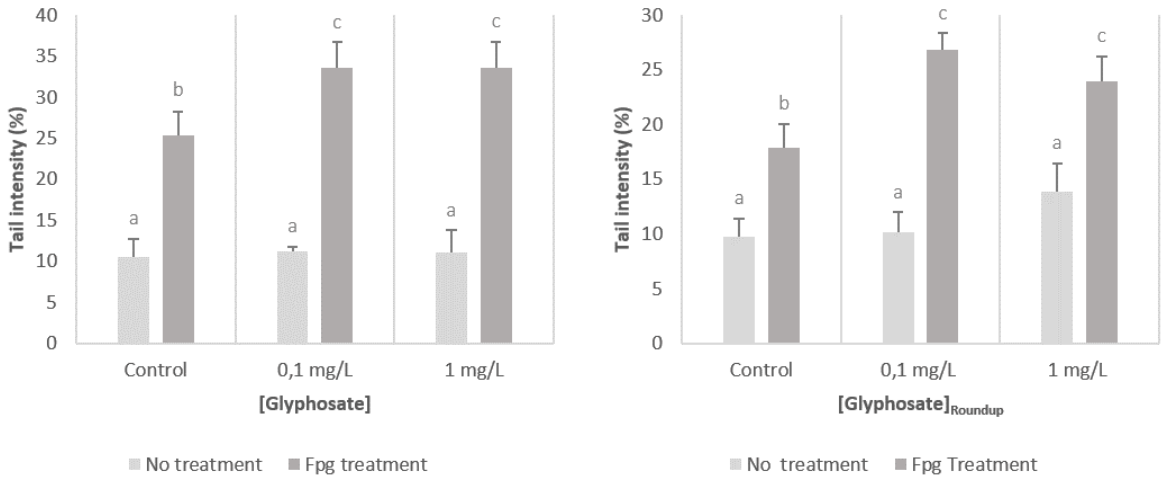

Figure 8: DNA damage in RTL-W1 cell line induced by glyphosate (A) and Roundup (B) measured by the comet assay with and without Fpg treatment. Values represent Mean ± SE. Different letters indicate significant differences. N=3, Kruskal-Wallis ($p<0.05$).

4. Conclusion

According to the World Health Organization (WHO, 1996), the acute toxicity of Roundup is considered to be low in vertebrates. Because of its widespread use, and its slow degradation, this herbicide is often found in aquatic environments at relatively high concentrations (Vera et al., 2010) and thus could represent a threat for pollutant sensitive species or early developmental stages. Several authors have studied the acute toxicity of glyphosate on ELS, fingerlings and adults of rainbow trout (Folmar et al., 1979; Hildebrand et al.,1982; Morgan and Kiceniuk, 1992; Anton et al., 1994). 96h LC_{50} for rainbow trout embryos and larvae was estimated to 16 and 3.4 mg/L glyphosate (a.i.) respectively (Folmar et al., 1979). However, acute toxicity varies according to the commercial formulation. For example, 96h LC_{50} on rainbow trout fingerlings was estimated to be 54.8 mg/L using Roundup® formulation (Hildebrand et al., 1982); and 10.42 mg/L using Vision formulation (Morgan and Kiceniuk, 1992). The work of Yusof et al. (2014) focused on glyphosate toxicity on Java medaka. Their results showed that 50% of embryos exposed to 100 mg/L of glyphosate died after 16 days of exposure, and a decrease on hatching rate in a concentration-dependent manner from 100 to 500 mg/L of glyphosate.

The *in vitro* study analyzed the toxicity of glyphosate using the rainbow trout liver cell line (RTL-W1) considering technical grade glyphosate and its commercial formulation Roundup. The toxicity test carried out on trout liver cells may provide additional information about the toxicity mechanistic of pollutants (Bols et al., 2005; Castaño et al., 2003). Besides, we can consider the RTL-W1 cell line as a suitable model considering that liver is the main organ of metabolism of pollutants (Belpaeme et al., 1998). The results obtained on RTL-W1 in this study highlights the cytotoxic effects of glyphosate but at

high concentrations above 200 mg/L. The cytotoxicity, revealed by the MTT test, indicates that the commercial formulation is slightly more toxic than the technical grade compound, which could be related to the presence of additives and especially surfactants (POEA) in the commercial formulation. Similar studies on human cell lines have shown that glyphosate-based formulations, are usually more cytotoxic that the technical grade compound (Gasnier et al., 2009; Koller et al., 2012; Martínez et al., 2007; Mesnage et al., 2013; Vanlaeys et al., 2018). In addition, the study of Gasnier et al. (2009) evidenced that the concentration of glyphosate in the commercial formulation is not related to toxicity. Indeed, the formulation containing 400 g/L of glyphosate (a.i.) (Grands Travaux®, homologation 8800425) has a lower LC_{50} than its homolog containing 450 g/L (Grands Travaux plus®, homologation 2020448) confirming that the nature and concentration of adjuvants has a real impact on the toxicity of the mixture. Very few studies have been done on fish cell lines regarding the toxic effects of glyphosate. The LC_{50} of glyphosate on diploid and triploid fin cell lines from *Misgurnus anguillicaudatus* (DIMF and TRMF) were 315.34 and 371.77 mg/L respectively (Qin et al., 2017). Cytotoxicity of Roundup was also studied on zebrafish cell line ZF-L regarding the integrity of the plasma membrane, mitochondrial activity and lysosomal integrity finding significant reductions on cell viability from 67.7 µg/L (a.i.) (Goulart et al., 2015). LC_{50} of mononuclear blood cells was determined at 56.4 mg/L for Roundup, and 1630 mg/L for technical grade glyphosate (Martínez et al., 2007). These differences of toxicity might depend on the concentration of the active agent but also the nature and concentration of its adjuvants, as well as the cell line used.

In the literature, there are few studies concerning the effects of glyphosate on fish growth. Rainbow trout fingerlings were exposed up to 100 µg/L of glyphosate (a.i.) using Vision formulation (Monsanto Co.) and no significant effect on length or weight was observed after two months exposure (Morgan and Kiceniuk, 1992). However, studies using Roundup formulations did find some effects on fish growth. *Leporinus obstusidens*, a South American fish species, was exposed to 1 and 5 mg/L of glyphosate (a.i.) using Roundup for 90 days and exhibited a lower growth rate (with reductions between 10 and 15 % respectively) and a lower weight gain (between 44 and 65 % respectively) when compared to control fish (Salbego et al., 2010). Similarly, Bridi et al. (2017) highlighted reduced body length of zebra fish larvae (*Danio rerio*) when exposed from 0.01 to 0.5 mg/L of Roundup (a.i.) for 96 h. Koakoski et al., (2014) also observed a reduction of the weight gain and biomass of *Rhamdia quelen* fingerlings when exposed to 1.21 mg/L of Roundup for 96 h and after 180 days of depuration. Another study using adult fishes (*Piaractus Mesopotamicus*) reported that glyphosate reduced food intake and therefore could have an impact on normal growth (Cardoso Giaquinto et al., 2017). Furthermore, some authors have stated that glyphosate may have an effect on growth hormone and cortisol levels in fishes (Cericato et al., 2008; El-Shebly and El-kady, 2008; Koakoski et al., 2014). Cericato et al. (2008) observed that

cortisol levels in fish exposed to glyphosate were higher than in non-exposed fish. Indeed, cortisol is released in response to stress and contributes to restore homeostasis (De Boeck et al., 2001), and some evidence suggest that elevated doses of cortisol might interfere with normal growth of fishes by stimulating energy-consuming processes (Bernier et al., 2004; De Boeck et al., 2001). In our study, a 3-week exposure with similar glyphosate concentrations as used in previous studies did not show significant reductions in total body length of exposed rainbow trout larvae. However, head length of larvae was significantly smaller for those exposed to the highest tested concentration, and the ratio of head to total body length showed a significant decrease in a concentration-dependent manner. Interestingly, Zebral et al., (2017) evaluated some cephalic morphological parameters, like eye diameter and distance between eyes, of pejerrey embryos (*Odontesthes humensis*) exposed to this herbicide (0.36-5.43 mg/L) for 96 h and observed that both parameters were significantly reduced in a concentration-dependent manner in exposed groups. Similar results were found by Zhang et al., (2017) in zebra fish embryo (*D. rerio*) but using higher concentrations (up to 400 mg/L) of glyphosate for 96 h. These results may demonstrate that exposure to the concentrations of glyphosate used in our study may induce reduce growth or future maxillofacial malformations. Zebral et al., (2017) proposed that glyphosate might altered the retinoic acid pathway, which plays a major role in growth and development, and it may be involved in morphological modifications in vertebrates (Paganelli et al., 2010). Our results showed a trend to increasing spinal deformities when rainbow trout embryos were exposed to 1 mg/L of glyphosate. Several studies have reported significant body malformations, spinal curvature, pericardial and yolk sac edemas on embryos of zebra fish (Sulukan et al., 2017; Zhang et al., 2017) and Java medaka (Yusof et al., 2014) using relatively high concentrations from 1 to 500 mg/L of Roundup® (a.i.).

Larvae exposed to 0.1 mg/L of Roundup® (a.i.) were more active under light stimulation. Several previous studies have also examined the effects of glyphosate on the swimming behaviour on fish. In agreement with our results, Morgan et al., (1991) observed that after one-month exposure to 45.75 µg/L of glyphosate, under Vision's commercial formulation, fry rainbow trout presented an erratic and agitated behaviour compared to unexposed fish. Similar abnormal behaviours and hyperactivity were also reported in Nile tilapia (Ayoola, 2008) and *Tilapia zillii* (Nwani et al., 2013) exposed from 2 to 310 mg/L for 4 days and from 216 to 540mg/L of glyphosate for 96h, respectively. A Neotropical hybrid fry fish, surubim, increased their swimming activity and their ventilation frequency 96 h after exposure to 7.5 and 15 mg/L of Roundup® (a.i.) (Sinhorin et al., 2014). Similarly, Zhang et al., (2017) remarked that low concentrations of Roundup® (0.01 and 0.5 mg/L a.i.) increased locomotive activity and decreased rest on zebrafish larvae. In the other hand, Bridi et al., (2017) observed that zebrafish larvae and adults exhibited significantly reduction of distance travelled and mobility when exposed to

glyphosate and Roundup® formulations (0.01 to 0.5 mg/L a.i.) for 96 h. The behavioural study of this work was performed in larvae after 21 days of glyphosate exposure. It was shown that chronic exposure to low concentrations of glyphosate (0.1 mg/L a.i.) induced hyperactivity in exposed rainbow trout larvae. However, no effect on swimming activity was observed at 1 mg/L.

In this study, the use of the enzyme Fpg allowed to improve the detection threshold of DNA lesions. The standard comet assay can detect single or double strand breaks and alkali-labile sites while the addition of Fpg enzyme can also detect lesions related to alkylation damage, abasic sites (apuric or apyrimidic) and oxidative damage (8-oxoGua) induced by ROS (Reactive Oxygen Species) production (Kienzler et al., 2012). In our exposure conditions, glyphosate did not induce any DNA strand breaks on blood cells of rainbow trout larvae after 21 days of exposure. However, some previous studies have demonstrated the genotoxic potential of Roundup® in different fish species like *Anguilla anguilla* (Guilherme et al., 2012, 2010), *Corydoras paleatus* (De Castilhos Ghisi and Cestari, 2013), *Prochilodus lineatus* (Moreno et al, 2014) and *Carassius auratus* (Çavaş and Könen, 2007). Guilherme et al*.,* (2010) showed Roundup®'s capacity to induce DNA single strand breaks and cytogenetic effects on blood cells of European eel using low concentrations (58 and 116 µg/L a.i.) after 1 and 3 days of exposure. Cavalcante et al, (2010) observed genotoxic potential of Roundup® on blood and gill cells after 6 h of exposure to 10 mg/L (a.i.) on fish (*Prochilodus lineatus*), but comet values returned to the baseline level of control after 24 and 96 h of exposure for erythrocytes and gill cells respectively. The activation of the antioxidant and DNA repair systems on cells after glyphosate exposure have already been demonstrated by Cavalcante et al., (2010) and Marques et al., (2014). In our case, we may assume ROS were produced but larvae were able to activate protective mechanisms such as DNA repair enzymes to prevent DNA damage on blood cells, as demonstrated by several articles (Marques et al., 2014; Ching et al., 2001; Kienzler et al., 2013).

In the other hand, Fpg-modified comet assay in RTL-W1 cell line indicated that both technical grade glyphosate and Roundup® induced abasic sites and oxidative DNA damage at concentrations of 0.1 and 1 mg/L (a.i.), but no significant increase in DNA damage was observed with the classical comet assay. The fact of observing a genotoxic on RTL-W1 (in a short exposure), and no on larvae (longer exposure) favors the hypothesis of the activation of *in vivo* repair systems. However, we must be cautious with this comparison because the studied cells are not the same *in vivo* and *in vitro*. No genotoxicity studies of glyphosate have been performed on RTL-W1 cell line before, but mostly using human-derived cells. Using the human hepatoma cell line, HepG2, no DNA damage was observed when glyphosate was tested as a pure form after an exposure of 4 h (Kašuba et al., 2017). In human buccal epithelial cells, TR146, glyphosate and Roundup induced DNA damage from 20 mg/L and DNA damage increased as a function of the exposure concentration (Koller et al., 2012). Differences in genotoxicity

activity was observed between *in vitro* and *in vivo* exposure in tilapia erythrocytes after exposure to glyphosate (a.i.) (0.0007 - 0.7 mM) (Alvarez-Moya et al., 2014). *In vitro*, DNA damage was proportional to glyphosate concentration; however, *in vivo*, glyphosate was genotoxic to fish erythrocytes but not in a concentration-dependent manner.

Malondialdehyde (MDA) is one of the secondary products that can be formed during lipid peroxidation of uncontrolled oxidative stress in cells (Ayala et al., 2014). It is considered as the most mutagenic product of lipid peroxidation, and once formed MDA can react on proteins or DNA to form adducts resulting in biomolecular damages (Ayala et al., 2014). Because of its easy reaction with thiobarbituric acid (TBA), MDA has been used as a convenient biomarker of lipid peroxidation using the thiobarbituric acid reacting substances test (TBARS) (Ayala et al., 2014). Lipid peroxidation (LPO) has already been studied in fish exposed to glyphosate based herbicide and results might be very variable according to fish species, exposure duration (Glusczak et al., 2007; Modesto and Martinez, 2010; Sinhorin et al., 2014), gender (Harayashiki et al., 2013) and tissues (Glusczak et al., 2007; Sinhorin et al., 2014). Juveniles of *Prochilodus lineatus* have significantly increased LPO levels in liver after 6 h of exposure to both 1 and 5 mg/L of Roundup Transorb. However, these alterations returned to control levels after 24 h of exposure (Modesto and Martinez, 2010). On the other hand, Glusczak et al. (2007) did not observed TBARS alterations in liver of silver catfish (*Rhamdia quelen*) when exposed to 0.2 and 0.4 mg/L, but they did in muscle tissue at both concentrations. Ferreira et al. (2010) also studied the oxidative stress of different pesticides (glyphosate, methyl parathion and tebuconazole) in *R. quelen* finding that these last two compounds enhanced TBARS levels in liver of fish, but glyphosate did not. The hybrid amazon fish surubim had significantly increased TBARS levels in both liver and muscle, but not in brain after an exposure from 2.25 to 15 mg/L of Roundup (Sinhorin et al., 2014). Even though several authors have studied TBARS levels on fish exposed to glyphosate, only few analyses have been done on whole larvae. Our results show that TBARS levels were reduced in whole larvae exposed to 0.1 mg/L of glyphosate when compared to control group. Fishes have a natural defense barrier against free radicals and they were able to weaken the oxidative damage underneath control levels (Marques et al., 2014). As hypothesized by Marques et al. (2014), a development of antioxidant systems may occur as a response to ROS reducing the vulnerability of cells and their constituents. Reduced levels of lipid peroxidation have already been observed in liver of male guppy exposed to 700 µg/L of Roundup (a.i.) (Harayashiki et al., 2013), in brain of piava fish (*Leporinus obtusidens*) exposed from 3 to 20 mg/L of glyphosate commercial formulation (a.i.) (Glusczak et al., 2011), in liver of catfish when exposed to paper mill effluent (Ahmad et al., 2000), and in brain of *Oreochromis niloticus* after one-day exposure to diazinon (Üner et al., 2006). However, Üner et al., (2006) observed that MDA content in *O. niloticus* was increased in brain after 7 and 15 days of exposure to diazinon and then returning to same control levels

after 30 days of exposure. Lipid peroxidation may not only depend on ROS production, but may be also affected by physiological transitions that occurs at different developmental stages of fish (Cao et al., 2010; Mourente et al., 1999). The presence of carbonyl groups in proteins induced by glyphosate was also studied in several reports (de Moura et al., 2017; Glusczak et al., 2011; Sinhorin et al., 2014) generally in liver since it is consider as the main site of protein carbonyl production (Sinhorin et al., 2014). In contrast, the absence of protein carbonyl changes in our results could also indicate, once again, that the antioxidant system of rainbow trout larvae might function efficiently to defend against the oxidative stress. As for TBARS, only few analyses have been done using whole fish larvae to analyze carbonyl groups in proteins. Considering that protein carbonyl formation is non-reversible (Zhang et al., 2008), it can be suggested that at this developmental stage of larvae, ROS formation in rainbow trout larvae exposed to low or moderate concentrations of glyphosate was weak or low enough to be detoxified by the antioxidant systems causing no changes in TBARS and protein carbonyls groups.

Among the enzymes involved in ROS detoxification are SOD (superoxide dismutase), CAT (catalase) and GST (glutathione-S-transferase). Inhibition of CAT and SOD activities in liver were observed following exposure to glyphosate by Ferreira (2010) in *R. quelen*, Modesto and Martinez (2010) in *Prochilodus lineatus* and by Sinhorin (2014) in surubim (*Pseudoplatystoma sp*). In contrast, CAT activity was induced in liver of *L. obtusidens* exposed up to 6 mg/L of Roundup® (a.i.). We observed that *cat* gene was significantly repressed in larvae exposed to 1 mg/L of glyphosate. Topal et al. (2015) studied both gene expression and enzymatic activity in liver of juvenile rainbow trout exposed to different concentrations of glyphosate (from 2.5 to 10 mg/L) from 6 to 96 h, observing that the expressions of *cat* and *sod* were induced the first 6 h and then significantly decreased after 24 h of exposure. In the same study, Topal et al. (2015), observed that the trend of the antioxidant enzymes activity of catalase was opposed to the level of gene expression.

Interestingly, Webster and Santos (2015) studied the transcriptional profile, using RNA-seq, of brown trout females exposed to glyphosate and Roundup (0.01, 0.5 and 10 mg/L) for 14 days. They identified differentially expressed genes that encode antioxidant system proteins (up-regulation of glutathione reductase, *gsr*) stress-responses proteins (heat shock proteins, *ddit*, *ddit4l* and *gadd4l*) and pro-apoptotic signalling proteins (transcription factor tumour suppressor protein *p53*). The nature of the response of the cell depends on the amount and the duration of the stress since cells respond in a variety of signalling pathways (Fulda et al., 2010; Webster and Santos, 2015). According to Webster and Santos (2015), low concentrations of ROS contribute to induce pro-survival signalling, while higher levels of oxidative stress and cellular damage might activate cell death signalling pathways as a protective mechanism. In addition, in this same study (Webster and Santos, 2015), few changes in pro-apoptotic

factors were observed suggesting a pro-survival stress response at lower concentrations of glyphosate producing low levels of oxidative stress.

The *cox1* gene code the cytochrome c oxidase subunit 1, which is one of the enzymes involved in the respiratory electron chain transport in mitochondrial membrane. The mitochondrial electron-transport chain is the main source of ROS during normal metabolism (Chen et al., 2003). Even though cytochrome oxidase is not a source of ROS, its inhibition may promote ROS production (Chen et al., 2003). Our results revealed a significant induction of *cox1* (x2) gene expression on whole larvae exposed to 1 mg/L of glyphosate. An induction of *cox1* could be a cell response to maintain the respiratory chain functioning (Arini et al., 2015). Induction of *cox*1 gene could be view as a mechanism to restore mitochondria activity and to efficiently consume O_2 and thus to limit ROS production. Induction of *cox1* gene expression could be considered as a mechanism to avoid ROS production (Achard-Joris et al., 2006).

5. Conclusions

This study provides an extensive evaluation of toxicological effects of glyphosate using an *in vivo* and *in vitro* approach. Results revealed that relative low concentrations of glyphosate induced hyperactivity on swimming behavior (0.1 mg/L) and morphological alterations on heads of larvae (1 mg/L). In parallel, the studied cell line, RTL-W1, exhibited a genotoxic impact while it was not observed on blood cells from exposed larvae using the same concentrations of glyphosate. This difference may be explained by the time of duration of exposure, which was longer, and could have led to an activation of the antioxidant and DNA repair system on blood cells. The decreased TBARS levels and the differential regulation of *cat* and *cox1* gene expression observed on whole exposed larvae could also confirm this. It is important to consider the adjuvants in commercial formulations, which increases the toxicity of glyphosate for vertebrates, and not only the active compound. Regarding the toxicity of glyphosate on both cell line and ELS of rainbow trout at concentrations that are also found in the ecosystems, we can conclude that there is a potential risk of glyphosate in natural environment especially for aquatic organisms that are directly exposed.

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Article 3

Low environmental concentrations of chlorpyrifos can induce subtle effects in early developmental stage of rainbow trout, *Oncorhynchus mykiss*

En préparation pour publication

Shannon Weeks Santos, Patrice Gonzalez, Nicolas Mazzela, Aurélie Moreira, Christelle Clerandeau, Bénédicte Morin, Jérôme Cachot

Principaux résultats de l'Article 3

- *Malgré la saturation préalable pendant 48 h de tout le système d'exposition par du chlorpyrifos (CPF) en solution, le dosage du CPF dans l'eau a révélé une décroissance importante de cette molécule après 48 h d'exposition. Cela peut indiquer une adsorption d'une partie de ces molécules sur les parois des unités d'exposition. Mais il est également possible que les larves aient absorbé et accumulé une partie du CPF présent dans l'eau.*

- *Après 21 jours d'exposition des embryons de truite arc-en-ciel à des concentrations faibles et modérées de CPF, aucun effet sur la mortalité, le développement, les dommages à l'ADN, la peroxydation des lipides et les protéines carbonylées n'a été observé.*

- *En revanche, des concentrations de chlorpyrifos aussi faibles que 3 µg/L ont réduit significativement la mobilité des larves de truite arc-en-ciel.*

- *L'expression du gène codant pour le récepteur aux œstrogènes ERβ a été régulée négativement chez les larves exposées aux deux concentrations de CPF. L'expression du gène cyp19a1 a également été réprimée de manière significative, mais seulement chez les larves exposées à la plus forte concentration de CPF.*

Low environmental concentrations of chlorpyrifos can induce subtle effects in early developmental stage of rainbow trout, Oncorhynchus mykiss.

Shannon Weeks Santos, Patrice Gonzalez, Nicolas Mazzela, Aurélie Moreira, Christelle Clérandeau, Bénédicte Morin, Jérôme Cachot

Abstract

Chlorpyrifos (CPF) is one of the most commonly used organophosphorus pesticides due to its action to control insects and other agricultural pests. This compound can found in aquatic ecosystems at concentration up to 0.3 µg/L because of water runoff from treated crops. Some studies revealed that low concentrations of CPF may have deleterious effects in aquatic organisms. To investigate the developmental effects of CPF, rainbow trout (*Oncorhynchus mykiss*) embryos were exposed in controlled conditions to environmental concentrations of CPF. Eyed-stage embryos were exposed in semi-static conditions to 0.3 and 3 µg/L of CPF up to the larval stage for 3-weeks at 12°C under laboratory-controlled conditions. Several endpoints were analysed including survival, hatching delay, hatching success, biometry, swimming activity, DNA damage (comet assay), lipids peroxidation (TBARS), protein carbonyl content, AChE activity and target gene transcription (*cat*, *sod*, *gst*, *ERα*, *ERβ*, *cyp19a1*, *cox*, *12S*, *ogg1*, *rad51*, *bax* and *p53*). Both CPF concentrations did not affect embryonic or larval survival, and no significant morphological abnormalities were observed. On the other hand, exposure to 0.3 µg/L of CPF had an inhibitory effect on hatching. At the end of the 3-weeks exposure, larvae exposed to the higher concentration of CPF were less mobile compared to control and the lowest CPF condition. No significant differences on AChE activity were observed on both CPF conditions compared to control, but it was significantly reduced for larvae exposed to 3 µg/L compared to those exposed to 0.3 µg/L of CPF. No significant induction of TBARS levels, protein carbonyl content and DNA damage were observed in exposed larvae. Expression of genes that encodes oestrogen receptors (*ERβ*) were down-regulated on larvae exposed to both CPF concentrations. *CP19a1* expression was also significantly repressed but only on larvae exposed to the highest concentration of CPF. Our results indicated that chronic exposure to environmental concentrations of CPF could lead to sub-lethal effects on early life stages of rainbow trout, and it might represent a risk for aquatic organisms.

Keywords: early life stages, rainbow trout, chlorpyrifos, biomarkers, swimming behaviour, gene expression.

1. Introduction

Chlorpyrifos (CPF) is an organophosphorus pesticide (OP) generally used for crop protection and pest control. CPF is used in a wide range of crops including cereal, fruit, cotton, almonds and corn. It is classified as very toxic and bioaccumulated to aquatic organisms (Benedetto et al., 2016; Lal et al., 1987). CPF has low solubility in water (2 mg/L at 25°C) and high LogKow (4.96) and its half-life in laboratory has been estimated in 53 days in distilled water (Odenkirchen and Eisler, 1988). The hydrolysis rate of CFP in water is influenced by the temperature and the pH and increases when both temperature and pH rises. The use of CPF is discontinued in urban and residential area, and its use in agricultural application has been reduced in Canada, United States and several European countries (Canadian environmental Quality Guidelines, 2008).

CPF has been detected in sediments and in stream water, and is usually transported by field runoff absorbed in suspended particles (Jergentz et al., 2005; Phillips and Bode, 2004). Maximum concentrations of CPF has been observed at 0.06 µg/L in Kisco river (south-eastern New York State, USA), and in the Pampa Ondulada (Argentina) 42 % of 26 tested samples exhibit detectable levels of CPF where concentrations varied from <0.2 to 10.8 µg/L in surface water (Marino and Ronco, 2005). Jergentz et al. (2005) characterized 3 insecticides, including CPF, from streams near a soybean region in Argentina finding concentrations up to 0.45 µg/L in Brown (floodwater) and 0.3 µg/L in Horqueta (runoff water).

The toxicity of CPF is initiated by its metabolic bioactivation in chlorpyrifos-oxon (CPF-oxon) (Faria et al., 2015; Jacobson et al., 2010; Kharabsheh et al., 2017) by oxidative desulfuration. This metabolite can bind to and inactivate the enzyme acetylcholine esterase (AChE) (Barron et al., 1993).The inhibition of this enzyme produces the accumulation of acetylcholine (ACh) at the choline receptors causing continual nerve stimulation (Fulton and Key, 2001; Van der Oost et al., 2003). The inhibition of AChE by OP may persist in the brain of fish since the recovery depends of new enzymes synthesized to restore normal activity (L. W. H. U. Chandrasekara and Pathiratne, 2007; Fulton and Key, 2001). Post and Leasure (1974) observed that recovery brain AChE could vary according to the fish species. For example, brook trout, rainbow trout and coho salmon, previously exposed to malathion, needed 25, 35 and 42 days, respectively, to return to levels of AChE similar to non-exposed fishes (Post and Leasure, 1974). Consequently, the study of AChE activity has been considered as a sensitive and reliable biomarker of exposure to OP. Several studies document the AChE inhibition after CPF exposure to fish (Rao Venkateswara et al., 2005; Rodríguez-Fuentes et al., 2015; Sandahl et al., 2005; Sledge et al., 2011; Faria et al., 2015; Topal et al., 2016). Other sub-lethal effects of CPF in aquatic organisms also includes locomotor alterations (generally correlated with AChE inhibition) (Jin et al., 2015; Levin et al., 2004, 2003; Richendrfer et al., 2012; Sastre et al., 2018; Selderslaghs et al., 2013, Faria et 2015), morphological

abnormalities (Sreedevi et al., 2014; Faria et 2015), oxidative stress (Oruç, 2010; Zahran et al., 2018) and DNA damage (Ali et al., 2009). At certain cases, it may interact with oestrogen and androgen pathways (Canesi et al., 2011; Filby et al., 2007; Yu et al., 2015).

Chlorpyrifos may cause long-term effects on the aquatic environment. Such is the case of the pond Bages-Sigean (Narbonne, France) where in December of 2004 an important pollution of CPF was detected and severe mortality of fishes was observed (Ifremer, 2005). In one study, Carr et al. (1997) described how CPF affected several populations of fish and crustaceans in a small pond near a construction site. Indeed, high mortality and an important inhibition of muscle and brain acetylcholinesterase (AChE) were observed on crayfish, large-mouth bass, golden shiners and bluegill sunfish; however, populations of mosquitofish were not affected demonstrating the different between-species sensibility. Toxicity values (96h-LC_{50}) for freshwater fishes ranges from 9 µg/L for rainbow trout to 806 µg/L for channel catfish (Phipps and Holcombe, 1985). Since CPF is very toxic to aquatic organisms, the Canadian Water Quality Guidelines for CPF in surface water were set to 0.02 µg/L and 0.002 µg/L as a short-term value and a long-term value respectively, for the protection of freshwater life.

The fish species rainbow trout (*Oncorhynchus mykiss*), native from North America, is considered as a biological model in aquatic ecotoxicology (OECD, 210) because its biology and culture is well known, and it presents a low tolerance to poor water quality. Because of its relative long embryo-larval development, toxicity assay with embryos permit longer sub-chronic exposure to pollutants. In addition, early life stages appeared to be more vulnerable stage to pollutants than the adult stage (Van Leeuwen et al., 1985).

The main purpose of this work was to characterize and evaluate the sub-acute effects of environmental concentrations of chlorpyrifos on early life stages of rainbow trout. An embryo-larval exposure was conducted using technical-grade chlorpyrifos for 3 weeks. Several end-points were studied such as viability, hatching success, biometric parameters, locomotion, DNA damage, AChE activity, lipid and protein oxidation, and target gene expression. For gene expression, we selected *cat* and *sod* (antioxidant defences), *gst* (detoxification), *ERα* and *ERβ* (oestrogen receptor), and *cyp19a1* (aromatase), *cox* and *12s* (mitochondrial metabolism), *ogg1* and *rad51* (DNA repair), *bax* (apoptosis) and *p53* (cell cycle arrest).

2. Materials and methods

2.1. Test chemicals

Chlorpyrifos-éthyl (CAS No. 2921-88-2) was purchased from ChemService (England). Chlorpyrifos (CPF) stock solutions were prepared at 0.1 and 1.0 g/L using DMSO as solvent. From stock solutions exposure solutions at 0.3 and 3 µg/L, respectively, were prepared in 5 L tanks. Each condition (control, 0.3 and 3 µg/L CPF) contained 0.0003% DMSO.

2.2. Exposure conditions

The exposure was performed in 1 L plastic tank (in polyethylene terephthalate) with recirculated water. As CPF has a low solubility in water (1.4 mg/L at 25°C) and a high LogKow (4.96); to reduce the loses of CPF in our exposure system, a saturation step was performed for 48 h, before test was initiated and water was renewed every 2 days. Besides, CPF concentrations were analyzed at T0 and T48 to verify the losses of CPF before water renewal.

