UNIVERSITÉ DU QUÉBEC À MONTRÉAL

EFFETS COMBINÉS DE LA TEMPÉRATURE ET DES HERBICIDES SUR LES PROCESSUS PHYSIOLOGIQUES DU PHYTOPLANCTON

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A Who's Who of pesticides is therefore of concern to us all. If we are going to live so intimately with these chemicals - eating and drinking them, taking them into the very marrow of our bones - we had better know something about their nature and their power.

Rachel Carson, *Silent Spring*

AVANT-PROPOS

J'ai découvert un intérêt pour l'écotoxicologie dès les premières années de mon baccalauréat, en particulier l'écotoxicologie aquatique. Ma décision de poursuivre dans ce domaine m'aura amené à l'UQÀM, puis dans le laboratoire de Philippe Juneau. Les premières expériences que j'y ai effectuées en tant que stagiaire à l'été 2007 auront permis de démontrer que des extraits d'eau de l'une des rivières les plus contaminées par les herbicides au Québec, la rivière Yamaska, produisaient une inhibition de la photosynthèse chez plusieurs espèces d'algues. De plus, les trois espèces d'algues étudiées n'avaient pas la même sensibilité aux extraits de pesticides. Ma curiosité avait été piquée. Je voulais comprendre comment les herbicides affectaient les algues et les cyanobactéries, pourquoi il y avait des différences entre les espèces, si les changements climatiques pourraient influencer la toxicité des herbicides et si les floraisons de cyanobactéries pouvaient être en lien avec la présence de pesticides dans les cours d'eau.

J'ai donc débuté une maîtrise, à l'hiver 2008, qui, sous les conseils de M. Juneau, visait à étudier l'effet combiné de la température et des herbicides. Les effets individuels de l'un ou l'autre facteurs étant bien connus, c'est l'interaction entre différents facteurs physiques et chimiques qui attirait à présent l'attention des scientifiques.

Cette maîtrise s'est éventuellement transformée en projet de doctorat, qui a officiellement débuté à l'été 2009. À l'automne 2009, je partais pour l'Autriche, dans le laboratoire de Martin Kainz, afin d'aller y étudier le lien entre les herbicides, la photosynthèse et la composition lipidique des algues. J'y ai appris à doser les acides gras tout en apportant mon expertise sur le dosage des microcystines aux étudiants de M. Kainz. Les expériences effectuées en Autriche n'ont malheureusement pas apportées toutes les réponses escomptées sur le lien entre photosynthèse et acides

gras, et, à mon retour au Québec, j'ai donc à la fois développé les protocoles pour mesurer le stress oxydatif et les enzymes de détoxification dans les algues, mais aussi, grâce à l'aide d'un collègue, les mesures de la composition pigmentaire des algues au HPLC. Des expériences ont été refaites, et des échantillons ont été envoyés au laboratoire de Michael Arts pour des analyses lipidiques supplémentaires qu'il n'était pas possible de faire à l'UQÀM.

Durant l'élaboration de mon projet de thèse, et après avoir complété la série d'expériences du chapitre II, je me suis interrogée sur l'importance que les algues, mais surtout les cyanobactéries, pouvaient avoir sur les herbicides présents dans les cours d'eaux. Après avoir étudié l'effet des herbicides sur le phytoplancton, je me suis donc demandée si le phytoplancton pouvait avoir un effet sur les herbicides, notamment en les dégradant. J'ai alors fouillé la littérature scientifique pour apprendre que plusieurs espèces d'algues et de cyanobactéries avaient des habiletés intéressantes pour capturer les contaminants et les dégrader. Le chapitre V prenait forme dans mon esprit.

Aujourd'hui, le domaine de la bioremédiation est celui qui m'intéresse le plus. Les scientifiques ont mis au point des dizaines de bioindicateurs de la santé des écosystèmes; sa détérioration par l'activité anthropique n'est plus à prouver. Je considère qu'il est maintenant temps de se consacrer aux solutions alternatives aux contaminants chimiques et à la remédiation des écosystèmes, afin de rendre notre environnement plus sain pour les organismes qui y vivent, mais aussi pour nous tous qui en dépendont étroitement.

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ABS RC^{-1}	Surface d'absorption effective par centre réactionnel
ANOVA	Analyse de variance
ANCOVA	Analyse de covariance
APC	Allophycocyanine
APX	Ascorbate peroxydase
ATP	Adenosine triphosphate
ATPase	Complexe de synthèse d'ATP
ATZ	Atrazine
BBM	Milieu de culture Bold Basal
Car	Caroténoide
CAT	Catalase
CEF	Transport cyclique des électrons photosynthétiques
Chl *x*	Chlorophylle (ou *x* peut correspondre à: *a*, *b*, *c* ou *d*)
CO_2	Dioxide de carbone
Cyt b_6f	Cytochrome b_6f
DCMU	Diuron
$DI_O\ RC^{-1}$	Dissipation d'énergie par centre réactionnel
DMSO	Diméthyl sulfoxyde
DW	Poids sec
EC_{50}	Concentration inhibant 50 % de l'activité d'une variable donnée
ELISA	Dosage d'immunoabsorption par enzyme liée
$ET_O\ RC^{-1}$	Transport d'électron par centre réactionnel
$ET_O\ TR_O^{-1}$	Efficacité de transfert d'électron au-delà de Q_A par le PSII
ETR	Taux de transport d'électron
FA	Acide gras
Fd	Fluridone

F'_M	Niveau maximal de fluorescence en présence de lumière actinique
F_M	Niveau maximal de fluorescence
F'_O	Niveau minimal de fluorescence en présence de lumière infrarouge
F_O	Niveau minimal de fluorescence à l'obscurité
F_S	Niveau minimal de fluorescence en présence de lumière actinique
FSC	Lumière diffusée en ligne droite avec le faisceau lumineux, indicateur de taille cellulaire
F_V/F_M	Rendement quantique maximal des PSII
GAT	Glyphosate N-acétyltransférase
GNAC	Enzymes reliées aux GCN5 N-acétyltransférases
GC/MS	Chromatographie gazeuse couplée à un spectromètre de masse
H_2DCFDA	2',7'-dichlorofluorescein diacétate, marqueur du stress oxydatif
HPLC	Chromatographie liquide à haute pression
HSM	Milieu de culture High Salt
LHC	Antenne collectrice de lumière
MDA	Malondialdéhyde
MeOH	Méthanol
MUFA	Acide gras mono-insaturé
n-3	Acide gras oméga-3
n-6	Acide gras oméga-6
$NADP^+$	Nicotinamide adénine dinucléotide phosphate
NADPH	Forme réduite de $NAPD^+$
NBT	Nitroblue tretrazolium
Nf	Norflurazon
NPQ	Dissipation non-photochimique d'énergie
PUFA	Acide gras poly-insaturés
R.u.	Unités relatives
O_2	Dioxygène
PAM	Fluorescence de type « Pulse Amplitude modulated »

PAR	Radiation photosynthétiquement active (entre 400 et 700 nm)
PBS	Phycobilisome
PC	Phycocyanine
PE	Phycoerytrine
PEA	Appareil d'analyse d'efficacité photochimique des plantes
PQ	Plastoquinone
PQH_2	Hydroplastoquinone
PS (I ou II)	Photosystème, I ou II
Q_A & Q_B	Quinone a et quinone b
qN_{REL}	Dissipation relative d'énergie de façon non-photochimique
qP_{REL}	Dissipation relative d'énergie par la photochimie
RC, I ou II	Centre réactionnel, associé au photosystème I ou II aussi appelé respectivement P700 et P680 ou P700* et P680* dans l'état oxydé
ROS	Espèce réactive oxygénée
RUBISCO	Ribulose-1,5-diphosphate carboxylase/oxygénase
SAFA	Acide gras saturé
SOD	Superoxyde dismutase
SSC	Lumière diffusée à 90° du faisceau lumineux, indicateur de complexité cellulaire
TBARS	Substances réactives avec les acides thiobarbituriques
$TR_O\ ABS^{-1}$	Probabilité de transfert d'un électron (correspond à F_V/F_M)
$TR_O\ RC^{-1}$	Taux maximal de transfert d'énergie par centre réactionnel
UQF_{REL}	Dissipation relative d'énergie sous forme de fluorescence
U	Unité (d'enzyme)
UI	Index du niveau d'insaturation des acides gras

LISTE DES SYMBOLES

Φ'_M	Rendement quantique opérationnel des PSII
Φ_M	Rendement quantique maximal des PSII
φ_{Eo}	Rendement du transport d'électrons
μ_{cell}	Taux de croissance exprimé par nombre de cellules par mL
$\mu_{\mu m3}$	Taux de croissance exprimé par biolovume de cellules par mL
μm^3	Volume cellulaire, biovolume

RÉSUMÉ

On retrouve dans les cours d'eau une panoplie de contaminants pouvant avoir des effets néfastes sur les organismes qui y vivent. Parmi ceux-ci, les herbicides sont particulièrement présents en raison de leur grande utilisation à l'échelle de la planète et de leur grande mobilité dans les sols. Or, les herbicides sont appliqués à différents moments de l'année et peuvent être lessivés vers des eaux qui ont différentes températures. Les herbicides n'étant pas spécifiques aux plantes qu'ils visent à inhiber, ils peuvent affecter les organismes autotrophes aquatiques, notamment les espèces de phytoplancton (algues et cyanobactéries). Cette thèse s'intéresse donc à l'effet combiné de la température et des herbicides sur les algues et les cyanobactéries.

Les résultats ont été obtenus à partir d'expériences effectuées en laboratoire en utilisant plusieurs espèces d'algues et de cyanobactéries. Dans un premier temps, une algue verte (*Scenedesmus obliquus*), une diatomée (*Navicula pelliculosa*) et deux souches de cyanobactéries (*Microcystis aeruginosa*) ont été acclimatées à trois températures (10, 15 et 25°C) et soumises à cinq concentrations d'atrazine (0, 0,01, 0,05, 0,1 et 0,15 µM). Les résultats obtenus ont permis de démontrer que la toxicité de l'atrazine sur la photosynthèse était plus élevée à faible température qu'à la température optimale de croissance pour l'algue verte et les cyanobactéries. Par contre, l'algue diatomée avait la même sensibilité à l'atrazine à toutes les températures étudiées. Des modifications physiologiques liées à l'acclimatation à la faible température chez les différentes espèces, comme des changements au niveau des pigments, ont augmenté l'inhibition de la photosynthèse par l'atrazine. Chez la diatomée toutefois, des caractéristiques propres à ce groupe d'algue leur permettant de faire face au stress lumineux lui ont conféré une plus grande tolérance à l'atrazine et ce, à toutes les températures étudiées.

Les caractéristiques physiologiques modifiées par la température et les herbicides ont ensuite été étudiées plus en profondeur chez l'algue verte *Chlamydomonas reinhardtii*. Celle-ci a donc été acclimatée à 8, 15 et 25°C et soumise à deux concentrations de norflurazon (1,25 et 2,5 µM) et une concentration de fluridone (1,25 µM). Les résultats obtenus ont montré que le norflurazon avait beaucoup plus d'effet à 15°C qu'à 25°C, inhibant notamment complètement la photosynthèse et la synthèse de la β-carotène à 15°C, mais que les deux herbicides n'avaient aucun effet à 8°C. Une augmentation dans le contenu en pigments photoprotecteurs, dans les enzymes de détoxification des ROS et un profil d'acide gras particulier peuvent expliquer cette absence d'effet à 8°C.

