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EDSMRE

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Intérêts de l'ovocyte de *Xenopus laevis* en écotoxicologie ?

Caractérisation des effets de contaminants environnementaux sur ce modèle alternatif

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Devant le jury composé de :

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Addunt ettiamnum alia magi, quae si vera sunt,
multo utiliores vitae existimentur ranae, quam leges.

Les mages ajoutent que, si c'est vrai, les grenouilles
seraient à juger beaucoup plus utiles à la vie que les lois.

Pline l'Ancien, Naturalis Historia, 32, 18

A mes parents, Maryline et Eric

A celle qui partage ma vie, Laureen

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ABREVIATIONS

AChE : Acetylcholinesterase

AMPA : Aminomethylphosphonic Acid

AMPHITOX : Set de tests de toxicité utilisant des embryons d'amphibien

ADN : acide désoxyribonucléique

ARLA : Amphibian Red List Authority

ASTM : American Society for Testing and Materials

ATSDR : Agency for Toxic Substances and Disease Registry

BChE : Butyrylcholinesterase

CAT : Catalase

CbE : Carboxylesterase

CEEA : Comité d'Ethique en Expérimentation Animale

Cdc2/Cdk1 : Cyclin-dependant kinase 1

Cdc42 : Cell division control protein 42 homolog

CYP450 : Cytochrome P450

DOC1R : Deleted in Oral Cancer One Related

e.a. : Equivalent acide

EEC : European Community Council

EC50 : Median Effective Concentration

EDTA : Ethylenediaminetetraacetic Acid

EGFP : Enhanced Green Fluorescent Protein

EPP : EndPlate Potential

EQS : Environmental Quality Standards

ERK2 : Extracellular signal-Regulated Kinase 2

FETAX : Frog Embryo Teratogenesis Assay – Xenopus

GSH : Glutathione (reduced)

GST : Glutathione S-transferase

GVBD : Germinal Vesicle Breakdown

H1 : Histone H1

H3 : Histone H3 (Serine 10)

hCG : Human Chorionic Gonadotropin

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSP70 : 70 kilodalton heat shock proteins

IARC : International Agency for Research on Cancer

ICl : Calcium-activated chloride currents

ICl1-S : Calcium-activated chloride current enhanced by calcium release from internal stores

ICl1-T : Calcium-activated chloride current enhanced by calcium release and calcium entry

ICl2 : Calcium-activated chloride current enhanced by calcium entry from external medium

ISO : International Organization for Standardization

LAGDA : Essai de croissance et de développement de larves d'amphibien

MAPK : Mitogen-Activated Protein Kinases

MAPKK : Mitogen-Activated Protein Kinase Kinase

MBMCR : Meat and Bone Meal Combustion Residues

MDA : Malondialdehyde

MEK : MAPK/ERK Kinase

MEPP : Miniature Endplate Potential

MISS : MAP kinase–Interacting and Spindle-Stabilizing protein

MMR : Mark's modified ringer

MPF : M-phase Promoting Factor

MS222 : Tricaine mesylate

MT : Metallothionein

MTOC : Microtubule Organizing Center

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF : Norme Française

NP : Nanoparticles

ND96 : Nathan Dasqual 96

NWS : Non-White Spot

OCDE : Organisation de coopération et de développement économiques

Pg : Progesterone

POEA : Polyethoxylated tallow Amine

QSP : Quantité Suffisante Pour

ROS : Reactive Oxygen Species

RSK/p90^{RSK} : Ribosomal S6 Kinase

SDS-PAGE : Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SEM : Standard Error of the Mean

SOD : Superoxide dismutase

T_3 : Triiodothyronine

TMA : Transient Microtubule Array

UICN : Union Internationale pour la Conservation de la Nature

USA : United States of America

USEPA : United States Environmental Protection Agency

UV : Ultra-Violet

WS : White Spot

Chapitre I - Introduction

1.1. Contexte bibliographique

Nous connaissons, aujourd'hui, une érosion de la biodiversité spectaculaire et jamais égalée auparavant. Depuis le siècle dernier, les pressions anthropiques ont modifié les écosystèmes de manière intensive et rapide et sont la cause de la sixième crise de la biodiversité. La précédente, il y a 65 millions d'années, ayant notamment provoqué la disparition des dinosaures. Il existe de nombreuses raisons au déclin actuel. Ainsi, comme principaux facteurs nous retrouvons la fragmentation des habitats, la contamination environnementale, le réchauffement climatique, l'introduction d'espèces exotiques invasives et la surexploitation des milieux.

Depuis de nombreuses années, les amphibiens sont considérés comme le groupe de vertébrés le plus menacé d'extinction. En 2018, 42 % des espèces connues possèdent selon l'UICN le statut « en danger critique » (exemple : *Werneria bambutensis* endémique d'une région montagneuse du Cameroun), « en danger » (exemple : *Hyloxalus sylvaticus* endémique de régions montagneuses du Pérou) ou « vulnérable » (exemple : *Pelophryne albotaeniata* endémique de l'île de Palawan aux Philippines) (IUCN, 2018). Ces catégories sont établies suivant des critères concernant la taille de la population, la répartition géographique, le nombre d'individus matures dans la population et la probabilité d'extinction à l'état sauvage en l'espace de 100 ans (IUCN, 2012). En comparaison, respectivement 25 % et 13 % des espèces décrites de mammifères et d'oiseaux sont menacées. Le problème de déclin de la diversité en amphibiens est reconnu au niveau mondial depuis environ 25 ans. Les quelques études réalisées auparavant étaient freinées par un manque de connaissances de la dynamique des populations de ce groupe (Linder et al., 2010). En effet, de nombreuses espèces sont migratrices et cela peut compliquer la dissociation entre des variations normales du nombre d'individus dans une zone donnée et un réel problème. C'est seulement depuis les années 1990, qu'un vaste programme mondial, nommé « *Global Amphibian Assessment* » (GAA) et mené par « *Amphibian Red List Authority* » (ARLA), a débuté pour fournir des informations précises et récentes à l'UICN sur le risque d'extinction des espèces d'amphibiens connues.

Evidemment, les causes classiques de l'érosion de la biodiversité, citées précédemment, sont responsables de l'état inquiétant des populations d'amphibiens dans le monde. Pour chacun de ces facteurs, nous pouvons citer, respectivement, comme exemple : la destruction d'habitats aquatiques, l'utilisation de phytosanitaires, la sécheresse, la pression de prédation exercée par l'amphibien invasif *Rana catesbeiana* (grenouille-taureau) et le braconnage pour l'alimentation. Toutefois, d'autres facteurs, plus spécifiques aux amphibiens, sont également reconnus comme responsables de leur disparition : les radiations UV, les

infections, comme la chytridiomycose provoquée par *Batrachochytrium dendrobatidis*, et le parasitisme, par exemple à *Ribeiroia ondatrae* (Alford, 2010; Alford and Richards, 1999; Blaustein et al., 2003; Blaustein and Wake, 1995, 1990). Il est important de préciser que les effets de ces pressions sur les populations d'amphibiens sont généralement plus importants lorsqu'elles sont associées.

La pollution est souvent citée comme un facteur principal du déclin global des amphibiens ou, *a minima*, l'un des cofacteurs. Avec la destruction des habitats, elle en est la raison majeure (IUCN, 2008). En interaction avec d'autres stress, les effets de contaminants chimiques peuvent être bien plus sévères. Cela a été démontré, par exemple, dans le cas d'expositions à des pesticides couplées à une pression de prédation (Relyea, 2005, 2004; Relyea and Mills, 2001). La sensibilité des amphibiens à la contamination environnementale est notamment liée à certaines de leurs caractéristiques biologiques (Sparling et al., 2010). Chez ces espèces, la fécondation et le développement embryonnaire sont externes, ce qui soumet les gamètes et les embryons aux mêmes concentrations en xénobiotiques qu'un adulte. Les œufs sont dépourvus de coquille, seule une gangue perméable protège l'œuf et l'embryon. Le cycle de vie est divisé en deux par la métamorphose. Cette période est, d'ailleurs, critique dans la vie d'un amphibien car il va connaitre de grands changements morphologiques, physiologiques et écologiques. La plupart des espèces vont, ainsi, évoluer après la métamorphose entre le milieu terrestre et le milieu aquatique, alors qu'elles ne vivaient exclusivement que dans l'eau avant la transformation. Comparé à un autre groupe taxonomique ne connaissant pas ces grands changements, un amphibien sera exposé tout au long de sa vie à une plus grande variété de contaminants, de concentrations et par des voies d'expositions qui changeront avec le temps. Enfin, la peau est hautement perméable à la diffusion du milieu aquatique environnant qui peut se révéler contaminé. Certaines de leurs caractéristiques écologiques participent également à cette sensibilité. Il est connu que les amphibiens ont gardé un lien très fort avec le milieu aquatique. La majorité des espèces se reproduisent d'ailleurs dans de petites étendues d'eau comme des étangs ou des mares, qui peuvent même être temporaires. Or, lorsqu'elles sont à proximité d'exploitations agricoles, de zones industrielles ou de routes, elles sont soumises, notamment par ruissellement, à de fortes contaminations chimiques (Bridges and Boone, 2003). Cela représente un réel problème étant donné que ces milieux sont habités par de nombreuses espèces d'amphibiens. Des travaux aux Etats-Unis ont montré que d'importantes concentrations en pesticides existaient dans des milieux normalement adéquats à leur vie (Battaglin et al., 2016, 2009; Fellers et al., 2013; Smalling et al., 2015). Néanmoins, les analyses des teneurs en contaminants de ces petits habitats aquatiques sont rares. Par exemple, en Europe, ces milieux, essentiels aux amphibiens et à de nombreux autres organismes, ne sont pas suivis de manière régulière dans

le contexte de la Directive Cadre sur l'Eau. Pourtant, cette dernière a été mise en place afin d'organiser une gestion de l'eau commune entre les pays de l'Union Européenne avec pour but d'en améliorer la qualité.

Quelques études ont décrit les effets de la pollution sur les populations d'amphibiens. Dans le parc national d'Acadia (Maine, USA), les populations de la salamandre sombre du Nord (*Desmognathus fuscus fuscus*) sont en déclin à cause de la contamination au mercure et à l'aluminium (Bank et al., 2006). Dans la Sierra Nevada (Californie, USA), les pesticides appliqués par voie aérienne ont contribué, avec l'introduction de poissons, à la réduction de la taille des populations de grenouilles des montagnes à pattes jaunes (*Rana muscosa*) (Carlos and Knapp, 2007). Enfin, toujours en Californie (USA), il a été démontré que les populations locales d'amphibiens de différentes zones disparaissaient à cause de l'exposition aux pesticides apportés par les vents (Sparling et al., 2001).

Après une reconnaissance tardive des problèmes liés aux contaminations environnementales, de nombreuses études ont été conduites depuis les années 2000 pour décrire les effets d'expositions d'amphibiens à des xénobiotiques (Linder et al., 2010; Sparling et al., 2010). Elles visent à établir un lien entre les contaminants et les impacts biologiques observés. Même si la majorité de ces travaux sont menés en laboratoire, déterminer les effets ainsi que les cibles des xénobiotiques apparait comme essentiel dans le but de mieux comprendre les réponses d'organismes exposés *in situ*. L'article de revue, intitulé « *Amphibian exposures to chemical contaminants: reproduction and development as key issues in the environmental toxicology literature* »[1] (Article 1), propose d'établir une synthèse d'études en toxicologie des amphibiens sur les effets d'expositions à des contaminants environnementaux (métaux, phytosanitaires et autres contaminants organiques émergents) en se focalisant sur les paramètres reproductifs et développementaux. Le moindre impact à ces niveaux affectera certainement la dynamique des populations de manière négative.

Les principales conclusions de cette revue mettent en évidence, premièrement, que peu de travaux visent à se rapprocher des conditions environnementales. Il existe de manière générale de fortes extrapolations entre ce qui est observé au laboratoire et ce qui existe dans le milieu naturel. Il est donc nécessaire d'apporter des efforts en ce sens en concevant des protocoles expérimentaux plus complexes et en prenant en compte d'autres paramètres abiotiques ou biotiques ou encore en travaillant sur le terrain. Ensuite, il apparait que les

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atteintes morphologiques sont les critères les plus utilisés pour évaluer la toxicité d'un contaminant. De la même manière, parmi les espèces modèles, quelques anoures sont surreprésentés par rapport à d'autres et surtout par rapport aux ordres des urodèles et des gymnophiones. Enfin, peu de travaux ont pour objet d'étude les gamètes alors que ces stades précoces du cycle de vie sont soumis à la contamination du milieu aquatique directement au moment de la fécondation et qu'une perturbation de leur biologie pourrait compromettre la viabilité des populations.

Amphibian exposures to chemical contaminants: reproduction and development as key issues in the environmental toxicology literature

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Abstract

Environmental contamination is one of the major factors or cofactors affecting amphibian populations. Since 2000, a broad number of lab studies were conducted in environmental toxicology using chemical exposures of these organisms in controlled conditions. They aimed to characterize biological effects on amphibians. This review proposes an overview of biological responses reported after exposures to metals, phytopharmaceuticals or emerging organic contaminants. The selected endpoints relate to amphibian reproduction and development. Due to their peculiar features, these species are especially sensitive to pollutant exposures. Despite the large range of tested compounds, the same species are often used as biological models and morphological alterations are the most studied observations.

From the results, the laboratory-to-field extrapolation remained uneasy and exposure designs have to be more elaborated to be closer to environmental conditions. Few studies proposed such experimental approaches. Nevertheless, field studies are still essential.

Lastly, gametes, embryos and larvae constitute key stages of amphibian life cycle that can be harmed by exposures to freshwater pollutants. Specific efforts have to be intensified on the earliest stages and notably germ cells.

Keywords: Amphibians; Reproduction; Development; Metals; Organic contaminants

Capsule: Lab studies were mainly conducted to describe pollutant effects on amphibians. Reproduction and development, affected by contaminant exposures, are key issues to address in amphibian toxicology.

1. Preamble

With 41% of known species listed by the International Union for Conservation of Nature and Natural Resources (UICN) at least as vulnerable in terms of extinction, amphibians are the most threatened animals (Monastersky, 2014). Many causes are responsible for their decline and all can act as a primary factor or a cofactor: ultraviolet radiations, habitat destruction, invasive species, human exploitations (food and pets), global warming, diseases, parasitism and also environmental contaminations (Alford, 2010; Alford and Richards, 1999; Blaustein et al., 2003; Blaustein and Wake, 1995, 1990). Concerning this last, the peculiar characteristics of amphibian species explain their high sensibility to chemical exposures. So, their fertilization and embryonic development are external, their eggs have no shell and most of species have their life cycle divided in two parts (aquatic/terrestrial or in some cases totally aquatic) separated by metamorphosis (Sparling et al., 2010). They likely spend their life and reproduce in small areas of water near roads and industrial or agricultural activities, where usually important chemical contaminations can be found (Bridges and Boone, 2003). It has been reported that environmental contaminations can contribute the decline of amphibian populations. The drastic decline of the northern dusky salamander (*Desmognathus fuscus fuscus*) population in the Acadia National Park (Maine, USA) has been related to mercury and aluminium leaching enhanced by episodic acidification events (Bank et al., 2006). In the Sierra Nevada (California, USA), both introduced fishes and especially airborne pesticide pollutions contributed to decrease the population of the mountain yellow-legged frog (*Rana muscosa*) (Carlos and Knapp, 2007). In the same region, Sparling *et al.* also reported that wind-blown pesticides played an important role in local amphibian population declines (Sparling et al., 2001). By studying the cholinesterase activity in field collected Pacific treefrog (*Hyla regilla*), they observed that this enzyme activity decreased in the same areas where status of *Rana spp.* populations was moderate or poor.

It appears essential to characterize the biological effects, the mechanisms and/or targets of chemicals to assess toxicity of contaminants and to better understand responses of exposed organisms *in situ*. That is the basis of development of biomarkers which could constitute predictive tools to preserve amphibian populations (Venturino et al., 2003). Since 2000, interest in impacts of pollution on amphibian increased and the number of studies multiplied (Sparling et al., 2010). They attempt to establish links between contamination exposures and biological impairments, but they are mainly lab works. This review proposes to focus on the possible impacts of contaminant exposures on reproductive parameters and precocious development of amphibians. They constitute critical steps of the life cycle. Any disorders at this level would disturb the reproduction and affect consequently the population

dynamic. All through our literature survey, we identified three prominent contaminant families. In fact, amphibian toxicology exposures are mainly conducted with metallic compounds, pesticides and emerging organic contaminants. A section will be devoted to each pollutant family.

2. Metal exposures

Essential and toxic metals are present naturally in the ecosystems. Most anthropic activities modify their environmental concentrations and contamination origins could differ according to the considered element. They are likely one of the most studied family in environmental sciences and the same ascertainment can be done regarding amphibian toxicology. The table S-1 reports the effects of metal exposures assess by reproductive and developmental endpoints.

2.1. Morphological development alterations

Growth inhibition is a frequent impact caused by metal (as cadmium, copper and lead) exposures (García-Muñoz et al., 2008; Herkovits et al., 1997; Mouchet et al., 2006; Pérez-Coll and Herkovits, 1990; Ranatunge et al., 2012) (Table S-1 (Parts 1 to 4)). On the contrary, an increase of the growth is uncommon. Nevertheless, that was observed for *Rana limnocharis* tadpoles exposed to cadmium in which the accelerated metamorphosis led to taller but lighter individuals (Patar et al., 2016) (Table S-1 (Part 1)).

Likewise, morphological anomalies are often observed whatever the species or the exposure conditions (metal ions, concentrations and durations). Exposures to cadmium or to lead during precocious development of *Bufo arenarum* result in severe abnormalities such as microcephaly, axial incurvation and underdeveloped gills (Pérez-Coll et al., 1988, 1986) (Table S-1 (Parts 1 and 4)). Similar effects have been reported in *Xenopus laevis* developed in cadmium contaminated media at different moments of their lifecycle. They were associated with abnormal tail, fin and eye, blisters, reduced pigmentation, gut malrotation, bend notochord, facial dysplasia, cardiac deformities, broadening of the dorsal fin. All these effects in *X. laevis* increased with the tested cadmium concentration (Herkovits et al., 1997; Sunderman et al., 1991) (Table S-1 (Part 2)). Blister, curved body axis and abnormal eyes were also observed in a dose-dependent manner in *Microhyla ornata* exposed to mercury from the gastrula stages (Ghate and Mulherkar, 1980) (Table S-1 (Part 4)).

2.2. Activity and behaviour

Tadpole swimming difficulties could be first explained by morphological deformities. That was observed for *B. arenarum* exposed to cadmium from stages 10 to 25 (Del Conte and Sirlin, 1951) for which severe axial incurvation and abnormal tail affected the swimming (Pérez-Coll et al., 1986) (Table S-1 (Part 1)). In the same species exposed to lead, tremble, swimming and equilibrium alterations were described as neurological disorders (Pérez-Coll and Herkovits, 1990) (Table S-1 (Part 4.)). Decrease of the swimming activity was also observed in *Duttaphrynus melanostictus* tadpoles exposed 10 days to cadmium (Ranatunge et al., 2012) (Table S-1 (Part 1)). After mercury exposures of tadpoles from the gastrula stage for 96 hours, *M. ornata* exhibited altered muscular movements in a dose-dependent manner (Ghate and Mulherkar, 1980) (Table S-1 (Part 4)). In these two last works, the authors observed in parallel growth inhibition or morphological malformations (Ghate and Mulherkar, 1980; Ranatunge et al., 2012).

For lower-sized abnormally swimming *X. laevis* tadpoles, lead exposures induced also a decrease in the food uptake (Mouchet et al., 2006) (Table S-1 (Part 4)).

In other studies, while no morphological and growth alterations have been observed, behavioural changes were reported. Lead exposed *Rana catesbeiana* tadpoles showed an increased latency of learning acquisition associated to a decrease of their avoidance behaviour (Strickler-Shaw and Taylor, 1991) (Table S-1 (Part 4)). Copper exposed *Bufo calamita* tadpoles were less reactive, their displacements were abnormal and reduced, even if a partial recovery was noted after a 96-hour post-treatment in uncontaminated conditions (García-Muñoz et al., 2008) (Table S-1 (Part 3)).

2.3. Sexual disorders

Secondary sexual characteristics and sexual behaviour could be modified by metal exposures. That was reported with cadmium-exposed *Pelophylax nigromaculata* (Table S-1 (Part 1)). Then, laryngeal formation and structure were abnormal in metamorphosed individual exposed from stages 19 to 46 (Gosner, 1960). Several effects were observed according to the tested concentrations: delayed development, decreased cross-section and the muscle fibre size and altered ultrastructure (Duan and Huang, 2016). For 60-day exposed male adults, calls were highly modified and also their responses to female call stimulation (Huang et al., 2015).

Table S-1. Reported reproduction and development effects after amphibian metal exposures (Part 1).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Cadmium	*Bufo arenarum*	St. 10[a]	6.10^{-7}, 3.10^{-6},$1.5.10^{-5}$ M 20, 30°C St. 25[a]	20 °C: ↗ development time during gastrulation, mild axial incurvation, microcephaly and underdevelopment of gills (st. 19, st. 25[a]). Folds in the ectoderm and more ciliated cells $1.5.10^{-5}$ M, 30 °C: closing of the blastopore failure, severe axial incurvation and malformation of the tail (swimming affected), atypical distribution of the ciliated cells in cords or clusters	(Pérez-Coll et al., 1986)
		Adult	0.5, 5 $mg.kg^{-1}$ Daily injection [dorsal lymph sac] 15 d	In female: dose- and time-dependent: ↘ *in vitro* oocyte maturation In ovaries, 5 $mg.kg^{-1}$: nuclear and cytoplasmic alteration of previtellogenic oocytes, dilated capillary lumens, edema, fibroblast proliferation in nodules, or strips and cumulus of cell with hyperchromatic to pyknotic nuclei, ↘ fully grown oocyte, ↗ atretic oocyte. In testis, 5 $mg.kg^{-1}$: dilated seminiferous tubule, germ cells showing hydropic tumefaction or signs of focal necrosis or dilated chromatin, enlargement of the vascular lumen in the interstitial tissue, ↘ sperm quality (concentration, viability and mobility)	(Medina et al., 2012)
	Duttaphrynus melanostictus	St. 24-26[b]	0.002, 0.02, 0.2, 1, 2 $mg.L^{-1}$ 10 d	≥ 0.2 $mg.L^{-1}$: ↗ mortality, ↘ body length, ↘ swimming activity	(Ranatunge et al., 2012)
	Pelophylax nigromaculata	St. 19[b]	4, 8, 16, 32, 64, 128 $\mu g.L^{-1}$ St. 46[b]	≥ 32 $\mu g.L^{-1}$: laryngeal development delayed ≥ 64 $\mu g.L^{-1}$: ↘ cross-section of larynx, ↘ male larynx muscle fiber size 128 $\mu g.L^{-1}$: altered ultrastructure of the larynx (mitochondrial swelling and ↘ number of mitochondria)	(Duan and Huang, 2016)
		Male adult	10^{-8}, 10^{-7}, 10^{-6} M 60 d	10^{-8} M: ↘ call duration, ↗ call rate ≥ 10^{-7} M: ↘ male % responding to the female receptive call, ↗ of the call latency, ↘ call rate, ↗ of the time of the first movements toward the source of the female call	(Huang et al., 2015)
	Pleurodeles waltl	St. 53[c]	0.5, 1, 2 $mg.L^{-1}$ 12 d	DNA breaks (different patterns according to parameters, exposure doses and durations)	(Mouchet et al., 2007)
			0.25, 0.5, 1, 2, 5, 10, 50 $mg.L^{-1}$ 12 d	≥ 5 $mg.L^{-1}$: ↗ mortality ≥ 10 $mg.L^{-1}$: 100 % mortality	(Mouchet et al., 2007)
	Rana limnocharis	St. 26-28[b]	18.5 $\mu g.L^{-1}$ 24 h	DNA breaks	(Patar et al., 2016)
			0.1, 0.2, 0.3 $mg.L^{-1}$ 96 h	≥ 0.2 $mg.L^{-1}$, dose- and time-dependent: ↗ micronucleated erythrocytes	(Patar et al., 2016)
			0.1, 0.2, 0.3, 0.4 $mg.L^{-1}$ 18 d	Dose- and time-dependent: ↗ mortality 0.2 $mg.L^{-1}$: accelerated metamorphosis, metamorphosed individuals: ↘ weight, ↗ length ≥ 0.3 $mg.L^{-1}$: 100 % mortality	(Patar et al., 2016)

Table S-1. Reported reproduction and development effects after amphibian metal exposures (Part 2).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Cadmium	*Rana limnocharis*	Male adult	2.5, 5, 7.5, 10 mg.L^{-1} 14 d	Dose-dependent: ultrastructural changes of spermatogenic and Sertoli cells, ↗ DNA breaks, ↗ GSH, ↗ expression of caspase-3 and Bax/Bcl-2 10 mg.L^{-1}: ↗ MDA, ↗ ROS	(Zhang et al., 2012)
	Xenopus laevis	St.VI oocyte[d]	0.136, 1.36, 5.4, 13.6, 65.5, 136 µM ± progesterone Overnight	+ progesterone, ≥ 65.5 µM : ↘ *in vitro* maturation % - progesterone: spontaneous maturations	(Marin et al., 2015)
			1.36, 13.6, 136 µM ± progesterone 13 h	± progesterone, 136 µM: disturbed MAPK cascade and MPF + progesterone: ↗ spindle and chromosomes abnormalities, 136 µM: delayed maturation process, ↘ *in vitro* maturation % - progesterone, 136 µM: spontaneous maturation	(Slaby et al., 2017)
			5.4 µM 24, 48 h	↗ ICl currents	(Marin et al., 2015)
			5.4 µM 48 h, 72 h	Time-dependent: ↗ resting potential (48 h, 72 h)	(Marin et al., 2015)
		Mature oocyte[d]	136 µM 7 min pre-exposure	35 min post-fertilization: ↘ *in vitro* fertilization	(Slaby et al., 2016b)
		Sperm	136 µM 7 min pre-exposure	35 min post-fertilization: ↘ *in vitro* fertilization	(Slaby et al., 2016b)
		Gametes	1.36, 13.6, 136 µM 35 min	↘ *in vitro* fertilization	(Slaby et al., 2016b)
			1.36, 13.6, 136 µM St. 4[e]	Delayed development	(Slaby et al., 2016b)
		St. 2 to 47[e]	0.1, 0.2, 0.5, 1, 2, 4, 6, 8, 10 mg.L^{-1} 72 h	Dose- and st.-dependent: ↗ mortality, axial incurvations, ↘ size, abnormal or underdeveloped developed tail, fin and eye, microcephaly, blisters, cellular dissociation, ↘ pigmentation, ↘ growth damaged apical surface of ectodermal cells 2 mg.L^{-1}: disappearance of the cilia	(Herkovits et al., 1997)
		Blastula	0.75, 1, 1.3, 1.8, 3, 5.6, 10, 18, 30 µM 101 h	Dose-dependent: gut malrotation, bent notochord, eye abnormalities, facial dysplasia, cardiac deformities, dermal blister, broadening of the dorsal fin	(Sunderman et al., 1991)

Table S-1. Reported reproduction and development effects after amphibian metal exposures (Part 3).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Cadmium	*Xenopus laevis*	St. 50[e]	0.25, 0.5, 1 mg.L^{-1} 12 d	↗ DNA breaks	(Mouchet et al., 2007)
			0.25, 0.5, 1, 2 mg.L^{-1} 12 d	2 mg.L^{-1}: ↗ micronucleated erythrocytes	(Mouchet et al., 2007)
			0.25, 0.5, 1, 2, 5, 10, 50 mg.L^{-1} 12 d	≥ 2 mg.L^{-1}, dose- and time-dependent: ↗ mortality ≥ 10 mg.L^{-1}: 100 % mortality	(Mouchet et al., 2007)
			2, 10, 30 µg.L^{-1} St. 54	↗ micronucleated erythrocytes, ↗ expression of detoxication genes and response to oxidative stress, ↘ expression of genes involved in DNA repair and apoptosis ≥ 10 µg.L^{-1}: dose-dependent: ↗ MT and expression of *mt1*	(F Mouchet et al., 2006)
		Adult female	0.5, 0.75, 1, 3, 5 mg.kg^{-1} ± hCG Daily injection [dorsal lymph sac] 21 d	± hCG: ↘ oocytes of different st., ↗ atretic oocytes	(Lienesch et al., 2000)
Cobalt	*Xenopus laevis*	St.VI oocyte[d]	0.192, 1.92, 7.7, 19.2, 96, 192 µM ± progesterone Overnight	- progesterone: spontaneous maturations	(Marin et al., 2015)
			7.7 µM 48 h	↗ ICl1-T current	(Marin et al., 2015)
Copper	*Bufo calamita*	St. 3[b]	0.1, 0.2, 0.3, 0.4 mg.L^{-1} 96 h	Dose-dependent: delayed development 0.2, 0.3 mg.L^{-1} ↘ length	(García-Muñoz et al., 2008)
		St. 19[b]	50, 80, 100, 200 µg.L^{-1} 96 h	Dose-dependent: delayed development, ↘ length	(García-Muñoz et al., 2008)
		St. 25[b]	70, 100, 130, 160 µg.L^{-1} 96 h	≥ 130 µg.L^{-1}: ↘ length	(García-Muñoz et al., 2008)
			60, 80, 100 µg.L^{-1} 96 h + 96 h (0 µg.L^{-1})	↘ reactivity, ↘ distance (totally recovered after 96 h), abnormal displacement (partially recovered after 96 h)	(García-Muñoz et al., 2008)

Table S-1. Reported reproduction and development effects after amphibian metal exposures (Part 4).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Lead	*Bufo arenarum*	St. 2[a]	0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32 mg.L^{-1} 72 h	0.5, 1 mg.L^{-1}: persistent yolk plug, uncomplete neurulation, microcephaly, pear shape embryos followed by mortality at neurula. Underdeveloped gills, microcephaly, delayed development, stunted tail and axial incurvation for survivors 2, 4 mg.L^{-1}: development arrested between late gastrula and early neurula 2 mg.L^{-1}: persistent yolk plug with pigmented and spherical cells 4 mg.L^{-1}: loss of intercellular contacts in ectodermal cells (dorsal and lateral lips) ≥ 8 mg.L^{-1}, dose-dependent: mortality at mid-late blastula, pigment displacement, cell dissociation	(Pérez-Coll et al., 1988)
		St. 12, 18, 20[a]	1 ppm 20 h	Mortality and according to the stage: failed closure of the neural tube, hydropsy, reduced and cylindered tail, under- and abnormal developed tail, axial incurvations, underdeveloped gills and growth inhibition, neurological disorder (trembles, swimming and equilibrium alteration)	(Pérez-Coll and Herkovits, 1990)
	Rana catesbeiana	*Not reported*	500, 625, 750, 1000 µg.L^{-1} 6 d	↗ latency of learning acquisition, ↘ avoidance	(Strickler-Shaw and Taylor, 1991)
	Xenopus laevis	St. VI oocyte[d]	0.9, 9, 90 µM ± progesterone 13 h	+ progesterone: ↗ spindle and chromosomes abnormalities 0.9 µM: ↘ *in vitro* maturation 9 µM: transient acceleration of *in vitro* maturation process	(Slaby et al., 2017)
			3.6 µM 24, 48 h	↗ ICl currents	(Marin et al., 2015)
			3.6 µM 48, 72 h	↗ resting potential (48 h)	(Marin et al., 2015)
		St. 50[d]	0.001, 0.01, 0.1, 1, 10, 30, 50, 100 mg.L^{-1} 0.1, 1, 10 mg.L^{-1} ± MBMCR 12 d	≥ 10 mg.L^{-1}, dose-dependent manner: ↗ mortality 1, 10 mg.L^{-1}: ↗ micronucleated erythrocytes 1, 10, 30 mg.L^{-1}: ↗ physical sign of anemia, ↘ size, ↘ food uptake, abnormal swimming behavior + MBMCR: ↘ toxicity and genotoxicity	(Mouchet et al., 2006)
Mercury	*Microhyla ornata*	Gastrula	50, 100, 150, 200, 250, 300 µg.L^{-1} 96 h	Dose-dependent: ↗ mortality, blister, delayed growth associated with underdeveloped eyes, altered muscular movement, curved body axis	(Ghate and Mulherkar, 1980)
	Rana pipiens	St. 7[b]	20, 40, 60, 80, 100 ppb 36 h	≥ 40 ppb: arrest of development, ↘ attained stages	(Dial, 1976)

Table S-1. Reported reproduction and development effects after amphibian metal exposures (Part 5).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Zinc	*Xenopus laevis*	St. VI oocyte[d]	0.183, 1.83, 7.3, 18.3, 91.5, 183 µM ± progesterone Overnight	- progesterone: spontaneous *in vitro* maturation	(Marin et al., 2015)
			0.479 µM 48, 72 h	↗ resting potential	(Marin et al., 2015)
		Sperm	31, 72, 155, 334, 759, 1417 µg.L^{-1} 4 min	Dose-dependent: ↘ total motility, ↘ progressive motility, ↗ straight-line velocity	(Christensen et al., 2004)
Cadmium + Lead	*Xenopus laevis*	St. VI oocyte[d]	5.4 + 3.6 µM 24, 48 h	↗ ICl currents	(Marin et al., 2015)
			5.4 + 3.6 µM 48, 72 h	Time-dependent: ↗ resting potential (48h, 72h)	(Marin et al., 2015)
		Gametes	13.6 + 9, 136 + 90 µM 35 min	136 + 90 µM: ↘ *in vitro* fertilization	(Slaby et al., 2016b)

St.: stage; a: (Del Conte and Sirlin, 1951); b: (Gosner, 1960); c:(Galien and Durocher, 1957); d: (Dumont, 1972) ; e: (Nieuwkoop and Faber, 1967)

3. Phytopharmaceutical exposures

Amphibians are non-target species of pesticides used for example in extensive agriculture or for disease vector control. These xenobiotics contaminated directly or indirectly freshwater ecosystems. Domestic uses of such products contributed also to surface water pollutions. Table S-2 presents the lab works related to phytopharmaceutical exposures (pure active ingredients and/or their commercial formulations) and described consecutive reproductive and developmental effects.

3.1. Morphological development alterations

As observed in metal conditions, pesticide exposures can induce a large range of morphological defects (Table S-2 (Parts 1 to 8)). Exposed to atrazine, *Bufo americanus*, *Rana pipiens* and *Rana sylvatica* larvae exhibited the same pattern of malformations in a dose-dependent manner, *i. e.* facial edema, blistering, axial shortening and latero-dorsal tail flexure (Allran and Karasov, 2001) (Table S-2 (Part 1)). No significant difference in sensitivity among species were reported in this study. For *Hyla versicolor* and *X. laevis* this herbicide exposures led to decrease of length and/or weight (Diana et al., 2000; Sullivan and Spence, 2003) (Table S-2 (Part 1)). For other herbicides several malformations were also described. In 2,4-D contaminated media, *X. laevis* developed intestine malformations and edemas (Lenkowski et al., 2010) (Table S-2 (Part 3)). For the same species, RoundUp® Power 2.0 (a commercial glyphosate-based formulation) exposures can induce gut miscoiling, edema, disappearance of the upper and lower jaws, damaged velar plate and branchial chamber, craniofacial defects, eye anomalies, altered morphology and localization of primitive organs, few pronephric tubules, poorly organized somatic musculature around notochord (Bonfanti et al., 2018) (Table S-2 (Part 7)).

The fungicide triadimefon exposures altered gills and pigmentation but also deformed cartilages (Papis et al., 2006) and caused intestine malformation and edemas (Lenkowski et al., 2010) in *X. laevis* tadpoles (Table S-2 (Part 8)). The organophosphorus insecticides malathion or parathion induced both in *B. arenarum* antero-posterior axis curved, tail folding, dropsy and edemas (Anguiano et al., 2001) (Table S-2 (Parts 5 and 6)). For malathion and its degradation product, abnormalities in pigmentation, gut, circulatory vessel and notochord have been observed, in a concentration-dependent manner, in *X. laevis* 96-hour exposed from the blastula (Snawder and Chambers, 1993) (Table S-2 (Part 6)). Concerning organochlorine insecticide exposure, chlorpyrifos caused flexure of the notochord of *X. laevis* larvae (Bonfanti

et al., 2004) (Table S-2 (Part 2)) and lindane induced profuse scaling, dropsy, organ displacement and bend tail in *B. arenarum* (Anguiano et al., 2001) (Table S-2 (Part 4)). In this species, less severe morphological alterations were observed after dieldrin exposures (Anguiano et al., 2001) (Table S-2 (Part 3)). For the carbamate family, exposures of *D. melanostictus* to carbosulfan based formulation led to bend tail and abnormal swelling (Samarakoon and Pathiratne, 2017) (Table S-2 (Part 5)). For carbaryl an inhibition of the growth of *H. versicolor* was only observed when exposures were associated with a predator pressure (Relyea and Mills, 2001) (Table S-2 (Part 2)).

3.2. Activity and behaviour

Studies reporting larvae abnormal activities are often related to insecticide treatments. Carbaryl exposures induced a reduction of the time spent being active associated with a decrease of the swum speed and distance in *Rana blairi* exposed at the stage 25 (Gosner, 1960) for 96 hours (Bridges, 1997) but also hypoactivity enhanced by a caged predator treatment for *H. versicolor* (Relyea and Mills, 2001) (Table S-2 (Part 2)). The exposures to another carbamate substance contained in Marshal® (carbosulfan) were responsible of abnormal swimming of *D. melanosticus* tadpoles (Samarakoon and Pathiratne, 2017) (Table S-2 (Part 5)). In parallel abnormal morphologies were detected in this study. Hypoactivity was recorded after malathion-based commercial formulation exposures of stage 32 (Gosner, 1960) *Phyllodytes luteolus* for 16 days (Egea-Serrano and Solé, 2017) (Table S-2. (Part 4)). Malathion and parathion induced both for *B. arenarum* exposed 96 hours after 6 days of development a lower activity (Anguiano et al., 2001) (Table S-2 (Parts 5 and 6)). The authors reported also circle-swimming movements of the tadpoles and morphological malformations. This species was more sensible to parathion exposures (effects occurred at a 4-time lower concentration than for malathion). For longer exposures to malathion another effect has been recorded in *R. castebeaina* tadpoles: equilibrium impairment (Fordham et al., 2001) (Table S-2 (Part 5)). For *B. arenarum*, *Physalaemus albonotatus* and *Rhinella fernandezae* after anti-mosquito specialty exposures, different responses in the activity level have been observed among species and insecticides (Abate®, Depe® and Introban®) (Junges et al., 2017) (Tables S-2 (Parts 1, 3 and 4)). *B. arenarum* seemed to be the most sensitive species and in some cases opposite effects were even noticed, as for example Depe® (permethrin-based formulation) exposures which induced hypoactivity for *B. arenarum* and hyperactivity for *R. fernandezea*. Hyperactivity was however a characteristic recorded in 6-day-old *B. arenarum* tadpole exposed to dieldrin or lindane for 96 hours (Anguiano et al., 2001) (Table S-2 (Parts 3 and 4)).

Herbicide substances can be harmful too regarding tadpole activity and behaviour. *X. laevis* exposed to atrazine from 2 days after the hatch until the stage 66 (Nieuwkoop and Faber, 1967) exhibited abnormal swimming (Carr et al., 2003) (Table S-2 (Part 1)). Associated to tachycardia, higher activity level was also induced by RoundUp® Original exposures (Costa et al., 2008) (Table S-2 (Part 7)). At last, the organotin compound called triphenyltin chloride, used as fungicide and antifoulant, induced in a concentration-dependent manner a decrease of the time spent swimming and opposite effects concerning the time spent feeding (Semlitsch et al., 1995) (Table S-2 (Part 9)).

3.3. Sexual disorders

The occurrence of sex reversal characteristics is one of the most impressive effects caused by environmental contaminants. It is well documented for amphibian atrazine exposures. In *X. laevis*, tadpole exposures to this herbicide (*i*) induced primary sexual organs defects as intersexual, multiple (up to 6) and altered gonads (Carr et al., 2003; Hayes et al., 2002; Tavera-Mendoza et al., 2002), (*ii*) decreased plasmatic testosterone level in mature male (Hayes et al., 2002) and (*iii*) altered the formation of secondary sexual organs such a decreased laryngeal size (Hayes et al., 2002) (Table S-2 (Part 1 and 2)).

Abnormal male gonads were also described for *R. pipiens* in metamorphosed individuals exposed to atrazine during a large part of tadpole development (Hayes et al., 2003) (Table S-2 (Part 1)). These authors observed similar effects for *R. pipiens* field-collected in atrazine-treated areas across the United States and then confirmed the endocrine-disrupting potential of atrazine for amphibians.

Likewise, estrogeno-mimetic effects were reported after DDT, dieldrin or toxaphene exposures that engendered an increase of vitellogenin levels in *X. laevis* male adults (Palmer et al., 1998; Palmer and Palmer, 1995) (Table S-2 (Parts 3 and 8)). Feminization cases had been noticed in *Hyperolius argus* exposed to DDT or derivatives in which a female-biased sex-ratio were assessed by the secondary sex coloration (Noriega and Hayes, 2000) (Table S-2 (Part 3)). Finally, environmentally relevant exposures to the fungicide vinclozolin altered the call behaviour of male *X. laevis* (Hoffmann and Kloas, 2010) (Table S-2 (Part 9)).