Eyed stage embryos of rainbow trout (*Oncorhynchus mykiss*) at 250 degree days were obtained from INRA-PEIMA (Sizun, FR). Each water exposure was prepared in a 5 L tank of spring water from Laqueuille (4.7 mg/L Ca, 1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, 7.5 pH, <1.2 mg/L Cl^-) and was renovated every two days to avoid too much loss of CPF by adsorption on plastic. Embryos were exposed to 0 (control solvent), 0.3 and 3 µg/L CPF in total darkness, with a controlled temperature at 12°C in a climate chamber. Each studied condition consisted in 3 replicates with for each 50 embryos in 1 L aquaria. A peristaltic pump (Watson Marlow, USA) was used to maintain a continuous water flow (9 mL/min) into the incubation aquaria. Dissolved oxygen concentration was measured every day with a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburf, Germany) and data was recorded with OxyView v6.02 software (PreSens Precision Sensor). Water samples were analyzed to determine the true concentration of CPF. Water samples were collected at T_0 (at the moment of exposure), T_{24} and T_{48} (before water was changed). Concentrations of CPF were analyzed by solid-phase microextraction and gas chromatography-mass spectrometry (SPME/GC-MS).

2.3. Embryo-toxicity

Viability of embryos and larvae were recorded daily and dead was removed. Embryonic, larval and half-hatched embryos denote the number of living individuals compared to the total number of individuals

at the beginning of the experiment. Half-hatched embryos were considered when a part of the body was still inside the corium. Hatching time expresses the number of degree days (DD) from fertilization to hatching. At the end of the experiment, larvae were sampled per replicate as follow: 15 larvae to record for biometrics and morphological anomalies, 6 larvae for the study of swimming behavior, 6 larvae for blood sampling for genotoxicity test. The remaining larvae were pooled and stored at -80°C for biochemical analysis and gene transcription. For the biometrics measurements, 15 larvae were sedate on iced with few drops of carbonated water and photos were taken of each individual with a stereomicroscope (MZ 7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). From the photos, total body size and head size was measured for each larva using Leica Microsystems software v3.8 (Nanterre, France). Larvae were also observed to locate possible malformations such as edemas, spinal and craniofacial deformities, cardiac anomalies, hemorrhages and yolks sac resorption.

2.4. Photomotor responses

At the end of the exposure, 12 larvae per replicate were randomly chosen to study their locomotion activity and their behavior in response to light stimulation. Before analysis, larvae were acclimated individually in 6-well microplate with 3 mL of exposure solution for 30 min in the dark at 12°C. Once acclimation was over, microplates were placed in the recording chamber (Daniovision Image Analysis System version 12.0 Noldus, with a temperature controller at 12 ± 1°C) where larvae were subjected to a light stress with a cycle of 30 min (10 min dark, 10 min light and 10 min dark). An infrared camera in the recording chamber records the motion of each larvae focusing on their center of gravity. The software, Ethovision, calculated the average velocity of each larva every 30 seconds. Average velocity, total distance swam, time of mobility and time spent in the peripheral area of the wells were determined individually for each larva.

2.5. Genotoxicity test with modified Comet assay

The alkaline comet assay was realized following the protocol of Singh et al. (1988) and adapted by Le Bihanic et al. (2014) and Weeks et al. (2019). Blood cells were sampled on heart of 6 larvae per replicate previously sedated with ice water and few drops of carbonated water. Blood samples were frozen in liquid nitrogen with 200 µL of cryoconservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) until analysis. Two aliquots of 50 µL of the blood cell suspension were placed on glass slides formerly coated with NMPA (Normal Melting Point Agarose,

0.8 %, w/w). Lysis treatment (10 mM Tris; 2.5 M NaCl; 100 mM EDTA; 1 % Triton x-100; 10 % DMSO; pH adjusted to 10 with NaOH) was performed for 90 min at 4°C in the dark. Afterwards, slices were immersed in two hellendahls, one containing 60 mL of buffer with 12 µL of the endonuclease Fpg (Biolabs, Avry, France) diluted in 1/5000; and the other one only containing 60 mL of buffer for 30 min at 37°C. Fpg is an endonuclease, which improve the sensitivity of this test by detecting oxidized bases that can be repaired. Following the enzymatic treatment, slices were horizontally placed in a tray with electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH> 13) for 20 min at 4°C and in the dark, to allow the DNA to unwind. Subsequently, electrophoresis was performed for 20 min with a voltage of 25 V and 300 mA. Slices were left in absolute ethanol for 20 min and then allowed to dry at a room temperature for at least 12 h. Slides were then stained with 20 µg/mL of ethidium bromide solution, and comet lecture was realized using an epifluorescence microscope using the Olympus BX51 microscope (zoom x20) equipped with an Olympus U-RFL-T reflected fluorescence system lamp. Comets were quantified using the Comet Assay IV software. The quantification corresponds to the counting of 100 randomly selected cells r per slide. Results are expressed as percentage of degradation of DNA tail.

2.6. Biochemical analyses

Preparation of supernatant

After 3-weeks exposure, pools of 2 whole-larvae (for TBARS and carbonylated proteins) and pools of decapitated heads of 2 larvae (for AChE activity) were frozen in liquid nitrogen and stored at -80°C until analysis. Pools were homogenized in a phosphate buffer (0.1 M; pH 7.5; 4°C) with a tissue homogenizer (UltraTurrax®) fitted with a potter (3 000 rpm, 4°C). Samples were centrifuged at 9 000 g for 25 min at 4°C. The supernatant (S9 fraction) obtained was divided in different tubes for total proteins and different measurements.

Total protein

The total protein concentration was analyzed after the method of Lowry et al. (Lowry et al., 1951) on S9 fraction. A standard of Bovine Serum Albumin (BSA) was used. Measurements were done at 750 nm using a spectrophotometer microplate reader (Synergy HT, BioTek).

Protein carbonyls

Content of carbonylated proteins was assessed following the method of Augustyniak et al., (Augustyniak et al., 2015). 50 µL of a solution containing 11% of streptomycin sulfate-phosphate buffer (100 mM pH 7.4) was added to 500 µL of S9 fraction, mixed and incubated at room temperature for 15 min.

Afterwards, mixture was centrifuged at 6000 g for 10 min. 400 µL of supernatant was divided equally into two tubes, one used as a control tube (200 µL of supernatant added to 800 µL of HCl 2.5 M) and the other used as a sample tube (200 µL of supernatant added to 800 µL 2.4-dinitrophenylhydrazine (DNPH) 10 Mm). Then, the mixtures were incubated for 1 h at room temperature and vortexed every 15 min. The proteins were precipitated with 20% of trichloroacetic acid, vortexed and centrifuged for 10 min at 10000 g. The pellets were rinsed 3-times with 1 mL of ehtanol-ethyl acetate (v:v), vortexed and centrifuged; and then they were solubilized with 500 µL of 6 M guanidine HCl and centrifuged for 10 min. The carbonylated protein content was analyzed using a UV-spectrophotometer (Synergy HT, BioTek) at 370 nm. The results are expressed as nanomoles of DNPH incorporated per mg protein.

Lipid peroxidation (TBARS)

Lipid peroxidation was analyzed following the technique of Buege and Aust (1978) with modifications. 500 µL of a solution containing 20 % of butylated hydroxytoluene (BHT) and 20 % of thrichloroacetic (TCA) was added to 500 µL of S9 fraction. Subsequently, the mixtures were centrifuged at 9000 g for 10 min. 600 µL of supernatant was added to 480 µL of TRISbase (25 mM)-TBA (thiobarbituric acid-100 mM) and 120 µL of HCl (0.6 N), and heated at 80 °C for 15 min. Then, the mixtures were cooled and mixed. Lectures of TBARS levels were done using a UV-spectrophotometer (Synergy HT, BioTek) in a microplate of 530 nm. Results are expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) equivalents per mg of protein.

AChE activity

Measurement of AChE activity was assessed in S9 fraction from heads of larvae and was performed following Ellman et al. (1961) adapted to a microplate reader. Each well contained 10 µL of S9, 180 µL of 5,5′-dithiobis (2 nitrobenzoic acid) (DTNB) 0.5 mM in 0.05M Tris Buffer pH 7.4 and 10 µL of acetyltiocholine iodide. The rate of increase of optical density (DO) was measured using a spectrophotometer (Synergy HT, BioTek) at 412 nm. Results are expressed as nmol/min/mg protein.

2.7. Gene expression

6 larvae per replicate were sampled and stored in RNA later buffer (Qiagen), at -80 °C until analysis.

RNA extraction

Total RNA extraction was performed on whole larvae with the kit “SV Total RNA Isolation system” (Promega) respecting the supplier’s recommendations. Larvae were homogenized using the MP fastprep®-24 (Biorad, 6 m/s, 40s) with ceramics beads (MP Biomedicals, Lyssing Matrix D bulk).

Retro-transcription of total RNA into cDNA

The retro-transcription of total purified RNA was prepared with the kit "GoScript Reverse Transcription System" (Promega). The Reverse Transcription was performed with 1 µL of oligodT (1 µM) and 1 µL of hexanucleotides (1 µM) added to 10 µL of total purified RNA (1 µg). The mixtures were heated at 70°C for 5 min with a thermocycler (Eppendorf Mastercycler) followed by 5 min at 4°C to allow primer annealing. Then, 1 µL of dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of $MgCl_2$ (25 mM), 1 µL of reverse transcriptase (1 U/µL) and 0.5 µL of RNAsine were added. The reverse transcription reaction was performed in a thermocycler at 42°C for 1 h. The cDNA obtained were stored at -20°C until analysis by quantitative real-time PCR reaction.

Quantitative real-time PCR

Twelve genes were studied with specific primer-pairs designed by Primer3plus software (Table 1). Real-time qPCR was performed using GoTaq® qPCR Master Mix kit (Promega). PCR reactions contained 17 µL of a mix consisted of Nuclease-Free Water and GoTaq® qPCR Master containing SyberGreen fluorescent dye, 2 µL of specific primer pairs mix (200 µM each) and 1 µL of cDNA. Real-time PCR reactions was done in a Mx3000P® qPCR system (Stratagene) using a first step at 95°C for 10 min and then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity was determined by for each reaction from the dissociation curve of the PCR products. The dissociation curve was obtained by following the SyberGreen fluorescent level during a gradual heating of the PCR products from 60 to 95°C.

Cycle thresholds (Ct) were calculated for each gene from the software MxPro™ qPCR. Two different reference genes (Rpl7 and EF1α) were analyzed. Consequently, relative quantification of each gene expression level was normalized with the mean of Ct value of reference genes according to the 2ΔCt methods explained by Livak and Schmittgen (2001). The induction factor (>2) or repression factor (<0.5) was determined by the ratio of transcription levels of each gene between exposed conditions and the control ones.

2.8. Statistics

Each condition had 3 identical replicates, and each replicate was considered as an independent sample. All data is indicated as mean ± SD (standard deviation). Statistical tests were analysed using R (http://cran.r-projet.org/). Normality of data distribution was analysed on the residues by the Shapiro-Wilk test ($p<0.01$) and the homogeneity of variances was verified by the Levene test ($p<0.05$). If the data followed a normal distribution, a one-way ANOVA test was used ($p<0.05$) followed by a Tukey HDS post-

hoc test. If the data distribution was not normal, data was analysed by non-parametric tests of Kruskal-Wallis ($p<0.05$).

Table 1: Accession number and specific primer pairs for the Oncorhynchus mykiss genes selected in our study.

Gene	Accession number	Primer (5' – 3')
rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
ef1α	NM_001124339.1	ATGGGCTGGTTCAAGGGATG[a] GATCATACCGGCCTTCAGGG[b]
cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG[a] GTCCAGGATGGGAAGTTGC[b]
sod	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
gst	BT073173.1	ATTTTGGGACGGGCTGACA[a] CCTGGTGCTCTGCTCCAGT[b]
er-a	AJ245219	AGAGGCATAGTCACTGCAGA[a] CCCAGCCAGTCATACTACCT[b]
er-b	AJ242741	AGCCCTCTCCTCCACCCTACCA[a] ACAGCTGGCTGAGGAGGAGTT[b]
cox	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12s	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
cyp19a1	XM_021598638	CTCTCCTCTCATACCTCAGGTT[a] AGAGGAACTGCTGAGTATGAAT[b]

[a]Forward primer

[b]Reverse primer

3. Results

3.1. Condition of exposure

Dissolved oxygen in the exposure water was above 85% for the different treatments throughout the embryonic and larval exposure. Measured concentrations of CPF in water is showed in Table 2. Water samples were analyzed at T_0, T_{24} and T_{48}, just before water exposure was changed, to estimate the losses of CPF by adsorption into the plastic of the system. For the low dose of CPF at 0.3 µg/L, the measured concentration at T_0 was comparable to the nominal concentration; however, the concentration was decreased to 42 % at T_{24} and was below limit of quantification at T_{48}. Similarly, reduction of CPF over time were observed for 3 µg/L condition. The measured concentration at T_0 was 50 % the nominal concentration, and reductions of CPF were also observed at T_{24} (37 %), and at T_{48} CPF was highly reduced below limit of quantification.

Table 2: Measured concentrations of CPF for both conditions at T_0, T_{24} and T_{48}.

Nominal concentration of Chlorpyrifos (µg/L)		Measured concentration of Chlorpyrifos (µg/L)
0.3	T_0	0.33
	T_{24}	0.16
	T_{48}	<0.01
3	T_0	1.40
	T_{24}	0.88
	T_{48}	<0.01

Limit of quantification: 0.13 µg/L

Limit of detection: 0.04 µg/L

3.2. Embryonic and larval survival and hatchability

No significant differences in embryonic and larval viability were observed between the control and both CPF treatments (Table 3). Both, embryonic and larval viability reached above 95 % in all conditions. Hatchability was high in all conditions, hatching rate was similar to embryonic viability. The duration of development (DD) was slightly longer for embryos exposed to 0.3 µg/L CPF (p value = 0.06) when compared to control embryos. In addition, when observing hatchings at days 7 and 8 from Figure 1, embryos from control condition were almost completed hatched while hatchability for embryos

exposed to 0.3 µg/L and 3 µg/L CPF only 59 and 73 % respectively. This difference was not more observed after day 9.

Table 3: Effects of chlorpyrifos exposure on viability and development of early life stages of rainbow trout. Values represent Mean ± SD. Results show no significant differences. (*) p-value = 0.06, N = 3, ANOVA/Kruskall-Wallis.

	Control	0.3 µg/L CPF	3 µg/L CPF
Acute toxicity			
Embryo viability (%)	99.3 ± 1.2	96 ± 5.3	100 ± 0.0
Larval viability (%)	96.7 ± 3.0	96.6 ± 4.2	99.3 ± 1.2
Cumulative viability (%)	96.0 ± 2.0	92.6 ± 3.0	99.3 ± 1.2
Hatching rate (%)	99.3 ± 1.2	96.0 ± 5.3	100.0 ± 0.0
Sub-lethal toxicity			
Duration of development (DD)	318.4 ± 7.2	329.5 ± 3.9*	322.0 ± 1.3
Total length (mm)	19.8 ± 0.3	19.6 ± 0.2	19.6 ± 0.3
Head length (mm)	4.6 ± 0.1	4.4 ± 0.1	4.3 ± 0.2
Ratio of head/body length (%)	23.2 ± 0.3	22.6 ± 0.2	22.1 ± 0.7
Area of yolk sac (mm^2)	11.4 ± 0.3	11.8 ± 0.5	11.5 ± 0.6
Developmental anomalies (%)			
Total abnormal larvae	15.6 ± 3.8	20.0 ± 0.0	22.2 ± 10.2
Oedemas	2.2 ± 3.8	4.4 ± 3.8	4.4 ± 7.7
Spinal	8.9 ± 7.7	17.8 ± 3.8	20.0 ± 6.7
Craniofacial	6.7 ± 6.7	4.4 ± 7.7	6.7 ± 11.5
Cardio-vascular	0.0 ± 0.0	2.2 ± 3.8	4.4 ± 7.7
Haemorrhages	0.0 ± 0.0	6.7 ± 0.0	4.4 ± 7.7

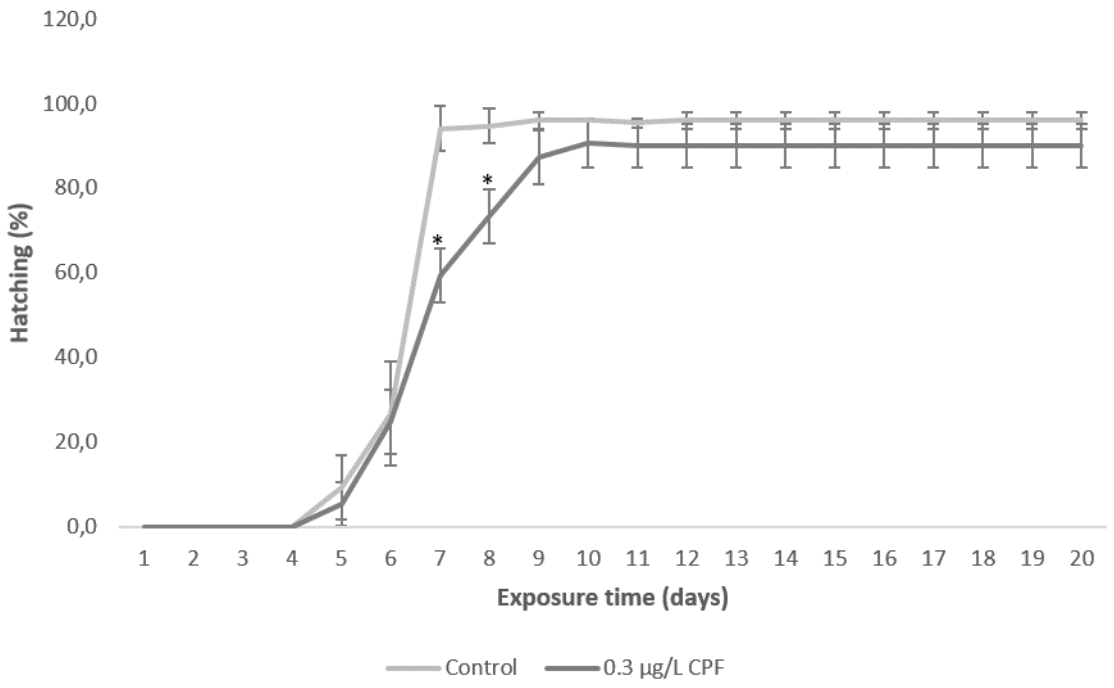

Figure 1: Hatching embryos from control, 0.3 and 3 µg/L CPF condition. Asterisks indicate significant differences between condition of 0.3 µg/L compared to control ($p < 0.05$). Values represent Mean ± SD. N = 3, ANOVA.

3.3. Phenotypic effects

Biometrics measurements did not show any significant differences in total size, head size, the ratio between head and total size, and the area of yolk sac on larvae exposed to both concentrations of CPF when compared to non-exposed larvae (Table 3). Likewise, the embryo-larval exposure to CPF showed a slight but not significant increase of abnormalities. Total malformation was 15.6 ± 3.8, 20.0 ± 0.0 and 22.2 ± 10.2 % for control, 0.3 and 3 µg/L conditions respectively. The most common malformation observed in all conditions was spinal abnormalities (kyphosis, lordosis, shaped-C larvae).

3.4. Photomotor response

Results of swimming behavior are presented in Figure 2. Distance traveled by larvae exposed to both chlorpyrifos concentrations, did not show significant differences when compared to control condition. Notwithstanding, at the moment of the light changing, significant differences were observed between exposure CPF treatments since larvae exposed to 3 µg/L of CPF covered a lower distance (3481 ± 830 mm) compared to larvae exposed to 0.3 µg/L of CPF (5877 ± 600 mm). Same trend on light

stimulation was observed on mobility of larvae, where both control and larvae exposed to 0.3 µg/L of CPF were significantly more mobile (46 ± 12.4 s and 47 ± 5 s, respectively) than larvae exposed to 3 µg/L of CPF (27 ± 4.5 s).

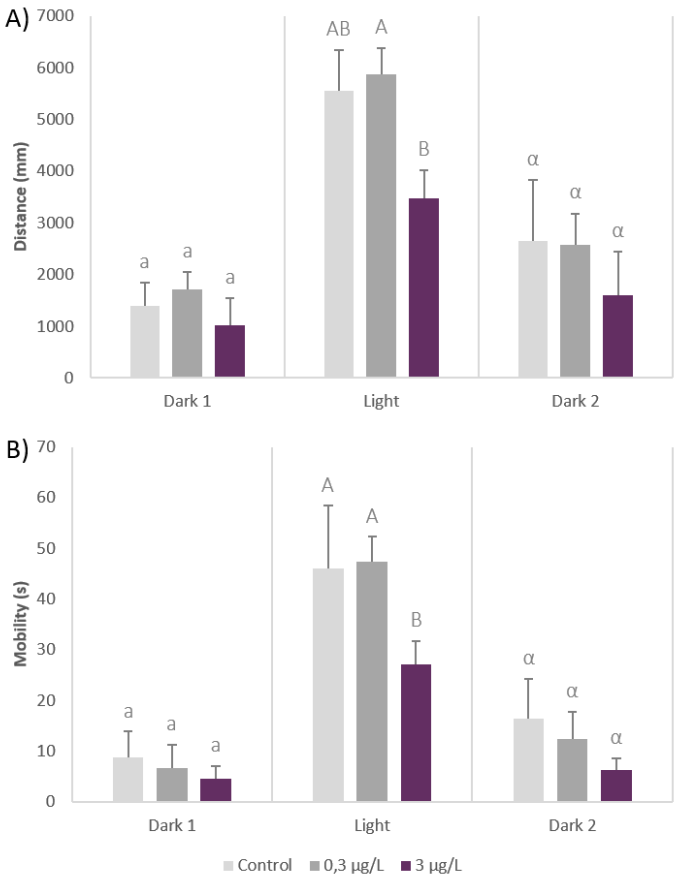

Figure 2: Mean distance travelled (mm) (A) and cumulative time of mobility (B) of rainbow trout larvae following chlorpyrifos exposure. Different letters indicate significant differences between each treatment for a same light/dark condition (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.5. AChE activity

AChE activity in head of larvae exposed to 3 µg/L was significantly reduced in comparison to larvae exposed to 0.3 µg/L (Figure 3) but no significant differences were observed between both CPF conditions and control group.

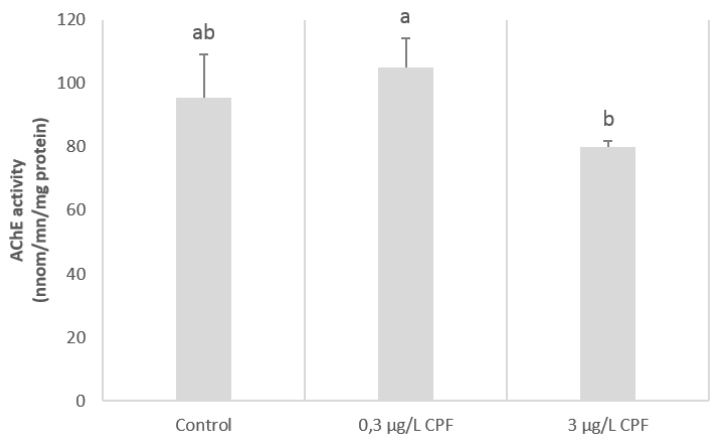

Figure 3: AChE activity in head of rainbow trout larvae following 3-weeks exposure to CPF. Different letters indicate significant differences between conditions (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.6. Genotoxicity

Figure 4 shows the average tail DNA for each studied condition, with- and without Fpg (formamidopyrimidine DNA glycosylase) treatment. Our results showed no significant differences in DNA damage between conditions, with or without treatment with Fpg, when compared to control condition.

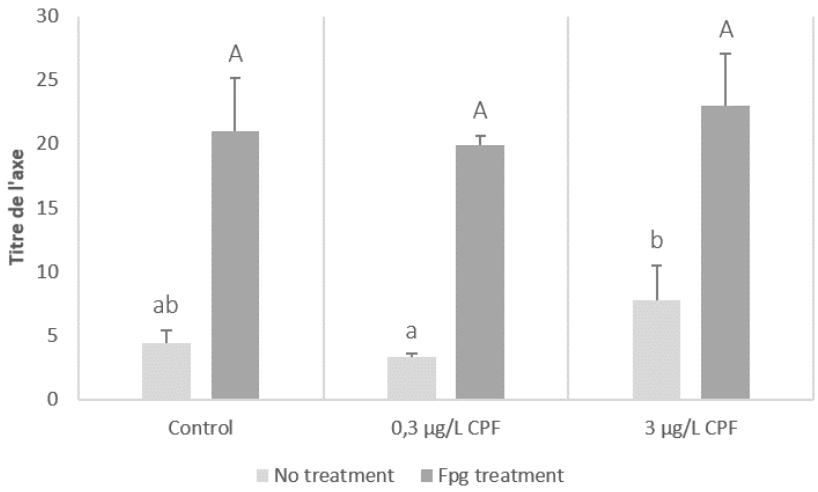

Figure 4: DNA damage in blood cells from rainbow trout larvae after exposure to 0.3 and 3 µg/L of CPF, with- and without FpG enzymatic treatment. Different letters indicate significant differences between conditions (N = 3, ANOVA, $p < 0.05$).

3.7. Lipid peroxidation and protein carbonyls

After exposure to 0.3 and 3 µg/L of CPF, no significant differences of thiobarbituric acid (TBARS) levels (Figure 5) and protein carbonyl content (figure 5) were detected in exposed larvae compared to non-exposed larvae.

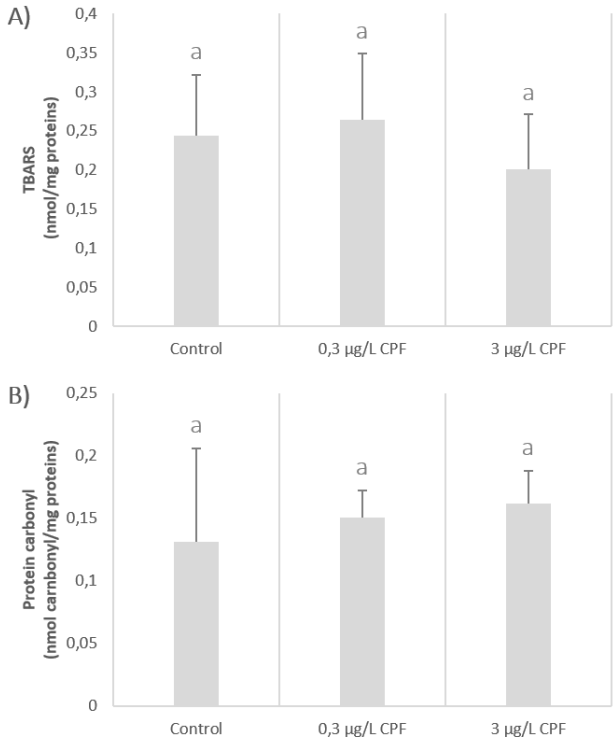

Figure 5 : Lipid peroxidation expressed as nanomoles of TBARS/mg of protein (A) and protein carbonyls expressed as nanomoles of carbonyl/mg of protein (B) on whole-body of rainbow trout larvae exposed to chlorpyrifos. The same letters at the top of the bars indicate no significant differences (N = 3, ANOVA, $p > 0.05$).

3.8. Gene expression

Gene expression was analysed on whole body of larvae after 3-weeks exposure to CPF. No statistical changes were observed in expression of the two gene of references *Rpl7* and *Ef1α* between conditions (data not shown). Among the 12 studied genes, only the *erb* (oestrogen receptor) was down regulated in both CPF conditions compared to control and the gene *cyp19a1* that codes the enzyme aromatase was also down-regulated only for larvae exposed to the highest CPF concentration (Table 4).

Table 4: Transcriptional expression levels in rainbow trout larvae exposed to 0.3 and 3 µg/L of CPF. Gene transcription values were normalized to housekeeping gene Ribosomal Protein L7 (RPL7) and Elongation Factor 1α (EF1α). Data was expressed in comparison to the control treatment as an induction (above 2) or repression (below 0.5) factors. Asterisks indicate significant differences with the control treatment (N = 3, ANOVA, $p < 0.05$).

Condition	cat	sod	gst	er-a	er-b	cox	12s	ogg1	rad51	bax	p53	cyp19a1
0.3 µg/L CPF	/	/	/	/	0.26*	/	/	/	/	/	/	/
3 µg/L CPF	/	/	/	/	0.27*	/	/	/	/	/	/	0.16*

4. Discussion

Organophosphates pesticides (OP) are used worldwide in agriculture and in domestic applications, where they constitute, approximately, half of total pest control agents used as insecticide (Atwood and Paisley-Jones, 2017). Because of its human and animal toxicity, US EPA restricted most of this kind of insecticides for residential use in 2001. However, their use in agriculture is still allowed. US EPA and World Health Organization (WHO) have classified CPF as moderately hazardous (Class II); and it was rank as the most commonly OP used in 2012 (Atwood and Paisley-Jones, 2017). In our study, CPF, which is hydrophobic as well as most OP, showed a strong adsorption into plastic of our exposure system. Nearly half of both CPF concentrations were adsorbed within the first 24 h of exposure; and after 48 h, both CPF concentrations were under limit of detection. In aquatic systems, CPF exhibits a strong partitioning from aqueous solutions to soils and sediments (Gebremariam et al., 2012). The k_d values of CPF was estimated at 40 L/kg from a vegetated agricultural ditch sediment (Rogers and Stringfellow, 2009), and 38.99 L/kg from Chaohu lake (China) indicating that CPF had the higher adsorption affinity when compared to triazophos and their metabolites (Liang et al., 2011).

Although a large range of aquatic species have been reported to be sensitive to CPF exposure very few studies investigated effect on early development stages. Our results showed that three weeks of exposure to CPF concentrations under 3 µg/L did not alter embryonic and larval survival. Acute toxicity of CPF was determined for different species of fish. A 96 h LC_{50} was reported at 140 and 120 µg/L of CPF on larvae of fathead minnow (*Pimephales promelas*) using technical Dursban and commercial Dursban® 10 CR respectively (Jarvinen and Tanner, 1982). Phipps and Holcombe (1985) estimated a 96 h-LC_{50} of CPF at 542 µg/L for fathead minnow, 806 µg/L for channel catfish (*Ictalurus punctatus*), 10 µg/L for juvenile bluegill (*Lepomis macrochirus*) and 9 µg/L for juvenile rainbow trout. It appears that

toxicity of CPF depends on species and also on the temperature of exposure; vulnerability of fish increased when temperature was augmented (Humphrey and Klumpp, 2003; Macek et al., 1969). A 96h-LC_{50} of CPF was estimated on juveniles of rainbow trout at 7.1 µg/L with a temperature of 12.7°C, 15 µg/L at 7.2°C, and 51 µg/L at 1.6°C using a commercial formulation Dursban® (Macek et al., 1969). Humphrey and Klumpp (2003) also studied the effects of temperature on CPF toxicity on rainbowfish (*Melanotaenia splendida splendida*). Embryos and larvae survival was significantly affected with concentrations from 125 and 160 µg/L, respectively, at 29°C, whereas no significant mortality was observed at 25°C. In the other hand, 96 h-LC_{50} was also determined for 16-day old larvae, adults and embryos of rainbowfish, which were 0.117, 0.396 and 2.019 mg/L of CPF, respectively (Humphrey and Klumpp, 2003).

Hatching on embryos exposed to 0.3 µg/L of CPF was delayed compared to control condition. Similar results were observed on zebrafish (*Danio rerio*) in the studies of Jin et al. (2015) and Sreedevi et al. (2014). Decreased hatchability was observed at 60 hpf in a concentration-dependent manner on zebrafish embryos exposed to 30 to 300 µg/L of CPF with 27.4 and 51.9 % of decrease hatchability when compared to control group (Jin et al., 2015). However, in our study, hatchability was no longer significant different after 96 h of exposure to CPF. Nevertheless, some discrepancies have been observed with other studies. For example, the study of Yu et al. (2015) exposed also zebrafish embryos to concentrations from 100 to 1000 µg/L of CPF where hatching rate was significantly increased in a concentration-dependent manner compared to control groups. The studies were performed using chlorpyrifos technical grade and further research is required to elucidate its potential effect on hatchability on fish.