Enfin, la capacité de *S. obliquus* et deux souches de *M. aeruginosa* à s'acclimater à la présence d'atrazine et de glyphosate a été étudiée en exposant ces microorganismes à

une concentration de 0,1 µM d'atrazine et 180 µM de glyphosate pendant 30 jours. Bien que le taux de croissance ait été augmenté jusqu'au niveau des cellules non exposées aux herbicides après 15 à 30 jours, cette acclimatation n'a pas permis d'augmenter l'EC_{50} des espèces acclimatées par rapport aux cultures non-acclimatées. Une diminution d'atrazine dans le milieu après 6 jours d'exposition à 0,01 µM n'a pas été observée, mais une diminution notable du glyphosate après 6 jours d'exposition à 1 µM a été mesurée en présence des cyanobactéries.

Ces expériences démontrent que la toxicité des herbicides dépend bien de la température à laquelle les microorganismes autotrophes sont acclimatés, mais aussi que cette interaction dépend du mode d'action des herbicides et des caractéristiques physiologiques des organismes étudiés.

Mots clés : Acclimatation, photosynthèse, pigments, stress oxydatif, acides gras, dégradation

Keywords : Acclimation, photosynthesis, pigments, oxidative stress, fatty acids, degradation

CHAPITRE I : REVUE DE LITTÉRATURE

1.1. Mise en contexte

La contamination des cours d'eau est l'une des grandes préoccupations environnementales de ce siècle. En effet, la qualité des eaux douces et marines s'est grandement dégradée au cours du siècle dernier en raison de l'agriculture, des rejets industriels et municipaux, et du manque de règlementation face à l'utilisation de produits toxiques par les populations riveraines (U.N. Water, 2013). Dans les pays en développement, plus de 80% des eaux usées sont déversées sans traitement dans les lacs, rivières et aires côtières (WWAP, 2013). À Montréal même, le traitement des eaux usées de la ville est très rudimentaire (Pilote, 2011). De plus, lors de fortes pluies, l'usine de traitement des eaux usées ne fournit plus et les eaux sont évacuées directement dans le fleuve Saint-Laurent. En plus de contribuer à la contamination en nitrates et en bactéries pathogènes, les eaux usées révèlent maintenant des quantités importantes de dérivés de médicaments, tels les anti-inflammatoires, les anti-bactériens et les anti-dépresseurs (Heberer, 2002 ; Lajeunesse, Gagnon et Sauvé, 2008).

L'activité industrielle, quant à elle, favorise le déversement de métaux toxiques résultant de l'activité minière ou de l'inondation des terres (Berryman, 2007 ; Gendron, Brunelle et Roy, 2007), des hydrocarbures aromatiques polycycliques (HAPs) provenant des fonderies d'aluminium et des industries chimiques, biphényles polychlorés (BPCs) provenant d'une gestion inadéquate des déchets et des huiles recyclées (Laliberté et Mercier, 2006 ; McDonald et Tourangeau, 1986), en plus des contaminants émergeants, tels les *polybrominated diphenyl ethers* (PBDE) servant de retardateurs de flammes dans la fabrication des plastiques, des meubles et des tissus (Environnement Canada, 2013).

C'est toutefois l'agriculture qui serait responsable de la plus grande source de contamination des cours d'eau (US EPA, 2009). En effet, les champs agricoles se trouvent souvent très près des cours d'eau. La contamination peut donc s'effectuer par épandage d'engrais ou de pesticides directement au-dessus des ruisseaux, suite au lavage de la machinerie agricole, ou encore par lessivage des champs après l'irrigation ou une pluie abondante (Carter, 2000 ; Zhao *et al.*, 2013). L'agriculture cause une pollution qui est diffuse et difficile à contrôler efficacement. De 2008 à 2010, des relevés d'échantillonnage d'eau de quatre rivières québécoises situées en zones agricoles ont démontré que, en moyenne, le pesticide s-métolachlore a été détecté dans 99% des prélèvements effectués, l'atrazine dans 97%, le glyphosate dans 86%, l'imazéthapyr dans 79% et le bentazone dans 75% des échantillons (Giroux et Pelletier, 2012).

Cette contamination des cours d'eau par les pesticides révèle de nombreux dangers pour les espèces qui y vivent ou font utilisation des plans d'eau, incluant l'humain. Ils vont, par exemple, engendrer une diminution dans la diversité des invertébrés (Barton et Metcalfe-Smith, 1992), des malformations chez les chironomides (Bird, 1994) et des troubles dans les rétinoïdes hépatiques des ouaouarons (Boily *et al.*, 2005). Chez les producteurs primaires, la présence de pesticides a aussi été corrélée avec des changements dans les communautés de microorganismes (Bérard *et al.*, 2003 ; Gustavson, Møhlenberg et Schlüter, 2003 ; Peterson *et al.*, 1994). Cela va affecter les invertébrés et poissons qui se nourrissent de ces producteurs primaires. Par exemple, le cladocère *Daphnia* se trouve très affecté par une diète riche en cyanobactéries et pauvre en algues, soit en raison de la présence de toxines, soit à cause de la pauvre valeur nutritive des cyanobactéries (Demott et Müller-Navarra, 1997 ; Ferrão Filho *et al.*, 2008). Chez les poissons, la présence de cyanotoxines peut causer une mortalité massive chez les poissons sauvages comme ceux d'élevage (Rodger *et al.*, 1994). C'est donc toute la chaine

alimentaire aquatique qui peut être affectée, directement ou indirectement, par la présence de pesticides dans les cours d'eau.

Évidemment, les pesticides ne sont pas le seul facteur pouvant entrainer des changements dans les communautés de phytoplancton. La plupart des facteurs physiques (lumière, température), chimiques (nutriments, pH) et biotiques (présence d'autres espèces) peuvent y contribuer (Behrenfeld *et al.*, 2004 ; Elser, 1999 ; Kardinaal et Visser, 2005 ; Mooij *et al.*, 2005 ; Shapiro, 1997 ; Visser *et al.*, 2005). Parmi ceux-ci, la température est particulièrement intéressante à cause des changements climatiques que cette planète connait (Mooij *et al.*, 2005). En effet, la température influence la diversité des espèces de phytoplancton (Coles et Jones, 2000 ; Litchman *et al.*, 2010). Si les cyanobactéries ont un taux de croissance beaucoup plus faible que les diatomées à basse température (10°C), elles peuvent rattraper et dominer les autres espèces à température plus élevée (25°C) (Mooij *et al.*, 2005 ; van der Grinten *et al.*, 2005). De plus, en utilisant un modèle développé pour un lac des Pays-Bas, il a été démontré que des étés plus chauds, reliés à une stabilisation de la colonne d'eau et une faible turbulence, déplaçait l'équilibre compétitif entre les espèces en faveur des cyanobactéries comme *Microcystis* (Jöhnk *et al.*, 2008).

Il est également intéressant de se demander si la contamination des cours d'eau par les pesticides pourra augmenter avec les changements climatiques. Il s'avère qu'une équipe de chercheurs britanniques a étudié cette question pour le Royaume-Uni (Bloomfield *et al.*, 2006). D'une part, un climat plus chaud et plus humide favoriserait la croissance des herbes invasives, des parasites et des maladies. Une plus grande utilisation d'herbicides, insecticides et fongicides est donc à prévoir. Une température plus chaude entraine également une plus forte volatilité des pesticides et conséquemment, un transport plus grand par voie aérienne depuis le point d'application. Par contre, les températures plus chaudes favoriseraient également la dégradation des pesticides. Toutefois, si les précipitations sont très abondantes, la décharge de pesticides vers les eaux de surface ou souterraines sera

plus importante. De plus, si les sols deviennent très secs à cause d'une sécheresse, les fissures créées dans le sol peuvent favoriser la perte de pesticide par drainage lors d'une pluie. Bref, l'augmentation de la présence des pesticides dans les cours d'eau est prévisible, mais dépendra de la compétition entre les différents processus sensibles au climat, par exemple la dégradation versus le drainage et la volatilisation.

La température et les herbicides sont donc deux facteurs qui vont influencer les algues et les cyanobactéries. Mais y a-t-il une interaction entre ces deux facteurs ? Est-ce que la température peut influencer la toxicité des herbicides ? Est-ce que cette sensibilité sera différente entre différentes espèces d'algues et de cyanobactéries ? Cette thèse s'intéressera en particulier à la capacité d'acclimatation de certaines espèces de phytoplancton à la température et aux herbicides, ainsi que sur les effets mesurables de l'un ou l'autre de ces deux facteurs sur la croissance et la photosynthèse (Chapitre II et V) ou sur des paramètres physiologiques plus précis tels que les enzymes antioxydantes, les pigments photoprotecteurs et les acides gras (Chapitre III et IV).

1.2. Les micro-algues et les cyanobactéries

Le phytoplancton représente l'ensemble des organismes pouvant effectuer la photosynthèse se retrouvant en suspension dans la colonne d'eau. Il est donc composé, principalement, des algues microscopiques et des cyanobactéries (Reynolds, 1984).

Les algues sont des organismes eucaryotes composés d'une ou de plusieurs cellules. Il existe plusieurs divisions et classes d'algues, identifiées généralement par la couleur des pigments photosynthétiques qu'elles possèdent : les Rodophytes (algues rouges), les Chrysophycées (algues dorées), les Phaeophycées (algues brunes), les Chlorophytes (algues vertes), etc. En effet, les types et la combinaison des pigments photosynthétiques ont un grand rôle à jouer dans la classification des

espèces (van den Hoek, Mann et Jahns, 1995). Dans cette thèse, des représentants de deux classes d'algues ont été étudiées. D'une part, les algues vertes, auraient probablement donné naissance aux plantes supérieures, considérant leur similarité au niveau des pigments (chlorophylle *a* et *b*) et de leur formes de réserves (amidon) (Bold et Wynne, 1985). D'autre part, les diatomées sont reconnues pour leur paroi cellulaire, aussi appelée frustule, composée de silice. Elles possèdent des fucoxanthines et des diatoxanthines, qui sont des pigments brun-verts leur donnant leur couleur caractéristique. Elles constituent une grande proportion du phytoplancton en milieu marin et pour cette raison, jouent un grand rôle dans la production primaire (van den Hoek, Mann et Jahns, 1995). En raison de leur grande distribution dans les cours d'eau et de leur facilité à être cultivées et utilisées en laboratoire, les algues vertes et diatomées sont les plus étudiées en recherche.

Les cyanobactéries quant à elles, sont des organismes procaryotes. Elles ne disposent donc pas de noyau ou d'organite cellulaire comme les algues, et n'ont qu'une seule membrane. Elles peuvent également être unicellulaires ou coloniales (van den Hoek, Mann et Jahns, 1995). On les appelle souvent algue bleu-vert à cause des pigments bleus, les phycocyanines, qui assistent la chlorophylle dans le captage de l'énergie lumineuse (Grossman *et al.*, 1993). Les cyanobactéries sont également reconnues parce qu'elles vont former des floraisons à la surface des lacs (Codd, Morrison et Metcalf, 2005). Les causes de ces floraisons sont encore peu connues, mais semblent souvent reliées à l'activité agricole, en particulier l'apport de nutriment dans les cours d'eau. Ainsi, un bas ratio azote : phosphore avantagerait les cyanobactéries par rapport aux algues (Smith, 1983). Les floraisons de cyanobactéries peuvent être très nuisibles car elles peuvent provoquer l'eutrophication du plan d'eau lors de la sénescence massive de cette biomasse, en plus de nuire aux activités touristiques et récréatives (Kardinaal et Visser, 2005). De plus, la plupart des cyanobactéries peuvent synthétiser des composés très toxiques pour les organismes aquatiques et les êtres humains, telles que les microcystines et les anatoxines

(Carmichael *et al.*, 2001 ; Chorus et Bartram, 1999 ; Codd, Morrison et Metcalf, 2005). C'est la présence d'un gène spécifique, la microcystine synthétase (*mcy*) qui est responsable de la production de microcystine, mais toutes les cyanobactéries ne le possèdent pas (Kardinaal et Visser, 2005). Certaines espèces de cyanobactéries sont donc non toxiques et même comestibles, telles *Spirulina platensis* (Ciferri, 1983). Chez d'autres espèces, telles *Microcystis aeruginosa*, on retrouve des souches toxiques et non-toxiques, telles CPCC299 (toxique) et CPCC632 (non-toxique), deux souches des collections du *Canadian Phycological Culture Collection* (CPCC) (Deblois et Juneau, 2010).