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 1).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Abate® *Temefos*	*Bufo arenarum*	St. 33[a]	16 mg[a.i.].L^{-1} 24 h	↘ global activity, ↘ distance moved, ↗ time spent immobile	(Junges et al., 2017)
	Physalaemus albonotatus	St. 33[a]	3.65 mg[a.i.].L^{-1} 24 h	↗ distance moved	(Junges et al., 2017)
Atrazine	*Bufo americanus*	St. 8-12[a]	0.02, 0.2, 2, 20 mg.L^{-1} 96h-post-hatching	Dose-dependent: ↗ deformed %, tail flexures, facial edema, axial shortening, blistering	(Allran and Karasov, 2001)
	Hyla versicolor	9 d	2, 20, 200, 2000 µg.L^{-1} Metamorphosis	≥ 200 µg.L^{-1}: ↘ length, ↘ weight	(Diana et al., 2000)
	Rana pipiens	St. 10-12[a]	0.02, 0.2, 2, 20 mg.L^{-1} 96 h post-hatching	Dose-dependent: ↗ deformed %, tail flexures, facial edema, axial shortening, blistering	(Allran and Karasov, 2001)
		2 d post-hatching	0.1, 25 ppb Tail resorption	In male: underdeveloped testis, closed or no testicular lobule, low to absent germ cells, varying degree of sex reversal (testicular lobule with oocytes, limited number of lobule)	(Hayes et al., 2003)
	Rana sylvatica	St. 10-12[a]	0.02, 0.2, 2, 20 mg.L^{-1} 96 h-post-hatching	Dose-dependent: ↗ deformed %, tail flexures, facial edema, axial shortening, blistering	(Allran and Karasov, 2001)
	Xenopus laevis	Oocyte St. VI[b]	50, 100 µg.L^{-1} 390 min + progesterone	Dose-dependent: accelerated maturation process	(Ji et al., 2016)
		48 h post-hatching	1, 10, 25 µg.L^{-1} St. 66[c]	25 µg.L^{-1}: abnormal swimming, intersex individuals, ↗ incidence of discontinuous gonads In intersex individual: flat and smaller testes	(Carr et al., 2003)
		St. 41[c]	0.35, 3.5, 10, 35 mg.L^{-1} 48 h	≥ 3.5 mg.L^{-1}: intestine malformations, edemas	(Lenkowski et al., 2010)
		St. 41, 42, 43[c]	10, 25, 35 mg.L^{-1} St. 47[c]	Exposure start stage differences: ↗ heart abnormalities, ↗ visceral hemorrhaging, ↗ reduced or aberrant intestinal rotation, ↗ curved body axes, ↗ edemas	(Lenkowski et al., 2008)
		St. 46-48[c]	20, 40, 80, 160, 320 µg.L^{-1} St. 66[c]	↘ weight	(Sullivan and Spence, 2003)
		St.48[c]	0.01, 0.1, 1, 10, 25 ppb St. 66[c]	≥ 0.1 ppb: multiple gonads (up to 6), hermaphroditism (with multiple testes and ovaries) In male, ≥ 1 ppb: ↘ laryngeal size In mature male, ≥ 25 ppb: ↘ plasma testosterone level (10-fold)	(Hayes et al., 2002)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 2).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Atrazine	*Xenopus laevis*	St.48[c]	0.1, 0.4, 0.8, 1, 2, 200 ppb St. 66[c]	In male, ≥ 1 ppb: ↘ laryngeal size	(Hayes et al., 2002)
		St. 56[c]	21 µg.L^{-1} 48 h	↘ testes volume, ↘ number of spermatogonial cell nests, ↘ number of nursing cells	(Tavera-Mendoza et al., 2002)
C-K Yuyos FAV® *Glyphosate*	*Bufo arenarum*	St. 36-38[a] + 48 h	1.85, 3.75, 7.5, 15, 30, 60, 120 mg[a.i.].L^{-1} 48 h	↘ AChE, BChE, GST and CbE activities	(Lajmanovich et al., 2011)
Carbaryl	*Hyla versicolor*	Tadpole (56 ± 5 mg)	0.045, 0.09 mg.L^{-1} ± caged predator 10 d	± predator: mortality + predator, 0.045 mg.L^{-1}: ↗ mortality	(Relyea and Mills, 2001)
			1, 2.1, 4.2, 8.3 mg.L^{-1} ± caged predator 16 d	± predator: mortality Dose-dependent: ↘ activity + predator, ≤ 2.1 mg.L^{-1}: ↘ activity	(Relyea and Mills, 2001)
			0.07, 0.14, 0.27, 0.54 mg.L^{-1} ± caged predator 16 d	± predator: mortality + predator: ↗ mortality, ↘ activity, ↘ growth	(Relyea and Mills, 2001)
	Rana blairi	St. 25[a]	3.5, 5, 7.2 mg.L^{-1} 96 h	↘ time spent being active, ↘ speed, ↘ distance swam, testicular resorptions, aplasia	(Bridges, 1997)
Chlorpyrifos	*Xenopus laevis*	St. 9[c]	0.05, 0.1, 0.25, 0.75, 1, 3, 6 mg.L^{-1} St. 47[c]	≥ 0.1 mg.L^{-1}, dose-dependent: malformed larvae, notochord flexure, smaller and distorted myotomes, myocyte abnormalities (smaller, completely distorted, not correctly oriented, rich in lipid droplets, with no clear cellular boundaries, with contractile apparatus with myofibrils with cross striations or heavy disarrangement of contractile structures and marked hypertrophies at the cell extremities), AChE inhibition	(Bonfanti et al., 2004)
Clomazone	*Rana catesbeiana*	St. 25[a]	0.5 mg.L^{-1} ± chitosan/alginate NP 96 h	± chitosan/alginate NP: ↗ melanomacrophage centers, lipidosis in the hepatic tissue + chitosan/alginate NP: ↘ hepatocytes	(de Oliveira et al., 2016)
2,4-D	*Xenopus laevis*	St. VI Oocyte[b]	10 mM 10 h ± progesterone	Spontaneous white spot not associated with germinal vesicle breakdown, asymmetric cell shape (cone coincident with white spot formation), short and depolymerized microtubules, Mos protein expression blocked	(Stebbins-Boaz et al., 2004)
			2.5, 5, 10 mM 10 h ± progesterone	MAPK phosphorylated by a progesterone-independent pathway and distinct from the Mos/MAPK/MPF pathway (probably activated by MAPKK), cytoplasmic polyadenylation blocked White spot cell, ≥ 2.5 mM, - progesterone: MPF activation (H1 kinase activity) inhibited White spot cell, 5 mM, + progesterone: MPF activation (H1 kinase activity) inhibited	(Stebbins-Boaz et al., 2004)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 3).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
2,4-D	*Xenopus laevis*	St. 41[c]	20, 40, 60, 70 mg.L^{-1} 48 h	≥ 60 mg.L^{-1}: intestine malformations, edemas	(Lenkowski et al., 2010)
o,p'-DDD	*Hyperolius argus*	Forelimb emerged (24 h)	0.28, 2.8 µM 20 d	2.8 µM: ↗ premature female	(Noriega and Hayes, 2000)
o,p'-DDE	*Hyperolius argus*	Forelimb emerged (24 h)	0.28, 2.8 µM 20 d	2.8 µM: ↗ premature female	(Noriega and Hayes, 2000)
o,p'-DDT	*Hyperolius argus*	Forelimb emerged (24 h)	0.028, 0.28, 2.8 µM 20 d	≥ 0.28 µM: ↗ premature female	(Noriega and Hayes, 2000)
	Xenopus laevis	Male adult	1, 250 µg.g^{-1} Daily injection [dorsal lymph sac] 7 d	↗ vitellogenin	(Palmer and Palmer, 1995)
Depe® *Permethrin*	*Bufo arenarum*	St. 33[a]	0.006 mg[a.i.].L^{-1} 24 h	↘ global activity, ↘ distance moved, ↗ time spent immobile	(Junges et al., 2017)
	Rhinella fernandezae	St. 33[a]	0.012 mg[a.i.].L^{-1} 24 h	↗ global activity, ↗ distance moved	(Junges et al., 2017)
Dieldrin	*Bufo arenarum*	Fertilized oocyte	0.02, 2, 20 ppm 168 h	0.2 ppm, 120 h and 168 h: ↗ GSH S-transferase activity 2 ppm, 120h: ↗ of GSH S-transferase activity	(Anguiano et al., 2001)
			0.02, 0.2, 2 ppm 96 h	≥ 0.2 ppm: moderate morphology alteration	(Anguiano et al., 2001)
		6 d	0.02, 0.2, 2 ppm 96 h	≥ 0.2 ppm: hyperactivity, hemorragia	(Anguiano et al., 2001)
	Xenopus laevis	Male adult	1 ppm 11 d	↗ vitellogenin	(Palmer et al., 1998)
Ectran® *Bispyribac-sodium*	*Bufo arenarum*	St. 29-30[a]	0.1562 mg[a.i.].L^{-1} 48 h	↘ AChE and BChE activities, ↗ erythrocyte nuclear abnormalities	(Lajmanovich et al., 2013)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 4).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Endosulfan	*Hyla pulchella*	St. 26 to 36[a]	2.5, 5, 10 µg.L^{-1} 96 h	≥ 5 µg.L^{-1}: ↗ micronucleated erythrocytes	(Lajmanovich et al., 2005)
Formitek® *Malathion*	*Phyllodytes luteolus*	St. 32[a]	0.112 mg[a.i.].L^{-1} 16 d	↗ mortality, ↘ activity, ↗ development	(Egea-Serrano and Solé, 2017)
Glifoglex® *Glyphosate*	*Bufo arenarum*	St. 36-38[a] + 48 h	1.85, 3.75, 7.5, 15, 30, 60, 120 mg[a.e.].L^{-1} 48 h	↘ AChE, BChE, GST and CbE activities	(Lajmanovich et al., 2011)
Glyphosate	*Leptodactylus latrans*	St. 36[a]	3, 15, 75, 300 mg.L^{-1} 96h	In liver, 15 mg.L^{-1}: lipidosis, ≥ 75 mg.L^{-1}: ↗ melanomacrophage cells, 300 mg.L^{-1}: ↗ melanomacrophage centers	(Bach et al., 2018)
	Rana catesbeiana	St. 25[a]	1 mg.L^{-1} 96h	↘ oxygen uptake Skin morphology: ↗ epidermis thickness, several layers of overlapping small cells and some chromatid fragmentation	(Rissoli et al., 2016)
	Xenopus laevis	St. 8[c]	7.5, 10, 20, 30, 50 mg.L^{-1} St. 46[c]	≥ 30 mg.L^{-1}: cardiac edema	(Bonfanti et al., 2018)
Infosato® *Glyphosate*	*Bufo arenarum*	St. 36-38[a] + 48 h	1.85, 3.75, 7.5, 15, 30, 60 mg[a.e.].L^{-1} 48 h	↘ AChE, BChE, GST and CbE activities	(Lajmanovich et al., 2011)
Introban® *Bt toxines*	*Bufo arenarum*	St. 33[a]	13.88 mg[a.i.].L^{-1} 24 h	↘ global activity, ↘ distance moved, ↗ time spent immobile	(Junges et al., 2017)
Lindane	*Bufo arenarum*	Fertilized oocyte	0.02, 2, 20 ppm 180 h	↗ GST activity (144 h)	(Anguiano et al., 2001)
			0.02, 0.2, 2 ppm 96 h	Erratic movement ad hyperactivity ≥ 0.2 ppm: segmentation altered	(Anguiano et al., 2001)
			2 ppm 96 h	↘ GSH content	(Anguiano et al., 2001)
		6 d	0.02, 0.2, 2 ppm 96 h	Hyperactivity, profuse scaling, dropsy, organ displacement and bent tail 2 ppm: high mortality	(Anguiano et al., 2001)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 5).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Marshal® *Carbosulfan*	*Duttaphrynus melanostictus*	St. 25[a]	0.3, 0.6, 1.2, 1.8, 2.4, 5, 10, 20, 25, 30, 40, 50 mg[a.i.].L^{-1} 96 h	Dose-dependent: ↗ mortality, ↘ ChE activity ≥ 0.6 mg[a.i.].L^{-1}: abnormal swimming and swelling, tail bending	(Samarakoon and Pathiratne, 2017)
Malaoxon	*Xenopus laevis*	Blastula	0.3, 1.5, 3 µM 96 h	Dose-dependent: abnormal pigmentation and gut, enlarged circulatory vessel, notochordal defects, ↘ total ascorbic acid, ↘ hydroxyproline, ↘ lysyl oxidase and collagen proline hydrolase *in vitro* activities	(Snawder and Chambers, 1993)
Malathion	*Bufo americanus*	St. 25[a]	0.1, 1, 5, 10, 20 mg.L^{-1} ± caged predator 16 d	5 mg.L^{-1}, ± predator: ↗ mortality ≥ 10 mg.L^{-1}, ± predator: high mortality	(Relyea, 2004)
	Bufo arenarum	Fertilized oocyte	2, 20 ppm 96 h	↘ pigmentation 20 ppm: ↘ gastrula %	(Anguiano et al., 2001)
			2, 20 ppm 384 h	2 ppm: ↗ GST activity (384 h) 20 ppm: ↗ GST activity (240 h, 384 h)	(Anguiano et al., 2001)
			20 ppm 96 h	↘ GSH content	(Anguiano et al., 2001)
		6 d	2, 20 ppm 96 h	20 ppm: hypoactive, antero-posterior axis curved, tail folding, circle-swimming movement, dropsy and edema	(Anguiano et al., 2001)
			20 ppm 96 h	↘ GSH content	(Anguiano et al., 2001)
	Hyla versicolor	St. 25[a]	0.1, 1, 5, 10, 20 mg.L^{-1} ± caged predator 16 d	5 mg.L^{-1}, ± predator: ↗ mortality ≥ 20 mg.L^{-1}, ± predator: high mortality	(Relyea, 2004)
	Rana catesbeaina	St. 26-28[a] + 10 d	0.5, 1, 2, 2.5, 3 mg.L^{-1} 28 d	≥ 2.5 mg.L^{-1}: mortality, development slowed 3 mg.L^{-1}: impaired equilibrium	(Fordham et al., 2001)
		St. 25[a]	0.1, 1, 5, 10, 20 mg.L^{-1} ± caged predator 16 d	≤ 1 mg.L^{-1}, ± predator: ↗ mortality ≥ 5 mg.L^{-1}, ± predator: high mortality	(Relyea, 2004)
	Rana clamitans	St. 25[a]	0.1, 1, 5, 10, 20 mg.L^{-1} ± caged predator 16 d	5 mg.L^{-1}, ± predator: ↗ mortality ≥ 10 mg.L^{-1}, ± predator: high mortality	(Relyea, 2004)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 6).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Malathion	*Rana pipiens*	St. 25[a]	0.1, 1, 5, 10, 20 mg.L^{-1} ± caged predator 16 d	≥ 5 mg.L^{-1}, ± predator: high mortality	(Relyea, 2004)
	Rana sylvatica	St. 25[a]	0.1, 1, 5, 10, 20 mg.L^{-1} ± caged predator 16 d	≤ 5 mg.L^{-1}, ± predator: ↗ mortality ≥ 10 mg.L^{-1}, ± predator: high mortality	(Relyea, 2004)
	Xenopus laevis	Oocyte st. VI[b]	50, 100 µg.L^{-1} + progesterone 390 min	Dose-dependent: accelerated maturation process	(Ji et al., 2016)
		Blastula	3, 15, 30 µM 96 h	Dose-dependent: abnormal pigmentation, gut and notochord, enlarged circulatory vessel, ↘ total ascorbic acid, ↘ hydroxyproline, ↘ lysyl oxidase and collagen proline hydrolase *in vitro* activities	(Snawder and Chambers, 1993)
		St. 9[c]	0.375, 0.75, 1.5, 3, 6 mg.L^{-1} St. 47[c]	≥ 1.5 mg.L^{-1}, dose-dependent: malformed larvae, smaller and distorted myotomes, myocyte abnormalities (completely distorted, rich in lipid droplets, no clear cellular boundaries and contractile apparatus with cross striated myofibrils), AChE inhibition	(Bonfanti et al., 2004)
Methoxychlor	*Xenopus laevis*	St. VI oocyte	62.5, 250, 4000 nM + progesterone	Dose-dependent: ↘ GVBD	(Pickford and Morris, 1999)
Metsulfuron 60® *Metsulfuron-methyl*	*Bufo arenarum*	St. 29-30[a]	80 mg[a.i.].L^{-1} 48 h	↘ AChE, BChE and GST activities, ↗ erythrocyte nuclear abnormalities	(Lajmanovich et al., 2013)
Parathion	*Bufo arenarum*	Fertilized oocyte	0.5, 2 ppm 384 h	2 ppm: ↗ GST activity (240 h, 384 h)	(Anguiano et al., 2001)
			0.5, 2 ppm 96 h	↘ pigmentation 2 ppm: ↘ gastrula %	(Anguiano et al., 2001)
		6 d	0.5, 2 ppm 96 h	2 ppm: hypoactivity, antero-posterior axis curved, tail folding, circle-swimming movement, dropsy and edema	(Anguiano et al., 2001)
RoundUp® *not specified name Glyphosate*	*Bufo americanus*	St. 25[a]	0.1, 1, 5, 10, 20 mg[a.i.].L^{-1} ± caged predator 16 d	≥ 5 mg[a.i.].L^{-1}, ± predator: high mortality	(Relyea, 2005)
	Hyla versicolor	St. 25[a]	0.1, 1, 5, 10, 20 mg[a.i.].L^{-1} ± caged predator 16 d	≥ 5 mg[a.i.].L^{-1}, ± predator: high mortality	(Relyea, 2005)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 7).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
	Rana catesbeiana	St. 25[a]	0.1, 1, 5, 10, 20 mg[a.i.].L^{-1} ± caged predator 16 d	≥ 5 mg[a.i.].L^{-1}, ± predator: high mortality	(Relyea, 2005)
	Rana clamitans	St. 25[a]	0.1, 1, 5, 10, 20 mg[a.i.].L^{-1} ± caged predator 16 d	≥ 5 mg[a.i.].L^{-1}, ± predator: high mortality	(Relyea, 2005)
	Rana pipiens	St. 25[a]	0.1, 1, 5, 10, 20 mg[a.i.].L^{-1} ± caged predator 16 d	≥ 5 mg[a.i.].L^{-1}, ± predator: high mortality	(Relyea, 2005)
	Rana sylvatica	St. 25[a]	0.1, 1, 5, 10, 20 mg[a.i.].L^{-1} ± caged predator 16 d	1 mg[a.i.].L^{-1}, + predator: ↗ mortality ≥ 5 mg[a.i.].L^{-1}, ± predator: high mortality	(Relyea, 2005)
	Xenopus laevis	St. 41[c]	0.25, 0.5, 1, 5 mg[a.i.].L^{-1} 48 h	5 mg[a.i.].L^{-1}: intestine malformations	(Lenkowski et al., 2010)
RoundUp Original® *Glyphosate*	*Rana catesbeiana*	St. 25[a]	1 mg.L^{-1} 48 h	Hyperactivity, tachycardia In liver: ↗ CAT, SOD and lipid peroxidation activity levels In muscle: ↘ CAT and SOD and ↗ lipid peroxidation activity levels	(Costa et al., 2008)
		St. 25[a]	1 mg[a.e.].L^{-1} 96 h	Skin morphology: ↗ epidermis thickness, several layers of overlapping swollen cells and extensive chromatin fragmentation	(Rissoli et al., 2016)
RoundUp® Power 2.0 *Glyphosate*	*Xenopus laevis*	St. 8[c]	1, 5, 7.5, 10, 20, 22.5, 25 mg[a.e.].L^{-1} St. 46[c]	Dose-dependent: gut miscoiling, edema, disappearance of upper and lower jaws (total at 20 mg[a.e.].L^{-1}), velar plate and branchial chamber damaged ≥ 5 mg[a.e.].L^{-1}: craniofacial defects ≥ 7.5 mg[a.e.].L^{-1}: eye defects ≥ 20 mg[a.e.].L^{-1}: affected primitive organs, completely closed intestine tract with abundant vitelline platelets, ↘ pronephric tubules, poor muscle around notochord ≥ 22.5 mg[a.e.].L^{-1}: hemorrhage 25 mg[a.e.].L^{-1}: ↗ mortality	(Bonfanti et al., 2018)
RoundUp® Ultra-Max *Glyphosate*	*Bufo arenarum*	St. 29-30[a]	10 mg[a.i.].L^{-1} 48h	↘ AChE, BChE and GST activities	(Lajmanovich et al., 2013)
		St. 36-38[a] + 48 h	1.85, 3.75 mg[a.e.].L^{-1} 48 h	↘ AChE, BChE, GST and CbE activities	(Lajmanovich et al., 2011)
	Leptodactylus latrans	St. 36[a]	0.37, 0.74, 2.22, 5.25 mg[a.e.].L^{-1} 96 h	In liver, 0.37 mg[a.e.].L^{-1}: ↗ melanomacrophage cells and center, 2.22 mg[a.e.].L^{-1}: congestion and lipidosis, 5.25 mg[a.e.].L^{-1}: congestion	(Bach et al., 2018)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 8).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
RoundUp Transorb R® *Glyphosate*	*Rana catesbeiana*	St. 25[a]	1 mg[a.e.].L^{-1} 96 h	↗ oxygen uptake Skin morphology: single layer with swollen cells and extensive chromatin fragmentation	(Rissoli et al., 2016)
Tordon 24-K® *Picloram*	*Bufo arenarum*	St. 29-30[a]	0.0195 mg[a.i.].L^{-1} 48 h	↘ AChE, BChE and GST activities, ↗ erythrocyte nuclear abnormalities	(Lajmanovich et al., 2013)
Toxaphene	*Xenopus laevis*	Male adult	1 ppm 11 d	↗ vitellogenin	(Palmer et al., 1998)
Triadimefon	*Xenopus laevis*	St. 9[c]	1.96, 3.91, 7.82, 15.63, 31.25, 45, 62.5, 125, 250 µM St. 47[c]	Hyperpigmentation, gills and buccal apparatus altered	(Papis et al., 2006)
		St. 10[c]	32 µM St. 37[c]	St. 22, 27, 37[c]: ↗ *HSP70* and *CYP26* expression	(Papis et al., 2007)
			125, 250, 500 µM 2 h	St. 47[c]: Alterations of the neural crest-derived (reduced, missing or incorrectly positioned), Meckel's suprarostral and infrarostral (badly articulated or fused) and ceratobranchial cartilages, gill lamellae underdeveloped	(Papis et al., 2006)
			500 µM 2 h	St. 22, 27, 37[c]: ↗ *CYP26* expression St. 46[c]: fusion of the anterior cartilages in a funnel-shape structure	(Papis et al., 2007)
		St. 15[c]	500 µM 2 h	St. 22, 27, 37[c]: ↗ HSP70 mRNA level, ↗ CYP26 expression	(Papis et al., 2007)
		St. 16[c]	125, 250, 500 µM 2 h	St. 47[c]: Severe alterations of the neural crest-derived (reduced, missing or incorrectly positioned), Meckel's suprarostral and infrarostral (badly articulated or fused) and ceratobranchial cartilages, gill lamellae underdeveloped	(Papis et al., 2006)
		St. 41[c]	7.29, 21.88, 36.47, 51.05 mg.L^{-1} 48 h	≥ 36.47 mg.L^{-1}: ↗ mortality ≥ 21.88 mg.L^{-1}: intestine malformations and edema	(Lenkowski et al., 2010)
Triphenyltin chloride	*Rana esculenta*	20 to 21 d	5, 10, 20 µg.L^{-1} 48 h	Dose-dependent: ↘ time spent swimming 5 µg.L^{-1}: ↗ time spent feeding 20 µg.L^{-1}: ↘ time spent feeding	(Semlitsch et al., 1995)
Vinclozolin	*Xenopus laevis*	Male adult	10^{-10}, 10^{-8}, 10^{-6} M 96 h	≥ 10^{-10} M: ↘ advertisement calls, ↘ duration of clicks in the slow trills of calls, ↘ accentuated clicks, ↗ growling call 10^{-6} M: ↘ calling activity, ↘ sexual arousing calls	(Hoffmann and Kloas, 2010)

Table S-2. Reported reproduction and development effects after amphibian phytopharmaceutical exposures (Part 9).

Contaminant	Specie	Stage	Exposure conditions	Effect	Ref.
Atrazine + malathion	*Xenopus laevis*	Oocyte st. VI[b]	50 + 50, 100 + 100 µg.L^{-1} + progesterone 390 min	Dose-dependent: accelerated maturation process 100 + 100 µM: delayed ↘ cyclin B2 level, ↘ Emi2 level	(Ji et al., 2016)
		Fertilized oocyte	50 + 50, 100 + 100 µg.L^{-1} St. 6[c](Nieuwkoop and Faber, 1967)	↗ mortality, abnormal cell division	(Ji et al., 2016)
Dieldrin + Chlordane	*Xenopus laevis*	Male adult	1 + 1 ppm 11 d	Reduced ↗ vitellogenin observed with dieldrin exposure alone	(Palmer et al., 1998)
Dieldrin + Toxaphene	*Xenopus laevis*	Male adult	1 + 1 ppm 11 d	Reduced ↗ vitellogenin observed with dieldrin or toxaphene exposures alone reduced	(Palmer et al., 1998)
RoundUp® Ultra-Max + Metsulfuron 60®	*Bufo arenarum*	St. 29-30[a]	2.5 + 2.5 mg[a.i.].L^{-1} 48 h	↘ AChE, BChE and GST activities inhibited, ↗ erythrocyte nuclear abnormalities	(Lajmanovich et al., 2013)
RoundUp® Ultra-Max + Ectran®	*Bufo arenarum*	St. 29-30[a]	39.05 + 39.05 µg[a.i.].L^{-1} 48 h	↘ BChE activity, ↗ erythrocyte nuclear abnormalities	(Lajmanovich et al., 2013)
RoundUp® Ultra-Max + Tordon 24-K®	*Bufo arenarum*	St. 29-30[a]	4.85 + 4.85 µg[a.i.].L^{-1} 48 h	↘ AChE and BChE activities, ↗ erythrocyte nuclear abnormalities	(Lajmanovich et al., 2013)
Toxaphene + Chlordane	*Xenopus laevis*	Male adult	1 + 1 ppm 11 d	Reduced ↗ vitellogenin observed with toxaphene exposure alone	(Palmer et al., 1998)

St.: stage; a: (Gosner, 1960); b: (Dumont, 1972); c: (Nieuwkoop and Faber, 1967)

4. Emerging organic contaminants exposures

Main selected studies in this section interest in endocrine disruption effects (Table S-3). That is the reason why the reported xenobiotics are essentially hormones and hormone mimetic compounds. The described effects are so related to sex reversal.

In *R. pipiens*, estradiol or ethinylestradiol induced intersex gonads and reduced the proportion of male individuals (Mackenzie et al., 2003) (Table S-3 (Part 1)). The increased of intersex individual occurrence was also detected in *R. pipiens* exposed to 4-nonylphenol or the antiestrogen ICI 182780 (Mackenzie et al., 2003) (Table S-3 (Part 2)). Similarly, for *R. sylvatica*, 4-nonylphenol exposures increased the proportion of intersexual individuals and enlarged the semiferous tubule lumina of testes (Mackenzie et al., 2003) (Table S-3 (Part 2)). Such abnormal structures in mice and frogs could correspond to a support to contain oocytes that has subsequently degenerated (Couse et al., 1999; Humphrey et al., 1950). *X. laevis* male adults exposed to ethynylestradiol showed severe alteration of the testes with the occurrence of oocytes within (Cevasco et al., 2008) (Table S-3 (Part 1)). Biased sex ratios (higher number of female and/or intersex) were detected after the exposure of *X. laevis* tadpoles to estradiol or bisphenol A (Carr et al., 2003; Levy et al., 2004) (Table S-3 (Parts 1 and 2)). Estradiol and diethylstilbestrol induced both increased of vitellogenin levels in injected male adults (Palmer et al., 1998; Palmer and Palmer, 1995) (Table S-3 (Parts 1 and 2)). Another female-biased sex ratio was assessed in *Xenopus tropicalis* exposed to ethynylestradiol and the female individuals exhibited altered or absent oviducts (Pettersson et al., 2006) (Table S-3 (Part 1)).

Concerning masculinization, it has been observed in *X. laevis* exposed to methyldihydrotestosterone or tamoxifen (Cevasco et al., 2008) (Table S-3 (Part 3)). In females, male germ cells were present and associated with atresia and a decreased number of oocytes. In the same work, exposed males developed an increased number of germ cell nests and even longer testes. Methyldihydrotestosterone exposures impacted also the call receptivity and emission, by increasing the latency to respond to the male stimuli and by increasing the percentage of advertisement calls in males and decreasing the rasping (Hoffmann and Kloas, 2012) (Table S-3 (Part 3)).

A5. Outcomes and perspectives

This work proposes a literature review of the studies assessing effects of contaminant exposures with reproductive and developmental endpoints in amphibians.

Table S-3. Reported reproduction and development effects after amphibian emerging organic contaminant exposures (Part 1).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
Estradiol	*Hyperolius argus*	Forelimb emerged (24 h)	0.00028, 0.0028, 0.028, 0.28, 0.367, 3.67 µM 20 d	≥ 0.0028 µM: ↗ premature female	(Noriega and Hayes, 2000)
	Rana pipiens	St. 25[a]	1, 10, 50, 100 $\mu g.L^{-1}$ St. 46[a]	Metamorphosis delayed, female-biased sex ratio, ↗ female w/ early vitellogenic oocytes 1 $\mu g.L^{-1}$: ↗ intersex gonads (ovotestis)	(Mackenzie et al., 2003)
	Xenopus laevis	48 h post-hatching	100 $\mu g.L^{-1}$ St. 66[b]	↗ length, ↗ female, ↗ intersex individual	(Carr et al., 2003)
		St. 42-43[b]	10^{-8}, 10^{-7} M 120 d	↗ female	(Levy et al., 2004)
		St. 50[b]	10^{-7} M 2 weeks	↗ estrogen receptor expression	(Levy et al., 2004)
		Male adult	1 $\mu g.g^{-1}$ Daily injection [ip] 7 d	↗ vitellogenin	(Palmer and Palmer, 1995)
			1 $mg.kg^{-1}$ Weekly injection [ip] 1 month	↗ vitellogenin	(Palmer et al., 1998)
Ethinylestradiol	*Rana pipiens*	St. 25[a]	1, 10 $\mu g.L^{-1}$ St. 46[a]	Metamorphosis delayed, female-biased sex ratio (no male), ↗ female with early vitellogenic oocytes 10 $\mu g.L^{-1}$: ↗ intersex gonads (ovotestis)	(Mackenzie et al., 2003)
	Rana sylvatica	St. 25[a]	1, 10 $\mu g.L^{-1}$ St. 46[a]	10 $\mu g.L^{-1}$: ↗ length, ↗ female with early vitellogenic oocytes	(Mackenzie et al., 2003)
	Xenopus laevis	Female adult	10^{-8} M 4 weeks	↗ ≥ st. III and atretic oocytes	(Cevasco et al., 2008)
		Male adult	10^{-8} M 4 weeks	↘ testis length, ↘ tubule diameter, thickening of the interlobular connective tissue, enlarged blood vessel, granulocyte infiltration, occurrence of small number of oocytes in testis, ↘ germ cell nest number	(Cevasco et al., 2008)
	Xenopus tropicalis	St. 47-49[b]	1, 10, 100 mM St. 66[c]	Female-biased phenotypic sex-ratio In female, 100 mM: ↗ oviduct weight, lacking one or both oviducts	(Pettersson et al., 2006)
Nonylphenol	*Rana catesbeiana*	St. 35-37[a]	234, 468, 936 $\mu g.L^{-1}$ 7 d	936 $\mu g.L^{-1}$: ↗ tail growth	(Christensen et al., 2005)

Table S-3. Reported reproduction and development effects after amphibian emerging organic contaminant exposures (Part 2).

Contaminant	Specie	Stage	Exposure conditions	Effect	References
4-nonylphenol	*Rana pipiens*	St. 25[a]	10, 100 µg.L^{-1} St. 46[a]	Metamorphosis delayed, biased sex ratio, ↗ female with early vitellogenic oocytes % 10 µg.L^{-1}: intersex gonads (ovotestis)	(Mackenzie et al., 2003)
	Rana sylvatica	St. 25[a]	10, 100 µg.L^{-1} St. 46[a]	Biased sex ratio 10 µg.L^{-1}: testes with enlarged seminiferous tubule lumina	(Mackenzie et al., 2003)
Bisphenol A	*Rana rugosa*	St. 10[c]	10^{-8}, 10^{-7}, 10^{-6} M 9 d ± T_3	≥ 10^{-7} M, dose-dependent: T_3-induced tail disappearance suppression 10^{-6} M, ± T_3: no DNA fragmentation and ladder formation in tails	(Goto et al., 2006)
	Xenopus laevis	*Not reported*	10^{-7}, 10^{-6} M 11 d ± T_3	T_3-induced EGFP activity in hindlimbs suppression	(Goto et al., 2006)
		St. 42-43[b]	10^{-8}, 10^{-7}, 10^{-6} M 120 d	10^{-7} M: ↗ female	(Levy et al., 2004)
		St. 50[b]	10^{-7} M 2 weeks	↗ estrogen receptor expression	(Levy et al., 2004)
	Xenopus tropicalis	St. 57[b]	10^{-6} M 10 d	Suppression of metamorphosis, tail disappearance and hindlimb growth	(Goto et al., 2006)
Diethylstilbestrol	*Xenopus laevis*	Male adult	1 µg.g^{-1} Daily injection [ip] 7 d	↗ vitellogenin	(Palmer and Palmer, 1995)
			1 ppm 11 d	↗ vitellogenin	(Palmer et al., 1998)
Dihydro-testosterone	*Xenopus laevis*	48h-post-hatching	100 µg.L^{-1} St. 66[b]	↗ laryngeal dilator muscle size	(Carr et al., 2003)
Flutamide	*Rhinella schneideri*	Male adult	1, 5 mg.kg^{-1} Daily injection [Subcutaneous] 7 d	↘ total locular area of the testicular tissue, ↗ spermatogonia, ↗ spermatocyte, ↘ spermatozoa area 1 mg.kg^{-1}: ↗ spermatid, in the Bidder's organ: ↗ oocytes in final diplotene stage, ↘ oocytes in initial diplotene, ↘ degenerating oocytes, ↘ atretic oocytes	(de Gregorio et al., 2016)
	Xenopus laevis	2-4 y-o	10^{-8}, 10^{-6} M 72 h	10^{-6} M: ↘ aromatase expression, ↘ calling activities	(Behrends et al., 2010)
ICI 182780	*Rana pipiens*	St. 25[a]	1, 10 µg.L^{-1} St. 46[a]	Metamorphosis delayed, biased sex ratio, ↗ female with early vitellogenic oocytes % 10 µg.L^{-1}: ↗ intersex gonads (ovotestis)	(Mackenzie et al., 2003)

Table S-3. Reported reproduction and development effects after amphibian emerging organic contaminant exposures (Part 3).

Contaminant	Specie	Stage	Exposure conditions	Effect	Ref.
Methyldi-hydrotestosterone	*Xenopus laevis*	7 y-o female adult	0.3045, 3.05, 30.45 µg.L^{-1} 96 h	3.05 µg.L^{-1}: ↗ male call receptivity on day 1 and 2 30.45 µg.L^{-1}: ↗ latency in respond to male stimulus	(Hoffmann and Kloas, 2012)
		3 y-o male adult	0.3045, 3.05, 30.45 µg.L^{-1} 96 h	↗ advertisement calls, ↘ rasping (sexual arousing)	(Hoffmann and Kloas, 2012)
		Female adult	10^{-8} M 4 weeks	↗ atretic oocyte, occurrence of male germ cells, ↘ oocytes, ↘ < st. III oocytes	(Cevasco et al., 2008)
		Male adult	10^{-8} M 4 weeks	↗ testis length, ↗ germ cell nest	(Cevasco et al., 2008)
Tamoxifen	*Xenopus laevis*	Female adult	10^{-8} M 4 weeks	↗ atretic oocyte, occurrence of male germ cells, ↘ oocytes, ↘ < st. III oocytes	(Cevasco et al., 2008)
		Male adult	10^{-8} M 4 weeks	↗ germ cell nest	(Cevasco et al., 2008)
Tetrabromo-bisphenol A	*Rana rugosa*	St. 10[c]	10^{-8}, 10^{-7}, 10^{-6} M 9 d ± T_3	≥ 10^{-7} M, dose-dependent: T_3-induced tail disappearance suppression In tail, 10^{-6} M, ± T_3: no DNA fragmentation and ladder formation	(Goto et al., 2006)
	Xenopus laevis	*Not reported*	10^{-8}, 10^{-7} M 11d + T_3	10^{-7} M: T_3-induced EGFP activity in hindlimbs suppression	(Goto et al., 2006)
	Xenopus tropicalis	St. 57[b]	10^{-6} M 10 d	Suppression of metamorphosis, tail disappearance and hindlimb growth	(Goto et al., 2006)
Tetrachloro-bisphenol A	*Rana rugosa*	St. 10[c]	10^{-8}, 10^{-7}, 10^{-6} M 9 d ± T_3	≥ 10^{-8} M, dose-dependent: T_3-induced tail disappearance suppression In tail, 10^{-6} M, ± T_3: no DNA fragmentation and ladder formation	(Goto et al., 2006)
	Xenopus laevis	*Not reported*	10^{-7}, 10^{-6} M 11 d + T_3	T_3-induced EGFP activity in hindlimbs suppression	(Goto et al., 2006)
	Xenopus tropicalis	St. 57[b]	10^{-6} M 10 d	Suppression of metamorphosis, tail disappearance and hindlimb growth	(Goto et al., 2006)

Table S-3. Reported reproduction and development effects after amphibian emerging organic contaminant exposures (Part 4).

Contaminant	Specie	Stage	Exposure conditions	Effect	Ref.
Tetramethyl-bisphenol A	*Rana rugosa*	St. 10[c]	10^{-8}, 10^{-7}, 10^{-6} M 9d ± T_3	≥ 10^{-7} M, dose-dependent: T_3-induced tail disappearance suppression In tail, 10^{-6} M, ± T_3: no DNA fragmentation and ladder formation	(Goto et al., 2006)
	Xenopus laevis	*Not reported*	10^{-7}, 10^{-6} M 11 d + T_3 exposure	T_3-induced EGFP activity in hindlimbs suppression	(Goto et al., 2006)
	Xenopus tropicalis	St. 57[b]	10^{-6} M 10 d	Suppression of metamorphosis, tail disappearance and hindlimb growth	(Goto et al., 2006)
Atorvastatin + bezafibrate + metformin	*Limnodynastes peronii*	St. 26[a]	0.5 + 0.5 + 0.5, 5 + 5 + 5, 50 + 50 + 50, 500 + 500 + 500 µg.L^{-1} 30 d	5 + 5 + 5 µg.L^{-1}: ↘ length 500 + 500 + 500 µg.L^{-1}: ↗ mass, ↗ development	(Melvin et al., 2017)

St.: stage; a: (Gosner, 1960); b: (Nieuwkoop and Faber, 1967); c: (Taylor and Kollros, 1946)

They dealt with lab works to characterise biological effects of pollutants and investigate their mechanisms of actions in controlled conditions. For this purpose, exposure designs were simplified. Few studies were conducted in conditions allowing to mimic environmental interactions as multiple contaminants and/or various stressors. Despite lab toxicity assessment with amphibians has a prominent necessity, it does not reproduce relevant environmental conditions (Linder et al., 2010). Major part of works studied effects of single contaminant exposures. In this way, exposure to complex cocktails of environmental contaminants have to be favoured. In this review, few co-exposures are reported. Cadmium and lead mixture exposures in *X. laevis* increased the ICl currents and the resting potential of oocytes and decreased the *in vitro* fertilization success (Marin et al., 2015; Slaby et al., 2016b) (Table S-1 (Part 5)). In the same species, oocyte exposures to atrazine and/or malathion accelerated the maturation process, but only in binary exposure conditions delayed the cyclin B2 level decrease and reduced of the Emi2 level (Ji et al., 2016) (Table S-2 (Parts 1, 6 and 9)). Dieldrin and toxaphene alone increased the level of vitellogenin of male *X. laevis*, but this effect was less important in cases of exposures to these pesticides associated together or to chlordane (Palmer et al., 1998) (Table S-2 (Part 3, 8 and 9)). Single and binary exposures of *B. arenarum* tadpoles to Ectran®, RoundUp® Ultra Max, Metsulfuron 60® and Tordon 24-K® led to different effects on the cholinesterase and the glutathione S-transferase activities as well as on erythrocyte nuclear abnormality occurrences (Lajmanovich et al., 2013) (Table S-2 (Parts 4 and 6 to 9)). Other protocols were designed to assess the effects of contaminant under predator pressure (Relyea, 2005, 2004) (Table S-2 (Parts 5 to 7)). So, some authors observed that the presence of a caged predator increased the mortality of *R. sylvatica* tadpoles exposed to a glyphosate-based commercial formulation for 16 days (Table S-2 (Part 7)) (Relyea, 2005).

The experimental complexity level could also be increased by using mesocosm exposure systems to minimize the laboratory-to-field extrapolation errors, and by conducting *in situ* ecotoxicological studies (Linder et al., 2010). In their chapter, Linder *et al.* reviewed mesocosm exposures and highlighted that mortality and/or growth impairments were observed in case of exposures to for example atrazine, carbaryl, RoundUp®, copper or mercury as tested chemicals (Linder et al., 2010). In certain works, contaminant exposures were also combined with other stressors such competition, predation, limited food, temperature or UV radiation. For field studies, feminization cases were reported in frogs exposed to environmental contaminants. It has been observed, in different areas of the United States characterized by low or high agricultural atrazine uses (and confirmed in laboratory conditions), where collected leopard frogs (*R. pipiens*) exhibited varying degree of sex reversal such oocytes inserted in testicular lobules, underdeveloped testes, closed or no testicular lobules or low to absent male germ cells (Hayes et al., 2003). The neotropical anuran male *Physalaemus cuvieri* exposed to

organochlorine, organophosphate and carbamate compounds in permanent water bodies near a farm in Marmeleiro (Brazil) showed high frequency of intersexual gonads with sperm and oocytes (Moresco et al., 2014). In laboratory conditions, *X. laevis* exposed to environmental samples of the Italian Lambro River containing agricultural run-off, domestic and industrial effluents exhibited also strong gonadal abnormalities in both sexes (Cevasco et al., 2008). To assess the impact of trematode infection correlated with contamination, the wood frog (*R. sylvatica*) were encaged in ponds near agricultural fields treated or not by organochlorine and organophosphorus compounds (Kiesecker, 2002). Limb deformities induced by trematode infection increased in the contaminated ponds.

Concerning the species some of them are more represented than the others. Moreover, since the ascertainment of Sparling *et al.* in 2010, there is still no interest in amphibian toxicology for the order Gymnophiona (Sparling et al., 2010). Among the most studied species, we can depict the particular case of *X. laevis*. This South African frog is considered as a classical lab model. It is an exclusive aquatic species easy to bread in the animal house facilities. For many decades xenopus has been used as a privileged model in biology, to study for example calcium signalling, cell cycle transitions, embryology or developmental biology (Delisle, 1991; Gurdon and Hopwood, 2000; Heikkila et al., 2007; Machaca, 2007; Marin, 2012; Marin et al., 2010). The wide literature about this frog is certainly the main reason why there is so many environmental toxicological studies with it. The standardized toxicological test FETAX (Frog Embryo Teratogenesis Assay: Xenopus) have been developed with *X. laevis*. This short screening assay (96h) allows the assessment of teratogenic potential of chemicals, mixtures or environmental samples (Dumont et al., 1983; Mouche et al., 2011). Besides, *X. laevis* is nowadays a worrisome invasive species observed in different parts of the world, where it disturbs the local populations by predating and enhancing the propagation of fungal pathogen as *Batrachochytrium dendrobatidis* (Lillo et al., 2011; Measey et al., 2012). It could be envisaged to catch this exotic species (from clutch to adult), first to regulate its invasive populations and then to biomonitor quality of freshwater ecosystems, without adding another treat to local amphibian species.

In amphibian toxicology, the most studied endpoints are largely the morphological developmental alterations. Venturino *et al.* in 2003 established the same statement (Venturino et al., 2003). This large use of morphological criteria, due to their relevance and their readily observability, allows to state that such deformities are not specific for any category of compounds (Venturino et al., 2003). However, it is necessary to develop analysis methods to generate more accurate biometric information. The development of complementary methods like histopathological analysis, automatic biometric assessment or microstructure and sub-

microstructure analyses, brings new informative data (Bach et al., 2016; Bonfanti et al., 2018; Slaby et al., 2016a).

Lastly, there is still a lack of experimental designs using amphibian gametes as model, despite the interests to use developmental precocious stages to assess toxic impacts of xenobiotic exposures. Any damage on oocytes and/or spermatozoa will disturb the fertilization or the development and so the dynamic of populations. Few studies dealt with this kind of exposures, but it has been proved that the formation of healthy eggs can be disturbed by metal or pesticide exposures (Ji et al., 2016; Marin et al., 2015; Slaby et al., 2017; Stebbins-Boaz et al., 2004). Concerning spermatozoa, their mobility can be inhibited by divalent zinc ion at environmental concentrations and in a dose-dependent manner (Christensen et al., 2004). To assess the impacts on gametes is needed in amphibian toxicology in addition of conventional analysis which could miss critical windows of exposures. Characterization of toxic effects on gametes is too consistent with the 3R's principles (Russell and Burch, 1959).

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1.2. Objectifs

Dans ce contexte de déclin des amphibiens, il existe un manque de données sur les effets d'expositions à des contaminants environnementaux sur les stades précoces de leur cycle de vie. Il est reconnu que les micropolluants peuvent interagir avec les systèmes de reproduction et de développement selon plusieurs modes d'action. Par exemples, l'exposition à l'atrazine de têtards de *X. laevis* provoque une féminisation des individus avec l'induction d'ovotestis (gonade ayant à la fois des caractéristiques mâle et femelle) ou encore, chez les mâles, la diminution de la taille du larynx et du niveau de testostérone (Hayes et al., 2002). Néanmoins, les travaux en toxicologie des amphibiens ne se sont que peu focalisés sur l'étude des atteintes toxiques des cellules germinales et des embryons.

X. laevis est très souvent l'espèce modèle de ces travaux en toxicologie de l'environnement. C'est d'ailleurs le cas dans de nombreux domaines de la biologie, comme le développe le chapitre suivant « *Modèle et approches utilisés* ». Il était intéressant d'exploiter ces avantages dans une démarche pluridisciplinaire, mettant en relation la biologie cellulaire, du développement, la toxicologie de l'environnement et l'écotoxicologie. Trois principaux objectifs autour de l'ovocyte de xénope ont pu alors être établis :

- Les effets d'expositions à un large éventail de contaminants environnementaux sur différents paramètres de la maturation ovocytaire, de la fécondation et du développement embryonnaire ont été évalués. Le but étant de caractériser un maximum de réponses afin de valider notre modèle. Les processus cellulaires bien connus de ce mécanisme ont été étudiés en conditions contaminées selon différentes approches (morphologique, cytologique et biochimique) ;

- Afin de compléter les biomarqueurs classiquement utilisés en toxicologie des amphibiens, des cibles cellulaires et des signatures spécifiques des expositions ont été identifiées. Ce développement de nouveaux endpoints, visait également à mieux comprendre les mécanismes d'action de nos contaminants ;

- Des effets d'échantillons prélevés dans l'environnement ont été caractérisés en utilisant les protocoles précédemment développés. Cette partie constitue le commencement de, et contribue à, la proposition d'un nouveau modèle, en écotoxicologie des amphibiens, basé sur l'ovocyte de xénope pour évaluer la qualité de milieux aquatiques.

Chapitre II - Modèle et approches utilisés

2.1. Matériel biologique : Xenopus laevis, son ovocyte et son développement précoce

X. laevis (Daudin, 1802) est une espèce de la famille des *Pipidae* (*Amphibia, Lissamphibia, Salienta, Anura)*. Originaire d'Afrique du Sud, le xénope lisse est également appelé dactylère du Cap. Son cycle de vie est exclusivement aquatique, ce qui présente un avantage pour son maintien en laboratoire. Les femelles (Figure 1A), d'une longueur d'environ 10 centimètre du rostre au cloaque, sont toujours plus grandes que les mâles et les deux sexes possèdent des griffes sur les trois doigts internes des pattes postérieures. *X. laevis* est une espèce classiquement utilisée en laboratoire dans des thématiques de recherche variées telles que la biologie cellulaire, le développement, l'embryologie, la cancérologie ou l'écotoxicologie. Deux prix Nobel de physiologie ou médecine ont d'ailleurs été obtenus pour des travaux utilisant cette espèce en tant que modèle. Les embryologistes, Hans Spemann et Hilde Mangold, ont découvert en 1924 le concept de centre organisateur dans l'embryon en développement (Spemann and Mangold, 1924). Seul H. Spemann reçu le prix Nobel en 1935, H. Mangold ne pouvant le recevoir à titre posthume. John Gurdon et Shinya Yamanaka, quant à eux, ont mis en évidence que des cellules différenciées peuvent être reprogrammées pour devenir des cellules pluripotentes (Gurdon, 1962; Gurdon et al., 1958). Pour leurs travaux, ils ont obtenu le prix Nobel en 2006. Sur le plan médical, les femelles xénopes ont constitué les premiers tests de grossesse jusque dans les années 1940-50 (Elkan, 1938). Aujourd'hui, dans l'environnement, *X. laevis* est considéré comme une espèce exotique invasive retrouvée dans différentes parties du globe (Afrique, Europe, Amérique et Asie) où ses populations sont problématiques pour les espèces locales à cause de la prédation et de la propagation de pathogènes (Measey et al., 2012).

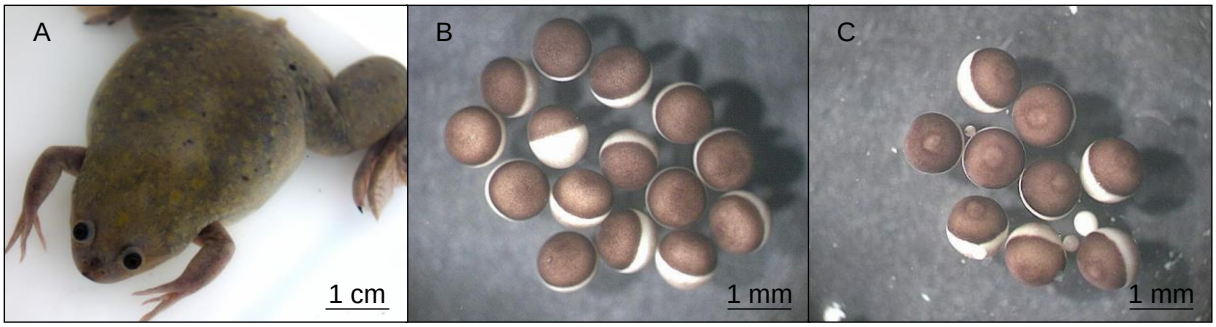

Figure 1. Photographies (A) d'un *X. laevis* femelle, (B) d'ovocytes de stade 6 immatures et (C) d'ovocytes matures présentant la tache de maturation.

L'ovogenèse de *X. laevis* comprend six stades. Durant son développement le gamète grandit, acquiert une pigmentation, accumule du vitellus et se régionalise pour constituer à terme un ovocyte de stade VI qui pourra subir le phénomène de maturation (Dumont, 1972).

Ce dernier stade est une large cellule sphérique d'environ 1,3 mm de diamètre, présentant un pôle animal sombre et pigmenté et un pôle végétatif plus clair (entre le crème et l'ocre) séparés sur le plan équatorial par une bande plus claire nommée anneau équatorial (Figure 1B). Plus de la moitié du volume de l'ovocyte est constituée de plaquettes vitellines destinées à la nutrition de l'embryon (pôle végétatif). Les ovocytes de stade VI, que nous nommerons dans le reste du document ovocytes immatures, présentent au niveau cytologique une vésicule germinale intacte excentrée vers le pôle animal (Figure 2A). Pour arriver à ce stade de leur cycle, les cellules sont passées par les premières étapes méiotiques. Ainsi après la phase S, consistant en la duplication des chromosomes pour donner des paires de chromatides sœurs. Par la suite, la première division de méiose débutera. Elle est appelée réductionnelle (méiose I) étant donné que les cellules filles sont haploïdes et donc ne possèdent qu'une seule copie de chaque homologue parental. Les ovocytes immatures précédemment évoqués sont tous arrêtés prophase I de méiose, ils sont synchronisés. Par induction hormonale, la méiose va reprendre et la deuxième division, nommée équationnelle (méiose II), va avoir lieu. Les chromatides sœurs vont se séparer dans des cellules haploïdes. Le traitement hormonal donnera des ovocytes matures et synchronisés en métaphase II de méiose (Ferrell, 1999).

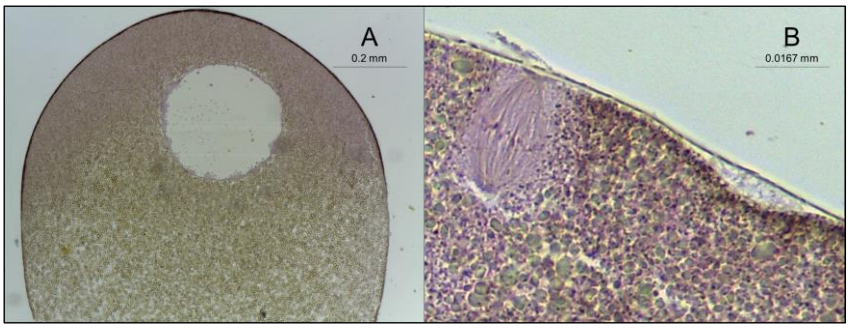

Figure 2. Photographies d'ovocytes colorés au rouge nucléaire et au picro-indigo-carmin (A) immature, présentant la large vésicule germinale et (B) mature au niveau du fuseau de division et de la poche du globule polaire de *X. laevis*.

La maturation ovocytaire est une étape essentielle préparant l'ovocyte à la ponte et à la fécondation. *In vitro*, la reprise méiotique peut être induite par différents composés, et notamment par des hormones stéroïdiennes, telle que la progestérone, ou l'hormone peptidique insuline (Haccard and Jessus, 2006). Cependant, *in vivo* cette action hormonale est encore un phénomène mal compris (Ferrell, 1999; Schmitt and Nebreda, 2002). Durant ce processus, une tache de maturation (white spot) apparait au sommet du pôle animal de la cellule (Figure 1C). Elle est causée par la montée puis la disparition de la vésicule germinale (GVBD, Germinal Vesicle Breakdown) qui écartera les pigments corticaux. Ce phénomène peut être découpé en trois phases : la rupture de l'enveloppe nucléaire, l'organisation d'un réseau de fibrilles dans la zone nucléaire basale et l'accumulation de granules sous l'extrémité

basale de l'enveloppe nucléaire (Huchon et al., 1981). Un premier fuseau de division va se former, l'ovocyte sera alors en prométaphase I. Par la suite, il y aura l'émission du premier globule polaire et formation du second fuseau de division accroché à la membrane cellulaire (Brachet et al., 1970; Huchon et al., 1981). Cette structure est en forme de losange (baril) et comporte les chromosomes alignés sur la plaque métaphasique (Figure 2B). Contrairement à des fuseaux méiotiques classiques, celui des ovocytes de xénope ne possède ni de centrosome ni de centromère (Gard, 1992). Les centrosomes sont inactivés au stade diplotène de première prophase de méiose.