The measurement of the activity of acetylcholinesterase (AChE) in brain and muscle is considered as a biomarker of exposure to certain organophosphate insecticides, as CPF, in fish and aquatic organisms (Sandahl et al., 2005). The toxicity of these insecticides is based on the inhibition of the AChE activity which could interfere with the proper cholinergic signaling in vertebrates. Numerous studies reveal the detrimental effects of CPF on the nervous system (Sledge et al., 2011). Regarding our results of AChE activity in brains of rainbow trout larvae, no significant differences were observed between both CPF concentrations and control larvae because of high inter-individual variability; however, larvae exposed to 3 µg/L of CPF had significantly reduced AChE compared to larvae exposed to 0.3 µg/L. Significant inhibition of AChE was already observed in brain of adults of rainbow trout after 72 h or 96 h exposure to 6.75 µg/L of CPF, but no significant differences were observed when fish were exposed for the same duration to 2.25 and 4.5 µg/L of CPF (Topal et al., 2016). After 96 h embryonic exposure to CPF, larvae of zebrafish exposed to 100 and 300 µg/L had significantly lower AChE activity than the control (Jin et al., 2015). Similarly, Jeon et al. (2016) observed a significant inhibition of AChE

activity in a concentration-dependent manner in zebrafish embryos exposed from 200 to 1000 µg/L; while the activity in adults was significantly enhanced for those exposed to 600 µg/L and inhibited at 1000 µg/L of CPF. The AChE activity was also measured in brain of different life stages of Nile tilapia (*Oreochromis niloticus*) for 14 days at concentrations from 0.5 to 12 µg/L of CPF (L. W. H. U. Chandrasekara and Pathiratne, 2007). In this study, AChE activity was significantly lower than control fish starting from 0.5 µg/L of CPF and was decreasing in dose-dependent manner. In addition, larvae were the most sensistive, lethality was observed at 8 and 12 µg/L of CPF exposure, and presented lower AChE activity in brains of larvae (39-85 %) than fingerlings (27-74 %) and sub-adults (18-47 %) (L. W. H. U. Chandrasekara and Pathiratne, 2007). Kavitha and Venkateswara Rao (2008) exposed adults of mosquitofish (*Gambusia affinis*) for 96 h to 297 µg/L of CPF, followed by 20 days of recovery. After 96 h, severely impairments were observed on locomotion (speed) of fish and AChE activity (with reductions of 82%) when compared to control. However, a successful recovery in both behavior and AChE activity was observed indicating that fish have the capacity to overcome the stress of CPF (Kavitha and Venkateswara Rao, 2008).

A decrease in AChE activity causes acetylcholine accumulation within synapses of cholinergic neurons, leading to deficiency of important functions as swimming and feeding (Topal et al., 2016). Several studies have documented the correlation between AChE activity with behavioral endpoints, as swimming performance, in fish after exposure to CPF (Faria et al., 2015; Kavitha and Venkateswara Rao, 2008; Sandahl et al., 2005). Our results showed that concentrations of CPF as low as 3 µg/L significantly decrease mobility of rainbow trout larvae. Several studies reported abnormal swimming behavior in different fish species exposed to CPF. For instance, adults of mosquitofish (*G. affinis*) exposed to 60 µg/L of CPF for 20 days had a reduce swimming speed in a duration-dependent manner (Rao Venkateswara et al., 2005). In another study, juveniles of coho salmon (*Oncorhynchus kisutch*) exposed lo low concentrations of CPF (0.6 to 2.5 µg/L) displayed impairment of swimming and feeding behaviors from 0.6 µg/L after 96 h of exposure (Sandahl et al., 2005). Larvae of zebrafish were exposed for 96 h to 30 to 300 µg/L of CPF had a significant reduction of swimming distance (Jin et al., 2015). In another study, also with zebrafish, where embryos were exposed up to the larval stage for 7 days to concentrations of CPF from 0.001 to 0.1 µM (0.35 to 35 µg/L), larvae showed a decreased of swimming speed and thigmotaxis (with preference to the edge of the well) from 0.01 µM (3.5 µg/L) (Richendrfer et al., 2012). Hypoactivity were also observed at 24 h and 96 h of exposure on *Jenynsia multidentata*, a fish species from South America, to similar concentrations of CPF as our study (4 µg/L) (Bonansea et al., 2016). Interestingly, at the same study, when fish were exposed to a mixture of CPF with cypermethrin (CYP) in low concentrations (0.4 µg/L of CPF and 0.04 µg/L of CYP), fish showed an increase in the speed and distance traveled after 96 h; and when exposed to higher concentrations (4 µg/L of CPF and 0.4 µg/L of

CYP) a significant decrease in speed and distance was observed indicating a differential behavioral effect according to the concentration of exposure (Bonansea et al., 2016). Moreover, a short term CPF exposure showed long-lasting behavioral impairments when zebrafish embryos were exposed to 10 and 100 µg/L from 1 to 5 days post-fertilization (Levin et al., 2003). After 5-days of exposure, fish were transferred and kept in clean water until age of 20-weeks for behavioral testing (Levin et al., 2003). Their results showed opposite behavioral effects, the lower CPF concentration reduced response latency, whereas the highest increased response latency. These results reveal that CPF may have persistent effects even when organisms have been shortly exposed at early life stage.

Our results from genotoxicity in blood cells (comet assay), lipid peroxidation (TBARS) and content of carbonyl proteins in whole larvae were not significant different from control, suggesting that the low to moderate environmental concentrations of CPF did not enhance damage on DNA, cellular membrane and proteins. In the literature, there is some evidence that CPF at moderate or high doses might have potential genotoxic properties. DNA damage was observed in lymphocytes and gill cells from *Channa punctatus* fish adults exposed to concentrations from 68 µg/L of CPF for 35 days, where maximum DNA damaged was remarked at day 5 and then, a decreased non-linear was observed (Ali et al., 2009). A significant induction of micronuclei was remarked by Ismail et al. (2018) in blood cells from fish adults *Labeo rohita* exposed to CPF from 73.8 µg/L.

Oruç (2010) observed significant reductions in AChE and GST activities, and an increase in SOD activity without lipid peroxidation in gonad tissue of adults of Nile tilapia (*O. niloticus*), exposed for 30 days at concentrations from 5 of CPF. They assumed that the antioxidant defense system was able to scavenge reactive oxygen species, preventing the increase in the gonadal oxidative damage. However, in another study, MDA levels were increased in gill and liver of Nile tilapia adults exposed to 15 of CPF for 14 days (Zahran et al., 2018). Similar results were obtained by Yonar (2018), who remarked that MDA levels were significantly induced in liver, kidney and gills from adults of common carp (*Cyprinus carpio*) exposed to concentrations from 40 µg/L of CPF for 14 days.

Transcription of selected genes was studied on liver of rainbow trout adults by Benedetto et al. (2016). In this study, fish were exposed to 50 µg/L of CPF for 96 h, and then they were kept in clean water for decontamination for 28 days. At the end of 96 h exposure, several genes involved in oxidative stress were up-regulated as *hsp70* (heat shock protein), *gssg*-r (glutathione reductase), *gsh-px* (glutathione peroxidase). In the present study, 12 genes of rainbow trout were analyzed using RT-PCR. Among these genes, the *erb* and *cyp19a1* were significantly down-regulated for larvae exposed to CPF. However, no significant differential expressions were observed for *er-a* gene and genes involved in the antioxidant defense (*sod*, *cat*), detoxification (*gst*), cellular metabolism (*cox*, *12S),* DNA reparation

(*rad51*, *ogg1*) and apoptosis (*p53*, *bax*). Given the post-transcriptional and post-translational regulation, the decrease expression of *er-b* and *cyp19a1* at the protein level should be confirmed.

The *er-b* encodes the estrogen receptor-β, that is a nuclear receptor activated by the sex hormone estrogen. The *cyp19a1* encodes the enzyme aromatase which catalyzes the estrogen biosynthesis from androgens, and has a role in sex-determination and in neuroprotection. Many of the physiological actions of estrogen are mediated by these receptors (Yu et al., 2015). Some studies indicate that CPF affects the thyroid hormones, and is an endocrine disruptor with both anti-androgenic and estrogenic properties (Brandt et al., 2015; Filby et al., 2007; Watts, 2012; Yu et al., 2015). *In vitro*, CPF was found to be a weak estrogenic inducer in two *in vitro* assays with 25 % activation of response in the estrogen receptor transactivation assay, and 36 % activation in MCF-7 cell proliferation assay of the maximum 17 β-estradiol response (Andersen et al., 2002). In Nile tilapia, significant lower serum cortisol, estradiol and testosterone levels were observed after exposure to 5, 10 and 15 µg/L of CPF for 30 days (Oruç, 2010)

5. Conclusion

Chlorpyrifos (CPF) is one of the most used insecticides in the world, and is considered as very toxic for aquatic organisms but effects of environmental concentrations on fish embryonic development were poorly documented. After 21 days of exposure of rainbow trout embryos to low and moderate concentrations of CPF, no effects on mortality, development, DNA damage, lipid peroxidation and protein carbonyls were observed. Nevertheless, larvae exposed to 3 µg/L of CPF were significantly less mobile and swam a reduced distance upon light stimulation when compared to control larvae. In addition, exposure to 0.3 µg/L of CPF induced down-regulation of *er-b* and *cy19a1* genes involved in reproduction of fish. This study demonstrates that environmental concentrations of CPF could trigger sub-lethal effects on early development stages of fish and thus might represent a risk for aquatic ecosystems.

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Chapitre IV

Étude des mécanismes de défense face à une exposition à un mélange de pesticides

Bien que la plupart des études écotoxicologiques se concentrent sur l'étude de pesticides individuels, plusieurs rapports confirment que les pesticides se retrouvent dans l'environnement en mélanges complexes et à faibles concentrations (Stone et al., 2014), et que les organismes aquatiques y sont directement exposés de façon chronique (Relyea, 2009; Relyea and Hoverman, 2006; Schäfer et al., 2012). L'exposition aux mélanges de pesticides peut déclencher des effets combinés, ou additif, lorsque les composés ont un mode d'action similaire et affectent la même cible moléculaire (Beyer et al., 2014). Il est également possible d'observer des effet de synergie ou d'antagonisme des composés, lorsqu'ils présentent un mode d'action différent qui affecte la même cible moléculaire ou une cible différente (Beyer et al., 2014).

Ce chapitre a pour objectif d'analyser l'impact d'un mélange de pesticides sur les premiers stades de développement de la truite arc-en-ciel (*Oncorhynchus mykiss*). Pour cela, les 3 molécules utilisés précédemment à savoir le cuivre, le Roundup et le chlorpyrifos, ont été mélangées dans les conditions suivantes :

Condition	CPF (µg/L)	Cu (µg/L)	GLY (µg/L)
A	0.0	0.0	0.0
B	0.1	0.67	33.3
C	**0.3**	**2.0**	**100**
D	**3.0**	**20.0**	**1000**

La méthodologie utilisée pour l'exposition et les analyses biologiques, ainsi que les résultats et les discussions de cette étude sont présentés dans l'**article 4**.

Article 4

Pesticides mixture induced sub-lethal responses in first stages of development of rainbow trout (Oncorhynchus mykiss)

En préparation pour publication

Shannon Weeks Santos, Patrice Gonzalez, Nicolas Mazzela, Aurélie Moreira, Christelle Clerandeau, Bénédicte Morin, Jérôme Cachot

Principaux résultats de l'Article 4

- *Dans cette étude, nos résultats ne montrent pas de mortalité significative sur les embryons et les larves de la truite arc-en-ciel exposées aux mélanges de Cu, CPF et GLY. L'ajout de CPF et de GLY semble limiter la biodisponibilité ou la toxicité du Cu chez la truite. Ce résultat pourrait s'expliquer par les interactions possibles entre les composés. En effet, le GLY est connu pour être un puissant chélateur de métaux lourds tels que le Cu. Il réduirait ainsi la biodisponibilité de ce métal dans nos conditions d'expérimentation.*

- *En ce qui concerne l'activité natatoire, les larves exposées dans la condition intermédiaire « C » étaient plus mobiles que les larves non exposées.*

- *Concernant les effets génotoxiques, le traitement par l'enzyme Fpg a révélé des cassures supplémentaires de l'ADN qui correspondent aux purines oxydées, en particulier pour les larves exposées à la concentration la plus forte de mélange (condition D). Cela pourrait suggérer que les mélanges du Cu, GLY et CPF pourraient induire des lésions oxydatives de l'ADN.*

- *Les niveaux de TBARS des larves exposées à la condition D étaient plus faibles par rapport au contrôle. L'absence de niveaux élevés de TBARS et de protéines carbonylées pourrait signifier une réponse efficace du système antioxydant de la larve pour réduire les radicaux libres.*

- *L'analyse en RT-qPCR a montré une induction de nombreux gènes après 3 semaines d'exposition et ceci dans toutes les conditions. En effet, l'exposition au mélange de ces trois composés a conduit à l'induction de l'expression des gènes impliqués notamment dans la détoxication, le métabolisme mitochondrial et la réparation de l'ADN.*

Pesticides mixture induced sub-lethal responses in first stages of development of rainbow trout (*Oncorhynchus mykiss*)

Shannon Weeks Santos, Jérôme Cachot, Bettie Cormier, Nicolas Mazzella, Pierre-Yves Gourves, Christelle Clérandeau, Bénédicte Morin, Patrice Gonzalez

The aim of this study was to analyze the impact of pesticides mixture on the first stages of development of rainbow trout (*Oncorhynchus mykiss*). For this, a mixture composed by glyphosate (GLY), chlorpyrifos (CPF) and copper (Cu), at different environmental concentrations, were exposed to eyed-stage of rainbow trout up to the larval stage at 12°C under laboratory-controlled conditions. During 3-weeks exposure, embryos were exposed to the following mixture concentrations: condition A (control-solvent), condition B (33 µg/L GLY, 0.1 µg/L CPF, 0.67 µg/L Cu), condition C (100 µg/L GLY, 0.3 µg/L CPF, 2 µg/L Cu) and condition D (1000 µg/L GLY, 3 µg/L CPF, 20 µg/L Cu). The lethal and sub-lethal effects were assessed through a number of phenotypic and molecular endpoints including survival, hatching delay, hatching success, biometry, swimming activity, DNA damage (Comet assay), lipid peroxidation (TBARS), protein carbonyl content and gene expression. Ten target genes involved in antioxidant defenses, DNA repair, mitochondrial metabolism and apoptosis were analyzed using real-time RT-qPCR. Our results show that after exposure to the three conditions, no significant mortality or half-hatch mortality were observed compared to control condition, suggesting a suppressive action of GLY and CPF to Cu toxicity. Behavior, analyzed by imaging analysis system Daniovision (version 12.0 of Noldus), showed significant hyperactivity of larvae, from condition C, at the basal level (before light stimulation). On the other hand, significantly increased DNA damage was observed on larvae exposed to the strongest pesticide mixture concentration when cells were treated with Fpg enzyme demonstrating an increase of oxidative damage (8-oxoguanine); however, no significant increases of DNA damage were observed in the standard comet assay. No significant differences were observed on protein carbonyl content on larvae from all conditions; nevertheless, larvae from condition D significantly decreased the TBARS levels when compared to control condition. With regard to gene transcription, the analyzed genes revealed up-regulations in all conditions, especially genes involved in detoxification (*gst* and *mt1*), DNA repair (*ogg1*), mitochondrial metabolism (*cox1* and 12S) and cholinergic system (*ache*). These genetic up-regulations may suggest an adaptation effect of larvae facing the chemical contamination.

Keywords: pesticide, copper, glyphosate, chlorpyrifos, early life stages, rainbow trout

1. Introduction

Nowadays, an increasing concern of chemicals contaminants found in natural environments are challenging the research to understand and to predict the impacts of these contaminants on the ecosystems. Because of its nature to protect cultures, pesticides are the only kind of chemicals that are directly release into the environment (Sánchez-Bayo, 2011). Currently, more than 400 chemicals compounds are used to treat crops from pests and weeds (Stone et al., 2014), and global pesticide usage is calculated in 4.6 million tons per year (Sánchez-Bayo, 2011; Zhang et al., 2011). It has been estimated that 98 % of pesticides that are applied in agricultural crops do not reach the principal target, arriving to impact terrestrial and aquatic ecosystems. Studies reveal that 40 % of global land area are in risk to transport pesticides molecules by runoff toward rivers and streams (Ippolito et al., 2015). While most ecotoxicological studies are focused on studying individual pesticides, several reports confirmed that pesticides are usually found in complex mixtures at low concentrations (Krishnan and Brodeur, 1994; Stone et al., 2014), and aquatic organisms are directly exposed to them (Relyea, 2009; Relyea and Hoverman, 2006; Schäfer et al., 2012). Exposure to pesticides mixtures may trigger combined effects by concentration addition, where similar mode of action affects the same molecular target, or by independent action, where dissimilar mode of action affects the same or different molecular target, and therefore, synergism or antagonism may occur (Beyer et al., 2014).

Because of its anti-microbial and anti-fungal properties, copper (Cu), also listed as a priority substance from water Framework Directive (WFD), is widely used to protect vineyards from fungal disease. With its intensive use, for both conventional and organic agriculture, Cu is an omnipresent compound in the environment and transfer from soils to aquatic ecosystems is likely to occur (Devez et al., 2005; Ruyters et al., 2013). Despite its essential role in numerous biological processes in living organism, in excess Cu becomes toxic by producing reactive oxygen species (ROS) which might cause cellular damage to lipids, proteins and nucleic acids leading to cell death (Manzl et al., 2004; Prousek, 2007). Previously, first stages of rainbow trout were found to be very sensitive to Cu after exposure for 3-weeks at environmental concentrations (2 and 20 µg/L Cu) (article 1), where inhibitory effect on hatching and significant malformations were observed. Also, several genes were down-regulated in rainbow trout exposed to Cu, especially those involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest (*p53*) genes.

Glyphosate (GLY), a commonly used herbicide and the active molecule of Roundup®, is a broad-spectrum, non-selective and systemic herbicide. Its mode of action consists in the inhibition of the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), the shikimic acid pathway that is common in all plants. Because of its extensive use, especially in genetically modified crops (Van Bruggen et al., 2018),

high concentrations (ranging from 7.52 to 700 µg/L) have been found in different streams and lakes near to agricultural basins (Aparicio et al., 2013; Coupe et al., 2012; Peruzzo et al., 2008). Several reports have highlighted the sub-lethal effects of GLY on fish, and effects have been observed on genotoxicity (Çavaş and Könen, 2007; Guilherme et al., 2012, 2010), on acetylcholinesterase (AChE) inhibition (Salbego et al., 2010), in swimming alterations (Bridi et al., 2017; Valéria D.G. Sinhorin et al., 2014), and antioxidant enzymes activity (de Moura et al., 2017; Glusczak et al., 2011). After 3-weeks of embryo-larval exposure to glyphosate (0.1 and 1 mg/L), rainbow trout larvae exhibited a decreased head size when compared to control larvae. Additionally, behavior was also altered and exposed larvae increased their mobility compared to non-exposed larvae. On the other hand, lipid peroxidation (measured as TBARS levels) were reduced on exposed larvae and it was suggested the intervention of the antioxidant system to counteract the damage caused (article 2).

Chlorpyrifos (CPF) is an organophosphorus insecticide (OP) which was widely used for crop protection and in domestic applications. However, because of its elevated toxicity on humans and animals, its residential use has been restricted (US EPA), and currently there is a declining trend of its global consumption (Zhang et al., 2011). Since it is part of the list of priority substances of the WFD, its presence in surface and groundwater is highly monitored. CPF has been detected mostly in sediments from cultivated areas, nevertheless low concentrations have been observed in streams water where maximum concentrations ranged between 0.06 and 0.45 µg/L in USA and Argentina, respectively (Jergentz et al., 2005; Marino and Ronco, 2005). However, concentrations as high as 10.8 µg/L was observed in Pampa Ondulada from Argentina (Marino and Ronco, 2005). As a OP (organophosphate) insecticide, CPF reacts on the nervous system by inhibiting the AChE enzyme (Barron et al., 1993), disrupting the transmission of nerve impulses at the level of synaptic terminals, impacting essential functions such as respiration and swimming behavior of fish (Fulton and Key, 2001; Van der Oost et al., 2003). In a recent study done with CPF at low concentrations (0.3 and 3 µg/L), rainbow trout embryo and larval viabilities were not affected. However, sub-lethal effects were observed on mobility of larvae which was reduced for those exposed to 3 µg/L compared to control condition. Low concentrations of CPF also down-regulated genes involved in reproduction such as *er-b* and *cyp19a1* (section 3).

These three compounds (Cu, GLY and CPF), which have dissimilar mode of action and function, could be applied all together into crops. Since they are easily transported into water bodies by runoff, spray drifts or groundwater, it could be valuable to consider the toxicity of their combined effect, using realistic environmental concentrations, in aquatic organisms. Indeed, to date data on the effects of pesticides mixtures on fish are still scarce. All three compounds are able to induce oxidative stress leading to induce long-term effects such as genotoxicity, biochemical alterations (AChE inhibition, lipid peroxidation and protein oxidation), endocrine and reproductive alterations. To assess the sub-lethal

effects of the pesticide mixture, the purpose of this study was to evaluate several endpoints after 3-weeks exposure on first developmental stages of fish. Fish are considered as a suitable experimental models in aquatic ecotoxicology, and the use of developmental stages of rainbow trout (*Oncorhynchus mykiss*) presents further advantages as its commercial availability, ease of breeding under laboratory conditions, it has been extensively studied, and it has low tolerance to pollutants. Besides, its slow embryo-larval development allows longer sub-chronic exposure to pollutants. The studied endpoints include survival, hatching delay, hatching success, morphological anomalies, swimming behavior, genotoxicity (measured by the comet assay), lipid peroxidation and protein carbonyl content, and gene transcription levels. The expression levels of ten genes were selected according to their biological function that are involved in antioxidant defenses (*cat*, *sod*), detoxification (*mt1*, *gst*), mitochondrial metabolism (*cox1*, 12S), cholinergic system (*ache*), DNA repair (*ogg1*, *rad*) and apoptosis (*bax*).

2. Materials and methods

2.1. Test chemicals

Chlorpyrifos-ethyl (CAS No. 2921-88-2) was purchased from ChemService (England). Copper sulfate ($CuSO_4 \cdot H_2O$, CAS No. 7758-99-8, 99.99 %) was purchased from Sigma Aldrich (Lyon, France). Glyphosate was purchase as a commercial formulation (available on the market) Roundup®GT Max. The active substance of Roundup®GT Max is 480 g/L of glyphosate acid which is equivalent to 588 g/L of potassium salt of glyphosate.

2.2. Exposure system

Eyed-stage embryos rainbow trout (*Oncorhynchus mykiss*) were purchased from INRA-PEIMA (Sizun, FR). Embryos were exposed for 3 weeks from stage 240 DD (Degree Days) to larvae stage 500 DD at 12 ± 0.5 °C. Stock solutions of copper (0.67, 2 and 20 mg/L) and glyphosate (33.3, 100 and 1000 mg/L) were prepared using osmosis water. Stock solutions of chlorpyrifos-ethyl (33.3, 100 and 1000 mg/L) were prepared using DMSO (dimethyl sulfoxide) as a solvent. Each condition (control-solvent, mixtures conditions) contained 0.0003 % DMSO.

Prior embryonic exposure, experimental units and tanks were saturated with chlorpyrifos diluted in distilled water for 2 weeks with the studied concentrations for each condition to ensure saturation of aquaria and avoid dramatic decrease of this compound during exposure. Test solutions were prepared by dilution of stock solutions in a 5 L tank of spring water (dechlorinated) from Laqueuille (4.7 mg/L Ca,

1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, 7.5 pH, <1.2 mg/L Cl^-) oxygenated and were renovated every two days. The experimental conditions were design as follow: condition A (solvent control), condition B (0.1 µg/L of CPF + 0.67 µg/L of Cu + 33.3 µg/L of GLY), condition C (0.3 µg/L of CPF + 2 µg/L of Cu + 100 µg/L of GLY) and condition D (3 µg/L of CPF, 20 µg/L of Cu + 1000 µg/L of GLY). Conditions C (3-fold condition B) and D (10-fold condition C), which agree with the medium and highest concentrations in this study, correspond to the lowest and highest concentrations previously studied (articles 1, 2 et 3). Condition B corresponds to the lowest concentrations in this study.

Each studied condition consisted of 3 replicates with 75 embryos in 1 L aquaria. A peristaltic pump (ISMATEC, ISM942) was used to maintain a continuous flow rate (9 mL/min) of exposure water from tanks into the incubation aquaria. Dissolved oxygen was measured each day with a fiber-optic mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburf, Germany) and data was recorded with OxyView v6.02 software (PreSens Precision Sensor).

2.3.Chemical analysis in water

Water samples were collected at T_0 (at the moment of exposure), T_{24} and T_{48} (before water was changed). Water samples were analyzed to determine Cu concentrations. 40 mL of water sample of each condition were acidified with 5 % of nitric acid (Nitric acid 65 %, Fluka). Copper concentrations in water of condition “20 µg/L” and fish samples were analyzed by inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES 720, Agilent Technologies), whereas copper concentrations in water of controls and “2 µg/L” conditions were analyzed by atomic absorption spectrophotometer (Varian SpectrAA 240Z, Agilent Technologies, Santa Clara, USA). Detection limits (DL) for ICP-OES was 2.26 µg/L Cu^{2+} and for atomic absorption was 0.5 µg/L Cu^{2+}. Water samples were analyzed to determine glyphosate concentration and its main metabolite aminomethylphosphonic acid (AMPA) using the method described by (Fauvelle et al., 2015). These samples were analyzed using an HPLC-ESI MS MS (Dionex Ultimate 3000, Thermo Fisher scientific – API 2000 ABSciex). Concentrations were determined with a calibration curve from 1 to 10 µg L^{-1}. To ensure accuracy of each analysis, isotope-labeled surrogates was quantified, derivatization blanks were analyzed and quality controls were performed every 10 samples during analysis. Finally, data treatment was performed with Analyst V1.6.2

2.4. Embryo-toxicity assay

Dissolved oxygen was supervised every day. Embryos and larvae viability was recorded daily and dead embryos were removed. Embryonic and larval viability denote the number of living individuals compared to the total number of embryos at the start of the experiment or total number of hatching larvae. Hatching rate is calculated by dividing the number of hatched embryos by the total number of embryos at the beginning of the experiment. Duration of development expressed in degree-days (DD) is the duration of embryonic development from fertilization to hatching. At the end of the experiment, 15 larvae were placed individually in Petri dish with ice water and few drops of carbonated water to sedate them. Photos were taken for each larvae with stereomicroscope (MZ 7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). From the photos, total body length, head length and yolk-sac area were measured for each larva. Larvae were also observed for the presence of developmental anomalies (including edemas, yolk-sac absorption, spinal malformations, craniofacial anomalies, presence of hemorrhages).

2.5. Swimming behavior analyses

Swimming behavior analyses was performed on 10 larvae per replicate at the end of the 3-weeks exposure. For 30 min, larvae were acclimated in the dark in 6-well microplates containing 3 mL of exposure solution at 12.0 ± 0.5°C. After acclimation, the microplates were placed in the recording chamber (Daniovision Image Analysis System with Ethovision software version 12.0 Noldus) coupled to a thermoregulatory system set at 12 ± 0.5°C (Pilot one®, Huber). Larvae were subjected to a light stress with a cycle of 30 min (10 minutes dark, 10 minutes light and 10 minutes dark). With this cycle, photomotor response of larvae was analyzed in response to light stimulation. An infrared camera records the motion of each larvae focusing in their center of gravity. The Ethovision software recorded the average velocity every 30 seconds, and average velocity, total distance swam, time of mobility was determined for each condition.

2.6. Genotoxicity test with modified enzyme test

Comet assay was performed on blood cells, sampled by decapitation of 6 larvae per replicate using a heparinized pipette. Prior decapitation, larvae were sedated using ice water and a few drops of carbonated water. Samples were immediately frozen in liquid nitrogen in microtubes with 200 µL of cryoconservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) until analysis. To improve the sensibility of the comet assay, slices were incubated

with formamidopyrimidine glycosylase (Fpg) enzyme for 30 min at 37°C, as described by (Kienzler et al., 2012). The comet assay was assessed following Le Bihanic et al. (2014) and Weeks et al. (2019). After 20 min of DNA unwind in alkaline solution, electrophoresis was realized with a voltage of 25 V and 300 mA for 20 min. After test, slides were stained with 20 µg/mL of ethidium bromide solution, and comet lecture was done using an epifluorescence microscope using the Olympus BX51 microscope (zoom x20) equipped with an Olympus U-RFL-T reflected fluorescence system lamp. 100 nuclei per slide were quantified using the Comet Assay IV software (Instrument Perspective LtD). Results are expressed as percentage of degradation of DNA tail.

2.7. Biochemical analysis

Preparation of supernatant

Pools of 2 larvae (approximately 250 mg) were homogenized in a phosphate buffer (0.1 M; pH 7.5; 4°C) using a UltraTurrax® tissue homogenizer with a potter at 9000 g at 4°C. The supernatant S9 fraction was obtain after centrifugation at 9000 rpm for 25 min at 4°C. The S9 fraction was placed in several tubes for total protein, TBARS and carbonyl protein analysis.

Total protein

The protein content was determined according to the method described by Lowry et al. (1951) on the S9 fraction. Measurements was performed using bovine serum albumin as standard, and absorbance was recorded at 750 nm using a spectrophotometer microplate reader (Synergy HT, BioTek).

Lipid peroxidation (TBARS)

Lipid peroxidation was performed as reported in the method of Buege and Aust (1978) adjusted to a microplate reader. A volume of 500 µL of a solution that contained 20 % of butylated hydroxytoluene (BHT) and 20 % of trichloroacetic acid (TCA) was added to 500 µL of S9 fraction. Mixture was centrifuged at 9000 g for 10 min. Then, 600 µL of the supernatant was added to 120 µL of HCl and 480 µL of TRISbase (25mM)-TBA (thiobarbituric acid – 100mM) and heated at 80°C for 15 min. Afterwards, samples were cooled in ice water and mixed. The absorbance of the mixtures was measured using a UV-spectrophotometer (Synergy HT, BioTek) at 530 nm. Results were expressed as nmol of thiobarbituric acid reactive substance (TBARS) equivalents per mg of protein.

Protein carbonyl assay

Protein carbonyl content was performed by the procedure described in Augustyniak et al. (2015). S9 fraction (500 µL) was added to 50 µL of a solution of streptomycin sulfate (11 %) - phosphate buffer

(100 mM pH 7.4), mixed and incubated for 15 min at a room temperature. Mixtures were centrifuged at 6000 g for 10 min. Then, mixtures were divided into two tubes, one used as a control tube where 200 µL of supernatant was reacted with 800 µL of HCl (2.5 M), and the other tube used as a sample where 200 µL of supernatant reacted with 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 Mm), and left incubated for 1 h. Proteins were precipitated with 20 % TCA (trichloroacetic acid), and the formed pellets were washed 3-times by resuspension with 1 mL of ethanol-ethyl acetate (v:v). Then, pellets were solubilized with 500 µL of 6 M guanidine hydrochloride and centrifuged at 10000 g for 10 min. The measure of carbonyl content was performed with a UV-spectrophotometer (Biotek Synergy HT) at 370 nm. Results were expressed as nanomoles of DNPH incorporated/mg of protein, using the molar absorption coefficient of 22000 $M^{-1}cm^{-1}$.

2.8.Gene expression

At the end of exposure, 6 larvae per replicate were sampled and kept individually in RNA later buffer (Qiagen). Samples were stored at -80°C.

RNA extraction

Total RNAs were extracted in whole larvae using the kit SV Total RNA Isolation system" (Promega) following the indications of the provider. Larvae were mixed and homogenized with the MP fastprep®-24 (Biorad, 6 m/s, 40s) using ceramics beads (MP Biomedicals, Lysing Matrix D bulk).

