UNIVERSITÉ DU QUÉBEC À MONTRÉAL

ÉVALUATION DE L'IMPACT D'UNE EXPOSITION MULTIGÉNÉRATIONNELLE SUR LA SENSIBILITÉ DE TROIS ESPÈCES PHYTOPLANCTONIQUES À L'HERBICIDE REFLEX®

MÉMOIRE

PRÉSENTÉ

COMME EXIGENCE PARTIELLE

DE LA MAÎTRISE EN BIOLOGIE

PAR

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*PPIX	Protoporphyrine IX en état excité
μ	Taux de croissance
^{3}Chl	Chlorophylle excitée à l'état triplet
ABS	Flux d'énergie lumineuse absorbée
ADP	Adénosine diphosphate
ANOVA	Analyse de la variance
APC	Allophycocyanine
ATP	Adénosine triphosphate
ATPase	Adénosine-triphosphate synthase
Chl *a*	Chlorophylle *a*
CPCC	Canadian Physiological Culture Center
CTE	Chaîne de transport d'électron
DCF	2',7'-Dichlorofluorescéine
DI	Flux d'énergie lumineuse dissipée
DMSO	Diméthylsulfoxyde
EPA	Agence de protection environnementale
ET	Flux de transport d'électrons

F'_0	Fluorescence maximale pour un organisme photosynthétique adapté à la lumière
F'_M	Valeur maximale de la fluorescence induite par un flash saturant de lumière actinique pour un organisme photosynthétique sous illumination continue
F_0	Fluorescence initiale mesurée à 50 µs
F_{2ms}	Fluorescence mesurée à 2 ms
$F_{300\mu s}$	Fluorescence mesurée à 300 µs
$F_{50\mu s}$	Fluorescence mesurée à 50 µs
FACHB	Freshwater Algae Culture Collection at the Institute of Hydrobiology
FL1	Fluorescence verte réémise après illumination avec de la lumière filtrée à 530nm dans un filtre d'une épaisseur de 30nm
F_M	Valeur maximale de la fluorescence induite par un flash saturant de lumière actinique pour un organisme photosynthétique adapté à l'obscurité
FQR	Ferrédoxine-plastoquinone oxydoréductase
F_s	Fluorescence variable induite par une illumination actinique continue
F_v	Fluorescence variable
FW	Freshwater
H_2DCF	2',7'-Dichlorodihydrofluorescéine
H_2DCF-DA	2',7'-Dichlorodihydrofluorescéine diacétate
LHCII	Complexe d'antennes collectrices en périphérie du PSII

LHCIIb	Fragment b du complexe d'antennes collectrices en périphérie du PSII
M_0	Taux net de fermeture des centres réactionnels du PSII
NADH	Nicotinamide adénine dinucléotide
NADP+	Nicotinamide adénine dinucléotide phosphate réduit
NADPH	Nicotinamide adénine dinucléotide phosphate
Ndh	Complexe NADPH/NADH déshydrogénase
N_g	Nombre de générations d'une culture algale
NOAEL	Concentration la plus élevée pour laquelle aucun effet toxique n'est observable
NPQ	Quenching non photochimique
O_2	Oxygène moléculaire
PAR	Radiations photosynthétiquement actives
PC	Phycocyanine
PEA	Plant Efficiency Analyzer
pH	Potentiel Hydrogène
PPGIX	Protoporphyrinogène IX
PPIX	Protoporphyrine IX
PPO	Protoporphyrynogène oxydase
PSI	Photosystème I
PSII	Photosystème II
Q	Quinone accepteuse principale

Q_A	Quinone A
Q_B	Quinone B
qE	quenching énergie-dépendant
qn_{rel}	Quenching non photochimique relatif
qp_{rel}	Quenching photochimique relatif
RC	Reaction center
ROS	Espèces réactives oxygénées
SSC	Complexité cellulaire
T_d	Temps de doublage d'une culture algale
TR	Flux de transport d'électrons
UQF_{rel}	Fluorescence non-''quenchée'' relative
UV	Ultraviolet
V_j	Densité de centre réactionnel PSII fermé après 2 ms d'illumination avec une lumière actinique
ϕ	Rendement photochimique

RÉSUMÉ

Il y a recrudescence de l'utilisation de l'herbicide fomésafène (5-[2-chloro-4-(trifluorométhyl) phénoxy] -N-(méthylsulfonyl) -2-nitrobenzamide) en réponse à l'émergence de plantes néfastes résistantes à l'impact du glyphosate. En réponse à ce phénomène, l'augmentation de l'utilisation de fomésafène entraîne inévitablement une hausse dans la probabilité que cette molécule se fraye un chemin vers les écosystèmes aquatiques. Ce projet de maîtrise vise à étudier l'impact d'un traitement et d'une exposition multigénérationnelle à un herbicide à base de fomésafène couramment utilisé, le Reflex®, sur la physiologie (croissance, contenu en pigments photosynthétique, photosynthèse, stress oxydatif, morphologie) du phytoplancton potentiellement retrouvé dans les eaux douces à proximité de zones à forte activité agricole. Pour ce faire, deux microalgues vertes *(Raphidocelis subcapitata* FACHB271, *Chlamydomonas snowii* isolée du réservoir Choinière) et une cyanobactérie (*Microcystis aeruginosa* CPCC632) furent soumises à deux séries d'expériences. En premier lieu, les différents biomarqueurs physiologiques furent évalués suite à une exposition à des concentrations croissantes de fomésafène (Reflex®, 5, 10, 40, 320 $\mu g\ L^{-1}$). Des courbes concentrations-réponse furent ensuite calculées et celles-ci démontrent une réduction dans la croissance de *R. subcapitata* suite à une exposition à plus de 10 $\mu g\ L^{-1}$. Chez cette espèce, cette inhibition fut reflétée par une diminution dans la production de pigment photosynthétique et protecteur, une baisse dans l'activité photosynthétique et une élévation drastique de la présence de ROS. Lors de la deuxième série d'expériences, la pression toxique de ces mêmes concentrations d'exposition fut maintenue sur plusieurs générations pour les trois espèces d'algues afin de vérifier l'impact de la persistance d'une contamination à pesticide sur la sensibilité du phytoplancton d'eau douce au fomésafène. Les résultats ont démontré

qu'une pré-exposition à plus de 10 µg L^{-1} a permis à *R. subcapitata* de retrouver des conditions physiologiques normales, et ce, même en présence de la plus forte concentration d'herbicide testée (320 µg L^{-1}). Ce projet met l'emphase sur la présence de différences inter- et intraspécifiques quant à l'impact d'un herbicide à base de fomésafène sur le phytoplancton. De plus elle permet d'entrevoir l'effet d'une exposition prolongée à des concentrations environnementales et supérieures sur la physiologie des microorganismes photosynthétiques retrouvés en eaux douces à proximité de milieux agricoles.

Mots-clés : herbicide, inhibiteur de la PPO, exposition multigénérationnelle, physiologie, chlorophytes, cyanobactérie

INTRODUCTION GÉNÉRALE

0.1 La problématique

À la base de tout réseau trophique se retrouvent les organismes photoautotrophes. À partir de l'énergie solaire, ces microorganismes photosynthétiques produisent la biomasse nécessaire pour combler l'apport nutritionnel requis par les premiers maillons de diverses chaînes trophiques (Falkowski *et al.*, 1998; Platt *et al.*, 1983). Simultanément, ces organismes sont responsables de la production d'une majeure partie de l'oxygène, transportent une grande quantité de carbone vers les sédiments et sont la pierre angulaire de cycles biogéochimiques importants (Chapman, 2013; Kump, 2008; Rost *et al.*, 2008; Stockner et Antia, 1986). Le phytoplancton se démarque comme étant particulièrement sensible à la présence de facteurs de stress environnementaux. En effet, de nombreuses sources de stress tels que les changements de température (Chalifour et Juneau, 2011; Eppley, 1972), les rayons UV (Holzinger et Lütz, 2006; Maske, 1984; Pessoa, 2012) ou le relargage de contaminants issus d'activités anthropiques comme l'agriculture (Downing *et al.*, 2008; Schindler, 1987) se sont révélé des facteurs de stress importants qui peuvent affecter la dynamique du phytoplancton. Suite à l'utilisation intensive de pesticides par l'industrie agroalimentaire, un large éventail de ceux-ci peuvent se retrouver dans les écosystèmes aquatiques par lessivage ou écoulement direct (Chekroun *et al.*, 2013; Guo *et al.*, 2003; Potter *et al.*, 2011). De plus, plusieurs recherches démontrent la persistance d'un vaste éventail de pesticides dans les écosystèmes aquatiques (Edwards et Adams, 1970; Feo *et al.*, 2010; Navarro *et al.*, 2004; Thurman *et al.*, 1991; Zhang *et al.*, 2017). Ces dernières années furent aussi marquées par une recrudescence de l'utilisation du fomésafène ou 5-[2-chloro-4-(trifluorométhyl) phénoxy] -N-(méthylsulfonyl) -2-

nitrobenzamide, un inhibiteur de la protoporphyrinogène oxydase couramment utilisé lors du contrôle des espèces néfastes dans la culture du soya (*Glycine max*), du coton (*Gossypium hirsutum*), le haricot vert (*Phaseolus vulgaris*), la tomate (*Solanum lycopersicum*), la patate (*Solanum tuberosum*) et le piment (*Capsicum* spp.) (Campbell *et al.*, 2012).

0.2 Risque écotoxicologique du fomésafène dans l'environnement

Le fomésafène (5-[2-chloro-4-(trifluorométhyle) phénoxy]-N-méthylsulfonyl-2-nitrobenzamide) est l'ingrédient actif de 27 produits enregistrés dont cinq appartiennent à la compagnie Syngenta Crop Protection LLC (Campbell *et al.*, 2012). Cette molécule est fréquemment utilisée à cause de son puissant effet toxique à faibles concentrations (Wu *et al.*, 2014). Ce mémoire met l'emphase sur l'herbicide Reflex®, une formulation commerciale contenant 22.8% de fomésafène sous forme de sel de sodium en tant qu'ingrédient actif. Dans le contexte de cette étude, les concentrations d'exposition à l'herbicide Reflex® font référence à la concentration de la molécule active présente en solution. Ce produit phytosanitaire est utilisé seul ou en combinaison avec d'autres pesticides pour le traitement de surface, au moment de la pré- et/ou post-émergence des dicotylédones néfastes à feuilles larges lors de la culture du coton, du soya (Caquet *et al.*, 2005), des haricots (Santos *et al.*, 2006), du pois mange-tout (Scott, 2016), de la tomate et la patate (Boyd, 2015; Monday *et al.*, 2015) avec une application maximale recommandée d'ingrédient actif de 0.375 lb/acre par année (Scott, 2016).

Le fomésafène est catégorisé comme étant un herbicide qui persiste dans le sol, car il possède une très longue demi-vie (entre 60 et 240 jours) (Guo *et al.*, 2003). La persistance du fomésafène contribue à une accumulation de cette molécule au fil des années et peut poser un problème pour les écosystèmes terrestres et aquatiques (Khorram *et al.*, 2016). Étant donnée l'existence d'un usage varié et répété de cette molécule dans l'industrie agricole, il existe un risque élevé de dissipation et d'accumulation dans l'environnement. Les principales routes de dissipation du

fomésafène sont le lessivage, le ruissellement et la dégradation microbienne (Feng *et al.*, 2012; Potter *et al.*, 2011). De plus, la recrudescence de l'utilisation du fomésafène augmente le nombre d'endroits nouvellement touchés par cet herbicide ainsi que le taux d'application de cette molécule ce qui accroit invariablement le risque d'introduire cette molécule dans les sources d'eau douce arborant les zones à forte activité agricole. Une fois dans les eaux de surfaces, le fomésafène peut potentiellement affecter la croissance d'organismes non-cibles tels que le phytoplancton. Malgré ce risque, il existe très peu d'information sur le devenir du fomésafène dans l'environnement aquatique. Entre 2006 et 2008, Environnement Canada a détecté la présence de fomésafène en concentrations allant de 0.66 à 873 ng L^{-1} dans plusieurs rivières et petit cours d'eau au Québec et en Ontario. Le rapport indique d'ailleurs que le fomésafène fût détecté dans 25.3% des échantillons analysés, soit la troisième molécule la plus fréquemment retrouvée dans les écosystèmes aquatiques testés (Struger *et al.*, 2011).

0.3 Le phytoplancton

Le phytoplancton est un groupe polyphylétique extrêmement diversifié. Celui-ci est composé de microalgues et de cyanobactéries photosynthétiques qui sont nécessaires dans l'alimentation de la quasi-totalité des réseaux trophiques et permettent le cycle biogéochimique d'un vaste éventail d'éléments et composés chimiques (Rousseaux et Gregg, 2013). De manière générale, le phytoplancton est considéré comme étant des protistes aquatiques photoautotrophes possédant la capacité de libérer de l'oxygène et pouvant être unicellulaire, colonial, filamenteux ou composé de tissus simples (Guiry, 2012). Les cyanobactéries sont quant-à-elles des procaryotes considérés comme étant des bactéries Gram-négatives qui, comme les eucaryotes photosynthétiques, possède la chlorophylle *a* (Chl *a*). Ainsi, les cyanobactéries sont capables de faire la photosynthèse et participent à la production d'oxygène (Taiz et Zeiger, 2006). De plus, la lumière peut être captée par plusieurs autres pigments produits par les cyanobactéries comme la phycocyanine, la phycoérythrine et l'allophycocyanine (Gantt et Lipschultz, 1973).

Des milliers d'espèces d'algues et cyanobactéries connus, il existe une large variété de combinaison de pigments photosynthétiques principaux et accessoires participants dans la production primaire (Bonilla *et al.*, 2009; Briand *et al.*, 2003; Guiry, 2012).

En écotoxicologie, le phytoplancton est régulièrement employé pour déterminer la présence d'un vaste éventail de polluants dans l'environnement (Lewis, 1995; Mohan et Hosetti, 1999). Le taux de division rapide du phytoplancton permet d'évaluer l'effet d'un toxique sur plusieurs générations, et ce, en très peu de temps et à moindres coûts. Différentes méthodes permettent de visualiser l'impact physiologique d'un pesticide sur le phytoplancton comme la mesure du taux de croissance ou la fluorescence réémise lors de la photosynthèse (Krause et Weis, 1984). Les résultats présentés dans ce mémoire sont souvent exprimés sous forme de pourcentages à la hausse ou à la baisse en fonction de la culture témoin obtenue à partir de courbes concentrations-réponse. Ce format permet de comparer les résultats à des paramètres de toxicité régulièrement utilisés en écotoxicologie comme le CE_{50}, soit la concentration d'exposition à un toxique nécessaire pour engendrer 50% de son impact sur une population.

0.4 Le mode de toxicité du fomésafène

Cette molécule exerce son action par l'inhibition de la protoporphyrinogène oxydase (PPO), une enzyme clé dans la biosynthèse de l'hème et la chlorophylle (Ma *et al.*, 2002; Matringe *et al.*, 1989). L'inhibition compétitrice de la PPO par le fomésafène entraîne ainsi une baisse de la production de la chlorophylle *a*. La Chl *a* étant une composante essentielle des complexes antennaires et des centres réactionnels, toute diminution dans la concentration de cette molécule peut avoir un impact sur le rendement photosynthétique. Une conséquence supplémentaire de l'exposition au fomésafène est l'inhibition de la PPO laquelle s'ensuit d'une accumulation du précurseur de la protoporphyrine IX (PPIX), la protoporphyrinogène IX (PPGIX). En s'accumulant, la PPGIX dérive ainsi librement de son lieu de synthèse et subséquent métabolisme pour ensuite être oxydée en PPIX par des peroxydases non spécifiques et

via auto-oxydation. En présence de lumière, la PPIX s'excite (*PPIX) et réagit fortement avec l'eau moléculaire entrainant ainsi la production d'espèces réactives de l'oxygène (ROS) (Wakabayashi, 1999). Si la production de ROS surpasse la capacité de protection des mécanismes de défense normalement activés en conditions de stress (Ledford et Niyogi, 2005; Niyogi, 1999, 2000), les pigments photosynthétiques, protéines et lipides déjà synthétisés subiront des dommages oxydatifs qui mèneront à la destruction des centres réactionnels, une baisse de l'activité photosynthétique, une perte de l'intégrité de la membrane photosynthétique et cellulaire puis, éventuellement, la mort de l'organisme (Fig. 0.1).

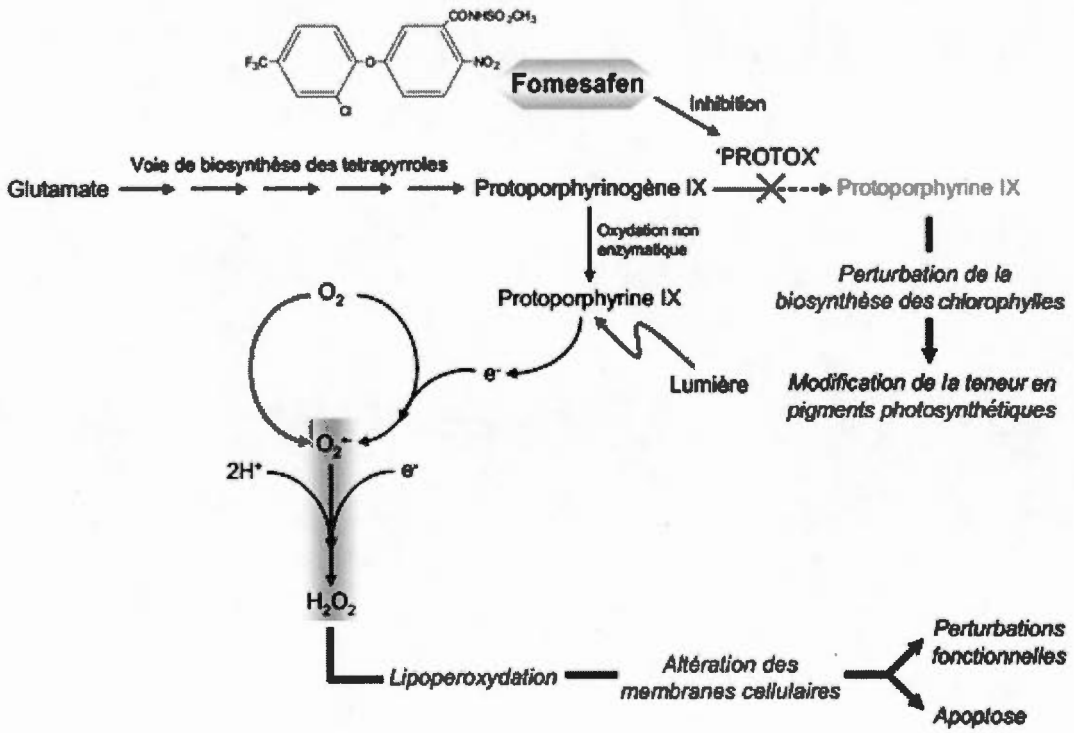

Figure 0.1 Représentation schématique démontrant le mode d'action du fomésafène et les conséquences à l'échelle cellulaire. Adapté de Lagadic et al. (2007).

0.5 La photosynthèse

La photosynthèse est un processus biologique par lequel la lumière est capturée et emmagasinée par un organisme afin de pouvoir alimenter les processus cellulaires de celui-ci (Roach et Krieger-Liszkay, 2014). La photosynthèse implique des pigments chlorophylliens et permet le transfert d'électrons à l'aide de processus réactionnels entrainés par l'énergie lumineuse. Seule la portion se situant entre 400 à 700nm représente la fraction des radiations considérées photosynthétiquement actives (PAR) du point de vue des organismes contenant des pigments chlorophylliens. Généralement, les phycobilines des cyanobactéries capturent efficacement la lumière entre 450 et 660 nm (Bennett et Bogorad, 1973).

Chez les microalgues, la photosynthèse se produit au niveau des chloroplastes, structures subcellulaires contenant l'ensemble des pigments photosynthétiques. Au sein du chloroplaste se trouve les thylakoïdes, un arrangement complexe de membranes où s'intègre les pigments nécessaires à la photosynthèse. L'absorption de la lumière ainsi que les réactions primaires nécessaires à la conversion de la lumière en énergie chimique se produisent ainsi au niveau de la membrane thylacoïdale. Le stroma, section aqueuse et non membranaire située à l'intérieur des chloroplastes, est principalement composé d'enzymes solubles impliquées dans le métabolisme du carbone nécessaire à la production de molécules destinées à être exportées hors du chloroplaste pour supporter d'autres mécanismes cellulaires. Étant morphologiquement distinctes, les cyanobactéries possèdent quelques différences au niveau de la photosynthèse. En absence d'organite, les thylakoïdes des cyanobactéries se situent dans le cytosol où la chaîne de transport d'électron respiratoire se retrouve. En conséquence, les processus physiologiques de la photosynthèse et la respiration chez les cyanobactéries sont associés l'un avec l'autre. À ce niveau se produisent les étapes principales de la photosynthèse soit le captage de la lumière et la génération de l'énergie nécessaire à la fixation du carbone (Moro *et al.*, 2016).

La photosynthèse est un processus complexe (Figs. 0.2 et 0.3) subdivisé en quatre phases : l'absorption de la lumière ainsi que le transfert de celle-ci via un système d'antennes, le transfert primaire d'électron vers les centres réactionnels, la stabilisation de l'énergie produite via les processus secondaires et la synthèse et l'exportation de produits stables (Roach et Krieger-Liszkay, 2014). Tous les processus, hormis la synthèse et l'exportation des produits stables, font référence à la phase claire de la photosynthèse. Les processus de synthèse et d'exportation des produits stables sont traditionnellement définis en tant que phase sombre de la photosynthèse puisque ces processus sont entièrement enzymatiques et se produisent indépendamment de l'illumination. Lors de ce travail de maîtrise, l'attention sera portée vers la phase claire de la photosynthèse.