Comme le présente la figure 3, la maturation ovocytaire correspond à la mise en place de différentes voies de signalisation amenant à l'activation du MPF (M-phase-Promoting Factor) (Schmitt and Nebreda, 2002). Ce facteur universel est composé d'une sous-unité régulatrice cycline B et d'une sous-unité catalytique Cdc2 (Cdk1, Cyclin dependant kinase 1) (Dunphy et al., 1988; Gautier et al., 1988). Son rôle est de réguler le passage en phase M (Norbury and Nurse, 1990). Dans l'ovocyte immature, il est contenu sous sa forme inactive, nommée pré-MPF. A ce moment, ses deux sous-unités sont associées entre elles. Son activation repose sur la déphosphorylation de Cdc2. Cette action est réalisée par la phosphatase Cdc25 qui enlève la phosphorylation Cdc2 et l'active. A son tour, Cdc2 activera Cdc25, permettant une auto-amplification rapide du MPF (Haccard and Jessus, 2006). La déphosphorylation de Cdc2 est également dépendante de l'inactivation de Myt1, elle-même régit par l'activation (phosphorylation) de $p90^{RSK}$ (Rsk 1, 2, p90 kDa ribosomal s6 kinase) (Ferrell, 1999; Haccard and Jessus, 2006; A. Palmer et al., 1998). Il existe donc une balance entre l'activité de Cdc25 et de Myt1 permettant l'activation de Cdc2 et donc du MPF. A propos de l'action de Myt1, un autre modèle propose qu'après l'augmentation du taux de cycline B en réponse au traitement hormonal à la progestérone, que le nouveau complexe Cdc2-cycline B soit responsable de l'inhibition de son propre inhibiteur et donc permette la reprise méiotique (Gaffré et al., 2011). Le MPF aura, par la suite, différentes cibles, par exemple l'histone H3.

La phosphorylation de Rsk est incluse dans la cascade MAPK (Mitogen-Activated Protein Kinase). Ainsi l'augmentation de la concentration en protéine Mos (Proto-oncogene serine/threonine-protein kinase) sera suivie de l'activation de MEK1, ERK2 (Extracellular signal-regulated kinase 2) et de Rsk1, 2 (Ferrell, 1999; Frödin and Gammeltoft, 1999). Associé à cette activité MAPK, le MPF sera donc activé (Figure 3). La voie MAPK est cependant également régulée par le MPF (Ferrell, 1999).

Les différents acteurs biochimiques cités précédemment ont un rôle crucial dans la formation du fuseau méiotique. Il a été prouvé dans l'ovocyte de *X. laevis* que la cascade MAPK est essentielle pour l'organisation bipolaire du fuseau (Bodart et al., 2005).

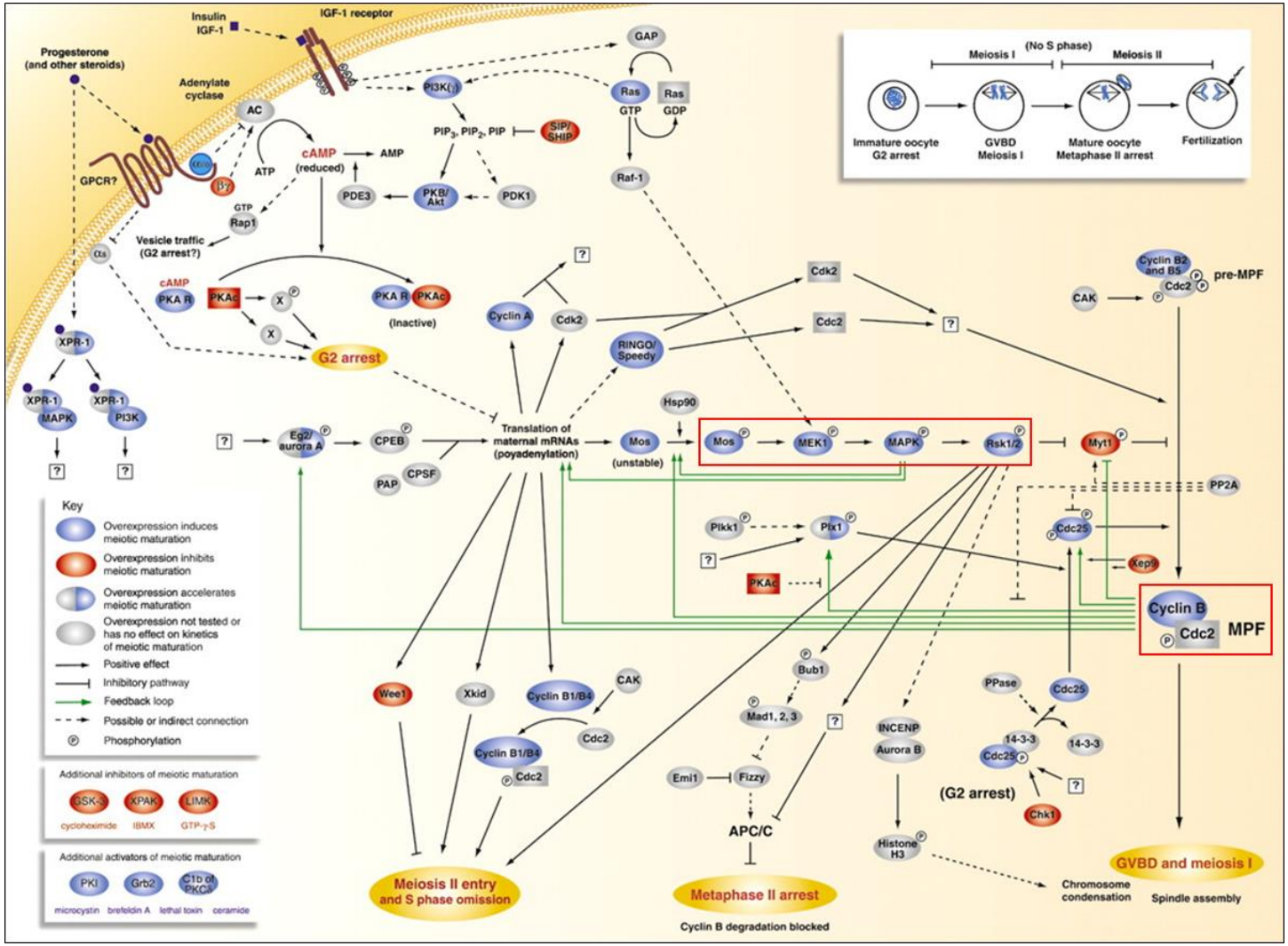

Figure 3. Activations des voies de signalisation impliquées dans la maturation ovocytaire. Encadrés en rouge : la cascade MAPK et le MPF. Illustration modifiée d'après Schmitt and Nebreda, 2002.

De même, chez *Xenopus tropicalis*, l'activité MEK est nécessaire à la formation du fuseau (Bodart et al., 2002). Dans l'ovocyte de souris, l'activité MAPK est également associée à l'organisation des microtubules et à la condensation de la chromatine durant la méiose (Verlhac et al., 1994). Les auteurs ont également montré que ces événements cytologiques étaient d'ailleurs plus corrélés à l'activité MAP kinase qu'à l'activité MPF. Toujours dans cette cellule germinale mammalienne, il a été prouvé que la morphogénèse du fuseau est régulée par deux substrats de la voie MAPK (MISS et DOC1R) (Lefebvre et al., 2002; Terret et al., 2003). L'activité MAPK n'a donc pas qu'un rôle de régulateur du MPF mais aussi une action directe sur la formation du fuseau méiotique. Une étude dans les ovocytes de l'amphibien *Rana japonica* a montré, d'ailleurs, que leurs fonctions étaient complémentaires (Kotani and Yamashita, 2002). Les auteurs ont conclu que le MPF induit la GVBD et la transformation les microtubules en une agrégation compacte, que MAPK permet la réorganisation du réseau de microtubules entourant la vésicule germinale au début de la GVBD et que leur « collaboration » est nécessaire pour la condensation des chromosomes, la formation du centre cellulaire organisateur des microtubules (MTOC) et du *transient microtubule array* (TMA). Enfin, concernant la cible du MPF nommée histone H3, sa modification conservée par phosphorylation est associée à la condensation des chromosomes (Hans and Dimitrov, 2001; Hendzel et al., 1997; Van Hooser et al., 1998).

Après cette maturation ovocytaire, les gamètes femelles sont pondus et sont prêts pour la fécondation externe. En milieu naturel, le mâle s'accroche à la femelle dans la région lombaire à l'aide des callosités kératinisées de ses pattes antérieures (amplexus). La femelle, aidée par les pressions exercées par le mâle, va pondre ses œufs qui seront fécondés rapidement. L'activation de l'œuf par un spermatozoïde, déclenche deux grands phénomènes morphologiquement visibles. La rotation d'équilibration correspondant au retournement du nouvel embryon, libéré de toute prise, en fonction de la pesanteur après formation de la membrane de fécondation et le réorientant pôle animal vers le haut. La rotation corticale, durant laquelle l'embryon acquiert une polarité dorso-ventrale, accompagnée de la formation du croissant gris à l'opposé du point d'impact du spermatozoïde et qui correspond à la face dorsale. Après fécondation, la tache de maturation disparait également. Par la suite, comme le présente la figure 4, le développement embryonnaire progressera par les étapes classiques de l'embryogenèse, à savoir la segmentation, la gastrulation et l'organogenèse. La segmentation est une succession de clivages embryonnaires holoblastiques (divisions mitotiques concernant la totalité de l'embryon) constituant une prolifération cellulaire organisée durant laquelle le volume embryonnaire restera inchangé ; la gastrulation correspond à la mise en place des feuillets embryonnaires (endoderme, mésoderme et ectoderme) ; et l'organogenèse fait référence à la formation des différents organes et débute par la

différenciation de cellules du neurectoderme et l'organisation du tube neural, ébauche du système nerveux (Bernardini et al., 1999). A 23 °C, le développement jusqu'à la métamorphose (apparition des membres antérieurs) s'accomplit en 58 jours (Nieuwkoop and Faber, 1967). Un individu deviendra sexuellement mature et pourra à son tour se reproduire entre 6 et 10 mois (Figure 4).

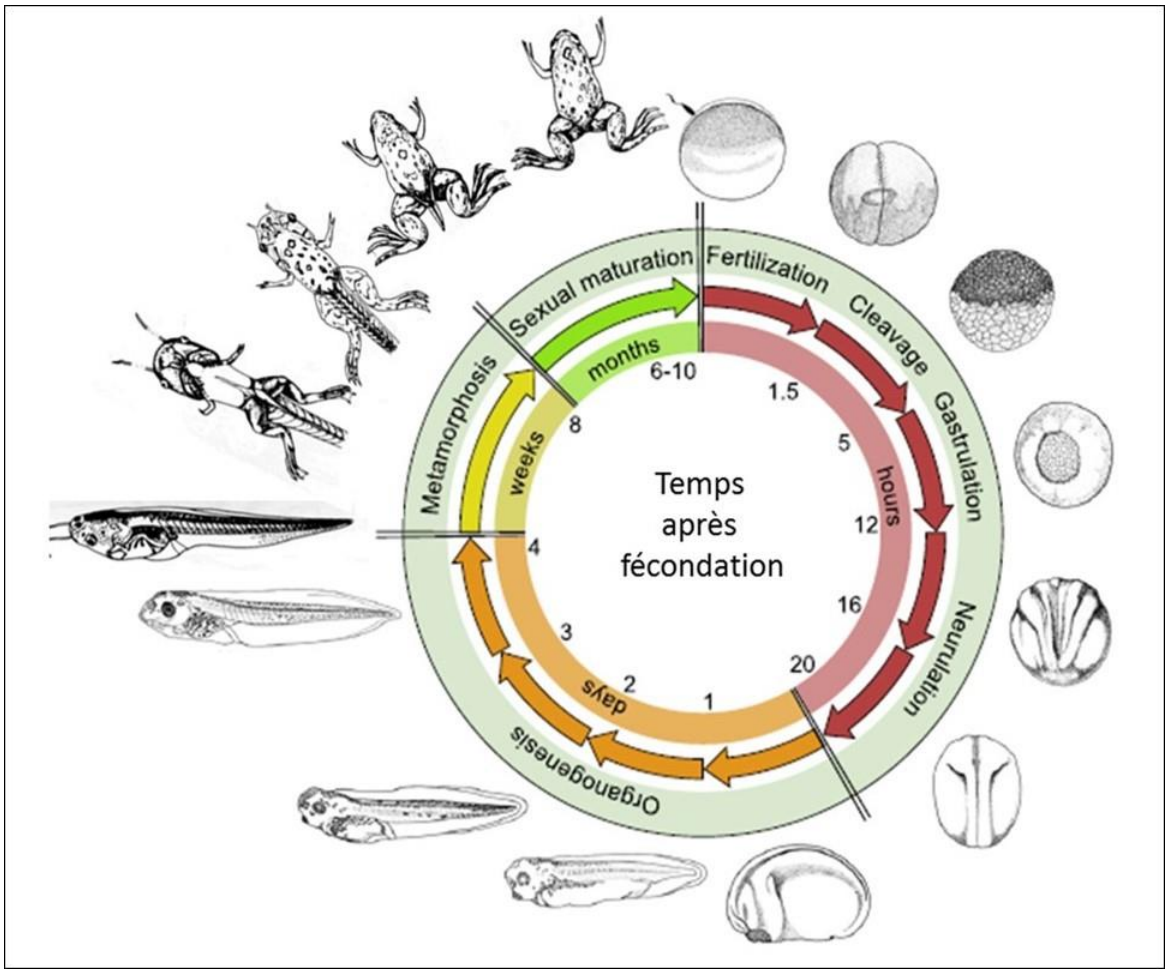

Figure 4. Cycle de vie de *Xenopus laevis* d'après Nieuwkoop and Faber, 1967.

2.2. Choix des contaminants

Nous avons choisi des contaminants selon deux critères principaux. Tout d'abord, ce sont des substances représentatives de leur famille respective : le cadmium, le plomb et le cuivre pour les métaux ainsi que la bouillie bordelaise, le glyphosate (pur et sous formulation commerciale) et la deltaméthrine pour les phytosanitaires. Elles sont également des substances à surveiller dans l'environnement, en raison de leur toxicité, de leurs utilisations et de leur distribution dans l'environnement. Au niveau régional, les Hauts-de-France sont très concernés par les problématiques de contamination des milieux aquatiques du fait de leur

population dense et des activités industrielles et agricoles importantes présentes ou passées. Le tableau 1 reprend les différents contaminants et concentrations utilisés dans cette étude.

Tableau 1. Concentrations des différents contaminants étudiés.

Contaminants		Concentrations				
$CdCl_2$	$mg.L^{-1}$	0,25	2,5	25		
	$mg[Cd^{2+}].L^{-1}$	0,153	1,53	15,3		
	$\mu M[Cd^{2+}]$	1,36	13,6	136		
$PbCl_2$	$mg.L^{-1}$	0,25	2,5	25		
	$mg[Pb^{2+}].L^{-1}$	0,186	1,86	18,6		
	$\mu M[Pb^{2+}]$	0,9	9	90		
$CuSO_4$	$\mu g.L^{-1}$	1,6	16	160	1600	
	$\mu g[Cu^{2+}].L^{-1}$	0.637	6,37	63,7	637	
	$\mu M[Cu^{2+}]$	0,00399	0,0399	0,399	3,99	
Bouillie Bordelaise	$\mu g.L^{-1}$	8	80	800	8000	
	$\mu M[Cu^{2+}]$	0,00399	0,0399	0,399	3,99	
Glyphosate	$mg.L^{-1}$	0,025	0,25	2,5	25	250
	µM	0,148	1,48	14,8	148	1480
RoundUp® GT Max	$mg[a.e.].L^{-1}$	0,025	0,25	2,5	25	250
	µM[a.e.]	0,148	1,48	14,8	148	1480
Deltaméthrine	$\mu g.L^{-1}$	0,015	0,15	1,5	15	
	µM	0,0003	0,003	0,03	0,3	

a.e. : acide équivalent

2.2.1. Métaux

2.2.1.a. Cadmium

Le cadmium est référencé dans plusieurs listes d'éléments chimiques à surveiller prioritairement dans l'environnement, établies par des organisations gouvernementales (US Environmental Protection Agency (USEPA) 2006, 2007 ; Agency for Toxic Substances and Disease Registry (ATSDR) 2017 ; Canadian Environmental Protection Agency (CEPA) 1999) et institutionnelles (Commission Européenne (CE) 2001, 2007) (Agency for Toxic Substances and Disease Registry, 2017; Grillitsch and Schiesari, 2010). Les sources de contamination peuvent être l'épandage d'engrais phosphatés mais aussi son utilisation dans des alliages, des revêtements, des accumulateurs ou en tant que pigment jaune (Bisson et al., 2011). C'est aussi un élément présent dans certains minerais (surtout de zinc mais de plomb et de cuivre

également). Dans ce travail, les expositions au cadmium ont été conduites en utilisant des solutions réalisées à partir de chlorure de cadmium. La détermination des concentrations s'est basée sur des données toxicologiques (EC50 = 1,6 $mg.L^{-1}$) obtenues par le test de toxicité standardisé FETAX (Frog Embryo Teratogenesis Assay – Xenopus) et reportées dans la littérature (Güngördü et al., 2010). Il est intéressant de noter que les concentrations testées, bien qu'élevées, ne sont pas complètement dénuées de pertinence environnementale. Ainsi, même si, par exemple, la concentration en cadmium a été mesurée à 191,1 $ng.L^{-1}$ dans le canal de la Deûle (France) (Lesven et al., 2008), il existe des zones extrêmement polluées par ce métal. Il a été dosé à 0,5 $mg.L^{-1}$ dans le canal de Suez (Egypte) (Sharaf and Shehata, 2015) et à, en moyenne, 67 $mg.L^{-1}$ dans la rivière Simiyu (Tanzanie) (Nyangababo et al., 2005).

2.2.1.b. Plomb

Tout comme le cadmium, le plomb est référencé parmi les éléments issus de différentes listes d'éléments chimiques à surveiller prioritairement dans l'environnement (USEPA 2006, 2007 ; ATSDR 2017 ; CEPA 1999 ; CE 2001, 2007) (Agency for Toxic Substances and Disease Registry, 2017; Grillitsch and Schiesari, 2010). Nous retrouvons également ce métal dans des accumulateurs ou en tant que revêtement mais aussi dans la plomberie, dans des peintures, en tant que pigment blanc ou qu'antidétonant (Amara et al., 2016). Concernant la forme du composé et les concentrations utilisées, le chlorure de plomb ($PbCl_2$) a été choisi et les concentrations ont été basées sur l'EC50 cadmium obtenue par le test FETAX (Güngördü et al., 2010). Dans l'environnement, les concentrations en plomb varient énormément, avec par exemple 5,17 $\mu g.L^{-1}$ dans le canal de la Deûle (France) (Lesven et al., 2008), 0,92 $mg.L^{-1}$ dans le canal de Suez (Egypte) (Sharaf and Shehata, 2015) ou encore 29 $mg.L^{-1}$ en moyenne, dans la rivière Simiyu (Tanzanie) (Nyangababo et al., 2005).

2.2.1.c. Cuivre

Contrairement au cadmium et au plomb, le cuivre, en concentrations optimales, est un métal essentiel à la vie de nombreux organismes (Festa and Thiele, 2011). Néanmoins, il s'agit également d'un contaminant comme le prouve sa présence sur des listes de substances à surveiller prioritairement dans l'environnement (USEPA 2006, 2007 ; ATSDR 2017) (Agency for Toxic Substances and Disease Registry, 2017; Grillitsch and Schiesari, 2010). Ce métal peut être utilisé dans des conducteurs électriques, en tant que pigment vert ou dans des substances fongicides, bactéricides et algicides (Pichard, 2005). Sa forme sulfatée a été

choisie pour notre travail ($CuSO_4$). Les concentrations ont été basées sur la valeur prédictive sans effet (PNEC) de l'ion cuivre en eau douce qui est de 1,6 $\mu g.L^{-1}$ (Pichard, 2005). En France, le cuivre a pu être détecté à 2,88 $\mu g.L^{-1}$ dans la rivière Riou Mort, à 1,25 $\mu g.L^{-1}$ dans le Lot ou encore à 1,4 $\mu g.L^{-1}$ dans la Garonne (Audry et al., 2004). Cependant, dans le réservoir Danjiangkou (Chine), la concentration de 13,32 $\mu g.L^{-1}$ a pu être retrouvée (Li et al., 2008).

2.2.2. Phytosanitaires

2.2.2.a. Bouillie bordelaise

La bouillie bordelaise est un pesticide aux propriétés fongicides. Dans ce travail, cette substance est un lien logique avec la famille des métaux, étant donné qu'elle est composée en majorité de $CuSO_4$. Le reste de la composition est constitué de chaux éteinte. Autorisée en agriculture biologique, la bouille bordelaise est principalement utilisée pour le traitement de la vigne (Pichard, 2005). C'est d'ailleurs une des raisons principales des fortes teneurs en cuivre dans les eaux de la Garonne et de ses affluents (*cf. 1.3.1.c.*). Comme le $CuSO_4$, les concentrations ont été basées sur la PNEC en eau douce de l'ion cuivre (Pichard, 2005).

2.2.2.b. Glyphosate et RoundUp® GT Max

A l'origine, le glyphosate ($C_3H_8NO_5P$) était utilisé pour ses fonctions de chélateur de métaux. Ce n'est qu'à partir de 1970 qu'il fut employé comme herbicide et en 1974 qu'il fut commercialisé pour la première fois sous le nom de RoundUp© (Duke and Powles, 2008). Il est non sélectif et a un effet foliaire. Son effet altère la synthèse d'acides aminés, en bloquant l'activité de l'enzyme 5-enolpyruvylshikimate-3-phosphate synthase de la voie du shikimate (Martinez et al., 2018). Il existe de nombreuses formulations commerciales du glyphosate de différentes compagnies d'agrochimie. Elles varient selon leurs teneurs en additifs, visant à améliorer l'efficacité de la substance active, comme le surfactant POEA (polyoxyéthylène amine) (Martinez et al., 2018). En mars 2015, le Centre International de Recherche sur le Cancer (CIRC) a classé cet herbicide dans le groupe 2A, celui des substances « cancérogènes probables ». Dans l'environnement, le glyphosate est également très préoccupant. En France, pays très gros consommateur de phytosanitaires, c'est la troisième substance pesticide la plus retrouvée dans les eaux de surface. Les deux premières étant des produits de dégradation : l'AMPA (acide aminométhylphosphonique), principal métabolite du glyphosate (Rueppel et al., 1977) et l'atrazine déséthyl, principal métabolite de l'atrazine (composé aujourd'hui interdit en

France) (Ministère de la Transition écologique et solidaire, 2017). Concernant les concentrations présentes dans les eaux de surface, le glyphosate a pu être retrouvé jusqu'à 40,8 $\mu g.L^{-1}$ dans le sud de l'Ontario (Struger et al., 2008), 8,7 $\mu g.L^{-1}$ dans le Midwest aux Etats-Unis (Battaglin et al., 2005) ou 1,082 $\mu g.L^{-1}$ dans la rivière Boële en France (Botta et al., 2009). Néanmoins, il peut être mesuré à des concentrations beaucoup plus importantes, comme dans certaines zones d'agriculture en Argentine, où elles peuvent atteindre 0,7 $mg.L^{-1}$ (Peruzzo et al., 2008). Dans notre travail, les effets du glyphosate seul et de sa formulation commerciale RoundUp® GT Max ont été évalués. La substance commerciale contient 588 $g.L^{-1}$ (43,78 % p/p) de sel de potassium de l'acide glyphosate, équivalent à 480 $g.L^{-1}$ d'acide glyphosate (35,75 % p/p). Les concentrations ont été déterminées selon les normes de qualité environnementale définies dans le contexte de la Directive Cadre sur l'Eau (INERIS, 2014). En eau douce, elles ont pour valeurs 28 $\mu g.L^{-1}$ (moyenne annuelle dans l'eau non destinée à la consommation) et 70 $\mu g.L^{-1}$ (concentration maximale acceptable dans l'eau).

2.2.2.c. Deltaméthrine

La deltaméthrine est un insecticide utilisé pour contrôler les populations d'insectes ravageurs ou vecteurs de maladies, mais aussi dans le cadre domestique et pour traiter les animaux de compagnie (Soderlund, 2012). De formule chimique $C_{22}H_{19}Br_2NO_3$, ce pyréthrinoïde de type II cause une ouverture prolongée des canaux sodium, ce qui dépolarise la membrane bloquant ainsi la propagation de potentiel d'action et induit une inflammation des organes sensoriels (Barlow et al., 2001). En France, son utilisation est très importante dans les départements et territoires d'outre-mer mais aussi dans certaines régions de la métropole comme en Gironde. Les concentrations en deltaméthrine utilisées dans nos expositions ont été basées sur la PNEC qui est de 1 $ng.L^{-1}$ (INERIS, 2018). Il n'existe aucune étude portant sur les teneurs dans l'environnement de ce pesticide.

2.3. Aspects méthodologiques

Les xénopes utilisés dans nos expérimentations proviennent de l'Université de Rennes. Les animaux sont maintenus dans des aquariums standards (XenopLus – Amphibian Housing System from Techniplast) à l'animalerie de l'Université de Lille (Villeneuve-d'Ascq, France) et sont nourris *ad libitum* avec des granulés expansés flottants (Aquatic 3, Special Diets Services). Quotidiennement, les paramètres de la qualité de l'eau sont mesurés avec précision et un renouvellement total de l'eau est effectué. Les expérimentations ont été effectuées selon

les recommandations du Conseil de l'Union Européenne (86/609/EEC) concernant les expérimentations animales et validées par le Comité d'Ethique en Expérimentation Animale des Hauts-de-France (CEEA 07/2010).

2.3.1. Obtention des ovocytes

Afin de récupérer les ovocytes immatures, une femelle est anesthésiée par balnéation dans une solution de tricaine mesylate (1 $g.L^{-1}$) pendant 45 minutes. Par ovariectomie, opération correspondant à une petite ouverture au niveau abdominal, les grappes d'ovocytes sont récupérées à l'aide d'une pince fine. Les morceaux d'ovaire prélevés sont ensuite soumis à un traitement à la collagénase (1 $g.L^{-1}$) pendant 45 minutes afin de faciliter ultérieurement la dissociation des cellules germinales des cellules folliculaires. Les gamètes sont ensuite stockés à 14 °C dans le milieu de culture ND96 (Nathan Dascal 96 : 96 mM NaCl ; 2 mM KCl ; 1,8 mM $CaCl_2$; 1 mM $MgCl_2$; 5 mM HEPES-NaOH ; pH 7,5) et débarrassés du reste des cellules folliculaires à l'aide de pinces fines sous loupe binoculaire, avant les différentes expositions. Les protocoles utilisant l'ovocyte comme matériel biologique, ainsi que les procédures des analyses cytologiques et des western blots sont décrits dans les sections matériels et méthodes des articles du chapitre « Résultats ».

2.3.2. Fécondations in vitro

L'évaluation des effets d'exposition à des contaminants environnementaux sur le succès de fécondation et les premières étapes du développement a été rendue possible par l'élaboration au laboratoire d'un protocole expérimental de fécondation *in vitro*. La veille de l'expérimentation, une femelle est stimulée par injection dans les sacs dorsaux lymphatiques d'hormone chorionique gonadotrope humaine (700 U). Elle est ensuite isolée dans un environnement calme pendant une nuit. Cette stimulation prépare la femelle à la ponte. Son cloaque est alors turgescent. Les œufs sont obtenus en exerçant de légères pressions au niveau des ovaires de l'animal. Ils sont matures et entourés d'une gangue mucilagineuse. Cette enveloppe a un rôle essentiel qui permet au spermatozoïde de féconder l'œuf et qui possède des propriétés protectrices (Olson and Chandler, 1999). En effet, elle est résistante et adhésive, rendant possible la fixation de la ponte à un substrat. Pour réaliser la fécondation *in vitro*, les œufs sont mis en contact avec un morceau de testicule prélevé après sacrifice d'un mâle. Cette étape se réalise en milieu contaminé pendant 10 minutes. Il est également possible d'exposer uniquement un seul des deux gamètes. Pour cela, il est nécessaire de

réaliser des pré-expositions des cellules germinales aux contaminants pendant un temps que nous avons défini à 7 minutes. Après élimination de la solution contaminée par rinçages, la fécondation est ensuite conduite dans le milieu témoin an associant le gamète exposé avec celui de l'autre sexe non exposé. La suite du protocole est la même que pour les expositions simultanées des gamètes. Les 10 minutes suivantes sont dédiées à un traitement à de la L-cystéine (2 %) dans les solutions contaminées, permettant une légère digestion de la gangue et facilitant ainsi les manipulations des embryons. Les milieux sont ensuite rincés trois fois afin d'éliminer la cystéine. A 35 minutes post fécondation, les taux de fécondation sont déterminés par analyse photographique grâce aux rotations caractéristiques des œufs fécondés. Ce moment a été choisi afin de permettre les différentes manipulations et pour que la ponte reste au repos quelques minutes, sans atteindre le premier clivage de la segmentation. La suite du développement est suivie et se déroule en milieu contaminé.

2.3.3. Analyse automatisée de la biométrie des têtards (Article 2)

Le but de ce travail spécifique est de proposer une nouvelle méthode pour quantifier les impacts de contaminants sur le développement de *X. laevis*. Elle est basée sur une technique de coloration au bleu alcian, associée à une analyse biométrique automatisée grâce à un programme développé spécialement pour nos travaux, en collaboration avec TisBio (UGSF UMR 8576 CNRS). Ce protocole a fait l'objet d'une publication intitulée : « *Biometric data assessment on Xenopus laevis tadpoles* »[2].

Après 6 jours, les têtards issus des fécondations *in vitro* décrites précédemment sont fixés (alcool 100 °). Ce moment permet un développement indépendant d'un apport alimentaire extérieur et limite la variabilité expérimentale. A ce stade, les formations cartilagineuses sont développées (Nieuwkoop and Faber, 1967) et une coloration au bleu alcian permet de les mettre en évidence. Elle est classiquement utilisée chez les embryons d'amphibiens et d'autres vertébrés (Klymkowsky and Hanken, 1991). Nous avons réalisé des adaptations de protocole afin d'optimiser l'intensité de coloration pour la suite de l'analyse. Après l'acquisition d'images des têtards sur les plans latéral, ventral et dorsal, l'utilisation d'un plugin conçu spécialement pour ce travail, sur le logiciel ImageJ, permet une amélioration de la qualité, la segmentation et l'obtention de mesures biométriques de manière automatisée.

[2] *Titre : Biometric data assessment on Xenopus laevis tadpoles*
Journal : Journal of Xenobiotics
Publié en 12/2018 (DOI : 10.4081/xeno.2016.6587)
Contribution : Mise en place de la technique, rédaction, relecture et correction

La mise en évidence des formations cartilagineuses par le bleu alcian et la haute qualité des images rendent possible la détermination de malformations anatomiques, concernant par exemple la mise en place des arcs branchiaux ou de la mâchoire mais aussi d'autres organes non colorés. Comme le montre la figure 5, la différenciation du cartilage du reste de l'individu est possible. Ensuite, elle fournit des données quantitatives automatiquement, tels que la longueur (peu importe la courbure de l'individu), la largeur, le périmètre, l'écart oculaire. Il est également possible de normaliser les données obtenues avec ceux d'individus témoins et/ou en fonction de la longueur du têtard. Des résultats préliminaires ont montré l'efficacité de cette technique pour discriminer des anomalies de croissance.

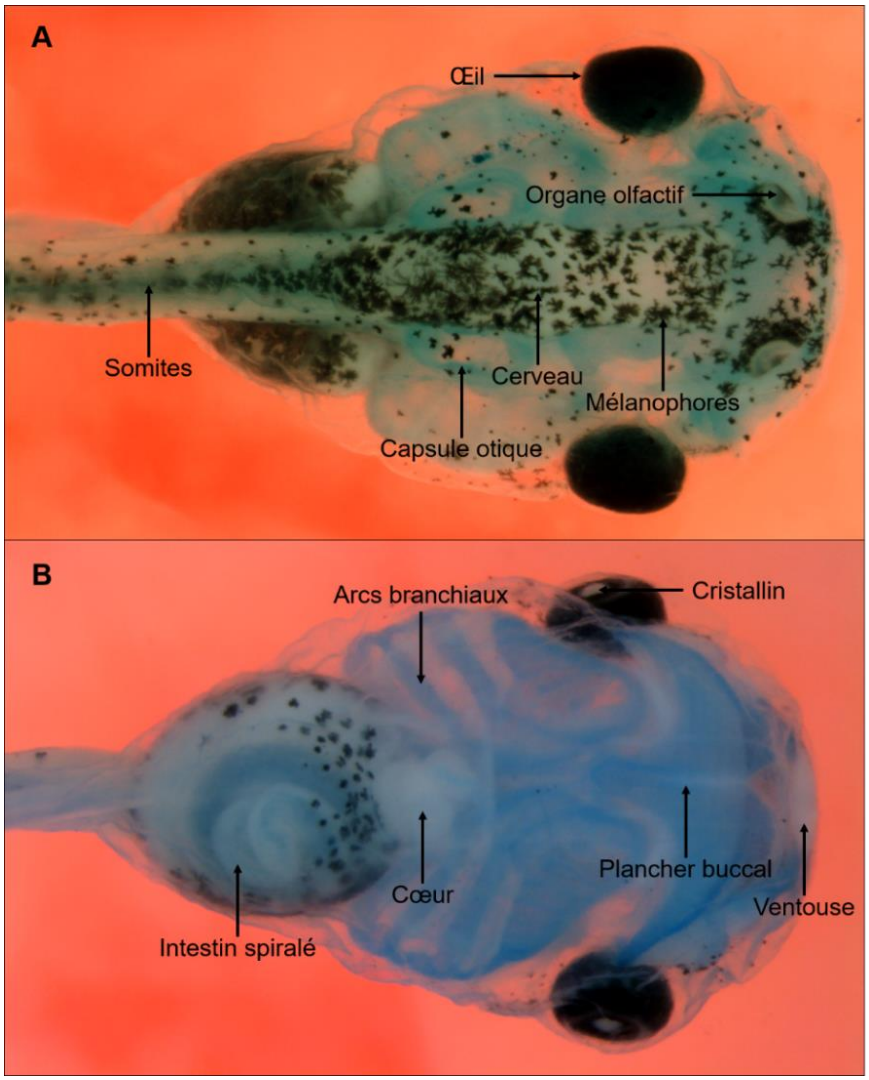

Figure 5. Photographies faces dorsale (A) et ventrale (B) d'un têtard de *Xenopus laevis* de 6 jours développé dans le milieu témoin et coloré au bleu alcian.

Ce travail propose une alternative aux méthodes d'évaluation traditionnelles des impacts morphologiques d'expositions de têtards de xénope à des contaminants. Elle est automatisée, reproductible et rapide. De plus, la mesure de multiples paramètres par individu

répond aux principes des trois R en réduisant le nombre de têtards nécessaires pour les mesures (Russell and Burch, 1959). Il apparait que l'évaluation de la biométrie par cette méthode pourrait être un outil performant en biosurveillance environnementale.

Biometric data assessment on *Xenopus laevis* tadpoles

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Introduction

Recent studies stated that 41% of known species of amphibians are threatened.[1] Indeed, their dramatic population decline is due to environmental conditions, including pollution.[2,3] Their permeable skin and their close relationship with aquatic compartment, make them particularly sensitive to contamination and stand as strong models in biomonitoring studies.[4] Consequently, the use of biomarkers in batrachians arose in the 80's[5]: from morphological and anatomical approaches (deformities, gonadic abnormalities) to biochemical measurements (vitellogenin, methallothionein, CYP450).[4] For example, embryotoxic and teratogenic damages on larvae were detected with the FETAX (Frog Embryo Teratogenesis Assay - *Xenopus*) test developed in *Xenopus laevis*.[6-8] In this regard, this specie appears as a very effective laboratory model for assays to dissect actions of chemical contamination. Indeed, profusion of data is available in cell cycle, embryology and development fields.[9-11] Moreover, our recent studies showed some different sub-individual modifications, especially a decrease of oocytes' capacity to perform the maturation and the fertilization in presence of cadmium.[12,13]

Here, we propose new methods to quantify the impacts of chemical contaminants on *Xenopus laevis* young tadpoles by recording biometric criteria.

Materials and Methods

All embryos were obtained by *in vitro* fertilization as described in our previous work.[13] They were maintained in healthy or contaminated media, until 6 days (stage 42)[11] and fixated in ethanol 100° at –20°C.

Then, tadpoles were stained in Alcian blue solution (0.1 mg.L^{-1} in acetic acid:ethanol 1:4) for 2 to 4 days according to the colour intensity wished. The background was removed by using a washing solution (chloridric acid:ethanol 70° 1:99) for 1 to 2 days and tadpoles were progressively dehydrated in ethanol baths (70°, 95° and 100°) for 2 h each. Before the analysis process, biological materials were rehydrated in: water:ethanol 1:4, water:ethanol 1:1, MEM:ethanol 4:1 and MEM (MEM: 0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, pH7.4). All steps were performed at room temperature. The tadpoles can be maintained in MEM for up to 3 days at 4°C. All animal experiments were performed at the animal facility of Lille 1 University according to the rules of the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The animal protocol was approved by the Local Institutional Review Board (*Comité d'Ethique en Experimentation Animale Nord-Pas- de-Calais*, CEEA 07/2010).

Image enhancement, segmentation and morphological analysis were fulfilled using homemade plugin developed for ImageJ,[14] as depicted in Figure 1. While image enhancement produces binary information such as spine integrity or brain appropriately developed regarding development stage, segmentation provides quantitative measurement of tadpole morphology. In this study, we focused on the tadpole's length (calculated based on the Feret's diameter, corresponding to the longest distance between two points of the selection boundary), perimeter, distance between eyes and position of the suction cup. Parameters can also be combined, normalizing measurements on animal and/or reference size to assess relative morphological differences.

Results and Discussion

Our new method provides numerous qualitative and quantitative data could be obtained. As depicted in Figure 2, a control tadpole (NT) was compared to treated ones (A, B and C). Morphological observations revealed abnormalities in terms of growth (tadpole A), dorsal curvature (tadpole B), or dorsal break and various edema (tadpole C).

While traditional approaches focus only on growth, mortality and qualitative data,[7] our method relies on extensive images enhancement automation and complete this qualitative approach with new biometric criteria, and thus for each suited tadpole. Figure 2D shows some of these measurements: length, perimeter, distance between the eyes (*eyes*) and the position of the suction cup.

To go even farther in the approach, in a second step of experiment, different tadpoles were compared. First normalization (grey bars, Figure 2D) was done in comparison with a control organism (yellow dotted line, Figure 2D). Then, in order to highlight subtle alterations, a second normalization was achieved: each measurement was expressed as a function of studied tadpole length (colored bars). Thus, for treated tadpole A, it appeared that even if the individual was smaller (length, grey bar) the other measured parameters were over-size compared to untreated tadpole (colored bars).

Conclusions

The new method we propose presents several advantages compared to the traditional ones. Indeed, automated segmentation and detection allow automated quantification of tadpole's morphology and anatomy. In this paper, we focused on several parameters such as perimeter or distance between eyes, but numerous others can be obtained from segmented images (*i.e.*, branchial arches or brain size and morphology, *etc.*). Thus, from the high quality of each individual description, it answers to the ethical principles of the 3R's, first stated by Russell and Burch in 1959,[15] by decreasing the number of tadpoles needed to

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Key words: *Xenopus laevis*; tadpole; biosensor; biomarker; image analysis; biometry.

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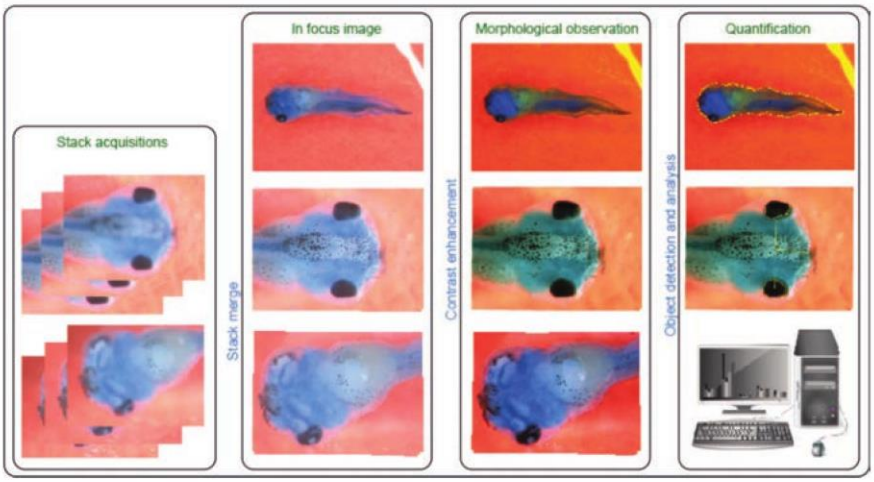

Figure 1. Logical scheme of image improvement and analysis for biometric criteria quantification. Morphological observation results in binary information such as spinal cord or organs integrity, while objects detection and segmentation provides quantitative biometrical description of each tadpole.

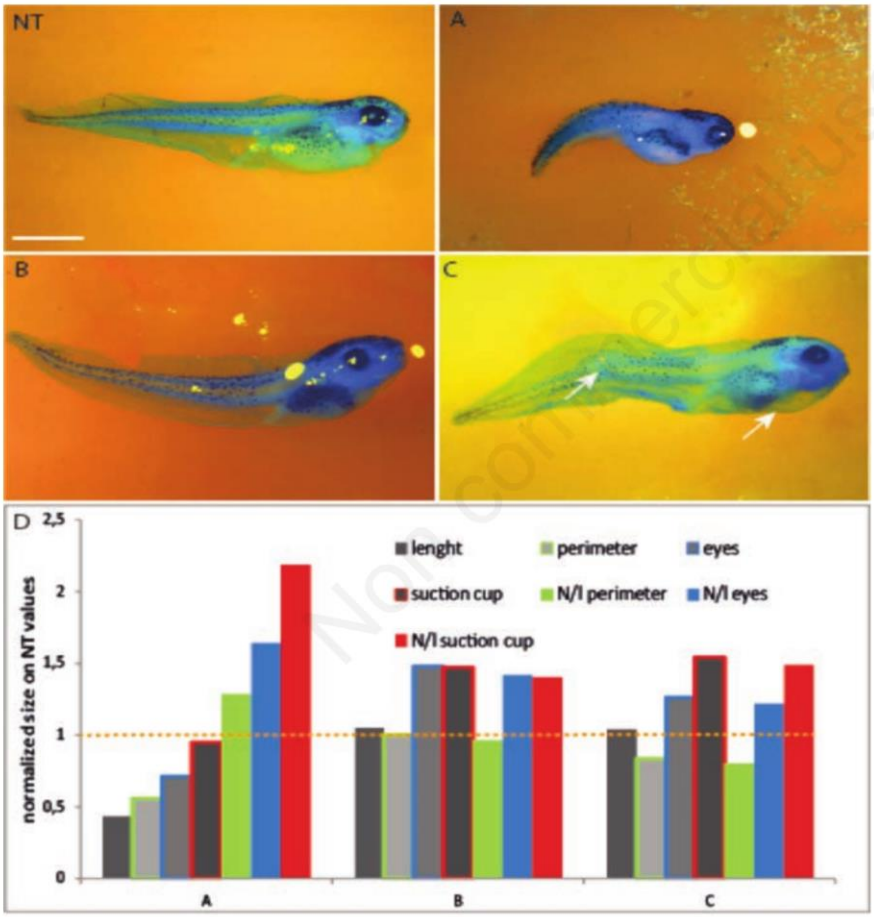

Figure 2. Biometric analysis of 4 different tadpoles. NT corresponds to non-treated animal while A-C are tadpoles grown in contaminated environment. White arrows illustrate some of the morphological defects enlightened by the image improvement step. D corresponds to biometrical quantification from A-C normalized on measurements performed on NT, with: i) length and perimeter; ii) eyes, corresponding to the distance between the both animal's eyes centroids in ventral position; iii) suction cup corresponding to the distance between the isobarycenter of both eyes and the suction cup. N/l stands for the parameters also normalized on the length of the concerned animal. Doted orange line corresponds to NT. Scale bar 1 mm.

achieve precise estimation of xenobiotic impact. Then, each contaminant results in a specific biometric signature, an even more efficient biomarker. Thus, this biometric data assessment appears as an effective tool for environmental biomonitoring.

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Chapitre III - Résultats

3.1. Expositions au cadmium et au plomb

3.1.1. Xenopus laevis oocyte maturation is affected by metal chlorides (Article 3)

Cette première étude, nommée « *Xenopus laevis oocyte maturation is affected by metal chlorides* »[3], avait pour but de montrer que l'ovocyte de xénope pouvait constituer un modèle cellulaire utilisable dans des études de toxicologie de l'environnement. En effet, peu d'études ont été conduites avec cette cellule germinale. Pourtant, elle présente de nombreux avantages, notamment la possibilité de combiner des enregistrements électrophysiologiques et des approches phénotypiques.

Ce travail s'intéressait donc aux effets d'exposition d'ovocytes à des chlorures de métaux (cadmium, zinc, plomb et cobalt) en utilisant des approches de biologie cellulaire. (1) Dans un premier temps, la survie a été évaluée en suivant visuellement l'intégrité des ovocytes pendant 72 heures et en mesurant leur potentiel de repos sur ce même temps en utilisant la technique du double électrode. (2) Dans une seconde série d'expériences, le processus de préparation à la ponte et à la fécondation – la maturation ovocytaire – a été étudié en suivant l'apparition de la tache de maturation au sommet du pôle animal et (3) en enregistrant des courants chlorures dépendants du calcium, associés à la maturation.

(1) Les résultats obtenus montrent que les chlorures de métaux n'affectent pas la morphologie cellulaire mais dépolarisent fortement le potentiel de repos des ovocytes.

(2) Les expositions au chlorure de cadmium sont capables d'inhiber la maturation ovocytaire. A l'opposé, les mêmes expositions, réalisées sans stimulation hormonale, ont montré que le zinc mais aussi dans une moindre mesure le cadmium et le cobalt, sont capables d'induire le phénomène de maturation ovocytaire.

(3) Les enregistrements électrophysiologiques de courants chlorures dépendants du calcium et connus pour être associés à la maturation ovocytaire ont été largement perturbés par les expositions.

L'ensemble de ce travail préliminaire montre alors la sensibilité importante de l'ovocyte de *X. laevis* aux expositions métalliques et particulièrement au chlorure de cadmium. Les effets observés, sans aucun doute, pourront enrayer la fécondation. Par ailleurs, il apparaît

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Contribution : Conduite des expositions au cadmium et au plomb, relecture et correction

également important de varier les approches pour définir des signatures spécifiques aux contaminants. Enfin, ce travail montre que l'ovocyte de xénope pourrait être utilisé comme modèle cellulaire dans des études de toxicologie environnementale.

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Xenopus laevis oocyte maturation is affected by metal chlorides

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ABSTRACT

Few studies have been conducted using *Xenopus laevis* germ cells as oocytes, though these cells offer many advantages allowing both electrophysiological studies and morphological examination.

Our aim was to investigate the effects of metal (cadmium, lead, cobalt and zinc) exposures using cell biology approaches. First, cell survival was evaluated with both phenotypical and electrophysiological approaches. Secondly, the effect of metals on oocyte maturation was assessed with morphological observations and electrophysiological recordings.

From survival experiments, our results showed that metal chlorides did not affect cell morphology but strongly depolarized *X. laevis* oocyte resting potential. In addition, cadmium chloride was able to inhibit progesterone-induced oocyte maturation. By contrast, zinc, but also to a lesser extent cadmium, cobalt and lead, were able to enhance spontaneous oocyte maturation in the absence of progesterone stimulation. Finally, electrophysiological recordings revealed that some metal chlorides (lead, cadmium) exposures could disturb calcium signaling in *X. laevis* oocyte by modifying calcium-activated chloride currents. Our results demonstrated the high sensitivity of *X. laevis* oocytes toward exogenous metals such as lead and cadmium. In addition, the cellular events recorded might have a predictive value of effects occurring later on the ability of oocytes to be fertilized. Together, these results suggest a potential use of this cellular lab model as a tool for ecotoxicological assessment of contaminated fresh waters.

1. Introduction

Among animal models used in biological research areas, amphibians play a prominent part. They have been regarded for decades as key species allowing to gather new insights and to unravel original concepts, which were extended to other animal models. One cannot doubt that *Xenopus*, or other amphibian species, have been since the mid-fifties models for developmental biology. Amphibians were first privileged by pioneer embryologists such as Hans Spemann, Nobel prized in 1935 for its work on the organizing effect of a cellular group during embryogenesis. Moreover, Nobel Prized Sir John Gurdon performed the first nuclear transfer with success in 1958 (Gurdon et al., 1958), opening a road driving to Dolly's birth after nuclear transplantation. At the same time, in the end of the sixties, beginning of the seventies, the existence of a cytoplasmic activity responsible for the onset of M-phase was discovered in amphibian (Brachet et al., 1975; Masui and Markert, 1971), laterly explored in other model, such as starfish (Kishimoto et al., 1984). Namely M-Phase Promoting Factor (MPF, characterized in 1988 in *Xenopus* oocytes (Lohka et al., 1988), was rapidly found be a universal key factor for division, not restricted to amphibian.