Retro-transcription of total RNAs into cDNA

The reverse transcription of total purified RNAs was performed using the kit "GoScript Reverse Transcription System" (Promega). A mixture of 1 µg of oligo dT (1 µM), 1 µL of hexanucleotides (1 µM) and 10 µL of total purified RNA (1 µg) were mixed and heated for 5 min at 70°C followed by 5 min at 4°C with a thermocycler (Eppendorf Mastercycler) to let primer annealing performed. Afterwards, mixtures were added 1 µL of dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of $MgCl_2$ (25 mM), 1 µL of reverse transcriptase (1 U/µL) and 0.5 µL of RNAsine. Then, the reverse transcription was realized using the thermocycler at 42°C for 1h. The obtained samples (cDNA) were kept at -20°C until analysis by quantitative real-time PCR.

Quantitative real-time PCR

The studied primers were designed using the Primer3plus software (Table 1). Real-time qPCR was performed using GoTaq® qPCR Master Mix kit (Promega). Each reaction mix was consisted on 1 µL of cDNA of samples, 2 µL of specific primer pairs mix (200 µM each) and 17 µL of a mix consisted of

Nuclease-Free Water and GoTaq® qPCR Master containing SyberGreen fluorescent dye. Real-time PCR reactions was done in a Mx3000P® qPCR system (Stratagene), and the amplification program was one cycle at 95°C for 10 min, then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity was obtained by the dissociation curve of the PCR products, and the dissociation curve was acquired by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C.

For each gene, the cycle thresholds (Ct) were obtained from the software MxPro™ qPCR. Two reference genes (*rpl7* and *ef1α*) were used, and level of gene expression was normalized with the mean of Ct value of reference genes according to the method of $2^{\Delta Ct}$ explained by Livak and Schmittgen (2001). Induction (>2) or repression (<0.5) factors were determined by the ratio of transcription levels of each condition and the control. The list of studied genes and primers pairs are presented in Table 1.

2.9.Statistics

Each condition was realized in 3 replicates which were considered as independents samples. Results are presented as mean ± SD (standard deviation). Normality of data distribution was verified by the Shapiro-Wilk test ($p < 0.01$), and the homogeneity of variances by the Levene test ($p < 0.05$). If data follow a normal distribution, a one-way ANOVA analysis was used ($p < 0.05$) followed by the Tukey post-hoc test. If no normality was accomplished, the non-parametric test of Kruskal-Wallis ($p < 0.05$) was used. All statistical analysis was performed using R (http://cran.r-projet.org/).

Table 1: Accession number and specific primer pairs for the Oncorhynchus mykiss used in our study.

Gene	Accession number	Primer (5' – 3')
rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG[a] GTCCAGGATGGGAAGTTGC[b]
sod Cu/Zn	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
gst	BT073173.1	ATTTTGGGACGGGCTGACA[a] CCTGGTGCTCTGCTCCAGT[b]
cox1	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12S	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
mt1	M18104.1	GTGGATCCTGCAAGTGCTCA[a] GTAATGCACCAGGCCTCACT[b]
ache	XM_021577686	AGGAGGGTTCTACAGCGGAT[a] TATCCTGGACCCACTGGAGG[b]

[a]Forward primer

[b]Reverse primer

3. Results

3.1. Condition of exposure

Table 2 reveals the measured concentrations of CPF (chlorpyrifos), GLY (glyphosate) and Cu (copper). Samples solutions of each condition were analyzed between water changes at T_0, T_{24}, T_{48} to estimate the losses of the three compounds. GLY was treated with a metal complex EDTA (ethylenediaminetetra-acetic acid), and Cu was acidified with HNO_3 to analyze the complexes between Cu-GLY. In all conditions, the measured concentrations of CPF were inferior to the nominal concentrations at T_0, being the condition B below the detection limit. At T_{24}, from condition C, CPF was below detection limit, and at T_{48} no CPF was detected. From condition D, CPF was reduced 93.9 and 97 % at T_{24} and T_{48} respectively. In the case of GLY, measure concentration was superior to the 3 nominal concentrations at T_0, and also reductions of GLY were observed at T_{24} and T_{48} but always higher than the nominal concentration. For condition D, GLY concentrations at T_{24} and T_{48} were higher than the concentration from T_0, and we attributed this to phenomenon of adsorption and desorption from the exposure system.

3.2. Embryonic and larval survival

Dissolved oxygen varied from 84 to 94.6 % throughout the embryonic and larval exposure. After 3-weeks exposure to mixture of pesticides, no significant mortality was observed on embryos and larvae from all studied conditions (Table 3). Embryonic and larval survival reached more than 95 % in all conditions. Hatchability was high and more than 90 % of embryos succeeded to hatch. No significant differences were also observed on the duration of development.

3.3. Phenotypic effects

Biometric measurements included total size, head size and yolk-sac area, and no significant alterations were observed on larvae exposed to mixtures of CPF, Cu and GLY compared to non-exposed larvae (table 4). Similarly, no significant anomalies were registered at the end of 3-weeks exposure on larvae, where percentages of total malformations observed for each condition were 15.0 ± 2.8, 23.7 ± 3.6, 15.0 ± 7.8 and 22.8 ± 11.3 % for control and conditions B, C and D, respectively (Table 3).

Table 2: Nominal and measured concentrations of chlorpyrifos (CPF), copper (Cu) and glyphosate (GLY) for each condition. Concentrations of samples were analyzed at T_0, T_{24} and T_{48}.

	CPF (µg/L)[1]			Cu^{2+} (µg/L)[2]			GLY (µg/L)[3]			
	Nominal concentration	Measured concentration		Nominal concentration	Measured concentration		Nominal concentration	Measured concentration		
					No acidified	Acidified (HNO_3)		No EDTA		EDTA
Condition B	0.1	T_0	0.01	0.67	T_0	<LQ	33.3	T_0	63.3	64.7
		T_{24}	0.01		T_{24}	<LQ		T_{24}	67.5	49.2
		T_{48}	0.01		T_{48}	<LQ		T_{48}	55.4	42.2
Condition C	0.3	T_0	0.18	2.0	T_0	2.03	100	T_0	160.0	175.8
		T_{24}	0.01		T_{24}	4.16		T_{24}	140.0	168.0
		T_{48}	0.00		T_{48}	4.81		T_{48}	154.0	137.25
Condition D	3.0	T_0	1.63	20.0	T_0	19.63	1000	T_0	1345.0	1785.0
		T_{24}	0.1		T_{24}	26.32		T_{24}	1645.0	2022.5
		T_{48}	0.05		T_{48}	26.65		T_{48}	1775.0	1790.0

[1]Limit of quantification: 0.13 µg/L, limit of detection: 0.04 µg/L.

[2]Limit of quantification: 1.1 µg/L, limit of detection: 0.1 µg/L.

[3]Limit of quantification: 30, 600 and 6000 ng/L

Table 3: Effects on viability and development of early life stages of rainbow trout exposed to a mixture of pesticides. Values represent Mean ± SD. No significant differences were observed, N = 3, ANOVA.

	Control- A	Condition B	Condition C	Condition D
Acute toxicity				
Embryo viability (%)	98.7 ± 2.3	96.8 ± 3.4	96.4 ± 1.5	94.2 ± 4.1
Larval viability (%)	97.7 ± 2.1	94.5 ± 4.8	96.0 ± 3.2	95.2 ± 4.9
Cumulative viability (%)	96.4 ± 0.8	91.7 ± 7.3	92.6 ± 3.1	89.5 ± 3.5
Hatching rate (%)	95.6 ± 2.0	92.0 ± 4.6	92.0 ± 4.8	90.2 ± 4.1
Sub-lethal toxicity				
Duration of development (DD)	301.1 ± 2.2	294.9 ± 12.2	305.7 ± 1.1	309.9 ± 8.3
Total length (mm)	19.5 ± 0.3	19.6 ± 0.2	19.8 ± 0.2	19.1 ± 0.2
Head length (mm)	4.5 ± 0.0	4.5 ± 0.1	4.6 ± 0.0	4.3 ± 0.2
Ratio of head/body length (%)	23.2 ± 0.1	23.1 ± 0.2	23.1 ± 0.1	22.4 ± 0.7
Area of yolk sac (mm^2)	10.8 ± 0.2	10.1 ± 0.4	10.4 ± 0.5	10.5 ± 0.1
Abnormalities (%)				
Total deformed larvae	15.0 ± 2.8	23.7 ± 3.6	15.0 ± 7.8	22.8 ± 11.3
Oedemas	7.5 ± 8.4	12.7 ± 6.2	9.3 ± 5.6	11.9 ± 6.5
Spinal	7.5 ± 8.4	16.6 ± 11.2	12.9 ± 11.3	16.5 ± 14.8
Craniofacial	1.8 ± 3.2	5.4 ± 5.3	11.3 ± 4.5	14.0 ± 4.5
Haemorrhages	7.6 ± 6.6	0.0 ± 0.0	9.3 ± 5.6	11.9 ± 6.5

3.4.Swimming behavior

Figure 1 presents the results of swimming behavior of larvae. The results correspond to the average velocity (mm/s) and to the mobility (s) of larvae subjected to a light stress. Average velocity did not show any significant difference between conditions, only a slight increase between condition A and C (p-value = 0.052) was observed at the basal period (dark 1, Figure 1-A). On the other hand, larvae exposed to the condition C significantly increased their mobility compared to larvae from condition A ($p < 0.05$) when larvae were not subjected to the light stress (dark 1 and 2, Figure 1-B). No significant differences were observed when larvae were subjected to the light stress a cause of their high variability.

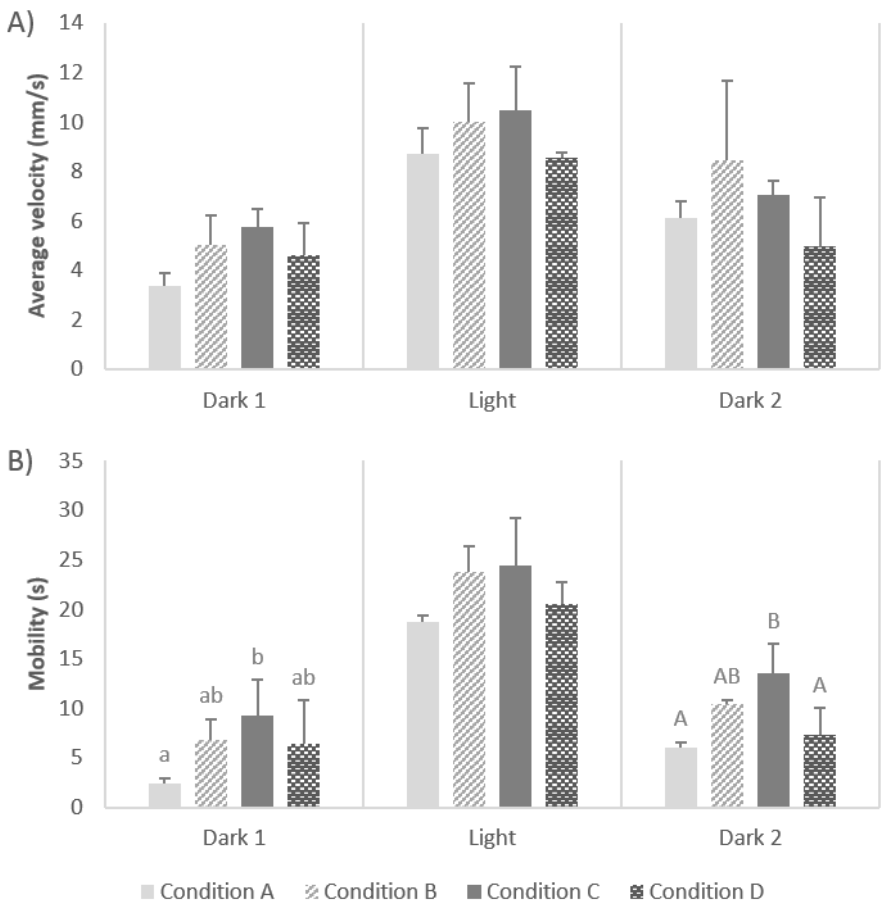

Figure 1: Velocity average (mm/s) (A) and cumulative time of mobility (s) (B) of larvae following exposure to mixture of pesticide. Different letters indicate significant differences between conditions. Values represent Mean ± SD (N = 3, ANOVA, $p < 0.05$).

3.5.Genotoxicity

Results of comet assay from blood cells are presented in Figure 2 for all studied conditions, treated or not with Fpg enzyme prior the alkaline treatment. Fpg treatment was used to increase the sensitivity of the test. No significant differences on DNA damage were observed in all conditions when determined by the classical comet assay, where DNA damage ranged between 3.6 and 6.4 % of tail intensity. When cells were treated with Fpg enzyme, an increase of DNA damage was observed in all conditions. Results evidenced in this case a significant difference between control (16.2 ± 3.0 %) and larvae from condition D (23.3 ± 1.5 %).

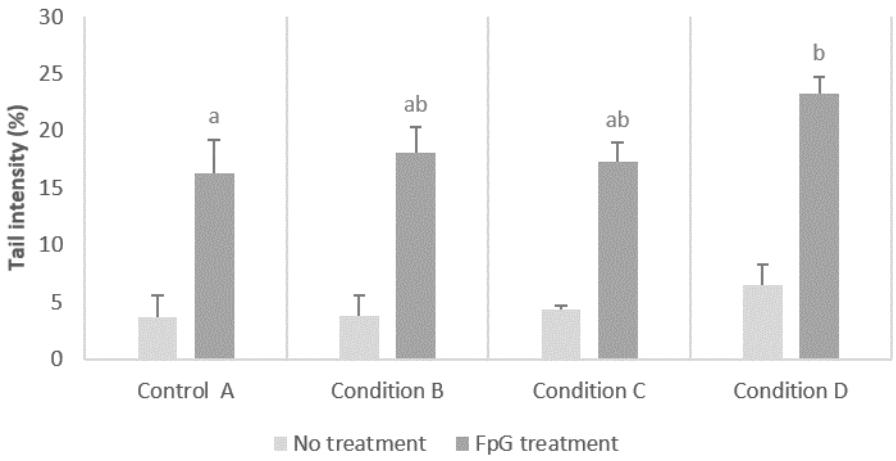

Figure 2: DNA damage on blood cells from rainbow trout larvae after exposure to mixture of pesticides (CPF, Cu and GLY), with- and without treatment of Fpg enzyme. Different letters indicate significant differences between conditions. Absence of letters indicate no significant differences (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.6.Lipid peroxidation and protein carbonyls

TBARS levels and protein carbonyl contents are presented on Figure 3. A significant reduction of TBARS levels was observed on whole-larvae from condition D when compared to control (Figure 3-A). However, no significant alterations in protein carbonyl contents was observed on larvae from all conditions when compared to non-exposed larvae (Figure 3-B).

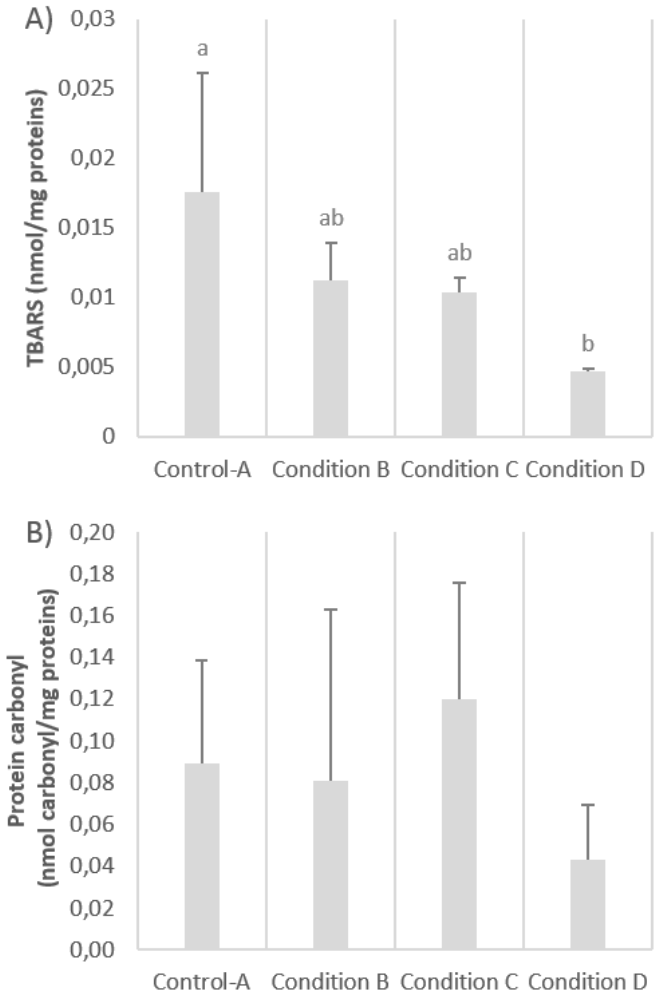

Figure 3: Lipid peroxidation expressed as nanomoles of TBARS/mg of protein (A), and protein carbonyl expressed as nanomoles of carbonyl/mg of protein (B) performed on whole larvae of rainbow trout exposed to mixture of pesticides (GLY, Cu and CPF). Different letters indicate significant differences between conditions. Absence of letters indicate no significant differences. Values represent Mean ± SD (N = 3, ANOVA, $p < 0.05$).

3.7.Gene expression

Gene expression levels from whole-larvae of rainbow trout revealed significant up-regulations in all conditions of pesticide mixture compared to control condition after 3-weeks exposure (Table 4). Mixture of GLY, Cu and CPF resulted in an induction of genes involved in detoxification (*gst* and *mt1*), DNA repair (*ogg1*), mitochondrial metabolism (*cox1* and *12S*) and cholinergic system (*ache*). High variability on gene transcription was observed on genes involved in oxidative stress (*cat*, *sod*) and not significant differences were observed. Gene involved in apoptosis (*bax*) was neither regulated. A strong genetic induction was remarked on larvae from condition D indicating the impact of strong concentrations of pesticides on the genetic response.

Table 4: Transcription levels of whole-larvae of rainbow trout after exposure to mixture of CPF, Cu and GLY. Data was expressed as induction (above 2) or repression (below 0.5) factors compared to the control condition. Asterisks refer to significant differences compared to control condition (N = 3, ANOVA, $p < 0.05$).

	Condition B	Condition C	Condition D
Cat	/	/	/
Sod	/	/	/
gst	2.5*	/	/
Cox1	5.3*	3.8*	23.8*
12s	4.9*	5.2*	32.4*
ogg1	4.4*	6.3*	34.8*
rad51	3.5	/	/
bax	/	/	/
mt1	9.4*	/	12.7*
ache	4.5*	7.0*	36.7*

4. Discussion

Numerous reports evidenced that most aquatic ecosystems, that are near agricultural lands, are usually contaminated with diverse kinds of pesticides at different concentrations which can have an impact on aquatic biota (Beyer et al., 2014; Relyea, 2009; Vidal-Dorsch et al., 2011). Commercial formulations that include copper (Cu), chlorpyrifos (CPF) and glyphosate (GLY) are widely used for the treatment of cultures and are frequently detected in wetlands and streams, usually transported by runoff (Aparicio et al., 2013; Coupe et al., 2012; Davis et al., 2001; Devez et al., 2005; Phillips and Bode, 2004). Therefore, the interaction between these molecules can lead to modifications of their overall toxicity as a function of the synergistic and/or antagonistic effects (Krishnan and Brodeur, 1994). Each studied compound has a different mode of action and its interaction may implicate an induction or an inhibition of a specific metabolic pathway (Krishnan and Brodeur, 1994).

Our results show no significant mortality of embryos and larvae of rainbow trout exposed to a mixture of Cu, CPF and GLY. In previous studies performed with individual pesticides, no mortality was observed on early life stages of rainbow trout exposed to GLY (article 2) and CPF (article 3). However, a significant embryonic mortality (about 10 %) was observed for embryos exposed to 20 µg/L of Cu (article 1). Additionally, a significant high mortality of half-hatched embryos (25 %) was also observed for those exposed to 20 µg/L of Cu. In this study, the addition of CPF and GLY seems to antagonize the toxicity of Cu for embryos and larvae of rainbow trout. This results could be explained by possible interactions between compounds. Indeed, GLY is known to be a strong chelator of heavy metals such as Cu (Glass, 1984; Subramaniam and Hoggard, 1988). The functional groups that formed GLY (amine, carboxylate and phosphate) react with metal ions to form complexes resulting in a decreased bioavailability of Cu (Tsui et al., 2005; Zhou et al., 2012). Our findings agree with similar studies that have been done with freshwater cladocera (*Ceriodaphnia dubia*) and earthworms (*Eisenia fetida*). Neonates of *C. dubia* were exposed to seven heavy metals, alone and in binary mixture with Roundup®, and most of the metals displayed less than additive toxicity (Tsui et al., 2005). At the same study, LC_{50}-48h for *C. dubia* was estimated at 10 µg/L of Cu^{2+}, but in the presence of GLY mortality was reduced at 95 %. GLY also influenced the bioaccumulation of metals differently, where it favored the uptake of Hg and decreased the uptake of Ag (Tsui et al., 2005). Zhou et al. (2012) experimented the interactions between Cu and GLY on the toxicity of earthworm (*Eisenia fetida*). Acute toxicity of Cu for *E. fetida* was calculated at 0.11 mg/L (LC_{50}-48h), but when it was combined with GLY at concentrations of 0, 2 and 10 mg/L, the worm mortalities were 57, 3 and 0 % respectively. The authors observed that the free Cu^{2+} were reduced with the presence of GLY preventing the accumulation of Cu in worms.

When we studied the swimming behavior of rainbow trout larvae exposed to single pesticides, we observed that GLY (100 µg/L) induced hyperactivity of larvae increasing their velocity and mobility under light stimulation (see article 2). Similar observations were obtained by Ayoola (2008); Sinhorin et al. (2014); Zhang et al. (2017) in different fish species. On the other hand, larvae that were exposed to CPF (3 µg/L) were significantly less mobile than non-exposed larvae (see article 3), and comparable results were observed by Kienle et al. (2009) and Richendrfer et al. (2012) usually related to a decreased of acetylcholinesterase (AChE) activity (Bonansea et al., 2016; Sandahl et al., 2005; Yen et al., 2011). In this study, at the basal level, larvae exposed to condition B and C (lowest and medium mixtures concentrations) were significantly more mobile than control larvae. Mobility of larvae exposed to condition D were not different to controls and no differences were also observed on the response to light stimulation a cause of high variability of response of larvae. Bonifacio et al. (2016) studied similar concentrations of CPF (0.1 to 1 µg/L) and GLY (0.2 to 2 mg/L) to expose adult females of ten spotted livebearer fish (*Cnesterodon decemmaculatus*) for 6-weeks. They observed that fish exposed to only CPF reduced their swimming activity, but no differences were observed on fish exposed to GLY and binary mixtures, arguing that GLY decreases the effect of CPF on swimming activity. In another study, the addition of Cu also diminished the impact of CPF on swimming behavior of adult zebrafish (*Danio rerio*) exposed to a mixture of Cu (6.3 to 40 µg/L) and CPF (35 to 220 µg/L) for 24 h (Tilton et al., 2011). Indeed, decreases in swimming rate was observed on zebrafish exposed to the strongest concentration of CPF suggesting that the addition of Cu may block biochemical neurological impacts of CPF or modify its uptake on action at sensitive neuronal sights. Responses of hyperactivity and hypoactivity were both observed on females of *Jenynsia multidentata* exposed to a binary mixture of CPF (0.4 and 4 µg/L) and cypermethrin (0.04 and 0.4 µg/L) for 24 and 96 h, where low mixture concentrations increased significantly their swimming activity in the upper area of the aquaria and higher mixture concentrations reduced their swimming activity showing also a preference of the bottom area of the aquaria (Bonansea et al., 2016). Alterations of normal behavior may also depend of the ratio of the concentrations of pollutants that are present in a mixture. For example, Kienle et al. (2009) exposed mixtures of CPF, at 0.25 and 1 mg/L, and nickel chloride ($NiCl_2$), at 7.5 and 15 mg/L, to zebrafish embryos for 11 days. When fish were exposed to individual compounds, opposed behavior was observed: CPF increased their swimming activity and nickel decreased it. Interestingly, when nickel was combined to low concentration of CPF, the swimming activity of larvae was also decreased; but combined with higher concentrations of CPF, larvae increased their activity. Kienle et al. (2009) argued that both compounds have different mode of action and in a mixture they might act independently of each other.

We can suppose that the swimming behavior of hyperactivity observed in our larvae may be an effect caused mostly by the dominance of GLY (in accordance with our results from article 2). However,

since there is an absence of significant response of activity to light change of larvae exposed to mixtures of pesticides we can assume that the presence of Cu and CPF antagonized or reduced the effect of GLY on larvae.

When embryos of rainbow trout were exposed separately to Cu, GLY and CPF, no significant increases of DNA damage on blood cells were observed after 3-weeks exposure compared to non-exposed larvae (see article 1, 2 and 3). Blood cells from larvae exposed individually to GLY and CPF were also treated with Fpg (formamidopyrimidine DNA glycosylase) enzyme (article 2 and 3) but no significant changes were observed. Fpg is a DNA base excision repair enzyme that allows to detect and remove lesions related to abasic sites (apuric or apyrimidic), alkylation and oxidative damage (8-oxoguanine) induced by Reactive Oxygen Species (ROS) (Kienzler et al., 2012). In this study, the addition of Fpg enzyme reveal additional lesions in particular for larvae exposed to the strongest mixture concentration (condition D), which presented the highest damage compared to control condition. This might suggest that strong mixture concentration of Cu, CPF and GLY induced earlier genotoxic effect detected by Fpg DNA repair enzyme treatment (Kienzler et al., 2012). DNA damage, assessed by comet assay, have already been observed in aquatic organisms exposed individually to Cu, GLY and CPF using environmental concentrations (Ali et al., 2009; Çavaş and Könen, 2007; Guecheva et al., 2001; Guilherme et al., 2010), but few studies have examined the genotoxicity effects of mixture of pesticides on fish. DNA damage was only observed on freshwater clam hemocytes, *Corbicula fluminea*, when exposed to an herbicide mixture of Roundup® (2 and 10 mg/L) and atrazine (2 and 10 µg/L) for 96 h, but no genotoxicity effects were observed when clams were exposed to herbicides separately using the same concentrations (dos Santos and Martinez, 2014). In another study, a mixture of endosulfan and CPF (0.94 to 1.88 µg/L) also caused high significant DNA damage to erythrocytes of fingerlings of Nile tilapia *Oreochromis niloticus*, after 70-days exposure (Ambreen and Javed, 2018). Genotoxicity of mixtures of metals have been studied using the micronucleus assay on erythrocytes of fish, *Synodontis clarias* and *Tilapia nitolica*, exposed to Cu and zinc (Zn) (Obiakor et al., 2010). In this case, micronuclei frequency was highly increased when fish were exposed to binary mixture compared to exposure of individual metals.

No significant protein carbonyls content was observed on exposed larvae when compared to control group. However, TBARS levels from larvae exposed to condition D were significantly low. Exposure to individual compounds did not enhance TBARS levels on whole larvae after 3-weeks exposure (see article 1, 2 and 3). On the other hand, we observed a significant reduction of TBARS levels on larvae exposed to 0.1 mg/L of GLY (article 2). As we hypothesized previously, the absence of increased TBARS levels and protein carbonyls may be an efficient response of development of

antioxidants systems to defend against the oxidative stress as observed by Ahmad et al. (2000), Glusczak et al. (2011), Marques et al. (2014) on their work.

With regard to the gene expression, 3-weeks exposure to all conditions were mostly associated with the up-regulation of genes and similar patterns were observed in each of the studied conditions. The exposure to the mixture of the three compounds led to the up-regulation of genes involved notably in detoxification, mitochondrial metabolism and DNA repair.

Cu, GLY and CPF are well known to produce individually reactive oxygen species (ROS), and the enzymes catalase (CAT) and superoxide dismutase (SOD) protect the cells from oxidative damage caused by ROS. In our study, no significant regulation of these genes were observed on exposed larvae because of their elevated variability. However, many studies showed evidence of the capability of these compounds to induce these antioxidant genes expressions (Benedetto et al., 2016; Casanova et al., 2013; Topal et al., 2015; Woo et al., 2009). Genes involved in detoxification, *gst* and *mt1*, were induced especially on larvae exposed to the lowest and the highest mixture concentrations. *Gst* (glutathione s-transferase) expression is usually up-regulated when fish are exposed to xenobiotics. In our study, *gst* expression was significantly increased on larvae exposed to the lowest mixture concentration (condition B), and this could indicate that larvae were able to implement mechanisms of defense from low concentrations of pesticides. *Gst* gene has an important role in detoxification of xenobiotic compounds producing endogenous and exogenous molecules that can be eliminated by catalyzing the conjugation or reduced glutathione (GSH) (Mukhopadhyay and Chattopadhyay, 2014). *Mt1* gene encodes the metallothionein proteins, usually induced by metals exposure such as copper, but it has also an antioxidant role (Ruttkay-Nedecky et al., 2013). Metallothionein proteins have the capacity to bind xenobiotic heavy metals through their thiol groups providing protection against metal toxicity and oxidative stress (Ruttkay-Nedecky et al., 2013). In the previous study done with Cu (see article 1), rainbow trout larvae showed a down-regulation of *mt1*, *mt2* and *gst* genes after 3-weeks Cu exposure. However, no significant changes of *gst* gene expression were observed on larvae exposed individually to GLY and CPF (article 2 and 3).

In our study, a significant induction of *cox1* (cytochrome c-oxidase) and *12S* genes expressions were observed in all conditions compared to control larvae. These up-regulations were even stronger on larvae exposed to the highest mixture concentration (condition D). An induction of these genes could reveal a mitochondrial chain disruption from the lowest mixture concentration and therefore the number of mitochondria is increased with the up-regulation of *12S* gene. The role of mitochondria is the production of energy for cellular metabolism by synthesizing ATP (adenosine triphosphate). Since the mitochondria is a major source of ROS (superoxide and hydrogen peroxide) through the disruption

of the electron-transport chain (Chen et al., 2003), the up-regulation of *cox1* could denote an increased demand for energy to combat the effects of contaminants, but this increase could also stimulate ROS production (Achard-Joris et al., 2006; Chen et al., 2003). The observed induction of *cox1* and *12S* genes could mean a high energy demand, in the form of ATP, to defend themselves from ROS production.

We also observed, in all conditions, an induction of DNA repair gene expression, *ogg1*, which is responsible for the removal of 8-oxoguanine as a result of ROS production. The over-expression of *ogg1* on larvae exposed to the highest mixture concentration (x34.8) is probably related with the significant induction of DNA damage that we observed on blood cells after treatment with Fpg-enzyme. Consequently, DNA lesions observed on condition D in blood cells were possibly from oxidative damage.

The up-regulation of DNA repair (*ogg1*) and detoxification (*gst* and *mt1*) genes expressions could reveal that the mechanisms of defense were effectively implemented to avoid the effects of ROS production in cells and DNA. This might be related with the high demand on energy with the up-regulation observed for 12S and *cox1*. When mitochondrial activity is increased, ROS is produced along the respiratory chain (Chen et al., 2003). However, in order to validate this relation, we should study the expression of mitochondrial superoxide dismutase gene (*MnSod*), which transforms the superoxide, from mitochondrial electron-transport chain, into hydrogen peroxide, and the expression of glutathione peroxidase (*gpx*), which is also located on mitochondria and detoxifies the hydrogen peroxide into water.