Le mécanisme photochimique primaire de la photosynthèse est exécuté par la chaîne de transport d'électron (CTE) composée de deux complexes moléculaires nommés photosystème II (PSII) et photosystème I (PSI). L'énergie lumineuse est d'abord captée par un amalgame de pigments de type chlorophylle (principalement la Chl *a*) et accessoires (les caroténoïdes) se regroupant sous forme de complexe d'antennes collectrices en périphérie du PSII (LHCII). Chez les cyanobactéries, les phycobiliprotéines comme la phycocyanine (PC), l'allophycocyanine (APC) ou la phycoérythrine (PE) peuvent se retrouver dans la composition pigmentaire des antennes collectrices leur permettent de capter la lumière à des longueurs d'onde différentes par rapport aux autres organismes photosynthétiques (Shevela *et al.*, 2013). L'énergie lumineuse captée par les antennes collectrices du PSII migre au travers du complexe antennaire par résonance entre les molécules de chlorophylle jusqu'à un centre réactionnel (Taiz et Zeiger, 2006). Le centre réactionnel du PSII est formé des protéines D1 et D2, deux protéines principales qui stabilisent les pigments et les acteurs de la chaîne de transport d'électrons associés au PSII (Takahashi et Badger, 2011). Lors du transfert vers les centres réactionnel, l'énergie est redistribuée en trois voies complémentaires. Elle sera principalement utilisée pour 1) des processus

photochimiques afin de permettre la production d'ATP et de NADPH utilisés lors de la photosynthèse (Figs. 0.2 et 0.3) alors que la fraction énergétique restante sera dissipée 2) sous forme de chaleur et 3) réémise sous forme de fluorescence (Taiz et Zeiger, 2006). Au centre réactionnel du PSII, les photons provoquent la photolyse d'une molécule d'eau qui libère un électron dans la CTE de l'appareil photosynthétique et la libération d'une molécule d'oxygène et un proton dans le lumen du thylakoïde. Les électrons libérés permettent l'excitation de la Chl *a* et leur transfert vers la phéophytine, le premier accepteur de la CTE. Les électrons sont ensuite transférés vers les quinones (Q_A et Q_B), puis vers une réserve de plastoquinone. Les électrons sont ensuite dirigés vers le cytochrome b_6f où la plastocyanine, un transporteur lipophile, va assurer le déplacement de ceux-ci vers le PSI. Ultimement, les électrons sont acheminés jusqu'à la ferrédoxine afin de permettre réduction du $NADP^+$ en NADPH. Cette dernière étape termine le transport dit non cyclique ou linéaire des électrons (Taiz et Zeiger, 2006).

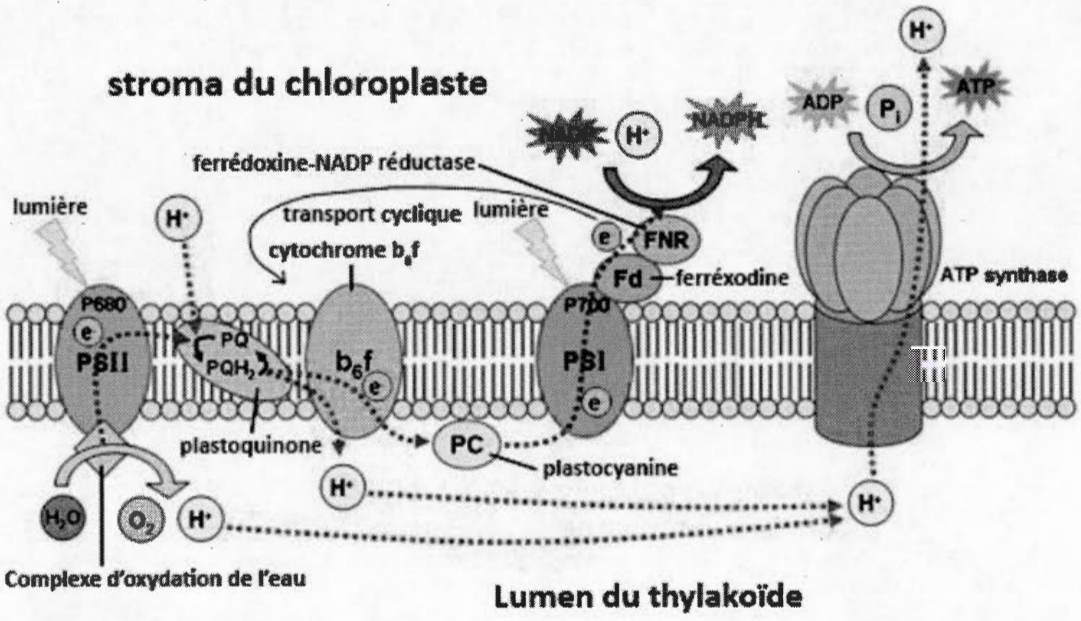

Figure 0.2 Mécanisme du transport linéaire et cyclique chez les chlorophytes. Adapté de Taiz et Zeiger (2006).

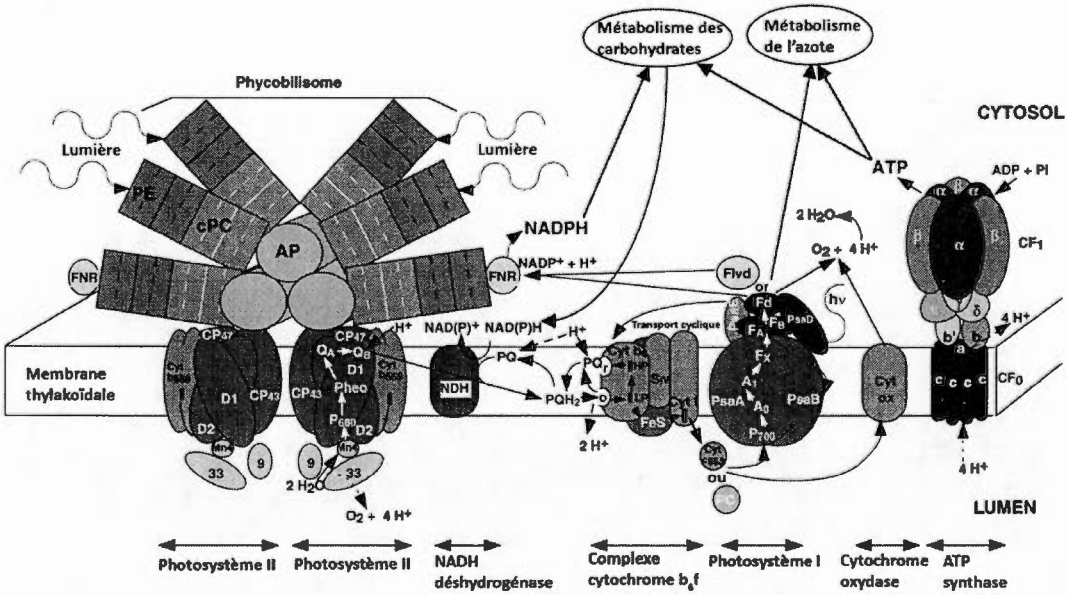

Figure 0.3 Mécanismes du transport linéaire et cyclique lors de la photosynthèse chez les cyanobactéries. Adapté de Campbell *et al.* (1998).

Le transport cyclique d'électrons est un type de transport d'électron impliquant le PSI qui, à la différence du transport linéaire, ne permet pas la production d'oxygène ni la réduction du $NADP^+$. Celui-ci opère au sein d'un supercomplexe composé du PSI, du cytochrome b_6f, d'une plastocyanine et d'une ferrédoxine. Il existe deux voies de transport cyclique autour du PSI, soit une voie impliquant l'enzyme ferrédoxine-plastoquinone oxydoréductase (FQR) et l'autre impliquant le complexe NADPH/NADH déshydrogénase (Ndh) (Bendall et Manasse, 1995). Le transport d'électrons s'effectue entre la ferrédoxine réduite du PSI ou le NADPH et la quinone accepteuse principale Q pour ensuite rejoindre le pool de plastoquinones et le cytochrome b_6f (Alric, 2010a; Bendall et Manasse, 1995; Joliot et Joliot, 2002).

Les transports linéaire et cyclique des électrons permettent la création d'un gradient de protons de part et d'autre de la membrane thylacoïdale. Le gradient de proton est utilisé par un complexe adénosine triphosphate synthase (ATPase) dans le but de produire de

l'adénosine triphosphate (ATP). L'énergie produite sous forme d'ATP et de NADPH est principalement utilisée lors du processus de fixation du carbone. Chez les cyanobactéries, l'ATP peut aussi être utilisée pour la fixation du N_2 et d'autres processus physiologiques (Shevela *et al.*, 2013).

Malgré la nécessité de la lumière pour les processus photosynthétiques, une quantité excessive de photons peut avoir une influence négative sur le phytoplancton. Lors du transfert de l'énergie lumineuse vers la chaîne de transport d'électrons, l'excitation des pigments photosynthétiques composant les LHCII peut mener à la formation de ROS à partir de l'oxygène moléculaire (O_2) (Ledford et Niyogi, 2005). Afin d'éviter la photoinhibition totale du PSII, les organismes photosynthétiques ont développé plusieurs mécanismes de photoprotection. Une fraction de l'énergie superflue sera réémise sous forme de fluorescence de la Chl *a* (Krause et Weis, 1991). L'énergie restante ne pouvant pas être transférée vers la chaîne de transport d'électrons sera dissipée par les mécanismes de *quenching* non photochimique comme le déplacement des LHCII vers le PSI (qT) et la dissipation de l'énergie en trop sous forme de chaleur (qE).

En réponse à un stress lumineux, en plus des mécanismes de protection non photochimiques, le phytoplancton peut favoriser davantage le transport cyclique d'électron autour du PSI dans le but de maintenir un apport stable d'ATP afin d'alimenter le cycle de Calvin. En effet, le transport cyclique rend possible l'établissement et le maintien du gradient de pH nécessaire à la production énergétique alors qu'il se produit une régulation négative du PSII via l'épuisement de l'énergie lumineuse superflue par libération de chaleur (Heber et Walker, 1992; Takahashi et Badger, 2011). Afin de pouvoir obtenir un rendement photosynthétique maximal, les microalgues et cyanobactéries requièrent une homéostasie au niveau de l'énergie d'excitation fournie aux deux photosystèmes ainsi qu'une quantité non limitante d'ATP et de pouvoir réducteur (NADPH) conformément au ratio requit pour la fixation

du carbone via le cycle de Calvin (Finazzi *et al.*, 1999). Cette balance est possible grâce au transfert entre les états de transition. Cette modification est un phénomène se produisant lorsque la phosphorylation réversible d'une fraction du LHCII provoque son transfert du PSII (état 1) vers le PSI (état 2) (Bonaventura et Myers, 1969).

Les différentes espèces d'algues et de cyanobactéries possèdent aussi la capacité de mitiger la photoinhibition par la synthèse d'antioxydants et de pigments caroténoïdes (Mallick et Mohn, 2000; Zhu *et al.*, 2010), la présence de mécanismes de réparation de dommages liés à la peroxydation lipidique et l'endommagement des centres réactionnels du PSII (Baier et Dietz, 1999; Barber et Andersson, 1992). Il existe d'autres mécanismes de défense en réponse à la photoinhibition comme l'induction de systèmes de régulation tels que la libération d'énergie sous forme de chaleur et les processus d'acclimatation comme la réduction de la taille des antennes des LHCII et la migration du LCHII vers le PSI pour diminuer la fluorescence de la photosynthèse (Mullineaux et Baker, 2010; Niyogi, 1999, 2000). La diversité de ces mécanismes de protection peut induire une disparité dans l'efficacité des processus impliqués dans la photosynthèse comme l'absorption de la lumière et la dissipation de l'énergie (Dubinsky et Stambler, 2009; MacIntyre *et al.*, 2002; Richardson *et al.*, 1983). La variation de cette efficacité est d'autant plus accentuée considérant que les algues possèdent la capacité de moduler leur activité photosynthétique en fonction des différents paramètres environnementaux qui influencent leur croissance comme l'intensité lumineuse, la température ou la biodisponibilité des nutriments (Huner *et al.*, 2003).

0.6 Exposition multigénérationnelle du phytoplancton

Tel que mentionné précédemment, les microorganismes photosynthétiques possèdent plusieurs mécanismes qui leur permettent de maintenir le bon fonctionnement de la

photosynthèse. Ainsi, différentes réponses émergent de la présence à long terme de sources de pression toxique et environnementale comme la mitigation des dommages causés par le stress oxydatif par la synthèse d'enzymes et molécules antioxydantes, la réparation de dommages causés par la peroxydation lipidique et les mécanismes de réparation en réponse à la dégradation des centres réactionnels du PSII (Baier et Dietz, 1999; Mullineaux et Baker, 2010; Niyogi, 1999, 2000; Subashchandrabose *et al.*, 2013; Takahashi et Badger, 2011).

Plusieurs études attestent de l'impact d'une exposition prolongée à divers facteurs de stress environnementaux sur la physiologie du phytoplancton. Ces recherches remarquent des changements dans les processus biochimiques des microorganismes photosynthétiques, l'apparition de résistance suite à des mutations pré-sélectives, la présence de résistance naturelle et parfois de mécanismes de détoxification en réponse à la présence de toxiques comme le glyphosate (López-Rodas *et al.*, 2007), l'atrazine (Chalifour *et al.*, 2016; Delorenzo *et al.*, 2004; Reboud *et al.*, 2007; Tang *et al.*, 1998), le diuron (Grizeau *et al.*, 1985; Stachowski-Haberkorn *et al.*, 2013), les hydrocarbures polycycliques aromatiques (Carrera-Martínez *et al.*, 2010; Huertas *et al.*, 2010; Kirso et Irha, 1998), la simazine et le diquat (Marvá *et al.*, 2010) et le lindane (González *et al.*, 2012).

Le développement naturel ou synthétique d'une tolérance à un herbicide inhibiteur de la PPO comme le fomésafène ou une formulation chimique à base de fomésafène a déjà été démontré chez certains organismes photosynthétiques comme l'amarante de Palmer (*Amaranthus palmeri* S. Watson, (Chahal *et al.*, 2017); Salas *et al.* (2016)), le soya (*Glycine max*, Skipsey *et al.* (2005)), le tabac (*Nicotiana tabacum*, Ichinose *et al.* (1995)), l'ambroisie élevée (*Ambrosia artemisiifolia*, Rousonelos *et al.* (2012)), la poinsettia (*Euphorbia heterophylla*, (Brusamarello *et al.*, 2016)) et la microalgue d'eau douce *Chlamydomonas reinhardtii* (Randolph-Anderson *et al.*, 1998).

0.7 Objectifs & hypothèses de travail

Objectif 1 : Évaluer l'impact d'un herbicide à base fomésafène (Reflex®) sur la physiologie (croissance, photosynthèse, stress oxydatif et morphologie) de :

a. *Raphidocelis subcapitata* FACHB271
b. *Chlamydomonas snowii* (Espèce isolée du réservoir de Choinière, Québec, Canada (Deblois *et al.*, 2013))
c. *Microcystis aeruginosa* CPCC632

Hypothèse 1 : Le fomésafène exerce une toxicité sur les algues et les cyanobactéries en diminuant la production de chlorophylle *a* et, conséquemment, la croissance et le rendement photosynthétique.

1.1 La photosynthèse sera le paramètre le plus affecté par l'exposition à un produit phytosanitaire à base de fomésafène à cause de l'effet inhibiteur du fomésafène sur la synthèse des pigments photosynthétiques, molécules nécessaires pour capter l'énergie lumineuse requise pour la croissance via la photosynthèse.

1.2 L'impact du fomésafène sera différent entre les espèces en raison des différences physiologiques intra- et interspécifiques.

Tel que discuté, le fomésafène exerce son effet toxique via l'inhibition de la PPO, une enzyme clé dans la voie de biosynthèse de la porphyrine chez les animaux et les plantes (Caquet *et al.*, 2005). Le substrat de la PPO, la protoporphyinogène IX, s'accumule dans le cytosol où il sera oxydé. La présence du produit oxydé mène à la formation d'espèces réactive de l'oxygène. Une exposition à différentes concentrations de fomésafène va donc induire une inhibition de l'activité photosynthétique et de la croissance chez le phytoplancton puisque les ROS endommagent les pigments chlorophylliens, les phycobilines ainsi que l'intégrité de la membrane cellulaire, composantes essentielles à la survie des organismes autotrophes (Caquet *et al.*, 2005).

Ces composantes se retrouvent ou non en différentes concentrations chez les espèces photosynthétiques sélectionnées. La mention du concept de sensibilité fait référence aux réponses qualitatives ou quantitatives à une substance potentiellement toxique de la part des différentes espèces testées (Juneau *et al.*, 2001; Nordberg *et al.*, 2009). L'impact de l'exposition à l'herbicide devrait être plus marqué pour *R. subcapitata* et *M. aeruginosa* que *C. snowii* puisque le faible ratio surface/volume de cette dernière devrait réduire l'absorption de l'herbicide au travers la membrane cellulaire (Delorenzo *et al.*, 2004; Stachowski-Haberkorn *et al.*, 2013; Tang *et al.*, 1998). De plus, les variations dans la composition en molécules et enzymes antioxydantes entre les chlorophycées et les cyanophytes devraient engendrer des différences dans la dynamique d'inhibition de ces organismes à l'herbicide.

Objectif 2 : Déterminer si, d'un point de vue physiologique, une pré-exposition à long terme à cet herbicide à base de fomésafène va provoquer une augmentation ou une diminution de la sensibilité de ces espèces phytoplanctoniques à ce polluant.

Hypothèse 2 : En présence d'un toxique dans le milieu de culture, le phytoplancton peut afficher une résistance suite à une acclimatation ou l'apparition de mutations présélectives

Suite à une exposition prolongée à un ou plusieurs facteurs de stress, un organisme peut développer une résistance via acclimatation ou suite à l'émergence de rares mutations bénéfiques. Dans l'optique de cet ouvrage, une résistance fait référence à certains traits génétiques acquis que possède certains organismes permettant à ceux-ci de proliférer dans des conditions environnementales autrement nuisibles ou fatales pour d'autres organismes de la même espèce n'ayant pas été préalablement adaptés. L'acclimatation est un processus réversible intra-générationnel pouvant s'amorcer dès les premiers instants suivant une exposition à un stress via lequel un organisme peut mitiger l'impact de changements environnementaux durables par l'ajustement de certains processus physiologiques via des changements dans l'expression de traits acquis (Flynn *et al.*,

2015). Ce phénomène découle du principe de plasticité phénotypique qui dicte qu'un génotype peut produire différents phénotypes en réponse aux conditions environnementales dans le but de maintenir l'homéostasie (Bradshaw et Hardwick, 1989). L'acclimatation diffère de l'adaptation, car ce dernier concerne un principe évolutif impliquant des changements héréditaires dans la séquence d'ADN qui altèrent l'expression de certains traits (Flynn *et al.*, 2015). Le terme ''tolérance'' fait référence à la capacité d'un organisme croître en présence d'un certain éventail de concentrations d'un toxique sans démontrer d'effets indésirables (Nordberg *et al.*, 2009). Dans certains cas, les modifications du patrimoine génétique des organismes soumis à un polluant sont principalement dues à la présence de mutations se produisant de manière aléatoire sur le génome. Lors d'une exposition multigénérationnelle, ce phénomène implique un potentiel d'adaptation qui peut mener à des résistances durables ou permanentes (Sniegowski *et al.*, 1995). Une exposition à long terme à la présence d'un herbicide à base de fomésafène (Reflex®) devrait induire des changements dans le génome des microorganismes photosynthétiques exposés qui vont induire des modifications dans l'expression de la PPO la rendant insensible à la présence de ce polluant. Ce phénomène favorisera le maintien de l'activité photosynthétique et la croissance chez les microalgues et les cyanobactéries puisque la synthèse des pigments photosynthétique ne devrait plus être affectée par la présence du polluant.

Objectif 3 : S'il y a présence d'une diminution dans la sensibilité à cette toxine suite à une pré-exposition à long terme, il sera question de déterminer si ces changements persistent après une période de dépuration.

Hypothèse 3 : Une souche phytoplanctonique ayant acquis une résistance suite à des mutations présélectives conséquemment à un prétraitement à un herbicide va préserver cette résistance suite à une période de dépuration.

Une période de dépuration, soit une phase durant laquelle les cultures phytoplanctoniques prétraitées à un toxique sont cultivées en l'absence de la substance

nocive, permet aux organismes de se réajuster à un milieu de culture frais et potentiellement d'éliminer ou dégrader le toxique internalisé (Jin *et al.*, 2012; Kabra *et al.*, 2014; Zhang *et al.*, 2010). Les microalgues et les cyanobactéries ayant démontré des changements de sensibilité suite à un prétraitement à un herbicide à base de fomésafène (Reflex®) peuvent conserver ces différences et les transférer d'une génération à l'autre malgré l'absence de contamination dans le milieu de culture ce qui indique essentiellement la création de nouvelles souches de cet organisme (Grizeau *et al.*, 1985; Stachowski-Haberkorn *et al.*, 2013). Ainsi, les microalgues et cyanobactéries qui ont acquis une résistance au fomésafène vont préserver leur résistance suite à une courte (1 semaine) et une longue (6 mois) période de dépuration.

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CHAPITRE I

EFFECT OF FOMESAFEN-BASED HERBICIDE REFLEX® ON THREE FRESHWATER PHYTOPLANKTONIC SPECIES

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1.1 Contributions

Jonathan Naoum a réalisé le plan d'expérience, les bioessais en laboratoire, l'analyse des résultats et la rédaction, alors que Philippe Juneau a participé à la conception du plan d'expérience, effectué la supervision des travaux de laboratoire et participé à la rédaction et la révision du texte.

1.2 Abstract

Pesticides leaching and run-off to nearby freshwater sources are a major ecological concerns. The emergence of herbicide-resistant weeds such as glyphosate-insensitive *Palmer Amaranth* led to the increased usage of the diphenyl ether fomesafen, a protoporphyrinogen oxidase (PPO) inhibitor. This recent rise in demand and uses diversity for this molecule invariably increase the chance of this herbicide entering freshwater environments and affecting non-target organisms. In plants, fomesafen is known to inhibit photopigment biosynthesis pathway through competitive inhibition with protoporphyrinogen IX (PPGIX) for the PPO enzyme. Subsequent accumulation of PPGIX leads to light-dependent ROS-generation, which causes disruption in membrane and pigment integrity and eventual death of the photosynthetic organism. As of today, there is still a lack of information concerning the impact of this diphenyl ether herbicide on the physiology of freshwater phytoplankton. This study aimed to confirm, compare and further understand the impact of increasing concentrations (5, 10, 40, 320 $\mu g\ L^{-1}$) of fomesafen-based herbicide (Reflex®) on the physiology of two species of green microalgae (*Raphidocelis subcapitata;* Chlamydomonas *snowii*) and one cyanobacteria (*Microcystis aeruginosa* CPCC632). This study shows that physiological biomarkers (growth, photosynthesis, pigment content, oxidative stress and morphology) of *R. subcapitata* were significantly affected by the fomesafen treatment. Moreover, exposure to this toxicant produced no significant changes in the physiology of *C. snowii* and *M. aeruginosa*. This resistance is most likely due to morphological and genetic discrepancies between species.