As prototypic lab animals, amphibians exhibit advantages that include a well-characterized physiology, tolerance to temperature and oxygen variations, and a greater resemblance to humans than many other animal models (Brown, 2004; Ferrell, 1999; Schultz and Dawson, 2003). Such assertion is especially relevant in physiological research and in evolutionary biology, but also in environmental studies (Burggren and Warburton, 2007). Thus, observations concerning growth, development, reproduction and physiology might be gathered easily and could be applied in aquatic ecotoxicology. In this respect, and by being aquatic throughout all

Abbreviations: FETAX, Frog Embryo Teratogenesis Assay Xenopus; GVBD, germinal vesicle breakdown; ICl, calcium-activated chloride currents; ICl1-S, calcium-activated chloride current enhanced by calcium release from internal stores; ICl1-T, calcium-activated chloride current enhanced by calcium release and calcium entry; ICl2, calcium-activated chloride current enhanced by calcium entry from external medium; MPF, M-phase Promoting Factor; MS222, tricaine methane sulfonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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its embryonic and post-embryonic life, *Xenopus laevis* is one of the most studied amphibians. This species is strictly dependent upon aquatic media (Hillman, 1980), and its embryos and larvae are directly exposed to freshwater environmental micropollutants. *X. laevis* is considered as a valuable sentinel species to evaluate environmental health (Collins and Storfer, 2003). Surprisingly, few studies were undertaken on this amphibian, though *Xenopus* tadpoles and adults are strictly aquatic living animals. Thus, adults' tissues including ovaries and testis, eggs and embryos could be indirectly or directly exposed to environmental pollutants. Most studies with *X. laevis* have been developed for many years with embryos to assess early developmental toxicity of environmental pollutants (Frog Embryo Teratogenesis Assay—*Xenopus*, FETAX) (ATSM, 1998).

X. laevis oocyte has been used for decades as a model for studying calcium signaling (Delisle, 1991; DeLisle and Welsh, 1992; Marin, 2012; Marin et al., 2010), cell cycle transitions (Bodart et al., 2002; Heikkila et al., 2007; Machaca, 2007). However, this model has been seldom used in toxicological studies in spite of the advantages provided by different species of *Xenopus* genus (Khokha, 2012; Schultz and Dawson, 2003). Forty years ago, Dumont described *X. laevis* oogenesis (Dumont, 1972). According to his classification, six stages (I to VI) may be distinguished, which are related to vitellogenesis. Fully-grown Stage VI oocytes have a diameter ranging from 1 to 1.3 mm. Referred as immatures, these oocytes are blocked at prophase of meiosis I, in a G2-like state. The release from the block at prophase I can be experimentally triggered by progesterone, mimicking the steroid stimulation provided by follicular cells *in vivo*. Then, the immature oocytes resume meiosis and a typical white spot appears at the top of the animal pole. This white spot is relative to the germinal vesicle breakdown (GVBD) and the migration of nuclear material to the apex of the cell. Oocytes exhibiting a white spot are often referred as mature oocytes. These oocytes progress from meiosis I until metaphase of meiosis II. Thus, mature oocytes are arrested in a second block, at metaphase of meiosis II, waiting for fertilization (Nebreda and Ferby, 2000). It is to note that meiotic resumption is analogous to the transition from G2-like state to a M-phase, and is therefore considered as an M-phase entry. At the molecular level, the meiotic resumption is enabled by the activation of a universal factor, the MPF (M-Phase Promoting Factor), which is activated by Cdc25, a pivotal enzyme in the regulation of M-phase progression (Nebreda and Ferby, 2000).

This hormonal-dependent maturation corresponds to morphologic events (appearance of a typical white spot at the top of the animal pole, relative to the GVBD and migration of nuclear material to the apex of the cell), and biochemical events (initiation of signaling pathways leading to the activation of the MPF). During this maturation (corresponding to G2/M transition), the oocyte also undergoes ionic changes, including calcium fluxes: calcium release from internal stores of endoplasmic reticulum and/or calcium entry from the outside of the cell. With electrophysiological approach, calcium sources/fluxes could be followed, in particular by recording calcium-activated chloride currents (also called ICl currents) which develop in order to block polyspermy during fertilization.

Many characteristics specific to the *X. laevis* oocyte make it an excellent experimental system, especially of interest for aquatic ecotoxicology. The large size of oocytes provides ease of amenability for manipulations varying from electrophysiology to micro-injection, large amounts of proteins facilitating biochemical studies, a system for heterologous expression and a model where cell cycle regulation mechanisms are evolutionary conserved. Easy to dissect manually, oocyte can be used to carry out various assays within a single cell (Cailliau and Browaeys-Poly, 2009; Weber, 1999). Noteworthy, fertilization can be easily reproduced *in vitro*, and external development offers opportunity to test the direct effects of various compounds.

In this first work, we chose metals as representative of freshwater contaminants. Indeed, it is well documented that in the Northern Hemisphere all the water bodies are contaminated with metals such as mercury, cadmium and lead due to long-range atmospheric transport and deposition from anthropic sources (Naimo, 1995). Contaminated environments (water bodies, ditches, ponds) are used by amphibians. Besides, studies on non-point sources revealed that urban storm water and highway runoffs are a major source of pollutants. Metals were found in this order: zinc > lead ≈ copper > cadmium (Davis et al., 2001).

Here, we intended to use *X. laevis* oocytes to evaluate the effect of metal chlorides on cells survival and on their ability to undergo into M-phase. In one hand, healthy oocytes were evaluated using both a phenotypic approach and electrophysiological recordings; in the other hand, M-phase entry was assessed by phenotypical examination of G2/M transition and by measuring associated calcium-dependent chloride channels.

2. Materials and methods

2.1. Reagents and test substances

All compounds were of molecular biology grade of purity. They were obtained from Sigma–Aldrich Chimie (Saint-Quentin Fallavier, France). All tested solutions and media (ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, pH 7.5) were freshly daily prepared or obtained extempore by appropriate dilutions of metal chloride stock solutions in ND96 medium. Metal salts were of 99.9% grade of purity (Sigma–Aldrich Chimie, Saint-Quentin Fallavier, France).

2.2. Handling of oocytes

The use of living organisms was conducted in accordance to the protocols submitted and approved by the French national guidelines for animal welfare. *X. laevis* females were anesthetized by immersion in 1 g L^{-1} tricaine methane sulfonate (MS222) solution for 45 min. An ovariectomy was performed: clusters of oocytes were surgically removed and placed in ND96 medium. Oocytes were isolated first, by a collagenase treatment (1 mg mL^{-1} of collagenase in ND96) for 45 min then, by a manual dissociation with tweezers under a binocular microscope. Only the stages V and VI oocytes (suitable for maturation, see Fig. 1) were defolliculated and used for all the experiments.

2.3. Metal chloride exposures for meiosis resumption

Oocytes were exposed overnight at 19 °C to metal chloride solutions: cadmium, zinc, lead, or cobalt (Table 1). Maturation was tested in presence of progesterone (4 μg mL^{-1}). The M-phase entry was associated with the appearance of the white spot. In standard conditions, *Xenopus* oocyte maturation occurs in about 12 h. Such experiment allowed obtaining M-phase entry percentage as a function of metal concentrations. Oocytes were placed in metal chloride solutions in 24-wells plates (10 oocytes per test well and 15 for the control one, in triplicates). For each metal, the following concentrations were tested (metal chloride concentrations): 25 mg L^{-1}, 12.5 mg L^{-1}, 2.5 mg L^{-1}, 1 mg L^{-1}, 250 μg L^{-1} and 25 μg L^{-1}. In order to choose the concentrations to be tested, we looked for the EC50 (embryo-toxicity) of metal ions in the FETAX test. The most toxic metal ion (with the lowest reported EC50) was cadmium (1.6 mg L^{-1}) followed by zinc (55.6 mg L^{-1}), lead (96.1 mg L^{-1}) and cobalt (613 mg L^{-1}) (Gungordu et al., 2012;

A

Stage	I	II	III	IV	V	VI
Diameter (µM)	50-300	300-450	450-600	600-1000	1000-1200	1200-1300
outward show	translucent	white	brownish	Individualization of 2 poles	Animal and vegetal poles clearly defined	Equatorial area without pigment
Pg maturation	-	-	-	-	+	+

B

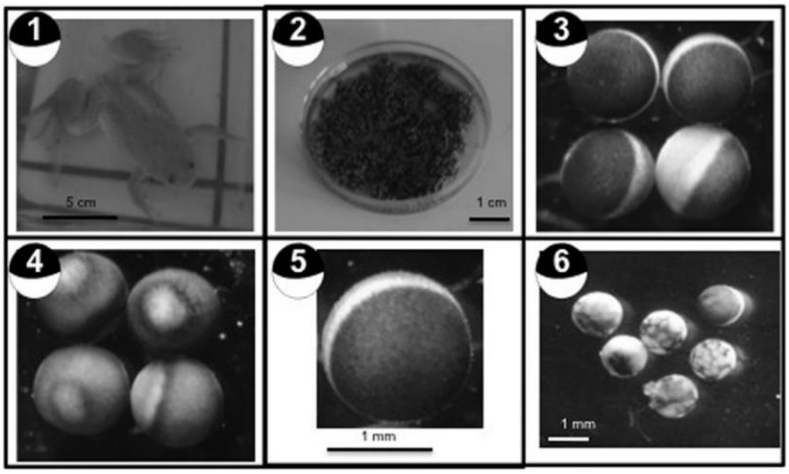

Fig. 1. Stages of *Xenopus laevis* oocyte. (A) typical feature of oocyte in terms of stage, diameter, look and the ability (+) or (−) to undergo maturation with progesterone (Pg). (B) Images showing *Xenopus laevis* (1), clusters of oocytes (2), immature (stage VI) oocytes (3), mature oocytes with a white spot on the top of the animal pole, after maturation with progesterone (4), intact oocyte (stage VI) (5) and ongoing dead (stage VI) oocytes (6). (Modified from Marin, 2012).

Table 1
Levels of metals used in the study (in mg L^{-1} and µM).

Metal chloride	mg L^{-1}	0.025	0.25	1	2.5	12.5	25
Cd^{2+}	mg L^{-1}	0.015	0.153	0.612	1.53	7.65	15.3
	µM	0.136	1.36	5.4	13.6	65.5	136
Pb^{2+}	mg L^{-1}	0.019	0.186	0.744	1.86	9.3	18.6
	µM	0.090	0.900	3.60	9	45	90
Zn^{2+}	mg L^{-1}	0.012	0.120	0.479	1.20	6	11.98
	µM	0.183	1.83	7.3	18.3	91.5	183
Co^{2+}	mg L^{-1}	0.011	0.113	0.452	1.13	5.65	11.3
	µM	0.192	1.92	7.7	19.2	96	192

Luo et al., 1993; Plowman et al., 1991). The EC50 of cadmium was 1.6 mg L^{-1} that corresponds in terms of metal salt to 2.61 mg L^{-1}. So, we chose as central concentration 2.5 mg L^{-1} of cadmium chloride and framed with two lesser concentrations (0.25 and 0.025 mg L^{-1}) and two higher concentrations (12.5 and 25 mg L^{-1}). In order to compare all metal chlorides we conducted experiments with the same concentrations for each salt tested. The concentration of 1 mg L^{-1} was chosen to compare results with the other experiments: oocyte survival and electrophysiology. Cadmium and lead were tested because of their well-known presence in the environment, zinc and cobalt were also studied as essential metals (Moger, 1983). Only the two non-essential metals were tested in mixture with equal levels to check potential additive or antagonist effects of these two exogenous metals on the biological events studied.

2.4. Oocyte survival

In order to evaluate the survival, 15 oocytes were maintained in Petri culture dishes (35 mm diameter) containing 4 mL of metal chloride solution (1 mg L^{-1}). Each 24 h, survival was assessed both by visual examination (according to the criteria presented in Fig. 1) and by electrophysiological recording of resting potential (24–72 h).

2.5. Electrophysiology

In order to complement the visual examinations, an electrophysiological approach was developed. To this extent, two parameters were measured: membrane potential and calcium-activated chloride currents. Measurements were performed with the double electrode technique. In this set of experiments, a defolliculated oocyte was put in an electrophysiology tank in ND96, and 2 electrodes were planted in the animal pole. The membrane potentials were measured after 24 h, 48 h, and 72 h of exposure to metal chlorides (concentration of 1 mg L^{-1}). Records were performed using Clampex 6.0 software (Invitrogen, Saint-Aubin, France).

Moreover, we used the "triple step" protocol developed by Yao and Tsien (Yao and Tsien, 1997). As depicted in Fig. 2, this stimulation consisted in a first depolarization of the cell from −40 mV to +40 mV, following by a hyperpolarization up to −140 mV and a third depolarizing step to +40 mV. At the end of the first step, ICl1-S was measured; this current is activated by calcium release from internal stores. At the end of the hyperpolarizing step, a current (called ICl2) mirroring calcium entry from the outside was measured. The last step allowed the measure of "a tail current" activated both by calcium release and calcium entry (this current is called ICl1-T).

2.6. Data analysis

All results are shown as mean ± standard error of the mean (SEM); N refers to the number of separate experiments performed

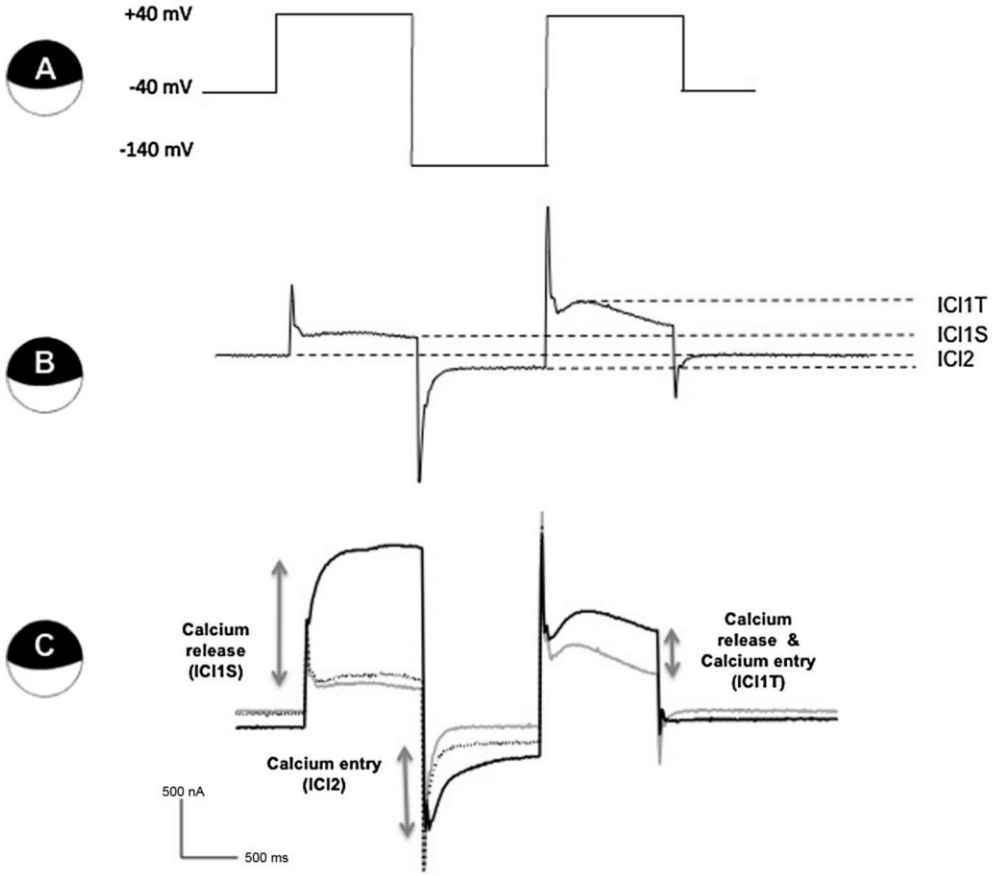

Fig. 2. Effects of intracellular calcium stores depletion on ICl currents. (A) the triple step protocol. Oocytes were held at −40 mV and stepped to + 40 mV for 1 s, to −140 mV for 1 s and back to + 40 mV (modified from (Kuruma and Hartzell, 1999)). (B) Calcium-activated chloride currents recorded after triple step protocol. Three currents were obtained: ICl1-S, measured at the end of the first pulse at + 40 mV, ICl2, measured at the end of the −140 mV pulse and ICl1-T, calculated by measuring the peak transient current during the second pulse at + 40 mV and subtracting ICl1-S. (C) Typical recordings before (black dotted line), during (black line) and after (gray line) calcium store depletion were illustrated here. (Modified from Marin et al., 2010; Marin, 2012).

(number of females) and n corresponds to the number of exposed oocytes. Electrophysiological data were analyzed using Clampfit 10.0 (Axon Instruments, Forster City, CA). For statistical analysis and graphical presentation, Excel, Power Point (Microsoft Corporation) and Origin (Microcal Software, Northampton, MA) softwares were used. The significant differences were assessed with SigmaStat 3.1 software (SysStat, Erkrath, Germany) by means of one-way analyses of variance followed by *post hoc* Tukey's tests. Statistical significance was accepted for $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

In a first step, *X. laevis* oocytes survival was assessed (Fig. 3). Oocytes were exposed to metal stress by incubation in metal chloride solutions (1 mg L^{-1}): zinc, cobalt, cadmium, lead and a mixture of lead and cadmium (1 mg L^{-1} each). For each experiment, 10–15 oocytes were exposed and survival measured after 24, 48 and 72 h of exposure. Firstly, survival was evaluated phenotypically. In that case, an oocyte was considered healthy when pigmentation, membrane and size of the cell were similar to those of freshly defolliculated oocytes. As depicted in Fig. 3A, after 48 h of exposure, cell survival was not affected at all in any of the tested conditions. Consequently, phenotypical examination after the longer exposure of 72 h was omitted.

Secondly, in order to further characterize the effect of metal chloride exposures on oocyte viability, the resting potentials of exposed and control oocytes were measured. Electrophysiological recordings were assessed on same oocytes as those used for phenotypical approach. Results are depicted in Fig. 3B. In good accordance with Weber (1999), resting potential in control oocytes was between −90 and −80 mV after 24 and 72 h of experiments. Regarding oocytes treated with metal chloride solutions, resting potentials were not significantly different from those of controls, fluctuating from about −70 mV (zinc exposure) to −110 mV (lead exposure) after one-day exposure. Nevertheless, after 48 h of treatment and with the exception of zinc and cobalt exposures, resting potential strongly depolarized (up to −20 mV for cadmium). This decline was larger after 72 h of exposure for all tested conditions.

In a second set of experiments, the effects of metal chloride solutions on *X. laevis* oocyte meiotic resumption were assessed (Fig. 4). Oocytes were bathed overnight in the metal chloride solutions (from 25 μg L^{-1} to 25 mg L^{-1} of cadmium, cobalt, zinc, lead and mixture Cd + Pb) with (Fig. 4A) or without (Fig. 4B) progesterone. For *X. laevis* oocytes, meiotic arrest in prophase is released *in vitro* in response to hormonal stimulation by progesterone. Thus, meiosis resumes, from prophase I to metaphase II, and is characterized by morphological modifications, including migration and breakdown of the germinal vesicle at the apex of the animal pole (GVBD) and occurrence of a white spot. In our investigations, white spot appearance was considered as a sign of irreversible entry of the oocytes in M-phase and beginning of the maturation process. As reported in Fig. 4A, only cadmium was able to significantly prevent the meiotic resumption of oocytes stimulated by progesterone, for concentrations above 12.5 mg L^{-1}. Thus, after

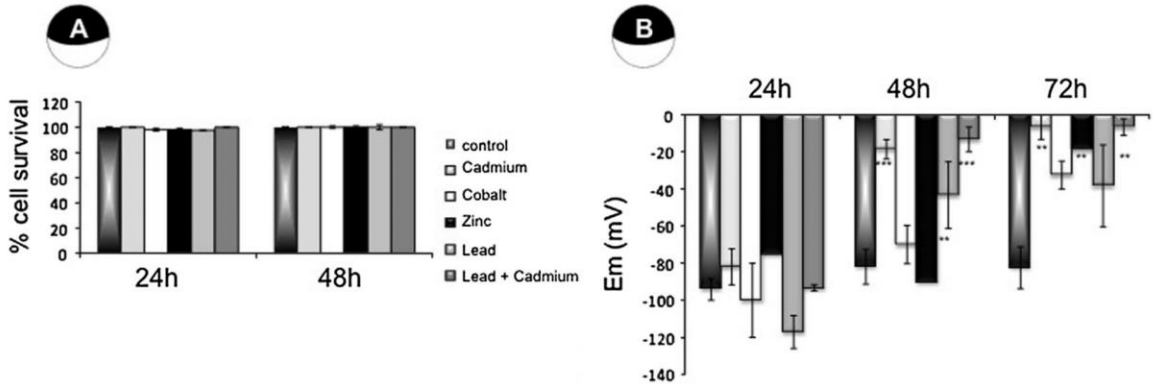

Fig. 3. Effects of metal chlorides on *Xenopus* oocytes survival. (A, B) Stage VI oocytes were exposed to 1 mg L^{-1} $CdCl_2$ (low gray), $CoCl_2$ (white), $ZnCl_2$ (black), $PbCl_2$ (middle gray) or a mixture of 1 mg L^{-1} of $PbCl_2$ and 1 mg L^{-1} $CdCl_2$ (dark gray), and compared to control conditions in ND96 alone (shaded off). Oocytes survival was assessed (A) phenotypically 24 h and 48 h after metal exposures, and (B) electrophysiologically by measuring the resting potential of the oocytes. Results are expressed as mean ± SEM ($N \geqslant 3$ and $n > 45$). $^{***}p < 0.001$; $^{**}p < 0.01$ (one-way anova, *post hoc* Tukey's tests).

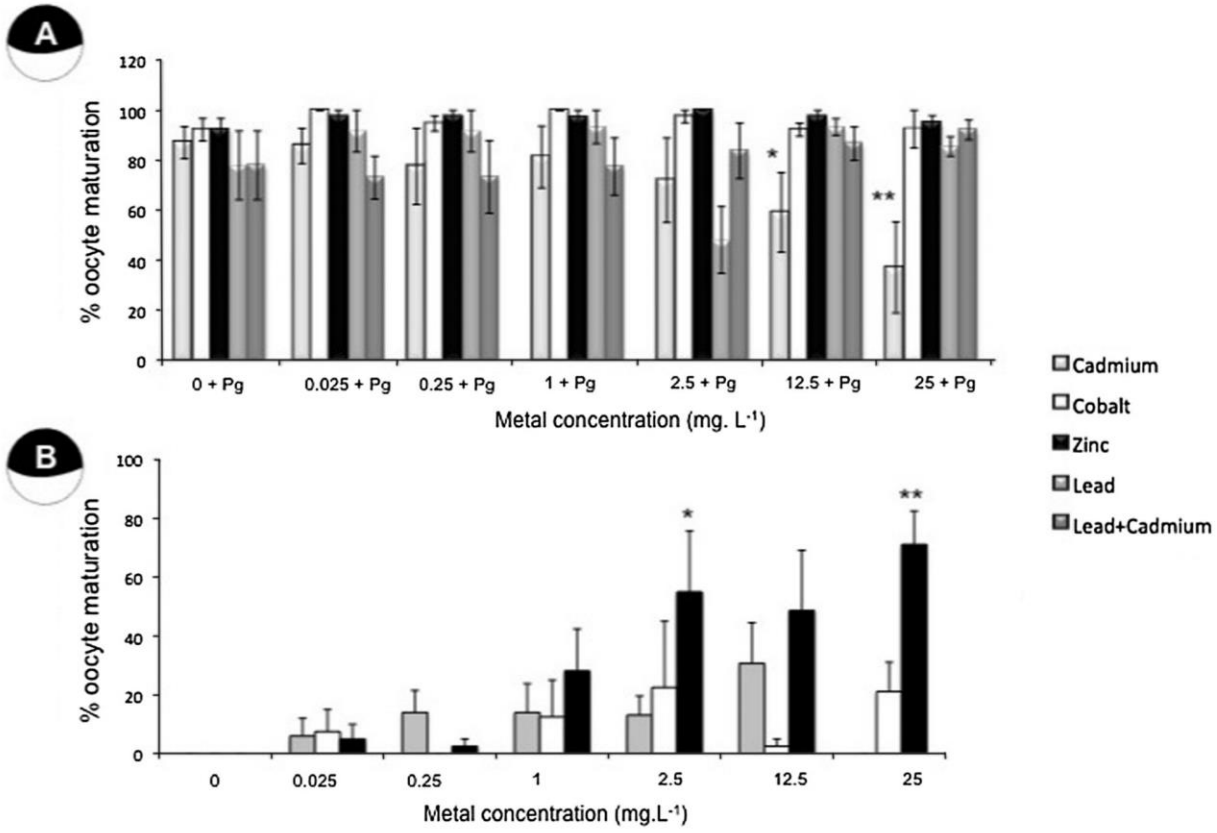

Fig. 4. Effects of metal chlorides on *Xenopus* oocytes maturation. Oocytes were exposed to increasing (25 and 250 μg L^{-1}, 1, 2.5, 12.5 and 25 mg L^{-1}) concentrations of metal chloride solutions (cadmium in low gray, cobalt in white, zinc in black, lead in middle gray and a mixture of lead and cadmium in dark gray), with (A) or without (B) progesterone (4 μg mL^{-1}), overnight. Maturation was assessed with white spot (GVBD) and results expressed as percentage ± SEM ($N \geqslant 3$ and $n > 45$) and compared to control conditions (ND96). $^{*}p < 0.05$; $^{**}p < 0.01$, one-way anova, *post hoc* Tukey's test).

25 mg L^{-1} exposure, less than 40% of oocytes underwent maturation and after 12.5 mg L^{-1} exposures less than 60% of maturation was observed. So, high-dose cadmium could be considered as an inhibitor of oocyte maturation.

In parallel, as presented in Fig. 4B, without progesterone, all tested concentrations of zinc, cobalt and cadmium revealed spontaneous maturations (M-Phase entry), only significant for zinc exposures (2.5, 12.5 and 25 mg L^{-1}). By contrast, in the absence of metal chlorides and progesterone, no maturation was observed.

The morphologic events of oocyte meiotic resumption are related to ionic actors: (i) calcium channels and/or (ii) calcium-activated channels (Marin, 2012; Sun et al., 2008). That is the reason why calcium-activated chloride currents (ICl) were recorded using the triple step protocol (Fig. 5). Such data allow identifying calcium mobilization and modification in *X. laevis* oocytes. Zinc did not increase ICl currents and cobalt only increased ICl1-T current. Typical recordings presented in Fig. 5 showed that cadmium, lead and their mixture strongly increased

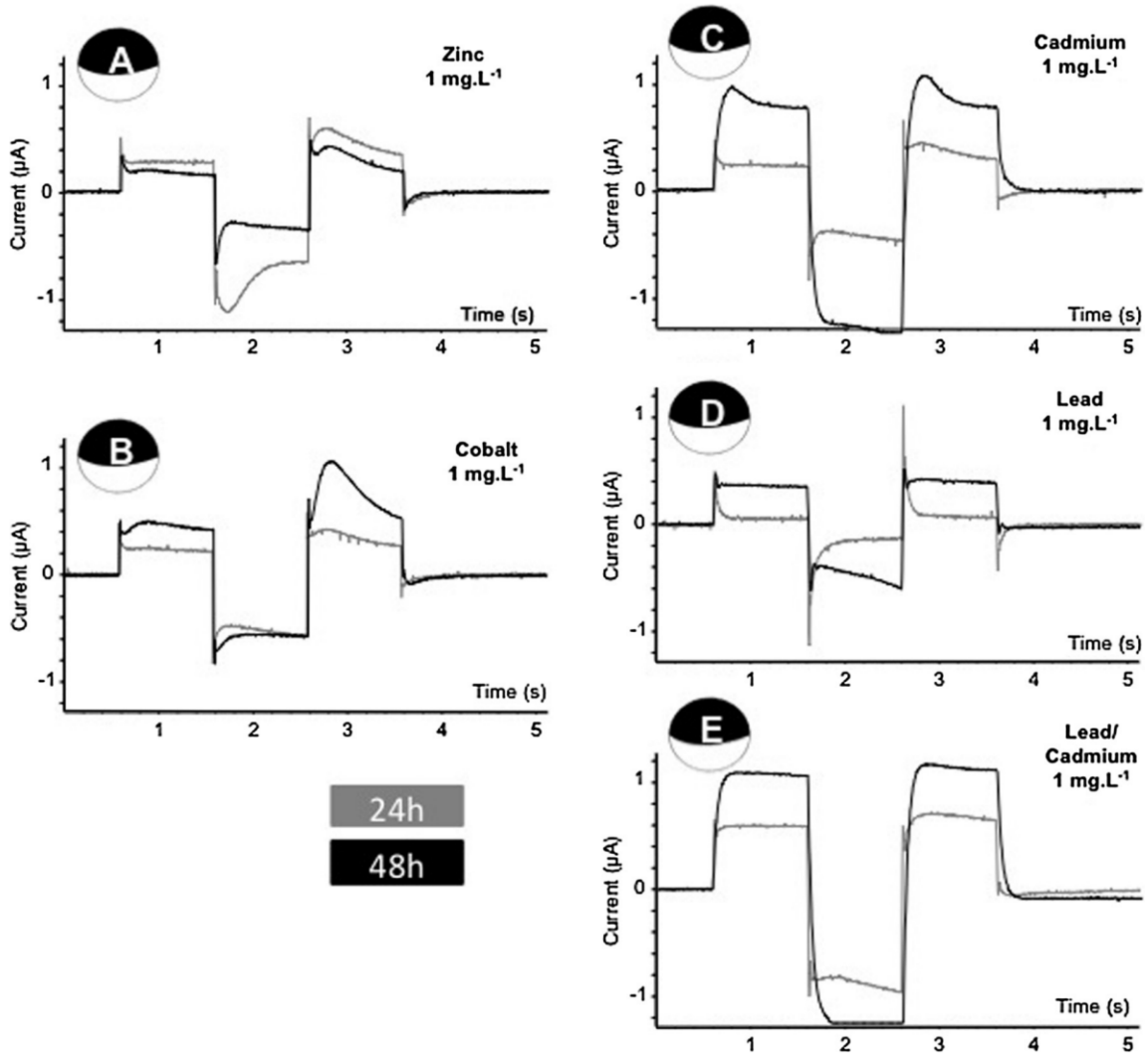

Fig. 5. Calcium-activated chloride currents (ICl) in oocytes exposed to metal solutions. Stage VI oocytes were exposed up to 2 days to 1 mg L^{-1} of chloride solutions of zinc ($ZnCl_2$, A), cobalt ($CoCl_2$, B), cadmium ($CdCl_2$, C), lead ($PbCl_2$, D) or a mixture of cadmium and lead (Pb/Cd, E). ICl currents were evoked using the triple step protocol and measure after 24 h of exposure (gray line, 24 h) and 48 h of exposure (black line, 48 h). This figure depicted typical recordings ($N \geqslant 3$ and $n > 9$).

ICl1-S, ICl2 and ICl1-T currents. The increase of ICl1-S and ICl1-T corresponds to calcium release from endoplasmic stores and the increase of ICl2 reflects calcium entry from extracellular medium.

In our experiments, except for zinc exposures, we observed that metal-treated oocytes exhibit increased ICl. This is consistent with our first observation on oocyte resting potential (Fig. 3), showing a depolarized resting potential (from −90 to −20 mV). The appearance of increased ICl currents after oocyte maturation could inhibit fertilization since they are normally developed to block polyspermy at fertilization.

4. Discussion

The goal of this work was to evaluate the effect of metal chlorides on *X. laevis* oocytes survival and their ability to undergo into M-phase using both a phenotypic approach and electrophysiological recordings.

From survival experiments, we observed after 24 h exposure a good accordance between the electrophysiological and phenotypic observations. Nevertheless, many differences were noticed after 48 h incubation, in particular for cadmium, lead and their mixture for which electrophysiological recordings revealed detrimental effects occurring to oocytes. Consequently, electrophysiological recording appeared as a more powerful approach than single phenotypic examination to evaluate cell survival.

Changes in the resting potential as a measure of a decrease of oocytes integrity were obtained as soon as 48 h of exposure to 1 mg L^{-1} (5.4 µM) of cadmium, 1 mg L^{-1} (3.6 µM) of lead or their mixture and after 72 h of exposure to 1 mg L^{-1} (7.3 µM) of zinc. When comparing our results with data from the literature, this response appeared as very sensitive. Indeed, by measuring neutral red uptake, Jorissen et al. (2013) showed that 100 µM of lead had no effect on T cells viability (Jorissen et al., 2013). Similarly, using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) other cell types like human lens epithelial cells and osteoblasts exposed for 24 h to 60 µM of cadmium were shown to decrease in viability (Qiu et al., 2012; Song and Koh, 2012). Regarding cobalt, periodontal ligament cells exposed to cobalt chloride exhibited a decreased viability (Song et al., 2009). Lastly, there was no changes observed in cell survival following zinc exposure up to 200 µM of zinc sulfate, in contrast to a previous study in human and porcine intestinal cell lines (Lodermann et al., 2012). It can be also outlined that the changes we observed for oocyte electrophysiological survival were obtained for metal concentrations far lower than the EC50 in the FETAX test.

Regarding the effects of metals on oocyte maturation, our data showed that metal chlorides were able to induce spontaneous maturation (i.e. without hormonal stimulation). Indeed, although our results were only significant for zinc, the total absence of spontaneous maturation in the case of control oocytes can allow us considering that all the metals tested affected oocytes.

Our observations on maturation are in agreement with studies reporting that cadmium (injection of females from 0.5 to 5 mg kg^{-1}) could significantly disrupt oogenesis in *X. laevis* (Lienesch et al., 2000), and that cobalt, as well as manganese, induce oocyte maturation in *Rana temporaria* (Nikiforova and Skoblina, 1992). Spontaneous M-phase entry may occur when external calcium and magnesium concentrations increased, in presence of a calcium ionophore, raising the possibility of a non-specific effect of divalent cations on meiosis resumption (Wasserman and Masui, 1975a,b) whereas increasing zinc concentration in external medium has already been reported to induce M-phase entry in the absence of hormonal stimulation (Wallace and Misulovin, 1980). Few studies were carried out both at electrophysiological and morphological levels in oocytes exposed to metals. Nevertheless, Szczerbik et al. (2006) showed that oocytes from Prussian carp exposed to cadmium (from 20 to 200 μM) underwent spontaneous maturation (Szczerbik et al., 2006). By contrast, in sheep, cadmium exposure strongly inhibited oocyte maturation (from about 97% to 32% at 20 μM) (Leoni et al., 2002).

Various studies showed how metals were toxic in somatic cells. Indeed, they were responsible for reactive oxygen species generation (Leonard et al., 2004; Yildiz et al., 2009), hypoxia (Wang and Semenza, 1995), cell cycle defects or arrest through genotoxicity and DNA damage (Bakka and Digernes, 1984; Cao et al., 2007; Wang and Semenza, 1995; Yildiz et al., 2009). Nevertheless, amphibian early embryos, and presumably oocytes, lack DNA damage checkpoint and fail to respond to DNA breaks (Anderson et al., 1997; Finkielstein et al., 2001). Then, due to the specificity of our cellular model (Bodart et al., 2002), other mechanisms than those including DNA damage response or transcription inhibition, have to be involved in the G2/M modulation observed. For instance, the inhibition of G2/M transition may rely on inhibition of protein synthesis, an effect that has been observed for cadmium exposure in liver (Smalinskiene et al., 2005). Dual effects of the metals tested, which in one hand inhibit meiotic resumption induced by hormonal stimulation and in the other hand, generate spontaneous M-phase entry, might relate to the plethora of proteins that might be affected by cadmium but also cobalt, lead or zinc. In their review, Henson and Chedrese (2004) highlighted the ability of cadmium to enhance or inhibit the biosynthesis of progesterone, to exert significant effects on ovarian and reproductive tract or to be responsible for low birth weight (Henson and Chedrese, 2004). In our study, cadmium alone inhibits progesterone-induced oocyte maturation, while exposure to the combination of cadmium and lead does not. As proposed in an Indian study on rat granulosa cells (Priya et al., 2004) and another on rat testicle (Pandya et al., 2012), it seems that cadmium and lead compete for one or more targets. These ones could be extra- (at the plasmic membrane) and/or intra-cellular (various signaling pathways).

Potential effects of lead on cell cycle regulators are seldomly documented while the effect of zinc on the cell cycle is more frequently described. In human prostatic cells, exposure to zinc increases the population of cells in G2 and M phases (Liang et al., 1999). Zinc exerts dual effects on MPF since in one hand, zinc overloading may either directly impair MPF activity or modulate the availability of functional MPF (Qian et al., 1999) and in other hand, zinc chelation prevents MPF activation through Cdc25 inhibition (Beaujois et al., 2013; Birck et al., 1996; Sun et al., 2007).

In our study, ionic events occurring in oocytes exposed to metals and more particularly calcium fluxes, measured using the triple step protocol (Marin, 2012; Yao and Tsien, 1997), demonstrated that calcium-activated chloride channels were activated after cadmium and/or lead exposures. In the early 1980s, calcium-activated chloride currents in *X. laevis* oocytes were shown to be activated during oocyte maturation in order to block polyspermy (Charbonneau et al., 1983; Guerrier et al., 1982; Vilain et al., 1989). This activation is responsible for depolarization of the resting potential (Parker and Miledi, 1988). In our study, since the activation of calcium-activated chloride currents concerned oocytes not yet fertilized, our results suggest that over a long term, reproductive parameters of amphibians could be affected by metal contamination. More investigations are now to be performed to check the incidence of metal exposure on the reproduction success of *X. laevis*. This could be conducted by the exposure of female individuals and the examination of their oocyte productions but also by the ability of eggs produced to be fertilized. Nevertheless, the high sensitivity observed toward metals regarding both cell survival and maturation based experiments makes *X. laevis* oocyte as a biological material of choice for the evaluation of the toxicity of contaminants affecting inland fresh waters.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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3.1.2. Maturation of Xenopus laevis oocytes under cadmium and lead exposures: Cell biology investigations (Article 4)

Au regard des effets décrits dans l'article précédent des expositions aux substances métalliques sur la maturation ovocytaire de *X. laevis*, le deuxième article de ce chapitre, intitulé « *Maturation of Xenopus laevis under cadmium and lead exposures: Cell biology investigations* »[4], vise à apporter de nouveaux éléments sur la toxicité du cadmium et du plomb sur ce phénomène. Parmi toutes les études existantes en toxicologie des amphibiens, très peu s'intéressent aux potentielles altérations du fonctionnement des cellules germinales femelles exposées à des contaminants environnementaux. Cependant, elles pourraient empêcher la reproduction et contribuer au déclin des populations observé de nos jours.

(1) Des cinétiques de maturation ont été réalisées en conditions contaminées. Les ovocytes ont été exposés durant 13 heures, aux concentrations suivantes de Cd^{2+} : 1,36 ; 13,6 ; 136 µM ou de Pb^{2+} : 0,9 ; 9 ; 90 µM. Les taux d'ovocytes présentant une tache de maturation ont été évalués toutes les 15 minutes après ajout de progestérone dans les milieux. (2) Ensuite, dans le but d'évaluer les impacts des expositions sur la morphogénèse du fuseau de division, les ovocytes ont été exposés aux mêmes concentrations de Cd^{2+} ou de Pb^{2+} sur la nuit à 19 °C après ajout de progestérone. Les gamètes présentant une tache de maturation ont été par la suite fixés et coupés. Une coloration au rouge nucléaire et au picro-indigo-carmin des coupes a été effectuée pour mettre en évidence le fuseau et les chromosomes. (3) Enfin, après avoir exposé les gamètes dans les mêmes conditions que précédemment, les états de phosphorylation de ERK2 (voie MAPK), de $p90^{RSK}$, de Cdc2 (sous-unité catalytique du MPF) et de l'histone H3 (cible du MPF) ont été détectés par western-blot.

(1) En présence de progestérone, les cinétiques ont montré que le processus de maturation ovocytaire en condition Cd^{2+} 136 µM était retardé. En effet, dans cette condition le moment où 50 % des ovocytes présentaient une tache de maturation ($GVBD_{50}$) était décalé comparativement aux ovocytes témoin. De plus, les taux de cellules avec cette tache étaient significativement plus faibles que dans la condition témoin bien avant la fin de l'expérimentation. Concernant le Pb^{2+}, de légères différences par rapport au témoin ont été détectées : une augmentation significative du taux de maturation à la concentration 90 µM, au milieu de la cinétique (rattrapée rapidement) et une diminution significative à la concentration de 0,9 µM en fin d'analyse. En l'absence de progestérone et dans toutes les conditions

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exposées au Cd^{2+}, l'apparition spontanée de taches de maturation a été observée. Il est d'ailleurs à noter que lorsqu'elle est provoquée par l'exposition au Cd^{2+}, le processus de maturation prend plus de temps à débuter. Aucune maturation spontanée n'a été observée chez les ovocytes exposés au Pb^{2+} durant ces tests.

(2) Concernant la mise en place du fuseau de division, des pourcentages plus importants d'anomalies ont été observés comparés au témoin, chez les ovocytes exposés au Cd^{2+}, eux-mêmes plus importants que ceux exposés au Pb^{2+}, à toutes les concentrations. Les problèmes les plus observés étaient : « chromosomes anormaux » et « fuseau désorganisé + chromosomes anormaux ».

(3) Les immunoempreintes ont révélé des anomalies de profils de phosphorylation de $p90^{RSK}$, ERK2, Cdc2 et de l'histone H3 en grand nombre lorsque les ovocytes étaient exposés au Cd^{2+} à 136 µM.

Il apparait intéressant d'augmenter la fréquence des mesures du taux de maturation lors des cinétiques. Ici, un relevé toutes les 15 minutes nous a permis d'obtenir plus précisément des données et de détecter des variations subtiles comparées à des mesures classiques toutes les heures. Cette étude a démontré que le cadmium pouvait empêcher et ralentir le processus de GVBD en présence de progestérone et l'induire en l'absence de l'hormone. Probablement, un mécanisme différent de celui induit par la progestérone en condition témoin doit être impliqué. Ces observations sont en accord avec les résultats des immunoempreintes, car ces retards/inhibitions ou ces maturations spontanées peuvent être expliqués par les états de phosphorylation anormaux des protéines étudiées. Ces problèmes biochimiques peuvent également être impliqués dans les morphotypes de fuseaux observés lorsque les ovocytes sont exposés aux métaux, et notamment au cadmium. En effet, le MPF joue un rôle essentiel dans sa mise en place. Ainsi, en perturbant l'activation MPF, ce métal dérèglerait la mise en place du fuseau.

Cette étude apporte de nouvelles données sur la maturation ovocytaire, une étape de préparation à la ponte et à la fécondation. Les résultats obtenus permettent de mieux comprendre les mécanismes d'action de ces contaminants sur l'ovocyte de *X. laevis* et confirment l'intérêt de considérer cette cellule germinale comme un modèle en écotoxicologie.

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Maturation of *Xenopus laevis* oocytes under cadmium and lead exposures: Cell biology investigations

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ABSTRACT

Since amphibians are recognised as good models to assess the quality of environments, only few studies have dealt with the impacts of chemical contaminants on their gametes, while toxic effects at this stage will alter all the next steps of their life cycle. Therefore, we propose to investigate the oocyte maturation of *Xenopus laevis* in cadmium- and lead-contaminated conditions.

The impacts of cadmium and lead ions were explored on events involved in the hormone-dependent process of maturation. In time-course experiments, cadmium, at the highest concentration, delayed and prevented the germinal vesicle breakdown. Even in the absence of progesterone this ion could also induce it. No such spontaneous maturation was observed after lead exposures. An acceleration of the process at the highest tested concentration of lead (90 µM), in presence of progesterone, was recorded. Cytological observations highlighted that cadmium exposures drove severe disturbances of meiotic spindle morphogenesis. At last, cadmium exposures altered the MAPK pathway, regarding the activation of ERK2 and RSK, but also the activation and the activity of the MPF, by disturbing the state of phosphorylation of Cdc2 and histone H3.

Xenopus laevis oocytes were affected by these metal ion exposures, notably by Cd^{2+}. Signatures of these metal exposures on the oocyte maturation were detected. This germ cell appeared to be a relevant model to assess the effects of environmental contaminants such as metals.

1. Introduction

Amphibian toxicology is a widely-investigated field, but the earlier stages of their life cycle seem to be left on the sidelines. In fact, only a few studies have dealt with the impacts of environmental contaminants on gametes. Regarding metal exposures, injection of cadmium in adults was reported to disrupt *X. laevis* oogenesis by decreasing the number of all oocyte stages and by increasing the number of atretic cells (Lienesch et al., 2000). These observations were similar to the results obtained by Medina et al. (2012). They highlighted that cadmium decreased the number of grown oocytes and increased the number of atretic ones in *Rhinella arenarum*. These authors noticed in this species that follicular oocytes presented nuclear and cytoplasmic alterations and that the germinal vesicle breakdown (GVBD) percentages were lowered in cadmium conditions. On the male germ cells of *R. arenarum*, hydropic tumefaction or sign of focal necrosis and fragmented chromatin have been observed associated with a decrease of the sperm concentration, and an alteration of the motility of spermatozoa (Medina et al., 2012). The sperm motility and velocity in *X. laevis* can be affected by zinc ions (Christensen et al., 2004). In this species, Fort et al. (2001) demonstrated that the fertilization efficiency was reduced after cadmium exposures. They also highlighted that the total number of oocytes was reduced together with an increase of necrotic ones in exposed female adults. Moreover, the proportion of the earlier stages of the oogenesis increased in these exposed females and the later stages decreased (in the conditions allowing oocyte survival) and also, they observed a concentration-dependent decrease of mature oocytes. For the males, cadmium exposures caused a decrease of the sperm counts and an increase of abnormal spermatozoa (Fort et al., 2001). Cadmium chloride injection altered the spermatogonia by causing a decrease in secondary spermatogonial and primary spermatocytic stages in the semineferous tubules of *Bufo melanostictus* and of *Rana hexadactyla* Lesson (Biswas et al., 1976; Kasinathan et al., 1987). In recent studies using *X. laevis* oocytes, cadmium and lead were reported to cause a resting potential

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depolarization, maturation impairments and calcium signalling disturbances (Marin et al., 2015). Moreover, the fertilization success in a cadmium-contaminated media was strongly reduced (Slaby et al., 2016b).

The objective of this study was to investigate the effects of cadmium and lead ions exposures on oocyte maturation of *X. laevis*. Because of their large size, synchronized stages, easy availability and physiological stability, frog oocytes constitute a good system for cell biology investigations (Cailliau and Browaeys-Poly, 2009; Gupta et al., 1984). Thus, the maturation process can be reproduced *in vitro* with these cells. During this hormone-dependent process, the oocytes arrested in prophase I resume the meiosis to be arrested in metaphase II (Bodart et al., 2002). The germinal vesicle moves toward the apex of the cell and its nuclear envelope breakdown. It is called the germinal vesicle breakdown (GVBD). The meiotic spindle formation occurred and the chromosomes condensated on the metaphase plate near the cell membrane. Consecutively, at the morphological level, a white spot occurs at the top of the cell. Composed of a catalytic subunit, called Cdc2 (Cyclin-dependent kinase 1) and a regulatory unit (Cyclin B), the MPF (M-Promoting Factor) promotes the M-phase entry (Norbury and Nurse, 1990). The serine 10 histone H3 is a downstream target of the MPF. Its phosphorylation is a conserved histone modification associated with chromosome condensation (Hans and Dimitrov, 2001; Hendzel et al., 1997; Van Hooser et al., 1998). During maturation, there is also activation of MAPKs (Mitogen-Activated Protein Kinases), which play a role in the regulation of the cell cycle. Among them, ERK2 (Extracellular signal-regulated kinase 2) is phosphorylated, and activates others kinases, notably $p90^{RSK}$ (p90 kDa ribosomal s6 kinases). (Ferrell et al., 1991; Frödin and Gammeltoft, 1999). In this work by assessing GVBD time-courses, meiotic spindle morphogenesis and phosphorylation states of proteins involved in the maturation signalling pathways, we investigated the impacts of cadmium and lead exposures on *X. laevis* oocyte maturation.

2. Material and methods

2.1. Reagents and test substances

All chemical compounds were obtained from Sigma-Aldrich Chimie® (Saint-Quentin Fallavier, France), except tricaine mesylate (MS222; Fluka®). Metal salts were of 99.9% grade of purity. Solutions of metal chlorides (cadmium or lead (II)) were daily diluted in the weekly prepared control medium ND96 (Nathan Dascal 96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, pH 7.5). The exposure concentrations of cadmium and lead ions were 1.36, 13.6 and 136 µM and 0.9, 9 and 90 µM, respectively, as in our previous study (Marin et al., 2015).