1.3. La photosynthèse

Malgré leurs différences, les algues et les cyanobactéries ont plusieurs points en commun, dont le plus important est sans doute celui de pouvoir produire de l'énergie chimique via la photosynthèse. La photosynthèse est divisée en deux étapes, dont la première se déroule dans des membranes appelées thylacoïdes (Figure 1.1). Ces membranes thylacoïdales se trouvent dans les chloroplastes chez les algues et associées à la membrane de la cellule chez les cyanobactéries. Dans ces thylacoïdes, on retrouve une quantité importante de chlorophylle, mais aussi de pigments accessoires comme les caroténoïdes ou les phycobilines, assemblés sous forme d'antennes collectrices. Ces antennes vont permettre de collecter la lumière via l'excitation des pigments et de canaliser l'énergie vers les centres réactionnels des photosystèmes II (PSII) et des photosystèmes I (PSI). Le PSII va se servir de cette énergie pour oxider l'eau, c'est-à-dire enlever quatre électrons à deux molécules d'eau, ce qui va générer une molécule d'oxygène et quatre protons. Cette oxydation a lieu grâce à un complexe enzymatique associé au PSII, le complexe d'oxydation de l'eau. Les protons seront relargués dans le lumen des thylacoïdes et vont servir à créer un gradient électrochimique avec le stroma. Les électrons quant à eux vont être transférés à une phéophytine puis à des quinones (Q_A puis Q_B) grâce à des réductions

successives de ces transporteurs d'électrons. Une série d'autres réductions, impliquant notamment le complexe du cytochrome b_6f vont amener les électrons jusqu'au PSI. Celui-ci transfèrera les électrons à une séries de protéines contenant du fer et du souffre (les FeS_x), puis à une ferredoxine qui permettra la réduction du $NADP^+$ en NADPH grâce à la ferredoxin-NADP reductase. Le gradient de protons créé à la fois par l'oxydation de l'eau et par les plastoquinones servira à la formation d'ATP via les ATP synthases (Taiz et Zeiger, 2006).

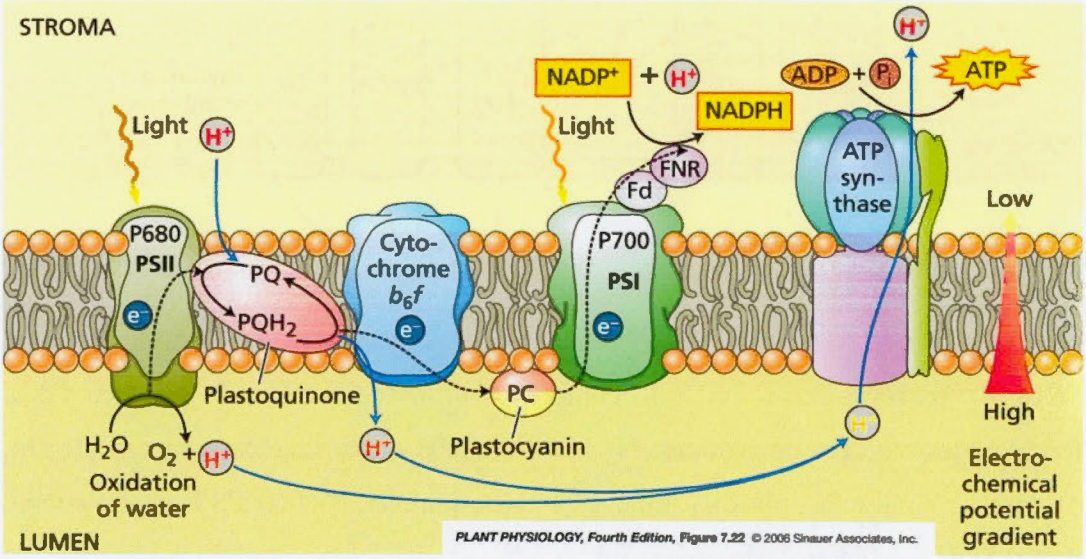

Figure 1.1. Structure de l'appareil photosynthétique et transport des électrons chez les plantes et les algues (tiré de Taiz et Zeiger (2006))

Ces étapes sont très similaires chez les algues et les cyanobactéries. Toutefois, l'absence de compartiments chez les cyanobactéries, c'est-à-dire de chloroplastes et de mitochondries, fait que la photosynthèse et la respiration sont interreliées. Ces deux processus vont parfois partager des électrons (Fig 1.2; Vermaas (2001)). De plus, les phycobilines sont organisées sous forme de phycobilisome, à la périphérie de

la membrane thylacoïdale plutôt qu'intégrée à celle-ci. Cela confère aux phycobilisomes une très grande mobilité, ce qui permet de diriger l'énergie préférentiellement au PSII ou au PSI, un phénomène appelé transition d'état.

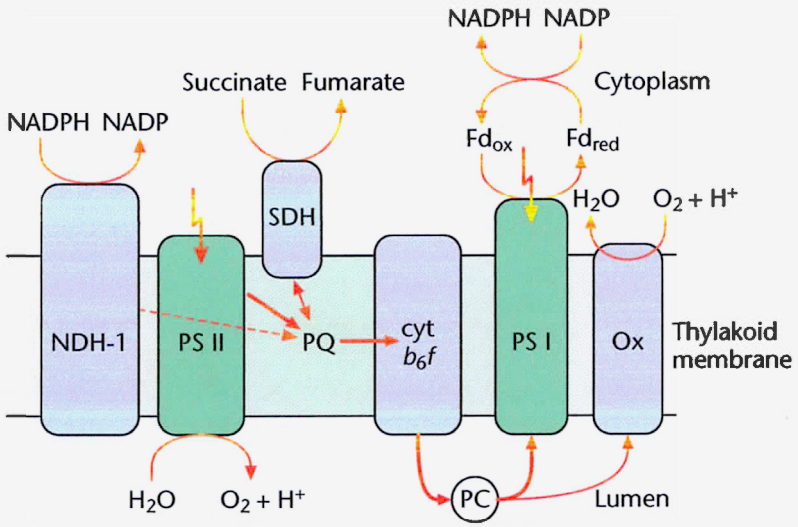

Figure 1.2. Transport des électrons chez les cyanobactéries (tiré de Vermass (2001)). Abbréviations : cyt b_6f, complexe du cytochrome b_6f; Fd_{ox} et Fd_{red}, ferredoxines oxydées ou réduites; NDH-1, NADPH déshydrogénase de type 1; Ox, oxidase terminale; PC, plastocyanine; PQ, plastoquinone; PS II et PS I, photosystème II et I, SDH, succinate déshydrogénase.

La deuxième phase de la photosynthèse, le cycle de Calvin, est aussi appelé réactions indépendantes de la lumière, ou fixation du carbone. Ces étapes se déroulent dans le stroma des chloroplastes chez les algues et dans des carboxysomes chez les cyanobactéries. Les sources d'énergie chimique (ATP et NADPH) produites par la chaine de transport des électrons seront utilisées afin d'incorporer le CO_2 en un triose-phosphate, le glyceraldehyde-3-phosphate. Les premières étapes consistent en la carboxylation du ribulose-1,5-bisphosphate par le dioxyde de carbone et sont

catalysées par l'enzyme RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase). Le 3-phosphoglycerate produit sera ensuite phosphorylé par l'ATP grâce à l'enzyme phosphoglycérate kinase, étape qui sera suivie par une réduction par le NADPH et l'enzyme glycéraldéhyde-3-phosphate déshydrogénase, résultant en la formation du glycéralgéhyde-3-phosphate. Les étapes subséquentes du cycle de Calvin serviront à la regénération du ribulose-1,5-bisphosphate. Deux molécules de glycéraldéhyde-3-phosphate pourront ensuite former un sucre à six carbones, tel le glucose, mais également servir à la production d'amidon, de sucrose ou de la cellulose, selon les besoins de la cellule (Taiz et Zeiger, 2006).

1.4. Les mécanismes de photoprotection

La lumière est donc essentielle au processus photosynthétique. Toutefois, un excès de lumière peut également être néfaste pour la cellule. La chaine de transport des électrons est une étape limitante; si elle n'arrive pas à gérer toute l'énergie lumineuse captée par les pigments photosynthétiques, des dommages seront occasionnés. Les molécules de chlorophylles excitées pourraient atteindre des niveaux d'énergie supérieurs et former de la chlorophylle triplet (^{3}Chl*), qui, réagissant avec l'oxygène moléculaire (O_2) provoquera la formation du très réactif oxygène singulet ($^1O_2$*) (Ledford et Niyogi, 2005). Ce dernier peut provoquer la peroxidation des lipides ainsi que la destruction des protéines, notamment celles du PSII (Girotti et Kriska, 2004 ; Mishra et Ghanotakis, 1994).

Afin d'éviter d'endommager son appareil photosynthétique, la cellule a toutefois développé de nombreux mécanismes de photoprotection. D'une part, l'énergie captée par les pigments pourra être dissipée sous forme de fluorescence, principalement la fluorescence associée à la chlorophylle *a* (Krause et Weis, 1991). En effet, lorsque la chlorophylle *a* excitée (^{1}Chl*)*a* par l'énergie d'un photon retombe à son niveau d'énergie fondamental, il y aura émission de fluorescence. D'autre part,

l'énergie excessive pourra également être dissipée sous forme de chaleur par plusieurs processus. Ce sont des processus qu'on regroupe souvent sous le nom de quenching non-photochimique (le quenching photochimique étant l'énergie envoyée dans la chaine de transport des électrons, la photosynthèse).

En premier lieu, certains caroténoïdes, les xanthophylles, vont participer à la dissipation de l'énergie de la chlorophylle. La conversion de la violaxanthine en anthéraxanthine puis en zéaxanthine par deux dé-époxydations successives permettrait en effet la dissipation de l'énergie sous forme de chaleur, soit parce que la zéaxanthine, ayant un niveau d'énergie inférieur à la chlorophylle, permettrait d'absorber l'énergie de cette dernière (Owens, Shreve et Albrecht, 1992), soit parce que cette conversion entrainerait des changements conformationnels dans la membrane permettant davantage de pertes thermiques (Horton *et al.*, 1991). La réaction inverse, soit la conversion de la zéaxanthine en violaxanthine, s'effectue par une époxydase (Yamamoto, Nakayama et Chichester, 1962). Ce processus est appelé le cycle des xanthophylles (voir Fig 1.3).

Chez les algues diatomées, ce processus sera principalement effectué par des pigments similaires, les diadinoxanthines. Dans ce cas, la diadinoxanthine sera convertie en diatoxanthine. Les diatomées peuvent donc effectuer le cycle de la diadinoxanthine et de la violaxanthine (Lohr et Wilhelm, 1999). Chez les cyanobactéries par contre, et bien qu'elles possèdent de la zéaxanthine, le cycle des xanthophylles ne semble pas exister (Hirschberg et Chamovitz, 2004). Les cellules dépendent donc d'autres mécanismes pour faire face au stress lumineux.