Keywords: Fomesafen, PPO-inhibitor, Reflex®, chlorophytes, cyanophytes, growth, photosynthesis, Chl *a*, ROS, biovolume, cell complexity

Highlights

- *The physiology of three phytoplanktonic species in response to a pulse exposure scenario to fomesafen (Reflex®) was assessed*
- *High concentrations of fomesafen (Reflex®) inhibited pigment production, photosynthesis efficiency and, ultimately, growth for R. subcapitata*
- *Sensitivity to fomesafen (Reflex®): R. subcapitata > C. snowii > M. aeruginosa*

1.3 Résumé

Les pesticides deviennent un souci écologique majeur lorsqu'ils contaminent les sources d'eau douce arborant les zones à fortes activité agricole via le lessivage ou le ruissellement par les pluies. L'émergence d'espèces photosynthétiques nuisibles nouvellement insensibles à la présence de glyphosate comme l'amarante de Palmer (*Palmer amaranth*) a donné lieu à une augmentation de l'usage du fomésafène, un inhibiteur de la protoporphyrinogène oxydase (PPO). Les récentes augmentations de la demande et la diversification des usages pour cette molécule augmentent invariablement la possibilité que cet herbicide se retrouve dans les eaux fraîches de surface et contaminent des organismes non-cibles comme le phytoplancton. À ce jour, il existe très peu d'information concernant l'impact de cet herbicide de type diphényle éther sur la physiologie du phytoplancton d'eau douce. Cette étude vise à confirmer, comparer et mieux comprendre l'effet d'une exposition à des concentrations croissantes d'une formulation à base de fomésafène, le Reflex® (5, 10, 40, 320 µg L^{-1}) sur la physiologie (croissance, photosynthèse, contenu pigmentaire, stress oxydatif, morphologie) de deux microalgues verte (*Raphidocelis subcapitata* FACHB271 ; Chlamydomonas *snowii*) et une cyanobactérie (*Microcystis aeruginosa* CPCC632).

Cette étude démontre que l'ensemble des biomarqueurs physiologiques de *R. subcapitata* sont significativement affectés par un traitement au fomésafène. De plus, l'exposition à ce toxique n'a provoqué aucun changement significatif dans la physiologie de *C. snowii* et *M. aeruginosa*. Cette résistance est probablement due à des différences morphologiques et génétiques entre les espèces.

Mots-clés : Fomésafène, inhibiteur de la PPO, Reflex®, chlorophytes, cyanophytes, croissance, photosynthèse, Chl *a*, ROS, biovolume, complexité cellulaire

1.4 Introduction

Pesticide applications in rural areas often lead to the contamination of nearby lakes and rivers. The protoporphyrinogen oxidase (PPO) inhibitor fomesafen (5-[2-chloro-4-(trifluoromethyl)phenoxy]-N-(methylsulfonyl)-2-nitrobenzamide) is used as an herbicide to control weeds in soybean, potatoes and cotton cultures and a growing number of studies now highlights the effectiveness of tank-mixing fomesafen to prevent the emergence of glyphosate-resistant weeds such as Palmer amaranth (*Amaranthus palmeri*) (Bond *et al.*, 2006; Chahal *et al.*, 2017; Salas *et al.*, 2016) and common waterhemp (*Amaranthus rudis*) (Sarangi *et al.*, 2017). This increase in its usage combined to the resulting runoffs and leaching, increases the risk of affecting non-target organisms, such as phytoplankton. Fomesafen toxicity in photosynthetic organisms results from inhibition of the chlorophyll biosynthesis pathway at the PPO complex (Matringe *et al.*, 1989). The herbicide competes with protoporphyrinogen IX (PPGIX) within the PPO complex resulting in an accumulation of free PPGIX that can be oxidized into protoporphyrin IX (PPIX). When PPIX is formed outside the PPO complex, it is photosensitive and generates, upon illumination, singlet oxygen, which causes subsequent lipid peroxidation (Matringe *et al.*, 1989). Thus, fomesafen is known to lower chlorophyll content, degrade membranes and consequently reduce

photosynthetic yields. While the impact of fomesafen is characterized in higher plants, very few studies focused on its effect on phytoplanktonic species. This study aimed to investigate the impact of increasing concentrations of a routinely used fomesafen-based herbicide (Reflex®) on the physiology of two species of chlorophytes and one cyanobacteria. The outcomes of fomesafen exposure on phytoplankton were assessed via various physiological biomarkers such as growth, photosynthesis, pigment content, oxidative stress and morphology changes.

1.5 Material and Methods

1.5.1 Phytoplanktonic cultures and growth conditions

Raphidocelis subcapitata (FACHB-271), *Chlamydomonas snowii* (species isolated from the Choinière reservoir in Quebec, Canada (Deblois *et al.*, 2013)) and *Microcystis aeruginosa* (CPCC632) were cultivated in batch culture with 100 ml Bold's Basal Medium growth medium (Stein, 1973). The growth temperature was 24°C with a 14/10h light-dark cycle at 100 µmol photons $m^{-2}s^{-1}$ supplied by white fluorescent lamps (Philips F72T8/TL841/HO, USA) and incandescent bulbs (Philips 60W). Axenic phytoplankton cultures prepared for the experiments were kept in their exponential growth phase by periodic transfers in fresh medium (every three days). Cells of *C. snowii* and *M. aeruginosa* were collected at normalized cell density (*R. subcapitata* mean biovolume) to account for total pigment and enzymatic content differences between species.

1.5.2 Herbicide solutions and fomesafen exposure

Stock solution of the herbicide fomesafen (Sodium salt of fomesafen; Reflex®, 22.8% a.i., 240g fomesafen l^{-1}, Syngenta, USA) (thereafter called fomesafen-based herbicide) was diluted in nanopure water and stored in darkness. For each species, cultures were

submitted for 72h to increasing concentrations of fomesafen treatment (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} a. i. sodium salt of fomesafen) before sampling.

1.5.3 Growth measurements and cell biovolume assessment

Cell density and mean cell biovolume were obtained using a particle analyzer (Multisizer 3, Beckman Coulter Inc., Fullerton, USA). From the plot of ln (cell ml^{-1}) in relation to time (days), the average growth rate (μ, $days^{-1}$) was calculated using $\mu = \ln \cdot (D_t/D_0)/t$; D = cell ml^{-1} at time (t); D_0 = cell ml^{-1} when the exposure started. The phytoplankton mean cell size was expressed in femtoliter (fl). Since analysis of cultures grown without toxic pressure showed no significant differences between pre-exposure and following the short depuration period, only results from the latter were taken into account to preserve equal sample size between data sets (Data not shown).

1.5.4 Photosynthetic activity evaluation

1.5.4.1 PSII Energy fluxes analysis

Prior to the analysis, each culture was sampled and dark-acclimated for 15 minutes to allow complete oxidation of PSII reaction centers. Then, measurement of fast polyphasic chlorophyll fluorescence kinetic was performed using a Plant Efficiency Analyzer fluorometer with the liquid compartment (PEA, Hansatech Ltd., King's Lynn, Norfolk, UK). Parameters resulting from the analysis of PSII energy fluxes were calculated following Force *et al.* (2003).

Table 1.1 PSII energy fluxes parameters calculated from the rapid rise in fluorescence of Chl *a* with their respective formula.

Parameter	Formula	Reference
ABS/RC	$(TR_0/RC)/(TR_0/ABS)$	Force *et al.* (2003)
TR_0/RC	(M_0/V_J)	
ET_0/RC	$(TR_0/RC) \times (ET_0/TR_0)$	
DI_0/RC	$(ABS/RC) - (TR_0/RC)$	
M_0	$(F_{2ms} - F_{50\mu s})/(F_M - F_{50\mu s})$	
V_J	$4 \times (F_{300\mu s} - F_{50\mu s})/(F_M - F_{50\mu s})$	

1.5.4.2 PAM fluorometry

Efficiency of PSII reaction center was measured according to Schreiber *et al.* (1986) by using a Water-PAM (Heinz Walz, GmbH, Effeltrich, Germany). After sampling and dark acclimation (15 minutes), the minimum (F_0) and maximum (F_M) fluorescent yields of PSII were determined under modulating light (2 μmol m^{-2} s^{-1}) and saturating light (700 ms, 3000 μmol $m^{-2}s^{-1}$) respectively. Then a continuous actinic light (125 μmol m^{-2} s^{-1}) and saturating pulses (700 ms, 3000 μmol m^{-2} s^{-1}, every 60 seconds) were applied to obtain respectively the steady state and the maximum fluorescence yields for light acclimated samples (F_S and F'_M) under illumination. After obtaining F_S and F'_M, the actinic light was then turned off to measure the minimal fluorescence yield of a light-acclimated sample (F'_0). Chlorophyll *a* fluorescence parameters were obtained according to the following equations:

Table 1.2 List of Chl *a* quenching parameters with their respective formulas.

Parameter	Formula	Reference
ϕ_M	$(F_M - F_0)/F_M = F_v/F_M$	(Kitajima and Butler, 1975)
ϕ'_M	$(F'_M - F_S)/F'_M$	(Genty *et al.*, 1989)
$qp_{(rel)}$	$(F'_M - F_S)/(F_M - F'_0)$	(Buschmann, 1995)
$qn_{(rel)}$	$(F_M - F'_M)/(F_M - F'_0)$	(Buschmann, 1995)
$UQF_{(rel)}$	$(F_S - F'_0)/(F_M - F'_0)$	(Juneau *et al.*, 2005)

1.5.4.3 Cyclic electron flow measurements

We estimated the proportion of cyclic electron flow during photosynthesis following an adapted procedure from (Joliot and Joliot, 2005). Cyclic electron flow around PSI was measured using a Joliot Type Spectrophotometer (JTS-10, BioLogic, France). A light-emitting-diode (LED) periodically radiated detection flashes of light between 705-740 nm. Meanwhile, an actinic far-red LED (720 nm) emitted excitation flashes through a 705 nm interference filter. The filtered light shine thought the culture sample and absorbance is then quantified using a measurement photodiode. To ensure only the recording of wavelengths over 695 nm, cut-off filters were placed in front of the measurement and reference photodiode. Prior to the analysis, cultures were dark-adapted for 15 min then analyzed a first time. Afterwards, this analysis was repeated on the same sample after light-acclimation for 3 min at 630 nm. Light acclimation is needed to activate the Benson-Calvin cycle where during this state the linear electron flow is predominant over cyclic transport (Joliot and Joliot, 2002). The change in absorbance is then interpreted as the change in rates of oxidation and reduction of the PSI ($\Delta I/I\ P_{700}$). Actinic light is switched off after 5s illumination, and then, we recorded

the half-time at which P_{700} absorption changes return to baseline levels. From this data, we estimate the proportion of cyclic electron flow during photosynthesis of the phytoplankton following exposure to fomesafen using the half-time difference between light adapted and dark-adapted-samples. The fraction of cyclic electron flow is expressed in percentage compared to the control.

1.5.5 Pigment content analysis

Following the 72h exposure to fomesafen-based herbicide, samples were collected under dim green light on 0.2 µm nitrocellulose filters (Xingya Puryfying Materials factory, Shanghai, China) then quickly frozen in liquid nitrogen and kept at -80°C. For each measurement, a minimum of 12 million cells (or the biovolume-normalized equivalent for *C. snowii* and *M. aeruginosa*) were harvested. Pigments were extracted in ethanol 95% at 78°C for 5 min. Samples were then stored 24 hours at -20°C to complete the extraction. Absorbance of the pigment extract was measurement with a Cary 300 ultraviolet-visible (UV-Vis) spectrophotometer (Varian Autralia Pty Ltd, Mulgrave, VIC, Australia). Chlorophyll *a* and carotenoid contents were evaluated following Lichtenthaler (1987) and expressed as % of pigment compared to control.

1.5.6 Reactive oxygen species content and cell complexity determination

The impact of fomesafen on the presence of intracellular ROS was revealed following Stachowski-Haberkorn *et al.* (2013) using a cell-permeable non-selective optical probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Data obtained from flow cytometry analysis were presented as FL1 ratios to normalize results (FL1 value of H_2DCF-DA stained cells divided by background FL1 fluorescence of fresh cells). The side scatter parameter (SSC) was also analyzed to measure the impact of fomesafen on phytoplanktonic cell complexity. Cell complexity results were expressed as SSC ratio ($SSC_{treated\ cells}/SSC_{control}$).

1.5.7 Statistical analysis

In order to test for significant differences in fomesafen's intraspecific impact on the phytoplankton, data were expressed as mean ± standard deviation (n = 9) and statistical analysis were performed using GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA). Shapiro-Wilk normality tests revealed that the data sets analyzed followed a normal distribution. Significant differences of the means of treated cultures were compared to their respective control by a one-way ANOVA followed by post-hoc Tukey multiple comparison test.

1.6 Results

1.6.1 Growth rate and photosynthesis

Throughout the experiment, *R. subcapitata* exposed to 40 and 320 µg L^{-1} of fomesafen-based herbicide showed a significant decrease in growth rate (15%; $P < 0.01$ and 54%; $P < 0.0001$, respectively) while cultures of *C. snowii* and *M. aeruginosa* remained unaffected (Fig. 1.1).

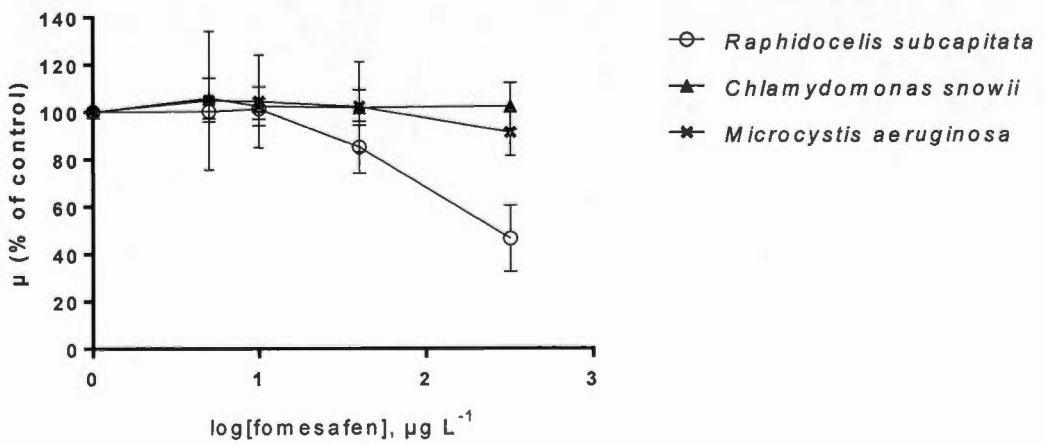

Figure 1.1 Growth rate (µ) for *R. subcapitata*, *C. snowii* and *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen). Mean ± SD; n = 9.

Upon light exposure, dark-acclimated cultures of *R. subcapitata* submitted to increasing concentrations of fomesafen-based herbicide showed a decrease in maximal (ϕ_M) and operational (ϕ'_M) PSII photochemical yields after 72h exposure to 320 μg L^{-1} (12%; $P < 0.001$ and 21%; $P < 0.001$, respectively). As for the growth rate, the herbicide induced no significant change on ϕ_M and ϕ'_M for *C. snowii* and *M. aeruginosa* (Fig. 1.2).

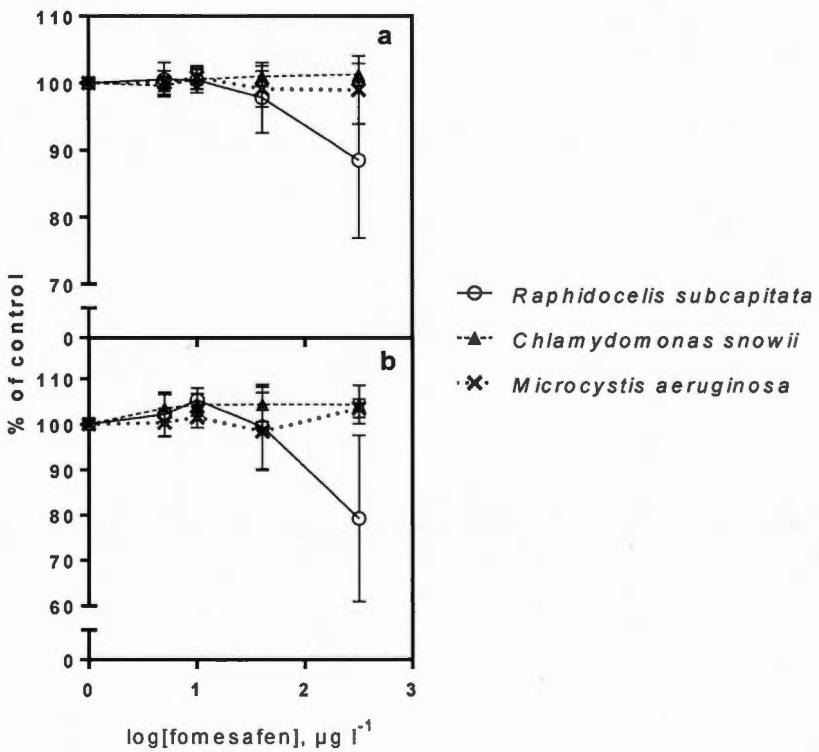

Figure 1.2 a) Maximal (ϕ_M) and b) operational (ϕ'_M) PSII photochemical yields of *R. subcapitata*, *C. snowii* and *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10, 40 and 320 μg L^{-1} fomesafen). Mean ± SD; n = 9.

Furthermore, exposure of *R. subcapitata* to high concentration of fomesafen (320 µg L^{-1} and over) yielded to significant changes in PSII energy fluxes (Fig. 1.3a, 1.3c and 1.3d). The observed effective antenna size per active reaction center (ABS/RC) was significantly higher following exposure to 320 µg L^{-1} (85%, $P < 0.0001$). The same exposure concentration resulted in a significant rise in TR_0/RC upon illumination compared to the control (40%, $P < 0.001$). Concomitantly, effective dissipation of an active RC, DI_0/RC, greatly increased (121%, $P < 0.001$) following exposure of *R. subcapitata to* fomesafen. On the other hand, none of the fomesafen-based herbicide concentrations tested produced significant change in PSII energy fluxes for *C. snowii* and *M. aeruginosa*.

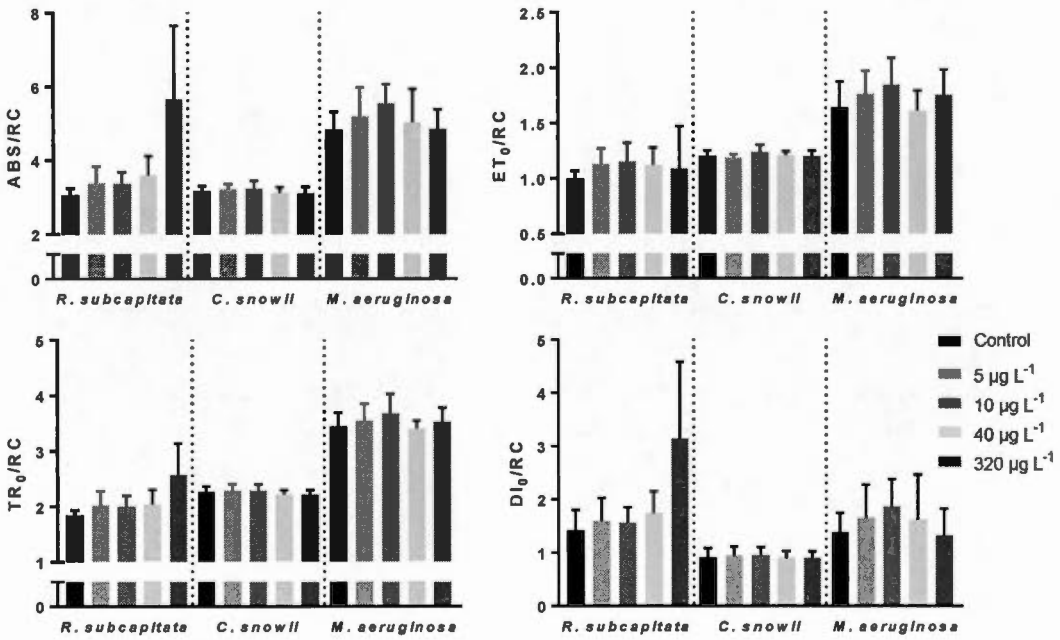

Figure 1.3 PSII energy fluxes of *R. subcapitata*, *C. snowii* and *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen). Mean ± SD, n = 9.

Energy dissipation pathways of *R. subcapitata* (Fig. 1.4a), *C. snowii* (Fig 1.4b) and *M. aeruginosa* (Fig. 1.4c) were also affected by the fomesafen treatment. Results for *R. subcapitata* showed that exposure of this species to 10 μg L^{-1} produced a significant increase in qp_{rel} (12%, $P < 0.05$) while treatment with 320 μg L^{-1} led to a significant decrease in this parameter (15%, $P < 0.01$). Treatment of *M. aeruginosa* with high concentration (320 μg L^{-1}) of fomesafen induced a significant increase (6%, $P < 0.01$) in this parameter. Only the qn_{rel} values of *R. subcapita* was also affected by the herbicide treatment. For *R. subcapitata*, exposure to the fomesafen-based herbicide produced a significant decrease in qn_{rel} at 10 μg L^{-1} (67%, $P < 0.05$) while exposure to 320 μg L^{-1} led to a significant increase in this parameter (70%, $P < 0.05$). For *M. aeruginosa*, qn_{rel} was significantly decreased following exposure to 10 μg L^{-1} (11%, $P < 0.05$), 40 μg L^{-1} (16%, $P < 0.001$) and 320 μg L^{-1} (16%, $P < 0.001$). Moreover, exposure of *C. snowii* and *M. aeruginosa* to the fomesafen-based herbicide significantly altered UQF_{rel}. This parameter decreased following treatment of *C. snowii* with 10 μg L^{-1} (25%, $P < 0.05$), 40 μg L^{-1} (26%, $P < 0.05$) and 320 μg L^{-1} (30%, $P < 0.05$) of the fomesafen-based herbicide while exposure of *M. aeruginosa* to 40 μg L^{-1} led to a 45% ($P < 0.05$) increase in this parameter.