It was not clear if the observed genes regulations on larvae were a consequence of a toxic effect or an adaptation effect. But, since no significant toxic effects were observed on exposed larvae, as mortality, genotoxicity, lipid/protein oxidation or regulation of apoptotic *bax* gene, we considered that the significant gene up-regulation were mostly an adaptive effect activated to confront the toxic exposure. At higher mixture concentration, the energy demand increases as does the DNA repair, *mt1* and *ache* gene expressions. This increase could mean an intensification of response according to the toxicants concentrations. In addition, it was observed fewer genes regulations on larvae from condition C and D when compared to larvae from condition B, probably indicating that other defenses functions were implemented and therefore, the studied genes were not involved in these adaptive responses. When we compared the pattern of gene expressions from the present work with previous work done with individual exposure of Cu, GLY and CPF (see article 1, 2 and 3), we consider that the joint action of the three pollutants had an additive or a synergistic effect since the observed effects were stronger than the isolated substances.

5. Conclusions

Sub-chronic exposure to a mixture of copper, glyphosate and chlorpyrifos on rainbow trout can induce hyperactivity on swimming behavior and genotoxicity, as when cells are treated with Fpg enzyme a significant increase of DNA damage was observed especially for the strongest mixture concentration. Besides, lipid peroxidation, measured as TBARS, were significantly reduced on larvae from the strongest concentration compared to control larvae. However, several genes were up-regulated especially those involved in detoxification, mitochondrial metabolism, cholinergic system and DNA repair. The up-regulations of genes suggests that exposure to mixtures of Cu, GLY and CPF promotes an adaptation effect since no lethal effects, and no inductions of TBARS and protein carbonyl were observed and no alteration of *bax* (apoptosis) gene transcription were observed. Our results give essential information about the mechanisms.

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Chapitre V

Étude des effets toxiques des échantillons environnementaux (eau et sédiment)

Cette partie de ce travail s'inscrit dans le cadre du projet PhytoCote (Labex Cote). Elle a pour objectif d'évaluer les effets des traitements phytosanitaires appliqués sur la vigne sur la qualité des masses d'eau.

Dans ce travail, nous nous sommes intéressés à l'évaluation du potentiel toxique d'échantillons environnementaux d'eaux de surface et de sédiments prélevés dans un petit cours d'eau, La Livenne, qui reçoit les eaux de lessivage d'un petit bassin viticole du Blayais (Sud-ouest).

Trois campagnes d'échantillonnage ont été menées en février, mai et août 2017. Des échantillons d'eau et de sédiments ont été collectés dans 4 sites situés le long de La Livenne : Menanteau (L1), Parodier (L2), Grand Village (L3) et Vignolles (L4) ; et un site de Reguignon aux Souches (SE) (petit ruisseau qui se rejette dans La Livenne).

Cette étude se divise en deux parties. Une étude de toxicité *in vitro*, sur cellules RTL-W1 exposées à des extraits d'eau ou de sédiments prélevés au cours des trois campagnes de 2017, avec mesure de la cytotoxicité (test MTT) et induction d'espèce réactive de l'oxygène (ERO). Dans la deuxième partie de l'étude, les larves de truites arc-en-ciel nouvellement écloses ont été exposées pendant 48 h à des échantillons d'eau et de sédiment prélevés en mai 2017 (qui corresponde à la saison d'épandage). Différents critères de toxicité tels que la viabilité, la biométrie, l'étude du comportement, la génotoxicité et le niveau d'expression de gènes cible ont été étudiés. L'ensemble de tous ces résultats est présenté dans l'article 5.

Article 5

Evaluation of the toxicity of environmental samples collected near vineyard parcels on rainbow trout larvae (Oncorhynchus mykiss) and Liver cell line rtl-w1

En préparation pour publication

Shannon Weeks Santos, Patrice Gonzalez, Johanna Groussin, Quentin Papin, Christelle Clérandeau, Bettie Cormier, Pierre-Yves Gourves, Bénédicte Morin and Jérôme Cachot

Principaux résultats de l'Article 5

- *Les résultats du test MTT sur les cellules RTL-W1, suggèrent une cytotoxicité des échantillons d'eau de surface de la campagne de février 2017 du site de Vignolles (L4). La campagne d'août a également montré une cytotoxicité légère, mais significative, sur le site de Grand Village (L3). À partir des mêmes échantillons d'eau collectés, aucune production d'ERO n'a été observée dans les cellules exposées.*

- *D'autre part, les résultats du test MTT indiquent que les élutriates de sédiment ne présentaient pas d'effet cytotoxique. Les polluants éventuellement présents dans les sédiments n'étaient pas en quantité suffisante pour provoquer une mort cellulaire importante. Cependant, une production d'ERO induisant un stress oxydant a été observée dans tous les sites mais à différentes périodes d'échantillonnage indiquant une variation temporelle.*

- *Par ailleurs, l'exposition pendant 48 h des larves de truite aux échantillons environnementaux (eaux de surface et sédiments) de La Livenne n'a pas entraîné une augmentation significative de la mortalité.*

- *Le sac vitellin des larves de la condition L4 était moins résorbé par rapport aux contrôles à la fin de l'exposition. La résorption du sac vitellin peut être considérée comme un paramètre important du développement de l'embryon, et le retard de consommation des réserves vitellines a déjà été observé chez les larves de poisson exposées à des polluants tels que les pesticides.*

- *Les larves, selon les conditions d'exposition, présentaient deux types de réponses comportementales. La réduction de la vitesse de nage a été observée pour les larves de la condition L3 par rapport aux autres conditions. En ce qui concerne la réponse des poissons lors de la stimulation lumineuse, les poissons des conditions L1 et L4 présentaient une augmentation significative de leur mobilité par rapport aux témoins et aux larves L3.*

- *Les modifications du comportement pourraient résulter de lésions ou d'inhibition au niveau du système nerveux. Dans notre étude, l'activité de l'AChE n'était pas réduite pour les larves exposées. Cependant, l'expression génique de l'ache était inhibée. Cela pourrait indiquer une perturbation des fonctions neuronales et neuromusculaires. Outre le gène ache, l'expression du gène rad51 a également été inhibée pour les larves exposées. Ce gène possède un rôle important lors de la réparation des cassures double brins de l'ADN.*

Evaluation of the toxicity of environmental samples collected near vineyard parcels on rainbow trout larvae (*Oncorhynchus mykiss*) and Liver cell line rtl-w1

Shannon Weeks Santos, Patrice Gonzalez, Johanna Groussin, Quentin Papin, Christelle Clérandeau, Bettie Cormier, Pierre-Yves Gourves, Bénédicte Morin and Jérôme Cachot

Abstract

Viticulture is one of the agricultural domain that uses the most pesticides. Aquatic ecosystems are usually the final receptacle of all pollutions by leaching, deposition or infiltration; but, because of its capacity of accumulation, sediments represent a reservoir of contaminants larger than the water column itself. The aim of this work was to assess the toxicity of environmental samples, waterborne and sediments, from La Livenne river in hepatic cell lines (RTL-W1) and rainbow trout (Oncorhynchus mykiss) larvae. Samplings were done in La Livenne's watershed near Bordeaux (Southwestern France), a region with a strong presence of vineyards, over three campaigns in February, May and August 2017. Waterborne and sediment samples were collected in 4 sites from La Livenne (Menanteau, Parodier, Grand Village and Vignolles) and one site Reguignon from Les Souches (a small stream highly impacted by viticulture activity that rejects in La Livenne). Pollutants from 1 L water column had been extracted by SPE (Solid Phase Extraction) method, and from sediments had been extracted by elutriates. In the first part of the study, RTL-W1 cells were exposed separately to extracts of water and sediment samples from the three campaigns and different toxicity tests were performed as cytotoxicity (MTT test) and ROS (Reactive Oxygen Species) induction. In the second part of the study, newly hatched larvae were exposed for 48 h to both water and sediment samples collected in May (during spreading season) 2017. Different toxicity criteria as viability, biometry and genotoxicity were studied. Waterborne extractions from Grand Village, Vignolles and Reguignon were cytotoxic but at 10 or 20 times the environmental concentration. No ROS production was observed. In the other hand, cells exposed to sediment elutriates were able to induce ROS; but no cytotoxicity was observed. In the case of rainbow trout larvae, different toxicological endpoints were studied such as genotoxicity (comet assay), lipid peroxidation (TBARS levels), carbonyl proteins, AChE enzymatic activity and gene expression. No mortality was observed after 48 h of exposure. However, differences in biometry was observed on larvae exposed to Vignolles when compared to non-exposed larvae. In addition, larvae from each site presented behavioral alterations where larvae from Grand Village decreased their velocity, and larvae from Menanteau and Vignolles showed hyperactivity increasing their mobility when compared to control condition. Our study demonstrated that environmental samples of water and sediments collected close to vineyards are toxic on *in vitro* and *in vivo* assays of rainbow trout.

Keywords: vineyard, rainbow trout, RTL-W1, toxicity, biomarkers

1. Introduction

Because of their widespread use in agriculture, phytosanitary products are a subject of many debates about their potential environmental impacts and health risks (J. N. Aubertot et al., 2005; Jas, 1998). In 2013, near 14 000 tons of phytosanitary products were used in the Adour-Garonne basin (France) (Adour-Garonne, 2014). Viticulture is one of the agricultural field that use the most of pesticides, and despite the fact that vines account for only 3.7 % of French agricultural surface area, vines are equivalent to nearly 20 % of national pesticide consumption (J. N. Aubertot et al., 2005). Due to its viticulture activity, the Garonne basin is under strong pesiticide pressure. Vineyard soils are generally very degraded and therefore undergo a greater contamination by pesticides (Komárek et al., 2010). These molecules, transported to surface water mainly by runoff, are often detected in waterborne and sediments habitually affecting negatively aquatic organisms such as fish (Ali et al., 2018; Coupe et al., 2012; Davis et al., 2001; Stone et al., 2014).

Many contaminants, such as phytosanitary products, are found transiently or permanently in sediments (Álvarez et al., 2013). In the aquatic environment, sediments represent a greater reservoir of contaminants than the water column itself. The accumulation of these compounds depend essentially on their hydrophobicity, the nature of the sediment, the load in organic matter, the reduction-oxidation potential and its pH, but also in the amount of each contaminant (Álvarez et al., 2013; Taylor and Owens, 2009). Contaminated sediments have a direct impact on biodiversity including benthic species, biofilm and macrofauna by reducing their diversity, structure and richness; but also can have an impact on the species in the water column (Chapman and Wang, 2001; Hyland et al., 2003). Indeed, compounds that are accumulated in the sediments could be released into the water column during resuspension of the sediments especially during floods (Knott et al., 2009; Taylor and Owens, 2009).

It is impossible to monitor all pollutants that may pose a threat to ecosystems (Van der Oost et al., 2003). However, to assess the quality of aquatic ecosystems, it is possible to examine biochemical responses that reflect the capacity of pollutants to alter the physiological processes of exposed organisms (Van der Oost et al., 2003). The analysis of biomarkers not only could provide information about the biological changes, but also provide early-warning signals of potential effects on the organism-level and environmental risks (Chapman and Wang, 2001; Van der Oost et al., 2003). Several of these biomarkers are indicators of mid-term and long-term adverse effects, and knowing this information could allow intervention before the harmful effects are irreversible (Van der Oost et al., 2003). For example, inhibition of the acetylcholinesterase (AChE) activity is one of the better characterized biochemical biomarkers used in ecotoxicology (Bocquené et al., 1995). It is associated with the toxic action of organophosphorus and carbamate pesticides on the nervous system (Bocquené et al., 1995;

Cajaraville et al., 2000). Antioxidant enzymes and lipid peroxidation are wildly studied biomarkers; however, they are more unspecific and can be altered by different pollutants, including pesticides (Valavanidis et al., 2006). Since DNA are susceptible to oxidative damage by reactive oxygen species (ROS), DNA damage is used extensively as a biomarker of genotoxicity and cellular oxidative stress (Valavanidis et al., 2006). Nevertheless, it could be of high interest to integrate these biomarkers with other more comprehensive sub-lethal variables, such as growth and behavior (Castro et al., 2004). Behavioral end-points are considered as individually based responses resulting from the integration of biochemical and physiological processes (Castro et al., 2004).

The aim of this study was to assess the toxicity of water and sediment samples from a small river, La Livenne. For this, two *in vitro* and *in vivo* toxicity assays have been carried out. La Livenne is a tributary of the Gironde estuary in the commune of Blaye, France. This region is characterized by a strong presence of vineyard and the use of large amounts of phytosanitary products. Water and sediment samples from La Livenne were collected at three different campaigns carried out in February, May and August 2017.

In a first step, rainbow trout hepatic cells lines (RTL-W1) were exposed to extracts from water and sediment samples. Two toxicity endpoints were analyzed on cells such as cytotoxicity by methyl thiasolyl tetrazolium (MTT assay) and reactive oxygen species (ROS) induction. The cell line RTL-W1 has been widely used for measuring toxic effects of pollutants through different biomarkers (Bols et al., 2005; Kienzler et al., 2012; Pannetier et al., 2018). It presents advantages, notably ethical, but also high throughput analyses, the quantity almost unlimited of cells, the low volume of test and the standardization of manipulations.

Since fish are constantly exposed to pesticides and other pollutants in the natural habitat, they are a good bioindicator of pollution, and a suitable experimental model to assess the effects of pollutants and environmental samples. For the second experiment, recently hatched larvae of rainbow trout (*Oncorhynchus mykiss*) were exposed for 48 h to both water and sediment samples from La Livenne River collected in May 2017. Different toxicity criteria were studied such as viability, biometry, swimming behavior, genotoxicity (comet assay) and biochemical analyses (Ache activity, lipid peroxidation and protein carbonyls content). In addition, the transcription levels of ten genes were selected according to their biological function in antioxidant defenses (*cat*, *sod*), detoxification (*mt1*, *gst*), mitochondria metabolism (*cox*, *12S*), neuro-cholinergic system (*ache*), DNA repair (*ogg1*, *rad*) and apoptosis (*bax*).

2. Materials and methods

2.1. Sampling sites

Water and sediment samples were collected from the river of La Livenne during the period of pesticide application in crops. La Livenne is a 42 km tributary of the Gironde estuary in the southwest France located in the Marcillac area. The sampling site is located at the level of the Blayais watershed, which is a region characterized by omnipresent of vineyard cultures and the use of a large amount of phytosanitary products. Four sites from La Livenne (Figure 1) were selected to study the contamination and the impact of pesticide inputs in aquatic ecosystem at the vicinity of vineyards: L1- Menanteau (which is the reference site in the upstream), L2- Parodier, L3-Grand Village and L4- Vignolles (the most downstream site). A fifth site in SE- Reguignon (a small tributary of La Livenne) was also studied. Water and sediment samples were collected during three campaigns carried out in February, May and August 2017. These campaigns were carried out before (February), during (May) and after (August) phytosanitary treatments. Sediments (800 g) from the superficial oxic layer were collected on the river bank with an aluminium spatula and stored in aluminium trays. Water (1 L) were sampled using plastic bottles (HDPE) previously submerged in 2 % HNO_3 solution for 48 h and washed 3 times with deionized water. Water and sediment samples for *in vitro* experiments were frozen at -20°C until extractions of pollutants. In addition, another 10 L of water from L1, L3 and L4 were collected in May for *in vivo* experiment. Physico-chemical parameters of water, such as temperature, pH, dissolved oxygen and turbidity using MPx (NKE) multiparameter probe were measured in each site (Table 1).

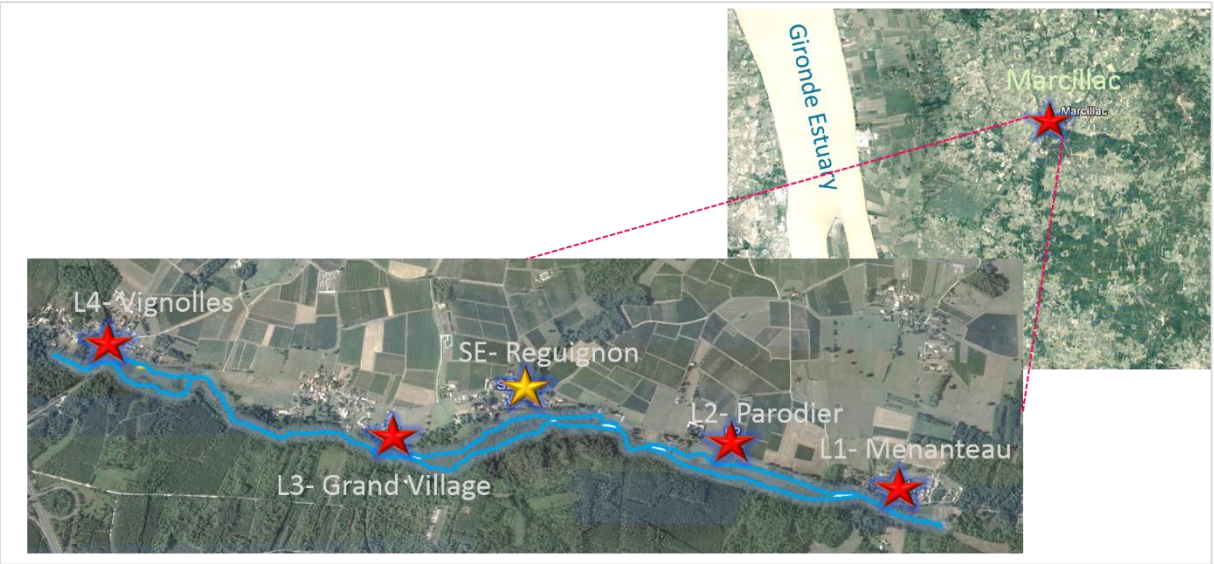

Figure 1: Study area showing the sampled sites: L1- Menanteau, L2- Parodier, L3- Grand Village, L4- Vignolles from La Livenne River, and SE- Reguignon from Les Souches tributary (New Aquitaine Région, Southwest, France).

Table 1: Physico-chemical parameters registered in La Livenne in each period time from each sampled site. L1-Menanteau, L2-Parodier, L3-Grad Village, L4-Vignolles, SE-Reguignon.

Site	Date	Temperature	pH	Oxygen (mg/L)	Conductivity (µS/cm)	Turbidity	Flow (L/s)
L1	2/2017	8.6	7.8	10.8	/	11	124.6
L2	2/2017	8.4	7.8	10.9	/	45	
SE	2/2017	8.7	6.9	10.6	/	0	4.8
L3	2/2017	7.7	7.4	11.1	/	14	
L4	2/2017	7.2	7.4	11.3	/	11	147.5
L1	5/2017	16	7.95	8.65	87	/	72.8
L2	5/2017	15.5	7.65	8.8	86.5	/	
SE	5/2017	14.3	6.85	8.55	83.5	/	1.19
L3	5/2017	15.1	7.3	8.2	80	/	
L4	5/2017	15.3	7.7	8.85	88.4	/	69
L1	8/2017	19.3	7.82	7.5	316	/	41.7
L2	8/2017	19.2	7.82	7.48	306	/	
SE	8/2017	18.5	7.39	16.6	196.4	/	0.25
L3	8/2017	18.8	7.74	11.3	390	/	
L4	8/2017	19.4	7.88	106	338	/	5.7

2.2. In vitro toxicity assay on rainbow trout cells

2.2.1. Pollutants extraction by Solid-Phase Extraction (SPE)

From 1 L of water sample from water column, pollutants were concentrated by Solid-Phase Extraction (SPE) from 1 X (environmental concentration) to 10,000 X in 100 µL of DMSO. Water samples were filtered through 0.8 µm membrane (cellulose acetate, Whatman®) and then pass through an OASIS HLB 500 mg solid-phase extraction cartridges, previously washed with 5mL of methanol and 5mL of Milli-Q water. The pollutants were eluted with 10 mL of methanol, then evaporated with a nitrogen flow, and finally solubilized in 100 µL of DMSO.

2.2.2. Preparation of sediment elutriate

The hydrophilic fraction of pollutants from the sediment was extracted with deionized water. The sediment samples were mixed with deionized water in a 1:4 and 1:1 (sediment: water, v/v) ratio based on the wet weight of sediment. The mixes were homogenized overnight in 50 mL Falcon® on a rotary shaker table at 190 rpm. After mixing, the samples were centrifuged for 15 min at 12,000 rpm and at

4°C. The supernatant, or the elutriate sample, was immediately removed and stored at 4°C for the *in vitro* toxicity test. Both cytotoxicity and ROS tests were performed within 24 h of elutriate preparation.

2.2.3. Cell exposure

Hepatic cell line derived from adult rainbow trout, RTL-W1 (L E J Lee et al., 1993), was used to study the toxicity of environmental samples. The cells were cultured in 75 cm^2 flask (Cell start® cell culture Flask Greiner) with L15 Leibovitz medium culture supplemented with 5 % FBS (Fetal Bovine Serum) and 1 % of Penicillin/Streptomycin (100 IU/mL) at 20°C without CO_2. All toxicity tests were conducted with cells from passage 65-72.

The exposure media from sediment samples was prepared with dilutions of 10 % of elutriates on medium culture. In the case of water samples, SPE extracts were diluted in water with 0.1 % final DMSO solvent, and the concentrations obtained were from 1X (environmental concentration) to 10 X. Control (medium) and solvent control (0.1 % DMSO) were also analyzed for each microplate.

The cell density was 200,000 cells/mL and were seeded 24 h before chemical exposure in 96- and 48-wells culture plates for cytotoxicity and ROS test, respectively. Cytotoxicity and ROS test were performed in triplicates.

2.2.4. MTT assay for cytotoxicity evaluation

The cytotoxicity test was performed by the MTT test using free L15 medium containing 10 % of 3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). After 24 h of water or sediment extracts exposure, cells from each well were rinsed with PBS and 100 µL of MTT solution were added. Then, the microplates were left one-hour incubated in the dark to allow cells to reduce MTT tetrazolium salts to MTT formazan product. Subsequently, MTT solution was discarded and isopropanol solution (4 % 1N HCl) was added for 15 min and left on a rotatory shaker table at 190 rpm to allow formazan crystals to dissolved. The formazan coloration was quantified in a Bio-Tek Synergy HT spectrophotometer at 570 nm and results were expressed as the percentage of mean absorbance compared to the solvent control.

2.2.5. Reactive Oxygen Species (ROS) production

To detect ROS production, the chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) probe (Molecular Probes TM; Life Technologies) was used. This probe specifically reacts with ROS

becomes fluorescent by cleavage of acetate groups. Therefore, the fluorescence measured at the end of the test is proportional to the amount of ROS present in the cells. A 48-cells microplate was seeded with 20,000 cells/mL for 24 h. Afterwards, cells were rinsed with Hank's Balanced Salt Solution (HBSS) and incubated with 125 µL of HBSS with 20 µM of CM-H2DCFDA dissolved in DMSO. After incubation for 20 min at 20°C, the cells were rinsed twice with culture medium phenol red-free, and were exposed to 500 µL of the surface water (at 10 X) and elutriate samples (at 1:1, v/v) diluted in a medium phenol red-free for 4 h. A positive control with 2 mM H2O2 solution was also analyzed in parallel to the samples. The production of DCF was read by fluorimetry (excitation at 485 nm and emission at 520 nm) using a microplate reader (Fluostar optima). Results were expressed as the percentage of ROS induction over a 240 min period.

2.3. In vivo toxicity assay on rainbow trout larvae

2.3.1. Experimental design

Eyed stage embryos of rainbow trout (*Oncorhynchus mykiss*) were obtained from INRA-PEIMA (Sizun, France). Embryos were maintained in aquaria with fresh spring water from Laqueuille (4.7 mg/L Ca, 1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, pH 7.5, <1.2 mg/L Cl^-) in total darkness at 12°C. Water was changed every two days and embryos were kept into these conditions up to the larval stage of 355 DD (degrees days) for the *in vivo* assay.

Larvae of 355 DD were exposed to environmental samples from La Livenne for 48 h. Surface water (5 L) and sediments of L1 (reference site), L3 and L4 from La Livenne were sampled in May 2017. Water samples were immediately filtered successively at 11, 1.2 and 0.45 µm, and left at 4°C (no more than 1 week) until *in vivo* exposure. In parallel, sediments were stocked at -20°C. Twenty-four hours before exposure, water and sediments were left at 12°C for temperature equilibration. Sediments were sieved to remove large particles (>2-3 mm) and to homogenize it. Each experimental unit consisted in aquaria with 1 L of filtrated water and 200 g of sediment from each studied site and 50 trout larvae. Each condition was triplicated. Larvae were not kept in direct contact with sediment, instead larvae were placed in a plastic basket perforated at the bottom and walls as observed in Figure 2. For control condition, silica sand and spring water from Laqueuille (4.7 mg/L Ca, 1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, 7.5 pH, <1.2 mg/L Cl^-) were used. A peristaltic pump (ISMATEC, ISM942), was used to maintain a continuous flow rate (9 mL/min) of water from each site to aquaria. Each day dissolved oxygen concentration was measured with fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburf, Germany) and data was recorded with OxyView

v6.02 software (PreSens Precision Sensor). The exposure conditions were maintained for 48 h at 12°C in the climatic chamber. At the end of the experiment, larvae were sampled per replicate as follow: 15 larvae to record for biometrics, 10 larvae for the study of swimming behavior, 6 larvae for blood sampling for genotoxicity test. The remaining larvae were pooled and stored at -80°C for biochemical analysis and gene transcription.

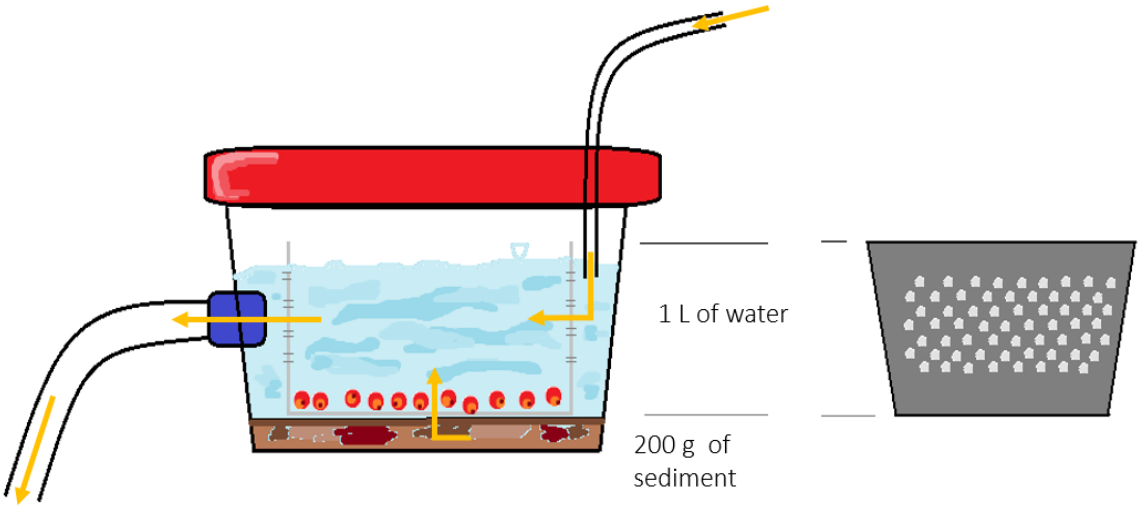

Figure 2: Experimental unit for rainbow trout larval exposure to sediment and water.

2.3.2. Viability

Larvae viability was recorded every day. Percentage of larvae mortality was calculated as the number of dead individual compared to the total number of larvae at the beginning of the experiment. At the end of the 48h-exposure, 15 larvae per replicate were placed in petri dish with ice water and few drops of carbonated water to sedate them. Photos were taken for each larvae with a stereomicroscope (MZ 7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). Afterwards, from each photo, total body and head length and yolk-sac area were measured using a Leica Microsystems software v3.8 (Nanterre, France).

2.3.3. Swimming behavior analysis

At the end of 48 h exposure, the swimming behavior analysis was performed using 10 larvae per replicate. Larvae were acclimated in 6-well microplates containing 3 mL of surface water at 12.0 ± 0.5°C in the dark for 10 min. Afterwards, the microplates were positioned in the recording chamber (Daniovision Image Analysis System with Ethovision software version 12.0, Noldus Netherlands) coupled to a thermoregulation system to keep water at 12 ± 0.5°C (Pilot ONE®, Huber). Larvae was subjected to a light/dark cycle of 5 min of dark, 5 min of light and 5 min of dark to study their photomotor responses.

In the recording chamber, an infrared camera records the movement of each larva focused on their center of gravity. Average velocity was calculated over 1 min. Time of mobility was also determined for each larva.

2.3.4. Genotoxicity test with modified FpG-enzyme test

The standard comet assay was performed following protocols described in Le Bihanic et al. (2014) and Weeks et al. (2019) on blood cells. Rainbow-trout larvae were anesthetized using ice water and few drops of carbonated water, and blood was sampled by decapitation of 6 larvae per replicate. Immediately, samples were added to 200 µL of cryoconservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) and frozen in liquid nitrogen until analysis. Electrophoresis was realized for 20 min after DNA unwind in alkaline solution with a voltage of 25 V and 300 mA. After DNA electrophoresis, slides were stained with 20 µg/mL of ethidium bromide solution, and comet lecture was done using an epifluorescence microscope using the Olympus BX51 microscope (zoom x20) equipped with an Olympus U-RFL-T reflected fluorescence system lamp. 100 nuclei per slide were quantified using the Comet Assay IV software (Instrument Perspective LtD). Results are expressed as percentage of DNA tail.

2.3.5. Biochemical analyses

Preparation of supernatant

Pools of 2 larvae (for TBARS and carbolynated proteins measurements) and pools of decapitated heads of 2 larvae (for AChE activity) were homogenized in a phosphate buffer (0.1 M; pH 7.5; 4°C) using an UltraTurrax® tissue homogenizer fitted with a potter at 3 000 rpm (4°C). To obtain the supernatant S9 fraction, samples were centrifuged at 9 000 g for 25 min at 4°C. The S9 fraction were divided in three tubes for measurements of total protein, TBARS and protein carbonyl content.

Total protein

Total protein concentration was measured from S9 fraction following the method described by Lowry et al. (1951). A standard calibration range with Bovine Serum Albumin (BSA) was used and measurements were assessed using a spectrophotometer microplate reader (Synergy HT, BioTek).

Lipid peroxidation (TBARS)

Measurements of lipid peroxidation was performed on S9 fraction after the method Buege and Aust (1978) adapted to a microplate reader. A volume of 500 µL of S9 fraction was added to 500 µL of a

solution containing 20 % of trichloroacetic acid (TCA) and 20 % of butylated hydroxytoluene (BHT). Mixtures were centrifuged at 9,000 for 10 min, and 600 µL of S9 fraction were added to 480 µL of TRISbase (25Mm)-TBA (thiobarbituric acid-100 mM) and 120 µL of HCl (0.6 N). Then, mixtures were heated at 80 °C for 15, cooled using iced water and mixed. Absorbances were read at 530 nm using a UV-spectrophotometer (Synergy HT, BioTek) in a microplate. TBARS levels were expressed as nmoles of thiobarbituric acid reactive substance equivalents per mg of protein.

Protein carbonyl content

The protein carbonyl content was assessed on S9 fraction after the method of Augustyniak et al. (2015). 50 µL of a solution of 11 % of streptomycin sulfate-phosphate buffer (100 mM pH 7.4) was mixed with 500 µL of S9 fraction and incubated at room temperature for 15 min. After centrifugation at 6,000 g for 10 min, supernatants were divided as follow: 200 µL added to 800 µL HCl 2.5 M, used as a control tube; and 200 µL to 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 Mm), used as a sample tube. Mixtures were incubated for 1 h at room temperature and vortexing every 15 min. Afterwards, proteins were precipitated with 1 mL of 20 % TCA (trichloroacetic acid), vortexed and centrifuged for 10 min at 10,000 g. The pellets were rinsed three times with 1 mL of ethanol-ethyl acetate (v:v), and then, solubilized with 500 µL of of 6 M guanidine HCl and centrifuged at 10,000 g for 10 min. The carbonyl content, expressed as nanomoles of DNPH incorporated/mg protein, was measured using a UV-spectrophotometer (Biotek Synergy HT) at 370 nm.