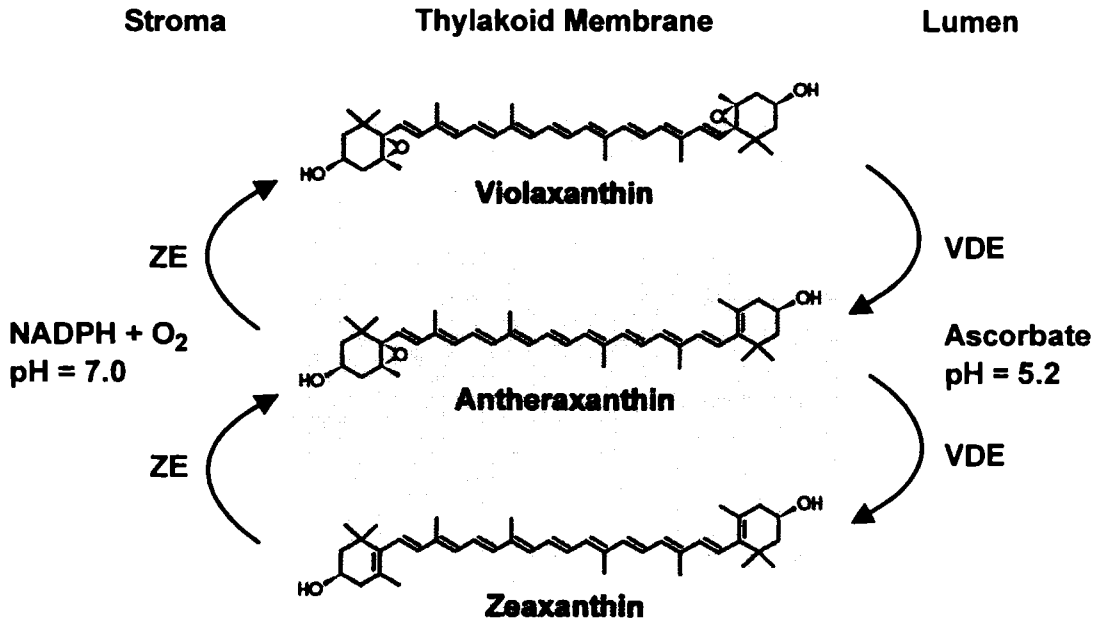

Figure 1.3. Cycle des xanthophylles. VDE : violanxanthine dé-époxydase, ZE : zéaxanthine époxydase (tiré de Hieber *et al.* (2004)).

Parmi ceux-ci, le déplacement des antennes collectrices de lumière du PSII vers le PSI, un phénomène appelé transition d'état (*state transition*), est particulièrement efficace chez les cyanobactéries (Campbell et Öquist, 1996). En effet, leurs antennes de phycobilisomes sont très mobiles et peuvent donc se déplacer rapidement du PSII au PSI. De cette façon, l'énergie transmise au centre réactionnel du PSII est diminuée et celle du PSI est augmentée, ce qui permet de drainer davantage d'électrons dans la chaine de transport et d'éviter l'accumulation d'énergie au PSII. La transition d'état existe aussi chez les algues, où les antennes collectrices se déplacent du PSII vers le PSI (Haldrup *et al.*, 2001). Par contre, comme les antennes sont imbriquées dans la membrane thylacoïdale, ce processus est moins efficace chez les algues.

Enfin, un autre mécanisme est mis en place lorsqu'une trop grande énergie lumineuse atteint le photosystème II : il s'agit de la photoinhibition. Lorsque le PSII est inhibé par une trop forte lumière, la protéine D1 du centre réactionnel sera dégradée. Comme cette protéine démontre un taux de renouvellement rapide, sa destruction permet d'inactiver le PSII sans endommager tout le centre réactionnel. Sa synthèse permettra de réactiver le PSII (Aro, Virgin et Andersson, 1993 ; Takahashi et Murata, 2008). Il s'agirait toutefois de la dernière ligne de défense du PSII contre les excès d'énergie (Krause et Weis, 1991).

1.5. Le stress oxydatif et les défenses antioxydantes

Malgré les différents mécanismes de protection contre le stress lumineux lié à la photosynthèse expliqués ci-haut, une certaine quantité d'espèces réactives oxygénées (ROS) est constamment produite par la cellule. Il s'agit même d'un moyen de signalisation permettant de contrôler des processus tels que la mort cellulaire programmée, la réponse aux stress abiotiques et les défenses contre les pathogènes (Mittler, 2002). Il a déjà été mentionné que la formation d'oxygène singulet était favorisée par le stress lumineux dans les thylacoïdes (Ledford et Niyogi, 2005). D'autres ROS seront également le produit du métabolisme cellulaire, notamment le radical superoxyde ($O_2^{\bullet-}$), qui serait produit notamment lorsque que le PSI réduit l'oxygène moléculaire plutôt que le $NADP^+$ (Asada, 2000). S'il n'est pas contrôlé, le superoxyde peut être converti, par la réaction de Haber-Weiss, en radical hydroxyl ($HO^{\bullet}$) soit le ROS le plus réactif et dommageable qui soit (Kehrer, 2000). Le peroxyde d'hydrogène (H_2O_2), quant à lui, est produit par la dismutation du superoxyde (Asada, Kiso et Yoshikawa, 1974) et, beaucoup plus stable, peut se déplacer à travers les membranes, diffuser dans la cellule et possiblement servir à signaler le stress et recruter des défenses (Bechtold, Karpinski et Mullineaux, 2005). Évidemment, une trop grande quantité de ROS va causer des dommages aux protéines, lipides et à l'ADN. Ainsi les défenses oxydatives incluent la présence de

molécules antioxydantes (les caroténoïdes, l'ascorbate, le glutathion et les tocophérols) et les enzymes de détoxification (Mittler, 2002). Parmi ces enzymes, la superoxyde dismutase (SOD) permet de transformer le superoxyde en oxygène moléculaire et H_2O_2. Il existe plusieurs isozymes de la SOD, dont certaines à base de cuivre-zinc et d'autres à base de manganèse (Giannopolitis et Ries, 1977). La catalase (CAT), l'ascorbate peroxydase (APX) et la glutathion peroxydase (GPX), quant à elles, vont permettre de réduire l'H_2O_2 en O_2 et H_2O (Mittler, 2002). Alors que la catalase se trouve presque seulement dans des peroxisomes, l'ascorbate peroxydase est particulièrement importante dans les chloroplastes (Asada, 1992). La glutathion peroxydase permettrait de détoxifier non seulement le H_2O_2, mais aussi les hydroperoxydes des acides gras et des phospholipides, provenant de la peroxydation des lipides. Cette enzyme serait présente dans le cytosol et les plastides des cellules (Dixon *et al.*, 1998).

1.6. La composition lipidique du phytoplancton

Enfin, les lipides ont un très grand rôle à jouer chez les algues comme chez les cyanobactéries. En effet, non seulement les lipides permettent le passage (ou non) des substances à travers la membrane de la cellule, ils forment également la matrice dans laquelle sont imbriqués les complexes photosynthétiques et les transporteurs d'électrons (la membrane thylacoïdale). Bien que le contenu lipidique des algues et des cyanobactéries change selon l'espèce et selon certains facteurs environnementaux, les lipides peuvent représenter de 10 à 30% du poids sec du phytoplancton (Olofsson *et al.*, 2012 ; Sallal, Nimer et Radwan, 1990). Les lipides qui composent les membranes de la cellule, autant la membrane cellulaire que la membrane thylacoïdale, sont principalement des glycérolipides polaires. Ceux-ci sont formés de deux chaines d'acide gras de 16 ou 18 carbones attachées à un glycérol (voir Fig. 1.4). Les membranes des tissus photosynthétiques contiennent des glycéroglycolipides, où le groupement de tête est un sucre, et les autres membranes

cellulaires sont principalement composées de glycérophospholipides, où le groupement de tête contient un phosphate (Taiz et Zeiger, 2006). On peut également retrouver des triacylglycérols, servant de réserve de carbone sous forme d'huile surtout dans les graines des plantes, mais aussi chez certaines algues étudiées pour leur capacité à produire du biocarburant (Liu *et al.*, 2013).

Au niveau de la photosynthèse, les lipides ont un important rôle à jouer. Ils vont influencer la fluidité de la membrane, ainsi que la stabilité et la fonctionnalité des PSII et PSI (Hölzl et Dörmann, 2007 ; Williams, 1998). Ils vont permettre le renouvellement de la protéine D1 et la stabilisation du complexe d'oxydation de l'eau (Allen et Ort, 2001 ; Sippola *et al.*, 1998). Le niveau d'insaturation des acides gras est également essentiel à la mobilité des plastoquinones entre le PSII et le PSI (Horváth *et al.*, 1986). Le degré d'insaturation, c'est-à-dire le nombre de doubles liaisons sur un acide gras, peut-être de 0 (acide gras insaturés), 1 (monoinsaturés) ou 2 et plus (polyinsaturés). L'ajout de doubles liaisons est effectué par des enzymes désaturases, et ces insaturations rendent la membrane plus fluide (Brown, Slabas et Rafferty, 2009). Tel que discuté plus bas, la désaturation des acides gras sera plus importante à basse température. Certains auteurs ont également mesuré une augmentation de l'insaturation des acides gras avec l'augmentation de l'intensité lumineuse (Klyachko-Gurvich *et al.*, 1999), mais également des modifications dans l'expression de plusieurs gènes reliés à l'insaturation des acides gras lors d'un stress de salinité chez *Chlamydomonas* (An *et al.*, 2013).

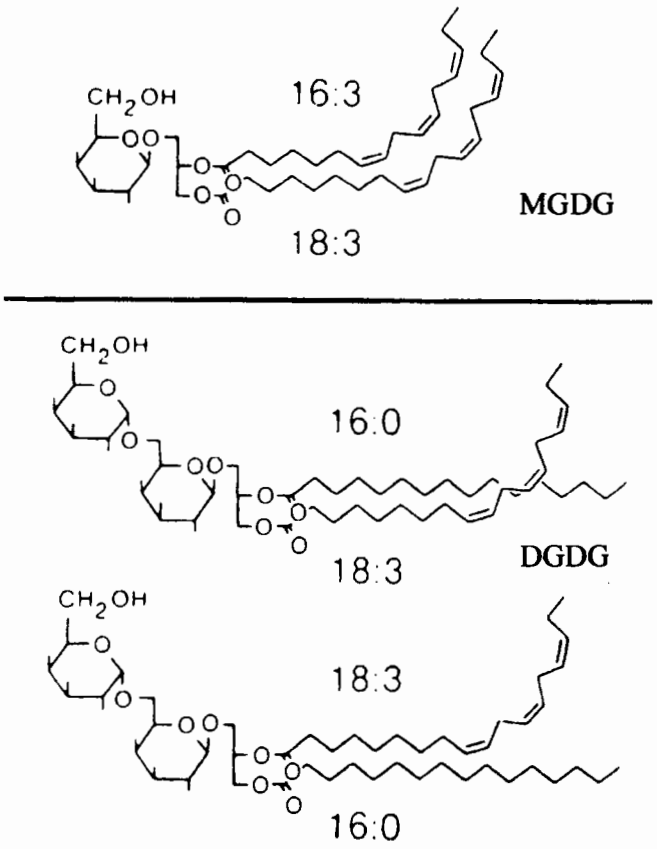

Figure 1.4. Les monogalactosyldiacylglycérols (MGDG) et digakactosyldiacylglycérols (DGDG) sont les principaux glycéroglycolipides retrouvés dans les membranes des tissus photosynthétiques. Les chaines d'acide gras sont indiquées par un chiffre représentant le nombre de carbones et le nombre de double liaisons. Ainsi, les acides gras 16 :0 sont des acides gras saturés à 16 carbones. Les acides gras 18 :3 sont des acides gras poly-insaturés à 18 carbones (tiré et adapté de Joyard *et al.* (1998))

Le stress oxydatif, en particulier celui créé par l'oxygène singulet ($^1O_2$*) peut entrainer la dégradation des acides gras, en particulier les acides gras insaturés, car le $^1O_2$* s'attaque directement à leur double liaison (Ledford et Niyogi, 2005). Des

peroxydes lipidiques seront formés (LOOH) qui eux-mêmes peuvent s'attaquer à d'autres acides gras, entrainant une cascade de peroxydation dans les lipides. Cette réaction en chaine ne pourra s'arrêter que grâce à la présence d'antioxydants, notamment les caroténoïdes, les tocophérols (vitamines E), l'ascorbate et le glutathion très présents dans les membranes cellulaires (Baroli *et al.*, 2004 ; Ledford, Chin et Niyogi, 2007 ; Maeda *et al.*, 2005).