2.2. Handling of Xenopus laevis and oocytes

Xenopus of our culture initially came from the University of Rennes. All animals were maintained in control tanks (XenopLus − Amphibian Housing System from Techniplast) in the animal house of our university (Lille 1, Villeneuve d'Ascq, France) and were fed with floating expanded pellets *ad libitum* (Aquatic 3, Special Diets Services). The water quality parameters were precisely controlled and maintained thanks to a 24-h complete water change. All animal experiments were performed at the university animal facility according to the rules of the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The animal protocol was approved by the local institutional review board (Comité d'Ethique en Experimentation Animale Nord-Pas-de-Calais, CEEA 07/2010).

Ovariectomies were performed every week on *Xenopus laevis* females. A bath of tricaine mesylate (1 g L^{-1}) solution was used to anesthetize animals for 45 min. To isolate cells from the collected clusters, a collagenase treatment (1 g L^{-1}) was done for 45 min before a manual dissociation with tweezers under a stereo microscope. At this time, all the cells were immature oocytes: only those in stage VI (Dumont et al., 1983), which were able to undergo maturation, were selected. All oocytes collected were stored in ND96 medium at 14 °C. Maturation process was induced by a progesterone (Pg) bath (4 mg L^{-1}).

2.3. Time-course experiments

The GVBD ratios were assessed by the appearance of the white spot on the animal pole of the cell. This endpoint indicated that the meiosis was resumed. In a culture plate, 30 oocytes per well were exposed at 19 °C for 13 h and GVBD ratios in each condition were assessed every 15 min. This test was conducted 4 and 6 times, respectively, for Cd^{2+} and Pb^{2+} exposures.

2.4. Cytological analysis

After an overnight exposure at 19 °C, the oocytes exhibiting a white spot were collected in each condition. Then they were fixed overnight in Smith reagent (Smith A: Potassium Bichromate 17 mM; Smith B: formol and acetic acid 80/20%) in the dark at room temperature. They were dried and paraffin-embedded, then sliced with a microtome (7 µm slice thickness). Nuclear red (0.1 g of nuclear red QSP in 100 ml 5% aluminium sulphate 5%) and picro-indigo-carmine (0.25 g of picro-indigo-carmine QSP in 100 ml saturated picric acid) colorations were conducted to reveal the nuclear structures and chromosomes, and the cytoplasmic structures, respectively (Flament et al., 1996). Slices mounted on slides were analysed by microscopy (×400).

2.5. Electrophoresis and western blotting

Cells were collected after overnight exposures at 19 °C. Electrophoresis and western blotting were conducted according to the protocol of Gelaude et al. (2015). Briefly, sampled oocytes were lysed in homogenization buffer and centrifuged (13.8g, 4 °C, 15 min) to collect the protein part. Supernatants were added to one volume of Laemmli 2X buffer with 4% beta-mercaptoethanol. Samples were heated at 75 °C for 5 min and stored at −20 °C before analysis. Proteins from oocytes were separated by SDS–PAGE (15% Chesnel modified) and transferred onto a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, United Kingdom). Blots were blocked with 5% low fat dry milk and incubated overnight with specific antibody. $p90^{RSK}$ was detected using the polyclonal rabbit antibodies (p90Rsk-1 C-21 sc-231 antibody, Santa Cruz Biotechnology®), ERK2 was detected using mouse monoclonal antibody (Erk2 D-2 sc-1647 antibody, Santa Cruz Biotechnology®), and pCdc2 and pH3 using rabbit polyclonal antibodies (Phospho-Cdc2 (Tyr15) antibody and Phospho-Histone H3 (Ser10) antibody, Cell Signaling® respectively). Nitrocellulose membranes with bounded primary antibody were then incubated with appropriate secondary antibodies (Sigma–Aldrich). Chemiluminescent assays (Clarity™ Western ECL Substrate, Bio-Rad®) were used to detect the signals according to manufacturer's instructions.

2.6. Statistical analysis

All statistical analyses and graphical representations were conducted with R software (version 3.3.2–The R Foundation for Statistical Computing, 2016). The GVBD ratios measured every 15 min during the 13 h of the kinetic analyses were compared performing Friedman's tests and post-hoc multiple paired comparison between conditions. $GVBD_{first}$ (time of the first occurrence of a white spot) and $GVBD_{50}$ were determined by graphical analyses. For all experiments, N refers to the number of replications (used females) and n to the number of oocytes per condition.

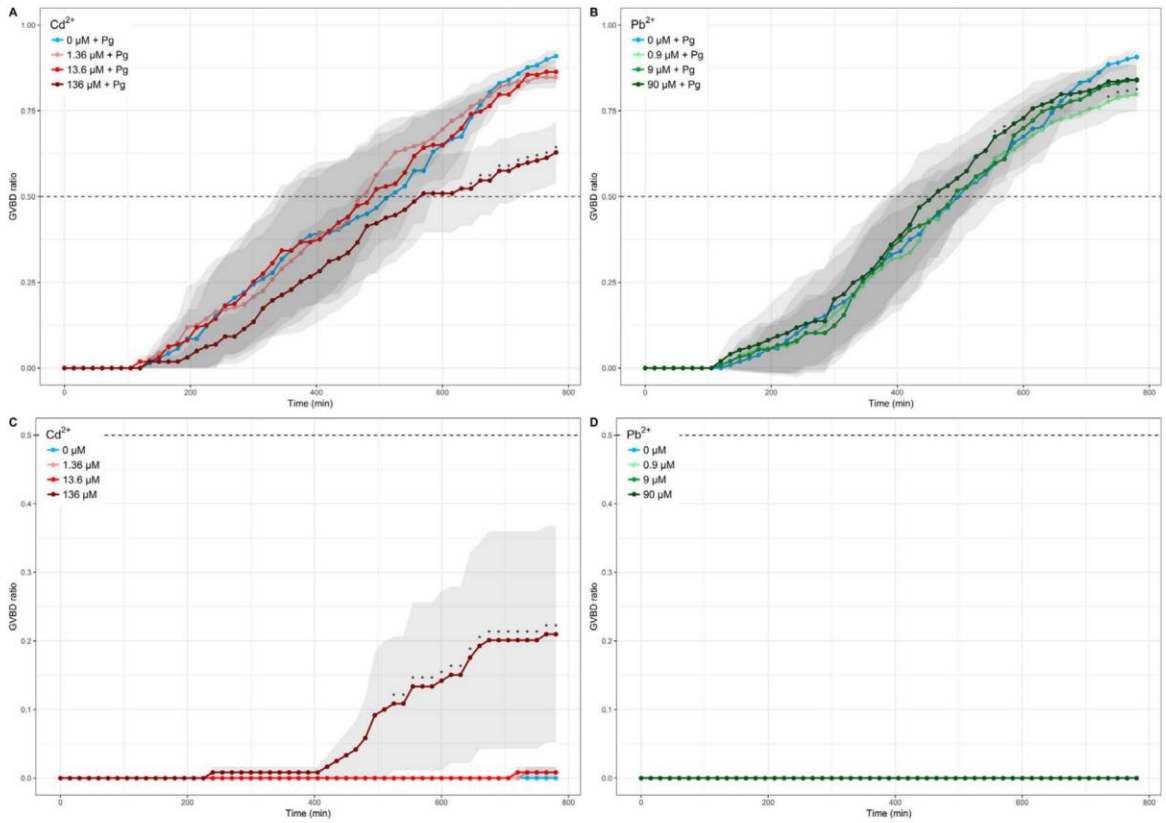

Fig. 1. Effects of cadmium and lead ion exposures on the meiosis resumption time courses. During 13 h, GVBD ratios were assessed by the white spot appearance every 15 min. Parts A and B correspond to Cd^{2+} exposures with or without Pg, respectively (N = 4 and n = 30). Used concentrations were 0 (blue line), 1.36, 13.6 and 136 µM (red lines). Parts C and D correspond to Pb^{2+} exposures with or without Pg, respectively (N = 6 and n = 30). Used concentrations were 0 (blue line), 0.9, 9 and 90 µM (green lines). Results are expressed as means ± SEM (grey areas). Significant differences were assessed by Friedman's tests and post-hoc multiple paired comparison between conditions (*: $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Time-course experiments

In a first set of experiments, the effects of metal ion exposures on the GVBD ratios were assessed. For this purpose, oocytes were exposed to increasing concentrations of cadmium and lead (0, 1.36, 13.6 and 136 µM and 0, 0.9, 9 and 90 µM, respectively) with or without progesterone. Every 15 min during 13 h, white spot appearance after Pg stimulation was scored (Fig. 1A & B). No difference between the survival ratio of control and contaminated conditions were found whatever the oocyte stage (data not shown), in good accordance with our previous results (Marin et al., 2015). For all Cd^{2+} concentrations, $GVBD_{first}$ occurred between 120 and 135 min (Fig. 1A). However, it appeared that $GVBD_{50}$ occurred earlier in low Cd^{2+} concentrations when compared to control conditions (respectively after 472 and 488 min for 1.36 and 13.6 µM compared to 514 min in control condition). $GVBD_{50}$ was interestingly delayed in the highest concentration (563 min). In addition, as soon as 645 min up to the end of the experiment, only for 136 µM of Cd^{2+}, GVBD ratios were significantly lower than in the control condition ($p < 0.05$). Concerning Pb^{2+} exposures, only slight differences were noticed. For the lowest concentration, the GVBD ratios from 735 to 780 min of experiment were significantly lower ($p < 0.05$) and for the higher concentration from 555 to 570 min the GVBD ratios were significantly higher ($p < 0.05$) (Fig. 1C) than in the controls.

In parallel, the same experiments were conducted without Pg stimulation (Fig. 1C & D). Whatever the tested concentration, Cd^{2+} exposures induced spontaneous maturation, compared to control and Pb^{2+} ones. The $GVBD_{first}$ were noticeably different from those measured with progesterone stimulation (735, 720 and 240 min for 1.36, 13.6 and 136 µM of Cd^{2+} without Pg, compared to 135, 120 and 135 min with Pg). At the highest concentration of Cd^{2+}, significant differences were observed from 525 min to the end of the time-course. $GVBD_{50}$ was never reached in any condition without Pg.

3.2. Cytological analysis

In a second set of experiments, cytological structures related to Pg-induced maturation were analysed. Table 1 and Fig. 2 summarize and illustrate the observed abnormal structures in case of cadmium and lead ion exposures. In the control condition with Pg (Fig. 2A) standard structures were observed: symmetric and perpendicular spindle near the cell membrane, and all the chromosomes lined up on the metaphase plate. The label "S" indicated that only spindle was disorganised or absent (with chromosomes detectable and without germinal vesicle), "C" referred to chromosomes not lined up on the metaphase plate or absent. The "E" and "GV" labels referred, respectively, to a spindle not anchored to the cell membrane and to the presence of a germinal vesicle. When several abnormal observations existed for one oocyte, it was counted in a subsequent category. For example, a cell "S + C" referred to an oocyte presenting both a disorganised spindle and non-lined up chromosomes (Table 1). When oocytes were exposed to Cd^{2+} or Pb^{2+} various abnormalities appeared more frequently compared to the control condition (from about 54.5% in 90 µM Pb^{2+} to 92.9% in 13.6 µM Cd^{2+}) (Fig. 2B–H & Table 1). Among these aberrations, "abnormal chromosomes" and "abnormal spindle + abnormal chromosomes" (Fig. 2B & D & Table 1) were the most widespread. Moreover, specific structures seemed to appear in cadmium-exposed oocytes: *i)* ectopic spindle (between 7.1 and 10%, Fig. 2E & Table 1) and *ii)*

Table 1
Observed cytological effects after cadmium and lead ion exposures.

Conditions (μM)			N	n	Normal	S	C	E	S + C	C + E	S + C + E	GV
Control	0	Pg	4	21	66.7%	–	–	4.8%	9.5%	–	19.0%	–
Cd^{2+}	1.36	Pg	2	10	10.0%	–	10.0%	10.0%	50.0%	–	20.0%	–
	13.6	Pg	2	14	21.4%	–	7.1%	7.1%	42.9%	7.1%	7.1%	7.1%
	136	Pg	2	13	23.1%	7.7%	7.7%	7.7%	15.4%	–	7.7%	30.8%
Pb^{2+}	0.9	Pg	2	9	44.4%	–	11.1%	–	33.3%	–	11.1%	–
	9	Pg	2	7	28.6%	–	14.3%	–	42.9%	14.3%	–	–
	90	Pg	2	11	45.5%	–	9.1%	–	9.1%	–	36.4%	–

Abbr.: Pg: presence of progesterone, N: number of used females, n: number of used oocytes, S: abnormal spindle, C: abnormal chromosomes; E: ectopic spindle; GV: germinal vesicle; +: and.

germinal vesicle (from 7.1 to 30.8%, Fig. 2H & Table 1) for oocytes with white-spot. Such germinal vesicle was also observed in all control and exposed germ cells without progesterone (data not shown). That was never observed for lead ion exposed oocytes (Table 1).

3.3. Electrophoresis and western blotting

The formation of these previously described cytological structures is related to various signalling pathways in *X. laevis* oocytes. So, in a third step, we decided to decipher some of them. In that way, immunodetection was used to characterize the phosphorylation states of MAPK proteins (RSK and ERK2) but also of proteins involved in the MPF pathway (Cdc2 and H3). In control condition, RSK and ERK2 were phosphorylated only in white spot oocytes (Fig. 3, condition 2). pCdc2 (Tyr15) is related to the activity of MPF, Cdc2 being the catalytic subunit. Histone H3 is a downstream target of MPF. Both the dephosphorylation of Cdc2 and phosphorylation of H3 indicate MPF activity within these oocytes (Fig. 3, condition 2). In contrary, when exposed to Cd^{2+} or Pb^{2+}, abnormal patterns were observed. These anomalies were numerous in case of cadmium exposures. As mentioned in Table 2, pCdc2 and pH3 profiles were often disrupted (from 16.7 to 100%). When spontaneous maturations were observed (WS without Pg stimulation), pCdc2 and pH3 profiles were all abnormal (Fig. 3, condition 9 & Table 2). After Pb^{2+} exposures, phosphorylation pattern abnormalities were less frequent (Table 2).

4. Discussion

Xenopus mature oocytes are blocked in metaphase II, in anticipation for fertilization. This phenomenon is marked by the white spot appearance due to the GVBD at the animal pole of the oocyte (Ferrell, 1999). Previously, cadmium but not lead ion exposures have been described to block or induce the GVBD (Marin et al., 2015). First, to better understand the mechanism of action of these two metals, maturation kinetics were investigated by evaluating the GVBD ratios every 15 min during 13 h. Such a monitoring had already been done and proven its usefulness to study maturation process (Gelaude et al., 2015; Sellier et al., 2006; Wakiyama et al., 2000), but here the measures were strengthened by determining precisely the $GVBD_{50}$ and the $GVBD_{first}$ (when the first oocyte underwent the GVBD). Significant subtle variations could be missed by hourly basis and final point evaluations. At the higher concentration of cadmium, the $GVBD_{50}$ was delayed. Moreover, despite the presence of progesterone, the GVBD ratios were significantly reduced at this concentration, largely before the other conditions reached their maximal GVBD ratios (from 630 min). So, cadmium not only prevented but also slowed down the meiosis resumption. Always in presence of progesterone, in the highest lead concentration (90 μM) the GVBD ratios significantly increased from 555 to 570 min of experiment and the $GVBD_{50}$ was reached faster, suggesting that Pb^{2+} could accelerate the maturation process. For the lowest lead concentration (0.9 μM), the final GVBD ratios were significantly lowered.

In the absence of progesterone, some cadmium-exposed oocytes exhibited a white spot. This effect was most pronounced for the highest concentration (136 μM), where the GVBD ratios differed significantly from the control condition during the maturation time-courses. Such spontaneous maturation was also highlighted when the oocytes of *Carassius auratus gibelio B.* were exposed to cadmium for one day (Szczerbik et al., 2008). Compared to the progesterone conditions, the meiosis resumption was noticeably delayed as when it was not hormone-induced (240 min). These findings were in accordance with the western blot analyses. In fact, delayed or spontaneous maturation could be caused by an alteration of essential pathways, or an activation of others. During the maturation, the MAPK pathway and the MPF are activated. Here, the MAPK activation was assessed by the detection of the phosphorylation states of ERK2 and RSK. Cadmium ion exposures disturbed the MAPK pathway when the oocytes resumed their meiosis

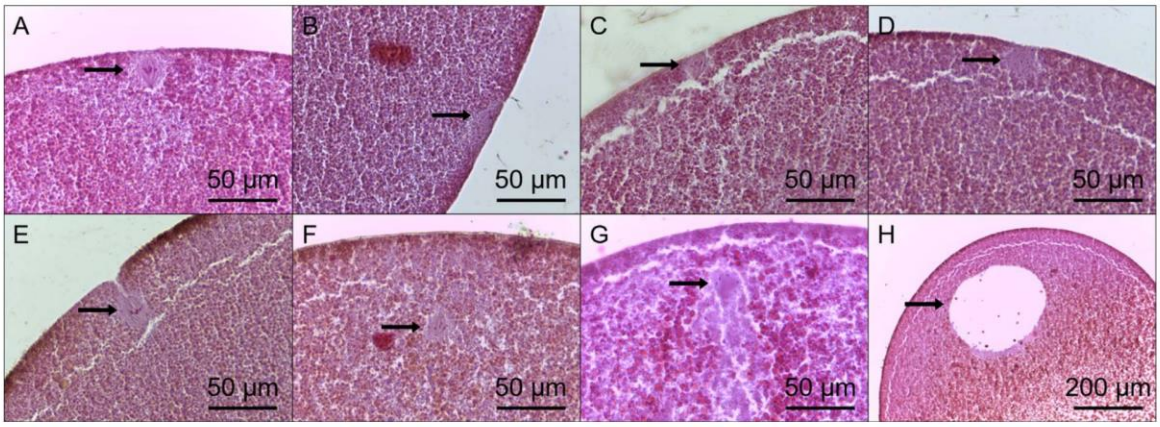

Fig. 2. Effects of metal exposures on spindle and chromosome formation. Typical photographs are presented (A: Control oocyte; B-H: exposed oocytes). Black arrows indicate meiotic structures. A: symmetric and perpendicular spindle and lined up chromosomes on the metaphasis plate, B: disorganised spindle, C: disorganised chromosomes, D: abnormal spindle and disorganised chromosomes, E: ectopic spindle, F: ectopic spindle and disorganised chromosomes, G: ectopic structure with abnormal chromosomes and spindle, H: germinal vesicle.

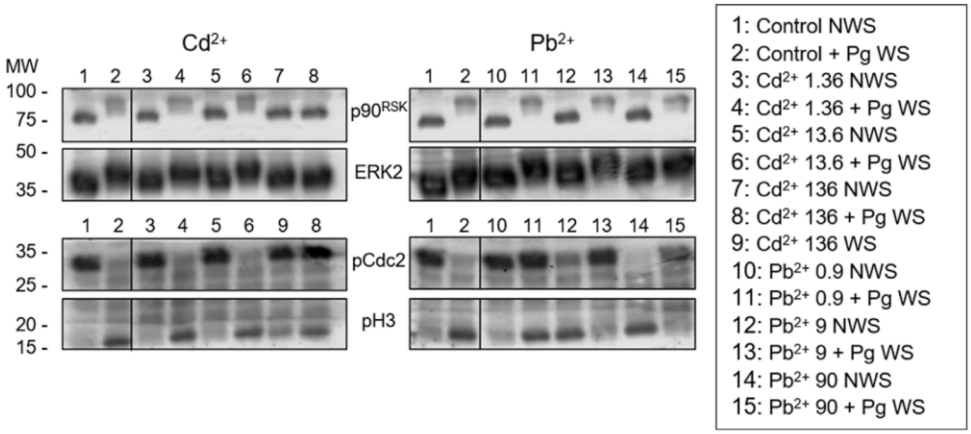

Fig. 3. Effects of cadmium and lead ion exposures on the MAPK pathway and the MPF. $p90^{RSK}$, ERK2 were representative of the MAPK pathway activity and pCdc2 (Tyr15) and pH3 corresponded to, respectively, the activation and the activity of the MPF. Representative blots are presented. Pg: progesterone, NWS: non-white spot, WS: white spot.

by a hormone induction or in a spontaneous way. Other studies underlined that Cd^{2+} exposure induced the phosphorylation of ERK1/2 in U-937 promonocytic human cells exposed to 200 µM for 2 h (Galán et al., 2000), in PC12 cells exposed to 5, 10 and 20 µM for 24 h and also in a time-dependent manner when the cells were exposed to 20 µM (Chen et al., 2008) or in 9L rat brain tumor cells exposed to 40, 60 and 80 µM for 2 h (Hung et al., 1998). However, these three examples referred to mammalian cell models.

Lead ion exposures did not alter the MAPK pathway as much as Cd^{2+} ones. In other studies, lead did not modify the phosphorylation state of ERK2 of C6 glioma cells at 0.1–1 µM for 20 and 48 h (Posser et al., 2007), but conversely increased its state in hippocampal slices of rat exposed *in vitro* (1, 5 and 10 µM for 3 h) or *in vivo* (daily intraperitoneal injection at the concentrations of 2, 8 and 12 mg kg^{-1} from the 8th to the 12th postnatal days) (Cordova et al., 2004) or in the cerebellum slices of *Rhamdia quelen*, *in vitro* (5 and 10 µM for 3 h) and *in vivo* (1 mg L^{-1} for 2 days) (Leal et al., 2006). Similar observations were done with human astrocytoma cells exposed to 10 µM for 15 min associated with an activation of RSK in a concentration-dependent manner (5–50 µM) (Lu et al., 2002). In GT1-7 cells, lead affected the state of phosphorylation of ERK2 in a concentration- (0–10 µM for 10 min) and time-dependent manner (5 µM for 30 min) for ERK2 and RSK (Zhang et al., 2003). Then, in the mouse CL3, the phosphorylation of ERK1/2 increased in concentration- (0–100 µM for 24 h) and time- (30 µM for 24 h) dependent manner (Lin et al., 2003). All these studies used lead acetate as salt source of lead ions. However, the comparison with these examples needs to consider the peculiarity of *X. laevis* oocyte, notably that it isn't a somatic cell.

The activation of the MPF by the phosphorylation states of one of its regulators called Cdc2 and its activity by the phosphorylation of the histone H3 were also analysed. Cadmium disturbed M-promoting factor activation/activity as well. Because of the essential role of the MPF in the spindle morphogenesis, these observations have to be linked to the cytological analysis results. In fact, abnormalities occurred for both metal exposures, but in higher proportion in Cd^{2+} exposures. These ones, by impacting the MPF, could disturb the spindle morphogenesis. We observed different kinds of abnormality and one oocyte could present several ones. Such deformities or a lack of the spindle structure will inevitably disturb the cell division and so the maturation and then the fertilization. These results could be related to those of Shimada et al. (1976) with cadmium (3 and 6 mg kg^{-1} body weight). This ion affected the number of chromosomes and can arrest the meiosis in mouse oocytes (hypoploidy, hyperploidy or diploidy) (Shimada et al., 1976). Germ cells with an intact germinal vesicle were observed in case of cadmium ion exposures, despite the presence of progesterone and the fact that only oocytes exhibiting a white spot were sampled. So, in such abnormal cases, the germinal vesicle would have moved toward the apex of the cell but did not breakdown. The germinal vesicle is normally present in immature cells and was observed in all oocytes from the without progesterone conditions (data not shown).

Table 2
Recorded percentages of abnormalities in the phosphorylation patterns following cadmium and lead ion exposures.

Conditions (µM)			WS/NWS	N	n per sample	Abnormalities			
						$p90^{RSK}$	pERK2	pCdc2	pH3
Control	0	–	NWS	6	$7 \le n \le 23$	–	–	–	–
		Pg	WS	9	$8 \le n \le 20$	–	–	–	–
Cd^{2+}	1.36	–	NWS	6	$5 \le n \le 20$	–	–	–	–
		Pg	WS	6	$12 \le n \le 20$	–	–	16.7%	–
	13.6	–	NWS	6	$4 \le n \le 20$	–	–	–	–
		Pg	WS	6	$14 \le n \le 21$	–	–	–	–
	136	–	WS	2	$1 \le n \le 12$	50%	–	100%	100%
		–	NWS	5	$3 \le n \le 20$	–	–	–	–
		Pg	WS	9	$7 \le n \le 19$	44.4%	22.2%	66.7%	22.2%
		Pg	NWS	5	$4 \le n \le 9$	–	–	–	20%
Control	0	–	NWS	$7 \le N \le 8$	$5 \le n \le 23$	–	–	–	–
		Pg	WS	$8 \le N \le 9$	$5 \le n \le 20$	–	–	–	–
Pb^{2+}	0.9	–	NWS	$7 \le N \le 8$	$5 \le n \le 21$	–	–	–	–
		Pg	WS	$7 \le N \le 8$	$5 \le n \le 18$	14.3%	–	25%	–
	9	–	NWS	$7 \le N \le 8$	$5 \le n \le 21$	–	–	–	–
		Pg	WS	$7 \le N \le 8$	$5 \le n \le 20$	–	–	12.5%	–
	90	–	NWS	$4 \le N \le 5$	$18 \le n \le 20$	–	–	–	–
		Pg	WS	$5 \le N \le 6$	$15 \le x \le 21$	–	–	–	–

Abbr.: Pg: presence of progesterone, WS: white spot, NWS: non-white spot N: number of used females, n: number of used oocytes.

Analysing gametes provided complementary information to standard studies with amphibians in environmental toxicology. It is essential, given that an impact at this stage will inevitably disturb the fertilization or the development of new organisms, and so affect the renewal of population. Our work focused on the oocyte maturation of *X. laevis*. The understanding of the mechanisms of action of environmental contaminants on germ cells will contribute to better assessment of the quality of ecosystems. The analysis of sperm quality parameters and of oocyte nuclear maturation in *R. arenarum* (Medina et al., 2012) and reproductive parameters of *X. laevis* such as breeding success, fertilization, segmentation, embryo-larval viability and biometric data on tadpoles (Fort et al., 2001; Slaby et al., 2016a, 2016b) have been proposed as relevant tools in biomonitoring studies. The present work on amphibian oocytes is consistent with the ethical principles of the 3R's (Russell and Burch, 1959), decreasing the number of necessary organisms needed to complete a set of experiments thanks to the high quantity of oocytes which can be obtained and *in vitro* exposed without sacrificing individuals.

5. Conclusions

Our work investigated effects of cadmium and lead ion exposures on the maturation process of *X. laevis* oocytes. Cd^{2+} effects were more pronounced than Pb^{2+} ones at the different studied levels (cell morphology, cytology and biochemistry). Obtained results bring new data that allow a better understanding of mechanisms of contaminant action in *X. laevis* oocyte. These results with environmental contaminants, associated with peculiarities of this germ cell confirm the *Xenopus* oocyte as a promising model in aquatic toxicology.

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3.1.3. Cadmium but not lead exposure affects Xenopus laevis fertilization and embryo cleavage (Article 5)

Comme l'ont montré les deux études précédentes, les mécanismes de la reprise de méiose peuvent être altérés par les expositions aux métaux. La maturation est une étape de préparation à la fécondation. De fait, notre travail s'est orienté vers l'étape de la reproduction. Cet article, intitulé « *Cadmium but not lead exposure affects Xenopus laevis fertilization and embryo cleavage* »[5], décrit les impacts d'expositions au cadmium et au plomb, seuls ou en mélange, sur la fécondation et les premières étapes de segmentation du modèle *X. laevis*.

(1) Tout d'abord, le succès de fécondation a été déterminé. Pour cela, des ovocytes matures pondus par une femelle préalablement stimulée ont été fécondés *in vitro* par mise en contact avec des morceaux de testicule, le tout en conditions contaminées au cadmium (Cd^{2+} : 1,36 ; 13,6 ; 136 µM), au plomb (Pb^{2+} : 0,9 ; 9 ; 90 µM) ou au mélange de ces deux substances (Cd^{2+} + Pb^{2+} : 13,6 + 9 ; 136 + 90 µM). Par analyse photographique, 35 minutes post-fécondation, les taux de fécondation ont été évalués grâce aux rotations d'équilibration et de symétrisation durant lesquelles la pesanteur oriente un œuf fécondé pôle animal vers le haut. (2) Ce paramètre a également été évalué en réalisant des pré-incubations des gamètes séparément dans une solution de Cd^{2+} à 136 µM pendant 7 minutes avant rinçages et fécondation *in vitro* dans le milieu contrôle. (3) Enfin, les délais d'apparition des stades de l'embryogénèse ont été mesurés sur les quatre premières étapes de segmentation issues des fécondations *in vitro*.

(1) Il s'avère que le succès de fécondation est réduit significativement lorsque cette étape est soumise à une concentration de 136 µM de Cd^{2+}. Aucune différence n'a été observée après les expositions au Pb^{2+}. Concernant les co-expositions à la plus forte concentration, les résultats ont montré un effet similaire à celui du cadmium seul.

(2) Afin de différencier une cible préférentielle du cadmium entre l'ovocyte et le spermatozoïde, des pré-incubations ont été réalisées. Les résultats ont montré, premièrement, que peu importe si les expositions concernent les gamètes mâles ou femelles : les succès de fécondation étaient réduits significativement dans les deux cas. Deuxièmement, en comparaison, ce paramètre est encore plus altéré lorsque les ovocytes sont exposés seuls.

[5] *Titre : Cadmium but not lead exposure affects Xenopus laevis fertilization and embryo cleavage*
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(3) Les embryons issus des fécondations *in vitro* exposés au cadmium ont montré un retard du développement embryonnaire qui augmente avec la concentration. De plus, les embryons issus des pré-incubations d'ovocytes seuls, n'ont jamais atteint le stade 2. Aucun effet similaire n'a été observé dans les conditions contaminées au plomb ou aux mélanges.

Les résultats ont montré principalement que les expositions au cadmium altèrent la reproduction en inhibant le succès de fécondation et en empêchant ou en ralentissant le développement embryonnaire. De plus, il apparait que l'ovocyte semble être la cible principale de ce métal.

Il apparait essentiel en toxicologie des amphibiens de s'intéresser aux stades précoces de développement. Comparé aux méthodes conventionnelles, ce travail apporte de nouvelles informations qui permettent de mieux appréhender les effets de contaminants environnementaux métalliques sur les amphibiens.

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Cadmium but not lead exposure affects *Xenopus laevis* fertilization and embryo cleavage

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ABSTRACT

Among the toxicological and ecotoxicological studies, few have investigated the effects on germ cells, gametes or embryos, while an impact at these stages will result in serious damage at a population level. Thus, it appeared essential to characterize consequences of environmental contaminant exposures at these stages. Therefore, we proposed to assess the effects of exposure to cadmium and lead ions, alone or in a binary mixture, on early stages of *Xenopus laevis* life cycle. Fertilization and cell division during segmentation were the studied endpoints.

Cadmium ion exposures decreased in the fertilization rates in a concentration-dependent manner, targeting mainly the oocytes. Exposure to this metal ions induced also delays or blockages in the embryonic development. For lead ion exposure, no such effect was observed. For the exposure to the mixture of the two metal ions, concerning the fertilization success, we observed results similar to those obtained with the highest cadmium ion concentration.

1. Introduction

Environmental contamination has significant effects on biodiversity, especially on Amphibians. This class, containing the highest number of known threatened species (Monastersky, 2014), is highly sensitive to chemical compounds (Blaustein and Wake, 1995; Lefcort et al., 1998). Until the early 2000s, few environmental toxicology studies were undertaken about chemical impacts on amphibians (Sparling et al., 2010). Moreover, in comparison with others pollutants like pesticides, the effects of metal contaminants on this group remain poorly understood despite their toxicity, dominance and persistence in the environment (Hopkins and Rowe, 2010).

However, recently studies about developmental toxicology of environmental toxicants such as trace metals have been published (Haywood et al., 2004; Mouchet et al., 2007b; Peles, 2013; Sztrum et al., 2011; Yologlu and Ozmen, 2015).

Cadmium and lead originating from natural processes but also discharged in large amount by human activities, as mining, industry or agriculture, are largely recognized as dangerous. Concerns and warnings from a large scientific community have often been expressed. Major environmental agencies, such as the *Agency for Toxic Substances and Disease Registry*, the *Canadian Environmental Protection Act* and the *US Environmental Protection Agency*, as well as the *European Community*, have ranked cadmium and lead on the priority list of dangerous substances, as metals of main focus and as chemical substances of priority concern (ATSDR, 2013; Grillitsch and Schiesari, 2010).

In a recent study, we demonstrated that metal exposures disrupted both meiotic resumption and meiosis progression in *Xenopus laevis* oocytes (Marin et al., 2015). We reported that both cadmium and lead exposures can strongly depolarize the resting potential of the cell, disturbs oocyte maturation and also impacts calcium signalling of the oocyte by modulating calcium-activated chloride currents (Marin et al., 2015). Because of those previous results, the aim of this work was to study biological responses, *i.e.* fertilization and the first stages of embryogenesis, to exposure to these metals further.

Whereas unfertilized eggs as well as spermatozoa are laid in the water, and consequently exposed directly to environmental

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pollutants, the earlier stages of the lifecycle of amphibians have yet remained unconsidered in environmental toxicology. Effects on further stages have been more thoroughly investigated with the use of the FETAX test. This assay with *Xenopus laevis* tadpoles have been used to determine lethal and teratogenic concentrations of complex mixtures (Dawson and Bantle, 1987; Dumont et al., 1983). For example, Vismara et al. used it to assess the efficiency of water processing plant in reducing ecotoxicological effects (Vismara et al., 1993). By means of this assay, cadmium has been reported to induce different types of malformations on tadpoles and also on juvenile *Xenopus laevis* (Plowman et al., 1994; Sunderman et al., 1991). As well, Mouchet et al. highlighted severe toxicity of lead chloride to *Xenopus* tadpoles (mortality, signs of anaemia, body size reduction and restricted food uptake) (Mouchet et al., 2007a). Their results are in accordance with the works of Sobotka and Rahwan which described serious adverse effects, as neural tube defects, tail curvature, and delayed appearance of lordoscoliosis, after short and long-term lead exposures (Sobotka and Rahwan, 1995).

Up to now, most studies have been performed on a few days old tadpoles or adults. The potential effects of compounds at earlier stages of embryogenesis (from the external fertilization) have been ignored. However, the success of breeding could be endangered in case of gamete and/or embryo exposures. In this context, the aim of this work was to assess the effects of metal exposure effects on the earliest key steps of *Xenopus* embryogenesis.

2. Materials & methods

2.1. Reagents and test substances

All compounds were obtained from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France). Tested solutions were prepared weekly. Solutions of cadmium and/or lead chlorides were obtained by dilutions in the control media MMR 1/10 (Mark's Modified Ringer: 0.01 M NaCl; 0.2 mM KCl; 0.1 mM $MgSO_4$; 0.2 mM HEPES; 0.01 mM EDTA). Metal salts were of 99.9% grade of purity (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France).

2.2. Handling of frogs

Adult males and females were kept separated in the animal house of our University Lille 1 in normal tap water at 18 °C with 12 h light dark cycle. They were fed with floating expanded pellets *ad libitum* (Aquatic 3, Special Diets Services). Individuals of our culture initially came from the University of Rennes. All animals were maintained in control tanks (Xenoplus—Amphibia Housing System from Tecniplast) in the animal house (Villeneuve d'Ascq, France). The water quality parameters are precisely controlled and maintained thanks to a complete water change over a 24-h period. All animal experiments were performed at the animal facility of Lille 1 University according to the rules of the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The animal protocol was approved by the local institutional review board (Comité d'Ethique en Experimentation Animale Nord-Pas-de-Calais, CEEA 07/2010).

Table 1
Levels of Metals used in the study (in mg L^{-1} and µM).

Metal Chloride	mg L^{-1}	0.25	2.5	25
Cd^{2+}	mg L^{-1}	0.153	1.53	15.3
	µM	1.36	13.6	136.6
Pb^{2+}	mg L^{-1}	0.186	1.86	18.6
	µM	0.900	9	90

2.3. Contaminants

Exposures were conducted with cadmium chloride ($CdCl_2$) and lead (II) chloride ($PbCl_2$), single or as binary mixture solutions. The concentrations were determined according to previous results (Marin et al., 2015), following the EC50 (embryo-toxicity) of metal ions in FETAX test ($CdCl_2$: 1.6 mg L^{-1}; $PbCl_2$: 96.1 mg L^{-1}) (Güngördü et al., 2010). We chose to test several concentrations for $CdCl_2$: 0.25 mg L^{-1}, 2.5 mg L^{-1}, 25 mg L^{-1}; for $PbCl_2$: 0.25 mg L^{-1}, 2.5 mg L^{-1}, 25 mg L^{-1} and for the binary mixtures: 2.5 mg L^{-1} and 25 mg L^{-1} of each contaminant (Table 1).

2.4. Collection of gametes

To stimulate the female to lay, a subcutaneous injection of human chorionic gonadotropin (HCG) was done in the eve of each fertilization. Depending on the female weight, the concentration of HCG ranged from 500 U to 700 U. This allowed oocyte maturation, an essential step in the reproduction. The next day, slight pressure was applied on the ovaries to help the laying, and eggs were collected.

Immediately, oocytes were placed in contact with sliced testicles, in the different exposure concentrations of contaminants. Male gonad explants were obtained every week, from an anesthetized *Xenopus* with a solution of MS222 (2 mg L^{-1}; 3-aminobenzoic acid ethyl ester methane sulfonate).

The media were slowly shaken to allow the fertilization during 10 min. Next, in order to weaken the jelly coat, enclosing eggs were bathed in a solution of L-cysteine (2%) with or without contaminants for 10 min. Then the egg in each condition was rinsed 3 times with its respective exposure solution.

2.5. Records

Thirty-five minutes after fertilization, pictures were taken to determine the fertilization rates. Only the ponds that reached a success rate of 0.5 of fertilization in the control condition were considered in the calculation of successful fertilization rates. Fertil-

Table 2
Percentages of fertilizations reaching the stages 2, 3 or 4.

Exposed gametes		Exposure conditions (mg L^{-1})		n	Fertilization reaching the stages			
Oocytes	Spermatozoa	$CdCl_2$	$PbCl_2$		1	2	3	4
X	–	25	–	3	100%	0%	0%	0%
–	X	25	–	3	100%	66.67%	66.67%	66.67%
X	X	0.25	–	4	100%	75%	75%	75%
X	X	2.5	–	13	100%	100%	100%	88.89%
X	X	25	–	14	100%	85.71%	78.57%	78.57%
X	X	–	0.25	7	100%	100%	100%	100%
X	X	–	2.5	12	100%	100%	100%	100%
X	X	–	25	11	100%	100%	100%	100%
X	X	2.5	2.5	4	100%	100%	100%	100%
X	X	25	25	4	100%	100%	100%	100%

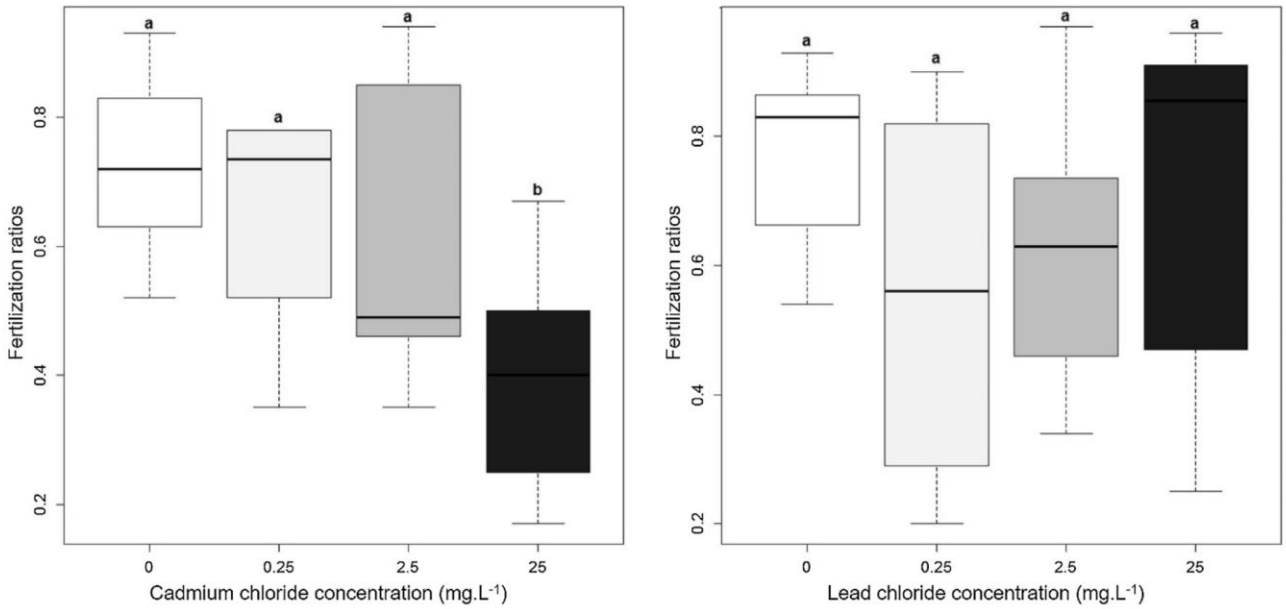

Fig. 1. Effects of cadmium (A) and lead (B) chloride on the fertilization success (fertilization ratios). Photographical analyses were performed 35 min after fertilization, to assess them in control media (MMR solution) containing increasing concentrations of $CdCl_2$ (A) or $PdCl_2$ (B) (0–0.25–2.5–25 mg L^{-1}). Results are expressed as boxplot and compared to others using Kruskal-Wallis rank sum test ($p < 0.05$). $A/3 \leq N \leq 20$ and $3 \leq n \leq 19$; $B/4 \leq N \leq 14$ and $4 \leq n \leq 13$.

ization was observed thanks to the cortical reaction, which causes the embryo to rotate according to gravity. Thus, vegetative hemispheres faced the ground while the animal poles were facing up. Any cell exhibiting such rotation was considered as a zygote, resulting from correct fertilization.

Then the occurrence times of the first steps of the *Xenopus* embryogenesis segmentation was recorded. Thanks to the large size of zygotes, we could distinguish the different stages of embryogenesis (Nieuwkoop and Faber, 1994) without interrupting its progress.

2.6. Gamete pre-incubation

Oocytes and pieces of testis were separately pre-incubated with a 25 mg L^{-1} cadmium solution for 7 min. After 3 bathes in MMR 1/10, they were associated with their unexposed gamete pairs in this control media.

2.7. Statistical analysis

All statistical analysis and graphical representations were carried out with the software R (The R Foundation for Statistical Computing, 2013). The significant differences were assessed perfoming Kruskal-Wallis tests and multiple comparison of treatments, Friedman tests and multiple comparison of treatments for the fertilization analysis and Wilcoxon Rank Sum and Signed Rank tests for the delay of the appearance of stages. On the boxplot figures, the presence of the same letter indicates no significant difference between conditions ($p > 0.05$).

3. Results

3.1. Fertilization

First, we assessed the fertilization success after metal exposures. Gametes were exposed, during the fertilization, to different concentrations of Cd and/or Pb. After 35 min of exposure, eggs were pictured and fertilization success monitored. Cadmium exposure had a significant negative impact on this reproductive success feature at 25 mg L^{-1} ($p < 0.001$), strongly decreasing this reproductive parameter at this higher concentration (from 0.748 to 0.401) (Fig. 1A). Moreover, the fertilization success decreased in a concentration-dependent manner with the cadmium exposure concentration from the 0.25 mg L^{-1} condition ($p < 0.05$) and the 2.5 mg L^{-1} one ($p < 0.01$) to the 25 mg L^{-1} exposure condition (Fig. 1A).

For lead exposures, no difference was ever observed (Fig. 1B). Regarding the co-exposures (cadmium and/or lead, paired experiment, 2.5 mg L^{-1} of each metal chloride), the fertilization success was significantly higher in the control condition than after the $PbCl_2$ alone condition ($p < 0.05$) or than after the metal co-exposure ($p < 0.05$) (Fig. 2A). For the 25 mg L^{-1} of $CdCl_2$ and $PbCl_2$ co-exposure, the fertilization success was significantly higher in the control and $PbCl_2$ conditions than in the two others ($p < 0.001$); no difference was observed between these two last ones, *i.e.* cadmium and the Cd and Pb binary co-exposure (Fig. 2B).

To discriminate whether the oocytes or the spermatozoa or both were impacted, in a second set of experiments, germ cells were pre-exposed separately before the induction of the fertilization in a control medium. As shown in Fig. 3, oocytes were more sensitive to cadmium exposure than spermatozoa ($p < 0.001$); although exposure of spermatozoa to cadmium also decreased the fertilization success signfantly ($p < 0.001$).

3.2. Segmentation

Next to the fertilization, embryos, from the fertilized eggs exposed to metal ions, were allowed to develop in control or contaminated media. Stages up to stage 4 (8 cells) were analyzed by recording the time of occurrence of each stage. Results obtained for cadmium, lead and binary mixture exposures are presented in Figs. 4 and 5. Cadmium exposure delayed the development, especially the appearance of the stage 3 for the 2.5 mg L^{-1} concentration ($p < 0.05$) and of the stages 2 and 4 in case of the 25 mg L^{-1} concentration ($p < 0.05$) (Fig. 4A). However, when the fertilization was successful, the embryogenesis generally progressed normally (Table 2). No difference was observed in case of lead and cadmium and lead exposures (Figs. 4 B and 5).

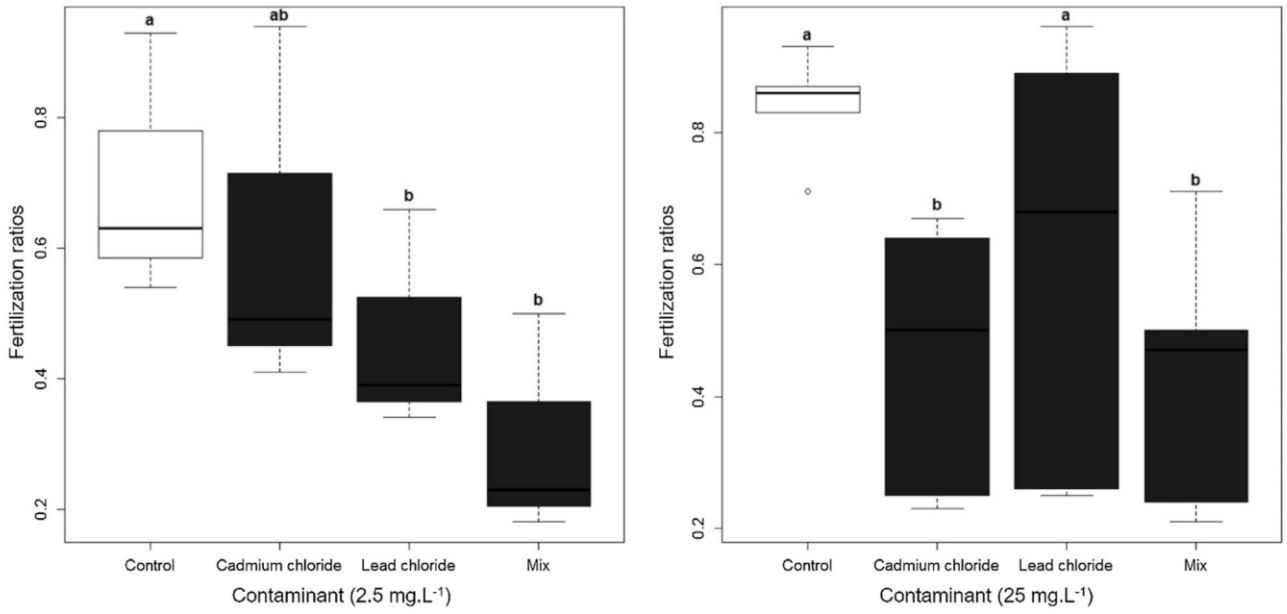

Fig. 2. Effects of low concentration (A) and high concentration (B) of cadmium chloride and lead chloride and their mixture on the fertilization success (fertilization ratios). Photographical analyses were performed 35 min after fertilization, to assess them in control media (MMR solution) containing 2.5 mg L^{-1} of $CdCl_2$, 2.5 mg L^{-1} of $PdCl_2$ or 2.5 mg L^{-1} of both metals (A) or 25 mg L^{-1} of $CdCl_2$, 25 mg L^{-1} of $PdCl_2$ or 25 mg L^{-1} of both metals (B). Results are expressed as boxplot and compared to others using Friedman rank sum test ($p < 0.05$). A/N = 3 and n = 3; B/N = 5 and n = 4.