AChE activity

AChE activity was measured on S9 fraction following the technique of Ellman et al. (1961) adapted to a microplate reader. Each well contained 10 µL of S9 and 180 µL of 5,5′-dithiobis (2 nitrobenzoic acid) (DTNB) 0.5 mM in 0.05M Tris Buffer pH 7.4. A volume of 10 µL of acetyltiocholine iodide was added. The rate of increase of optical density (DO) was measured using a spectrophotometer (Synergy HT, BioTek) at 412 nm. Results are expressed as nmol/min/mg protein.

2.3.6. Gene expression

After 48h of exposure, 6 larvae per replicate were sampled in a storage buffer (RNA later, Qiagen), frozen in liquid nitrogen and stored at -80°C until analysis.

RNA extraction

Total RNA extraction from whole larvae was assessed using the kit "SV Total RNA Isolation system" (Promega) according to supplier's description. Larvae was homogenized with the help of MP fastprep®-24 (Biorad, 6 m/s, 40s) with 14.4 mm diameter ceramics beads (MP Biomedicals, Lysing Matrix D Bulk).

Retro-transcription of total RNA into cDNA

The reverse transcription was performed with 10 µL of total purified RNA (1 µg) using the kit "GoScript Reverse Transcription System" (Promega) following the manufacturer's instructions. After the reagents addition, samples were incubated using a thermocycler at 42°C for 1 h. The obtained cDNA was kept at -20°C until analysis by real-time quantitative PCR.

Quantitative real-time PCR

The qPCR was realized in a Mx3000P® qPCR system (Stratagene), using the kit GoTaq® qPCR Master Mix kit (Promega). The amplification program was one cycle at 95°C for 10 min, then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity was checked by the dissociation curve of each PCR product, The dissociation curve was acquired by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95°C.

The cycle thresholds (Ct) was obtained for each gene from the software MxPro™ qPCR. Level of gene expression was normalized with the mean of Ct value of reference genes (*Rpl7* and *Ef1α*) according to the method of ΔCt explained by Livak and Schmittgen (2001). The induction factor (>2) or repression (<0.5) were calculated for each gene by the ratio of transcription levels of each condition and the control. The list of studied genes and primers are presented in Table 1, and includes genes involved in oxidative stress (*cat*, *sod*), detoxification (*gst*, *mt1*), mitochondrial metabolism (*cox1*, *12S*), DNA damage repair (*ogg1*, *rad51*), apoptosis (*bax*) and cholinergic system (*ache*).

2.4. Statistical analysis

All results are expressed as mean ± SD (standard deviation). Normal distribution of data and homogeneity of variance were verified using Shapiro-Wilk ($p < 0.01$) and Levene's test ($p < 0.05$), respectively. If both conditions were verified, a one-way ANOVA analysis was used followed by the Tukey post-hoc test. If not, the non-parametric test of Kruskal-Wallis was used. Statistical analyses were carried out using R (http://cran.r-projet.org/). Differences were considered significant when $p < 0.05$.

Table 2: Accession number and specific primer pairs for the 11 selected genes of Oncorhynchus mykiss.

Gene	Accession number	Primer (5' – 3')
Rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
Cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG[a] GTCCAGGATGGGAAGTTGC[b]
Sod	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
Gst	BT073173.1	ATTTTGGGACGGGCTGACA[a] CCTGGTGCTCTGCTCCAGT[b]
Cox	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12S	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
Ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
Rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
Bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
Mt1	M18104.1	GTGGATCCTGCAAGTGCTCA[a] GTAATGCACCAGGCCTCACT[b]
Ache	XM_021577686	AGGAGGGTTCTACAGCGGAT[a] TATCCTGGACCCACTGGAGG[b]

[a]Forward primer

[b]Reverse primer

3. Results

3.1. *In vitro* study

3.1.1. Cell viability

The potential cytotoxicity of water samples on RTL-W1 cell line are shown in Figure 3. Sediment elutriate samples did not show significant impaired cell viability on RTL-W1 after 24 h exposure (data not shown). However, a significant cytotoxicity effect was observed on samples from surface water collected on

February 2017 from L4 site at 1X and 10 X concentrations, where cell viability was above 70 % when compared to control condition (Figure 3-D). For L3 site, a significant reduction on cell viability was also observed at 2.5 X and 10 X above 90 % relative to control (Figure 3-C).

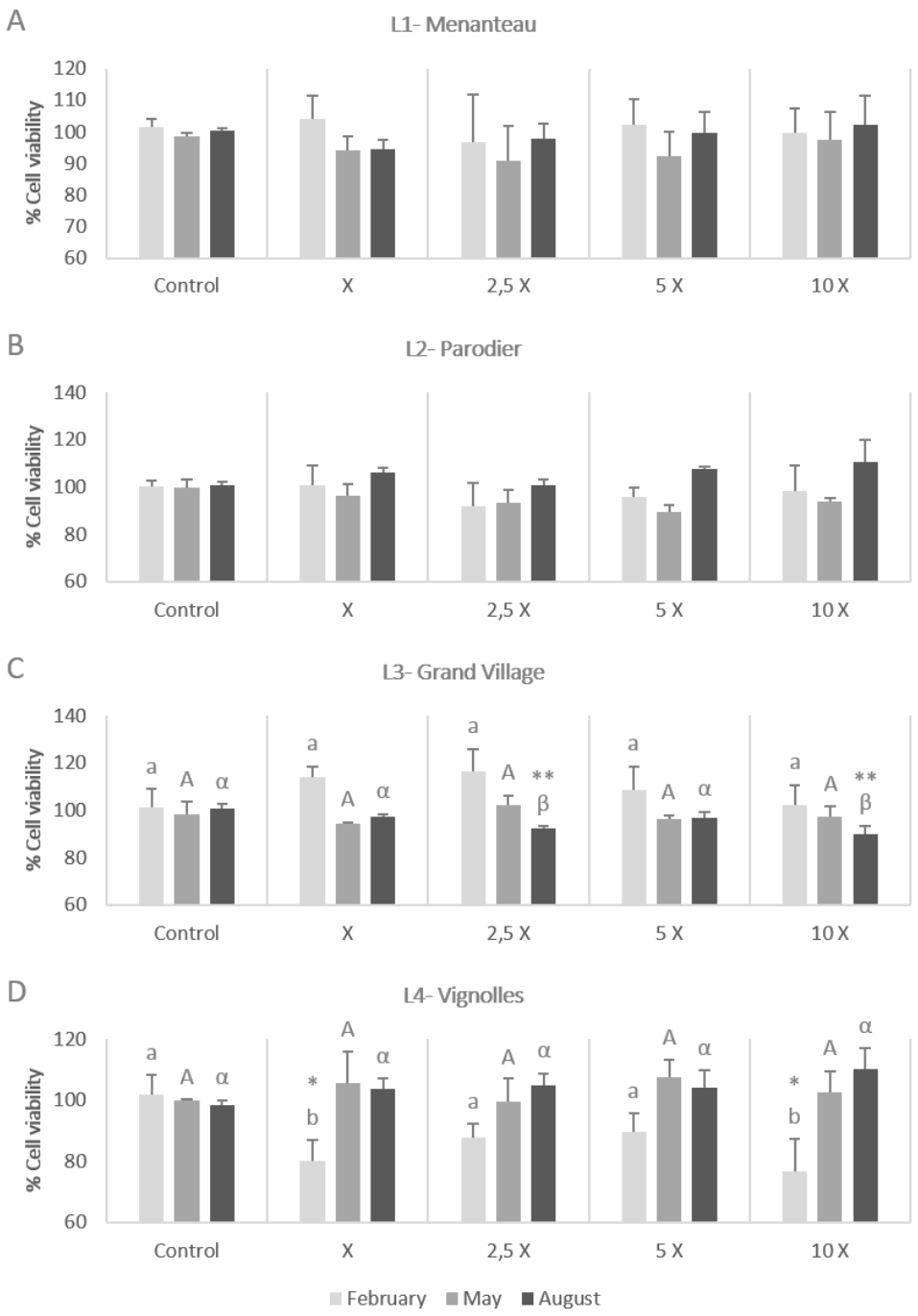

Figure 3: Cellular viability of RTL-W1 cell line exposed to different extracts of surface waters from La Livenne River sampled at Menanteau (L1- reference site), Parodier (L2), Grand Village (L3) and Vignolles (L4), at different periods of time. Cell viability was estimated with MTT assay. Data are expressed as Mean ± SD, N = 3, ANOVA. Different letters indicate significant differences between conditions. *$p < 0.05$, **$p < 0.01$ compared to control.

3.1.2. Production of reactive oxygen species (ROS)

To confirm the ROS production, a positive control was performed. After a 24 h-exposure of RTL-W1 cell line to1 µM H_2O_2 in RTL-W1, a significant ROS production was observed between 53 and 75 % compared to control condition (data not shown). ROS production was not evaluated from the site of L2. Exposure to surface water sample extracts at 10 X, did not significant induced ROS production on RTL-W1 (data no showed). In the contrary, after exposure to sediment elutriates, significant increased ROS production was observed in all sites from La Livenne depending on the period of sampling (Figure 4). Indeed, ROS production was increased in February and May 2017 particularly in the sites of L1, L3 and SE. However, in August ROS production was decreased markedly in the site of SE but and no more difference was observed compared to control condition. In the site of L4, a slight but significant increased ROS production was observed in May and August, but no difference was in February compared to control condition.

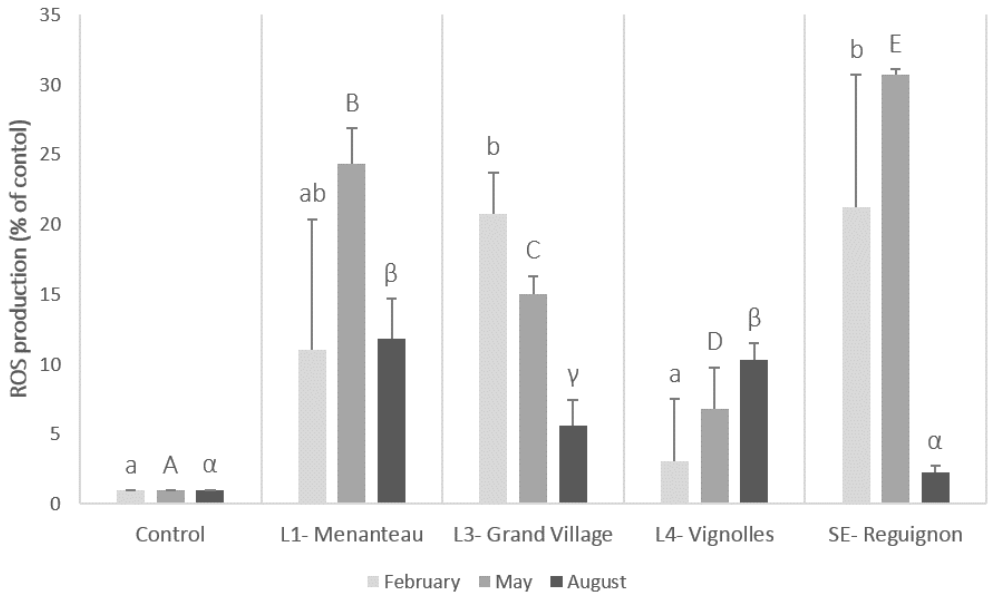

Figure 4: Percentage of ROS production on RTL-W1 cell lines exposed for 24h to elutriates of sediments from La Livenne River. Values represent Mean ± SD, N = 3, Kruskal-Wallis, $p < 0.05$.

3.2. *In vivo* study

3.2.1. Survival and growth of rainbow trout larvae

No significant mortality was observed on larvae whatever the treatments considered (Data not shown). Regarding larvae biometry after 48 h exposure, no significant differences were observed on total length of larvae; however, there were significant differences on head size and yolk-sac area for L4 sample

exposure (Figure 5). Indeed, larvae exposed to L4 had significant reduce head size and higher yolk-sac area when compared to non-exposed larvae ($p < 0.05$). Similarly, the rapport between total length and the head size of larvae exposed to L4 was also significantly reduced when compared to non-exposed larvae ($p < 0.05$).

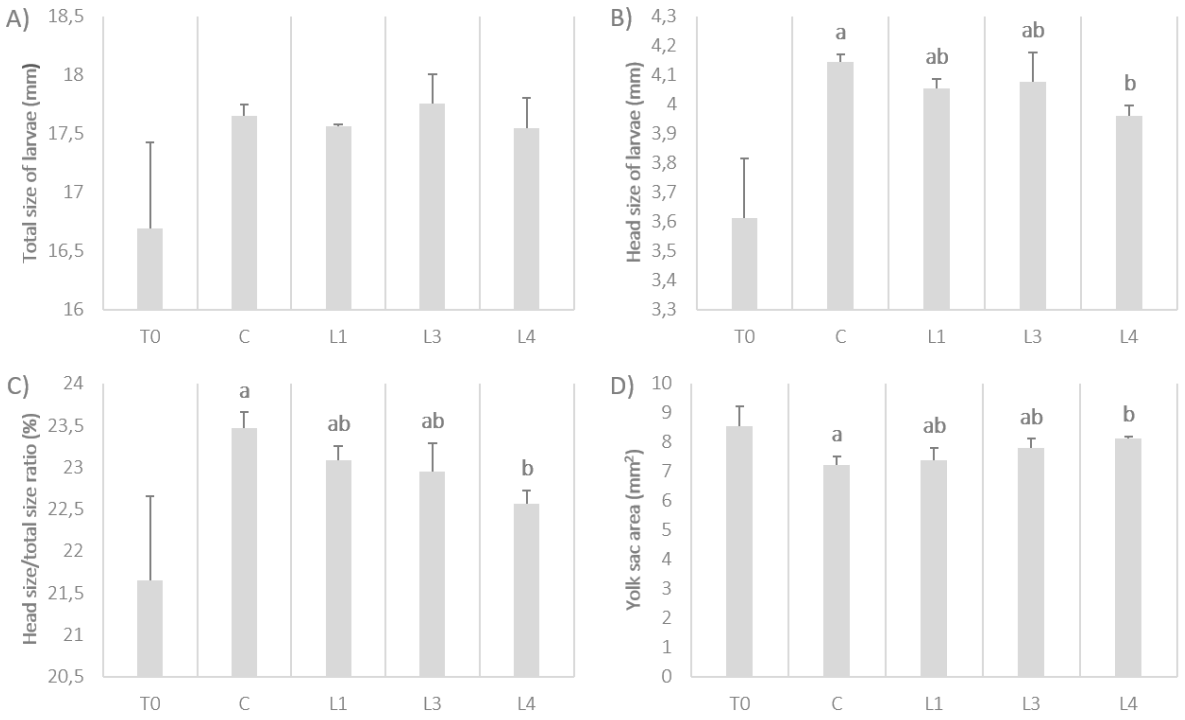

Figure 5: Biometric analyzes of larvae after exposure for 48h to sediment and water samples from La Livenne River: T_0 (biometrical measure before exposure), C (control), L1 (Menanteau), L3 (Grand Village), L4 (Vignolles). (A) Total body length (mm), (B) head size of larvae (mm), (C) ratio of head size to total length of larvae (%) and (D) yolk-sac area (mm^2) are showed. Different letters indicate significant differences between conditions (Mean ± SD N = 3, Tukey, $p < 0.05$).

3.2.2. Swimming behavior

Results of swimming behavior of larvae are presented in Figure 6, which corresponds to average velocity (cm/s) and mobility (s) of larvae subjected to a light/dark cycle. Velocity of larvae exposed to L3 were significantly reduced at the basal period compared to control and larvae from condition L1 and L4. However, this difference was no longer observed at light stimulation where all larvae increased their velocity without any significant differences (Figure 6-A). On the other hand, larvae from conditions L1 and L4 were significantly more mobility in light conditions compared to larvae from control and L3 treatments. At the basal level (dark 1), larvae from condition L3 were significantly less mobile than

control condition and larvae from condition L1. At the second dark period, larvae from condition L4 was still more mobile than control condition (Figure 6-B).

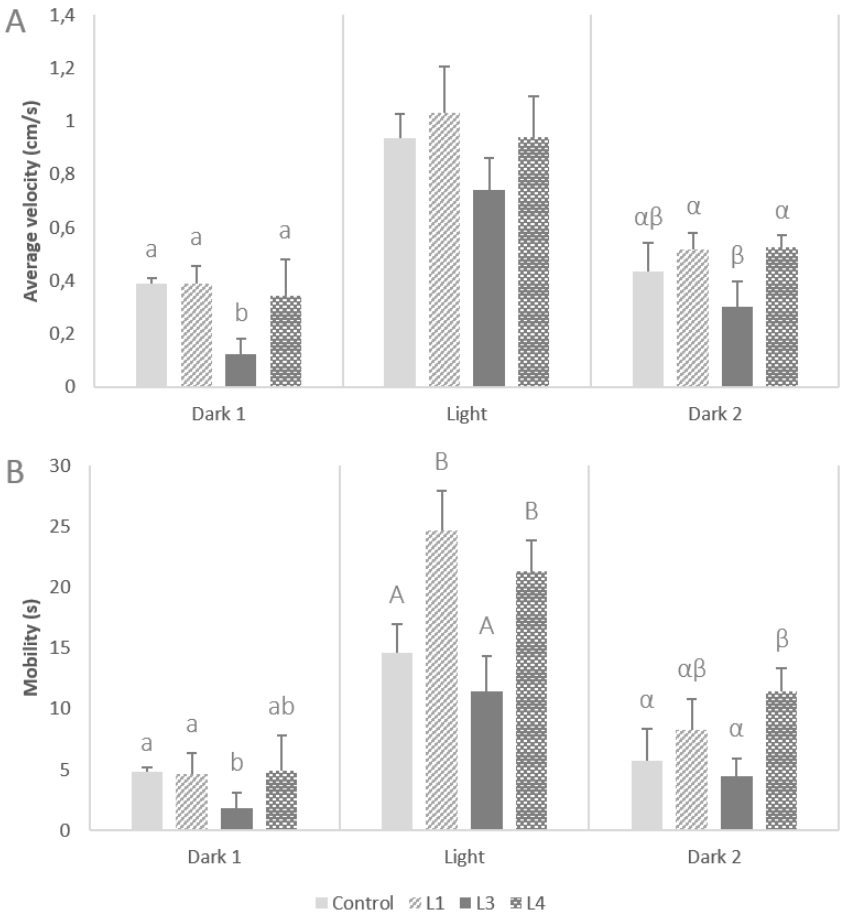

Figure 6: Average velocity (cm/s) (A) and cumulative time of mobility (s) of larvae exposed for 48h to sediment and water samples from La Livenne River. Different letters indicate significant differences between each period (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.2.3. AChE activity

AChE activity from rainbow trout head exposed to environmental samples from La Livenne are shown in Figure 7. No significant alterations on AChE activity were observed on larvae from all conditions when compared to control.

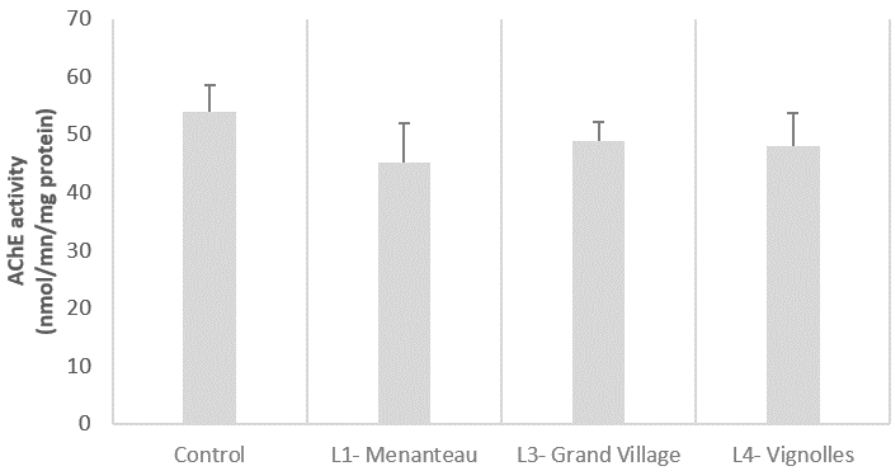

Figure 7: AChE activity from head of rainbow trout larvae after 48h exposure to environmental samples from La Livenne river. No letters indicate no significant differences between conditions (Mean ± SD, N = 3, ANOVA, $p > 0.05$).

3.2.4. Genotoxicity

Figure 8 shows the results of standard comet assay from blood cells of rainbow trout. Our results indicate no significant differences in DNA damage for all studied conditions when compared to non-exposed larvae.

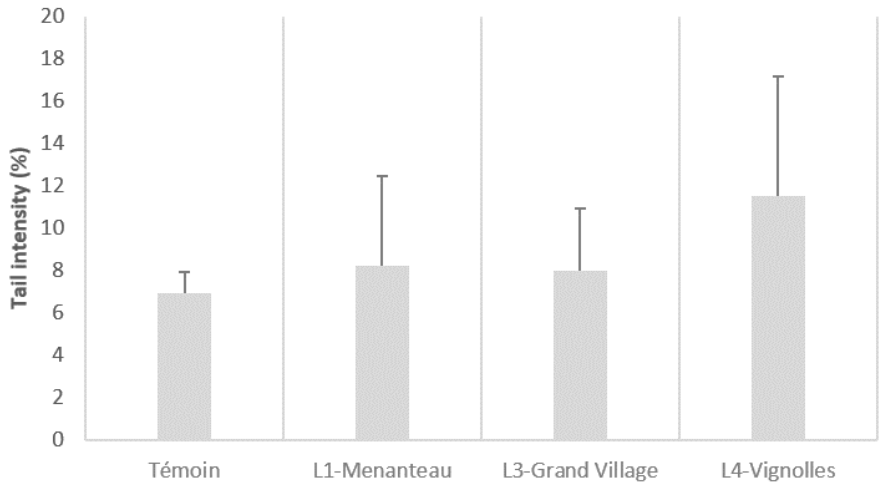

Figure 8: DNA damage in blood cells from rainbow trout larvae after exposure to water and sediment samples from La Livenne River. No letters indicate no significant differences (Mean ± SD, N = 3, ANOVA, $p > 0.05$).

3.2.5. Lipid peroxidation and protein carbonyls

Thiobarbituric (TBARS) levels and protein carbonyl content from larvae exposed to environmental samples from La Livenne river are presented in Figure 9. Larvae exposed to L4 condition resulted in reduced levels of TBARS when compared to non-exposed larvae (Figure 9-A). In contrary, no significant differences of protein carbonyl content were observed on larvae from all conditions compared to control (Figure 9-B).

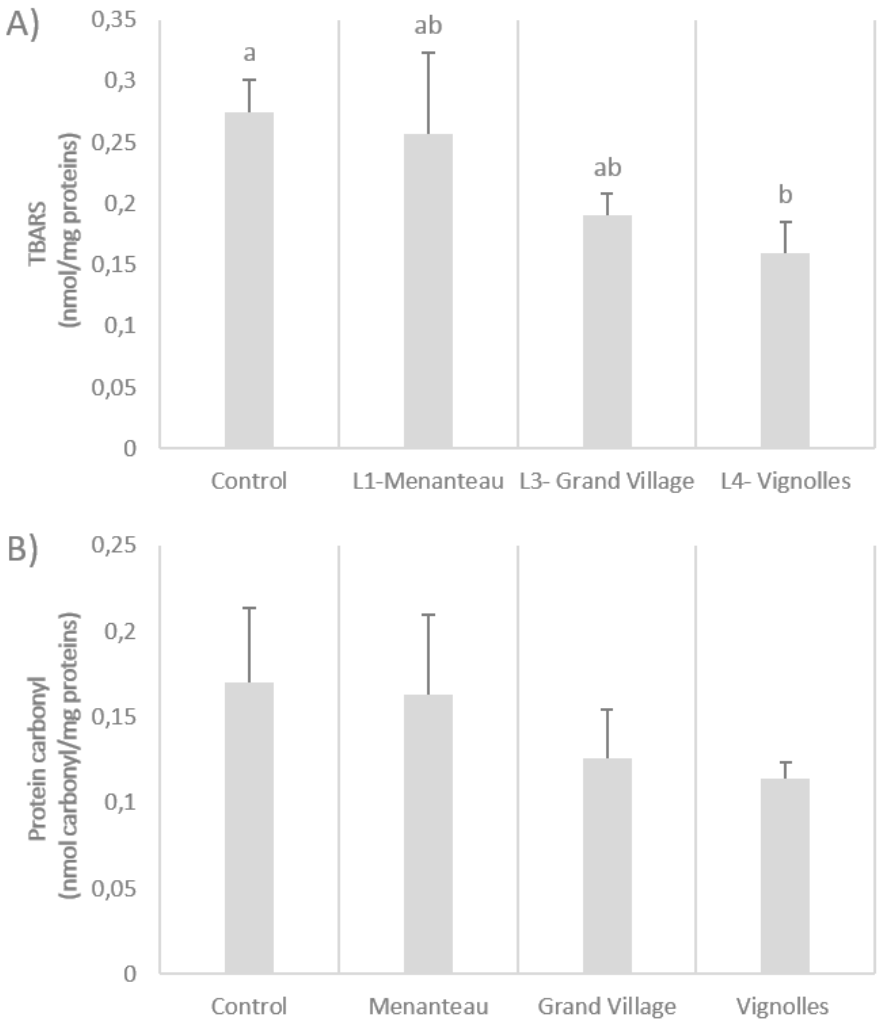

Figure 9: Lipid peroxidation expressed as nanomoles of TBARS/mg of protein (A) and protein carbonyl content expressed as nanomoles/mg of protein (B) in rainbow trout larvae exposed for 48h to sediment and water samples for La Livenne River. Different letters indicate significant differences between conditions (values represent Mean ± SD, N = 3, ANOVA, $p < 0.05$).

3.2.6. Gene expression

Gene transcription profile on whole-larvae after 48 h exposure to environmental samples from La Livenne was indicated in Table 3. No statistical changes were observed on expression of the two reference genes *Rpl7* and *Ef1α* between conditions (data no shown). Exposure to environmental samples resulted in down-regulation of genes involved in DNA repair (*rad51*) and cholinergic system (*ache*) on larvae from L1 and L3 conditions compared to non-exposed larvae. Larvae from L4 condition showed down-regulation of *ache* gene compared to control condition. The expression of *cat*, *sod*, *gst*, *cox*, *12S*, *ogg1*, *bax* and *mt1* were not significantly deregulated.

Table 3: Transcription levels of selected genes in comparison to mean reference genes, in whole-larvae of rainbow trout after 48h-exposure to environmental samples from La Livenne (L1= Menanteau, L3= Grand Village, L4= Vignolles). Gene expression values were normalized using housekeeping gene Ribosomal Protein L7 (Rpl7). Data was expressed compared to the control condition as induction (above 2) or repression (below 0.5) factors. Asterisks refer to significant differences compared to control condition (N = 3, ANOVA, *p < 0.05, **p < 0.01).

Gene	L1	L3	L4
cat	/	/	/
sod	/	/	/
gst	/	/	/
cox	/	/	/
12s	/	/	/
ogg1	/	/	/
rad51	0.19*	0.05**	/
bax	/	/	/
mt1	/	/	/
ache	0.20*	0.06**	0.3*

4. Discussion

In this work, we were interested in assessing the toxic potential of two environmental samples, surface water and sediments, from La Livenne using *in vivo* and *in vitro* methods.

In the study conducted by Gaillard et al. (2018) in 2017, 29 phytosanitary substances were detected in La Livenne watershed which 75 % of these molecules corresponds to fungicides. Among the detected pesticides, 5 fungicides (benalaxyl, dimethomorph, metalaxyl, spiroxamine and tebuconazole) and one herbicide (propyzamide) were found between 49 and 86 % of water samples. In addition, glyphosate, and its metabolite AMPA, were quantified at 100 % of water samples. This study found that the highest pesticide concentrations were mostly detected at the site of SE (the stream that drains from vineyard plots), and the lowest pesticide concentration were at the upstream of La Livenne (sites L1 and L2). In the other hand, the highest concentrations of the metabolite AMPA were mostly measured in the downstream of La Livenne (sites L3 and L4) with concentrations in the order of µg/L, while in the other sites it was found in the order of ng/L.

In vitro study

The results of MTT test, for cell viability assessment, suggest cytotoxicity of surface water samples of the campaign of February 2017 from the site of L4 at X (environmental concentration) and 10 X. The campaign of August 2017 also exhibited a significant slight cytotoxicity in the site of L3, but cytotoxicity was no longer observed on L4. From the same collected water samples, no significant ROS production was observed in any exposed cells when compared to control condition. Aquatic ecosystems are considered as a receptacles of contamination usually from urban, industrial and agricultural activities (Van der Oost et al., 2003). However, surface water is the reflection of a point of contamination, and the absence or presence of pollutants in the water column might be very variable depending on the season and the hydrology (such as sedimentation, transport by the water flow, dilution or degradation) at the moment of sampling (Knott et al., 2009; Sasakova et al., 2018). In addition, the sampling of surface water was performed once punctually, and pollutants may be diluted or not present. Indeed, punctual sampling of surface water makes it difficult to consider the temporal variability of the contamination (Le Dréau et al., 2015), thus peaks of contamination may go unnoticed. The spring rainfall of 2017 was low in the days before the sampling campaign, despite a monthly average ranked in the normal. This absence of precipitation reduces the phenomenon of leaching and, thus, contamination in the water column.

Several studies have detected cytotoxicity in cell cultures of environmental samples from areas under the influence of agricultural activities. Cytotoxic and genotoxic responses were observed in water samples from the Sinos river (southern Brazil), which is impacted by urban, agricultural and industrial

activities, using *Salmonella*/microsome assay (Lemos et al., 2009), HEp-2 epithelioid-type cells (Bianchi et al., 2017) and the Vero cell line (derived from the kidney of the African green monkey) (Bianchi et al., 2015). Lemos et al. (2009) observed a toxic effect in all sites sampled in Sinos River, especially in autumn (which corresponded to September 2005). Cytotoxicity was revealed at 50 and 83 % along the lower section of the river, meanwhile 33 % of cytotoxicity was observed in the upper section.

In the other hand, results of MTT test indicate that on the three sampling dates, February, May and August, sediment elutriates content did not exhibit cytotoxic effects. The pollutants that may be in the sediment were not sufficient to induce significant cell death. Since MTT test is a reliable and sensitive test, it allows us to affirm that the elutriates fractions collected were not cytotoxic. Although no cytotoxicity was revealed on elutriates, stress inducing ROS production was observed in all sites but at different period of sampling indicating a temporal variation. ROS production was observed in L3 and SE in February, L1, L3, L4 and SE in May, and in L1, L3 and L4 in August when compared to control condition. ROS production was generally more consistent in elutriates from May compared to February and August. Between February and May, L1 and L4 underwent changes in ROS production. For L4, this difference can be explained at least in part by the difference in particle size of the sediment. The collected sediments from all sites have been fine, except for the one collected at L4 in February, which was a coarse sediment. Indeed, the granulometry of sediment plays a very important role in the retention of metals and contaminants. The thinner the particle is, the higher the specific surface area has (Duong et al., 2010), the higher the fine organic matter, the greater the sorption of pollutants (Froehner et al., 2010; Orescanin et al., 2004). According to the meteorological data, February sampling took place after two weeks without rain, and the sampling of May was realized 3 days after rain. It is likely that after rain, soil leaching watershed, enriched with organic matter and pollutants, was more extensive. On the contrary, the dry period observed in August could have strongly limited the intake of organic matter and pollutants. Without the contribution of runoff, and with the decrease of water flow, the sediment could have gradually been emptied of pollutants.

The so-called soft extraction method was interesting for assessing the toxicity of sediments. As Geffard, (2001) reveals, the agitation water extraction is a method that can be compared to a natural resuspension of sediments in the water column, related to an increase of flow. The extractable, water-soluble, and potentially bioavailable contaminants are extracted during resuspension of the sediments. These contaminants are probably represented by hydrophobic, slightly hydrophilic organic compounds and metals.