1.7. Les herbicides

Parmi les nombreux contaminants pouvant se retrouver dans les cours d'eau, cette thèse s'intéresse principalement aux herbicides. En effet, les herbicides sont abondamment utilisés pour l'agriculture partout dans le monde, représentant 59 % de tous les épandages du Québec (Tellier, 2006). En outre, ils sont les plus souvent retrouvés dans les cours d'eau par les programmes de suivis de la qualité des eaux (Carter, 2000 ; Giroux et Pelletier, 2012) en raison des épandages multiples au courant de l'année et de leur temps de demi-vie relativement long (Shaner et Henry, 2007). De plus, la plupart des herbicides étant non-spécifiques, ils vont affecter les plantes aquatiques (incluant les algues) tout comme les plantes terrestres qu'ils visent à inhiber.

Il existe plusieurs classes d'herbicides, qui sont regroupés selon leur mode d'action (HRAC, 2010). Certains herbicides vont inhiber la synthèse de la chlorophylle (oxyfluorfen, oxadiazon), d'autres vont inhiber les auxines végétales (2,4-D, MCPA), la synthèse des acides aminés (glufosinate, chlorsulfuron) ou encore la synthèse des acides gras (alachlore, métolachlore) (Fedtke et Duke, 2004). Parmi les plus efficaces et les plus utilisés, les herbicides inhibiteurs de la photosynthèse, tels le DCMU et l'atrazine, vont agir principalement en bloquant la chaine de transport des électrons. Ils vont se lier au site de liaison de la plastoquinone Q_B sur la protéine D1 (Böger et Sandmann, 1998). Q_B ne pouvant être réduite par les électrons,

ceux-ci resteront bloqués au niveau de Q_A. Une accumulation d'énergie au niveau du PSII sera provoquée et, si elle n'est pas dégagée par les mécanismes de protection mentionnés plus haut, elle endommagera de façon importante les composants cellulaires, entrainant éventuellement la mort de la plante (Fedtke et Duke, 2004). Dans le cas de l'atrazine, des concentrations allant jusqu'à 10 µg/L ont été détectées dans certains cours d'eau au Québec, dépassant de plus de 5 fois le critère de protection de la vie aquatique (Giroux et Pelletier, 2012). Aux États-Unis, des concentrations allant jusqu'à 30 µg/L ont été retrouvées dans les régions de culture du maïs (Sullivan *et al.*, 2009). La fréquence de détection de l'atrazine dans quatre rivières situées en milieu agricole au Québec est en moyenne de 97% pour les années 2008 à 2010 (Giroux et Pelletier, 2012).

Certains herbicides vont affecter la synthèse des pigments photoprotecteurs, les caroténoïdes. Ainsi, le norflurazon et le fluridone sont tous deux des inhibiteurs de l'enzyme phytoène désaturase, une enzyme clé dans la voie de synthèse des caroténoïdes (Bartels et Watson, 1978). La phytoène désaturase catalyse en effet la conversion du phytoène en phytofluène, qui par la suite sera converti en ζ-carotène. Ces molécules sont des précurseurs des caroténoïdes plus importants dans la cellule tels que la β-carotène, la lutéine et les xanthophylles (Norris, Barrette et DellaPenna, 1995). Lorsque la phytoène désaturase est bloquée, le phytoène, un caroténoïde incolore s'accumule et l'appareil photosynthétique n'est plus protégé; la chlorophylle s'oxyde à cause du stress oxydatif et la plante devient blanchâtre (Dalla Vecchia *et al.*, 2001). Selon Breitenbach *et al.* (2001), le norflurazon compétitionnerait avec la plastoquinone, un cofacteur essentiel à l'action de la phytoène désaturase, sur son site de liaison. De plus, il a été noté par plusieurs auteurs que le norflurazon aurait également un effet sur les désaturases des acides gras (Abrous *et al.*, 1998 ; Abrous-Belbachir *et al.*, 2009 ; Di Baccio *et al.*, 2002). Ainsi, le norflurazon inhiberait la Δ^{15}-désaturase, une enzyme permettant la désaturation des acides gras 18 :2 en 18 :3 (Abrous *et al.*, 1998). Le mode d'action exact du fluridone n'a pas été étudié en

détail, mais, bien que ses effets soient très similaires au niveau des caroténoïdes (Arias *et al.*, 2005), son effet sur les lipides n'est pas connu. Le fluridone est utilisé principalement pour le contrôle des herbes aquatiques telles *Hydrilla* sp. ou le myriophylle en épis. Il est appliqué par pulvérisation directe à la surface de l'eau, injection sub-aquatique ou encore sous forme de granules contenant l'herbicide (HSDB, 2013a). Des concentrations allant jusqu'à 150 µg/L par cycle de vie des plantes visées peuvent être utilisées dans les cours d'eau aux États-Unis. Le norflurazon, quant à lui, est plutôt utilisé en pré ou post-émergence pour le contrôle des herbes invasives dans les champs de coton et d'arbres fruitiers (cerises, pêches, etc.) (HSDB, 2013b). Des concentrations allant jusqu'à 360 µg/L ont été retrouvées dans les eaux de drainage des champs d'agrumes en Floride (Wilson, Boman et Foos, 2007).

Le glyphosate, quant à lui, est devenu un herbicide extrêmement populaire et utilisé après que la compagnie Monsanto ait commercialisé un herbicide à base de glyphosate en 1974. Sa popularité tient au fait qu'il serait un herbicide peu toxique pour l'animal, peu mobile dans les sols et très biodégradable par l'activité microbienne (Duke, 1988). En outre, son utilisation a grandement augmenté après le développement de plantes transgéniques résistantes au glyphosate, dont le maïs, le soja, le coton et le canola (Duke et Powles, 2008). Au Québec, on assiste à une augmentation considérable des superficies de cultures génétiquement modifiées, celles-ci représentant 62,5% des superficies de maïs et 49 % des superficies de soya (Institut de la statistique du Québec 2011). Comme le glyphosate s'adsorbe facilement à la matière organique, il serait peu lessivé vers les cours d'eau (Duke et Powles, 2008). Toutefois, des concentrations maximales retrouvées dans les rivières étaient de 29 µg/L en 2010 au Québec (Giroux et Pelletier, 2012), et des concentrations allant jusqu'à 99 µg/L ont été retrouvées dans la rivière Mississipi aux États-Unis (Scribner *et al.*, 2007). Le glyphosate agit en inhibant l'enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) dans la voie de synthèse du

shikimate. Le glyphosate est un analogue du phosphoenylpyruvate, un substrat de l'EPSPS. Cette voie de synthèse mène à la production de trois acides aminés importants dans la cellule : la phénylalanine, la tyrosine et le tryptophane. Ces acides aminés sont impliqués dans la production de nombreuses protéines, de lignine et de phytoalexines (Duke et Powles, 2008). Par conséquent, l'action du glyphosate a aussi été reliée à la dégradation des chloroplastes et des membranes cellulaires, l'altération de la synthèse des acides nucléiques et l'inhibition de la photosynthèse et de la respiration (Ali et Fletcher, 1978 ; Bellaloui *et al.*, 2008 ; Foley *et al.*, 1983 ; Hernando *et al.*, 1989).

1.8. La température

La température est l'un des facteurs ayant le plus d'effet sur le métabolisme des cellules. La plupart des espèces d'algues et de cyanobactéries ont une température de croissance optimale se situant entre 20 et 25°C. Bien que le taux de croissance augmente généralement avec l'augmentation de la température, des variations selon les espèces sont observées. De plus, la plupart des organismes phytoplanctoniques verront leur taux de croissance diminuer à partir de 30-35°C (Reynolds, 1984). À basse température, les cellules vont prendre plus de temps pour se diviser (Vítová et Zachleder, 2005). Selon Vitovà *et al.* (2011), cette baisse du taux de croissance s'accompagne d'une augmentation dans la longueur du cycle cellulaire. Chez certaines classes d'algues telles que les chlorococcales ou les volvocales, dont font partie *Scenedesmus* et *Chlamydomonas*, les algues mères vont se diviser par fission multiple, résultant en des cellules filles (souvent 2, 4, 6 ou 8 cellules) connectées par un coenobium (Vítová et Zachleder, 2005). Parce que la longueur du cycle cellulaire est plus grande à basse température, ces algues vont souvent produire plus de cellules filles par division qu'à température plus élevée (Vítová *et al.*, 2011).

Afin d'évaluer le changement dans les processus physiologiques des organismes à la température, le coefficient Q_{10} a été élaboré. Celui-ci mesure le taux de changement d'une réaction chimique ou biologique lorsqu'on augmente la température de 10°C. Raven et Geider (1988) ont résumé les Q_{10} de plusieurs processus dans la cellule algale. Ainsi, la plupart des enzymes verront leur activité diminuer avec la température, mais également le transport passif et actif des substances à travers les différentes membranes de la cellule.

La température affectera notamment la photosynthèse. Il a été démontré par plusieurs auteurs que la basse température avait des effets similiaires à un stress de haute intensité lumineuse sur la photosynthèse (Huner, Öquist et Sarhan, 1998 ; Maxwell, Falk et Huner, 1995 ; Miskiewicz *et al.*, 2000). En effet, lorsque les algues ou les cyanobactéries sont placées à faible température, les processus enzymatiques reliés à la photosynthèse tels que le complexe de clivage de l'eau et le cycle de Calvin seront ralentis (Raven et Geider, 1988). Par contre, la quantité de photons arrivant aux antennes et l'absorption d'énergie par les pigments est indépendante de la température. La cellule doit donc gérer une quantité d'énergie similaire avec des processus moins efficaces. Afin d'éviter le stress oxydatif causé par une trop grande accumulation d'énergie au niveau de ses photosystèmes, la cellule autotrophe développera donc des mécanismes de protection pour faire face à la faible température similaires à ceux mis en place pour faire face à une forte intensité lumineuse. Ainsi, le contenu en chlorophylle et l'abondance en polypeptides reliés aux antennes collectrices de lumière sera diminué afin de réduire l'absorption d'énergie lumineuse (Maxwell *et al.*, 1994). Le contenu en pigments photoprotecteurs (β-carotène et xanthophylles) sera augmenté pour dissiper l'excès d'énergie. En outre, la quantité de zéaxanthine sera 16 fois plus élevée à 5°C qu'à 27°C, résultant en un état d'époxidation plus faible (Maxwell, Falk et Huner, 1995). Chez les cyanobactéries, telles *Plectonema boryanum*, le contenu en phycobilisomes sera réduit, et l'augmentation d'un caroténoïde, le myxoxanthophylle, sera observée

(Miskiewicz *et al.*, 2000). Ainsi, les algues et les cyanobactéries ayant été acclimatées à faible température exhibent une plus grande résistance à la photoinhibition que les cellules ayant été maintenues à température optimale (27°C) (Falk, Samuelsson et Öquist, 1990 ; Maxwell, Falk et Huner, 1995 ; Miskiewicz *et al.*, 2000).