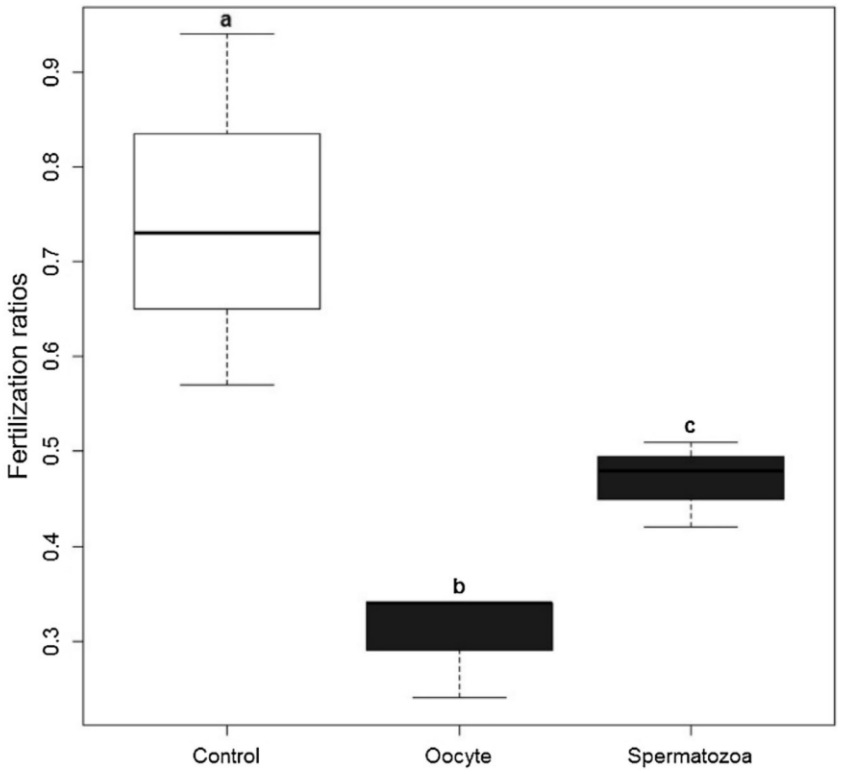

Fig. 3. Effects of high concentration of cadmium chloride on the fertilization success (fertilization ratios) of pre-incubated gametes. Oocytes or spermatozoa were exposed to 25 mg L^{-1} of $CdCl_2$ during 7 min before rinsing (thrice) and then mixing of gametes. Photographical analyses were performed 35 min after fertilization. Results are expressed as boxplot and compared to others using Friedman rank sum test ($p < 0.001$). N = 3 and n = 3.

When the oocytes were exposed to cadmium (25 mg L^{-1} concentration) before the fertilization, no embryos reached the stage 2 of embryogenesis (Table 2).

4. Discussion

In a context of global pollution and amphibian decline, the aim of our work was to assess and describe the effects of cadmium and lead

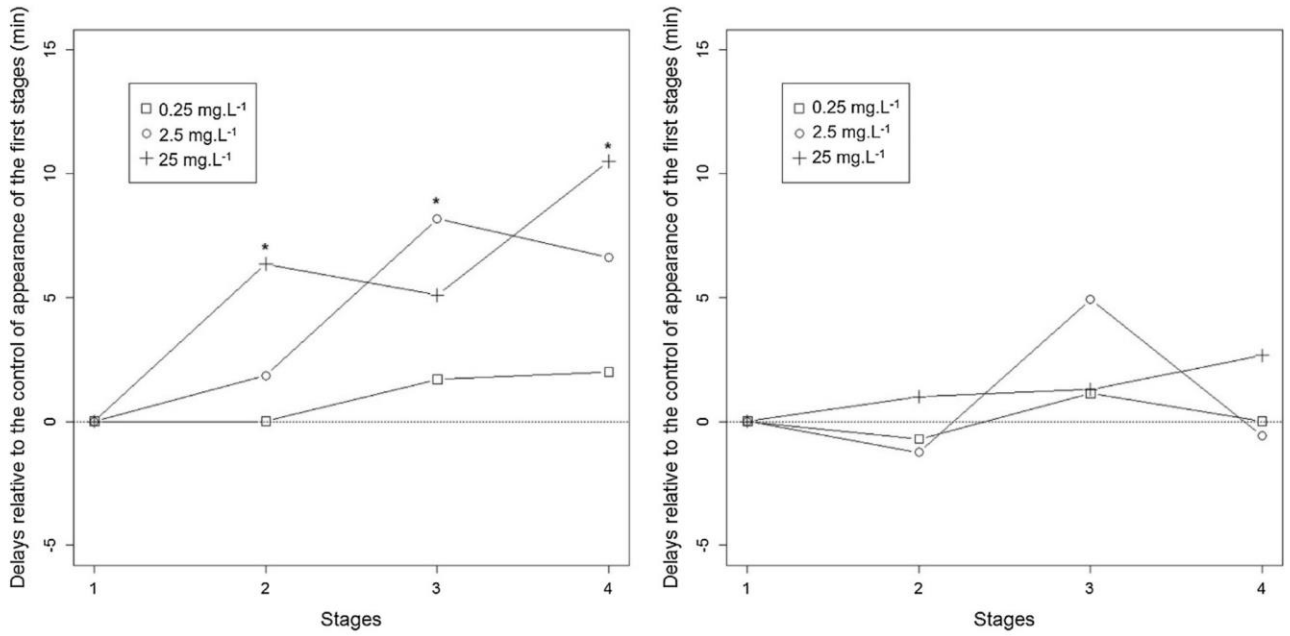

Fig. 4. Effects of cadmium (A) and lead (B) chloride on the occurrence times of the *X. laevis* embryogenic stages 1, 2, 3 & 4. Delays relative to the control condition are expressed in minutes. The fertilizations were exposed to increasing concentrations of $CdCl_2$ (A) or $PbCl_2$ (B) (0.25–2.5–25 mg L^{-1}). Results are compared to the control condition using Wilcoxon Rank Sum and Signed Rank test (*$p < 0.05$).

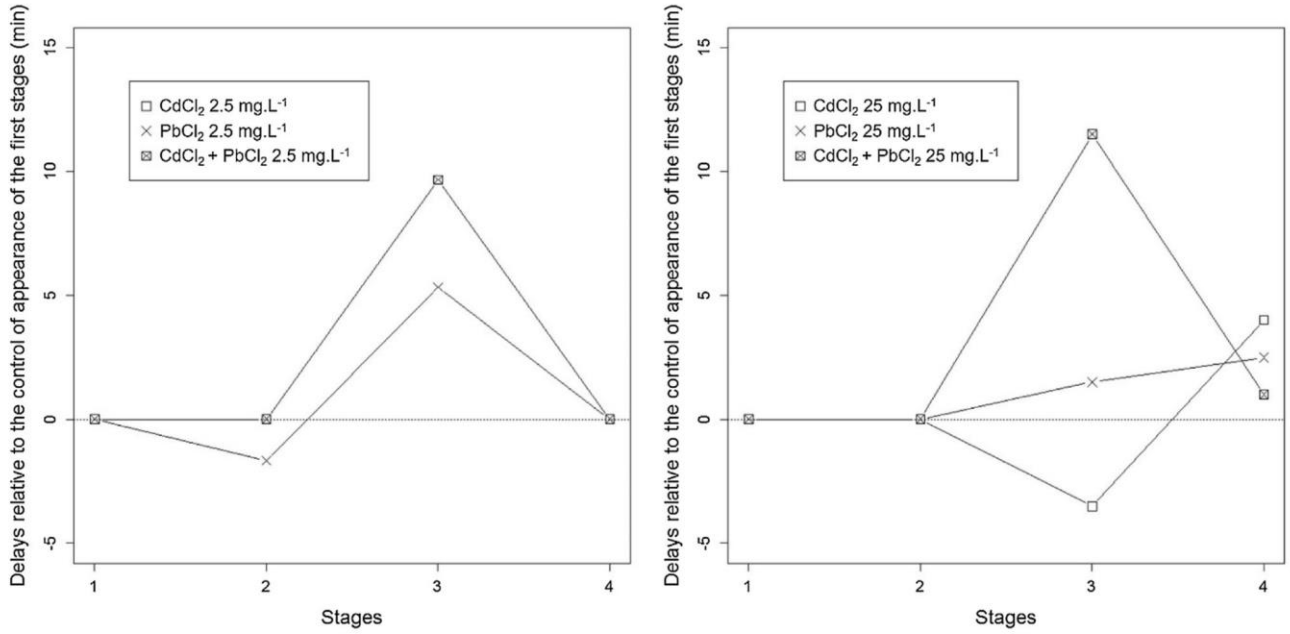

Fig. 5. Effects of low concentration (A) and high concentration (B) of cadmium chloride and lead chloride and their mixture on the occurrence times of the *X. laevis* embryogenic stages 1, 2, 3 & 4. Delays relative to the control condition are expressed in minutes. The fertilizations were exposed to solutions containing 2.5 mg L^{-1} of $CdCl_2$, 2.5 mg L^{-1} of $PdCl_2$ or 2.5 mg L^{-1} of both metals (A) or 25 mg L^{-1} of $CdCl_2$, 25 mg L^{-1} of $PdCl_2$ or 25 mg L^{-1} of both metals (B). Results are compared to the control condition using Wilcoxon Rank Sum and Signed Rank Test.

on the early stages of *Xenopus laevis* embryogenesis. This study represents the logical continuation of our previous works, where we observed that whereas cadmium exposure had an adverse impact on the ability of oocytes of *Xenopus laevis* to restart meiosis, in contrast to lead exposure (Marin et al., 2015). Here, our experiments involved conducting fertilization in metal-contaminated media and following the exposure during the embryo development. We observed that, unlike lead exposures, cadmium exposures drove drastic decreases of the fertilization success, correlated to the tested concentrations. Furthermore, when gametes were pre-incubated separately, we observed that cadmium decreased fertilization success significantly. However, in experiments where oocytes were exposed alone to cadmium (and then rinsed before fertilization in control condition), the fertilization success was significantly lower than in similar experiments with spermatozoa. As for oocyte maturation (Marin et al., 2015), we did not record additive or antagonistic effect after co-exposure to the mixture of the two metal chlorides; in fact results were similar to those obtained after single cadmium exposures. Then the first steps of embryogenesis, *i.e.* the segmentation cleavages, were also examined; due to their large

sizes, allowing stereomicroscope observations, development of *Xenopus laevis* is well-characterized (Nieuwkoop and Faber, 1994). We observed that cadmium delayed the embryogenesis of *X. laevis*. Furthermore, when oocytes were bathed in cadmium solutions before the beginning of the fertilization, embryogenic development did not reach the stage 2, although fertilization occurred.

In contrast to the majority of environmental toxicological studies using *Xenopus laevis*, metal exposures were conducted here from the first steps of fertilization and continued all along early development. These experimental designs embodied a new kind of test. *Xenopus laevis* is mainly used, through in the FETAX test, to assess teratogenic potentials of solutions, compounds or pollutants (Dawson and Bantle, 1987; Dumont et al., 1983; Plowman et al., 1994; Sunderman et al., 1991; Vismara et al., 1993). However, such tests use a few days old tadpoles and excludes fertilization and earlier development stages. Since it is crucial to understand why and how the pollutants could disturb amphibian reproduction and therefore their populations, it is essential to also consider these stages. They represent a true critical window of exposure, as gametes are directly exposed to any surrounding contaminants, and thus, vulnerable.

Few environmental toxicological or ecotoxicological studies have examined the effects of contaminants on amphibian gametes. We can, however, mention the development and validation of an inhibition of sperm motility test of *Xenopus laevis* using divalent zinc ion as reference substance (Christensen et al., 2004). Risley and Pohorenec developed a culture of explants of *Xenopus* testicles to study the formation of micronuclei (genotoxicity marker) and to screen potentially genotoxic agents on germ cells (Risley and Pohorenec, 1991). Concerning the oocytes, in their review, Venturino et al. reported that exposure of *Bufo arenarum* oocytes to dieldrin or azinphosmethyl could reduce the fertilization rate, increase the phosphoinositide turnover or block the response of phospholipase C (Venturino et al., 2003). They also mentioned that in *Xenopus laevis* oocytes, maitotoxin exposure (a phytotoxin of marine origin) caused cleavage of phosphatidylinositol 4,5-bisphosphate, precursor of inositol triphosphate (IP3) involved in the signalling of the fertilization process (Venturino et al., 2003). It has also been reported that cadmium chloride disturbed oogenesis of *Xenopus laevis*, increasing cellular death (Lienesch et al., 2000).

For many years, effects of contaminant exposures on reproduction have mostly focused on mammals and notably on the male reproductive system (Lienesch et al., 2000). From a medical point of view, Li et al. demonstrated that cadmium exposure could decrease semen volume, sperm motility and sperm morphology (Li et al., 2015), Xu et al. found that the concentration of cadmium in seminal plasma was negatively correlated to the sperm density and number, whereas changes in lead concentration did not affect the semen quality (Xu et al., 2003). Wildt et al. showed that men who are exposed to lead, presented a decrease in the stability of sperm chromatin (Wildt et al., 1983). In rat, cadmium also decreased the production of spermatozoa in a concentration-dependent manner and reduced or altogether prevented motility (Xu et al., 2001). The lack of data on the toxicological effects of contaminant exposures on female amphibian gametes led us to the present work. As cited above, cadmium chloride exposure disturbed or stopped the fertilization, preventing the first mitotic division from occurring. Our results demonstrated that, as spermatozoa (Xu et al., 2001), amphibian oocytes (Lienesch et al., 2000) and an early embryos could be considered as early and sensitive model to assess environmental contaminant effects.

Regarding the segmentation, the results were in accordance with studies reporting that cadmium induces the break of the actin cytoskeleton, by activating kinases in mouse mesangial cells (Liu and Templeton, 2010). Moreover, in a previous work, we observed that oocytes exposed to cadmium during maturation exhibited atypical structures compared to control oocytes: pronuclei, ectopic and disorganized spindles (data not shown) were seen. Such disturbance may account for the cell division delay observed in the present study, through the activation of cell cycle checkpoint, driving its arrest. Similarly, observations were also reported in *Hordeaum vulgare* and in *Vigna unguiculata*, where cadmium exposure decreased the frequency of cell division and caused chromosomal aberrations (Amirthalingam et al., 2013; Zhang and Yang, 1994). Considering the further development in the majority of cases, mortality wasn't the reason of the delays. These effects weren't observed with lead exposure. Then we can hypothesize that cadmium had a specific signature.

Concerning the binary mixture exposures, we didn't observe a joint effect of cadmium and lead. Results are similar to those obtained with cadmium alone. Since the metals are usually present concomitantly in the environment, effects of the co-exposures might have differed from those observed after single metal exposures. In contrast to our observation, most earlier studies have reported additive effects with frogs. For example, after co-exposures of *Xenopus laevis* tadpoles (stage 46) to lead and cadmium ions, Yologlu and Ozmen (2015) reported synergistic interactions of Cd and Pb mixtures at environmental concentrations on glutathione S-transferase, glutathione reductase and carboxylesterase activities, but not on acetylcholinesterase, glutathione peroxidase and catalase activities or on the MT levels. They only discussed the additive effects of metal binary mixtures and related them with results obtained in plants by Montvydiene and Marčiulioniene (Montvydiene and Marciulioniene, 2004). Another work reported additive effects as Cd and Pb interactions after frog exposures. Cooper and Manalis studied the evoked and spontaneous acetylcholine release in neuromuscular junction of *Rana pipiens* by means of endplate potential (EPP) amplitude and miniature endplate potential (MEPP) frequency, respectively (Cooper and Manalis, 1984). They conducted short-term *in vitro* exposures to 2 μM Pb^{2+} or Cd^{2+} and both. They observed additive interactions of the two metals on the EPP but Cd seemed to decrease Pb effects on MEPP. Thus, the authors hypothesized an inhibition of the entry of Pb in the terminal nerve through the calcium channels by Cd. It seemed that cellular availability of lead is affected by the concomitant exposure to cadmium.

Then, it appeared essential, in context of an environmental toxicological study, to consider all steps of the embryo development, from fertilization. Here we reported that cadmium exposure principally affected these early steps of *Xenopus laevis* embryogenesis. Conventional tests would have missed these disturbances. The other steps of embryo development, *i.e.* gastrulation and organogenesis, and also the first days of tadpoles, in contaminated environments are likewise essential to be investigated.

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3.2. Expositions au cuivre, au glyphosate et à leurs formulations commerciales

3.2.1. Effects of copper o the early development of Xenopus laevis: the case of $CuSO_4$ and Bordeaux mixture solutions (Article 6)

Le cuivre est un métal largement utilisé comme base de substances fongicides, algicides et bactéricides. Sa forme sulfatée entre notamment dans la composition de la bouillie bordelaise. Cette substance, autorisée en agriculture biologique, est principalement employée dans le traitement des vignes pour combattre le mildiou. C'est pourquoi, de fortes concentrations en cuivre sont souvent détectées dans l'environnement avoisinant ces cultures. Par ruissèlement, les eaux de surface sont contaminées et souvent de petits volumes d'eau temporaires ou non (mares, étangs, ruisseaux) accueillent d'importantes teneurs en contaminants. Or, ces milieux constituent généralement un espace privilégié de vie et de reproduction pour les amphibiens et où va se dérouler exclusivement leur cycle de vie depuis l'émission des gamètes jusqu'à la métamorphose. L'article nommé « *Effects of copper on the early development of Xenopus laevis: the case of $CuSO_4$ and Bordeaux mixture solutions* »[6], propose d'évaluer les effets du cuivre, sous forme de $CuSO_4$ et de bouille bordelaise sur les premières étapes de *X. laevis*, *i.e.* la maturation ovocytaire, la fécondation et le développement.

Les expérimentations ont été conduites dans des conditions contaminées au $CuSO_4$ ou à la bouillie bordelaise aux concentrations suivante : 0,00399 ; 0.0399 ; 0.399 ; 3.99 µM[Cu^{2+}]. (1) Tout d'abord, des cinétiques de reprise de méiose ont été réalisées. Pour cela des ovocytes immatures ont été exposés à la gamme de concentrations de nos contaminants en présence ou non de progestérone (hormone induisant *in vitro* la maturation). Un relevé des taux de GVBD, indice phénotypique indiquant la maturation, a été réalisé toutes les 15 minutes pendant 13 heures. (2) Les profils de phosphorylation de ERK2 et de RSK (cascade MAPK) et de Cdc2 (sous unité catalytique du MPF) et de H3 (une des cibles principales du MPF) ont été évalués par western blot dans des ovocytes exposés sur la nuit à 19 °C aux différentes formulations de cuivre avec ou sans progestérone. (3) Les succès de fécondation ont été déterminés par analyse photographique, 35 minutes après avoir mis en contact des ovocytes pondus et des spermatozoïdes. (4) Enfin, une étude du développement a été réalisée à l'aide

[6] *Titre : Effects of copper o the early development of Xenopus laevis: the case of $CuSO_4$ and Bordeaux mixture solutions*
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d'une coloration au bleu alcian et d'une méthode d'évaluation automatisée de la biométrie sur des têtards de 6 jours exposés depuis la fécondation. Dans cette étude, l'écart oculaire a été retenu comme critère d'analyse.

(1) En présence de l'hormone, il est apparu que la reprise de méiose n'est pas affectée par les expositions. En revanche, en l'absence de progestérone, les ovocytes immatures exposés à la plus forte concentration de cuivre dans les deux formulations ont présentés des maturations spontanées (non induites par une hormone).

(2) Les états de phosphorylation des protéines étudiées n'ont pas montré de différence avec les conditions témoins. La cascade MAPK et le MPF ne semblent pas affectées par les expositions au cuivre.

(3) De même, les expositions au $CuSO_4$ ou à la bouille bordelaise ne provoquent pas de variation du taux de fécondation.

(4) Concernant le développement des têtards de 6 jours, une augmentation de leur taille a pu être détectée. En effet, les individus exposés depuis la fécondation à la plus forte concentration de $CuSO_4$ ont présenté des écarts oculaires significativement plus important que ceux de la condition témoin.

En comparaison avec les expositions au cadmium, celles au cuivre, sous ses deux formulations, montrent certaines différences. Tout d'abord, elles ne provoquent pas de diminution du taux de GVBD en présence de l'hormone. Ensuite, même si nous avons observé des maturations spontanées, elles étaient différentes de celles dans les conditions exposées au cadmium. En effet, dans ce travail les cinétiques ont montré qu'elles arrivaient simultanément à celles induites par la progestérone dans les conditions témoin et contaminées contenant l'hormone, suggérant que des mécanismes différents de ceux supposés dans nos études sur les expositions cadmium soient impliquées. De plus, contrairement au cadmium, la cascade MAPK et le MPF ne semblent pas affectés par les expositions au cuivre.

Ces résultats montrent que les premières étapes du cycle de vie de *X. laevis* peuvent être perturbées par des expositions au cuivre. Du fait, du manque de connaissances en toxicologie des amphibiens sur les effets de contaminants sur les étapes précoces de leurs cycles de vie, il apparait important de considérer ces moments sensibles.

Effects of copper on the early development of Xenopus laevis: The case of $CuSO_4$ and Bordeaux mixture solutions

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Keywords: *Xenopus laevis*; oocyte; tadpole; maturation; fertilization; copper; Bordeaux mixture

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1. Introduction

Copper is an essential metal for the organism but it can be toxic at high concentrations (1,2). This metal is a part of several crop-protecting fungicides, algaecides and bactericides. Allowed in organic agriculture in Bordeaux mixture formulation ($CuSO_4$ and slaked lime), it is widely used to treat grapevine mildew (3). Copper concentrations in French wine-producing regions soils and water are indeed higher. In water, copper has been detected at levels of 2.88 $\mu g.L^{-1}$ in the Riou Mort river, 1.25 $\mu g.L^{-1}$ in the Lot river and 1.4 $\mu g.L^{-1}$ in the Garonne (4). In the soils of wine-producing Nouvelle Aquitaine region, copper concentration can be found at 508 $mg.kg^{-1}$ (5). This metal is also referenced in a chemical watchlist established by the United States Environmental Protection Agency (6). The continuous use of crop-protecting solution applications, among which copper solutions, is one of the main causes of amphibian decline (7). As a matter of fact, their life cycle is dependent on the quality of aquatic habitats, in which phytopharmaceutical products could eventually end up.

This study aims at defining the effects of $CuSO_4$ and Bordeaux mixture expositions on the early stages of *Xenopus laevis* development. In this purpose, oocyte maturation (time-courses and signaling pathways), fertilization success as well as development were assessed in Cu-contaminated conditions.

2. Materials and methods

Animal experiments were performed at the animal facility of the University of Lille in accordance with the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The protocol was approved by the local institutional review board (*Comité d'Ethique en Expérimentation Animale Hauts-de-France*, CEEA 07/2010). *Xenopus laevis* oocyte maturation was investigated by different protocols described in our previous study (8). Time-courses of the process were assessed under contaminant exposures (± progesterone) by recording the Germinal Vesicle Breakdown (GVBD) ratios every 15 minutes for 13 hours. The phosphorylation states of RSK ($p90^{RSK}$) and ERK2 from the Mitogen Activated Protein-Kinases (MAPK) cascade and Cdc2 and H3, the catalycal sub-unit of the M-Promoting Factor (MPF) and one of its principal target respectively, were evaluated in exposed oocytes by western blots. Then, fertilization success was determined following experimental design already conducted in previous work (9). To assess the fertilization success (% of fertilized eggs) photographic analyses were realized after 35 minutes of exposure. At last, developmental analysis were done using an automatic biometric data recording procedure (10). Here, distance between eyes were determined in 6-days old tadpoles exposed from the fertilization. $CuSO_4$ and Bordeaux mixture solutions were prepared weekly by dilution in ND96 (oocyte maturation) or in dechlorinated tapwater (fertilization and development). Concentrations used in the experiments are environmentally relevant and were derived from the environmental quality standards defined in France in the water framework directive context (3). The following range of concentrations has been tested: 0, 0.00399, 0.0399, 0.399 and 3.99 µM of Cu^{2+} contained in $CuSO_4$ and Bordeaux mixture solutions. Statistical analyses and graphical representation were performed with R software (Version 3.3.2; The R Foundation for Statistical Computing, 2016). The GVBD ratios were analyzed by Friedman's tests and post-hoc multiple paired comparison between conditions. Fertilization success and eye gap were assessed by Kruskal-Wallis tests and multiple paired comparison between treatments.

3. Results and discussion

The maturation process was assessed by kinetics approach with measurements taken at 15 min interval during 13 hours (Figure 1). The appearance of the white spot at the animal pole was checked. GVBD occurred at approximatively 165 min in controls and in contaminated conditions when gamete maturation was stimulated by progesterone (figure 1A & 1B). Same experiments were conducted without progesterone (figure 1C & 1D). In presence of the hormone, neither the $CuSO_4$ nor the Bordeaux mixture affected the maturation rate. Indeed, in all exposure conditions, the final rates of maturation ranged between 75 % and 90 %. These results did not differ from those obtained in the controls (without Cu^{2+}) or for lead exposures in a previous work (8). By contrast, in the absence progesterone, the highest concentration of copper in the both forms was able to induce the GVBD (figure 1C & 1D). These observations are called spontaneous maturations (up to 70 % of oocytes undergone GVBD). Previous work demonstrated same results when oocytes were exposed to cadmium or zinc ions (11). The time-course experiments provided also information on the beginning of maturation. Whatever the experimental conditions (with or without progesterone and with or without contaminant) the maturations began simultaneously (after 165 min of treatment). This suggests that the same molecular mechanisms or signaling pathways are involved in hormone-dependent maturation and spontaneous maturation induced by $CuSO_4$ or Bordeaux mixture exposures at the higher concentrations compare to what we observed after cadmium exposures ($CdCl_2$) where spontaneous maturation occurred much later than hormone-dependent ones (8).

Two major regulation pathways involved in the maturation process were also studied by immunoblotting: MAPK (ERK2 & RSK) and MPF (Cdc2 & H3) signaling pathways. No anomaly of phosphorylation patterns was detected after the exposures, whether the maturation was hormone-dependent or spontaneous (data not shown). These results differ from other studies concerning oocyte maturation of *Xenopus laevis*. Cadmium was shown to disrupt numerous phosphorylation steps in the MAPK pathway, in the activation of MPF and its activity (8).

In a second set of experiments, *in vitro* fertilizations were performed in increasing concentrations of $CuSO_4$ or Bordeaux mixture. After 35 minutes of exposure, the number of fertilized eggs was measured. The data revealed no effect of the both forms of Cu^{2+} on fertilization success (data not shown). Similar results have already been observed with lead. Indeed, $PbCl_2$ did not modify fertilization success (9).

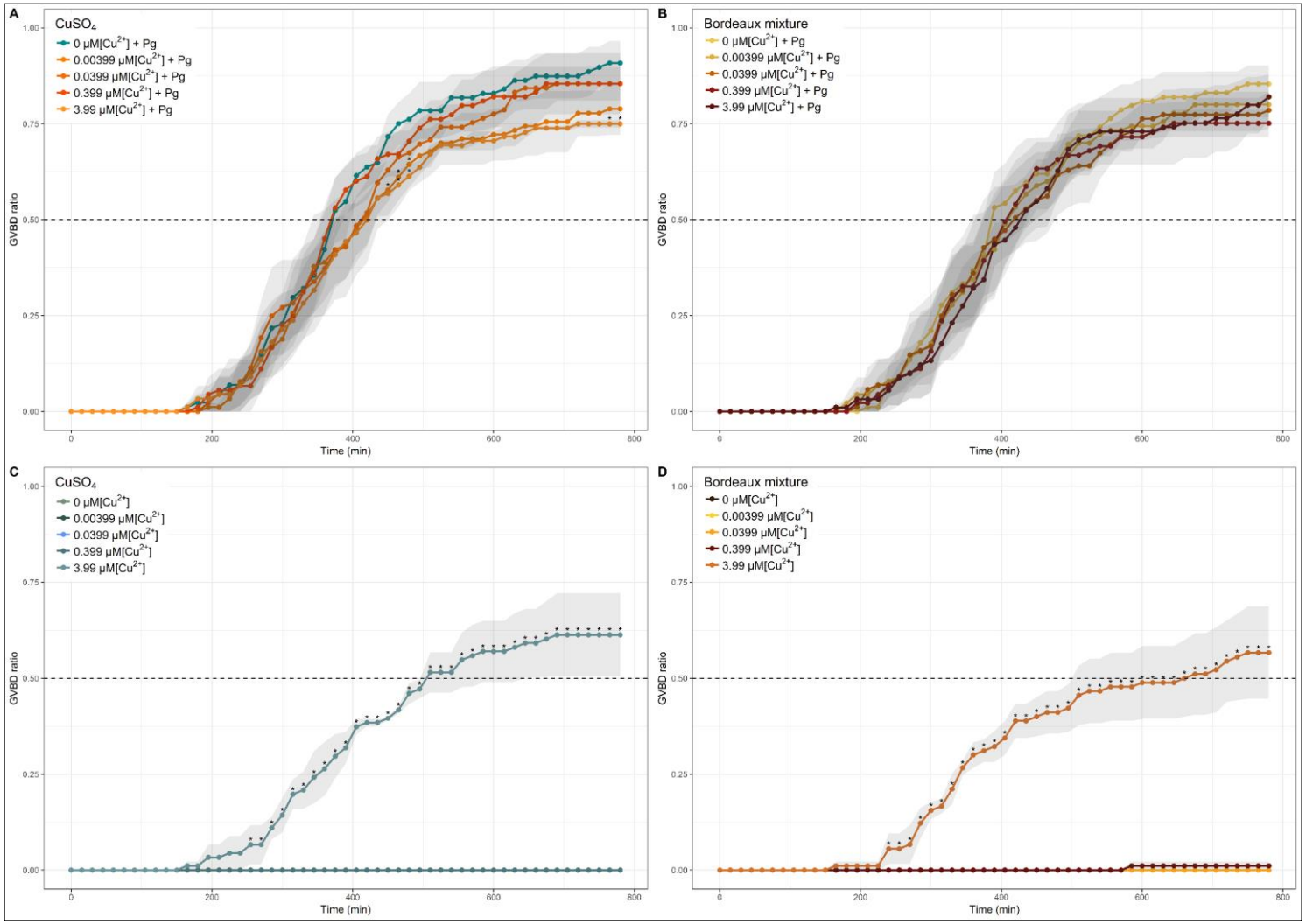

Figure 1. Effects of CuSO4 and Bordeaux mixture exposures on X. laevis oocyte's maturation. Oocytes were exposed to increasing concentrations of CuSO4 (A, C) or Bordeaux mixture (B, D) in presence (A, B) or not (C, D) of progesterone (Pg) for 13 h. Every 15 minutes, the maturation was assessed according to the white spot appearance. Results are expressed as mean ± SEM (grey areas) and compared to other treatments using Friedman rank sum test (*: $p < 0.05$).

In order to study the effects of $CuSO_4$ on the tadpoles' growth, after 6 days of exposure from the fertilization, tadpoles were fixed and stained in Alcian blue (10). Data for distance between the eyes (eye gap) are shown in figure 2 in embryos exposed to $CuSO_4$. At the highest concentration of $CuSO_4$ an increase in the ocular distance which suggest that the tadpoles became larger (or at least that the individual will have a larger head) was observed.

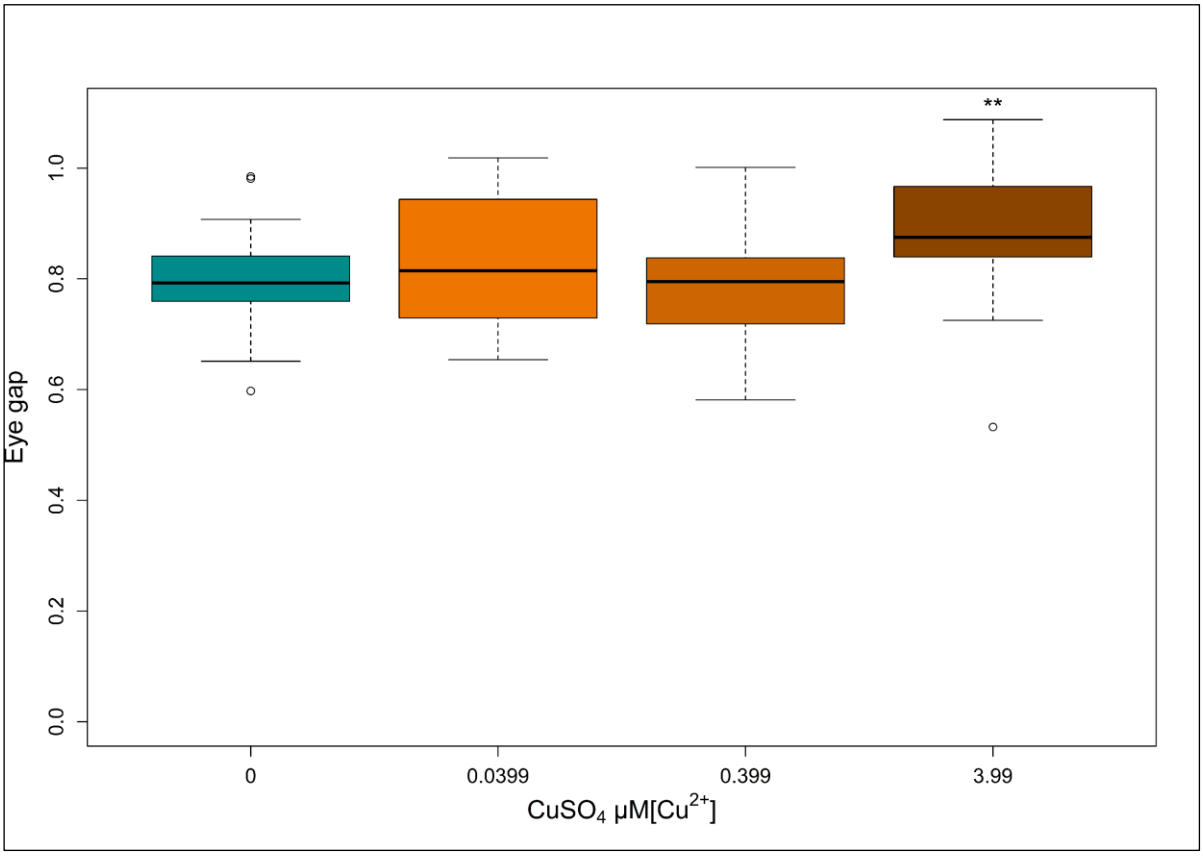

Figure 2. Effects of CuSO4 exposures on eye gap in 6-days old tadpoles. Tadpoles were exposed increasing concentrations of CuSO4 from the fertilization. After alcian blue staining, automatic image analysis was performed under Image J software (see Slaby et al., 2016 for details). Results are expressed as boxplots and compared to others using Kruskal-Wallis rank sum test (**: $p < 0.01$).

4. Conclusion

Although progesterone-stimulated maturation and fertilization ratio were not affected by copper exposures, these results showed that both forms of Cu^{2+} induced spontaneous maturation at the highest concentration and affected tadpoles' biometry. This study shows the importance of early development stages in amphibian toxicology because of their high sensitivity to contaminant at this early stage in life.

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3.2.2. Effects of glyphosate and a commercial formulation RoundUp® exposures on maturation of Xenopus laevis oocytes (Article 7)

Le glyphosate est un herbicide systémique à large spectre utilisé depuis les années 70. Parmi ses nombreuses variétés commerciales, le RoundUp® est certainement la plus connue. L'efficacité de cet herbicide sur nos cultures agricoles est incontestable mais il est difficile de nier les préoccupations en termes de santé humaine et d'écologie. Le Centre International de Recherche sur le Cancer a catégorisé le glyphosate dans le groupe 2A regroupant les cancérigènes probables. D'un point de vue environnemental, c'est la troisième substance la plus retrouvée dans les eaux de surface en France, sachant que la première est son principal produit de dégradation, l'acide aminométhylphosphonique. Cette contamination environnementale par de nombreux produits constitue une des raisons pour lesquelles les amphibiens sont menacés d'extinction. Beaucoup d'études ont été menées en toxicologie des amphibiens sur les effets du glyphosate, mais très peu s'intéressent aux expositions de stades précoces comme les gamètes. Cet article, intitulé « *Effects of glyphosate and a commercial formulation RoundUp® exposures on maturation of Xenopus laevis oocytes* »[7], propose d'évaluer les impacts de cet herbicide sous forme pure et sous une formulation commerciale nommée RoundUp® GT Max sur la maturation ovocytaire de *X. laevis*.

Tout comme nos études sur les métaux, nos investigations se sont concentrées sur des paramètres de la maturation à différents niveaux biologiques. Les expositions ont été menées aux concentrations suivantes de glyphosate ou de RoundUp® : 0,148 ; 1,48 ; 14,8 ; 148 ; 1480 µM[a.e.]. (1) Des suivis temporels des maturations ovocytaires en conditions contaminées avec ou sans progestérone ont été réalisés. Les modalités de ces cinétiques consistaient à évaluer les taux de GVBD toutes les 15 minutes pendant 13 heures. (2) La morphogénèse du fuseau de division a également été examinée en microscopie optique dans des ovocytes avec une tache de maturation exposés sur la nuit à 19 °C après ajout de progestérone. (3) Enfin, les profils de phosphorylation de ERK2 et de RSK (voie MAPK) et de Cdc2 (sous unité catalytique du MPF) et de H3 (une des cibles principales du MPF) ont été évalués par western blot.

(1) Un retard du processus de maturation ovocytaire a été observé dans les conditions contenant la plus forte concentration des deux formes de glyphosate. Néanmoins, cette

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différence n'a été significative que dans les expositions au glyphosate pur. Aux concentrations 14,8 et 148 µM de glyphosate pur, des taux de GVBD significativement plus faibles ont été détectés pendant 30 minutes mais ces retards ont été rattrapé ensuite en cours de cinétique et jamais observés à nouveau. En l'absence de progestérone, des maturations spontanées ont été détectées chez les ovocytes exposés aux deux formes de l'herbicide.

(2) Une augmentation de l'occurrence d'anomalies dans la mise en place du fuseau méiotique a été observée dans les conditions contenant les plus fortes concentrations pour les deux types de solutions (substance active pure ou en formulation). Concernant les types de malformations observées, des signatures des expositions ont été observées. Ces particularités étaient la présence de double structure cytologique.

(3) Aucun profil de phosphorylation anormal des protéines étudiées n'a été observé dans les ovocytes exposés. Cela suggère que la voie MAPK et le MPF ne sont pas affectés.

Il apparait que des mécanismes différents de ceux supposés dans nos études sur les expositions au cadmium soient mis en jeu pour expliquer les maturations spontanées. Ce constat est établi étant donné que, dans cette étude, elles sont induites au même moment que les maturations hormono-dépendantes et que ni la voie MAPK, ni le MPF ne semblent affectés par les expositions. Mais une étude plus approfondie des mécanismes est nécessaire pour confirmer cette hypothèse. Au niveau cytologique, des structures jamais observées en condition témoin ont été détectées suite aux expositions. La présence d'une vésicule germinale intacte a déjà été mise en évidence après des expositions au cadmium dans des ovocytes présentant également une tache de maturation. Dans ce cas, cette structure est montée vers le pôle animal, expliquant l'apparition d'une zone claire comparable à une tache de maturation. Il est à noter qu'aucune rupture de l'enveloppe n'est observée. Concernant les doubles structures, ces dernières n'ont, historiquement, jamais été observées lors de nos précédentes expositions. D'autres auteurs suggèrent une séparation du fuseau méiotique en deux parties, amenant ainsi deux nouveaux fuseaux. Les résultats de cette étude cytologique suggèrent des effets génotoxiques associés à la morphogénèse du fuseau plutôt qu'à des cassures à l'ADN.

Le nombre d'études s'intéressant aux gamètes en toxicologie des amphibiens est très limité. Pourtant, le moindre effet à ce moment pourrait avoir des effets critiques et empêcher la reproduction. Ce travail apporte de nouvelles données sur les effets du glyphosate et de l'une de ses formes commerciales sur la maturation ovocytaire. Ces dernières, associées aux nombreux avantages apportés par l'ovocyte de xénope dans la conduite des expérimentations, confirment l'intérêt de considérer cette cellule germinale comme modèle pour évaluer la qualité de solutions aqueuses en toxicologie de l'environnement.

Effects of glyphosate and a commercial formulation RoundUp® exposures on maturation of *Xenopus laevis* oocytes

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Abstract

Pesticides are often found at high concentrations in small ponds near agricultural field where amphibians are used to live and reproduce. Even if there are many studies on the impacts of phytopharmaceutical active ingredients in amphibian toxicology, only a few are interested in the earlier steps of their life cycle. While, their populations are highly threatened with extinction. Aim of this work is the characterization of the effects of glyphosate and its commercial formulation RoundUp® GT Max on the *Xenopus laevis* oocyte maturation which is an essential preparation for the laying and the fertilization. Glyphosate is an extensively used herbicide, recognized for its effectiveness but also for its indirect impacts on non-target organisms. Our results showed that exposures to both forms of glyphosate delayed this hormone-dependent process and were responsible for spontaneous maturation. Severe and particular morphogenesis abnormalities of the meiotic spindle were also observed. The MAPK pathway and the MPF did not seem to be affected by exposures. The xenopus oocyte is particularly affected by the exposures and appears, as a relevant model for assessing the effects of environmental contamination.

Keywords: Amphibian toxicology; Glyphosate; RoundUp; Pesticide; Oocyte; Maturation; Xenopus

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1. Introduction

Since the 70's, glyphosate (*N*-(phosphonométhyl)glycine, $C_3H_8NO_5P$) is recognized as a potent herbicide but it was initially patented for its metal chelator properties. Its first commercialization was in 1974 under the name of RoundUp® (Duke and Powles 2008). The glyphosate is a broad-spectrum systemic herbicide, which acts through leaves and inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate pathway essential for the amino acid synthesis (Martinez et al. 2018). There are various commercial formulations of glyphosate. They differ from the proportion of active ingredient (a.i.) inside and the different additives or surfactants, as polyethoxylated tallow amine (POEA), adding to improve the efficiency of treatment (Martinez et al. 2018). The International Agency for Research on Cancer (IARC) has classified glyphosate in the group 2A as a probable carcinogen to humans (IARC 2017). There are also major concerns on the impacts of this herbicide substance in the environment. It is highly soluble in water and it is the third pesticide found in the surface water in France, knowing that the first is the aminomethylphosphonic acid (AMPA) (French Minister for the Ecological and Inclusive Transition 2017), its principal degradation product (Rueppel et al. 1977). In aquatic environments the concentrations vary according the area and the season. For example, glyphosate concentration was detected at 1.082 $\mu g.L^{-1}$ in Boële river (France) (Botta et al. 2009), 8.7 $\mu g.L^{-1}$ in Midwestern United States streams (Battaglin et al. 2005), 40.8 $\mu g.L^{-1}$ in surface waters of Southern Ontario (Struger et al. 2008), but can reach approximatively 0.1 to 0.7 $mg.L^{-1}$ in Argentina culture areas (Peruzzo et al. 2008).

Amphibian is uncontestably the group the most threatened with 41 % of known species concern by the risk of extinction (Monastersky 2014). Pollution, destruction of habitats, climate change, invasive species, human uses, diseases and parasitism are known to be primary or interacting causes of the decline (Blaustein and Wake 1990, 1995; Alford and Richards 1999; Blaustein et al. 2003; Alford 2010). Due to their particular life cycle, amphibians are especially affected by environmental contaminations. Their biological particularities, as aquatic external fertilization, no shell-eggs and metamorphosis, make them very vulnerable and dependent those water ecosystem quality (Sparling et al. 2010). Small ponds or ditches, likely to receive in agricultural or industrial areas high levels of xenobiotics, are known to support their life and reproduction (Bridges and Boone 2003).

Various effects have been described in amphibian species exposed to glyphosate or its commercial formulations. Serious skin morphology changes and reduced oxygen uptakes were observed in *Rana catesbeiana* tadpoles exposed 96 hours to 1 $mg.L^{-1}$ of glyphosate (pure or acid equivalent (a.e.) in RoundUp Original® or in RoundUp Transorb R®) (Rissoli et al. 2016). *Xenopus laevis* tadpoles exposed from the stage 8 to 46 (Nieuwkoop and Faber 1967) to glyphosate (> 30 $mg.L^{-1}$) showed cardiac edemas while those exposed to RoundUp® Power 2.0 at a range of concentrations between 1 and 25 $mg[a.e.].L^{-1}$ exhibited in a dose-dependent manner critical morphological alterations as disappearance of the upper and lower jaws, edemas or gut miscoiling (Bonfanti et al. 2018). AChE, BChE, GST and CbE activities decreased in *Bufo arenarum* tadpoles exposed to different glyphosate-based formulations C-K Yuyos FAV®, Glifoglex®, Infosato® or RoundUp® Ultra-Max at concentrations higher than 1.85 $mg[a.e.].L^{-1}$ for 48 hours (Lajmanovich et al. 2011).

Overall in amphibian toxicology, the effects of xenobiotic exposures on gametic stages are still scarce. Incidences at this level would affect consequently the reproduction, future individual fitness and thus population viability. This work proposed to characterize impacts of glyphosate and its commercial formulation RoundUp® GT Max on the oocyte maturation process of *X. laevis*. After a hormonal induction, females produce mature oocytes which are blocked in metaphase of meiosis II and prepared for ovulation and fertilization (Ferrell 1999). Then, at the animal pole of the cell, a mature oocyte will exhibit a white spot (WS) caused by the Germinal Vesicle Breakdown (GVBD) and the formation of the meiotic spindle supporting condensed chromosomes lined up on the metaphasic plate. The oocyte maturation is enhanced by different signaling pathways. The activation of the Mitogen-Activated Protein Kinases (MAPKs) cascade involves successive phosphorylations of protein kinases such Extracellular signal-Regulated Kinase 2 (ERK2) and p90 kDa Ribosomal S6 Kinases (RSK) (Ferrell 1999; Frödin and Gammeltoft 1999). This phosphorylation cascade is associated with the activation of the M-Promoting Factor (MPF) which promotes the M-phase entry. The MPF is a complex composed of a catalytic subunit (Cyclin-Dependent Kinase 1; Cdc2) and a regulatory unit (Cyclin B) and its activation requires of the dephosphorylation of Cdc2 (Norbury and Nurse 1990; Ferrell 1999). The serine 10 histone H3 is one of the principal targets of the MPF. Its phosphorylation is a conserved histone modification essential to the condensation of chromosomes (Hendzel et al. 1997; Van Hooser et al. 1998; Hans and Dimitrov 2001). In the present work the oocyte maturation was studied by time-courses experiments, cytological analyses of the meiotic spindle morphogenesis and assessments phosphorylation states of ERK2, RSK Cdc2 and H3, as previously described in our previous work with cadmium and lead as contaminants (Slaby et al. 2017).

2. Material and methods

2.1. Chemicals

All chemical compounds were obtained from Sigma-Aldrich Chimie® (France), except tricaine mesylate (MS222; Honeywell Fluka®, France), glyphosate (GLY; Molekula Ltd, UK) and RoundUp® GT Max (RUp; Monsanto Europe, Belgium; French local reseller). RUp contains 588 $g.L^{-1}$ (43.78 % w/w) of potassium salt of glyphosate, equivalent to 480 $g.L^{-1}$ of glyphosate acid (35.74 % w/w). Contaminants were daily diluted in the weekly prepared control medium ND96 (Nathan Dascal 96: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, pH 7.5). Concentrations of GLY and RUp used in the experiments are expressed in acid equivalent: 0.148, 1.48, 14.8, 148, 1480 µM. This concentration range frames the environmental quality standards (EQS) defined in France in the Water Framework Directive context (INERIS 2014). These values are 28 $µg.L^{-1}$ (0.166 µM) and 70 $µg.L^{-1}$ (0.414 µM). They correspond respectively for freshwater to the Annual Average concentration-EQS and to the Maximal Acceptable Concentration-EQS.

2.2. Handling of Xenopus laevis oocytes

Animals used came originally from the University of Rennes 1 and were maintained in control tanks (XenopLus − Amphibian Housing System from Techniplast) in the animal house of our campus (ULille Sciences et Technologie, Villeneuve d'Ascq, France). Adults were fed with floating expanded pellets (Aquatic 3, Special Diets Services) *ad libitum*. Water quality parameters were controlled and a daily water removal was done. All animal experiments were conducted at the campus animal facility according to the rules of the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The animal protocol was approved by the local institutional review board (Comité d'Ethique en Experimentation Animale Nord-Pas-de-Calais, CEEA 07/2010). Collection of xenopus oocytes were weekly realized by ovariectomy after the anesthesia of the female in a bath of tricaine mesylate (1 $g.L^{-1}$) for 45 minutes. Collected oocytes were treated with collagenase solution (1 $g.L^{-1}$) for 45 minutes. Remaining follicular cells were then removed manually with tweezers. Only stage VI oocytes (Dumont 1972) were selected and stored in ND96 medium at 14 °C before the experiments beginning. Oocyte maturation was *in vitro* induced by progesterone (Pg; 4 $mg.L^{-1}$).