In vivo study

Assessing the adverse ecological effects of environmental samples subjected to anthropogenic pollutants is a challenge due not only to the incidence of synergistic or antagonistic effects caused by the combination of pollutants, but also to the complex physico-chemical nature of water and sediments and the influences that may have on bioavailability (Costa et al., 2011). Evaluating both surface water and sediments provide a more realistic approach to estimate the possibility of adverse effects occurring on organisms exposed directly to contaminated ecosystems.

Regarding our results from the *in vivo* experiment, 48 h exposure of environmental samples (surface water and sediments) from La Livenne did not induce significant mortality on rainbow trout larvae after the short-term toxicity test. Novelli et al. (2016) also did not observed significant mortality on juveniles of zebrafish (*Danio rerio*) after one-week exposure to a runoff samples, from a simulated experimental agricultural field containing abamectin (an insecticide) using the commercial formulation of Vertimec® 18EC. In another study, the sum of total concentrations of 25 pesticides measured in Larra, in the confluence with Save river in Gascogne (France), was between 0.27 and 1.82 µg/L according to the season of sampling (where metolachlor, isoproturon, chlorotoluron, atrazine and metabolites were the most abundant) and no mortality was observed on Crucian carp (*Carassius carassius*) after 4-days exposure to these samples (Polard et al., 2011). The ten spotted live-bearer fish (*Cnesterodon decemmaculatus*) was exposed to water samples from Reconquista river (Argentina). Pesticides concentrations from samples were lower than analytical detection limits, but several heavy metals were above Argentine guidelines such as Pb (0.0096 mg/L), As (0.033 mg/L) and Cu (2087 mg/L). After 96 h of exposure to these samples, mortality, at 15 and 10 %, were only observed on samples treated with Cd and cyclophosphamide, simulating a contamination pulse, respectively (Ossana et al., 2016).

In our study, the concentrations of pollutants presented in the water and sediment from La Livenne are not directly associated with mortality on fish. However, sub-lethal effects were perceived after 48 h of exposure. Subtle significant differences were observed on the biometry of larvae exposed to L4. Indeed, larvae presented significant smaller head size, and the ratio between head size and total length was reduced when compared to control larvae. Likewise, the area of yolk-sac of larvae from condition L4 were significantly bigger than controls after 48 h of study. Since larvae were exposed on a such short time, we were quite suspicious about these differences of biometry on larvae. However, yolk-sac absorption could be considered as an important endpoint of embryo development, and the delay in resorption has already been observed on fish early-stages exposed to pollutants as pesticides (Amblard et al., 1998; Coelho et al., 2011; Kalasekar et al., 2015) and metals (Johnson et al., 2007; Witeska et al., 2010). For instance, reduced yolk-sac was perceived in zebrafish larvae after 96 h exposure to

thrichlorfon, an organophosphate insecticide, from 20 mg/L (Coelho et al., 2011). Yolk-sac absorption from the northern pike (*Esox lucius*) was also reduced after exposure to 250 µg/L of carbofuran (Amblard et al., 1998). According to Witeska et al. (2010), copper (100 µg/L) and cadmium (100 µg/L) may also reduce the yolk absorption rate and growth in barbel (*Barbus barbus*) larvae. However, in another study from the same authors (Witeska et al., 2014), when these concentrations of Cu and Cd were exposed to ide embryos (*Leuciscus idus*), the yolk-sac perimeter area at hatching was reduced on those exposed to Cu and unchanged in those from Cd, suggesting a higher metabolic cost of energy. Kalasekar et al. (2015) studied different chemicals that could influence the absorption of yolk-sac on zebrafish, where larvae treated with butralin (an herbicide) increased the yolk absorption while no differences were observed on those exposed to imazalil (a fungicide) compared to control. In our study, since no edemas were observed on treated larvae in all conditions, we may suggest that the increase yolk-sac area on larvae from L4 condition is due to a reduction in yolk utilization. In early-life stages of fish, yolk is an inactive source of nutrients, which is used for growth and metabolism. A reduced absorption might implicate a delayed of development.

Fish swimming behavior has an important ecological relevance and its alterations are considered as a “early warning signal” for toxic exposure to fish (Hellou, 2011). Indeed, behavioral endpoints are used as a bioindicator that represents the individual level of a biological response (Hellou, 2011; Triebskorn et al., 1997; Weis et al., 2001). In our study, after 48 h of exposure, larvae from all conditions presented two different kinds of responses according to the period of dark/light exposure. The first dark period (10 minutes of duration) corresponded to the basal activity of larvae, and a significant decreased on swimming velocity was observed on larvae from condition L3 when compared to larvae from control, L1 and L4 conditions. However, this response was not evident at light stimulation where no significant differences were observed. In the other hand, when regarding the fish activity, at light stimulation, fish from L1 and L4 conditions resulted on a significant increased mobility compared to control and L3 larvae. Many pesticides, metals and other toxic pollutants can alter the behavior of fish, which is caused by the intervention of different physiological systems such as metabolic and neurological systems (Scott and Sloman, 2004). The decreased on swimming velocity observed on larvae from condition L3 may be related to a metabolic dysfunction, or an increase of metabolic demand resulting in less energy for locomotion (Scott and Sloman, 2004; Triebskorn et al., 1997). For instance, Handy et al. (1999) found that the decreased swimming activity of rainbow trout adults, previously fed with a Cu-supplemented diet for 3 months, was related to an increase of metallothionein concentration on liver and intestine, suggesting that the metabolic effort for detoxification of Cu resulted in a less energy for swimming and growth. Triebskorn et al. (1997) also observed a decreased on swimming performance of brown trout (*Salmo trutta fario*) exposed to highly polluted water from a test stream

(southern Germany) contaminated with pesticides, PAHs, PCBs and metals. This reduction on swimming activity was anti-correlated to an increase of metabolic enzymes, such as cytochrome-c-oxidase. In another study, a decrease in swimming performance was also reported in benthic fish (*Erimyzon sucetta*) exposed to coal ash-contaminated sediments (Hopkins et al., 2003). However, authors did not attribute this reduction to condition factor, lipid storage or fin erosion but mostly to physiological disruptions, such as detoxification or increase energy demands.

In the other hand, the increased on swimming activity observed on larvae from L1 and L4 conditions, could be interpreted as a reaction to a peak of pollution. Signs of hyperactivity has also been reported in Sacrameto splittail (*Pogonichthys macrolepidotus*) larvae exposed for 96 h to runoff samples from a French prune orchard in California (Teh et al., 2005). Besides reductions on swimming behavior, Triebskorn et al. (1997) also observed that fish exposed to a samples from a less contaminated site increased their locomotor activity, indicating that hyperactivity behavior could be used as a marker of short-term pollution. Several reports have documented that exposure to low concentrations of pesticides could increase swimming activity of fish. For example, red drum larvae (*Sciaenops ocellatus*) swam faster after exposure for 4 days to environmental levels of atrazine (40 and 80 µg/L) (Alvarez and Fuiman, 2005). Rainbow trout adults also showed hyperactivity behavior when exposed to 1 and 10 µg/L of atrazine, but this behavior was not observed at 100 µg/L (Tierney et al., 2007). Abnormal burst swimming reactions was observed on silver catfish (*Rhamdia quelen*) exposed to high concentrations of metsulfuron methyl for 96 h (Dos Santos Miron et al., 2005). Bluegill sunfish (*Lepomis macrochirus*) became hyperactive after 24 h exposure to 16 µg/L of malathion (Richmonds and Dutta, 1992). However, Dos Santos Miron et al. (2005) and Richmonds and Dutta (1992) observed that this hyperactivity was correlated to a decreased of acetylcholinesterase activity (AChE).

Modifications of behavior could also be a result of damage to the nervous system (Scott and Sloman, 2004; Weis et al., 2001), and correlations between neurotransmitter levels and behavior have been already observed in several studies (Scott and Sloman, 2004). The measure of the acetylcholinesterase (AChE) activity is one of the most studied indicators of altered neural function, and it is considered as a biomarker of exposure to certain organic chemicals such as organophosphorus and carbamates pesticides in fish and aquatic organisms (Bocquené et al., 1995; Cajaraville et al., 2000; Van der Oost et al., 2003). Indeed, these substances produce toxicity by altering the activity of AChE which interferes with the normal cholinergic signaling in vertebrates. In our study, no significant alterations of AChE activity were observed in larvae head exposed to all contaminated conditions when compared to control. However, several authors have evaluated the response of AChE activity in fish exposed to contaminated sites. For example, Brodeur et al. (2017) observed a reduction of ChE (cholinesterase) activity on one-side livebearer fish (*Jenynsia multidentata*) collected on a watercourse near an

agricultural region in Argentina compared to fish from unpolluted watercourse. An *in situ* assay was performed by Castro et al. (2004) where mosquitofish (*Gambusia holbrooki*) and guppy (*Poecilia reticulata*) were exposed to a watercourse close to a mine effluent. After 4-days exposure, inhibition of AChE was only observed for guppy while mosquitofish rested insensitive for this biomarker. The tilapia fish (*Tilapia tilapia*), captured New Damietta canal in Egypt, presented low activity of AChE which was in parallel with high concentrations of chlorpyrifos, malathion, diazinon, pirimiphos-methyl and profenobos detected in fish (Abdel-Halim et al., 2006). AChE activity was also inhibited in brains of adult females of *Cnesterodon decemmaculatus* collected in Reconquista River, a peri-urban polluted river in Argentina compared to fish from a reference site (De La Torre et al., 2005). However, this inhibition was mostly due to the presence of metals since no detectable insecticides were found in water after chemical analysis.

The effects of environmental samples from La Livenne on gene transcription were analyzed on whole-larvae of rainbow trout. Our results revealed that only genes involved in detoxification (*rad51*) and cholinergic system (*ache*) were significantly down-regulated on exposed larvae when compared to control condition. Down-regulation of *ache* indicates a perturbation in neural and neuromuscular functions. However, there is a discrepancy between enzymatic activity and levels of gene expression since no inhibition was observed in AChE activity. These discrepancies have already been observed by Rodríguez-Fuentes et al. (2015) and Pereira et al. (2012). The inhibition of gene expression could explain the behavioral changes observed on larvae, particularly on larvae from L3 condition which presented low levels of *ache* expression and reduced velocity compared to control. *Rad51* gene expression has an important role implicated to repair double-strand break in DNA (Kienzler et al., 2013). The repression of this gene could lead to accumulation of DNA damage in the form of double-strand breaks as a consequence of exposure to DNA damaging agents (Singh et al., 2017). Besides, accumulation of these damages could lead cells to be subjected to apoptosis.

In our study, the standard comet assay did not show any significant DNA damage in blood cells of rainbow trout after 48 h of exposure to environmental samples from La Livenne. However, since inhibition of rad51 gene expression was observed, probably different mechanisms of DNA repair has been activated to avoid significant damage. Several authors have demonstrated the capacity of many pesticides to cause genotoxicity in aquatic organisms. Moreover, the measure of DNA alterations in fish is a good indicator of exposure to genotoxic substances in polluted environments, and it is considered as a biomarker of response (Frenzilli et al., 2009; Hariri et al., 2018; Van der Oost et al., 2003). Significant DNA strand breaks was observed on blood cells of Sacramento Sucker fish (*Catostomus occidentalis*), after a laboratory exposure and field-caging experiment, exposed to runoff from an agricultural area of San Joaquin River (California, USA) (Whitehead et al., 2004). At this study, induction of DNA damage was

related to the agricultural runoff after a storm event. Genotoxicity was observed on zebrafish embryos after 96 h exposure to freeze-dried sediment and sediment extract from different sites of Laguna Lake (Philippines) (Kosmehl et al., 2008). Sediments from Laguna Lake presented high levels of perylene and heavy metals which could explained the induction of DNA damage. (Costa et al., 2011) demonstrated genotoxicity on blood cells after juvenile Senegalese soles (*Solea senegalensis*) were exposed for 28-days to sediments from the Sado Estuary (Portugal) in both field and laboratory bioassays. European carp (*Cyprinus carpio*) were used to study the genotoxicity of water samples from Noyyal River after 72 h exposure (Rajaguru et al., 2003). Authors observed induction of DNA damage in erythrocytes, liver and kidney cells, particularly from samples collected downstream industrialized and urban areas. In a field-study performed in the Morcille River, highly polluted by vineyard pesticides, a significant response of DNA damage was observed in blood cells of brown trout (*Salmo trutta fario*) larvae after 1-month exposure (Bony et al., 2008). Indeed, DNA damage was mostly exhibited on larvae from downstream station compared to the upstream station.

Lipid peroxidation and protein carbonyl content are biomarkers that are highly studied to assess the field contamination. Several authors have observed induction of lipid peroxidation in fish after exposure to environmental samples (Chen et al., 2012; Fonseca et al., 2011; López-López et al., 2011). In our case, no changes in lipid peroxidation on whole larvae was observed on those exposed to L1 and L3 compared to control condition, and no significant changes were also observed on protein carbonyls on all conditions. However, larvae exposed to L4 condition significantly reduced TBARS levels compared to non-exposed larvae. Since elutriates from sediments were able to induce ROS production on RTL-W1, particularly on samples from May 2017, our results suggest that the antioxidant defense system were able to prevent oxidative damage on lipids and proteins of rainbow trout larvae. Moreover, these findings are in accordance with previous studies performed on rainbow trout larvae exposed to glyphosate alone and in mixture with copper and chlorpyrifos (See article 2 and article 4, Weeks et al., 2018). Accordingly, (Tsangaris et al., 2011) did not observed any significant changes in lipid peroxidation on liver of Prussian carp (*Carassius gibelio*) exposed to water samples from 3 polluted Ukrainian rivers. However, authors did observe that enzyme GST on liver was significantly induced.

5. Conclusions

In this work, we studied the impacts of viticulture activity using two biological models: the cell line RTL-W1 and rainbow trout larvae. Our results highlighted significant effects of environmental samples from waterborne and sediment on both models. The *in vitro* assay permitted the study of toxicological effects according to the spatial and temporal variations observing significant differences in

cytotoxicity, particularly on water samples, and ROS production of elutriates. The *in vivo* assay allowed to integrate other toxicological endpoints such as molecular biomarkers, where significant alterations were observed on lipid peroxidation and notably, the expression levels of *rad51* and *ache* on larvae. Even if no significant inhibition was observed on AChE activity, the decreased genetic expression of *ache* could explain the alterations observed on behavior of larvae. After all these responses, we can conclude that water courses that have been impacted by vineyard treatment could represent a risk to natural environment especially for aquatic organisms like fish.

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Chapitre VI

Discussion générale

Discussion générale

La question sur l'exposition et les dangers des pesticides est l'une des problématiques d'actualité les plus documentées. Il existe une grande quantité d'études et de rapports portant sur les pesticides, dans différents domaines, allant de la toxicologie à la chimie de l'environnement jusqu'aux études socio-économiques. L'utilisation de ces substances a souvent suscité la controverse au sein de la société en raison de son impact sur la santé et l'environnement. De nombreuses productions agricoles dépendent du traitement par les pesticides en raison de leur "efficacité" pour traiter et lutter efficacement contre les parasites qui seraient sinon dommageables pour la productivité et la qualité des récoltes (Aktar et al., 2009; Fernandez-Cornejo et al., 2014). Cependant, en raison d'une utilisation intensive, et souvent incorrecte, les pesticides ont considérablement endommagé l'environnement, en particulier les écosystèmes aquatiques. Des études menées dans le monde entier ont montré la contamination généralisée des écosystèmes aquatiques par la présence d'une ou de plusieurs molécules phytosanitaires dans les écosystèmes aquatiques (Carr et al., 1997; Ifremer, 2005; Köhler and Triebskorn, 2013). De ce fait, le problème des pesticides est devenu un problème et une préoccupation globale.

En raison de la forte activité viticole dans le département de la Gironde, de nombreuses questions se posent concernant l'impact que les produits phytosanitaires pourraient avoir sur les écosystèmes aquatiques. En effet, de nombreuses études ont montré la présence de ces molécules dans plusieurs rivières et fleuves tels que la Garonne (Cruz, 2015; Grousset et al., 1999), et d'autres cours d'eau plus petits comme la Livenne (Gaillard et al., 2018). Par conséquent, pour répondre à cette question, une série expérimentations ont été effectuées au cours des trois années de cette thèse, en utilisant les premiers stades de poissons exposés à différentes substances phytosanitaires. De plus, des échantillons d'eau et de sédiments de La Livenne ont été collectés et analysés pour déterminer leur toxicité et les réponses des larves de truites à cette contamination, en complément de nos approches expérimentales.

Pour cela, la truite arc-en-ciel a été choisie comme modèle, car elle présente plusieurs caractéristiques avantageuses telles que :

- facile à élever dans des conditions de laboratoire, largement étudiée et caractérisée.
- faible tolérance aux eaux de mauvaise qualité.
- espèce sentinelle très utilisée en écotoxicologie aquatique.

De plus, les premiers stades de la truite arc-en-ciel ont un développement embryonnaire lent, ce qui permet des expositions sub-chroniques suffisamment longues. Ils constituent une composante

importante du réseau trophique et, en même temps, les premiers stades de développement sont une étape critique du cycle de vie du poisson permettant le recrutement et la persistance des populations de poissons. Parallèlement aux études avec la truite arc-en-ciel, nous avons également souhaité intégrer une analyse *in vitro* à l'aide d'une lignée cellulaire de cette même espèce. En effet, les nouvelles réglementations en matière d'expérimentation animale imposent le respect de la règle des 3R (Réduire, Remplacer, Raffiner). Les lignées cellulaires représentent donc une alternative crédible à l'utilisation d'animaux en écotoxicologie. Cependant, leurs sensibilité et fiabilité doivent être démontrées avant de pouvoir généraliser leur utilisation. La lignée cellulaire choisie était la lignée RTL-W1, qui provient de cellules hépatiques de truite arc-en-ciel adulte (Lucila E. J. Lee et al., 1993). Dans notre étude, l'utilisation de cette lignée cellulaire a permis d'obtenir des informations pertinentes sur la cytotoxicité et la génotoxicité de quelques composés chez cette espèce.

Un autre aspect qui a été pris en compte était le choix des molécules à étudier. Sur ce point, 3 molécules phytosanitaires représentatives des traitements classiquement utilisés en viticulture, et qui sont détectées dans des écosystèmes aquatiques, ont été étudiées. Parmi ces molécules, on trouve le cuivre, qui a largement utilisé comme fongicide dans les vignes et qui est accepté en viticulture biologique. Cependant, de nombreuses études ont montré les dommages que le cuivre peut causer aux organismes aquatiques (Kamunde et al., 2002; Marr et al., 1996; Vardy et al., 2013). Une autre molécule étudiée est le glyphosate, qui est l'herbicide le plus utilisé à travers le monde. Pour cette raison, le glyphosate, ou son produit de dégradation l'AMPA, sont fréquemment retrouvées dans les écosystèmes aquatiques (Aparicio et al., 2013; Coupe et al., 2012; Peruzzo et al., 2008). De nos jours, de nombreuses voix s'élèvent contre son utilisation en raison des répercussions environnementales, mais également pour ses effets néfastes pour la santé humaine. L'actualité récente, notamment en Europe, montre une prise de conscience et une démarche visant à interdire son utilisation. Enfin, le chlorpyrifos est un insecticide qui fait partie de la liste des substances toxiques prioritaires (DCE, 2013), a été restreint d'utilisation dans plusieurs pays. Il a été interdit pour un usage urbain et résidentiel, et son utilisation dans les applications agricoles a été réduite (Canadian environmental Quality Guidelines, 2008).

Chacun des bio-essais effectués avec les molécules, individuellement ou en mélange, a été réalisé dans les mêmes conditions d'expérimentation, c'est-à-dire en utilisant les mêmes stades embryonnaires, les mêmes conditions physico-chimiques, la même eau, la même durée d'exposition, le même type d'unités d'expérimentation, etc. Cela a permis de comparer chacune des réponses des larves à la fin de l'expérimentation.

1. Caractérisation des effets toxiques de 3 pesticides en fonction de leur mode d'action aux concentrations environnementales pertinents.

La survie, la croissance, le développement et la reproduction des poissons dépendent beaucoup de la qualité de l'habitat dans lequel ils vivent. Comme nous l'avons vu dans cette thèse, la présence de contaminants peut poser des risques pour les organismes aquatiques. Un des composés étudiés est le cuivre, qui étant un métal ubiquiste et essentiel, peut être présent de manière naturelle à des concentrations très variables en fonction de la couche géologique (Flemming and Trevors, 1989; Willis and Bishop, 2016). Cependant, de nombreuses espèces aquatiques et notamment les poissons ont une sensibilité élevée à ce métal (article 1). Par exemple, Vardy et al. (2013) ont déterminé la CL_{50} à 96-h pour des larves de truite arc-en-ciel, d'esturgeon blanc (*Acipenser transmontanus*) et du tête-de-boule (*Pimephales promelas*). Elle était respectivement de 40, 22 et 102 µg/L de Cu. Dans une autre étude, les réponses létales au Cu ont été observées à partir de 22 µg/L pour les embryons et alevins de la truite arc-en-ciel (Besser et al., 2005). Le cuivre exerce sa toxicité en induisant la formation d'espèces réactives de l'oxygène (ROS) et causent des dommages cellulaires aux lipides, aux acides nucléiques et aux protéines pouvant entraîner la mort de la cellule (Bopp et al., 2008; Manzl et al., 2004; Prousek, 2007). La concentration la plus forte de cuivre étudiée (20 µg/L) a entraîné une mortalité élevée, principalement chez les embryons semi-éclos. Le pourcentage de mortalité embryonnaire était beaucoup moins important, ce qui peut se justifier par la nature protectrice du chorion des œufs des poissons (Arukwe et al., 1997; Jezierska et al., 2009). Cependant, lors de l'éclosion, les embryons entrent en contact direct avec le contaminant, ce qui entraîne un choc qui affecte négativement leur survie (Jezierska et al., 2009). Une hypothèse est que le cuivre pourrait avoir un effet inhibiteur sur l'activité de la chorionase, qui dégrade la zone interne du chorion pour faciliter l'éclosion par des processus mécaniques et osmotiques (Jezierska et al., 2009; Johnson et al., 2007). Dans l'étude de Johnson et al. (2007), il a été observé des effets négatifs sur la viabilité embryo-larvaire du poisson zèbre à partir de concentrations supérieures à 96 µg/L Cu. De plus, un effet inhibiteur sur l'éclosion a été aussi observé à partir de 53 µg/L de Cu. A la fin de l'exposition au cuivre, les larves présentaient un pourcentage élevé de malformations. Les effets ont été observées à des concentrations de quelques µg ou 10 µg/L, de telles valeurs peuvent être détectées dans différents environnements aquatiques. Cela pourrait signifier que de nombreux organismes vivant dans les écosystèmes aquatiques pollués par le cuivre, pourraient subir de graves conséquences sur leur santé, en particulier les premiers stades de vie, qui sont plus sensibles que les stades adultes.

Malgré la forte mortalité observée au début de l'éclosion, la biométrie larvaire n'a pas été affectée à la fin des 3 semaines d'exposition. Cela peut être lié à la faible accumulation du cuivre dans le corps de la larve au cours de cette phase. Le comportement de nage n'a pas non plus été affecté. De

même, aucune induction de dommage à l'ADN, de peroxydation lipidique ou de protéines carbonylées n'a été observée, alors que de nombreuses études démontrent ce type de modifications chez les poissons exposés au cuivre et qui sont induites par la génération de ERO (Barjhoux et al., 2012; Oss et al., 2013; Romeó et al., 2000; Santos et al., 2010; Vutukuru et al., 2006). D'autre part, les résultats en RT-qPCR ont montré une dérégulation de gènes impliqués dans différentes fonctions qui protègent la cellule, telle que la superoxyde dismutase (*sod*) qui protège la cellule contre le stress oxydatif provoqué par les ERO. Les gènes impliqués dans la détoxification (*gst, mt1, mt2*), l'arrêt du cycle cellulaire et l'apoptose (*p53* et *bax*) après 3 semaines d'exposition ont également été réprimés.

Dans le cas du glyphosate, dans sa formulation commercial Roundup®, les réponses des larves ont été différentes de celles exposés au cuivre. Malgré les concentrations relativement fortes de glyphosate utilisées (0,1 et 1 mg/L), la viabilité embryonnaire et larvaire n'a pas été affectée. En examinant les études écotoxicologiques menées sur le glyphosate, il a été constaté que la grande majorité des études utilisaient des concentrations élevées de l'ordre du mg/L. Peu d'études ont utilisé des concentrations plus pertinentes de l'ordre de la 10 à la 100 µg/L. Guilherme et al (2010, 2012) en exposant 3 jours des anguilles européennes à des concentrations de 58 et 116 µg/L, ont pu observer des effets génotoxiques sur les cellules sanguines. La faible toxicité du glyphosate pour les espèces animales peut être expliquée par son mécanisme d'action. En effet, le glyphosate, en tant qu'herbicide, agit en inhibant la voie du shikimate, présente uniquement dans les plantes et certains micro-organismes (Giesy et al., 2000; Myers et al., 2016). Cette voie est commune à toutes les plantes et c'est pourquoi le glyphosate agit comme un herbicide non sélectif. Les animaux, n'ayant pas cette voie métabolique, sont moins sensibles à l'action de cet herbicide. L'étude sur lignée cellulaire RTL-W1 confirmerait cette explication, étant donné que des effets cytotoxiques ont été observés mais seulement à partir de concentrations supérieures à 100 mg/L, permettant d'obtenir une CL_{50} de 710 mg/L. Dans la littérature, très peu d'études ont été menées sur des lignées cellulaires du poisson concernant les effets toxiques du glyphosate. La CL_{50} du glyphosate a été calculée en 315,34 and 371,77 mg/L dans les lignées cellulaires DIMF et TRMF des nageoires de *Misgurnus anguillicaudatus*, respectivement (Qin et al., 2017). La cytotoxicité de Roundup® a également été étudiée sur la lignée cellulaire du poisson zèbre, ZF-L, avec une réduction significative de la viabilité cellulaire à partir de 67.7 µg/L de glyphosate (Goulart et al., 2015).

Le glyphosate, ingrédient actif de nombreuses formulations commerciales, y compris le Roundup®, est devenu l'un des herbicides les plus largement utilisés dans le monde. Malgré sa faible toxicité, l'un des problèmes du glyphosate réside dans son utilisation intensive dans de nombreuses cultures. En raison de sa forte solubilité dans l'eau, ses molécules sont facilement transportées par ruissellement vers les cours d'eau. Ainsi, c'est l'un des herbicides les plus fréquemment détectés, même

à des concentrations supérieures à 100 µg/L (Coupe et al., 2012; Peruzzo et al., 2008). Dans le cas du bassin Adour Garonne, les concentrations maximales du métabolite AMPA dans les eaux de surface, ont diminué à 10 µg/L en 2013 par rapport à 2012 avec 80 µg/L (Agence de l'eau Adour-Garonne, 2012, 2014). Cependant, l'AMPA était toujours une des molécules d'origine phytosanitaire la plus détectée (près de 50% des échantillons des eaux de surface) (Agence de l'eau Adour-Garonne, 2014).

Dans notre étude, les concentrations de glyphosate étudiées ne se sont pas révélées létales chez nos larves. Cependant, certaines altérations ont pu être observées à la fin des 3 semaines d'exposition. Par exemple, les larves exposées à 100 µg/L se sont révélées hyperactives par rapport aux larves non exposées. Cette hyperactivité a également été observée dans plusieurs études avec la truite arc-en-ciel et des autres espèces de poisson (Ayoola, 2008; Morgan et al., 1991; Nwani et al., 2013; Valéria D.G. Sinhorin et al., 2014; Zhang et al., 2017). Cependant, il convient de mentionner l'étude de Morgan et al. (1991), qui ont observé un comportement erratique et agité chez les larves de truite arc-en-ciel après un mois d'exposition à des concentrations de glyphosate de 45 µg/L. Des hyperactivités similaires ont également été rapportées chez le tilapia du Nil (Ayoola, 2008) et le tilapia zillii (Nwani et al., 2013) à partir de 2 mg/L de glyphosate pendant 4 jours, et de 216 mg/L de glyphosate pendant 96 h. Par rapport à la croissance des poissons, il existe peu d'études dans la littérature sur les effets du glyphosate. Bien que l'on n'ait pas observé de malformations significatives, les larves ont une tête plus petite, ce qui suggère que des expositions chroniques au glyphosate pourraient provoquer des atrophies cranio-faciales comme rapporté par Zebral et al. (2017) et Zhang et al. (2017). Il est intéressant de noter que Zebral et al. (2017) ont évalué certains paramètres morphologiques au niveau céphalique, tels que le diamètre de l'œil et la distance entre les yeux d'embryons de pejerrey (*Odontesthes humensis*) qui ont été exposés au Roundup® (0.36 - 5.43 mg/L). Ces deux paramètres étaient significativement réduits de manière dépendant de la concentration chez les groupes exposés. Egalement, des têtes et des yeux plus petits ont été observés par Zhang et al. (2017) dans les embryons de poisson zèbre exposés pendant 96h a concentrations plus élevées (à partir de 100 mg/L) de glyphosate.

En ce qui concerne les études de génotoxicité, l'essai sur les comètes n'a révélé aucun dommage significatif dans les cellules sanguines des larves de truite. Cependant, les cellules hépatiques de la lignée RTL-W1, préalablement traitées avec l'enzyme FpG, ont montré des dommages importants à l'ADN. En effet, l'enzyme FpG permet d'améliorer le seuil de détection des lésions de l'ADN (Kienzler et al., 2012). À notre connaissance, aucune étude de génotoxicité n'a été réalisée sur la lignée RTL-W1 exposée au glyphosate. La comparaison entre ces 2 modèles biologiques est peu documentée et peut être compliquée. En effet, les effets observés avec des lignées cellulaires correspondent aux effets directs du contaminant sur les structures et/ou le fonctionnement cellulaire, contrairement à l'exposition

embryo-larvaire où l'organisme entier est exposé. Ces résultats, à la fois chez les larves et dans la lignée cellulaire, indiquent que, même si une exposition aux concentrations environnementales de glyphosate n'est pas létale pour les organismes aquatiques, elle peut entraîner des altérations morphologiques, comportementales ou génotoxiques qui peuvent à moyen ou long termes impacter les capacités biotiques des individus.

Il est important de considérer les adjuvants dans les formulations commerciales des pesticides, qui peuvent augmenter la toxicité pour les vertébrés, et pas seulement le composé actif. C'est donc le cas du glyphosate, où plusieurs études ont montré que sa formulation commerciale, Roundup®, est plus toxique que son composé technique. Cela pourrait être lié à la présence d'additifs et tensioactifs (tels que le POEA) dans la formulation commerciale (Gasnier et al., 2009; Koller et al., 2012; Martínez et al., 2007; Mesnage et al., 2013; Vanlaeys et al., 2018).

Au sujet du chlorpyrifos (CPF), les concentrations étudiées n'ont pas entraîné d'effet létal significatif après 3 semaines d'exposition. Les molécules de CPF sont transportées par ruissellement vers les écosystèmes aquatiques. Compte tenu de son caractère hydrophobe, elle est généralement adsorbée sur les particules en suspension, ainsi que sur les sédiments. Comme notre système d'exposition est composé de différents types de plastiques, il est possible qu'une partie importante du CPF ait été adsorbée, comme l'ont montré les analyses chimiques qui notent une décroissance importante de ce composé dans nos unités expérimentales. Il est également possible que les larves aient absorbé les molécules de CPF en réduisant la concentration du contaminant dans l'eau (Barron et al., 1993). Pour contrecarrer cette diminution, le système d'exposition a été saturé avec du CPF pendant 48 h préalable à l'étude. En plus, l'eau d'exposition a été renouveler toutes les 48 h, pour assurer une exposition continue des truites.