En raison du stress que peut causer la lumière sur la chaine de transport des électrons (tel que discuté plus haut), les auteurs ont souvent remarqué une augmentation du stress oxydatif, notamment le contenu en ROS et la peroxydation des lipides, chez les algues et cyanobactéries soumises à la faible température (Choo, Snoeijs et Pedersén, 2004 ; Collén et Davison, 1999 ; El-Sheekh et Rady, 1995). Une augmentation des enzymes de détoxification sera également mesurée, en particulier la SOD et l'APX, qui auraient un grand rôle à jouer dans la détoxification du superoxyde et du H_2O_2 lors du stress à basse température (Lee *et al.*, 2000 ; O'Kane *et al.*, 1996). En effet, une grande partie du H_2O_2 produit se situe près des membranes (Levitt, 1980), et l'APX possède des isoformes imbriquées dans la membrane (Asada, 2000 ; Caverzan *et al.*, 2012). La capacité des espèces ou des souches à gérer efficacement le stress oxydatif leur donnera un net avantage sur les autres espèces. Par exemple, lorsque deux souches de la cyanobactérie *Arthrospira platensis* ont été transférées de 33°C à 15°C, la souche « Kenya », ayant augmenté plus rapidement l'activité de ses enzymes SOD, CAT, déhydroascorbate réductase et glutathione réductase, eut un temps de latence plus court et un taux de croissance plus élevé que la souche « M2 » (Chien et Vonshak, 2010).

1.9. Acclimatation à la température et aux herbicides

Dans les sections précédentes, les effets d'une exposition soudaine, aiguë aux herbicides ou à la faible température ont été décrits. Toutefois, cette thèse s'intéresse également aux effets à long terme que peuvent avoir ces deux facteurs sur les algues et les cyanobactéries, notamment sur les changements physiologiques mesurables

après une exposition chronique à la basse température ou aux herbicides. Le terme acclimatation, plutôt qu'adaptation, est utilisé car les changements phénotypiques et physiologiques sont mesurés, mais pas les changements génotypiques.

Si la plupart des enzymes sont moins actives à basse température, la cellule peut compenser cette diminution en augmentant leur concentration dans la cellule (Somero, 1995). À titre d'exemple, Davos *et al.* (1998) ont obtenu une concentration en Rubisco près de deux fois plus importante chez deux *Chloromonas* psychrophiles (cultivées à 5°C) que chez *Chlamydomonas reinhardtii* et d'autres algues mésophiles (cultivées à 25°C). De plus, l'activité des enzymes, notamment celles du cycle de Calvin, peut également être modifiée. En effet, l'activité de la Rubisco chez *Laminaria saccharina* et de la RuBO carboxylase chez *Skeletonema costatum* était plus élevée lorsque ces organismes étaient acclimatés à faible température (Davison, 1987 ; Mortain-Bertrand, Descolas-Gros et Jupin, 1988). Un contenu en protéines total plus important dans les cellules cultivées à faible température est souvent observé (Jørgensen, 1968 ; Morris et Farrell, 1971 ; Schöner et Heinrich Krause, 1990). Jørgensen (1968) a en effet mesuré deux fois plus de protéines total dans les cellules de *Skeletonema costatum* acclimatées à 7°C comparativement aux cellules acclimatées à 20°C. Il peut également y avoir synthèse d'une isozyme, c'est à dire un isomère d'une enzyme comme la Rubisco, ayant une activité plus importante à la faible température (Davison, 1991). En outre, la Rubisco aurait une affinité plus grande pour le CO_2 à basse température (voir revue par Sage et Kubien (2007)).

Afin de maintenir les membranes fluides à basse température, le niveau d'insaturation des acides gras des membranes sera augmenté (Guschina et Harwood, 2006 ; Los et Murata, 2004 ; Murata et Siegenthaler, 1998). Cela permettra évidemment de maintenir l'activité des photosystèmes et le déplacement des transporteurs d'électron dans la membrane. Un niveau élevé d'acides gras polyinsaturés permet également de mieux tolérer la photoinhibition (Gombos, Wada et Murata, 1992 ; Wada et Murata, 1990). Ainsi, l'expression des gènes des

désaturases sera augmentée à plus faible température (Los et Murata, 2004). En plus de l'insaturation des acides gras, la faible température peut augmenter la longueur des acides gras, modifier la proportion des différentes classes de lipides et augmenter le ratio lipides/protéines des membranes (Harwood, 1998).

Lors de l'acclimatation à la base température chez certaines plantes supérieures, la résistance au froid et au gel est augmentée en augmentant les défenses antioxydantes. Ainsi, la proportion d'antioxydants comme l'ascorbate et les tocophérols seront augmentés (Dai *et al.*, 2009 ; Maeda et DellaPenna, 2007) ainsi que l'activité des enzymes détoxifiantes comme la SOD, l'APX et la GR (Collén et Davison, 2001 ; Dai *et al.*, 2009 ; Suzuki et Mittler, 2006). Puisque le métabolisme cellulaire est ralenti, moins de ROS seront produits. De plus, la solubilité du CO_2 étant plus forte que celle de l'O_2 à faible température, la photorespiration et la production de H_2O_2 y étant associée sera moins importante (Collén et Davison, 2001 ; Sage et Kubien, 2007). Toutefois, puisque les ROS s'attaquent principalement aux doubles liaisons des lipides, l'augmentation de l'insaturation des acides gras à basse température rendra les membranes plus susceptibles à la peroxydation des lipides (Girotti et Kriska, 2004). Le contenu en tocophérols sera augmenté lors de l'acclimatation à la faible température (Leipner, Fracheboud et Stamp, 1997 ; Maeda *et al.*, 2005 ; Maeda *et al.*, 2006). Cela résultera en une augmentation de la résistance au gel ou à la forte lumière (Leipner, Fracheboud et Stamp, 1997). De plus, les tocophérols interagissent fortement avec la membrane lipidique, modifiant sa viscosité un peu comme le cholestérol chez les cellules animales (Fryer, 1992). En outre, l'augmentation des caroténoïdes permettra non seulement de jouer un rôle d'antioxydant, mais aussi d'influencer la fluidité de la membrane (Havaux, 1998). En effet, la β-carotène, entièrement incluse dans la partie lipophile de la membrane, a une orientation aléatoire et permet une certaine mobilité de la membrane. À l'inverse, la zéaxanthine a ses deux groupements de tête bien ancrés sur les deux bords de la membrane, dans la partie hydrophile, avec une orientation presque perpendiculaire à

celle-ci. Elle va donc permettre la stabilisation de la membrane (Gabrielska et Gruszecki, 1996).

L'acclimatation aux herbicides a également été étudiée par certains auteurs. Il semblerait qu'une acclimatation aux herbicides inhibiteurs de la photosynthèse comme le DCMU et le terbutryn induirait des effets similaires à l'acclimatation à la faible lumière chez la cyanobactérie *Anacystis nidulans* (Hatfield *et al.*, 1989). Les auteurs ont remarqué une augmentation des phycocyanines chez les cultures acclimatées aux herbicides, permettant donc une plus grande absorption de la lumière. De plus, une augmentation de 19% et 53% des lipides polaires en présence de terbutryn et DCMU, respectivement, a été mesurée. À l'échelle d'une communauté d'algues, une pré-exposition aux herbicides augmenterait la tolérance à une dose additionnelle d'herbicides (García-Villada et Reboud, 2007 ; Kasai, 1999 ; Nyström *et al.*, 2000 ; Seguin *et al.*, 2002). Toutefois, ces changements sont davantage attribués à la modification de la communauté phytoplanctonique vers des espèces plus tolérantes aux herbicides plutôt qu'à des changements physiologiques chez une même espèce. En effet, une pré-exposition chronique à 1 µg/L d'atrazine pendant 67 jours chez la diatomée *Craticula cuspidata* n'a pas permis d'augmenter sa tolérance à une exposition aigue d'atrazine allant de 83 à 3250 µg/L (Nelson, Hoagland et Siegfried, 1999). Une exposition à long terme à l'atrazine augmenterait plutôt la sensibilité des organismes à un pulse d'herbicide, tel que démontré chez l'algue *Pavlova* sp. (Pennington et Scott, 2001). Les effets à long terme et l'acclimatation au glyphosate ont été peu étudiés, possiblement en raison de sa forte dégradation en milieux naturels (Borggaard et Gimsing, 2008 ; Duke et Powles, 2008).

Enfin, il a été proposé que l'acclimatation à un stress puisse augmenter la tolérance à un second stress, un phénomène appelé « résistance croisée ». À titre d'exemple, les plants de tabac soumis à un choc thermique (2 heures à 38°C) étaient plus résistants à un choc osmotique subséquent causé par le NaCl (Harrington et Alm, 1988). De plus, l'acclimatation à une température sous-optimale permettrait

d'augmenter la résistance de certaines plantes à des pathogènes (Plazek et Zur, 2003). Il semblerait que la production de H_2O_2 plus importante lors d'un stress aurait un rôle à jouer dans cette augmentation de la résistance, puisqu'il permettrait de réguler l'expression des gènes associés aux défenses antioxydantes (Lamb, 1994). De plus, des changements dans les membranes induites par la faible température, par exemple au niveau de l'insaturation des acides gras, modifie les interactions protéines-lipides et pourrait modifier l'effet des herbicides tels l'atrazine (Siegenthaler et Trémolières, 1998).

1.10. Dégradation des contaminants et bioremédiation

Les cyanobactéries sont reconnues pour avoir une forte tolérance à toutes sortes de stress chimiques (Barton *et al.*, 2004 ; Coulon *et al.*, 2007 ; Gustavson, Møhlenberg et Schlüter, 2003). Par exemple, certaines cyanobactéries se sont montrées très tolérantes à la présence d'atrazine dans leur milieu (Bérard, Leboulanger et Pelte, 1999 ; Campos *et al.*, 2013). Cela pourrait être dû à leur plus grand ratio PSI/PSII ainsi qu'à la connexion plus rapprochée de la respiration et de la photosynthèse, permettant d'utiliser les électrons et transporteurs de la respiration et ainsi contourner le blocage des électrons par l'atrazine (Vermaas, 2001). Certaines cyanobactéries se sont également montrées très tolérantes au glyphosate (Forlani *et al.*, 2008 ; López-Rodas *et al.*, 2007 ; Powell, Kerby et Rowell, 1991), que ce soit à cause d'une forme insensible de l'enzyme-cible, l'EPSPS, ou alors une possible capacité à dégrader cet herbicide (Forlani *et al.*, 2008). L'utilisation du phytoplancton, en particulier les cyanobactéries, pourrait donc s'avérer intéressante pour la bioremédiation d'un milieu contaminé.