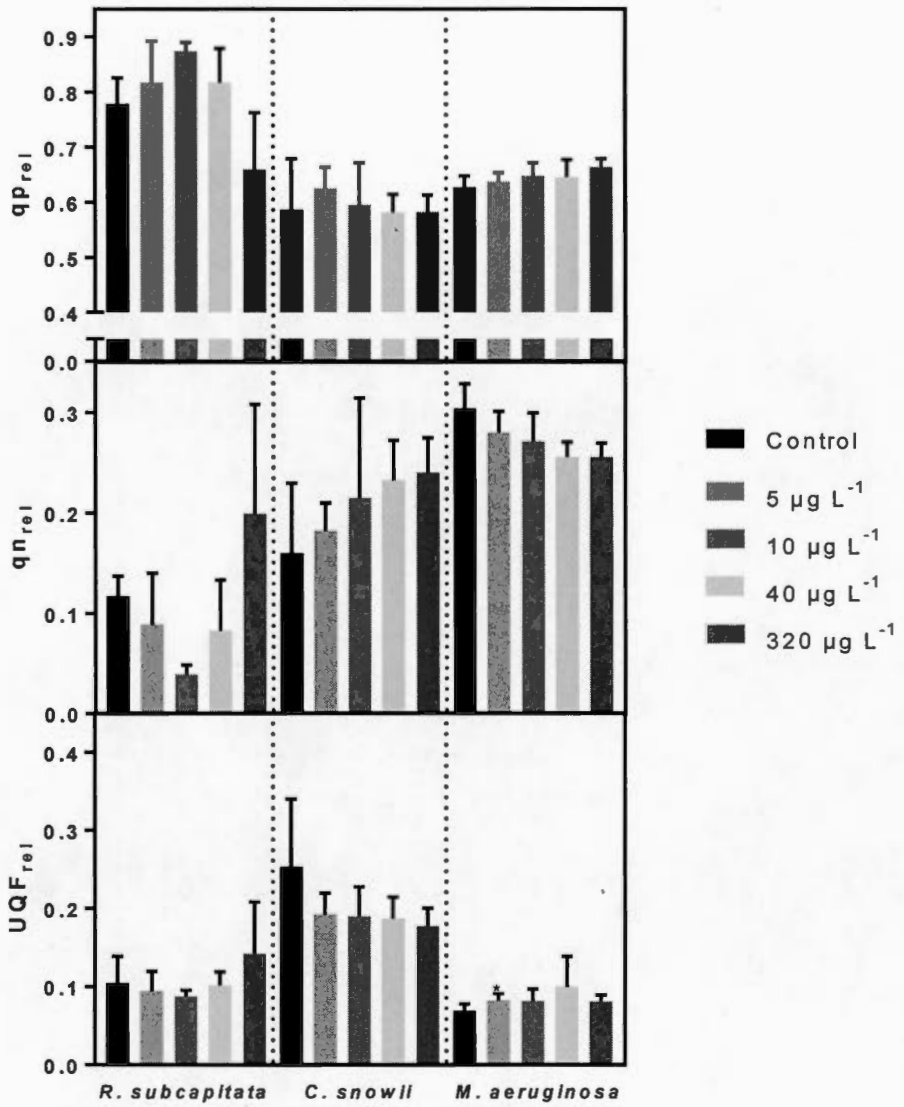

Figure 1.4 Relative photochemical quenching (qp_{rel}), relative non-photochemical quenching (qn_{rel}) and relative unquenched fluorescence (UQF_{rel}) of a) *R. subcapitata*, b) *C. snowii* and c) *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen). Mean ± SD, n = 9.

Measurement of cyclic electron transport of *R. subcapitata* showed that electron transport around PSI upon illumination was slightly higher after exposure to 10 μg L^{-1} and significantly higher following treatment with 40 μg L^{-1} ($P < 0.0001$) fomesafen formulation compared to control (Table 1.3). Results for *C. snowii* and *M. aeruginosa* revealed no clear trends (Data not shown).

Table 1.3 Cyclic electron transport for *R. subcapitata* following 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10 and 40, 320 μg L^{-1} fomesafen). N.d. stands for not determined since no signal was obtained at high fomesafen concentration. Mean ± SD; n = 9.

[Exposure]	% Cyclic electron transport
0 μg L^{-1}	7.5 (6.0)
5 μg L^{-1}	4.5 (6.6)
10 μg L^{-1}	16.5 (4.3)
40 μg L^{-1}	31.7 (15.6)
320 μg L^{-1}	N.d.

1.6.2 Pigment content

Chlorophyll *a* content of phytoplanktonic cultures significantly changed upon fomesafen-based herbicide treatment (Fig 1.5a). For *R. subcapitata*, Chl *a* content diminished following exposure to 40 μg L^{-1} (18%, $P < 0.05$) and 320 μg L^{-1} (26%, $P < 0.001$). For *C. snowii*, concentration of Chl *a* was significantly decreased after exposure to 10 (15%, $P < 0.01$), 40 (11%, $P < 0.05$) and 320 μg L^{-1} (18%, $P < 0.001$). *M. aeruginosa* Chl *a* content was not significantly affected by the fomesafen-based herbicide at the tested concentrations. Pigment analysis also showed that carotenoid

content was impacted by the herbicide-contaminated culture media (Fig. 1.5b). Lower concentration of carotenoids was detected in *R. subcapitata* cultures following exposure to 320 μg L^{-1} (31%, $P < 0.0001$). *C. snowii* carotenoid content was also significantly decreased following treatment with 10 (16%, $P < 0.001$), 40 (14%, $P < 0.01$) and 320 μg L^{-1} (20%, $P < 0.0001$) fomesafen-based herbicide treatment. At tested concentrations, exposure of *M. aeruginosa* to the fomesafen-based herbicide produced no significant impact on the cyanobacteria carotenoid content.

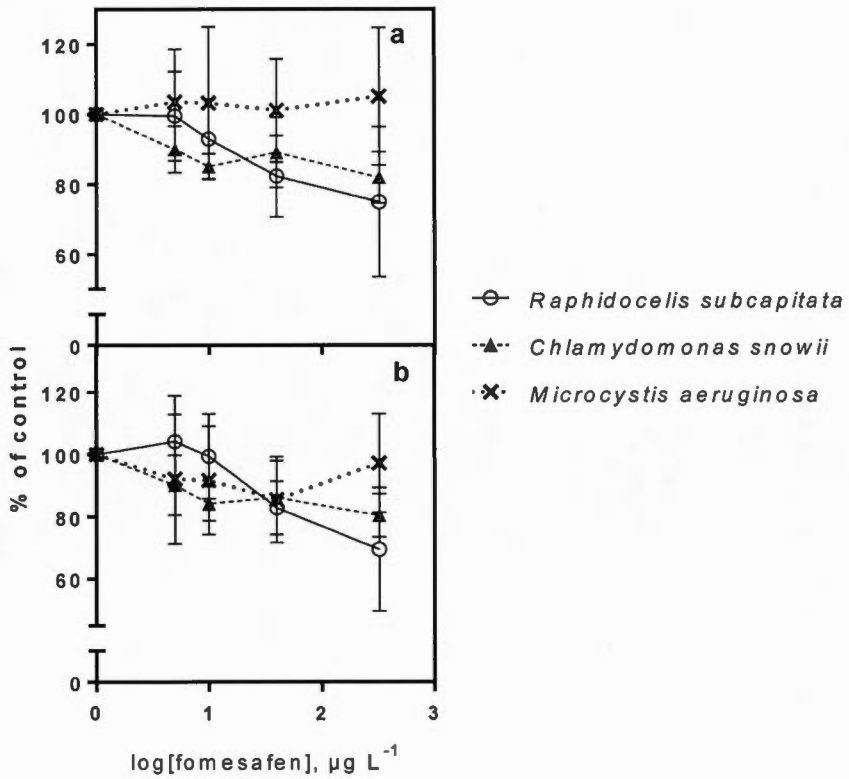

Figure 1.5 a) Chlorophyll *a* and b) carotenoid content normalized by cell density and cell volume for *R. subcapitata*, *C. snowii* and *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 μg L^{-1}). Mean ± SD; n = 9.

1.6.3 Oxidative stress and cell complexity

To investigate the impact of the fomesafen-based herbicide on intracellular ROS content we measured DCFH conversion to the fluorescent DCF by reactive oxygen species (Fig. 1.6a). Exposure to the fomesafen-based herbicide caused a significant increase in ROS content for the green microalgae *R. subcapitata* at 320 $\mu g\ L^{-1}$ (185%, $P < 0.0001$). We also showed that the fomesafen formulation caused no increase of cellular ROS content for *C. snowii* and *M. aeruginosa*. The relationship between the fomesafen-based herbicide and ROS-induced intracellular complexity (interpreted as a rise in the mean SSC parameter) was also investigated. Cellular complexity of *R. subcapitata* was found to be affected by the herbicide treatment (Fig. 1.6b) with a significant increased intracellular complexity when exposed to 320 $\mu g\ L^{-1}$ (33%, $P < 0.001$). The fomesafen-based herbicide treatment did not produce any significant change in mean SSC for the green microalgae *C. snowii* and *M. aeruginosa*.

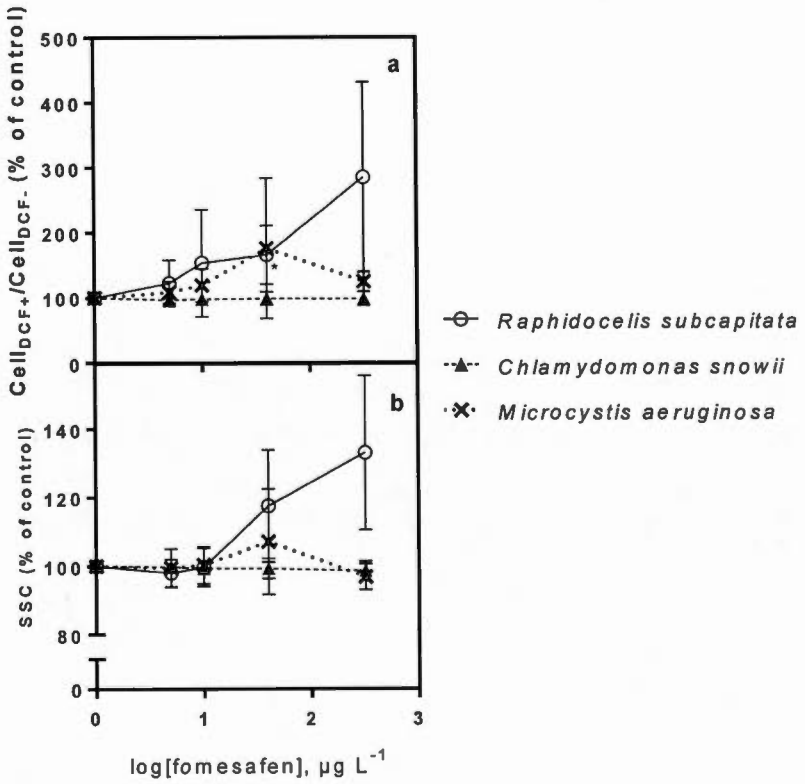

Figure 1.6 a) Intracellular ROS content and b) cell complexity of *R. subcapitata*, *C. snowii* and *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}). ROS content was measured as mean FL1 fluorescence of DCF positive cells divided by background mean FL1 fluorescence of fresh control culture. Cell complexity is represented by the mean side scatter parameter (SSC). Mean ± SD; n = 9.

1.6.4 Cell biovolume

Exposure to the fomesafen-based herbicide-induced changes in cell volume for *R. subcapitata* cultures (Fig. 1.7). Interestingly, cells of *R. subcapitata* were significantly larger after 72h exposure to 40 (31%, $P < 0.01$) and 320 µg L^{-1} (51%, $P < 0.0001$)

fomesafen. On the other hand, the tested concentrations did not produce variation in cell volume for *C. snowii* and *M. aeruginosa*.

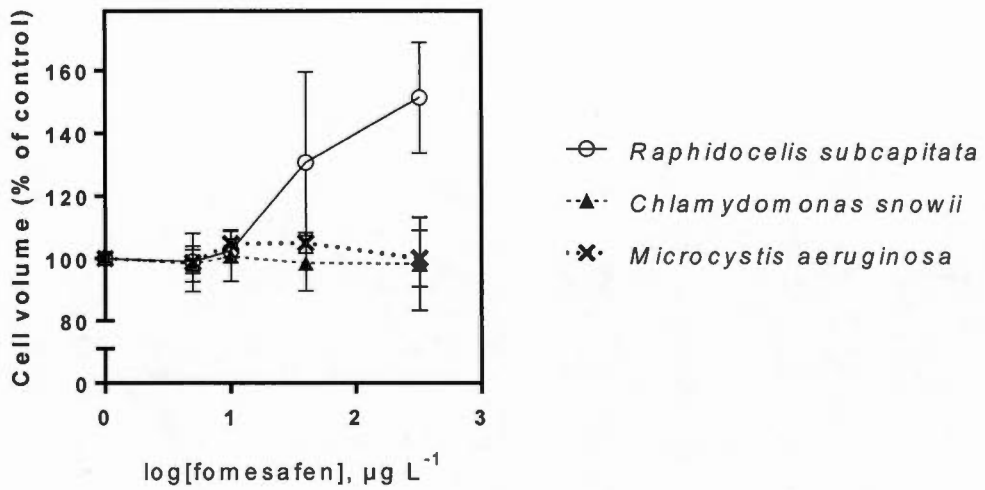

Figure 1.7 Cell volume of *R. subcapitata*, *C. snowii* and *M. aeruginosa* after 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}). Cell volume is expressed as mean femtoliter in relation to the control. Mean ± SD; n = 9.

1.7 Discussion

This experiment was conducted to study the impact of increasing concentrations of the fomesafen-based herbicide Reflex® on the physiology two green microalgae and one cyanobacteria. Results presented in this paper showed that the physiological impacts of environmentally relevant concentrations (5-10 µg L^{-1}) and higher (40-320 µg L^{-1}) of fomesafen vary among phytoplanktonic species. While exposure to the fomesafen-based herbicide significantly impaired the growth of *R. subcapitata* and *C. snowii*, the growth of the cyanobacteria *M. aeruginosa* remained unaffected (Fig. 1.1). These results are in accordance with US EPA literature which describes inhibition of growth of freshwater phytoplankton by fomesafen. Indeed, it was reported by MRID46673804

(cited in EPA (2009)) that *R. subcapitata* growth-NOAEC was 10 µg L^{-1} for sodium salt of fomesafen.

More insight of the physiological impact of the fomesafen-based herbicide on the growth of phytoplankton was obtained through analysis of photosynthetic efficiency and related physiological processes. Indeed, inhibition of growth is most likely related to the known impacts of PPO-inhibitors, like fomesafen, on photopigment production (Caquet *et al.*, 2005; Wakabayashi, 1999). As observed, exposure of *R. subcapitata* to the fomesafen-based herbicide induced a significant decrease in Chl *a* concentration. Chl *a* being an essential constituent of photosystems antennas and reaction centers, any decrease in their concentrations may have impacts on the photosynthetic yields. Another consequence of the exposure to fomesafen is that inhibition of PPGIX leads to the production ROS (Wakabayashi, 1999). As we can expect, our results demonstrate that exposure to high concentrations of fomesafen-based mixture enabled strong intracellular generation of ROS for *R. subcapitata*. If ROS production overpasses the oxidative stress defense mechanisms usually triggered under stress conditions (Ledford and Niyogi, 2005; Niyogi, 1999, 2000), the already synthesized chlorophylls, proteins and lipids will suffer oxidative damages leading to destruction of the reaction centers, disruption of the photosynthetic (and ultimately cell) membrane integrity, decreased in photosynthetic activity and, eventually, to the phytoplankton death (Matringe *et al.*, 1989). The rise in relative cell complexity measured for *R. subcapitata* exposed to high concentrations of fomesafen further suggests oxidative degradation of intracellular components. Similar results of higher complexity and degradation of intracellular components (interpreted from higher cell complexity) in presence of ROS was observed in *Tetraselmis suecica* exposed to 5 µg L^{-1} diuron, a concentration representative of a highly polluted environment (Stachowski-Haberkorn *et al.*, 2013). While there is a clear physiological response from the studied phytoplanktonic species following exposure to the fomesafen-based herbicide, it is important to note that most effects resulted from exposure to very high pesticide concentration unlikely to be found

in large bodies of water. Indeed, the aforementioned effect of fomesafen on the freshwater phytoplanktonic species studied thus may be detected where pesticides largely exceed expected environmental concentrations as in agricultural drainage ditches although, to our knowledge, there is no available data on fomesafen field run-offs concentration.

Concomitantly to the decrease in pigment concentrations and the increase in ROS content, we observed an inverse correlation between herbicide concentration and maximal and operational PSII quantum yields (ϕ_M and ϕ'_M) following exposure of *R. subcapitata* to the fomesafen-based formulation. This response may be due to the previously detailed fomesafen inhibitory effects on physiological processes linked to the photosynthetic activity of the phytoplankton. Genty *et al.* (1989) established that quantum yield of linear electron transport is relative to the efficiency at which the open PSII reaction centers capture and use trapped excitation energy. The lower photochemical quantum yields recorded following the fomesafen treatment may be due to a decrease in light capture efficiency from open PSII RCs, this is further evidenced by an increase in ABS/RC which may result from a higher number of inactivated reaction centers (Strasser *et al.*, 2000). Similarly, the lower TR_0/RC and DI_0/RC observed in our exposure conditions could also have originated from a lower density of active reaction centers (Force *et al.*, 2003). For *R. subcapitata*, electron transport in an active RC (ET_0/RC) remained unchanged for all tested concentrations. As the ET_0/RC parameter is influenced by TR_0/RC (Force *et al.*, 2003), this result can be partly explained by a reduction in open reaction centers which proportionally reduce the influx of electrons through the electron transport chain (ETC). This phenomenon coupled with a relative increase in the maximum electron trapping rate of the PSII can explain the stability of ET_0/RC.

The study of relative quenching coefficients can effectively reveal the proportion in which each quenching mechanisms are being actively solicited by the photosynthetic

organism in a stressful situation (Buschmann, 1995; Juneau *et al.*, 2005). Perturbations of the energy distribution pathways following exposure of *R. subcapitata* to the fomesafen herbicide is evidenced by the recorded lower qp_{rel} value and increase in qn_{rel} and UQF_{rel}. As we observed with ϕ'_M, qp_{rel} was only affected when treated with the higher concentrations of the fomesafen-based herbicide. Furthermore, the recorded increase in qn_{rel} for *R. subcapitata* can be attributed to the phytoplankton ubiquitous response to physiological stress (Niyogi, 1999, 2000). Indeed, phytoplankton under physiological stress will dissipate excessive light energy through non-photochemical quenching mechanisms such as the xanthophyll cycle, state transition and photoinhibition (Allen and Forsberg, 2001; Govindjee, 2002). Higher UQF_{rel} values following herbicide exposure suggest the inability of the PSI photosystem to drain electrons from the PSII (Juneau and Harrison, 2005). Overall, the fomesafen-induced changes in photochemical yields, PSII energy fluxes and quenching parameters may be attributed, in part, to structural modification at the PSII antenna level (Strasser *et al.*, 1999).

In freshwater phytoplankton, the various roles of cyclic photophosphorylation are not yet fully understood but enhancement of this process is often observed in response to a rise in physiological stress such as with the presence of an herbicide which limits electron flow from the PSII (Alric, 2010a; Alric *et al.*, 2010b; Bendall and Manasse, 1995; Finazzi *et al.*, 1999). It is suggested that to maintain proper electron output from the ETC to balance ATP and NADPH, hampered electron transfers between PSII and PSI can be compensated by an increase in cyclic electron flow. This is also to prevent over excitation of the PSII. Thus, in response to light-induced stress, on top of non-photochemical quenching mechanisms, phytoplankton may up regulate cyclic electron transport to maintain stoichiometric requirements of the Calvin cycle (Alric, 2010a). Our results did reflect this principle as the green microalgae *R. subcapitata* exhibited higher cyclic electron transport around PSI after being exposed to increasing concentrations of fomesafen-based herbicide (Table 1)

Together with the previous effects of increasing concentrations of the fomesafen-based herbicide, this herbicide significantly increased cell volume for *R. subcapitata*, compared to the control. Cell biovolume increase in the presence of a toxicant was previously reported for some phytoplanktonic strains, including *R. subcapitata* (Yamagishi *et al.*, 2017). Comparable results have been observed in multiple studies with a focus on microalgae sensitivity to photosynthesis-inhibiting herbicides such as diuron (Stachowski-Haberkorn *et al.*, 2013) and atrazine (Delorenzo *et al.*, 2004; Tang *et al.*, 1998). Our results suggest that median biovolume of *R. subcapitata* cells was shifted toward larger cells under the toxic pressure from fomesafen. This can be explained by the fact that a lower surface/volume ratio (obtained with larger cells) decreases the relative contact of the intracellular machinery to the pollutant. Kent et Currie (1995) established that cell biovolume play a significant role in the interaction between various phytoplanktonic species and lipophilic pesticides which subsequently affect the microorganism sensitivity. This may explain the difference in sensitivity to fomesafen between *Chlamydomonas snowii* and *R. subcapitata,* both green microalgae but with contrasting morphology and growth dynamics. Although other mechanisms may explain the higher resistance to the tested concentrations of the fomesafen-based herbicide, we showed that the intrinsic large cells of *C. snowii* (low surface/volume ratio compared to *R. subcapitata*) were more resistant than the other green algal species. Caquet *et al.* (2005) demonstrated that chlorophytes and cyanobacteria react differently to fomesafen using exposures in mesocosms that were supplemented five times (once every three weeks for three months) with nominal concentrations of fomesafen (40 $\mu g\ L^{-1}$) and a mixture of fomesafen and the polyethoxylated nonylphenol-based adjuvant Agral 90® (40 and 90 $\mu g\ L^{-1}$, respectively). Results for Caquet *et al.* (2005) showed that exposure to fomesafen with or without adjuvant caused a similar significant decrease in cell density for chlorophytes while abundance of cyanobacterial cells continued to increase after three months exposure. Moreover, Yu *et al.* (2014) reported that three-day exposure of *Microcystis aeruginosa* (FACHB-905) to concentrations of fomesafen of 20 $mg\ l^{-1}$ was needed to induce more than 40%

inhibition of growth rate. USEPA (2008) reported a vast discrepancy for fomesafen EC_{50} between green microalgae (92 $\mu g\ L^{-1}$, MRID 46673804) and cyanobacteria (710 $\mu g\ L^{-1}$). Although fomesafen targets the PPO which is an essential enzyme for photopigment biosynthesis in both green microalgae and cyanobacteria, Kato *et al.* (2010) suggested that a unique and unidentified bacterial gene encoding PPO in cyanobacteria may confer a natural resistance to the impact of PPO-inhibiting herbicides. As most results observed in this study resulted from exposure to very high concentration of the herbicide, it would be necessary to study the possibility of bioaccumulation and/or biodegradation of the contaminant. Moreover, the potential toxicity of fomesafen degradation by-products should be assessed if biodegradation of this chemical by the phytoplankton is discovered.