Les organismes aquatiques sont très sensibles aux expositions au CPF (Carr et al., 1997; Humphrey and Klumpp, 2003). En tant que pesticide organophosphoré (PO), son mécanisme d'action consiste à inhiber l'activité de l'acétylcholinestérase (AChE), présente à la fois chez les insectes et chez les vertébrés, entraînant également des modifications du comportement. Plusieurs études ont révélé les effets négatifs du CPF sur le système nerveux des organismes (Sledge et al., 2011). Cependant, dans notre étude, peu de modifications ont été observées chez les larves de truite arc-en-ciel exposées au CPF. L'inhibition de l'activité AChE dans la tête des larves exposées n'a pas été observée après 3 semaines d'exposition. Cependant, en analysant le comportement de nage des larves, il a été observé que les larves exposées à 3 µg/L étaient moins mobiles. Dans ce cas, il aurait été intéressant de pouvoir analyser les niveaux d'AChE dans les muscles des larves, car comme le suggèrent certaines études, ils

pourraient être responsable de la réduction de la mobilité (Fulton and Key, 2001). Mais, en raison de limitations techniques liées à la taille des larves, cela n'a pas été possible.

D'autre part, malgré le fait que le CPF n'est pas considéré comme un perturbateur endocrinien (Juberg et al., 2013; US EPA, 2015), les faibles concentrations de CPF inhibent l'expression de gènes codant pour le récepteur aux œstrogènes-β (*ER-β*) et de l'aromatase (*arom* ou *CYP19A1*). Bon nombre des actions des œstrogènes sont médiées par leurs récepteurs (Yu et al., 2015). Certaines études indiquent que le CPF affecte les hormones thyroïdiennes, et qu'il a des propriétés anti-androgéniques et œstrogéniques (Brandt et al., 2015; Filby et al., 2007; Watts, 2012; Yu et al., 2015). Oruç (2010) avait constaté que le CPF provoquait une perturbation du système endocrinien chez la tilapia du Nil, où des taux de cortisol, d'oestradiol et de téstostérone sériques étaient considérablement inférieurs après une exposition de 30 jours à 5, 10 et 15 µg/L de CPF.

Il convient de mentionner que la lignée RTL-W1 a également été exposée à différentes concentrations de CPF (jusqu'à 1000 µg/L). Bien que les microplaques aient été préalablement saturées de CPF, aucun effet sur la cytotoxicité n'a été observé. Cela peut être dû à une adsorption du CPF sur les parois de la microplaque (en polypropylène) en raison de sa forte hydrophobicité. Mais cela peut également venir de l'adsorption du CPF sur les composants du milieu de culture. Comme plusieurs études l'ont indiqué, le milieu d'exposition peut également influer sur la viabilité des cellules (Bols et al., 2005; Pannetier et al., 2018). Le milieu de culture est en générale enrichi en sérum tels que le sérum de veau fœtal (FBS = Fetal Bovine Serum), dont les composants peuvent adsorber des polluants organiques hydrophobes (Bols et al., 2005) telles que le CPF, et réduire sa biodisponibilité dans les cellules. En plus, le FBS contient des composés, telles que des antioxydants, qui pourrait inhiber la toxicité provoquée par les ERO (Bols et al., 2005). L'étude de Doostdar et al. (1988) a montré que la viabilité cellulaire de la lignée cellulaire humaine d'origine hépatique, Hep G2, exposé au bromobenzène variait significativement selon la composition et le milieu d'exposition. Donc, l'utilisation d'un milieu de culture riche peut conduire à une sous-estimation de la toxicité de la substance étudiée en la rendant moins biodisponible pour les cellules.

2. Etude des mécanismes de défense et des effets délétères induits lors d'une exposition de mélange de pesticides.

Comme de nombreuses formulations commerciales contenant du Cu, du glyphosate et du CPF sont largement utilisées pour le traitement des cultures, ces molécules ont souvent été détectées dans les affluents et les rivières proche de ces cultures (Aparicio et al., 2013; Coupe et al., 2012; Davis et al.,

2001; Devez et al., 2005; Phillips and Bode, 2004). C'est pourquoi les effets individuels de chacune de ces molécules ont été étudiés chez les embryons et les larves de truite arc-en-ciel. Cependant, ces pesticides peuvent se trouver combinés avec des autres molécules (Krishnan and Brodeur, 1994). En outre, de nombreux facteurs abiotiques peuvent influer sur la biodisponibilité et la toxicité de ces molécules. De ce fait, le deuxième objectif du présent travail était d'étudier l'effet combiné de ces trois pesticides en utilisant les mêmes stades de développement du poisson.

Contrairement aux expositions faites avec des pesticides individuels, un mélange de pesticides peut entraîner de nombreuses interactions pouvant modifier leur biodisponibilité, leur persistance et leur toxicité. Par exemple, un groupe de pesticides ayant un mode d'action similaire affectera la même cible moléculaire, pouvant entrainer des effets additifs. Ou, au contraire, un groupe de pesticides peut avoir différents modes d'action qui peuvent ou pas affecter la même cible moléculaire. Dans ce cas, des effets de synergie ou d'antagonisme peuvent avoir lieu. Le cuivre s'est révélé toxique chez les embryons de truite arc-en-ciel. Cependant, lorsque le cuivre est combiné avec le CPF et le glyphosate, le mélange ne s'est pas montré létal. Des études montrent que la quantité de Cu^{2+} peut être réduite en présence de glyphosate, ce qui réduit la biodisponibilité du cuivre pour les organismes (Tsui et al., 2005; Zhou et al., 2012). Par exemple, l'étude de Tsui et al. (2005) montre que les juvéniles de cladocera (*Ceriodaphnia dubia*), exposés à différents métaux (Ag, Cd, Cr, Cu, Ni, Pb and Zn), présentaient une diminution moins importante de la viabilité en présence du Roundup® que ceux exposés aux métaux seuls. Dans ce cas, la CL_{50} à 48 h pour *C. dubia* exposé au cuivre était estimée en 10 µg/L Cu^{2+}, mais en présence de glyphosate, la mortalité était réduite en 95 %. Zhou et al. (2012) ont expérimenté les interactions entre le Cu et le glyphosate sur la toxicité pour le ver de terre (*Eisenia fetida*). La CL_{50}-48 h du Cu pour *E. fetida* a été calculé à 0.11 mg/L, mais lorsque combiné avec le glyphosate à des concentrations de 0, 2 et 10 mg/L, le taux de mortalité des vers étaient de 57,3 et 0 %, respectivement.

Cependant, certains effets sub-létaux ont pu être observés tels que l'hyperactivité des larves exposées. Lors de l'étude des mélanges de composés xénobiotiques, il est difficile de prévenir quel type de modification du comportement peut influencer les organismes étudiés, car cela dépend non seulement de la concentration, mais également de la proportion de ces polluants et des espèces de poissons étudiées. Par exemple, plusieurs études ont observé, principalement dans des mélanges binaires de ces composés, une hyperactivité ou une hypoactivité chez les poissons en fonction des concentrations et des proportions de ces pesticides (Bonansea et al., 2016; Bonifacio et al., 2016; Kienle et al., 2009; Tilton et al., 2011). Il a été observé que les expositions au CPF réduisaient les activités de nage dans les poissons femelles chez *Cnesterodon decemmaculatus* (Bonifacio et al., 2016) et les adultes de poissons zèbre (Tilton et al., 2011). Cependant, dans les deux études, l'ajout de glyphosate (Bonifacio et al., 2016) ou de cuivre (Tilton et al., 2011) a réduit l'impact du CPF sur les poissons. En effet, l'ajout

de Cu ou glyphosate pourraient bloquer les effets neurologiques du CPF, ou modifier son absorption au niveau des neurones sensibles (Bonifacio et al., 2016; Tilton et al., 2012, 2011). Dans l'étude de Kienle et al. (2009), lors de l'exposition d'embryons de poisson zèbre à des combinaisons binaires de CPF et de $NiCl_2$, les auteurs ont montré que lorsque le nickel était combiné à de fortes concentrations de CPF, l'activité de nage augmentait. Mais, en combinant le nickel à de faibles concentrations de CPF, l'activité de nage diminuait. Étant donné que les deux composés ont des modes d'action différents, lorsqu'ils sont mélangés ils peuvent agir indépendamment l'un de l'autre ou rentrer en compétition et se neutraliser.

Fait intéressant, l'exposition à la concentration la plus élevée de pesticides a endommagé les molécules d'ADN après traitement par l'enzyme FpG lors du test des comètes. Ces dommages n'ont pas été observés chez les larves exposées individuellement à des pesticides. Bien que ces dommages n'aient été observés que dans le mélange à des concentrations élevées, l'association de ces 3 composés, peut induire des dommages à l'ADN de type lésions oxydatives car mises en évidence par le traitement à la FpG.

Notre analyse de l'expression génique a révélé de manière générale une induction significative des gènes impliqués dans la détoxification (*gst* et *mt1*), la réparation de l'ADN (*ogg1*), le métabolisme mitochondrial (*cox1* et *12S*) et le système cholinergique (*ache*). Les gènes impliqués dans la détoxification, *gst* et *mt1*, ont été induits en particulier chez les larves exposées aux concentrations de mélange la plus basse et la plus élevée. L'expression du *gst* (glutathion S-transférase) est généralement induite lorsque les poissons sont exposés aux xénobiotiques, tandis que le gène *mt1*, codant une métallothionéine, est généralement induit par l'exposition aux métaux tels que le cuivre, mais également lors d'un stress antioxydant. Ces changements dans l'expression de gènes pourraient indiquer que les larves ont mis en place des mécanismes de défense aux plus faibles concentrations de pesticides. En regardant les résultats d'expression de *cox1* (cytochrome c-oxydase) et *12S*, on observe une induction de l'expression de leurs gènes dans les larves exposées au mélange de pesticides. Ces dérégulations étaient encore plus sévères chez les larves exposées à la plus forte concentration du mélange. Une induction de ces gènes pourrait révéler un dysfonctionnement de la chaîne respiratoire. L'induction observée de *cox1* et *12S* pourrait être une réponse à une demande énergétique élevée, sous forme d'ATP, pour se défendre de la production de ERO (Achard-Joris et al., 2006; Chen et al., 2003). Par ailleurs, l'induction de l'expression du gène de réparation de l'ADN, *ogg1*, a été observée dans toutes les conditions. Ce gène est responsable de l'élimination de la 8-oxoguanine à la suite de la production d'ERO. La surexpression du gène *ogg1* sur les larves exposées à la concentration de mélange la plus élevée (x34,8) est probablement liée à l'induction de lésions de l'ADN que nous avons observées sur les cellules sanguines après traitement avec l'enzyme Fpg.

Ces profils d'expression génique confirment que la présence de faibles concentrations d'un mélange de pesticides dans les écosystèmes aquatiques pourrait également avoir des répercussions, qui pourraient se manifester à long terme, chez les organismes qui l'habitent. Il a également été constaté que l'induction de certains gènes est une réponse spécifique à l'exposition à certaines familles de polluants. Par exemple, le gène *mt1* est généralement induit par certains métaux tels que le cuivre (Gonzalez et al., 2006; Olsson et al., 1989; Sheader et al., 2006; Woo et al., 2006) ; puisque cette protéine est précisément responsable de la liaison aux métaux xénobiotiques par le biais de ses groupes thiol, offrant une protection à la cellule contre le stress oxydatif. Un autre gène est l'*ache*, qui est généralement altérée par l'exposition à des pesticides organophosphorés tels que le CPF (Rodríguez-Fuentes et al., 2015).

En observant le profil d'expression de ces gènes, on peut considérer qu'ils représentent une réponse adaptative des larves de truite arc-en-ciel exposées aux pesticides. En effet, les inductions de gènes observées n'ont pas été corrélées avec des augmentations de mortalité ou des taux de malformations importantes. Cette réponse adaptive pourrait être activé pour faire face à l'exposition aux toxiques. Cela peut être la raison pour laquelle aucune oxydation de lipides ou de protéines n'a été observeé. Le caractère adaptatif pourrait, de plus, confirmer les faibles niveaux de TBARS mesurés chez les larves exposées.

3. Etude des effets toxiques des échantillons environnementaux (eau et sédiment)

Un autre objectif de ce travail était d'étudier les effets sub-létaux d'échantillons environnementaux prélevés à proximité d'un vignoble. Pour cela, des échantillons d'eau et de sédiments La Livenne ont été analysés dans la lignée RTL-W1 et dans les larves de truite arc-en-ciel. Les échantillons ont été collectés au cours des mois de février, juin et août 2017. L'utilisation des lignées cellulaire a non seulement permis d'analyser un plus grand nombre d'échantillons, mais également a permis de réaliser une analyse spatio-temporelle de la toxicité de chaque site.

Dans le cas de la truite arc-en-ciel, la reproduction a lieu en hiver. Cependant, dans les sites d'élevage de truites, il est possible de réaliser une fécondation décalée pendant l'été. Puisque nous nous avons voulu analyser les échantillons les plus impactés par les pesticides, nous avons décidé d'exposer des larves des truites à des échantillons prélevés en juin 2017, qui sont conformes à la période de traitement par les produits phytosanitaires. Un autre facteur limitant dans l'étude sur la truite était la durée d'exposition qui devait être réduite à 48 h pour éviter le développement de champignons et de bactéries dans l'eau collectés de La Livenne. L'idéal aurait été de procéder à des collectes d'eau

hebdomadaires afin de prolonger le temps d'exposition. Une autre limite de cette étude est l'absence de données de contamination chimique, qui sont toujours en cours d'analyse.

Lorsque les compartiments eau et sédiments ont été évalués séparément sur la ligne RTL-W1, les profils de toxicité de ces deux compartiments étaient différents. Certains échantillons d'eau se sont révélés cytotoxiques. Cependant, aucune induction significative d'ERO n'a été observée. Comme nous l'avons déjà mentionné dans l'article 5, les eaux de surface sont le reflet d'une pollution ponctuelle. La présence ou l'absence de contaminants dans la colonne d'eau peut varier en fonction de la saison, de la fréquence de traitement et de l'hydrologie du tributaire (sédimentation, transport, dilution ou dégradation) au moment de l'échantillonnage. L'échantillonnage des eaux de surface présente plusieurs limites, car, lorsqu'ils sont effectués ponctuellement, les contaminants peuvent être très dilués. En outre, l'échantillonnage ponctuel des eaux de surface ne prend pas en compte la variabilité temporelle de la contamination (Le Dréau et al., 2015), et les pics de pollution peuvent passer inaperçus. L'absence de précipitations peut également influer sur le rejet de pesticides par les cultures en raison de la réduction du lessivage des sols.

Dans le cas des sédiments, les élutriats n'étaient pas cytotoxiques pour les lignées RTL-W1, mais ils étaient capables de produire des ERO. Les échantillons de tous les sites ont été en mesure d'induire ERO selon la période d'échantillonnage, à savoir les mois de février et mai. Cette période, qui coïncide avec la période de traitements phytosanitaires, sont ceux qui ont induit le plus de ROS. Les réductions d'ERO pour le mois d'août peuvent également être liées à la réduction des précipitations et donc du débit de La Livenne. La granulométrie et le type de sédiment jouent également un rôle important dans la rétention des métaux et des contaminants organiques hydrophobes, car plus la particule est fine, plus la surface spécifique est grande (Duong et al., 2010), et plus la quantité de matière organique est importante, plus l'adsorption de contaminants est grande (Froehner et al., 2010; Orescanin et al., 2004).

L'évaluation des effets écotoxicologiques d'échantillons environnementaux pollués constitue un défi. Cela est dû non seulement aux effets des mélanges de polluants, à leur concentration généralement faible mais également à la nature physico-chimique complexe de l'eau et des sédiments, et à l'influence qu'ils peuvent avoir sur la biodisponibilité et persistance des polluants (Costa et al., 2011). Une étude expérimentale intégrant les deux compartiments, l'eau et le sédiment, présente l'avantage de proposer une approche plus réaliste des conditions d'exposition environnementale des organismes directement exposés aux aquifères contaminés. Dans l'étude *in vivo*, les larves de truite ont été exposées aux deux compartiments pendant 48 h. Au bout de ce temps, certaines manifestations sub-létales ont été observées chez les larves. Par exemple, les larves exposées aux échantillons du site à l'aval de La Livenne (L4-Vignolles) présentaient malabsorption du sac vitellin, L'absorption du sac

vitellin peut être considérée comme un marqueur important du développement embryonnaire puisqu'un retard dans la résorption pourrait impliquer un retard dans le développement du poisson. Cela a déjà été observé pour des premiers stades de poissons exposés aux pesticides (Amblard et al., 1998; Coelho et al., 2011; Kalasekar et al., 2015), et aux métaux (Johnson et al., 2007; Witeska et al., 2010). D'autre part, lors de l'analyse du comportement de nage des larves, des réponses variables sont observées selon le site analysé. Par exemple, les larves exposées au site L3 (Grand Village) étaient moins rapides, tandis que les larves des sites L1 (Menanteau) et L4 étaient beaucoup plus mobiles. La diminution de la vitesse de nage peut être liée à un dysfonctionnement métabolique ou à une augmentation de la demande métabolique entraînant une allocation d'énergie insuffisante pour la nage (Scott and Sloman, 2004; Triebskorn et al., 1997). D'autre part, une augmentation de l'activité natatoire peut être interprétée comme une réaction à un stress et notamment à un pic de pollution. Les modifications du comportement peuvent aussi être le résultat de dommages sur le système nerveux (Scott and Sloman, 2004; Weis et al., 2001). En effet, de nombreuses études ont établi une relation entre les modifications du comportement de nage et une diminution de l'activité de l'AChE (Faria et al., 2015). Certains pesticides, tels que les organophosphorés et les carbamates, peuvent altérer l'activité de l'AChE en interférant avec la signalisation cholinergique normale chez les vertébrés, c'est le cas notamment du chlorpyrifos-oxon (Faria et al., 2015). Dans notre étude, aucune diminution de l'activité AChE n'a été observée chez les larves exposées quelles que soient les conditions d'exposition. Cependant, les résultats en RT-qPCR ont révélé que l'expression du gène de l'*ache* était diminuée chez toutes les larves exposées par rapport au témoin. Cette régulation négative était encore plus intense chez les larves du site L3, pouvant peut-être expliquer la diminution de la vitesse de nage. Par ailleurs, il a été montré que la régulation négative de l'ache implique une perturbation des fonctions neuronales et neuromusculaires.

Conclusions et perspectives

Conclusion et perspectives

Ce travail de thèse a permis de fournir une évaluation de l'impact de différents composés phytosanitaires, utilisés dans le domaine de la viticulture, sur les premiers stades de développement de la truite arc-en-ciel en prenant en compte à la fois des paramètres phénotypiques, comportementaux, génétiques et biochimiques. Pendant 3 semaines, des embryons de truites ont été exposés en conditions contrôlées de laboratoire au cuivre, au glyphosate et au chlorpyrifos, seuls et en mélange.

Les résultats sur le cuivre ont révélé des effets toxiques sur le développement de la truite, principalement sur les embryons exposés à la plus forte concentration (20 µg/L). Les principaux effets incluent l'augmentation des embryons demi-éclos, du taux de malformations, et la dérégulation des gènes impliqués dans la détoxication et l'arrêt du cycle cellulaire. Au moment de l'éclosion, la mortalité des embryons était élevée, mais les larves écloses ont survécu et semblaient s'adapter au milieu contaminé. Cependant, l'altération des gènes observée, principalement ceux impliqués dans la détoxication (*gst*, *mt1* et *mt2*), pourrait compromettre la survie à moyens et long termes de ces individus. Les résultats sur le glyphosate ont révélé que les plus faibles concentrations étudiées (0.1 mg/L) induisent une hyperactivité des larves et des altérations morphologiques de la tête. En parallèle, les études sur la lignée cellulaire RTL-W1, a montré des effets génotoxiques alors qu'aucun dommage à l'ADN n'a pas été observée sur les cellules sanguines de larves exposées aux mêmes concentrations de glyphosate. Cette différence peut s'expliquer par l'intervention des systèmes antioxydant et de réparation de l'ADN dans les cellules sanguines, car une diminution des taux de TBARS et une régulation différentielle du gène *cat* et *cox1* ont été observées dans les larves exposées. Le chlorpyrifos (CPF) est l'un des insecticides les plus utilisés en agriculture et est considéré comme très toxique pour les organismes aquatiques. Cependant, les effets de concentrations environnementales de CPF sur le développement embryonnaire des poissons ont été peu documentés. Après 21 jours d'exposition d'embryons de truite arc-en-ciel à des concentrations faibles et modérées de CPF, aucun effet sur la mortalité, le développement, les dommages à l'ADN, la peroxydation des lipides et les protéines carbonylées n'a été observé. Néanmoins, les larves exposées à 3 µg/L de CPF étaient nettement moins mobiles lors de la stimulation par la lumière. De plus, l'exposition au CPF a réduit le niveau d'expression des gènes *erb* et *cy19a1*, qui sont impliqués dans la reproduction des poissons. Dans ce cas, il serait utile de pouvoir étudier d'autres biomarqueurs en tant que marqueurs de perturbateur endocriniens. La vitellogénine (Vtg) et les protéines de zona radiata (Zrp) pourraient être utilisées comme biomarqueurs des effets des composés perturbateurs endocriniens dans les premiers stades de vie des poissons.

L'ensemble de ces résultats démontre que l'exposition individuelle au Cu, glyphosate et chlorpyrifos, aux concentrations qui peuvent se trouver dans les écosystèmes, pourraient représenter un risque potentiel en milieu naturel pour les poissons notamment aux premiers stades de vie. Il serait intéressant de poursuivre ces travaux avec des exposition chronique à des concentrations plus faibles (de l'ordre de la LOEC (low observed effect concentration) ou la PNEC (predicted no-effect concentration)) pour évaluer les effets à long terme sur la croissance et la reproduction voire même des effets transgénérationnels.

L'exposition au mélange de pesticides a induit une hyperactivité natatoire des larves, et une génotoxicité sur les cellules sanguines. En effet, lorsque les cellules ont été traitées avec l'enzyme Fpg, une augmentation significative des dommages à l'ADN a été observée, en particulier pour la concentration de mélange la plus forte. En outre, l'expression de plusieurs gènes a été induite, en particulier ceux impliqués dans la détoxication, le métabolisme mitochondrial, le système cholinergique et la réparation de l'ADN. L'induction de ces gènes suggère que l'exposition à de faibles concentrations d'un mélange de Cu, glyphosate et chlorpyrifos a un effet plutôt adaptatif pour les embryons et larves de truite car aucun effet létal n'a été observé.

Dans ce travail, nous avons également étudié l'impact des traitements phytosanitaires sur la vigne en utilisant les deux modèles biologiques, la lignée cellulaire RTL-W1 et les larves de la truite arc-en-ciel. Nos résultats ont mis en évidence les effets importants des échantillons environnementaux prélevés dans l'eau et les sédiments sur ces deux modèles. L'exposition *in vitro* a permis d'étudier les variations spatio-temporelles de toxicité des échantillons d'eau et de sédiment de la Livenne avec notamment des effets cytotoxiques, en particulier sur les échantillons d'eau, et la production d'ERO pour les élutriats. Le test *in vivo* a permis d'intégrer d'autres critères écotoxicologiques, tels que les biomarqueurs moléculaires, où des altérations importantes ont été observées sur la peroxydation lipidique et notamment, l'expression différentielle des gènes *rad51* et *ache* chez les larves. Même si aucune inhibition significative n'a été observée sur l'activité enzymatique de l'AChE, la diminution de l'expression du gène de l'*ache* pourrait expliquer les altérations observées sur le comportement des larves. En intégrant toutes ces résultats, nous pouvons conclure que les traitements phytosanitaires sur la vigne pourraient représenter un risque pour les espèces aquatiques notamment les premiers stades de développement de poissons vivants à proximité des parcelles viticoles. Pour compléter ces résultats, il serait nécessaire d'analyser les effets génotoxiques de ces échantillons sur la lignée cellulaire RTL-W1.

Ces expositions en conditions contrôlés de laboratoire, ne reflètent pas complétement les scénarios naturels. En effet, les tests de laboratoire ignorent les facteurs de stress physiques et biologiques que les organismes subissent dans leur environnement naturel (Connon et al., 2012). De

même, les expositions en continue aux échantillons environnementaux d'eau et de sédiment ne reflètent souvent pas les scénarios d'exposition intermittente aux pesticides dans l'environnement du fait de l'application ponctuelle et répétée des phytosanitaires sur les cultures (Connon et al., 2012). La mesure des biomarqueurs d'exposition ou d'effet chez les organismes collectés sur le terrain ou exposés *in situ* est donc plus pertinente du point de vue écologique que les essais de laboratoire (Connon et al., 2012). Dans cette optique, il serait judicieux de proposer des expositions en conditions contrôlées de laboratoire avec des mélanges de pesticides et en faisant varier des facteurs physico-chimiques tels que la température et le taux d'oxygène. Il serait également intéressant de proposer des expositions *in situ* dans un cours d'eau impacté par la viticulture, tel que La Livenne en encageant des embryons ou des larves de truite (Bony et al., 2008). De même, dans le cadre du projet PhytoCote, il serait utile de réaliser des collectes de poissons natifs *in situ* sur différents sites impactés et non impactés par les pesticides, et analyser les mêmes biomarqueurs déjà étudiés au cours de cette étude.

Deux différents modèles biologiques de la même espèce ont été utilisés au cours de ce travail. Les premiers stades de vie de la truite arc-en-ciel apportent plusieurs avantages par rapport à leur sensibilité aux polluants, leur facilité de manipulation, la durée d'exposition et la disponibilité d'embryons grâce aux écloseries de poissons. Cependant, étant une espèce non indigène du territoire européen, il pourrait être plus pertinent d'étudier des espèces de salmonidés qui sont natives de nos cours d'eau comme la truite fario (*Salmo trutta*) ou le saumon atlantique (*Salmo salar*). De même, nous pourrions proposer d'étudier d'autres familles de poissons qui sont communes de petits cours d'eau de la Gironde comme la perche commune (*Perca fluviatilis*) ou le brochet (*Esox lucius*), ce dernier étant considéré comme un poisson bioaccumulateur des polluants (Sandheinrich and Drevnick, 2016). Par ailleurs, la lignée cellulaire RTL-W1 a mis en évidence le caractère toxique de certains échantillons environnementaux de La Livenne ainsi que le caractère génotoxique du glyphosate. Néanmoins, il est possible de proposer l'utilisation d'autres lignées cellulaires provenant d'autres tissus des poissons, qui permettraient d'étudier divers biomarqueurs.

Les larves de truites arc-en-ciel disposent d'un système de défense antioxydant efficace. Cela est suggéré par la réduction significative de la peroxydation des lipides observées dans les larves exposées au glyphosate, aux mélanges de pesticides et aux échantillons environnementaux de La Livenne. Par ailleurs, nous avons observé une absence de carboxylation des protéines, et l'absence de dommage à l'ADN. Afin de vérifier cette hypothèse, il serait nécessaire d'étudier d'autres biomarqueurs enzymatiques tels que la catalase (CAT), le superoxyde dismutase (SOD), la glutathion s-transférase (GST), ainsi que les métallothionéines (MT), qui sont généralement induites en présence des métaux.

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Annexes

Liste des publications et communications

Publications :

S. Weeks Santos, J. Cachot, P-Y Gourves, C. Clérandeau, B. Morin, P. Gonzalez (2019). **Sub-lethal effects of waterborne copper in early developmental stages of rainbow trout (*Oncorhynchus mykiss*)**. *Ecotoxicology and Environmental Safety*. 170, 778-788.

S. Weeks Santos, P. Gonzalez, B. Cormier, N. Mazzela, C. Clérandeau, B. Bonnaud, S. Morin, B. Morin, J. Cachot. **A glyphosate-based herbicide induces sub-lethal effects in earlylife stages and liver cell line of rainbow trout, *Oncorhynchus mykiss***. *Soumis dans Aquatic Toxicology*.

S. Weeks Santos, P. Gonzalez, N. Mazzela, C. Clérandeau, B. Morin, J. Cachot. **Effects of sub-chronic exposure to the insecticide chlorpyrifos-ethyl to early stages of development of rainbow trout (*Oncorhynchus mykiss*)**. *En préparation*.

S. Weeks Santos, J. Cachot, B. Cormier, N. Mazzela, C. Clérandeau, B. Bonnaud, S. Morin, P-Y. Gourves, B. Morin, P. Gonzalez. **Effects of environmental concentrations of a mixture of copper, glyphosate and chlorpyrifos exposed to early life stages of rainbow trout (*Oncorhynchus mykiss*)**. *En préparation*.

S. Weeks Santos, P. Gonzalez, B. Cormier, C. Clérandeau, P-Y. Gourves, B. Morin, J. Cachot. **Evaluation of the toxicity of environmental samples collected near vineyard parcels on rainbow trout larvae (*Oncorhynchus mykiss*) and liver cell line RTL-W1**. *En préparation*.

Communications orales :

Shannon Weeks, Bénédicte Morin, Patrice Gonzalez, Jérôme Cachot. **Etude de l'expression génique et des dommages à l'ADN des premiers stades de développement de la truite arc-en-ciel (*Oncorhynchus mykiss*) exposés au fongicide $CuSO_4$.** 12th ECOBIM- Au Havre, France (30 mai – 1 juin 2016).

Shannon Weeks, Bettie Cormier, Bénédicte Morin, Kéwin Gery, Nicolas Mazzela, Patrice Gonzalez, Jérôme Cachot. **Environmental concentrations of glyphosate can induce sublethal effects in early life stages of rainbow trout (*Oncorhynchus mykiss*).** 30th ESCPB 2016- Barcelone, Espagne (4 – 7 septembre 2016).

Shannon Weeks, Patrice Gonzalez, Johanna Groussin, Quentin Papin, Christelle Clérandeau, Bénédicte Morin, Pierre-Yves Gourves and Jérôme Cachot. **Evaluation de la toxicité des échantillons environnementaux prélevés près de parcelles viticoles sur les larves de truite arc-en-ciel (*Oncorhynchus mykiss*) et la lignée cellulaire RTL-W1.** 14th ECOBIM 2018- Bordeaux, France (22 – 25 mai 2018).

Communications affichées :

Shannon Weeks, Bénédicte Morin, Patrice Gonzalez, Jérôme Cachot. **Sublethal effets of copper in early life stages of development of rainbow trout (*Oncorhynchus mykiss*).** 26th SETAC Europe 2016- Nantes, France (22 – 26 mai 2016).

Shannon Weeks, Bénédicte Morin, Kéwin Gery, Nicolas Mazzela, Patrice Gonzalez, Jérôme Cachot. **Etudes des effets sublétaux dans les premiers stades de développement de la truite arc-en-ciel**

(*Oncorhynchus mykiss*) exposés à trois pesticides : sulfate de cuivre, glyphosate et chlorpyrifos-éthyl. Colloque SEFA 2017- Lille, France (29 – 30 juin 2017).

Shannon Weeks, Patrice Gonzalez, Johanna Groussin, Quentin Papin, Christelle Clérandeau, Pierre-Yves Gourves, Bénédicte Morin, Jérôme Cachot. Evaluation of the toxicity of environmental samples collected near vineyard parcels on rainbow trout larvae (*Oncorhynchus mykiss*) and liver cell line RTL-W1. 28th SETAC Europe- Rome, Italie (13 – 17 mai 2018).

Animation scientifique :

Animation atelier : Pauline Pannetier, Shannon Weeks, Jérôme Cachot. Les embryons et larves de poisson, des sentinelles de la qualité des eaux. Journée Portes Ouvertes, Fête de la Science, Station Marine d'Arcachon. Arcachon, 10 octobre 2015.

Présentation orale. Étude des réponses adaptativesou délétères aux premiers stades de développement des poissons exposés à des pesticides organiques et inorganiques. Séminaire EPOC- EA. Talence, 28 avril 2017.

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Animation atelier. La pollution des eaux par les pesticides. Colloque jeune public (lors du congrès ECOBIM 2018- Bordeaux). 25 mai 2018.