La biodégradation de l'atrazine par les bactéries vivant dans le sol a déjà été mesurée. Cette dégradation s'effectue par une N-déalkylation, hydrolyse et

déchlorination de la molécule (Struthers, Jayachandran et Moorman, 1998). La N-déalkylation permet de produire les sous-produits déethylatrazine (DEA) et déisopropylatrazine (DIA), selon qu'elle s'effectue du coté éthyle ou isopropyle de la molécule. La déchlorination permet d'enlever l'ion chlore de l'atrazine ou des deux métabolites (DEA, DIA) (voir Fig. 1.5 et revue par Lin *et al.* (2008)). Le DEA aurait un effet sur la photosynthèse 4 à 10 fois plus faible que l'atrazine, et le DIA une toxicité 2 à 7 fois plus faible que le DEA lorsque ces métabolites ont été testés sur 5 espèces d'algues et de cyanobactéries (Stratton, 1984). Bien que la N-déalkylation d'un autre herbicide, le fluométuron par plusieurs espèces d'algues ait été mesurée, la production de déethylatrazine par ce même processus semble marginal (Zablotowicz, Schrader et Locke, 1998). En effet, peu d'études ont permis de démontrer une dégradation efficace de l'atrazine par le phytoplancton. En milieu aquatique, c'est donc davantage l'absorption d'atrazine par les cellules qui permet de décontaminer le milieu. En effet, la cyanobactérie *Microcystis novacekii* a permit d'enlever en moyenne 27,2 % de l'atrazine du milieu lorsqu'elle était exposée à des concentrations de 50, 250 ou 500 µg/L d'atrazine pendant 96 heures (Campos *et al.*, 2013). Gonzales-Barreiro *et al.* (2006) ont quant à eux mesuré une diminution de 80 et 93% de l'atrazine dans le milieu par *Synechococcus elongatus* et *Chlorella vulgaris*, respectivement, après seulement une exposition de 24 heures.

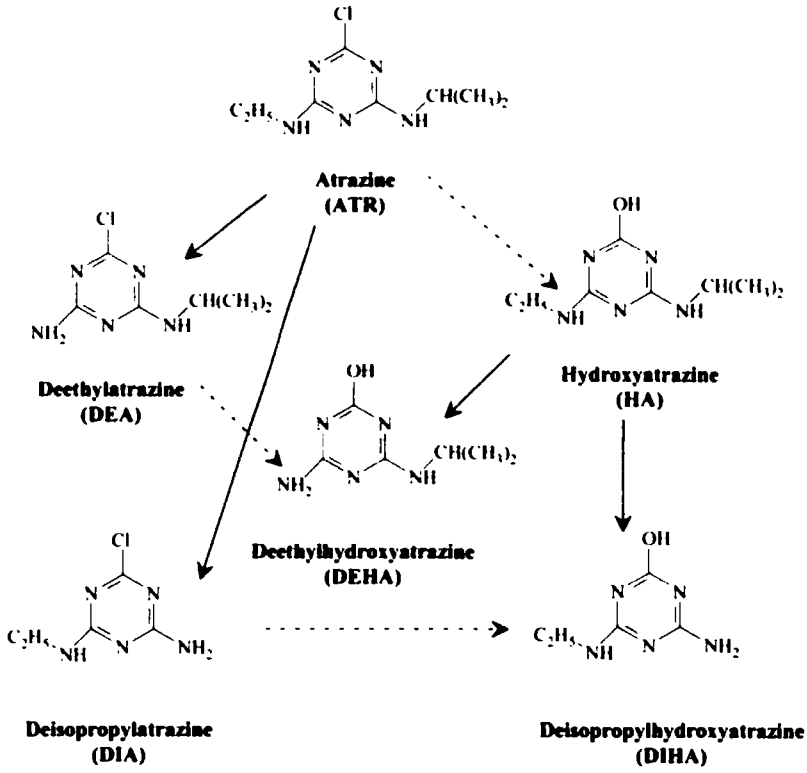

Figure 1.5. Voies de dégradation de l'atrazine. Les flèches pleines représentent une N-déalkylation et les flèches pointillées, une déchlorination de la molécule (Tiré de Lin *et al.* (2008)).

La dégradation du glyphosate par les bactéries peut se faire selon deux voies. Dans le premier cas, une enzyme oxydoréductase du glyphosate (GOX) vient briser le lien C-N du glyphosate pour produire l'acide aminométhylephosphonique (AMPA) et le glyoxylate (Schuette, 1998). L'AMPA peut ensuite être clivé par une enzyme C-P lyase, ce qui produit du phosphate et de la méthylamine (Van Herd *et al.*, 2003). Dans un deuxième cas, une C-P lyase peut directement cliver le lien C-P du glyphosate, produisant ainsi du phosphate et de la sarcosine (Kishore et Jacob, 1987). La sarcosine quant à elle serait dégradée en glycine et formaldéhyde par une sarcosine

oxydase. Ces voies de dégradation seraient présentes chez les plantes supérieures ((Duke, 2010); voir Fig. 1.6), mais à ce jour, la capacité des algues à dégrader le glyphosate ne semble pas avoir été mesuré. Néanmoins, Forlani *et al.* (2008) ont démontré que certaines espèces de cyanobactéries pourraient utiliser le glyphosate comme source de phosphore. Toutefois, comme l'AMPA n'a pas été détecté dans les cellules ou le milieu, il se pourrait que la voie de dégradation diffère de celle élucidée chez les bactéries. En cultivant la cyanobactérie *Microcystis aeruginosa* à une concentration létale en glyphosate (110 mg/L), pendant 60 jours, Lòpez-Rodas *et al.* (2007) ont démontré qu'il y avait une sélection d'individus résistants au glyphosate suite à des mutations spontanées. Ils ont ainsi pu démontrer que la résistance au glyphosate était due à une variabilité génétique aléatoire et non à une adaptation au glyphosate.

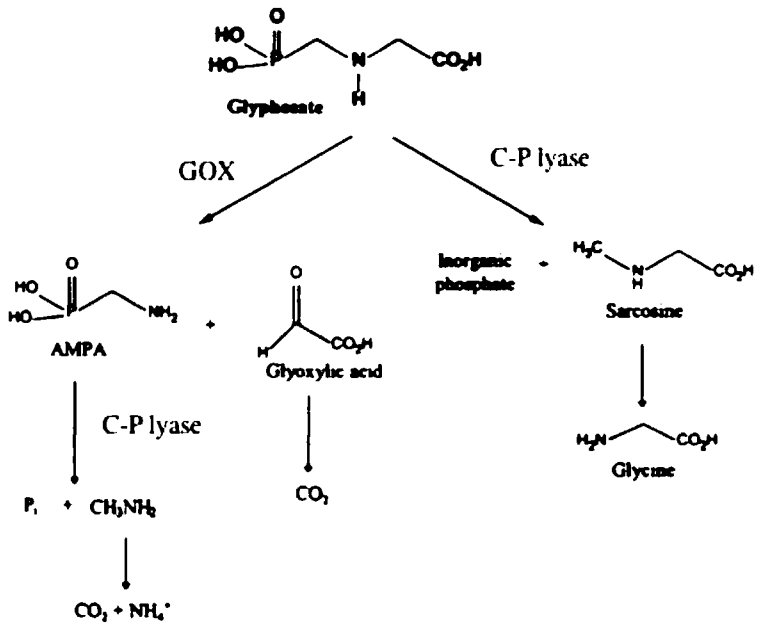

Figure 1.6. Voies de dégradation du glyphosate (adapté de Franz *et al.* (1997))

1.11. Objectifs du projet de recherche

La température et les herbicides sont donc deux facteurs qui vont influencer la photosynthèse et la croissance des algues et des cyanobactéries. Ce projet de recherche s'articulait donc autour de l'effet individuel, mais aussi combiné de ces deux facteurs sur la photosynthèse et sur le taux de croissance de quelques espèces d'algues et de cyanobactéries. De plus, ce projet s'est intéressé à certains paramètres physiologiques tels que le stress oxydatif, le contenu en pigments, la composition des acides gras, en plus de la capacité des algues et des cyanobactéries à dégrader les herbicides. L'objectif principal du projet de recherche est donc de vérifier si la sensibilité du phytoplancton aux herbicides est modifiée par la température du milieu dans lequel il se multiplie.

Dans le chapitre II, les effets de l'acclimatation à la température, en combinaison avec l'herbicide atrazine, sont étudiés sur deux espèces d'algues et deux souches de cyanobactéries. Cette étude a pour objectifs de 1) comparer la sensibilité des différents organismes utilisés à la basse température (10, 15°C) et à l'atrazine, 2) vérifier si la sensibilité à l'atrazine est modifiée par la température d'acclimatation des organismes et 3) identifier les raisons de cette différence de sensibilité selon la température utilisée.

Les chapitres III et IV sont très interreliés. En effet, les résultats présentés découlent d'une même série d'expériences. Toutefois, en raison de l'ampleur des données récoltées et des informations à présenter, les résultats ont été présentés dans deux chapitres distincts.

Dans le chapitre III, les effets de l'acclimatation à trois températures (8, 15 et 25°C) sur plusieurs paramètres physiologiques chez *Chlamydomonas reihnardtii* sont étudiés afin de comprendre pourquoi les herbicides norflurazon et fluridone ont des effets très variables aux différentes températures. Ce chapitre a donc pour objectifs de 1) vérifier les changements dans la croissance et la photosynthèse de *C. reihnardtii* à

basse température, 2) évaluer l'importance des pigments photoprotecteurs et des défenses antioxydantes dans la gestion du stress oxydatif et 3) mesurer les différences dans le profil d'acides gras aux différentes températures.

Dans le chapitre IV, les effets des herbicides norflurazon et fluridone seront examinés, et ce, aux trois températures d'acclimatation de l'algue *C. reinhardtii*. Les objectifs de cette étude seront donc de 1) mesurer l'effet de ces herbicides sur le contenu en pigments photosynthétiques et photoprotecteurs, 2) évaluer les conséquences d'une inhibition de la synthèse des caroténoïdes sur le stress oxydatif, 3) mesurer les effets de ces herbicides sur l'insaturation des acides gras et 4) identifier les facteurs qui peuvent expliquer la différence de sensibilité de *C. reinhardtii* aux deux herbicides, aux différentes températures.

Enfin, le chapitre V s'intéresse à la capacité d'une algue verte et de deux souches de cyanobactéries à s'acclimater à la présence des herbicides atrazine et glyphosate. Les objectifs de ce chapitre sont donc de 1) évaluer les changements dans la sensibilité des organismes à l'atrazine ou au glyphosate pendant une acclimatation de 30 jours aux herbicides, 2) comparer la tolérance des organismes aux herbicides après l'acclimatation à celle avant l'acclimatation, 3) évaluer la toxicité du milieu après croissance des organismes dans celui-ci pendant six jours et 4) mesurer la concentration d'herbicides dans le milieu après croissance des organismes dans celui-ci pendant six jours.

CHAPITRE II

TEMPERATURE-DEPENDENT SENSITIVITY OF GROWTH AND PHOTOSYNTHESIS OF *SCENEDESMUS OBLIQUUS*, *NAVICULA PELLICULOSA* AND TWO STRAINS OF *MICROCYSTIS AERUGINOSA* TO THE HERBICIDE ATRAZINE

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Contribution des auteurs

J'ai effectué toutes les expériences de cette étude, analysé les résultats et rédigé l'article. Philippe Juneau m'a supervisé dans le déroulement des expériences et a apporté ses commentaires et corrections sur l'article.