1.8 Conclusion

This study shows that exposure to the fomesafen-based herbicide Reflex® provokes different physiological responses among the three species of chlorophyte and cyanophyte studied. Indeed, only the green microalga *R. subcapitata* was significantly affected by this diphenyl ether herbicide. Fomesafen-induced inhibition of growth in *R. subcapitata* was linked to a decrease in photosynthetic efficiency, which originated from the herbicide mode of action: inhibition of photopigments biosynthesis pathway and subsequent ROS generation. Differences in sensitivity between chlorophytes could be associated to morphological distinctions as pesticide absorption rate is expected to decrease as the cell radius increase. Lack of sensitivity to fomesafen for *M aeruginosa* is suspected to originate from its small size and/or a genetic discrepancy in the PPO enzyme between cyanobacteria and green microalgae, but further studies need to be done to elucidate the mechanism involved in this high resistance. Although significant results presented in this paper originated from exposure to fomesafen concentrations undocumented and unlikely to be found in large aquatic ecosystems, similar result may

be expected to be found in areas close by point source pollution such as in a pipe ditch in an agricultural field.

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CHAPITRE II

MULTIGENERATIONAL EXPOSURE OF THREE FRESHWATER PHYTOPLANKTONIC SPECIES TO FOMESAFEN-BASED HERBICIDE REFLEX®

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2.1 Contributions

Jonathan Naoum a réalisé le plan d'expérience, les tests en laboratoire, l'analyse des résultats et la rédaction, alors que Philippe Juneau a participé à la conception du plan d'expérience, effectué la supervision des travaux de laboratoire et participé à la rédaction et la révision du texte. Disney Izquierdo et Catherine Ayotte ont participé aux tests en laboratoire et l'analyse des résultats concernant la longue dépuration.

2.2 Abstract

Massive use of pesticides such as glyphosate led to the emergence of resistance in weeds (e.g. *Palmer amaranth*). This phenomenon was successfully counteracted by tank-mixing or additional application of herbicides which inhibit protoporphyrinogen oxidase (PPO) such as fomesafen (5-[2-chloro-4-(trifluoromethyl) phenoxy]-N-(methylsulfonyl)-2-nitrobenzamide). This spike in fomesafen usage also raised the probability that this molecule leaks to freshwater bodies near agricultural fields. The presence of PPO-inhibitors in aquatic ecosystems is known to hamper the chlorophyll synthesis pathway in eukaryotic and prokaryotic phytoplankton thus having impact on growth. Additionally, control of glyphosate-resistant weeds by means of fomesafen treatments led to the recent development of PPO-inhibitor resistant strains of numerous weeds. From this context, we investigated the impact of multigenerational exposure to increasing concentrations (Reflex®; 5, 10, 40, 320 $\mu g\ L^{-1}$) of a commonly used fomesafen-based formulation on the physiology of two algal species *(Raphidocelis subcapitata,* FACHB271 and *Chlamydomonas snowii*) and one cyanobacteria (*Microcystis aeruginosa,* CPCC632). Also, the persistence of potential changes in physiology following fomesafen pre-exposure was explored after a few growth cycles in clean media. For *R. subcapitata,* pretreatment to high concentrations of fomesafen herbicide led to the complete restoration of every physiological biomarker evaluated (growth, pigment content, photosynthesis, ROS content and morphology). While growth of *C. snowii* was naturally unaffected by this herbicide, fomesafen-induced

pigment content inhibition was still noted for this species and was entirely restored by fomesafen pretreatment. Altogether, changes in phytoplankton physiology following multigenerational exposure remained after a short and long term depuration period which suggests the emergence of a fomesafen-resistant strain of *Rs* and, to a lesser extent, *C. snowii.*

Keywords: multigenerational exposure, fomesafen, Reflex®, microalga, cyanobacteria

Highlights

- *Multigenerational pre-exposure to high concentrations of a fomesafen-based herbicide (Reflex®) induced lasting resistance in R. subcapitata FACHB271*

2.3 Résumé

L'utilisation massive de pesticides tels que le glyphosate a mené à l'émergence de résistances chez certaines mauvaises herbes (e.g. *Palmer amaranth*). Ce phénomène a été contrebalancé avec succès par un mélange en cuve ou une application supplémentaire d'herbicides inhibiteurs de la protoporphyrinogène oxydase (PPO) comme le fomésafène (5- [2-chloro-4- (trifluorométhyl) phénoxy] -N- (méthylsulfonyl) -2-nitrobenzamide). Cette hausse dans l'utilisation du fomésafène augmente également la probabilité de retrouver cette molécule dans les sources d'eau douce en bordure des champs à forte activité agricole. La présence d'inhibiteurs de la PPO dans les écosystèmes aquatiques entrave la voie de synthèse des pigments photosynthétiques chez le phytoplancton eucaryote et procaryote. De plus, le contrôle des mauvaises herbes résistantes au glyphosate à l'aide de traitements au fomésafène a conduit à l'émergence de plusieurs souches photosynthétiques résistantes à l'impact d'inhibiteurs de la PPO. De ce contexte, cette étude vise à étudier l'impact d'une

exposition multigénérationnelle à des concentrations croissantes (Reflex®; 5, 10, 40, 320 µg L^{-1}) d'une formulation à base de fomésafène couramment utilisée sur la physiologie de deux espèces de microalgues vertes (*Raphidocelis subcapitata*, FACHB271 et *Chlamydomonas snowii*) et une cyanobactérie (*Microcystis aeruginosa*, CPCC632). De plus, la persistance de potentiels changements physiologiques suite à une pré-exposition au fomésafène a été explorée après quelques cycles de croissance dans un milieu de culture non contaminé. Dans l'ensemble, un prétraitement au fomésafène a mené à la restauration complète de tous les biomarqueurs physiologiques évalués pour *R. subcapitata* (croissance, teneur en pigments, photosynthèse, teneur en ROS et morphologie). Bien que la croissance de *C. snowii* ne fût naturellement pas affectée par le traitement, une diminution de la teneur en pigment a été notée et un retour à la normale du contenu pigmentaire a été observé suite à une pré-exposition au fomésafène. Dans l'ensemble, les changements dans la physiologie du phytoplancton suite à une exposition multigénérationnelle au fomésafène sont restés présents suite à une courte et une longue période de dépuration ce qui suggère l'émergence d'une souche phytoplanctonique résistante au fomesafène chez *R. subcapitata* et, dans une moindre mesure, chez *C. snowii.*

Mots-clés: exposition multigénérationnelle, fomésafène, Reflex®, microalgue, cyanobactérie

2.4 Introduction

The agricultural field has seen a recent renewed interest for fomesafen (5-[2-Chloro-4-(trifluoromethyl) phenoxy]-*N*-(methylsulfonyl)-2-nitrobenzamide), a protoporphyrinogen oxydase (PPO) inhibitor, following positive results in controlling unwanted weeds which developed resistance to glyphosate (Aulakh *et al.*, 2016; Chahal *et al.*, 2017; Ganie and Jhala, 2017; Knezevic *et al.*, 2009). As a diphenyl ether herbicide, fomesafen competitively inhibits the activity of the PPO, a key enzyme in the biosynthesis of photosynthetic pigments, thus preventing the growth of problematic glyphosate-resistant weeds (Sarangi *et al.*, 2017). The application of herbicides invariably led to their migration into aquatic ecosystems through leaching or direct runoffs and may affect non-target organisms such as phytoplankton (Chekroun *et al.*, 2013; Guo *et al.*, 2003; Potter *et al.*, 2011). In addition, the repeated use of pesticides can lead to the emergence of pesticide-resistant plants as seen with glyphosate (Powles *et al.*, 1998; Pratley *et al.*, 1999), atrazine (Gustavson and Wängberg, 1995; Ryan, 1970) and, more recently, fomesafen (Salas *et al.*, 2016; Wuerffel *et al.*, 2015). To our knowledge, no study focused on the outcome of multigenerational exposures to this chemical on the physiology of phytoplankton. Widespread use of fomesafen also increases the potential of this chemical to be found in rivers and streams nearby agricultural fields. In fact, very little is known on the impact of fomesafen on freshwater phytoplankton. Caquet *et al.* (2005) exposed phytoplanktonic communities to pure fomesafen (40 $\mu g\ L^{-1}$) and fomesafen mixed with the polyexothylated nonylphenol-based adjuvant Agral 90® (40 and 90 $\mu g\ L^{-1}$, respectively) in mesocosms and noted the persistence of fomesafen in aquatic bodies, together with a phytoplankton community shifts and high fomesafen-sensitivity of chlorophytes. We also shown recently that *Raphidocelis subcapitata* was more sensitive than *Chlamydomonas snowii* and *Microcystis aeruginosa* to a fomesafen-based herbicide from a series of physiological and biochemical indicators (Naoum and Juneau, 2018). Due to the potential for phytoplankton physiology to be altered by long-term exposure to pesticides (Huertas

et al., 2010; Pennington and Scott, 2001; Stachowski-Haberkorn *et al.*, 2013), we aimed to better understand under which fomesafen concentration exposure we may detect changes in the physiology of photosynthetic microorganisms to alter its sensitivity to fomesafen.

In this study, we aim to compare the impact of multigenerational exposure to a fomesafen-based herbicide (Reflex®) on growth, photosynthetic activity, pigment content. Oxidative stress and morphology of two green microalgae (*Raphidocelis subcapitata* FACHB271, *Chlamydomonas snowii* isolated from the Choinière reservoir) and one cyanobacteria (*Microcystis aeruginosa* CPCC632). We therefore evaluate if long-term pre-exposure to fomesafen herbicide caused an increase or a decrease in the sensitivity of the phytoplanktonic species to this toxicant. If present, we aimed to confirm the persistence of those changes in sensitivity by re-evaluating every physiological parameters following a short and long term depuration period.

2.5 Material and Methods

2.5.1 Test conditions

Three phytoplanktonic species (*Raphidocelis subcapitata* (FACHB271), *Chlamydomonas snowii* and *Microcystis aeruginosa* (CPCC632)) were maintained axenicaly in semi-batch culture and grown at 24°C in 250 ml Erlenmeyer flasks with 100 ml Bold's Basal Medium growth medium (Stein, 1973). The illumination cycle was 14/10h (light/dark; 100 µmol photon $m^{-2}s^{-1}$) provided by white fluorescent lamps (Philips F72T8/TL841/HO, USA) and incandescent bulbs (Philips 60W). All phytoplanktonic cultures were maintained in exponential growth phase by periodic transfers when cell density reached 1.0×10^6 cells/ml for *R. subcapitata* to preserve exponential growth. *C. snowii* and *M. aeruginosa* were transferred at normalized cell density (*R. subcapitata* mean biovolume) to maintain exponential growth while taking into consideration total pigment and enzymatic content differences between species.

2.5.2 Fomesafen exposure

The herbicide fomesafen was obtained from Syngenta (Sodium salt of fomesafen; Reflex® 22.8% a.i., 240g fomesafen l^{-1}) (thereafter called fomesafen-based herbicide) and was prepared in nanopure water and kept in darkness until use. The toxic impact of fomesafen on phytoplanktonic cultures was screened at three points in time during this experiment: at the beginning to establish the sensitivity baseline of the phytoplankton to this herbicide, following multigenerational exposure and subsequent to a short depuration period. Each time, cultures were submitted for 72h to increasing concentrations of fomesafen treatment (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} a. i. sodium salt of fomesafen) before sampling.

2.5.3 Multigenerational pre-exposure period

The multigenerational pre-exposure was adapted from (Stachowski-Haberkorn *et al.*, 2013). For each species tested, a daughter culture was maintained in exponential growth phase prior of being split into five fomesafen pre-treatments (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} a. i. sodium salt of fomesafen). Each treatment was maintained in exponential growth phase by periodic transfers in medium containing the same fomesafen concentration for up to 55 generations. The number of generations (N_g) was determined as: $N_g = T_d/2$; where $T_d = \text{Ln}(2)/\mu$ (Stachowski-Haberkorn *et al.*, 2013). Pre-exposed cultures are thereafter referred as $\text{Species}_{P[\text{fomesafen; µg } L^{-1}]}$ (e.g. *R. subcapitata*$_{P320}$).

2.5.4 Physiological measurements

2.5.4.1 Growth rate and cell size

Following exposure, cell concentration and cell size was determined using a Coulter Counter particle analyzer (Multisizer 3, Beckman Coulter Inc., Fullerton, USA). From the plot of ln (cell ml^{-1}) in relation to time (days), the growth rate (µ, $days^{-1}$) was

calculated using $\mu = \ln \cdot (D_t/D_0)/t$; D = cell ml^{-1} at time (t); D_0 = cell ml^{-1} when the exposure started. Mean cell biovolume was recorded in femtoliter (fl).

2.5.4.2 Pigment content analysis

The procedure for chlorophyll *a* and carotenoid content determination was taken from Lichtenthaler (1987). After each fomesafen exposure period, cultures were sampled under dim light and cells were separated from the growth media on a 0.2 µm nitrocellulose filter (Xingya Puryfying Materials factory, Shanghai, China). For the extraction, a minimum of 12 million cells were filtered for *R. subcapitata.* For *C. snowii* and *M. aeruginosa*, the number of cells collected was normalized from the mean biovolume of *R. subcapitata* to take into consideration enzymatic and pigment content differences between species. Pigments were extracted with ethanol 95% in a dry-heat bath at 78°C for 5 min. The pigment extracts were kept in the dark at -20°C overnight to complete the extraction. Absorbance of the pigment extract was measurement with a Cary 300 ultraviolet-visible (UV-Vis) spectrophotometer (Varian Autralia Pty Ltd, Mulgrave, VIC, Australia). The pigment contents were calculated according to Lichtenthaler (1987) and expressed as % of the control.

2.5.4.3 Photosynthesis measurements

2.5.4.3.1 Pulse amplitude modulated fluorescence evaluation

Photosynthetic electron transport yield was obtained according to Schreiber *et al.* (1986). Cultures were sampled then dark-acclimated for 15 minutes prior to the analysis to allow complete oxidation of PSII reaction centers. Under extremely weak modulated light (2 µmol m^{-2} s^{-1}), we recorded the minimum fluorescent yield (F_0) of the sample, then maximum fluorescent yield of PSII (F_M) was obtained after a saturating flash was triggered (700 ms, 3000 µmol $m^{-2}s^{-1}$). To record chlorophyll *a* fluorescence rise resulting from electron exchanges between PSII and PSI we maintained continuous actinic light exposure (125 µmol m^{-2} s^{-1}). Simultaneously, the

maximal fluorescence yield for a light-acclimated culture (F'_M) was induced under saturating flashes (700 ms, 3000 μmol $m^{-2}s^{-1}$) applied at 60-second intervals. Actinic light was turned off when fluorescence yield reached steady state (F_S). Background fluorescence of a light-acclimated sample (F'_0) is measured subsequent to a brief dark period. The equations used to calculate chlorophyll *a* fluorescence parameters were as follows:

2.5.4.3.2 Chl *a* fluorescence quenching parameters

Table 2.1 Chl *a* quenching parameters with their respective formula.

Parameter	Formula	Reference
ϕ_M	$(F_M - F_0)/F_M = F_v/F_M$	(Kitajima and Butler, 1975)
ϕ'_M	$(F'_M - F_S)/F'_M$	(Genty *et al.*, 1989)
qp_{rel}	$(F'_M - F_S)/(F_M - F'_0)$	(Buschmann, 1995)
qn_{rel}	$(F_M - F'_M)/(F_M - F'_0)$	(Buschmann, 1995)
$UQF_{(rel)}$	$(F_S - F'_0)/(F_M - F'_0)$	(Juneau *et al.*, 2005)

As previously stated, samples were dark-acclimated for 15 minutes prior to the analysis of the fast polyphasic chlorophyll fluorescence kinetics using a liquid-compartment adapted Plant Efficiency Analyzer fluorometer (PEA, Hansatech Ltd., King's Lynn, Norfolk, UK). Parameters resulting from this analysis permitted to evaluate the PSII energy fluxes (Force *et al.*, 2003; Strasser et Srivastava, 1995):

Table 2.2 PSII energy fluxes parameters calculated from the rapid rise in fluorescence of Chl *a* with their respective formula.

Parameter	Formula	Reference
ABS/RC	$(TR_0/RC)/(TR_0/ABS)$	(Force *et al.*, 2003)
TR_0/RC	(M_0/V_J)	
ET_0/RC	$(TR_0/RC) \times (ET_0/TR_0)$	
DI_0/RC	$(ABS/RC) - (TR_0/RC)$	
M_0	$(F_{2ms} - F_{50\mu s})/(F_M - F_{50\mu s})$	
V_J	$4 \times (F_{300\mu s} - F_{50\mu s})/(F_M - F_{50\mu s})$	

2.5.4.3.3 Cyclic electron flow measurements

To obtain an estimation of the proportion of cyclic electron flow during photosynthesis, we adapted a procedure taken from Joliot and Joliot (2005). Cyclic electron flow around PSI was measured using a Joliot Type Spectrophotometer (JTS-10, BioLogic, France). The apparatus was affixed with a light-emitting-diode (LED) which periodically radiates detection flashes in the 705-740 nm range. Actinic far-red LED (720 nm) provided excitation flashes through a 705 nm interference filter. Filtered light then goes thought the culture sample and hit a measurement photodiode which records the light absorbance. Cut-off filters are placed in front of the measurement and reference photodiode to ensure only the recording of wavelengths over 695 nm. This analysis was first done on cultures which were dark-acclimated for 15 min prior to the experiment. Then, these same samples were light-acclimated for 3 min at 630 nm before another round of measurements. Light acclimation is needed to activate the Benson-Calvin cycle where during this state the linear electron flow is predominant over cyclic transport (Joliot and Joliot, 2002). The measured absorbance variations are interpreted as the change in rates of oxidation and reduction of the PSI ($\Delta I/I\ P_{700}$).

Following 5s illumination, actinic light is then switched off and we recorded $T_{1/2}$ at which P_{700} absorption changes return to baseline levels. From this measurement, the half-time difference between light adapted and dark-adapted-samples is used to estimate the proportion of cyclic electron flow during photosynthesis of the phytoplankton following exposure to fomesafen. The fraction of cyclic electron flow is expressed in percentage compared to the control.

2.5.4.4 Reactive oxygen species content and cell complexity determination

The effect of fomesafen exposure on ROS content and intracellular damage was determined following Stachowski-Haberkorn *et al.* (2013). The oxidative stress probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used to quantify intracellular ROS. The results were normalized through division of mean FL1 results of H_2DCF-DA–stained cells by FL1 fluorescent of fresh cells. Cell complexity (SSC) was also analyzed to determine the impact of fomesafen-induced oxidative stress on phytoplanktonic membrane integrity. Cell complexity results were expressed as SSC ratio ($SSC_{treated\ cells}/SSC_{control}$) to normalize results.

2.5.5 Statistical analysis

In order to test for significant difference in fomesafen's impact on the phytoplankton, data was expressed as a mean ± standard deviation (n = 6~9) and statistical analyses were performed using GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA). To compare the impact of the herbicide between treated and control populations for each species and pre-exposed cultures, the data was analyzed with a one-way ANOVA followed up by post-hoc Dunnett's multiple comparisons test when significant differences were found.

2.6 Results

2.6.1 Growth rate and photosynthesis

Change is expressed in percentage of the difference between control (not pre-exposed to fomesafen) and pre-exposed cultures submitted to corresponding herbicide concentrations. We previously determined that the growth of *R. subcapitata* is significantly affected by an exposure to over 10 $\mu g\ L^{-1}$ fomesafen (Naoum and Juneau, 2018). Multigenerational pre-exposure of *R. subcapitata* to over 10 $\mu g\ L^{-1}$ fomesafen induced a higher resistance to the herbicide as shown by a reduced impact of the herbicide on growth rate after a 72h exposure to corresponding concentrations (Fig. 2.1a). On the other hand, pre-exposure of *C. snowii* (Fig. 2.1c) and *M. aeruginosa* (Fig. 2.1e) to the highest experimental concentration induced no significant change in growth rate. A second exposure following a short term depuration had no significant impact on the sensitivity to fomesafen for all three pre-exposed species (Figs. 2.1b, 2.1d and 2.1f). The pre-exposed phytoplankton growth response to fomesafen exposure did not change following a short term (Figs. 2.1b, 2.1d and 2.1f) and a long term depuration period (Fig. 2.13a).