2.3. GVBD time-courses

Kinetics of the GVBD was conducted monitoring the appearance of the WS at the top of the exposed gametes. For each concentration of GLY or RUp, 30 stage VI oocytes (Dumont 1972) were exposed with or without Pg (4 $mg.L^{-1}$), at 19°C. The GVBD ratios were assessed every 15 minutes for 13 hours from the beginning of the exposures. Time-courses were repeated 3 times for GLY and 4 times for RUp exposures.

2.4. Meiosis spindle formation analyses

Oocytes were exposed to GLY or RUp in presence of Pg overnight at 19 °C. Only germ cells exhibiting the WS were sampled. Fixation were conducted overnight in Smith reagent (80 % Smith A: Potassium Bichromate 17 mM, 20 % Smith B: formol and acetic acid) in the dark at room temperature. Then, oocytes were dried, paraffin-embedded and sliced with a microtome (7 µm slice thickness). Nuclear structures, chromosomes and cytoplasmic structures were revealed thank to a nuclear red (0.1 g of nuclear red QSP in 100 ml 5 % aluminium sulphate) and picro-indigo-carmine (0.25 g of picro-indigo-carmine QSP in 100 ml saturated picric acid) stainings. Slices mounted on slides were analyzed by optical microscopy.

2.5. MAPK and MPF activity investigations

Oocytes exposed overnight to GLY or RUp with or without Pg at 19°C were used. For each concentration, WS oocytes and non-WS oocytes were sampled separately. Electrophoresis and western blotting were conducted according to the protocol previously described by our lab (Gelaude et al. 2015). Briefly, after the lysis of pooled oocytes in the homogenization buffer following by a centrifugation (13.8g, 4 °C, 15 min), the proteic part was extracted. Supernatants were added to one volume of Laemmli 2X buffer with 4 % beta-mercaptoethanol. Samples were heated at 75 °C for 5 minutes and stored at −20 °C before SDS-PAGE (15 % Chesnel modified). Then transfer was done onto a nitrocellulose membrane (Hybond, Amersham Pharmacia Bio- tech, UK). Immunoblots were saturated with 5 % low fat dry milk and incubated overnight with specific antibodies. Rabbit polyclonal antibodies were used to detect $p90^{Rsk}$ (p90Rsk-1 C-21 sc-231 antibody; Santa Cruz Biotechnology®, USA), pCdc2 (Phospho-Cdc2 (Tyr15) antibody; Cell Signaling Technology®, Netherlands) and pH3 (Phospho-Histone H3 (Ser10) antibody; Cell Signaling Technology®, Netherlands). Mouse monoclonal antibody was used to detect ERK2 (Erk2 D-2 sc-1647 antibody; Santa Cruz

Biotechnology®, USA). After incubation with the appropriate secondary antibodies (Sigma-Aldrich®, France), chemiluminescent revelations (Clarity™ Western ECL Substrate; Bio-Rad®, USA) were conducted according to manufacturer's instructions. Every phosphorylation states were assessed at least 3 times with pools of oocyte from different females.

2.6. Statistical analysis

All statistical analyses and graphical representations were conducted with R software (version 3.3.2; The R Foundation for Statistical Computing, 2016). The GVBD ratios from the time-courses were compared performing Friedman's tests and post-hoc multiple paired comparisons between conditions. Cytological data were analyzed using Kruskall Wallis' tests and post-hoc multiple paired comparisons. For all experiments, N refers to the number of replications (used females) and n to the number of oocytes per exposure concentrations.

3. Results

3.1. GVBD time-courses

Kinetics of the maturation process were assessed by determining GVBD ratios every 15 minutes for 13 hours. Figure 1A presents the GVBD time-courses under GLY exposures in presence of Pg. Proportions of oocytes with a WS significantly decreased ($p < 0.05$) after 420 and 435 minutes of exposure to 14.8 and 148 µM of GLY but these delays quickly disappeared and were not observed again until the end of the experiments. At 1480 µM of GLY, more important delays were recorded. GVBD ratios were significantly lower after 375 ($p < 0.01$), 390 ($p < 0.05$), 405 to 450 ($p < 0.01$), 465 to 570 and 695 to 785 ($p < 0.05$) minutes. The last ratio measures at 800 minutes was not significantly different from the control condition. RUp exposures with Pg induced approximatively the same response profiles but no significant difference was found after statistical analyses (Fig. 1b). Without Pg, spontaneous GVBD were observed in GLY and RUp conditions and especially for the concentration 1480 µM[a.e.] of RUp where GVBD ratios were significantly higher from 570 minutes to the end of the exposure (Fig. 1c and 1d).

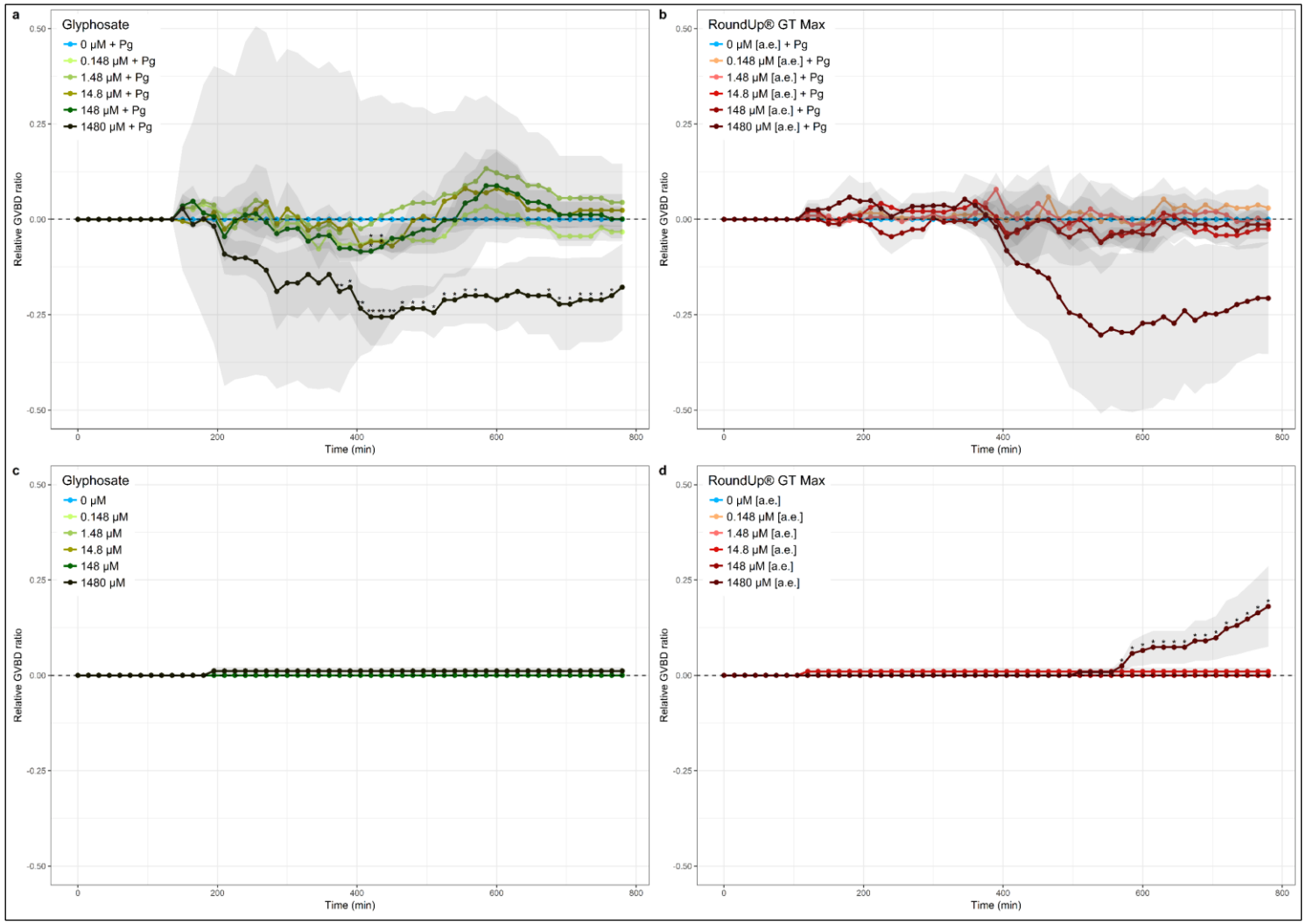

Figure 1: Oocyte maturation time courses under GLY and RUp exposures. WS appearances were assessed every 15 minutes for 13 hours. GLY (green lines) or RUp (red lines) exposures were conducted at 0 (blue line), 0.148, 148, 14.8, 148 and 1480 µM[a.e.] in presence (a, b) or absence (c, d) of Pg. Results are expressed as mean ratios relative to the control ± SEM (grey areas). Significant differences were assessed by Friedman's tests and post-hoc multiple paired comparisons between conditions (*: $p < 0.05$, **: $p < 0.01$).

3.2. Meiosis spindle formation analyses

Control immature oocytes show at the cytological level a large germinal vesicle (Fig. 2a). After the GVBD, in a control mature oocyte, there is the formation of the meiotic spindle. This barrel shape structure close to the cell membrane supports the condensed chromosomes lined up on the metaphase plate (Fig. 2b).

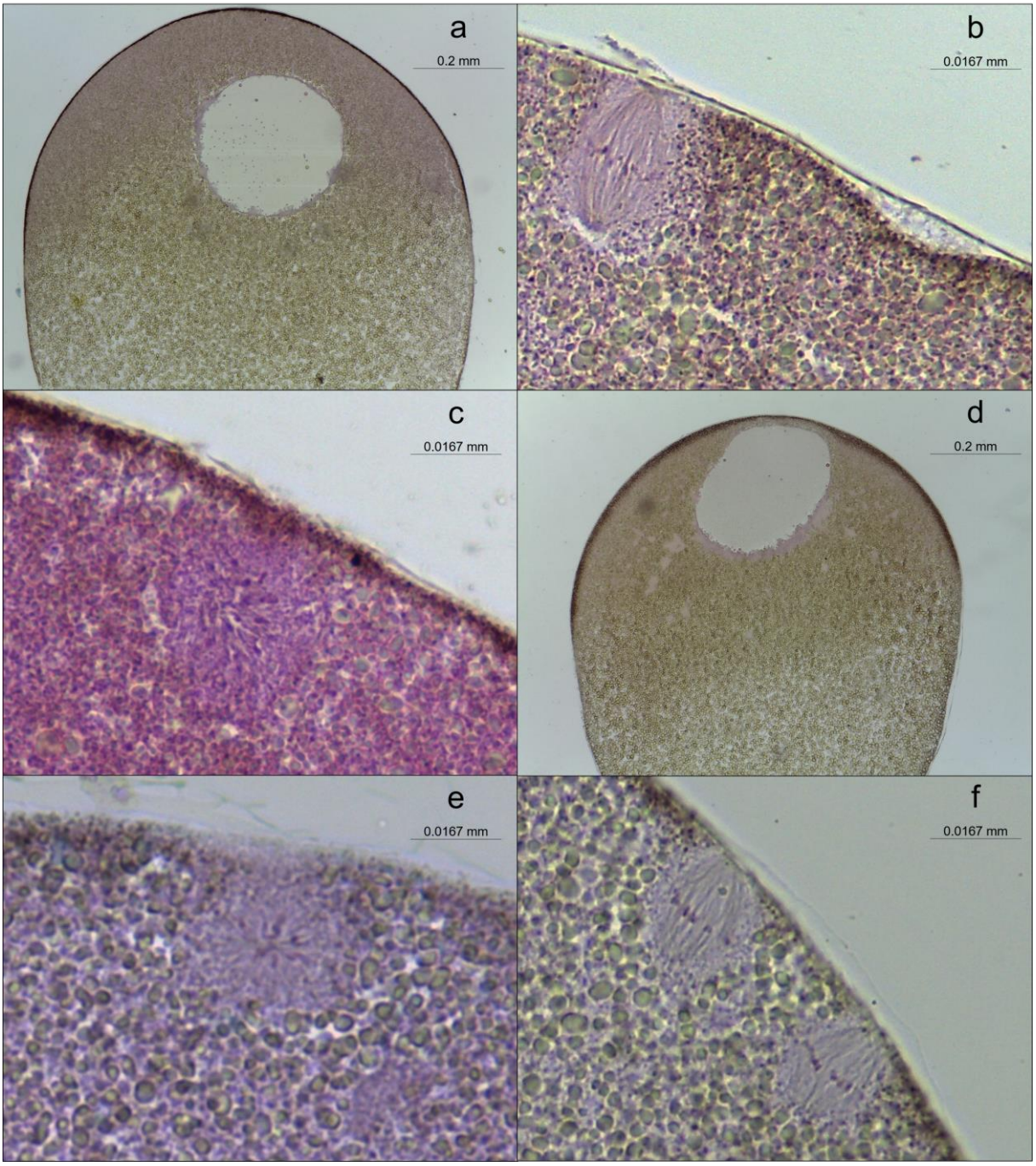

Figure 2: Optical microscopy typical photographs after glyphosate-based form exposures of *X. laevis* oocytes. a: control immature oocyte; b: control mature oocyte (Normal); c: dS+dC+E; d: GV; e: A; f: 2S.

Abnormal structures of the meiotic spindle formation can be observed in the control mature oocytes (30.10 %). For the RUp concentration of 1480 µM[a.e.] this percentage significantly increased to 76.19 % ($p < 0.05$; Fig. 3). A similar increased was observed for the same concentration of GLY (56.25 %) but did not differ significantly from the control condition ($p = 0.0572$; Fig. 3). Table 1 reports the proportion of the different kinds of abnormality found in WS oocytes after the exposures. The following categories were defined: disorganized spindle (dS), disorganized chromosomes (dC), ectopic spindle (E), no spindle (nS), no chromosomes (nC), double spindles (2S), aster (A), double asters (2A), germinal vesicle (GV). As indicated in the table 2 by the symbol "+", many abnormalities of the previous list could be observed in one oocyte, for example dS+dC+E (Fig. 2c). It appeared that specific structures never found in control mature oocytes was formed after GLY or RUp exposures, i.e. GV (Fig. 2d) and A (Fig. 2e). Moreover, the double structures 2S (Fig. 2f) and 2A were only observed in oocyte exposed to RUp.

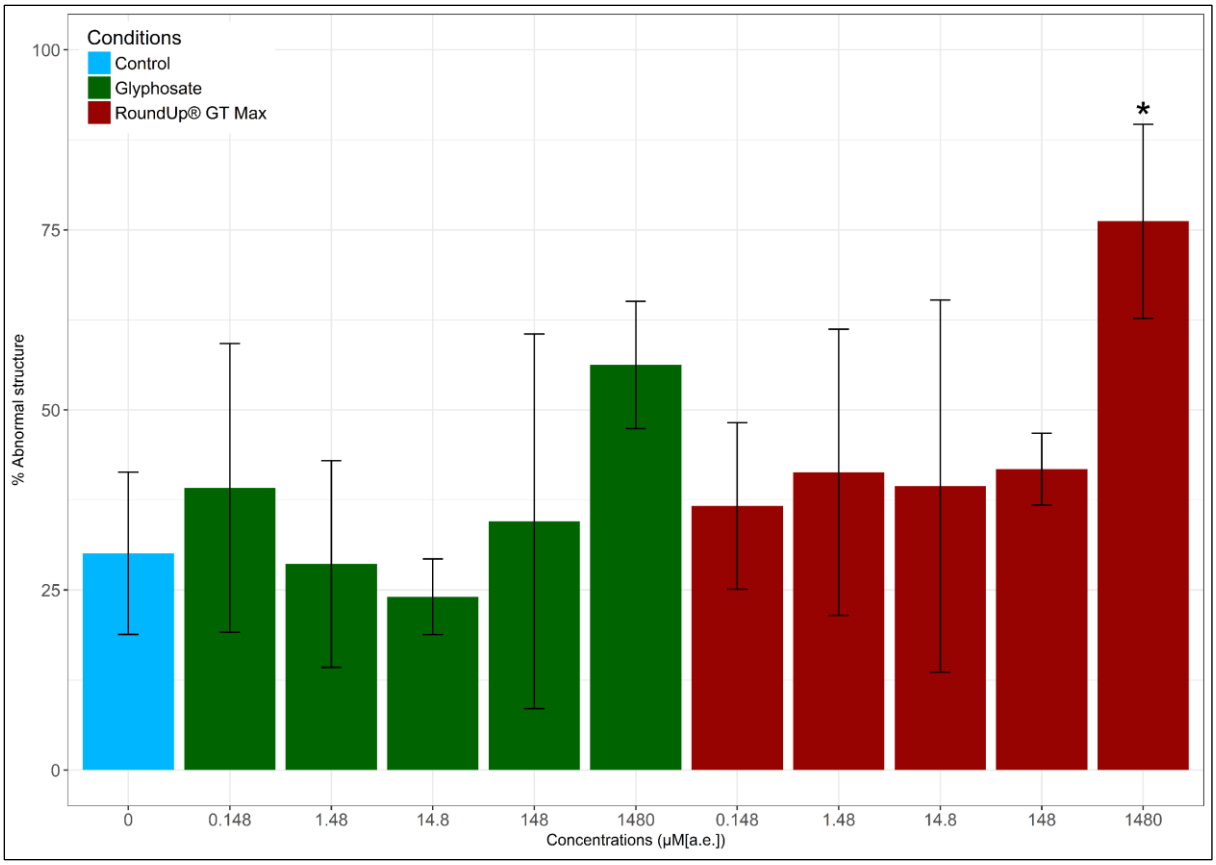

Figure 3: Percentages of cytological abnormalities after oocyte GLY or RUp exposures. Results are expressed as average percentages (± SD) per condition. Significant differences were assessed by Kruskal Wallis' tests and post-hoc multiple paired comparisons between conditions (*: $p < 0.05$).

Table 1: Observed abnormalities in WS oocytes after the GLY or RUp exposures.

Conditions	(µM[a.e.])	N	n	Normal	dS	dC	dS+dC	E	dS+E	dC+E	dS+dC+E	nS+nC	2S	A	2A	GV
Control	0	6	60	68.4 %	1,7 %	10 %	8.3 %	3.3 %	1,7%	-	3.3 %	3.3 %	-	-	-	-
GLY	0.148	3	28	60.8 %	-	7.1 %	7.1 %	3.6 %	3,6%	3.6 %	7.1 %	7.1 %	-	-	-	-
	1.48	3	35	70.36 %	-	3.7 %	7.4 %	7.4 %	-	-	3.7 %	7.4 %	-	-	-	-
	14.8	3	38	76 %	-	-	6.9 %	-	-	-	10.3 %	-	-	3.4 %	-	3.4 %
	148	3	38	66.7 %	-	3.7 %	3.7 %	11.1 %	-	3.7 %	-	7.4 %	-	-	-	3.7 %
	1480	2	20	44.4 %	-	16.7 %	11.1 %	11.1 %	-	-	11.1 %	5.6 %	-	-	-	-
RUp	0.148	3	28	64.4 %	-	10.7 %	3.6 %	7.1 %	-	-	7.1 %	-	-	7.1 %	-	-
	1.48	3	27	62.7 %	-	8.6 %	11.4 %	-	-	2.9 %	8.6 %	2.9 %	-	2.9 %	-	-
	14.8	3	29	57.9 %	-	10.5 %	5.3 %	7.9 %	-	-	5.3 %	-	2.6 %	7.9 %	2.6 %	-
	148	3	27	57.9 %	2,6 %	13.2 %	13.2 %	-	-	-	5.3 %	2.6 %	2.6 %	-	2.6 %	-
	1480	2	18	20 %	-	35 %	10 %	-	-	-	-	15 %	-	5 %	10 %	5 %

N: number of females, n: number of oocytes, dS: disorganised spindle, dC: disorganised chromosomes, E: ectopic spindle, nS: no spindle, nC: no chromosomes, 2S: double spindle, A: aster, 2A: double aster, GV: germinal vesicle

3.3. MAPK and MPF activity investigations

No particular abnormality in the phosphorylation states of ERK2, RSK, Cdc2 and H3 was detected on the immunoblots (Fig. 4). Only one experiment exhibited an aberrant phosphorylation state in pooled white spotted oocytes of Cdc2 (GLY 1480 µM) and H3 (RUp 1480 µM[a.e.]) (data not shown). This suggested that the MAPK pathway and also the activation and the activity of the MPF were not impacted by GLY or RUp exposures.

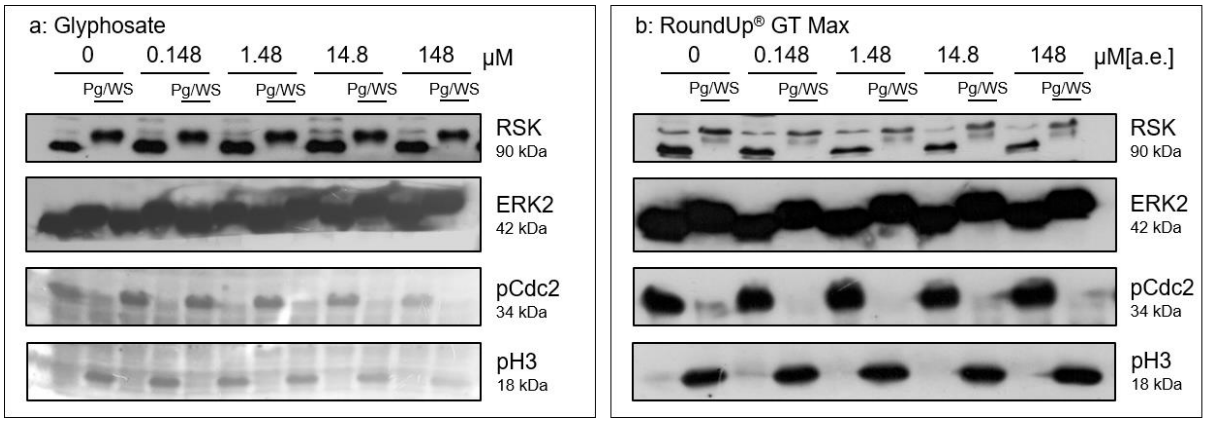

Figure 4: Effects of GLY (a) or RUp (b) exposures on the phosphorylation states of RSK and ERK2 of the MAPK cascade and of the catalytic subunit Cdc2 of the MPF and H3, a target of the MPF. No bar: non-WS oocytes not exposed to Pg; Pg/WS: WS oocytes exposed to Pg.

4. Discussion

This work proposed to investigate mechanisms of action of glyphosate and one of its commercial formulation on an essential and early step of the amphibian reproduction. To this purpose, *X. laevis* oocyte maturation was examined under exposure conditions. Main results and especially cytological analyses showed that this preparation to the fertilization was seriously disturbed by both forms of glyphosate (pure and in formulation). Concerning the time-course experiments, we observed that glyphosate alone at 1480 µM significantly delayed the maturation process. At the same dose (a.e.) similar but not significant delay was also induced by RoundUp® GT Max. Others toxicological studies showed disturbances of this parameter. Cadmium also delayed the hormone-dependent maturation process at 136 µM (Slaby et al. 2017) but in opposite atrazine and malathion alone or in mixture at 50 and 100 $\mu g.L^{-1}$ each shortened the time of appearance of the white spot in a dose-dependent manner (Ji et al. 2016). Interestingly, the last measure in glyphosate kinetic experimentations at 1480 µM did not differ from the control condition, suggesting that maturation timing impairment would have been missed if only final GVBD ratio was recorded. That underlines the interest of time-courses

approaches to assess *X. laevis* oocyte maturation. In absence of progesterone, spontaneous maturation occurred after RoundUp® GT Max or glyphosate exposures. Even if significant results were only detected at the highest concentration of the commercial formulation, any WS oocytes not induced by a hormone stimulation is abnormal. In previous works, we observed that cadmium, cobalt and zinc ions similarly induced this biological abnormality (Marin et al. 2015; Slaby et al. 2017). However, different mechanisms seem to be imply by the different contaminants. The first WS oocyte observed in cadmium conditions without progesterone appeared largely latter than the first under progesterone stimulation (Slaby et al. 2017). Here, pesticide exposures induced spontaneous maturation at around the same moment as in hormone presence conditions. These results is associated with no particular MAPK pathway or MPF disorder in contrary to abnormalities of the phosphorylation states of the same protein studied observed after metal ion exposures (Slaby et al. 2017). Further experiments are needed to understand how glyphosate induces such spontaneous GVBD.

Cytological analyses underlined an increase in the occurrence of abnormalities during spindle morphogenesis (1480 µg[a.i.].L^{-1} for the two tested forms of glyphosate). Some abnormalities were only detected in herbicide exposed oocytes as asters, double asters, double spindles and germinal vesicles in WS oocytes. This last cytological anomaly corresponded to an ascent of the germinal vesicle without its breakdown but the pigments located at the surface of the animal pole have been displaced. Similar effects were observed after 2,4-D exposure (10 mM for 10h). Actin-dependent modifications of the cell shape and the spontaneous WS appearance without GVBD but also depolymerization of microtubules were induced (Stebbins-Boaz et al. 2004). For the other abnormalities (2S, A, 2A) such structures were never reported in oocytes exposed to cadmium and lead ions (Slaby et al. 2017). Double spindle formations were also observed in co-exposed oocytes to Pregnyl® (Human chorionic gonadotropin; 150 IU.mL^{-1}) and hydroxyurea (1 mg.L^{-1}) for 30 minutes (Brachet et al. 1970). The authors hypothesized a splitting of the meiotic spindle affecting the chromosomes and also cytoplasmic constituents, possibly the centrioles, to explain such structures. For our exposures, double structures seemed to be specific anomalies, never observed in previous control or exposed oocytes historically in our lab and could correspond to signature of glyphosate (in both forms) exposures.

Several authors observed genotoxicity after glyphosate exposures of aquatic species. For example, the Indian skittering frog exposed for 24 to 96 hours to glyphosate-based herbicide (concentrations range from 1 to 3 mg[a.e.].L^{-1}) exhibited an increased incidence of micronucleated erythrocytes (Yadav et al. 2013). From these results the authors reaffirmed the genotoxic potential of glyphosate rather associated to clastogenicity. Similar conclusions have been drawn from exposure data of the European eel to 18 and 36 µg.L^{-1} of glyphosate in form

of RoundUp® solution (Guilherme et al. 2010). These authors studied DNA breaks by means of comet assay and erythrocyte nuclear aberrations for chromosomal and cytological damage. They hypothesized the oxidative stress contribution as a mechanism of genetic damage. In case of our work, cytological results suggest genotoxic effects associated with the spindle morphogenesis instead of DNA breaks.

Few studies deal with the impacts of glyphosate on aquatic specie gametes. In male, sperm quality of zebra fish exposed *in vivo* for 24 and 96 hours was reduced (Lopes et al. 2014). For both concentrations (5 and 10 $mg.L^{-1}$) sperm motility and motility period decreased and only for the highest dose the authors observed mitochondrial functionality and DNA damage. These concentrations are largely superior to those tested *in vitro* on oyster spermatozoa (Akcha et al. 2012). No cytotoxicity nor DNA damage has been observed after glyphosate or RoundUp express® exposures (1h) to concentrations ranging from 0.5 to 5 $\mu g[a.i.].L^{-1}$. For oocytes, we did not find any study. However, our results highlighted that oocyte maturation could be critically affected by contaminant exposures. Any effect as abnormal spindle morphogenesis, during this process may prevent the fertilization.

5. Conclusions

This work presents new information on the impacts of glyphosate and one of its commercial formulation on the oocyte maturation. It appears that this essential step of the reproduction can be seriously altered by these herbicide exposures. The *X. laevis* oocyte seems to be an efficient model to assess quality of aqueous solutions in environmental toxicology.

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Chapitre IV - Discussion

4.1. La maturation ovocytaire

La GVBD, premier événement visible de la maturation ovocytaire, a été évaluée dans un premier temps morphologiquement à l'aide de l'apparition de la tache de maturation. Parmi les contaminants étudiés dans le cadre de cette thèse, il est apparu que seul le cadmium pouvait inhiber la reprise du cycle cellulaire. Les ovocytes immatures exposés sur la nuit à la concentration de 136 µM de Cd^{2+} en présence de progestérone, présentaient des taux de reprises de méiose réduits de manière significative. Cet effet n'a pas été détecté lors des expositions au plomb, au mélange cadmium + plomb ou au cuivre (ainsi que pour le cobalt et le zinc). De même, parmi les phytosanitaires, ni les expositions à la bouillie bordelaise, au glyphosate, au RoundUp® GT Max ou à la deltaméthrine (Annexe I) n'ont engendré de telles inhibitions.

En l'absence de stimulation hormonale, aucune reprise de méiose ne devrait être observée. Néanmoins, cela s'est produit à la suite des expositions à certains contaminants. Pour les métaux, des maturations spontanées ont été détectées dans les conditions cadmium et cuivre (mais aussi au cobalt et au zinc) et pour les phytosanitaires dans les conditions bouillie bordelaise, glyphosate et RoundUp® GT Max. En fonction des concentrations, le pourcentage de gamètes femelles ayant repris leur cycle cellulaire pouvait varier, mais il est important de préciser que la moindre maturation non induite par l'hormone est anormale. L'induction de la maturation spontanée par des expositions métalliques a déjà été détectée chez d'autres espèces. Ainsi, le cobalt et le manganèse chez *Rana temporia* (Nikiforova and Skoblina, 1992) ou encore le cadmium chez *Carassius auratus gibelio* B. (Szczerbik et al., 2008) la provoque. En revanche, nos expérimentations conduites dans les conditions contaminées au plomb, au mélange cadmium + plomb ou à la deltaméthrine (Annexe I) n'ont jamais présenté ce phénomène.

Les cinétiques d'apparition des taches de maturation ont également été étudiées. Elles apportent de nombreuses informations et ont déjà été appliquées en routine au laboratoire (Gelaude et al., 2015; Sellier et al., 2006). Dans le cadre de cette thèse nous avons choisi d'effectuer un suivi sur 13 heures avec un relevé des taux de GVBD toutes les 15 minutes. Cette méthode a permis de mesurer des variations de manière plus précise qu'avec un protocole classique basé sur des relevés toutes les heures. Grâce à ces expérimentations, nous avons pu mettre en évidence, qu'en plus d'inhiber la reprise de la méiose, les expositions au cadmium ralentissaient ce processus à la concentration de 136 µM. Le même type d'effet s'est produit suite aux expositions glyphosate et RoundUp® GT Max à 1480 µM[e.a.] (non significatif pour la formulation commerciale). L'effet inverse, à savoir une accélération de l'apparition de la tache de maturation au sommet du pôle animal de l'ovocyte de xénope, a, au

contraire, déjà été rapporté à la suite d'expositions à l'atrazine et au malathion seul ou en mélange (50 et 100 µg.L^{-1} de chaque) (Ji et al., 2016). Ces auteurs ont également retrouvé la même variation en examinant la cinétique d'expression de la cycline B2 dans les ovocytes exposés au mélange (100 µg.L^{-1} de chaque). La diminution du niveau de cette protéine, observée dans le cas d'une maturation ovocytaire normale, se produisait de manière plus précoce en condition contaminée. Nos suivis temporels dans les milieux contenant du glyphosate ont montré un autre intérêt majeur. Aucune différence significative n'a été mesurée pour le dernier point de mesure, suggérant que l'impact de l'herbicide n'aurait pas été mis à jour si la mesure du taux de GVBD avait été appréciée uniquement en point final. Dans les cas des expositions au plomb, au cuivre ou à la bouillie bordelaise, les effets sur les cinétiques de reprise de la méiose étaient bien moins importants. Les possibles variations durant les expérimentations ont été très vite rattrapées durant le reste des expérimentations.

Les maturations spontanées précédemment évoquées ont été retrouvées durant les cinétiques. Néanmoins, leurs apparitions n'ont pas suivi le même profil en fonction des contaminants. Deux groupes sont discernables : les maturations induites par les expositions au cadmium qui débutent très tardivement par rapport aux maturations liées à la progestérone, et celles provoquées par les substances à base de glyphosate ou à base de cuivre qui arrivent en même temps que dans la condition témoin avec hormone. Le fait que, dans toutes les conditions avec hormone, la première cellule présentant la tache de maturation apparait approximativement au même moment, montre que ce décalage n'est pas dû au retard du processus provoqué par le cadmium. Il existerait donc des mécanismes différents amenant cette apparition de la tache selon le contaminant.

Les résultats de nos expérimentations sur les voies de signalisation donnent d'ailleurs du poids à cette hypothèse. En effet, la voie MAPK et le MPF ont été fortement perturbés par les expositions au cadmium. Les pourcentages d'ovocytes présentant des profils de phosphorylation anormaux de ERK2, RSK (cascade MAPK), Cdc2 (sous-unité du MPF) et H3 (cible du MPF) étaient plus importants en conditions contaminées à ce métal. Des inductions anormales de phosphorylation de ERK1/2 ont également été rapportées suite à des expositions au cadmium dans des lignées cellulaires U937 (Galán et al., 2000), PC12 (Chen et al., 2008) ou gliomes 9L (Hung et al., 1998). Les voies MAPK et MPF ne semblent pas anormales au regard des états de phosphorylation des protéines étudiées pour les expositions aux formulations de cuivre ou de glyphosate.

Du fait du rôle essentiel de la voie MAPK et du MPF dans la maturation ovocytaire, leurs altérations provoquées par les expositions au cadmium pourraient expliquer les anomalies de formation du fuseau méiotique. En condition normale, cette structure, en forme

de baril, est accrochée à la membrane cellulaire et supporte les chromosomes. Or, plusieurs anomalies de morphogénèse du fuseau ont été répertoriées à la suite des expositions au Cd^{2+} contrairement au Pb^{2+}. Dans une autre étude, il a été montré que le cadmium pouvait également affecter le nombre de chromosomes (hypoploïdie, hyperploïdie ou diploïdie) et arrêter la méiose dans des ovocytes de souris injectés (3 et 6 $mg.kg^{-1}$) (Shimada et al., 1976). En revanche, ce lien ne peut être établi entre les altérations cytologiques et l'état de la cascade MAPK et du MPF dans les conditions contaminées au glyphosate et au RoundUp® GT Max. Ces substances herbicides altèrent la mise en place du fuseau, en augmentant le pourcentage d'incidence (à la plus forte concentration) mais aussi en induisant des structures jamais observées auparavant. En plus des altérations « classiques », comme par exemples un fuseau déformé ou des chromosomes non alignés, des formations de doubles structures (deux fuseaux dans un seul ovocyte) ont été observées. Cela pourrait correspondre à une signature spécifique de cet herbicide dans l'ovocyte de xénope. Des doubles fuseaux ont déjà été rapportés par contre dans une autre étude à la suite d'une co-exposition d'ovocytes de xénope à de la hCG et de l'hydroxyurée (Brachet et al., 1970). L'hypothèse apportée par les auteurs est que le fuseau méiotique aurait été séparé en deux parties. Néanmoins, nous pouvons également nous interroger sur le premier globule polaire dans ces ovocytes présentant une double structure. Cette cellule, largement plus petite que l'ovocyte, est normalement issue d'une division cellulaire asymétrique permettant la conservation d'un maximum de réserve pour le futur développement embryonnaire (Maro and Verlhac, 2002). Le globule polaire contient un ensemble complet des homologues chromosomiques. La non émission de cette cellule haploïde pourrait donc, de manière hypothétique, expliquer la conservation d'un double matériel génétique, et la présence d'un deuxième fuseau. Dans l'ovocyte de *X. laevis*, il a été montré que l'inhibition de l'activation de la protéine Cdc42 empêche l'émission du globule polaire, sans affecter la position asymétrique du fuseau, qui est une condition à l'activation de la protéine, ou la séparation des chromosomes (Ma et al., 2006; Zhang et al., 2008). Ce mécanisme apparaît donc comme une potentielle cible du glyphosate. Il serait intéressant de l'étudier en conditions contaminées.

Une autre atteinte à la formation du fuseau provoquée par le cadmium et par les formulations de glyphosate est la persistance d'une vésicule germinale dans des ovocytes présentant une tache de maturation. Nous avons observé, dans ces cas, que la vésicule était montée vers le pôle animal tout en restant intacte, occasionnant une zone blanche au pôle animal par déplacement des pigments. Un effet similaire a été observé dans des ovocytes de *X. laevis* exposés au 2,4-D à 10 mM pendant 10 heures (Stebbins-Boaz et al., 2004). Dans ce cas, il a été prouvé que le contaminant a induit une dépolymérisation des microtubules occasionnant la montée de la vésicule germinale, la modification de la forme de la cellule et

l'apparition d'une tache au pôle animal. Les auteurs ont montré également que les expositions au 2,4-D empêchaient l'expression de Mos, l'activation du MPF (histone H1) et la polyadénylation cytoplasmique de l'ARNm cycline B1, et donc la maturation ovocytaire normale induite par de la progestérone, tout en activant MAPK. Cette activation était indépendante de la voie classique induite par la progestérone Mos/MAPK/MPF mais dépendante de MAPKK (MEK). Il existerait donc une voie indépendante, induite par le 2,4-D, pour l'activation de MAPK. Dans le cadre de cette thèse il serait donc intéressant de vérifier si l'activation de la voie MAPK et du MPF ne serait pas dû également à une voie alternative, en contrôlant dans un premier temps le niveau de Mos dans les ovocytes exposés. Cette hypothèse est à émettre notamment pour les expositions aux deux formes de glyphosate, étant donné que ces produits induisent de la maturation spontanée et de lourdes anomalies cytologiques sans perturber, a priori, MAPK et le MPF. Pour le cadmium, nos résultats suggèrent que ce métal pouvait induire et/ou perturber la voie « normale » de maturation. Les expositions au plomb, au cuivre, à la bouillie bordelaise et à la deltaméthrine (Annexe II) n'ont pas présenté de telles altérations de la voie MAPK ni du MPF, au regard des protéines étudiées.

4.2. La fécondation et le développement

A la suite de nos expérimentations, nous avons observé que les expositions au cadmium induisaient à la plus forte concentration une réduction significative du taux de fécondation. En effet, le nombre d'œufs fécondés, ayant opéré les réactions de symétrisation et corticale et s'étant orienté par la pesanteur, était drastiquement réduit dans cette condition. Concernant les autres expositions, ni le plomb, le cuivre, la bouillie bordelaise, le glyphosate (Annexe III) et ni le Roundup® GT Max (Annexe IV) n'ont eu cet effet. Au regard de l'impact du cadmium sur ce paramètre, nous avons recherché si ce métal affectait plus particulièrement le gamète mâle ou le gamète femelle. Pour cela, des préexpositions des morceaux de testicule et des œufs ont été conduites séparément avant de réaliser la fécondation dans le milieu contrôle avec les cellules germinales paires non exposées. Les résultats ont montré que, tout d'abord, quel que soit le type de gamète exposé, les taux de fécondation étaient significativement réduits dans les deux cas. Mais il a été également observé que ces taux étaient significativement plus faibles lorsque seuls les ovocytes étaient exposés, suggérant que la cellule germinale femelle est plus sensible que la cellule germinale mâle aux expositions au cadmium.

Dans la suite du développement, les premières étapes de l'embryogenèse ont également présenté une certaine sensibilité aux expositions à cet ion cadmium. Contrairement à celles au plomb, un retard d'apparition des premiers clivages embryonnaires ou même des arrêts de développement ont été observés. Un retard de développement augmente la durée de vie larvaire. Dans l'environnement, ce décalage dans le temps peut être dommageable pour l'individu qui sera soumis plus longtemps à une forte pression de prédation. De plus, nous pouvons émettre l'hypothèse que si la métamorphose arrive en retard, les conditions environnementales, comme la nourriture disponible, ne seront pas optimales pour le nouveau juvénile.

Arrivés au sixième jour de développement, les têtards sont fixés, colorés au bleu alcian et analysés à l'aide de notre protocole afin d'obtenir des données biométriques. Nous avons observé que les expositions au cuivre ont conduit à des individus plus grands d'une manière significative. Les écarts oculaires des individus exposés étaient plus importants. En comparaison, ce paramètre n'a présenté aucun changement suite aux expositions aux deux formulations de glyphosate (Annexes V et VI). La variation de la morphologie des têtards est également un facteur qui va modifier leur chance de survie. En fonction des altérations (anomalie de développement, croissance modifiée), les capacités motrices d'un individu peuvent changer, ainsi que les comportements de recherche de nourriture ou de fuite face à un prédateur, par exemples.

4.3. Intérêts et limites de nos protocoles expérimentaux

Une des principales qualités de nos différents endpoints pour évaluer la toxicité de composés est sans doute leur diversité. En effet, la maturation ovocytaire est analysée sous des aspects morphologique, physiologique, cytologique et biochimique. De même, plusieurs paramètres liés à la fécondation et au développement sont suivis, *i.e.* le succès de fécondation, l'apparition des stades embryonnaires, la croissance ou encore la formation des organes. Pour un contaminant donné, ces multiples analyses sont réalisées en limitant le nombre d'expositions. Par exemple, les voies de signalisation peuvent être étudiées après avoir recueilli les informations sur les taux de maturation, ou alors, le taux fécondation, le suivi de développement et l'analyse biométrique sont obtenus sur une même ponte. Ceci est en accord avec le principe des trois R (Réduire, Remplacer, Raffiner) en limitant le nombre d'individus exposés (Russell and Burch, 1959). Il est apparu, de plus, que ces différents paramètres étaient complémentaires. Ils ne réagissent pas forcément tous à la suite d'une même exposition. Pour illustrer, nous pouvons prendre le cas du glyphosate, car sous ses deux

formes testées, aucune altération de la fécondation et du développement n'a été observée à la suite des expositions (Annexes III à VI). Néanmoins, de graves anomalies de morphogénèse du fuseau méiotique et des retards du processus de maturation ont été détectés. D'ailleurs, nous pouvons noter que si nos analyses s'étaient limitées à des paramètres morphologiques, comme la plupart des études en toxicologie des amphibiens, ces effets sur la maturation ovocytaire n'auraient pas été mis à jour alors qu'ils constituent un réel problème pour la reproduction de notre amphibien modèle.

Nous avons vu que l'ovocyte de *X. laevis* est un modèle apportant de nombreux avantages en termes de manipulation et de connaissances (chapitre « *Approches et modèle utilisés* ») et qu'il est efficace pour évaluer la toxicité et mettre en évidence des signatures de contamination. Le tableau 2 décrit différents essais normalisés ayant pour modèle un amphibien ainsi que nos méthodes d'analyse. De manière générale, il est intéressant de noter que les tests standardisés utilisent presque tous *X. laevis*. Les nombreux avantages énoncés dans le chapitre « *Approches et modèle utilisés* » en font effectivement un modèle biologique de choix en toxicologie de l'environnement. Les deux exceptions concernent le test AMPHITOX (Herkovits and Perez-Coll, 2003) avec *Bufo arenarum* et le test d'évaluation de la génotoxicité à l'aide de larves d'amphibiens qui propose de travailler avec *X. laevis* ou *Pleurodeles waltl*. Nos expérimentations proposent deux méthodes d'évaluation de la toxicité complémentaires à celles normées déjà existantes et cela pour différentes raisons. En comparaison, nos expositions se concentrent sur des stades plus précoces que les techniques conventionnelles. Elles commencent soit au stade gamétique soit au moment de la fécondation. Les autres tests, utilisant le xénope lisse, LAGDA (OCDE, 2015), FETAX (ASTM International, 2012), EMA (OCDE, 2009) et celui d'évaluation de la génotoxicité (*X. laevis*) débutent soit à la gastrula (stade 8-10/11 (Nieuwkoop and Faber, 1967)) soit à des stades têtard beaucoup plus avancés (stades 50 ou 51 (Nieuwkoop and Faber, 1967)). Il en est de même pour l'essai AMPHITOX et l'évaluation de la génotoxicité à l'aide de larves d'amphibien (*P. waltl*) qui emploient également des stades têtards (respectivement stades 23-25 (Del Conte and Sirlin, 1951) et 53 (Galien and Durocher, 1957)). Ensuite, l'étude de la maturation ovocytaire présente une alternative ne nécessitant aucun sacrifice animal. Cette qualité respecte donc, encore une fois, les recommandations de la règle des trois R (Russell and Burch, 1959). S'intéresser aux stades parmi les plus précoces du cycle de vie, nous procure encore un autre avantage, cette fois-ci technique. Comparées aux autres essais, les questions classiques en toxicologie de l'environnement concernant l'alimentation ne se posent pas dans nos études. La nutrition d'individus exposés occasionne généralement des soins supplémentaires et surtout des interrogations sur les voies d'exposition à prendre en compte.

Tableau 2. Comparaison de techniques standardisées d'évaluation de la toxicité utilisant un amphibien comme modèle biologique à nos méthodes d'analyse.

Nom	Réf.	Espèce	Début	Fin	Voie d'exposition	Alim.	Sacrifice	Mesures
Maturation ovocytaire	/	*Xenopus laevis*	Ovocyte st. VI[a]	13 h	Aquatique ND96 ± progestérone	Non	Non	- En continu et fin : mortalité cellulaire, taux de maturation - Fin : morphogénèse du fuseau méiotique, activation et activité de la voie MAPK et du MPF
Fécondation et développement	/	*Xenopus laevis*	Fécondation	6 j	Aquatique Eau du robinet	Non	Oui	- 35 min : taux de fécondation - En continu : mortalité, anomalies externes, délai d'apparition des st. embryonnaires - Fin : mesures biométriques variées
EMA – Essai de métamorphose des amphibiens	OCDE n° 231	*Xenopus laevis*	St. 51[b]	21 j	Aquatique Eau disponible localement	Oui	Oui	- Quotidiennement : mortalité - A 7 j et fin : longueur des pattes postérieures, longueur museau-cloaque, stade de développement, poids humide - Fin : histopathologie de la glande thyroïde
LAGDA – Essai de croissance et de développement de larves d'amphibien	OCDE n° 241	*Xenopus laevis*	St. 8-10[b]	- Délai médian st. 62[b] + 10 sem : 16 à 17 sem - Sous échantillon st. 62[b]	- Aquatique Eau disponible localement - Alimentaire Possible/non testée	Oui	Oui	- Quotidiennement : mortalité, comportement - Sous échantillon : Délai jusqu'au st. 62, anomalies externes, morphométrie (longueur et poids), histopathologie de la glande thyroïde - Fin : morphométrie (longueur et poids), histopathologie des gonades, conduits gonadiques, reins et du foie, développement anormal, vitellogénine plasmatique, sex-ratios génotypiques et phénotypiques, indice hépato-somatique
AMPHITOX – Set de tests de toxicité utilisant des embryons d'amphibien	ASTM STP1443	*Bufo arenarum*	St. 23-25[c]	- 96 h : AMPHIACUT - 7 j : AMPHISHORT - 14 j : AMPHICHRO	Aquatique Eau disponible localement	Oui	Oui	- Mortalité - Test supplémentaire AMPHIEMB : malformation
FETAX – Frog Teratogenesis Assay - *Xenopus*	ASTM E 1439 - 12	*Xenopus laevis*	St. 8-11[b]	96 h (st. 46)	Aquatique Milieu FETAX	Oui	Oui	- Quotidiennement : mortalité - Fin : malformation, croissance - Additionnels : pigmentation (fin), locomotion, éclosion (48 h)
Évaluation de la génotoxicité à l'aide de larves d'amphibiens	NF ISO 21427-1:2006	- *Xenopus laevis* - *Pleurodeles waltl*	- *X. laevis* : St. 50[b] - *P. waltl* : St. 53[d]	12 j	Aquatique Eau disponible localement	Oui	Oui	- Toxicité aigüe : mortalité, croissance, nutrition, mobilité - Taux d'érythrocyte avec micronoyau

a : Dumont et al., 1983 ; b : Nieuwkoop and Faber, 1967 ; c : Del Conte and Sirlin, 1951 ; d : Galien and Durocher, 1957

Enfin, concernant les effets mesurés nous apportons de nouveaux endpoints qui ont montré à travers notre travail un réel intérêt. Sur la maturation ovocytaire, les aspects de ce phénomène à différents niveaux biologiques ne sont pas du tout abordés dans les autres tests. C'est d'ailleurs un constat général, comme nous le présente le chapitre « *Introduction* » de ce document, en toxicologie des amphibiens. L'essai LAGDA propose tout de même de vérifier la qualité des gonades par des techniques d'histopathologie et ainsi d'apprécier visuellement d'éventuelles anomalies de présence (par exemple le cas des individus intersexués) et de formation des gamètes (par exemple le développement des ovocytes). Concernant la fécondation et le développement, leurs évaluations ne sont pas possibles avec les tests standardisés proposés. L'appréciation des atteintes morphologiques est un critère plus commun et présent dans tous les autres essais. Dans ce travail, nous avons développé pour ces critères une méthode de mesures automatisées de nombreux paramètres biométriques basée sur une coloration des formations cartilagineuses. Cette coloration permet également d'obtenir plus facilement des informations sur leurs mises en place et, plus largement, sur l'ensemble du têtard. En s'intéressant à des stades précoces et à d'autres paramètres biologiques, nos analyses visent donc à apporter de nouvelles données essentielles dans la compréhension des effets de contaminants environnementaux sur la reproduction des amphibiens et dans l'évaluation de la toxicité de xénobiotiques et/ou qualités des milieux.