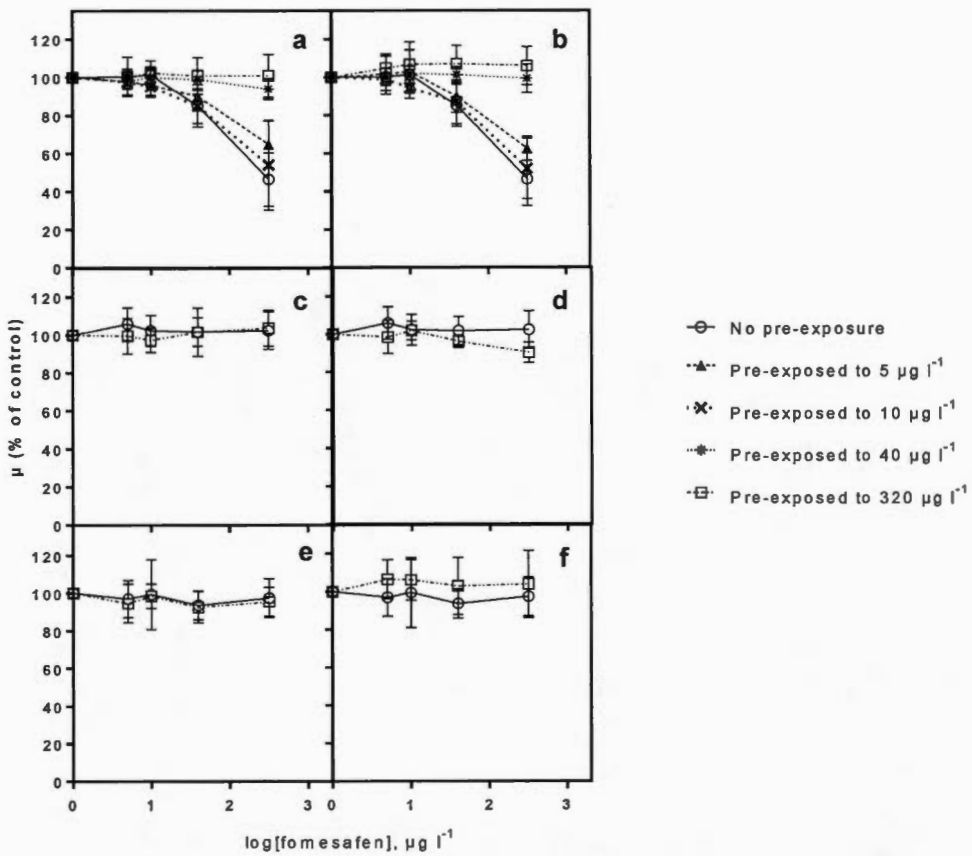

Figure 2.1 Effect of pre-exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 μg L^{-1}) and subsequent 72h exposure on growth rate (μ). The $exposure_{72h}$ was performed before and after a short-depuration period on a) *R. subcapitata*, b) *R. subcapitata*$_{post\text{-}depuration}$, c) *C. snowii*, d) *C. snowii*$_{post\text{-}depuration}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{post\text{-}depuration}$. Mean ± SD; n = 6~9.

Our results also indicate that pre-exposure to fomesafen had a significant impact on fomesafen-induced photosynthesis sensitivity in phytoplankton. Fomesafen pretreatment of *R. subcapitata* to the highest experimental concentration (320 μg L^{-1}) diminished fomesafen-induced photosynthesis inhibition as shown by a reduced impact

of 40 and 320 μg L^{-1} fomesafen on ϕ_M and ϕ'_M after 72h exposure (Figs. 2.2a and 2.3a). The herbicide pretreatment and subsequent exposure showed no sensitivity to the herbicide and no significant change in photosynthesis following pre-exposure for *C. snowii* (Figs. 2.2c and 2.3c) and *M. aeruginosa* (Figs. 2.2e and 2.3e). The pre-exposed phytoplankton photosynthesis response to fomesafen exposure did not change following a short term (Figs. 2.2b, 2.2d and 2.2f) and a long term depuration period (Fig. 2.13c).

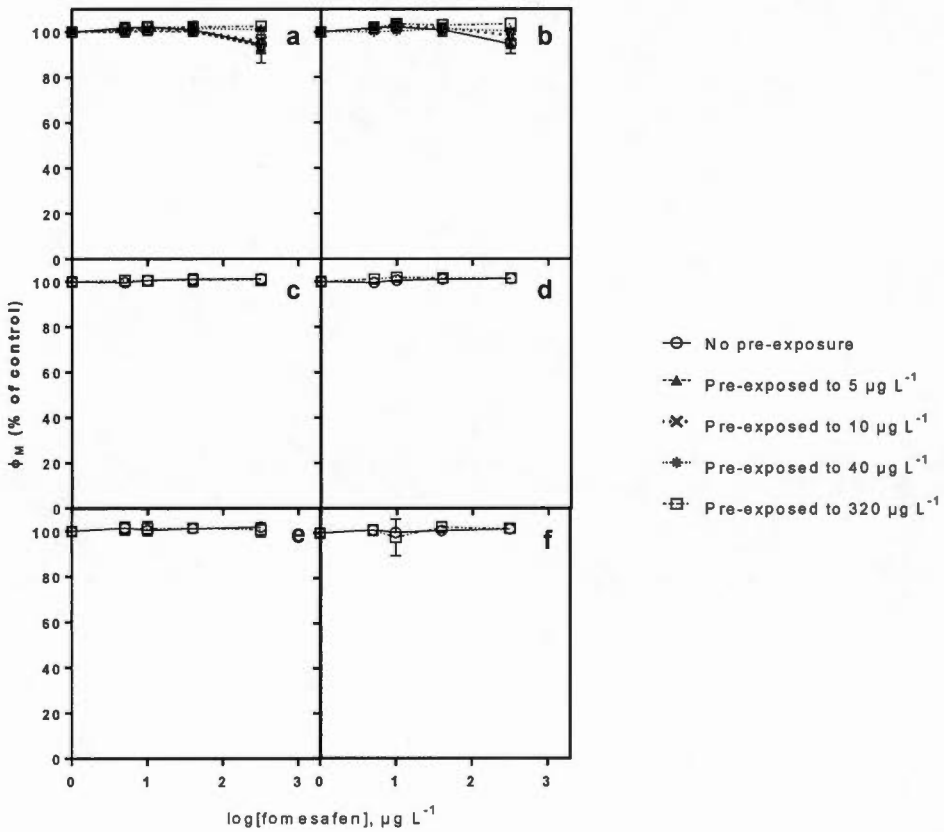

Figure 2.2 Effect of pre-exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 μg L^{-1}) and subsequent 72h exposure on maximal photochemical yield (ϕ_M). The $exposure_{72h}$ was performed before and after a short-depuration period on a) *R.*

subcapitata, b) *R. subcapitata*$_{post\text{-}depuration}$, c) *C. snowii*, d) *C. snowii*$_{post\text{-}depuration}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{post\text{-}depuration}$. Mean ± SD; n = 6~9.

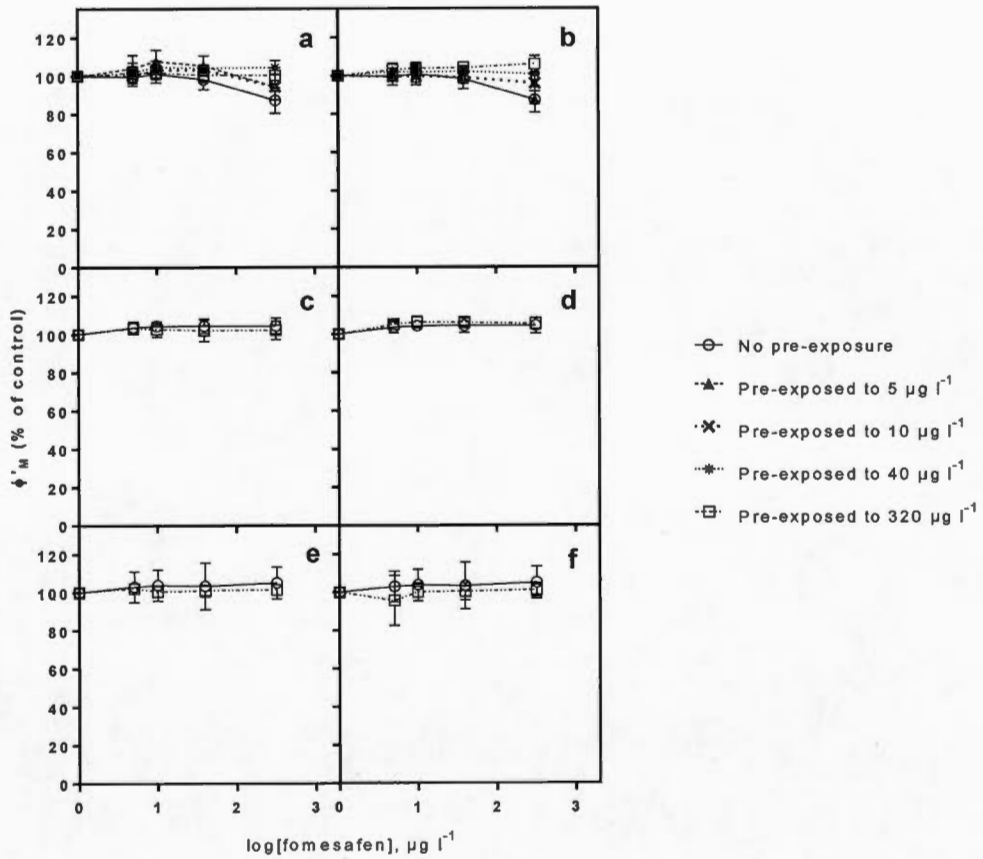

Figure 2.3 Effect of pre-exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}) and subsequent 72h exposure on operational photochemical yield (ϕ_M). The exposure$_{72h}$ was performed before and after a short-depuration period on a) *R. subcapitata*, b) *R. subcapitata*$_{post\text{-}depuration}$, c) *C. snowii*, d) *C. snowii*$_{post\text{-}depuration}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{post\text{-}depuration}$. Mean ± SD; n = 6~9.

More precisely, multigenerational exposure induced significant changes in the way the phytoplankton used light energy (Fig. 2.4a). Our results demonstrate that *R. subcapitata*$_{P10}$ no longer showed variations in light energy fluxes, as observed in

Naoum and Juneau (2018), in response to herbicide exposure at every tested concentration. Indeed, pre-exposure of *R. subcapitata* to over 10 µg L^{-1} fomesafen significantly reduced ABS/RC to a level matching unexposed cultures as shown by a subsequent exposure to corresponding concentrations of the herbicide. Photon-trapping of *R. subcapitata* was also affected by fomesafen pre-exposure. As seen, TR_0/RC for cultures of *R. subcapitata* pre-exposed to over 10 µg L^{-1} herbicide showed no significant change following exposure to these same herbicide concentrations. Every culture, beside *R. subcapitata*$_{P10}$ submitted to 320 µg L^{-1}, showed no significant impact of the herbicide on ET_0/RC. Our results showed that *C. snowii* and *M. aeruginosa* were naturally insensitive to fomesafen (Naoum and Juneau, 2018) and pre-exposure led to minor changes in PSII energy fluxes following exposure to the herbicide (Fig. 2.4b and 2.4c). While being inherently resistant to tested concentrations of fomesafen (Naoum and Juneau, 2018), a small but significant decrease in ET_0/RC between *C. snowii*$_{P320}$ and the control was seen after treatments to every fomesafen concentration. Furthermore, while being intrinsically resistant to fomesafen (Naoum and Juneau, 2018), *M. aeruginosa*$_{P320}$ dissipated three times less energy than control cultures when exposed at every concentration of the herbicide. The pre-exposed phytoplankton PSII energy fluxes response to fomesafen exposure did not change following a short term depuration period (Figs. 2.5a, 2.5b and 2.5c).

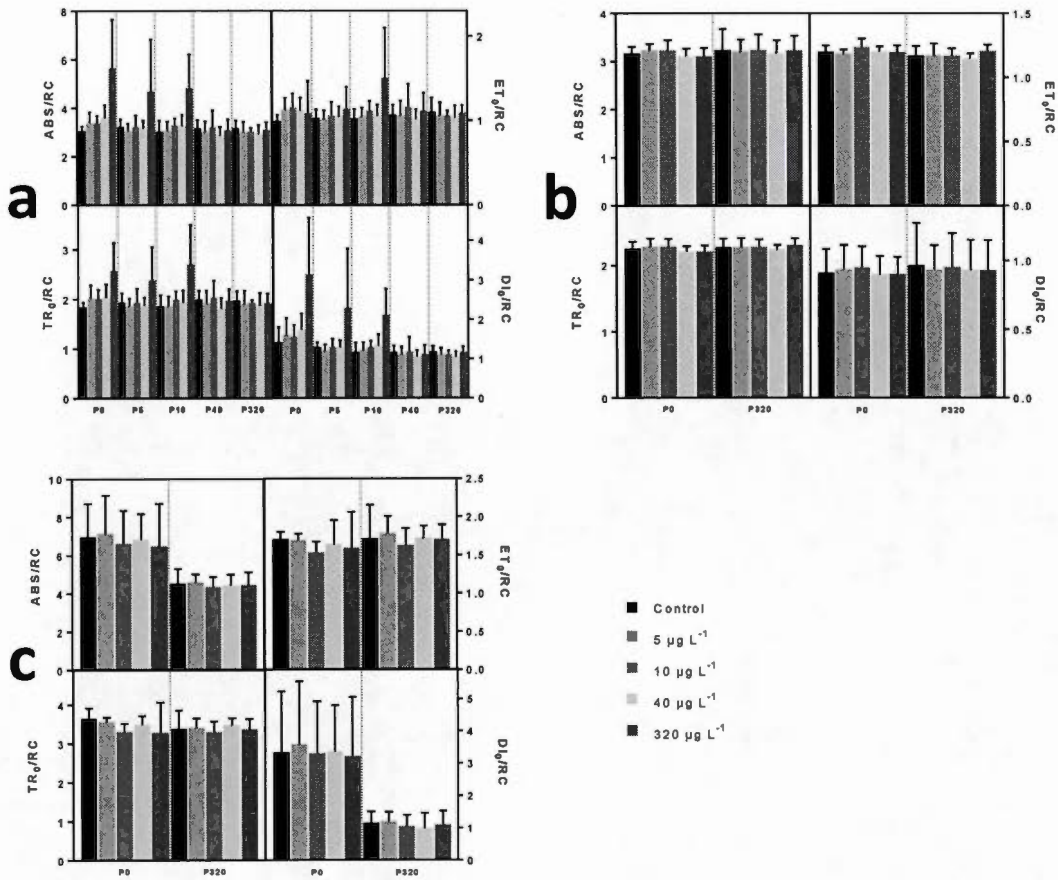

Figure 2.4 Impact of pre-exposure and subsequent 72h exposure of a) *R. subcapitata*, b) *C. snowii* and c) *M. aeruginosa* to increasing concentrations of fomesafen (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen) on PSII energy fluxes (ABS/RC; TR_0/RC; ET_0/RC; DI_0/RC). Mean ± SD; n = 6~9.

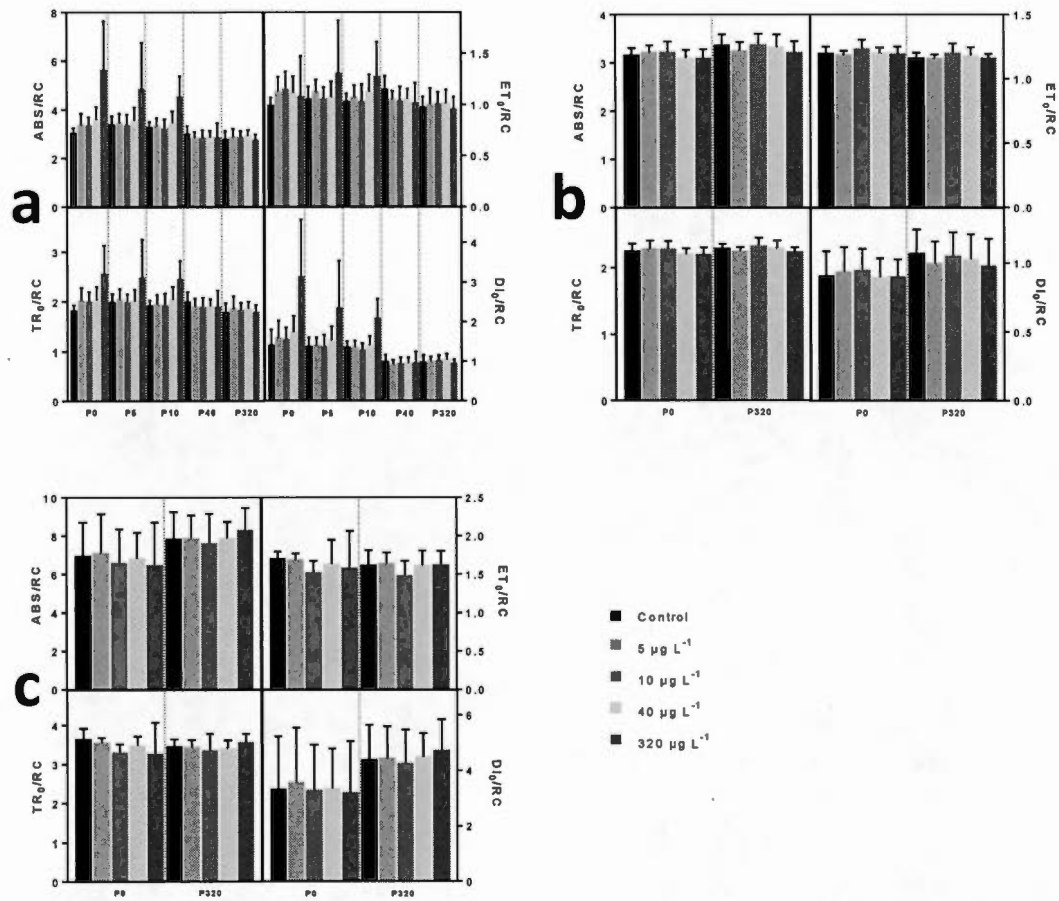

Figure 2.5 Impact of short term depuration and subsequent 72h exposure of pretreated cultures of a) *R. subcapitata*, b) *C. snowii* and c) *M. aeruginosa* to increasing concentrations of fomesafen (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen) on PSII energy fluxes (ABS/RC; TR_0/RC; ET_0/RC; DI_0/RC). Mean ± SD; n = 6~9.

Our data showed that energy dissipation pathways of *R. subcapitata* were significantly affected by a long-term multigenerational exposure to the fomesafen formulation (Fig. 2.6). Indeed, pre-exposure induced significant changes in the way *R. subcapitata* photochemically dissipate light energy. As we previously determined in Naoum and Juneau (2018), control cultures of *R. subcapitata* showed a significant increase and decrease in qp_{rel} following exposure to 10 and 320 µg L^{-1} fomesafen, respectively. Pre-

treatment of *R. subcapitata* with 5µg L^{-1} fomesafen (Fig. 2.6b) induced a small but significant rise in qp_{rel} for cultures subsequently submitted to 5 and 40 µg L^{-1} fomesafen and exposure to 320 µg L^{-1} revealed a restoration of qp_{rel} to levels comparable with unexposed cultures. Meanwhile, cultures pre-exposed to over 5 µg L^{-1} (Figs. 2.6c, 2.6d and 2.6e) now showed no variation in qp_{rel} following exposure to every concentration of fomesafen. On the other hand, qp_{rel} of *C. snowii* (Figs. 2.6f and 2.6g) and *M. aeruginosa* (Figs. 2.6h and 2.6i), previously determined as a fomesafen-insensitive parameter for these species (Naoum and Juneau, 2018), showed no change in sensitivity following pre-exposure to fomesafen. For all three species, our results showed that the non-photochemical quenching pathway was slightly affected by fomesafen exposure (Naoum and Juneau, 2018) and subsequent pre-exposure. *R. subcapitata*$_{P5}$ (Fig. 2.6b) and *R. subcapitata*$_{P40}$ (Fig. 2.6d) showed an overall significant reduction in qn_{rel} after exposure to the herbicide. The same species, but pretreated to 10 (Fig. 2.6c) and 320 µg L^{-1} (Fig. 2.6e), showed no change in qn_{rel} following exposure to all concentrations of fomesafen. In contrast, qn_{rel} of *C. snowii* was generally unsolicited following contact with the herbicide (Figs. 2.6f and 2.6g). Indeed, only pre-exposure and subsequent exposure to 320 µg L^{-1} (Fig. 2.6g) of *C. snowii* induced a small but significant increase in qn_{rel}. A slight but significant decrease in qn_{rel} following exposure to 10 and 40 µg L^{-1} fomesafen was previously established for *M. aeruginosa* (Naoum and Juneau, 2018). Pre-exposure of *M. aeruginosa* to the highest concentration of fomesafen restored this parameter when exposed to 10 µg L^{-1} (Fig. 2.6i). As seen in Naoum and Juneau, (2018), fomesafen exposure differently affected UQF_{rel} for all three species. Pre-exposure of *R. subcapitata* showed no UQF_{rel} response following fomesafen pre-exposure to all concentrations, except the highest (320 µg L^{-1}) (Figs. 2.6a, 2.6b, 2.6c and 2.6d). Indeed, pre-exposure of *R. subcapitata* to 320 µg L^{-1} (Fig. 2.6e) led to a significant rise in UQF_{rel} when exposed to concentrations higher than 5 µg L^{-1}. Pre-exposure of *C. snowii* to the tested concentrations of fomesafen completely restored herbicide-induced decrease in UQF_{rel} following the exposure at all concentrations (Figs. 2.6f and 2.6g).

UQF_{rel} for *M. aeruginosa* was not affected by exposure and pre-exposure to fomesafen (Figs. 2.6h and 2.6i).

Pre-treated cultures submitted to a short depuration period exhibited changes in previously observed herbicide-induced variations in light energy quenching for all three species (Fig. 2.7). Short term depuration restored qp_{rel} for *R. subcapitata*$_{P5}$ (Fig. 2.7b) and subsequently grown in contaminated media. Non-photochemical quenching, qn_{rel}, showed the most change following the short term depuration. Growth of *R. subcapitata*$_{P5}$ (Fig. 2.7b) and *R. subcapitata*$_{P40}$ (Fig. 2.7d) in clean media for a week completely restored qn_{rel} as evidenced by the insensitivity of this parameter following exposure to every concentration of fomesafen. For both *C. snowii* (Figs. 2.7f and 2.7g) and *M. aeruginosa* (Figs. 2.7h and 2.7i), no change in qn_{rel} response to fomesafen treatment was observed for pre-exposed cultures following a short term depuration period. For UQF_{rel}, short term depuration of *R.* subcapitata$_{P320}$ (Fig. 2.7e) restored previously seen rise in unquenched fluorescence following exposure to over 5 $\mu g\ L^{-1}$ fomesafen.

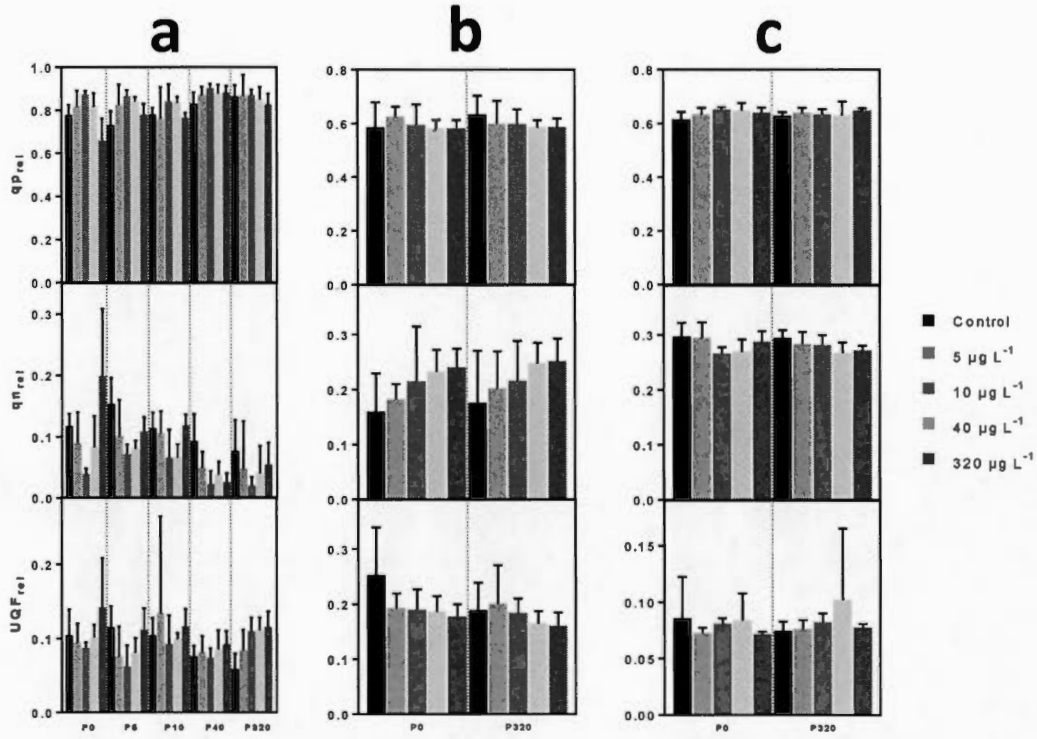

Figure 2.6 Relative photochemical quenching (qp_{rel}), relative non-photochemical quenching (qn_{rel}) and relative *unquenched* fluorescence (UQF_{rel}) of a) *R. subcapitata*$_{P0\text{-}320}$, b) *C. snowii*$_{P0\text{-}320}$ and c) *M. aeruginosa*$_{P0\text{-}320}$ after 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen). Mean ± SD; n = 6~9. *Significant difference compared to the control; one-way ANOVA followed by post-hoc Dunnett's multiple comparisons test.

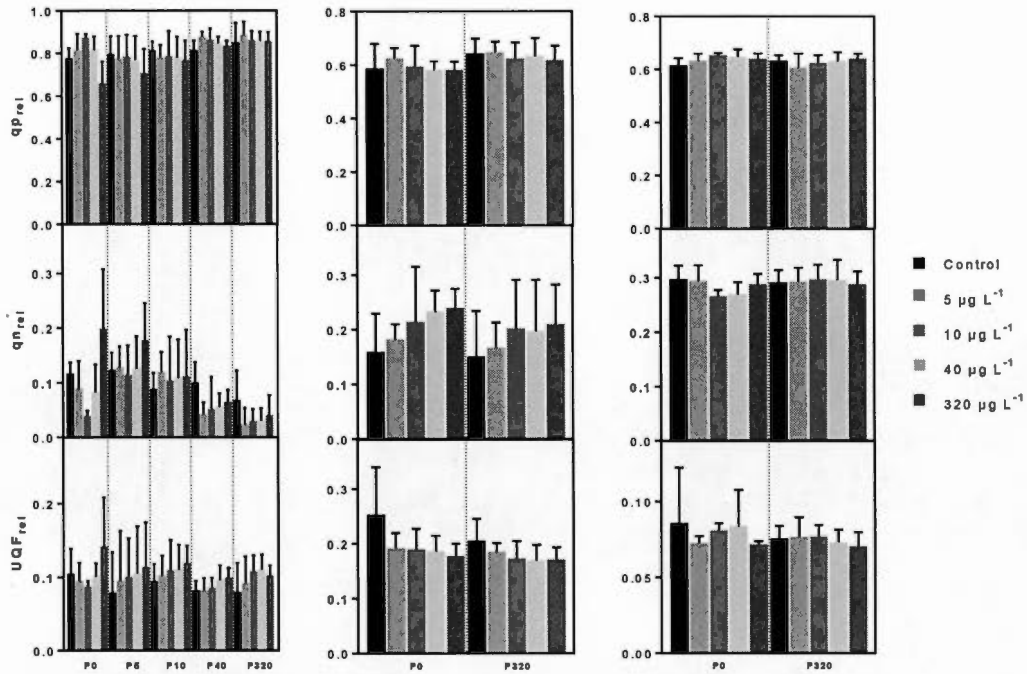

Figure 2.7 Effect of a short-term depuration period on relative photochemical quenching (qp_{rel}), relative non-photochemical quenching (qn_{rel}) and relative *unquenched* fluorescence (UQF_{rel}) of a) *R. subcapitata*$_{P0-320}$, b) *C. snowii*$_{P0-320}$ and c) *M. aeruginosa*$_{P0-320}$ after 72h exposure to increasing concentrations of fomesafen-based herbicide (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen). Mean ± SD; n = 6~9. *Significant difference compared to the control; one-way ANOVA followed by post-hoc Dunnett's multiple comparisons test.

For *R. subcapitata*, pre-exposure to every concentration of herbicide reduced the cyclic transport response by a significant margin in the presence of fomesafen (Table 2.3). Exposure of *C. snowii* and *M. aeruginosa* to fomesafen produced no significant response or trend concerning cyclic electron transport and subsequent pre-exposure to the herbicide did not produce noticeable changes in cyclic electron transport response (Data not shown). The pre-exposed phytoplankton cyclic electron transport ratio to

linear transport response to fomesafen exposure did not change following a short term depuration period (Table 2.4).

Table 2.3 Effect of pre-exposure and subsequent 72h exposure of *R. subcapitata* to increasing concentrations of fomesafen (Reflex®; 0, 5, 10, 40 and 320 μg L^{-1} fomesafen) on the solicitation of cyclic electron transport over linear transport. N.d. stands for not determined since no signal was obtained at high fomesafen concentration. Mean ± SD; n = 6~9.

[Exposure]	Non pre-exposed	Pre-ex 5 μg L^{-1}	Pre-ex 10 μg L^{-1}	Pre-ex 40 μg L^{-1}	Pre-ex 320 μg L^{-1}
0 μg L^{-1}	7.5 (6.0)	13.5 (8.4)	9.5 (4.7)	5.4 (5.4)	0(5.1)
5 μg L^{-1}	11.1 (10.9)	11.3 (9.5)	17.8 (13.6)	2.1 (3.8)	1.7 (1.4)
10 μg L^{-1}	16.5 (4.3)	16.2 (8.7)	16.3 (6.6)	11.6 (8.0)	0 (5.4)
40 μg L^{-1}	31.7 (15.6)	33.4 (8.5)	22.1 (5.6)	17.6 (9.8)	2.4 (6.3)
320 μg L^{-1}	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2.4 Impact of short term depuration period and subsequent 72h exposure of pretreated cultures of *R. subcapitata* to increasing concentrations of fomesafen (Reflex®; 0, 5, 10, 40 and 320 µg L^{-1} fomesafen) on the solicitation of cyclic electron transport over linear transport. N.d. stands for not determined since no signal was obtained at high fomesafen concentration. Mean ± SD; n = 6~9.

[Exposure]	Non pre-exposed	Pre-ex 5 µg L^{-1}	Pre-ex 10 µg L^{-1}	Pre-ex 40 µg L^{-1}	Pre-ex 320 µg L^{-1}
0 µg L^{-1}	7.2 (5.2)	9.8 (8.2)	6 (5.3)	4.9 (3.6)	0.9 (2.3)
5 µg L^{-1}	10.5 (7.8)	8.2 (11.4)	13.7 (7.0)	3.4 (3.0)	2.4 (5.0)
10 µg L^{-1}	15.6 (5.0)	12.7 (4.9)	12.1 (5.9)	16 (9.5)	0.9 (2.1)
40 µg L^{-1}	28.5 (12.4)	21.7 (7.7)	17.3 (6.9)	26.1 (8.0)	5.9 (5.1)
320 µg L^{-1}	n.d.	n.d.	n.d.	n.d.	n.d.

2.6.2 Pigment content

Photosynthetic pigment determination showed that pre-exposure of *R. subcapitata* to fomesafen induced changes in pigment content following exposure to increasing concentrations of the herbicide formulation (Figs. 2.8a and 2.9a).

Chl *a* content of *R. subcapitata* pre-exposed to fomesafen remained unchanged by an exposure to 40 µg L^{-1} and, in the case of *R. subcapitata*$_{P10}$ and *R. subcapitata*$_{P320}$, to the highest herbicide concentration tested (Fig. 2.8a). For *C. snowii*, our results showed that Chl *a* content was restored by a pre-treatment to fomesafen (Fig. 2.8c). *M. aeruginosa* pigment content was previously determined to be unaffected by fomesafen exposure (Naoum and Juneau, 2018), and showed no changes in sensitivity to fomesafen toxicity following pre-exposure to the highest concentration of the herbicide (Fig. 2.8e). The same pattern observed for Chl *a* content was also observed for

carotenoids of pre-exposed cultures of *R. subcapitata* (Fig. 2.9a) and *M. aeruginosa* (Fig. 2.9e). However, inhibition of carotenoids content we observed in Naoum and Juneau (2018) for *C. snowii* exposed to 10 and 320 µg L^{-1} was restored following pre-exposure to 320 µg L^{-1} fomesafen (Fig. 2.9c). The pre-exposed phytoplankton pigment content response to fomesafen exposure did not change following a short term (Figs. 2.8b, 2.8d and 2.8f; Figs. 2.9b, 2.9d and 2.9f). and a long term depuration period (Fig. 2.13b).

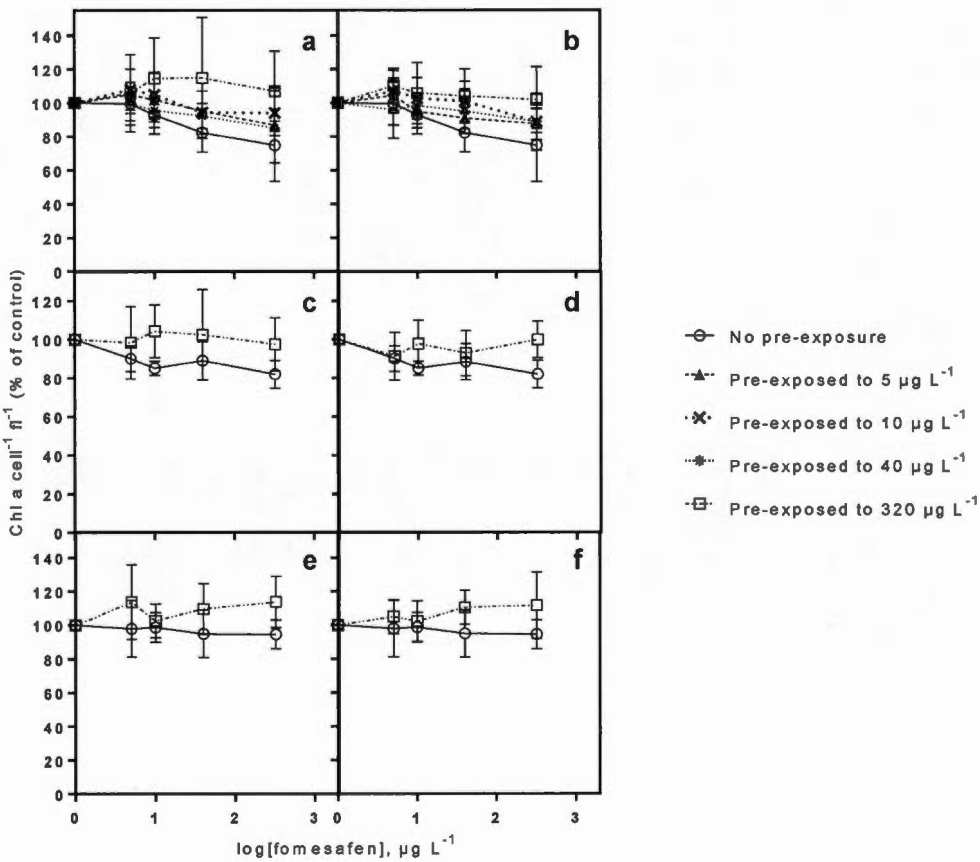

Figure 2.8 Effect of pre-exposure and subsequent 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}) on chlorophyll *a* (Chl *a*) content. The $exposure_{72h}$ was performed before and after a short-depuration period on

a) *R. subcapitata*, b) *R. subcapitata*$_{\text{post-depuration}}$, c) *C. snowii*, d) *C. snowii*$_{\text{post-depuration}}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{\text{post-depuration}}$. Results are normalized by cell density and size. Mean ± SD; n = 6~9.

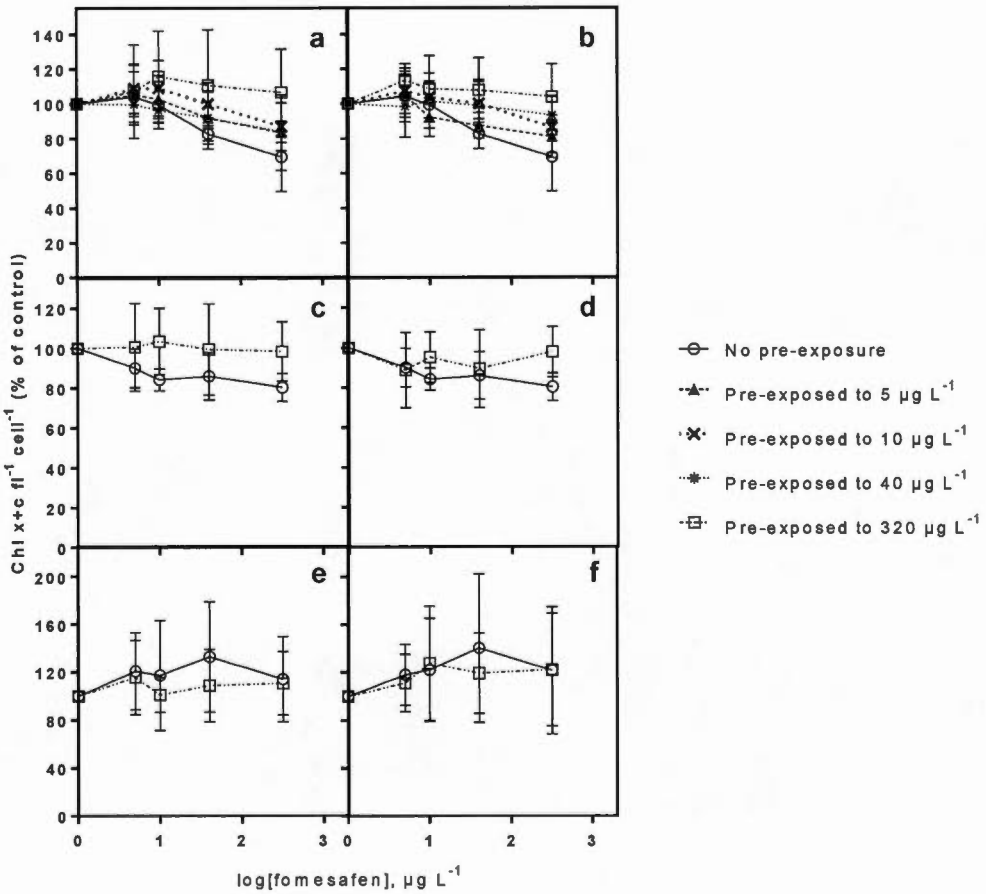

Figure 2.9 Effect of pre-exposure and subsequent 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}) on carotenoid (chl *x+c*) content. The exposure$_{72h}$ was performed before and after a short-depuration period on a) *R. subcapitata*, b) *R. subcapitata*$_{\text{post-depuration}}$, c) *C. snowii*, d) *C. snowii*$_{\text{post-depuration}}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{\text{post-depuration}}$. Results are normalized by cell density and size. Mean ± SD; n = 6~9.

2.6.3 Oxidative stress and cell complexity

Pre-exposure of *R. subcapitata* to concentrations over 10 µg L^{-1} of fomesafen significantly reduced ROS production, as shown by a decrease in intracellular ROS concentration after 72h exposure to the same concentrations of this herbicide (Fig. 2.10a). Long-term pre-exposure of *R. subcapitata* to 10 µg L^{-1} fomesafen led to a very large increase in ROS content following exposure to 320 µg L^{-1}. On the other hand, cultures of *C. snowii* and *M. aeruginosa* did not produce a significant amount of ROS following exposure to fomesafen (Naoum and Juneau, 2018) and pre-exposure did not alter this intrinsic resistance to the herbicide (Fig. 2.10c and 2.10e). With the exception of *R. subcapitata*$_{P5}$, the pre-exposed phytoplankton ROS-response to fomesafen exposure did not change following a short term (Figs. 2.10b, 2.10d and 2.10f) and a long term depuration period (Fig. 2.13d).

The rise in intracellular complexity we observed following exposure of *R. subcapitata* to fomesafen in Naoum and Juneau (2018) was also significantly altered by pre-exposure to fomesafen. Pre-treatment of this specie to over 10 µg L^{-1} was enough to restore cell complexity to levels comparable to unexposed cultures following exposure to 40 and 320 µg L^{-1} fomesafen (Fig. 2.11a). For *C. snowii* (Fig. 2.11c) and *M. aeruginosa* (Fig. 2.11e), pre-exposure to fomesafen induced no change in cell complexity following treatment to fomesafen. The pre-exposed phytoplankton cell complexity response to fomesafen exposure did not change following a short term depuration period (Figs. 2.11, 2.11b, 2.11d and 2.11f).

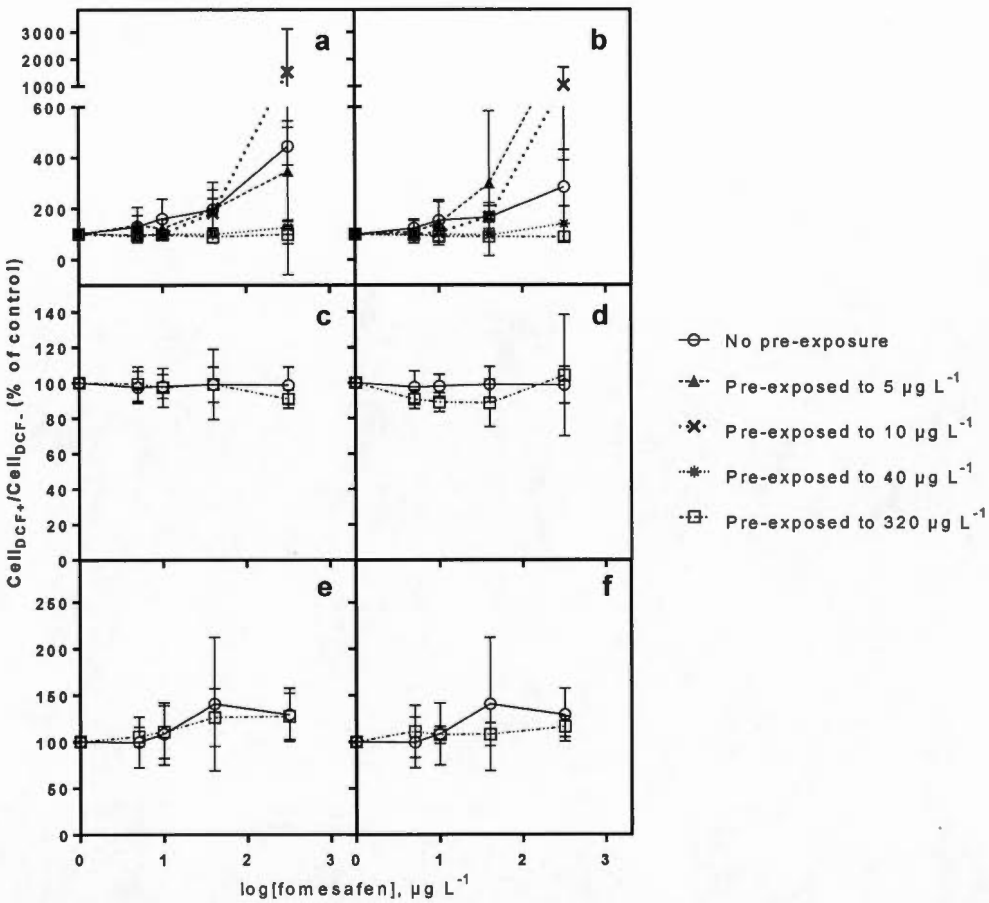

Figure 2.10 Effect of pre-exposure and subsequent 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}) on intracellular ROS content ($Cell_{DCF+}/Cell_{DCF-}$). The $exposure_{72h}$ was performed before and after a short-depuration period on a) *R. subcapitata*, b) *R. subcapitata*$_{post\text{-}depuration}$, c) *C. snowii*, d) *C. snowii*$_{post\text{-}depuration}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{post\text{-}depuration}$. Mean ± SD; n = 6~9.