Concernant les effets détectés il est difficile de les mettre directement en lien avec le déclin des amphibiens observé dans l'environnement sans émettre d'hypothèses. Il est nécessaire de toujours garder à l'esprit que notre travail est strictement mené au laboratoire et que différents aspects sont à améliorer afin de limiter les extrapolations hasardeuses laboratoire/environnement. En effet, même si l'évaluation de la toxicité en milieux contrôlés est plus que nécessaire en toxicologie des amphibiens, elle ne reproduit pas des conditions écologiques pertinentes (Linder et al., 2010). Tout d'abord, en milieu naturel un individu ou un gamète (lorsque la fécondation est externe) ne sera jamais exposé à un seul contaminant mais à un cocktail de polluants, ayant des interactions les uns avec les autres. Cette notion est à intégrer dans nos études. Des efforts ont déjà été fournis en ce sens, et des expositions binaires (cadmium et plomb) ont, dans un premier temps, été réalisées. C'est un mélange simple mais précurseur d'autres expérimentations. Il ne faut pas oublier également, les autres conditions abiotiques (température, pH, ensoleillement…) et les conditions biotiques (compétition, prédation…) auxquelles un individu est soumis dans l'environnement. Ce dernier point est difficile à intégrer dans les protocoles expérimentaux mais certains travaux proposent, par exemple, des méthodes pour évaluer les effets d'un contaminant associé à une pression de prédation (Relyea, 2005, 2004). L'utilisation de mésocosmes est aussi un moyen efficace pour ajouter des paramètres d'exposition tout en gardant un certain contrôle sur

l'expérimentation (Linder et al., 2010). Les études à l'échelle environnementale sont donc toujours nécessaires. Pour cela, des techniques d'encagement peuvent être employées. Les méthodes d'échantillonnage d'individus dans le milieu naturel sont aussi utilisées à travers le monde mais ajoutent, quand elles sont trop invasives, une pression anthropique supplémentaire sur les populations locales (protégées en France). Dans cette optique, il est possible d'envisager de travailler sur les populations invasives de *X. laevis* (discuté dans la suite du document). Enfin, cette volonté de se rapprocher des conditions environnementales peut aussi se réaliser en échantillonnant de l'eau dans des milieux d'intérêt et en y analysant nos endpoints au laboratoire. Dans notre objectif de développer un nouveau modèle en écotoxicologie, ce travail a déjà débuté et fait l'objet de la partie suivante.

4.4. Analyse d'échantillons environnementaux

A travers la mise en place de nouveaux protocoles et endpoints pour évaluer les effets toxiques de différents contaminants environnementaux sur l'ovocyte, l'un des objectifs de cette thèse était de contribuer à l'élaboration d'un nouveau modèle en écotoxicologie, basé sur l'ovocyte de xénope. Ainsi, après avoir établi les schémas expérimentaux, leurs utilisations en routine et la caractérisation d'un panel de réponses en conditions exposées, des premiers essais ont été conduites avec des échantillons environnementaux.

Les eaux ont été prélevées dans quatre environnements différents, chacun soumis à des pressions anthropiques différentes (Figure 6). Nous avons sélectionné un milieu urbain (La Risle (Ri), Pont-Audemer, Eure ; 49.356579, 0.516506), un parc périurbain (étendue d'eau (He), Villeneuve-d'Ascq, Nord ; 50.636296, 3.163341), une zone d'activité périurbaine (ruisseau (HB), Villeneuve-d'Ascq, Nord ; 50.604548, 3.157221) et une zone agricole (La Corbie (FM, Fort-Moville, Eure ; 49.332210, 0.420843).

Les échantillons ont été filtrés grossièrement, congelés à -20 °C puis filtrés finement (0,45 µm) avant utilisation. Des fécondations *in vitro* ont donc été réalisées dans ces milieux. Les taux de fécondation et un exemple de la quantification de la biométrie des têtards de 6 jours sont présentés respectivement dans les annexes VII et VIII. Les résultats ne présentent pas particulièrement d'atteinte sur ces endpoints, suggérant ainsi la bonne qualité des eaux testées. Ces évaluations sont évidemment des pré-expérimentations, mais l'essentiel réside dans le fait que nos techniques sont applicables dans le cadre d'une biosurveillance des milieux aquatiques.

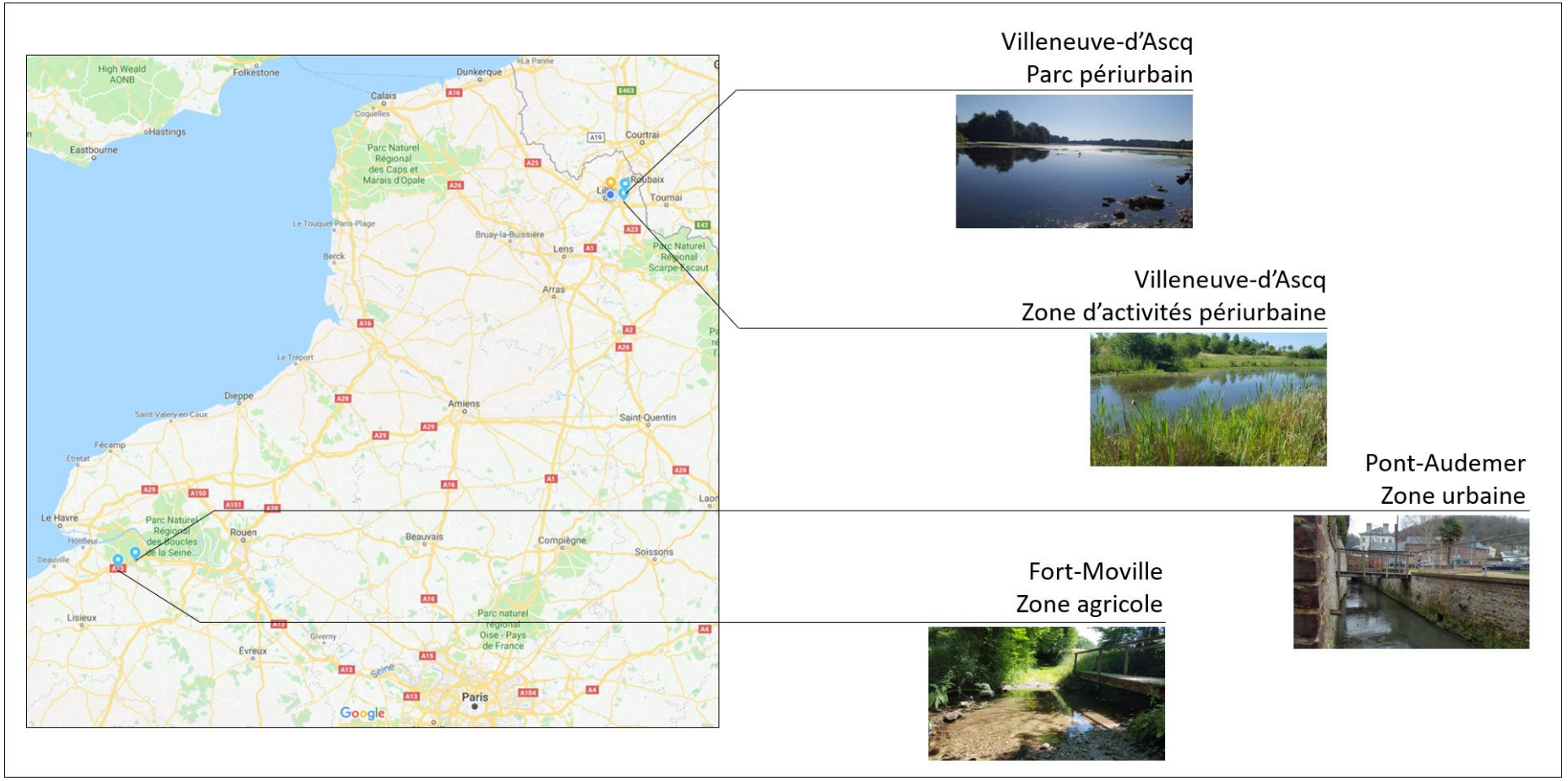

Figure 6 : Zones échantillonnées dans le cadre des pré-expérimentations d'évaluation de la qualité de milieux aquatiques.

Afin de réaliser une réelle analyse d'échantillons environnementaux, plusieurs points seront à respecter. Tout d'abord, les milieux à cibler prioritairement devront être des zones reconnues comme polluées et nos expérimentations devront être mises en relation avec des analyses physico-chimiques de l'eau mais aussi avec des données de recensement de populations d'amphibiens. Dans cette optique une collaboration avec des institutions locales (Agence de l'Eau, associations…) serait appréciable. Ensuite, l'échantillonnage devra se faire avec beaucoup de rigueur, en mettant en place un suivi temporel et en prélevant l'eau sur plusieurs points pour une même zone. Par ailleurs, ces expérimentations pourront s'élargir à divers effluents (station d'épuration, effluents industriels), avec pour objectif d'améliorer la maîtrise des risques et/ou la protection de l'environnement.

4.5. Xenopus laevis, une espèce invasive

L'utilisation des xénopes femelles pour diagnostiquer des grossesses dans les années 1930, dans les laboratoires de recherche ou comme animal de compagnie ont été à l'origine d'exportations de xénopes depuis l'Afrique du Sud vers des laboratoires de différentes régions du monde et, par la suite, d'apparitions de populations invasives (Measey et al., 2012). Mais, comme le mentionnent également les auteurs de cette revue, il a été prouvé que cette espèce très robuste a un réel impact négatif sur les populations locales d'amphibiens et de poissons et propage des maladies. En France, c'est dans les Deux-Sèvres que des populations de xénopes sont retrouvées. Dans le cadre de notre projet de recherche, utiliser ces groupes invasifs pourrait être une bonne perspective. En effet, récupérer les individus sur le terrain permettrait de répondre à deux grandes questions. Tout d'abord, de savoir si la maturation ovocytaire, la fécondation et de développement d'individus échantillonnés sont influencés de la même manière par nos expositions que nos individus de laboratoire. Ensuite, en corrélant avec des informations sur la qualité de l'eau de ce département, nous pourrions évaluer directement nos endpoints chez les xénopes provenant de ces milieux et les utiliser en tant qu'espèce bioindicatrice. Ce travail pourrait être effectué chez les adultes et les têtards pour lesquels notre protocole d'évaluation d'atteinte morphologique serait appliqué. Il serait également possible d'envisager de nouveau paramètre, comme la qualité des gonades ou d'autres organes (histologie). La détection des concentrations en contaminant retrouvées dans les ovaires serait aussi très intéressante afin d'avoir des données avec une réelle relevance écologique, inexistante dans la littérature scientifique. Enfin, ce projet aurait un impact positif indirect dans la lutte contre les populations invasives de *X. laevis*.

Conclusions

Les effets d'expositions à des contaminants environnementaux de gamètes n'a montré que trop peu d'intérêt jusqu'à aujourd'hui en toxicologie de l'environnement. Cette thèse de doctorat avait pour but d'apporter de nouvelles données sur les effets de la pollution sur l'ovocyte de *X. laevis*, en recherchant des cibles au sein de ce gamète et également de participer au développement d'un nouveau modèle efficace en écotoxicologie pour évaluer la qualité de milieux aquatiques.

Les connaissances et les différents avantages que nous offrent l'ovocyte de xénope ont permis l'élaboration de protocoles adaptés pour évaluer la toxicité. Grâce à ces schémas expérimentaux la maturation ovocytaire peut être analysée à différents niveaux biologiques (morphologique, cytologique et biochimique). Il est également possible de déterminer le succès de fécondation et de réaliser le suivi d'apparition des stades embryonnaires. Enfin, différentes données biométriques d'individus développés dans des milieux contaminés peuvent être appréciées précisément et automatiquement.

Dans leur ensemble, nos principaux résultats, présentés sous forme graphique dans le tableau 3, montrent que :

- l'ovocyte est un gamète sensible aux contaminants. Les expositions à l'ion cadmium diminuent fortement le succès de reproduction en altérant la maturation ovocytaire, la fécondation et le développement. Le glyphosate sous sa forme pure et une de ses formulations commerciales altèrent de manière importante la maturation. Le plomb, le cuivre et la bouillie bordelaise perturbent également, mais de manières différentes, cette préparation à la ponte et à la fécondation. Seule la deltaméthrine n'affecte pas ce phénomène.

- les expositions aux RoundUp® GT Max et bouillie bordelaise présentaient respectivement les mêmes effets sur les endpoints testés que le glyphosate et le cuivre. Ces observations laissent envisager que les réponses induites par ces formulations commerciales sont provoquées par leur composant principal.

- différentes signatures d'expositions sont apparues. Par exemple, les doubles structures cytologiques et les maturations spontanées non associées à des perturbations de la voie MAPK et du MPF provoquées par les substances à base de glyphosate.

De nombreuses perspectives ont vu le jour à l'issue de cette thèse. Ainsi, l'exploration d'autres mécanismes de signalisation cellulaire est envisagée pour expliquer les effets observés. Les expérimentations avec des échantillons environnementaux vont continuer et l'utilisation de xénopes issus de populations invasives serait un projet à mettre en place.

Ce travail a permis non seulement de mettre en évidence des effets jamais décrits en toxicologie des amphibiens sur des étapes précoces du cycle de vie de *X. laevis* mais aussi

de montrer que son ovocyte est un modèle pertinent à prendre en compte en écotoxicologie, au vu des avantages que cette cellule présente et des réponses observées suite à des expositions à des contaminants environnementaux. Ses intérêts tant d'un point de vue fondamental qu'appliqué sont illustrés par les résultats obtenus dans ce travail. Il existe donc un réel intérêt à s'intéresser aux premières étapes du cycle de vie qui constituent de véritables périodes critiques d'exposition.

Tableau 3. Synthèse graphique des principaux résultats.

Contaminants µM		Cadmium			Plomb			Cuivre				Bouillie bordelaise				Glyphosate					RoundUp® GT Max					Deltaméthrine			
		1,36	13,6	136	0,9	9	90	0,00399	0,0399	0,399	3,99	0,00399	0,0399	0,399	3,99	0,148	1,48	14,8	148	1480	0,148	1,48	14,8	148	1480	0,0003	0,003	0,03	0,3
Maturation ovocytaire	Maturation + Pg																												
	Maturation																												
	Cinétique + Pg			+																									
	Cinétique																												
	Cytologie	+	+	+				/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	/	/	/	/
	ERK2																												
	RSK																												
	pCdc2																												
	pH3																												
Fécondation et développement	Fécondation																									/	/	/	/
	Segmentation																									/	/	/	/
	Biométrie																									/	/	/	/

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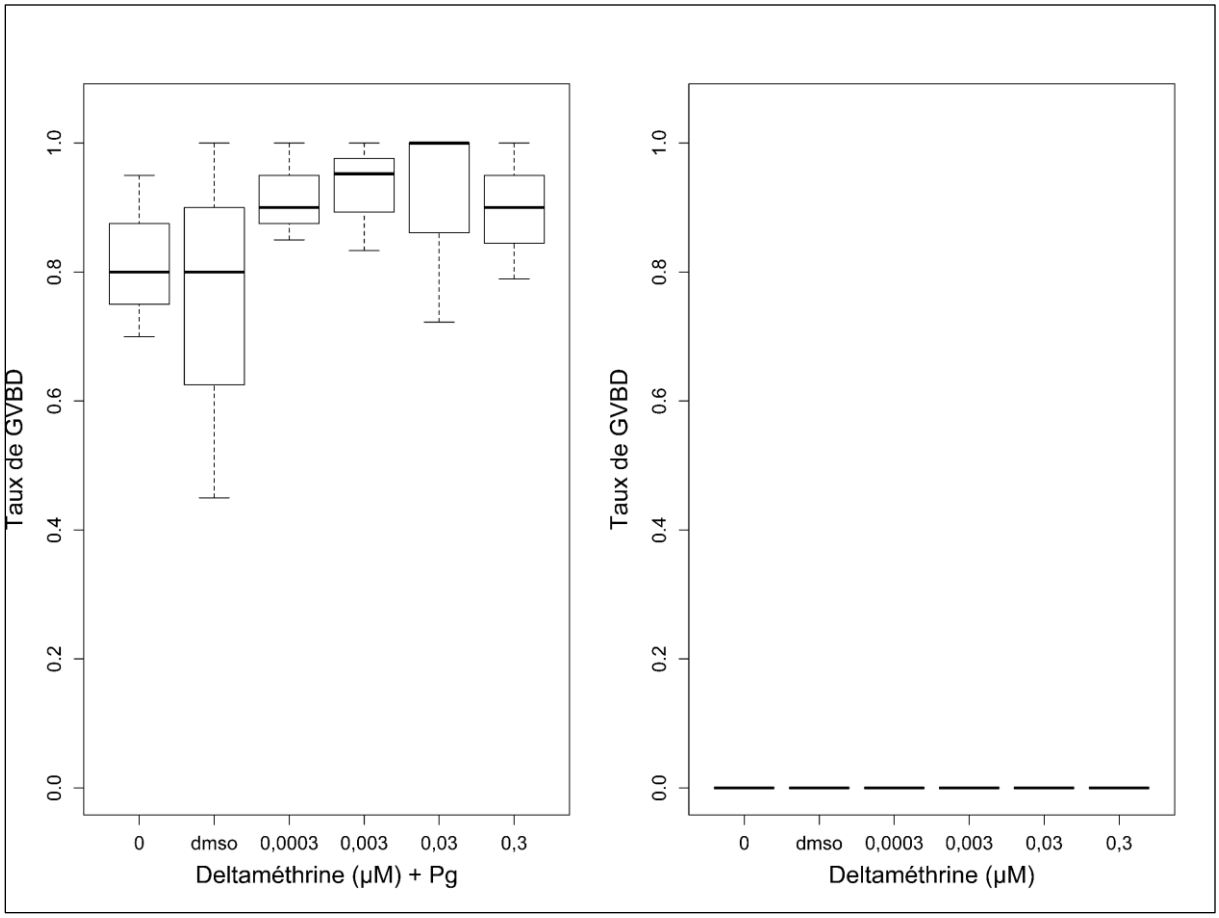
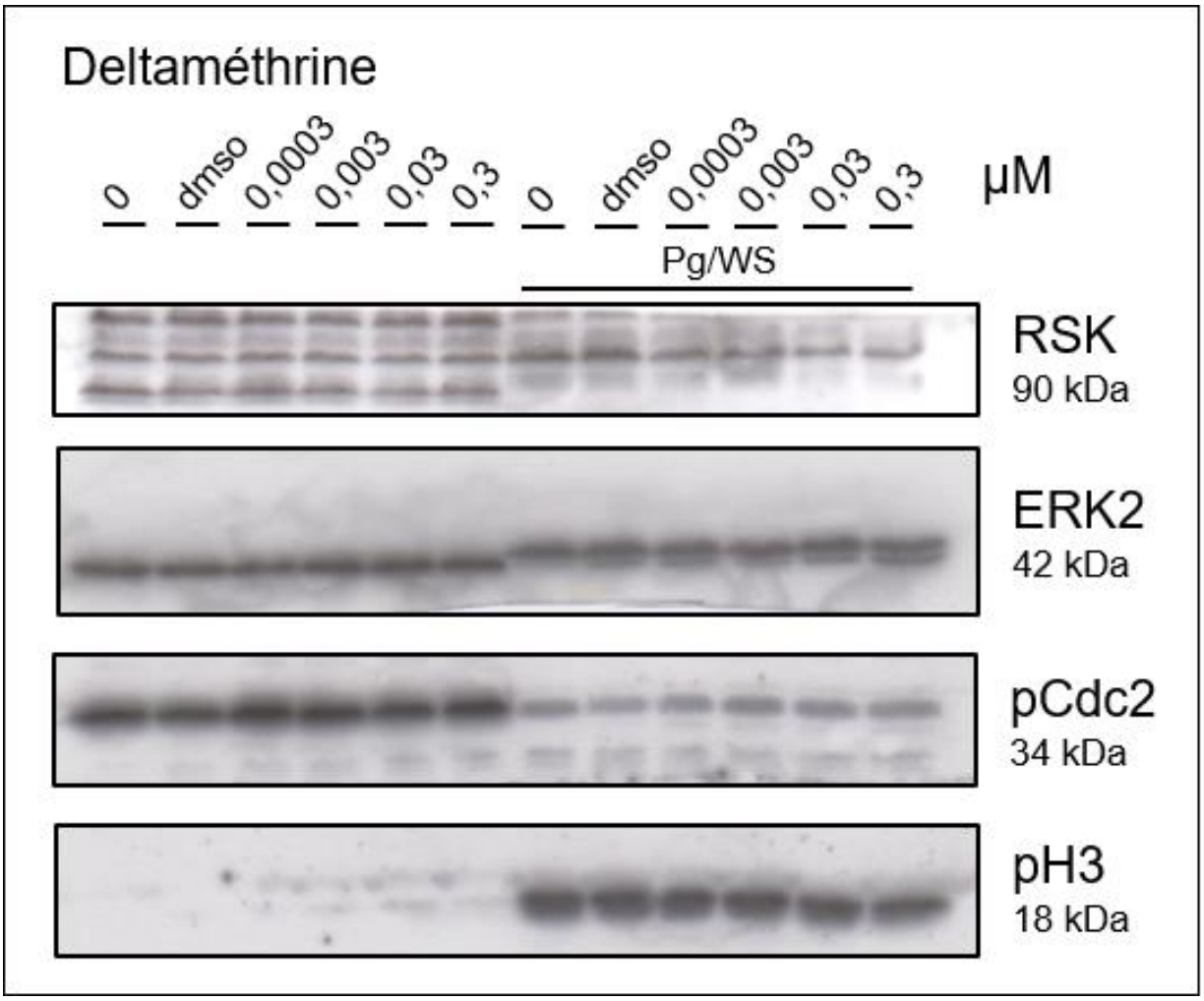
Annexes

ANNEXE I.



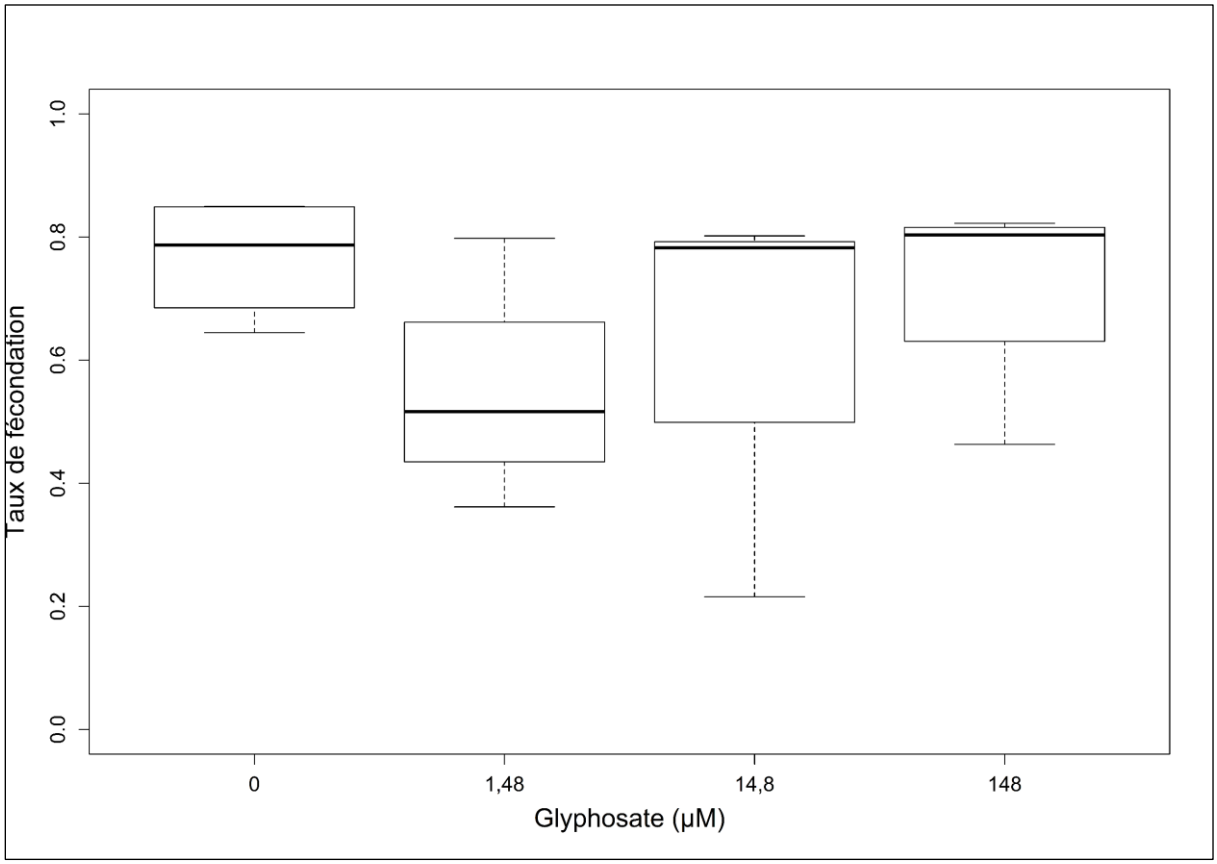
Annexe I. Effets de la deltaméthrine sur les taux de GVBD d'ovocytes de *Xenopus laevis*. Les cellules germinales ont été exposées sur la nuit à 19 °C à des concentrations croissantes de deltaméthrine (0 ; 0,0003 ; 0,003 ; 0,03 ; 0,3 µM) en présence (A) ou non (B) de progestérone. Un test de Friedman a été appliqué pour comparer les résultats. N = 3.

ANNEXE II.



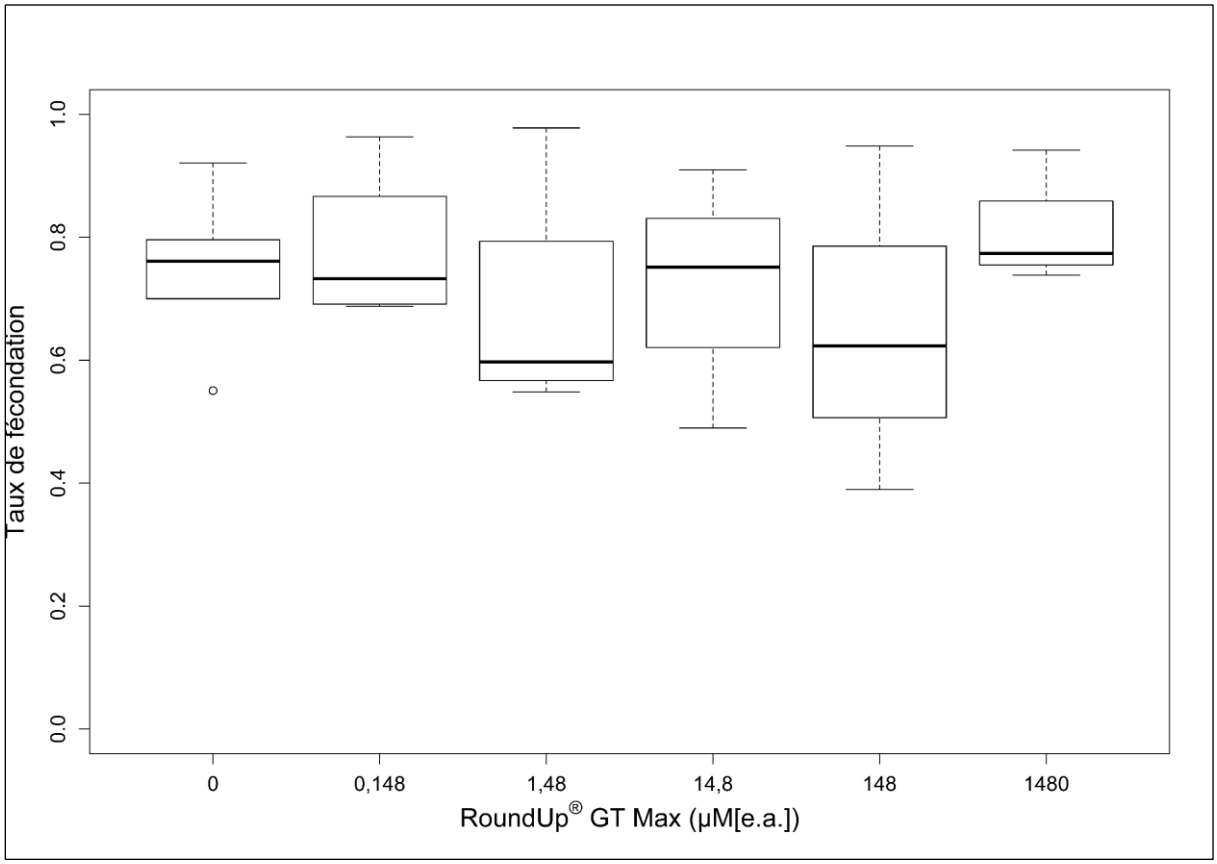
Annexe II. Effets des expositions à la deltaméthrine sur les états de phosphorylation de RSK et ERK2 de la cascade MAPK and de la sous unité catalytique Cdc2 du MPF et H3, cible du MPF. Absence de barre : ovocytes non exposés à la progestérone et sans tache de maturation ; Pg/WS : ovocytes avec tache de maturation exposés à de la progestérone. N = 3.

ANNEXE III.



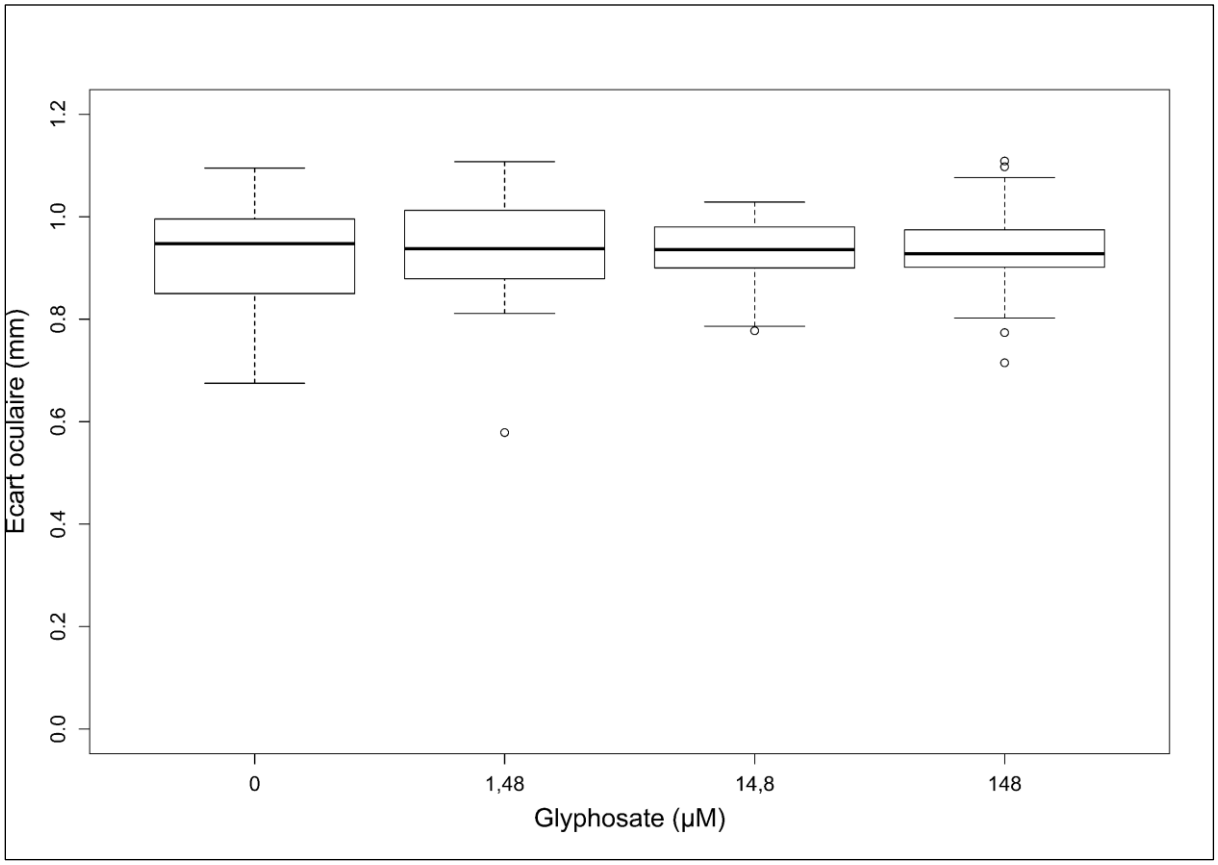
Annexe III. Effets des expositions au glyphosate sur les taux de fécondation de *Xenopus laevis*. Les analyses photographiques ont été effectuées 35 minutes après fécondation dans les milieux contaminés ou non au glyphosate (1,48 ; 14,8 ; 148 µM). Un test de Kruskal-Wallis a été appliqué pour comparer les résultats. N = 3.

ANNEXE IV.



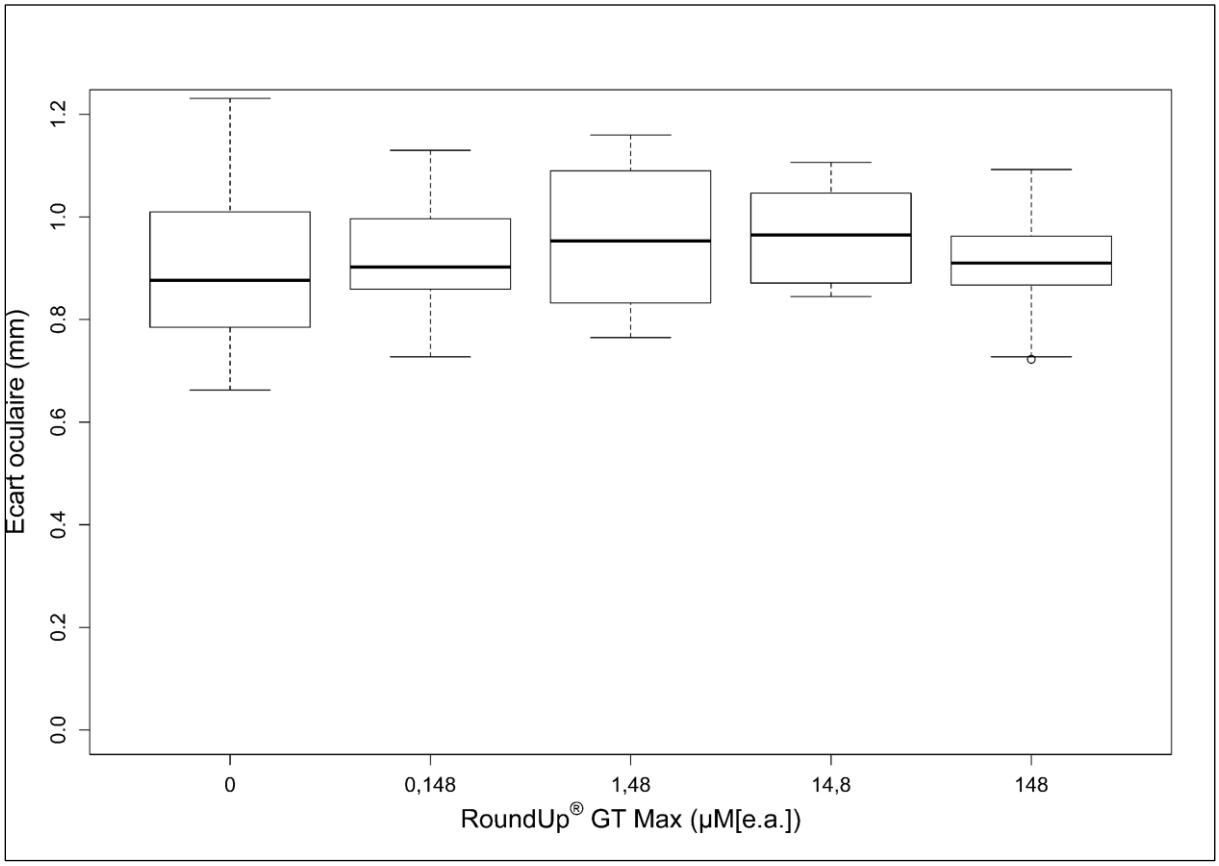
Annexe IV. Effets des expositions au RoundUp® GT Max sur les taux de fécondation de *Xenopus laevis*. Les analyses photographiques ont été effectuées 35 minutes après fécondation dans les milieux contaminés ou non au glyphosate (1,48 ; 14,8 ; 148 µM). Un test de Kruskal-Wallis a été appliqué pour comparer les résultats. N = 3.

ANNEXE V.



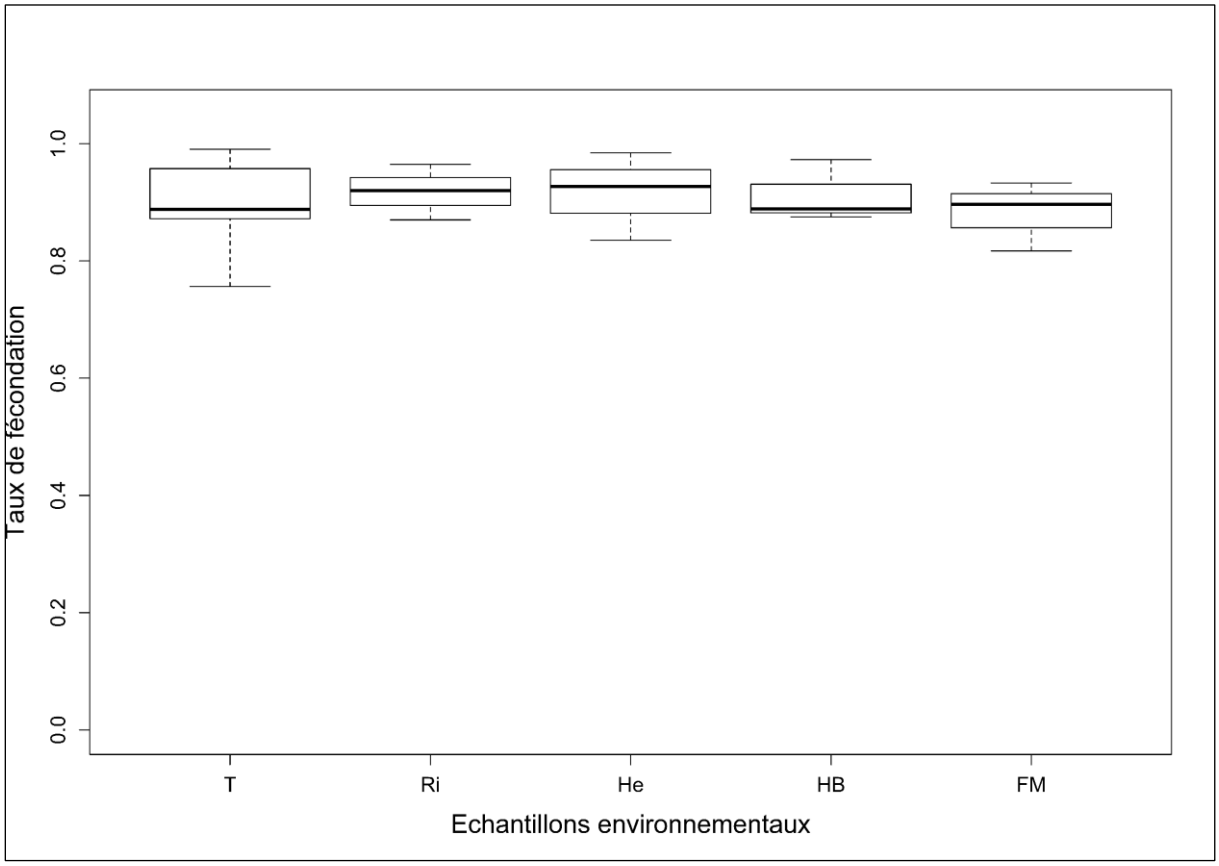
Annexe V. Effets des expositions au glyphosate sur l'écart oculaire de têtards de 6 jours. Les individus ont été exposés depuis la fécondation *in vitro*. Après une coloration au bleu alcian, une analyse automatisée des images a été conduite sous ImageJ. Un test de Kruskal-Wallis a été appliqué pour comparer les résultats. N = 3.

ANNEXE VI.



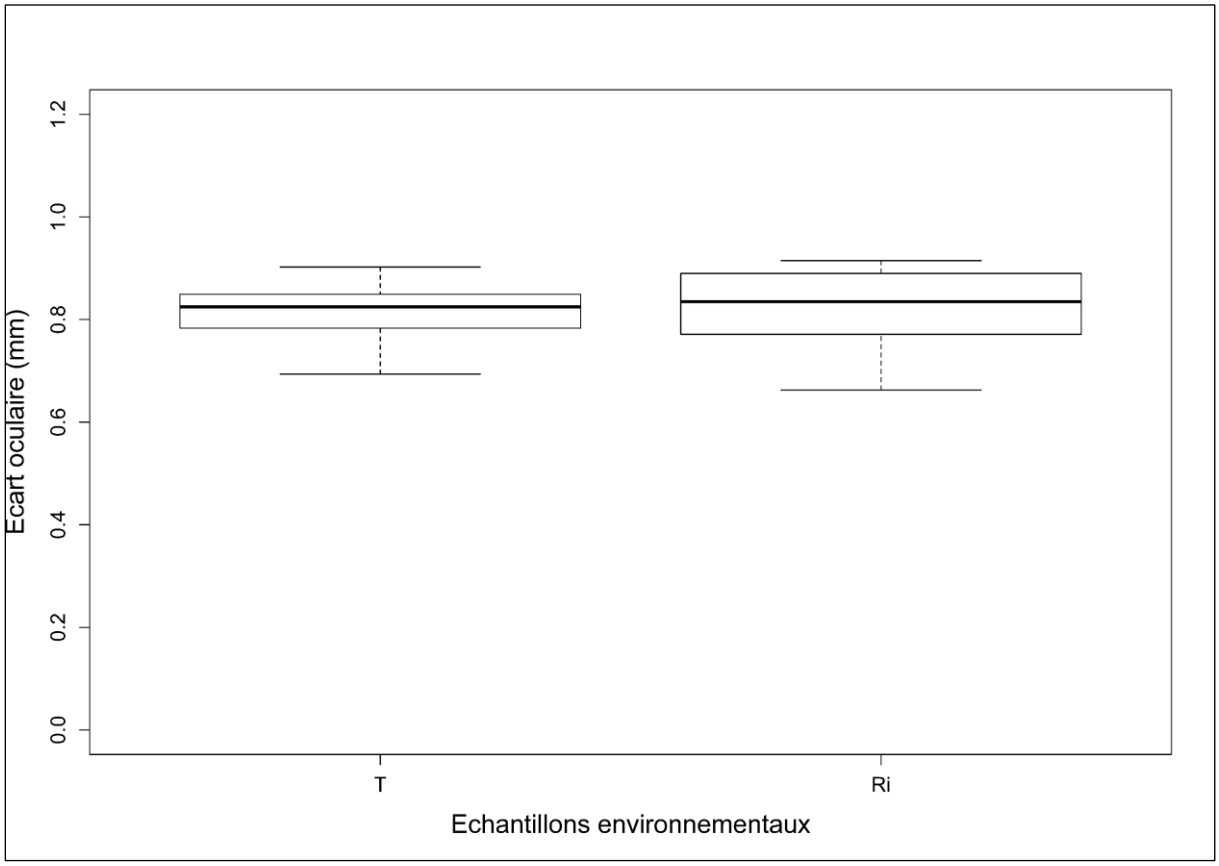
Annexe VI. Effets des expositions RoundUp® GT Max sur l'écart oculaire de têtards de 6 jours. Les individus ont été exposés depuis la fécondation in vitro. Après une coloration au bleu alcian, une analyse automatisée des images a été conduite sous ImageJ. Un test de Kruskal-Wallis a été appliqué pour comparer les résultats. N = 2.

ANNEXE VII.



Annexe VII. Effets des expositions aux échantillons environnementaux sur les taux de fécondation. Les analyses photographiques ont été effectuées 35 minutes après fécondation dans les différentes conditions. T : Témoin ; Ri : La Risle, Pont-Audemer, Eure ; He : Etendue d'eau, Villeneuve-d'Ascq, Nord ; HB : Ruisseau, Villeneuve-d'Ascq, Nord ; FM : La Corbie, Fort-Moville, Eure. Un test de Kruskal-Wallis a été appliqué pour comparer les résultats. N = 3.

ANNEXE VIII.



Annexe IX. Effets des expositions à de l'eau prélevée dans la Risle (Ri, Pout-Audemer, Eure) sur l'écart oculaire de têtards de 6 jours. Les individus ont été exposés depuis la fécondation *in vitro*. Après une coloration au bleu alcian, une analyse automatisée des images a été conduite sous ImageJ. Un test de Kruskal-Wallis a été appliqué pour comparer les résultats. N = 2.

Résumé : Les amphibiens constituent aujourd'hui le groupe le plus menacé d'extinction parmi les vertébrés. Néanmoins, peu de travaux en toxicologie des amphibiens tiennent compte des stades précoces de leur cycle de vie. Pourtant, un individu est exposé directement aux substances présentes dans le milieu aquatique depuis l'émission des gamètes. Dans ce contexte, cette thèse de doctorat a pour objectifs d'apporter de nouvelles données sur les effets d'expositions à des xénobiotiques d'ovocytes de *Xenopus laevis*, de rechercher des cibles au sein de ce gamète et de participer au développement d'un nouveau modèle en écotoxicologie pour évaluer la qualité de milieux aquatiques. Ces ovocytes présentent de nombreux avantages qui nous ont permis de développer des protocoles efficaces pour appréhender la toxicité de substances. Des endpoints ont pu être définis autour de la maturation et de la fécondation de l'ovocyte, puis autour du développement embryonnaire et de la formation de jeunes têtards. Les effets d'expositions au cadmium, au plomb, au cuivre, à la bouillie bordelaise, au glyphosate, au RoundUp® GT Max et à la deltaméthrine ont été déterminés avec nos paramètres. Des essais ont été également conduits pour des échantillons de milieux soumis à différentes pressions anthropiques. Il est apparu que l'ovocyte de xénope est sensible aux expositions, notamment au cadmium ou au glyphosate, sous ses deux formulations. Des effets, jamais rapportés ont également été observés. Différentes signatures d'expositions sont apparues, comme la formation de doubles structures cytologiques induites par le glyphosate. Les réponses mises en évidence et aussi les expérimentations menées avec de l'eau prélevée dans l'environnement prouvent que l'ovocyte de *X. laevis* est un modèle pertinent et permettent de recommander l'étude des premières étapes du cycle de vie de l'amphibien en toxicologie aquatique.

Mots-clés : Contamination aquatique ; Amphibien ; *Xenopus laevis* ; Ovocyte ; Maturation ; Fécondation ; Développement

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Abstract: Amphibians are one of the most imperiled group of extinction. Nevertheless, few toxicological studies are interested in the earliest steps of their life cycle, even if gamete emission, fertilization and embryogenesis are directly exposed to water pollution. In this context, this PhD thesis aims to bring new data about xenobiotic exposure effects on *Xenopus laevis* oocytes, to highlight targets inside this germ cells and to contribute to the elaboration of a new model in ecotoxicology to assess aquatic environment quality. As a well-known gamete, the xenopus oocyte makes possible to establish suitable experimental designs to assess toxicity. Many endpoints were defined regarding the oocyte maturation, the fertilization and also the development. The experiments were conducted in metal (cadmium, lead, copper) and in phytopharmaceutical (Bordeaux mixture, glyphosate, RoundUp® GT Max, deltamethrin) contaminated conditions, but also in environmental samples from various aquatic habitats. The xenopus oocyte appeared to be sensitive to contaminant exposures and specially to cadmium and both formulations of glyphosate. Never observed effects were reported. Pollutant signatures were also pointed up, like the double cytological structures induced by glyphosate exposures. The observed responses and results from environmental water experimentations show that *X. laevis* oocyte is a pertinent model in ecotoxicology and allow to recommend the first steps of the amphibian life cycle in aquatic toxicology.

Keywords: Aquatic contamination; Amphibian; *Xenopus laevis*; Oocyte; Maturation; Fertilization; Development