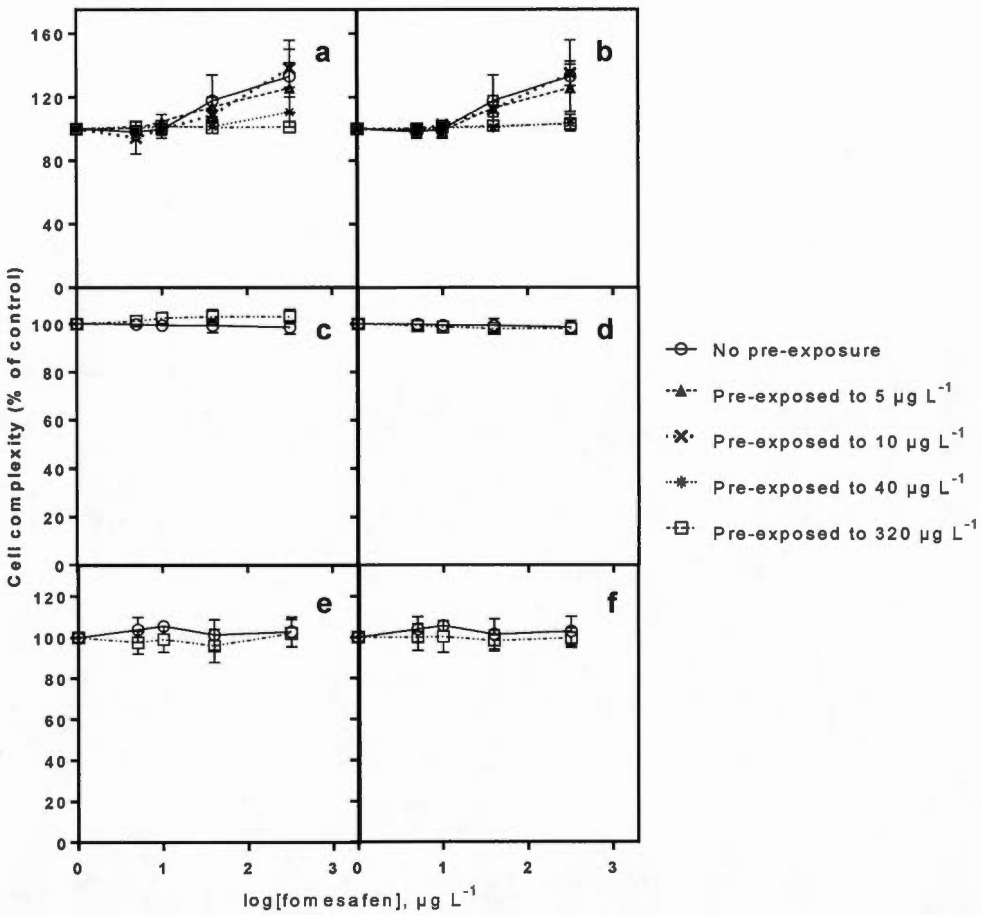

Figure 2.11 Effect of pre-exposure and subsequent 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}) on cell complexity ($SSC_{treated}/SSC_{fresh\ cells}$). The $exposure_{72h}$ was performed before and after a short-depuration period on a) *R. subcapitata*, b) *R. subcapitata*$_{post\text{-}depuration}$, c) *C. snowii*, d) *C. snowii*$_{post\text{-}depuration}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{post\text{-}depuration}$. Mean ± SD; n = 6~9.

3.2.5. Cell biovolume

Our results showed that pre-treatment of *R. subcapitata* to increasing concentrations of fomesafen led to significant changes in cell biovolume following exposure to the herbicide (Fig. 2.12a). Cultures of *R. subcapitata* pre-exposed to concentrations lower than 40 μg L^{-1} fomesafen showed significant increase in biovolume following exposure to the herbicide. Pre-exposure of *R. subcapitata* to concentrations equal and over 40 μg L^{-1} was sufficient to restore biovolume to a level similar to the control. Indeed, cultures pre-exposed to 40 μg L^{-1} were now unaffected by exposure to the same concentration of fomesafen and *R. subcapitata*$_{P320}$ showed no change in cell biovolume following exposure to every concentration of herbicide. For *C. snowii* (Fig. 2.12c) and *M. aeruginosa* (Fig. 2.12e), pre-exposure to fomesafen produced no change in cell complexity following exposure to fomesafen. The pre-exposed phytoplankton biovolume response to fomesafen exposure did not change following a short term depuration period (Figs. 2.12b, 2.12d and 2.12f).

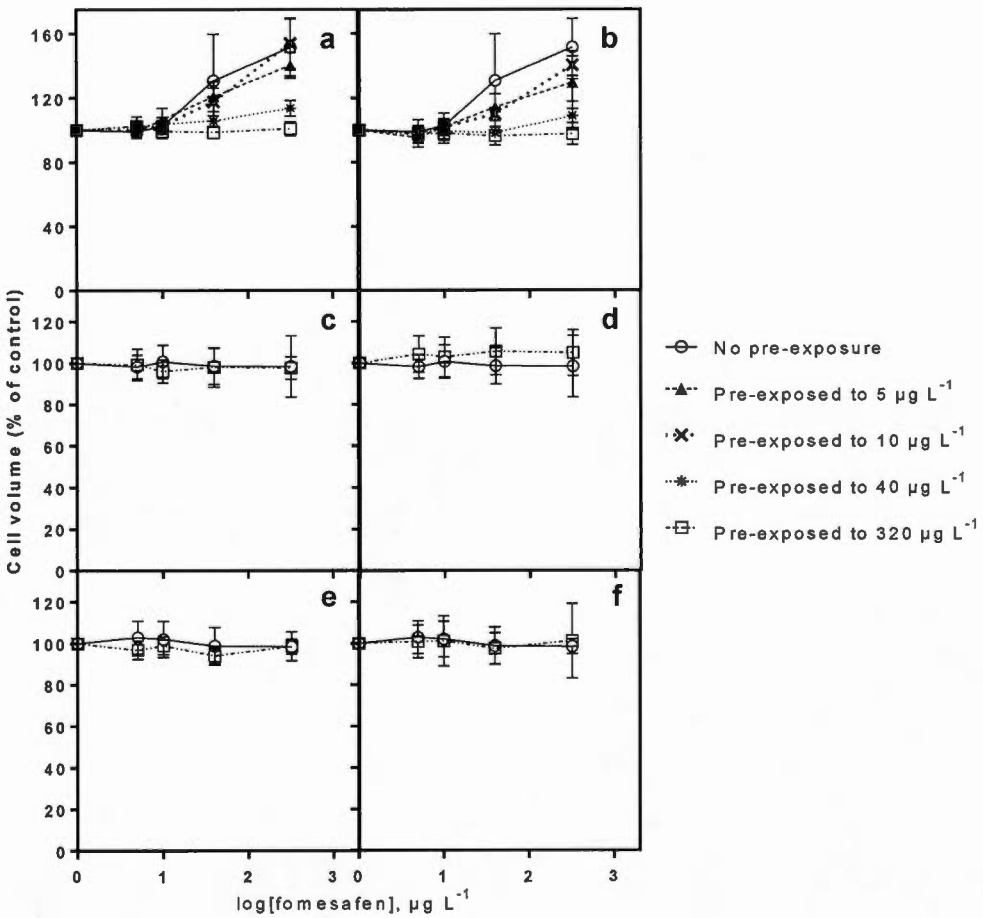

Figure 2.12 Effect of pre-exposure and subsequent 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 µg L^{-1}) on cell biovolume. The $exposure_{72h}$ was performed before and after a short-depuration period on a) *R. subcapitata*, b) *R. subcapitata*$_{post\text{-}depuration}$, c) *C. snowii*, d) *C. snowii*$_{post\text{-}depuration}$, e) *M. aeruginosa* and f) *M. aeruginosa*$_{post\text{-}depuration}$. Mean ± SD; n = 6~9.

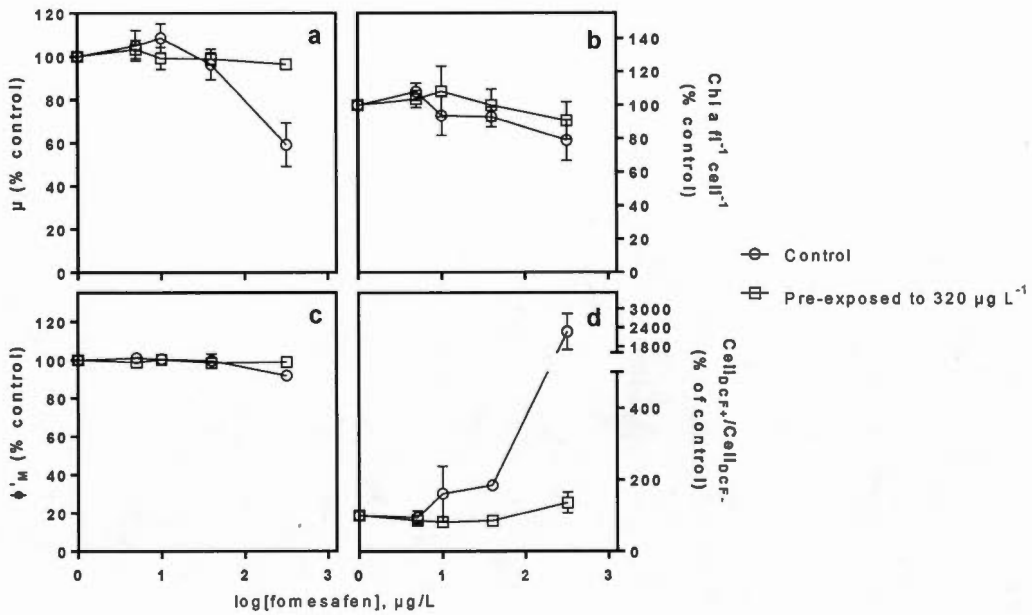

Figure 2.13 Effect of pre-exposure and subsequent 72h exposure to increasing concentrations of fomesafen (0, 5, 10, 40 and 320 μg L^{-1}) on a) growth, b) Chl *a* content, c) operational photochemical yield (ϕ'_M) and d) intracellular ROS content ($Cell_{DCF+}/Cell_{DCF-}$) of *R. subcapitata* and *R. subcapitata*$_{P320}$ following a long-term (6 months) depuration period. Mean ± SD; n = 9.

3.7 Discussion

3.7.1 Effect of pre-exposure to fomesafen on phytoplankton physiology

As was observed in Naoum and Juneau (2018), exposure of *R. subcapitata* to over 10 μg L^{-1} fomesafen in Reflex formulation led to significant inhibition in growth, pigment content and photosynthetic efficiency while inducing a strong generation of ROS. On the other hand, *C. snowii* and *M. aeruginosa* remained largely unaffected by the fomesafen treatment. Complementary to these results, the present paper aimed to

investigate the impact of pre-exposure to fomesafen on phytoplankton sensitivity to this herbicide.

As popularity of fomesafen and other diphenyl ether herbicides increased, latest literature pointed out the emergence of new photosynthetic strains which developed resistance to PPO-inhibitors. Dayan *et al.* (2010) recently listed 11 known weed species which, through various mechanisms, evolved resistance to PPO-inhibitors. The first species in which researchers have identified such resistance is believed to be the commonly known Tall waterhemp (*Amaranthus tuberculatus*). This resistance is thought to have originated from a deletion of a glycine amino acid (ΔGly210) in the PPO enzyme following repeated exposure to a PPO-inhibitor. This deletion enabled the mutant organism to be insensitive to a wide array of different PPO-inhibitors. In the present study we demonstrated that following 55 generations exposure to concentrations over 10 μg L^{-1} of fomesafen *R. subcapitata* developed clear signs of resistance to this herbicide even at the highest tested concentrations. We first noticed complete unhindered growth for pretreated cultures of *R. subcapitata* followed by exposures to 40 and 320 μg L^{-1} herbicide. Investigation of the impact of fomesafen pre-exposure on photosynthetic and protective pigments showed that pigment content of *R. subcapitata* was similar to unexposed algae even after an exposure to high concentrations of fomesafen. For *C. snowii*, pre-exposure to high concentration of fomesafen led to a complete restoration of the slight inhibition in photosynthetic and protective pigment content observed before the pre-exposure. Growth rate is known to be intimately related to photosynthetic yield which itself is affected by pigment content (Cullen, 1990; Falkowski *et al.*, 1985; Ryther and Yentsch, 1957). This is reflected in our results as fomesafen-pretreated cultures of *R. subcapitata* showed a complete restoration of every photosynthesis-related parameters following exposure to concentrations over 10 μg L^{-1} fomesafen. Furthermore, we noticed a lack of ROS content in fomesafen pretreated cultures of *R. subcapitata* which suggests that competitive inhibition of PPO by fomesafen is much less effective to a point where *R.*

subcapitata does not, or accumulate much less free PPIX, thus eliminating or drastically reducing the amount of ROS generated by the photo-oxidation of this pigment and subsequent energy transfer to oxygen. One can advance two main scenarios to explain the emergence of apparent resistance to an environmental stressor such as fomesafen. First, physiological acclimation to low toxicity herbicides is known to be sufficient to completely restore phytoplankton growth as a result of a change in gene expression (Bradshaw and Hardwick, 1989). Thus, the reduced physiological impact of the fomesafen-based herbicide on *R. subcapitata* could be explained by changes in phenomic and/or genomic traits induced by fomesafen's multigenerational toxic pressure. Also, changes in the physiological response to a stressor following long-term exposure to this stressor may also arise through adaptation processes. Indeed, spontaneously occurring point mutations and oxidative stress-induced mutagenesis during multigenerational exposure brings the potential for genetic adaptation which can lead to lasting or permanent resistance (Belfiore and Anderson, 2001; Sniegowski *et al.*, 1995). Thus, the decrease in sensitivity to fomesafen for *R. subcapitata* following multigenerational exposure to this herbicide could originate from spontaneous or ROS-induced change in the expression of enzymes and molecules involved in defense mechanisms normally activated in the presence of stress. Alternatively, changes in the expression of PPO could have occurred thus reducing fomesafen's affinity to this enzyme. This process would limit competitive inhibition of PPO by the fomesafen molecule consequently restoring photopigments biosynthesis thereby allowing normal photosynthesis and growth.

3.7.2 Conservation of changes in sensitivity

To gain insights on if this newly developed resistance is the result of physiological acclimation or genetic adaptation, each physiological parameter was re-evaluated following a short and a long-term depuration period in clean growth media to allow complete turnover of phytoplanktonic cells. Following these depuration periods, our

results show that pretreated cultures of *R. subcapitata* still exhibit a strong resistance to high concentrations of fomesafen following a short term depuration in uncontaminated media indicating that the induced resistance is most likely due to the emergence of a spontaneous mutation during the phytoplankton multigenerational exposure to the fomesafen-based formulation. This type of resistance was also noticed for other PPO-inhibiting herbicides following pre-exposure of green microalgae. Indeed, long-term exposure of a wild strain of *C. reinhardtii* to the *N*-phenylimide-based experimental herbicide S-23142 induced the emergence of a resistant mutant strain (rs-3). Cells of the rs-3 strain of *C. reinhardtii* treated with 0.3 µM of S-23142 showed temporary accumulation of porphyrins followed by normal growth (Sato *et al.*, 1994). Further analysis of rs-3 by Randolph-Anderson *et al.* (1998) showed that the resistance to the PPO-inhibitor S-23142 originated from a point mutation in the PPO gene which induced a transition from the amino acid valine to methionine. This transition yielded viable polymorphism of the PPO gene thus allowing the microalgae to produce enzymes insensitive to PPO-inhibitors and achieve normal growth. Recently, genetically engineered cells of the mutant rs-3 showed resistance to up to 136x the lethal concentrations of oxyfluorfen, a PPO-inhibitor (Bruggeman *et al.*, 2014). Similar rise in resistance for phytoplankton was observed for other herbicides which inhibit photosynthesis. Stachowski-Haberkorn *et al.* (2013) showed that long-term multigenerational exposure of the microalga *Tetraselmis suecica* to 5 µg L^{-1} of diuron induced a significant rise in doubling time for some cultures. Using chlorophyll fluorescence as a ecotoxicological endpoint, Seguin *et al.* (2002) showed that chronic exposure in mesocosms (25 days) to 30 µg L^{-1} of atrazine led to a significant increase in the apparent tolerance of the phytoplanktonic communities to this herbicide.

Additional evidence of the adaptation of *R. subcapitata* to the fomesafen-based herbicide is shown by the conservation of resistance following a third exposure after 6 months of depuration in clean growth media (Fig. 2.13). These results are comparable to Stachowski-Haberkorn *et al.* (2013) which confirm the capacity of microalgae to

maintain resistance to photosynthesis-inhibiting herbicides after a 12-month depuration period in fresh media as subsequent exposure to diuron did not provoke any growth inhibition for *T. suecica.*

3.8 Conclusion

This paper presents evidence that multigenerational exposure to high concentrations of the fomesafen-based phytosanitary product Reflex® can reduce the sensitivity of *R. subcapitata* to this herbicide. The absence of a variety of phytoplankton capable of withstanding the presence of PPO-inhibitors limit the development of phytoremediation methods for this herbicide. The emergence of a phytoplanktonic strain (such as the one developed in the present work) which can tolerate low to high concentrations of fomesafen herbicide bring the potential to create a phytoremediation tool for this toxicant. This is interesting knowing that depuration of very high volume of water showing low to moderate concentrations of toxicants can be very costly (Hultberg *et al.*, 2015; Levi, 2013; Pimentel, 2005) and, depending on the source and location of the contamination, is often deemed impracticable (Azubuike *et al.*, 2016; Levi, 2013). Certainly, more research is needed to better understand the full scope of fomesafen toxicity on phytoplankton and their adaptation. Further analysis to quantify intracellular concentration of fomesafen in phytoplankton post-exposure is needed to verify the presence of mechanisms to bioaccumulate or detoxify the molecule. If herbicide degradation is seen, characterization of by-products of fomesafen degradation is needed and the metabolic pathway of biodegradation of fomesafen in newly resistant phytoplanktonic species should be mapped.

3.9 Acknowledgments

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3.10 References

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CONCLUSION GÉNÉRALE

Lors de cette étude, nous avons évalué la toxicité d'un herbicide à base de fomésafène, le Reflex®, sur la physiologie des microalgues vertes et cyanobactéries à l'aide de deux stratégies d'exposition complémentaires. En premier lieu, le chapitre I de ce mémoire présente l'impact d'une simple exposition au fomésafène sur différents biomarqueurs physiologiques du phytoplancton (croissance, contenu pigmentaire, photosynthèse, stress oxydatif, morphologie). Cette étude démontre qu'une exposition au Reflex®, un herbicide à base de fomésafène, a provoqué différentes réponses physiologiques entre les chlorophycées et les cyanophytes. En effet, ce projet atteste d'un impact significatif du fomésafène sur l'ensemble des paramètres évalués chez *R. subcapitata* et, dans une moindre mesure, chez *C. snowii*. Une inhibition de la croissance chez *R. subcapitata* induite par une exposition à des concentrations de plus de 10 $\mu g\ L^{-1}$ fomésafène a été liée à une diminution de l'efficacité photosynthétique en réponse au mode d'action de l'herbicide soit l'inhibition de la voie de biosynthèse des photopigments et la génération subséquente de ROS. Les différences de sensibilités entre les chlorophytes furent associées à leurs disparités morphologiques. De plus, il est possible que l'absence de sensibilité au fomésafène pour *M. aeruginosa* soit issue d'une différence au niveau du gène codant pour la PPO entre les cyanobactéries et les microalgues vertes.

La deuxième stratégie d'exposition, présentée au chapitre II, met l'emphase sur l'impact d'une exposition multigénérationnelle à l'herbicide à base de fomésafène Reflex® sur les différents indicateurs physiologiques du phytoplancton soit le taux de croissance, le contenu en pigments photosynthétiques, la performance de la photosynthèse, le contenu en ROS et la morphologie. Ainsi, ce projet confirme que le prétraitement de *R. subcapitata* à de fortes concentrations de l'herbicide (plus de 10 μg

L^{-1}) permet de réduire drastiquement la sensibilité de cette microalgue à cet herbicide. L'inhabilité du fomésafène à induire une baisse dans la teneur en photopigments suggère le développement d'une résistante issue de mutations présélectives sous l'impact de la pression toxique de cet herbicide. Cette supposition est d'autant plus confirmée par la préservation des changements de sensibilité suite à une courte et longue période de dépuration. L'émergence de souches phytoplanctoniques capable de résister à l'impact du fomésafène ouvre la porte à la mise au point d'un outil de détoxification de l'eau à cet herbicide. Dans l'ensemble, ces résultats sont importants puisqu'ils témoignent du devenir et de l'impact de concentrations élevées en herbicide à base de fomésafène sur le phytoplancton en eau douce. Néanmoins, malgré la présence évidente d'interactions nuisibles entre cette formulation à base de fomésafène et la physiologie du phytoplancton d'eau douce, les concentrations étudiées dépassent les concentrations environnementales attendues dans les écosystèmes aquatiques comme les rivières et les lacs. Ainsi, il serait plus probable de constater des résultats similaires chez les espèces phytoplanctoniques retrouvées dans les petits écosystèmes aquatiques comme les tranchées à proximité de zones à fortes activités agricoles où se recueillent les eaux de ruissellement souvent fortement contaminées de pesticides (Elias *et al.*, 2018). À notre connaissance, il n'existe pas de données accessibles quant aux concentrations environnementales en fomésafène dans les eaux de ruissellement qui se retrouvent dans ces tranchées agricoles. La toxicité du fomésafène pourrait être plus forte lorsque le phytoplancton est cultivé sous la lumière naturelle du soleil comparativement à une croissance sous lumière artificielle (Matringe *et al.*, 1989). Ceci suggère la nécessité de sonder davantage la physiologie du phytoplancton d'eau douce suite à une croissance dans du milieu contaminé au fomésafène et supplémenté de lumière UV.

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