2.1. RÉSUMÉ

L'effet de la température sur la sensibilité de deux espèces d'algues et de deux souches de cyanobactéries à l'herbicide inhibiteur de photosynthèse atrazine a été évaluée afin de comprendre comment l'interaction entre la température d'acclimatation et l'atrazine peuvent affecter la croissance et la photosynthèse des microorganismes aquatiques. L'algue verte *Scenedesmus obliquus*, la diatomée *Navicula pelliculosa* et une souche toxique et non-toxique de la cyanobactérie *Microcystis aeruginosa* ont été acclimatées à trois différentes températures (10, 15 et 25°C) et exposées à cinq concentrations de l'herbicide atrazine (0-0,15 µM) pendant 72 heures. La croissance, les rendements photosynthétiques, les flux d'énergie dans le photosystème II et le contenu en pigments ont ensuite été mesurés pour toutes ces conditions. À l'exception de *N. pelliculosa*, la toxicité de l'atrazine était plus élevée lorsque les microorganismes étaient acclimatés à de plus basses températures. *N. pelliculosa* était non seulement la plus tolérante à l'atrazine, mais avait une sensibilité similaire à cet herbicide à toutes les températures d'acclimatation. Les différences observées quant à la sensibilité de la croissance à l'atrazine à basse température peuvent être associées avec la capacité des algues et des cyanobactéries à réagir à une forte pression d'excitation, notamment en augmentant le contenu en caroténoïdes photoprotecteurs et par la dissipation de l'énergie sous forme non-photochimique. Nos résultats démontrent que les critères de protection de la vie aquatique devraient considérer la température de l'eau comme un facteur important pouvant influencer la toxicité de l'atrazine envers les microorganismes aquatiques.

Mot clés : Algues, cyanobactéries, fluorescence de la chlorophylle *a*, rendements photosynthétiques, acclimatation

2.2. ABSTRACT

The temperature-dependent sensitivities of two algal species and two strains of cyanobacteria to the photosynthesis-inhibiting herbicide atrazine were evaluated in order to understand how the interaction between acclimation temperature and herbicide will affect growth and photosynthesis of aquatic microorganisms. The green alga *Scenedesmus obliquus*, the diatom *Navicula pelliculosa* and a toxic and non-toxic strain of *Microcystis aeruginosa* were acclimated to three different temperatures (10, 15 and 25°C) and exposed to five concentrations of the herbicide atrazine (0 – 0.15μM) for 72h. Growth, photosynthetic yields, energy fluxes within photosystem II and pigment content were then measured as potential responses to each treatment. With the exception of *N. pelliculosa*, the toxicity of atrazine was higher when microorganisms were acclimated to lower temperatures. *N. pelliculosa* was not only the most tolerant to atrazine, but also had a similar sensitivity to this herbicide at every temperature. The observed differences in growth sensitivity to atrazine at low temperature are associated with the ability of algae and cyanobacteria to cope with high excitation pressure, by increasing its protective carotenoid content and non-photochemical energy dissipation. Our results demonstrate that future guidelines for the protection of aquatic life should consider water temperature as an important factor influencing the toxicity of atrazine to aquatic microorganisms.

Key index words: Algae, Cyanobacteria, Chlorophyll a fluorescence, PSII quantum yields, Acclimation

2.3. INTRODUCTION

In agricultural areas, phytoplankton is exposed to both anthropogenic and naturally occurring environmental factors. Atrazine is the most widely used photosynthesis-inhibiting herbicide in the world because of its low cost and effectiveness as a control of annual broadleaf weeds and grass plants (Graymore *et al.*, 2001). Atrazine is therefore one of the most frequently detected pesticides in aquatic ecosystems (Sullivan *et al.*, 2009; 2010), because it is present in surface run-off and/or leaches into groundwater (Carter, 2000). Atrazine interacts at the Q_B binding site on the D1 protein of photosystem II of affected plants and phytoplankton (Fedtke & Duke, 2004). This results in an inhibition of photosynthetic electron transport and ultimately in the formation of reactive oxygen species that damage pigments, proteins and lipids in the vicinity of the photosynthetic apparatus (El-Sheek *et al.*, 1994; Gonzáles-Barreiro *et al.*, 2004).

Phytoplankton photosynthesis may also be influenced by natural factors such as temperature (Raven & Geider, 1988; Davison, 1991). Indeed, these organisms may modify their pigment composition (Huner *et al.*, 2003), enzymatic activity (Davison, 1991; Necchi Jnr, 2004), lipid composition and membrane fluidity (Harwood, 1998b) in response to temperature variation. Several authors (Maxwell *et al.*, 1995; Huner *et al.*, 1998; Miskiewicz *et al.*, 2000), have proposed that acclimation of algal and cyanobacterial photosynthesis to low temperature is basically an acclimation to high photosystem II (PSII) excitation pressure. Under constant light intensity, algae and cyanobacteria must decrease light-harvesting capacity and increase dissipation processes in order to compensate for the high PSII excitation pressure (and reduction of temperature dependant processes such as carbon assimilation) associated with low temperature (Davison, 1987; Savitch *et al.*, 1996; Miskiewicz *et al.*, 2000). All of

these modifications will result in changes in photosynthetic electron transport activity and other processes related to photosynthesis (Huner *et al.*, 1998).

Atrazine and temperature are environmental factors affecting photosynthetic electron transport and the combination of both factors can modify their individual effects. Most guidelines for the protection of aquatic life are based on published studies of experiments carried out between 20 and 30°C (US EPA, 2004; MDDEP, 2008). However, atrazine is usually applied to crop fields in pre- or post-emergence periods when water temperatures in the northern hemisphere are much colder than those used in previous lab experiments. Very few studies have evaluated the possible modulation of the atrazine effect by temperature. Bérard and collaborators (1999) have shown that growth of *Oscillatoria limnetica* was more inhibited by atrazine when cultivated at cold temperatures. In contrast, Mayasich et al. (1986) showed a strong inhibition of growth by atrazine at higher temperatures for the diatom *Nannochloris oculata* and no temperature sensitivity for *Phaeodactylum tricornutum*. Given these contradictory results, a better understanding of how the interaction of temperature and atrazine affects growth and photosynthesis of different phytoplankton species is required. Furthermore, understanding the combined mode of action of these two environmental factors on various phytoplankton species may shed light on the dominance of one species on another in watersheds impacted by agricultural activities.

The objective of this study is to determine how atrazine-sensitivity for four different strains of algae and cyanobacteria may be modified by acclimation temperature. To this end, we determined the growth rate, pigment content and photosynthetic energy fluxes of various aquatic microorganisms.

2.4. MATERIAL AND METHODS

2.4.1. Cultures

The green algae *Scenedesmus obliquus* (CPCC5), the diatom *Navicula pelliculosa* (CPCC552) and two strains of *Microcystis aeruginosa* (CPCC299 and CPCC632) were cultivated in semi-continuous culture, in Erlenmeyer flasks containing 200 ml of Bold's Basal Medium, pH 6.8 (green algae and cyanobacteria) or modified CHU-10 medium, pH 6.4 (diatoms; (Stein, 1973). CPCC299 is known to produce microcystins while CPCC632 is a non-toxic strain of *Microcystis aeruginosa* (CPCC, 2009). The cultures were acclimated to different temperatures (10, 15 or 25°C) for at least three weeks in order to achieve constant growth rates. The light intensity in the growth chambers was 100 µmol photons$\cdot m^{-2} \cdot s^{-1}$ and provided by a combination of incandescent bulbs and white fluorescent tubes (Philips F72T8/TL841/HO, USA) with a 14-h light/10-h dark cycle. Measurements were done at the same period of the day to minimize possible cell cycle effect.

2.4.2. Algal and cyanobacterial toxicity tests

Cells were collected during exponential phase, transferred to 500 ml Erlenmeyer flasks at an initial density of 200 000 cell/ml and exposed to 5 concentrations of atrazine (0, 0.01, 0.05, 0.1 and 0.15 µM) for 72 hours. The atrazine solution was a dilution (in distilled water) of the commercial Aatrex 480 liquid herbicide (Syngenta, Plattsville, Canada). Cell density and cell volume were evaluated at the end of the experiment with a Multisizer 3 Coulter Counter particle analyser (Beckman Coulter Inc., USA). Culture growth rates were calculated as the slope of the linear regression of the natural logarithm transformed cell density versus time.

In order to monitor the effectiveness and degradation of atrazine over the 72h period, a multiple time point experiment was carried out. Cells of *M. aeruginosa* CPCC299, acclimated to 15°C and 25°C, were exposed to 0.4µM of atrazine in 500ml Erlenmeyer flasks at initial cell density of approximately 200 000 cells/ml. Cell density and photosynthetic efficiency were measured at 0, 6, 24, 48 and 72 hours of exposure. The concentration of atrazine in the media was also measured at each time-step using an ELISA kit (Abraxis, Warminster, USA).

2.4.3. Fluorescence measurements

At 72h, the maximum (Φ_M) and operational PSII quantum yields (Φ'_M) were calculated from the fluorescence induction curves obtained with a Water-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany; see Table 2.1) . Prior to each fluorescence measurement, samples were dark adapted for 15 min at their specific growth temperature in order to completely re-oxidise the PSII primary electron acceptors. The PAM fluorescence yields (Table 2.1) were obtained as follows: the F_O fluorescence level represents the fluorescence yield when all PSII reaction centres are open, and Q_A is fully oxidized. Maximal fluorescence yield (F_M) was induced by a short (800 ms) saturation pulse of light (4000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) which triggers the reduction of all primary electron acceptors (Q_A and Q_B). The change of fluorescence yield (F_S) during the following illumination by actinic light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was recorded. Simultaneously, the change of the maximal fluorescence yield (F'_M) was induced by saturating pulses given periodically (every 60 s). At the same time, a Plant Efficiency Analyser fluorometer (PEA, Hansatech Ltd., King's Lynn, Norfolk, UK) was used to measure the rapid and polyphasic chlorophyll *a* fluorescence transients under 3600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Strasser *et al.*, 2004). On a time span from 50 µs to 1 s, we used the following fluorescence steps to calculate the parameters shown in Table 2.1 : the minimal fluorescence yield F_O at 50 µs, the fluorescence intensity at 300 µs (F_{300}), the fluorescence intensity at 2 ms, corresponding to the J-step, the fluorescence

intensity at 30 ms, corresponding to the I-step and maximal fluorescence (F_M or P-step). The kinetics were normalized to the relative variable fluorescence after subtracting F_O for every data point ($F = (Ft - F_O)/(F_M - F_O)$, where Ft is the fluorescence at time t, F_O the fluorescence at 50 μs and F_M the maximal fluorescence of the curve.

Table 2.1. Fluorescence parameters used in this study with their definitions and equations

Parameter	Definition	Equation	Reference
PAM parameters			
Φ_M	Maximal PSII quantum yield	$\Phi_M = (F_M - F_O)/F_M$	Kitajima and Butler, 1975
Φ'_M	Operational PSII quantum yield	$\Phi'_M = (F'_M - F_S)/F'_M$	Genty et al., 1989
NPQ	Non-photochemical quenching	$NPQ = (F_M - F'_M)/F'_M$	Bilger and Björkman, 1990
PEA parameters			
ABS/RC	Effective antenna size of an active RC	$ABS/RC = (TR_0/RC)/(TR_0/ABS)$ $TR_0/ABS = (F_M - F_{50\mu s})/F_M$	Force et al., 2003
TR_0/RC	Maximal trapping rate of PS II	$TR_0/RC = (M_0/V_J)$ $M_0 = 4 \times (F_{300\mu s} - F_{50\mu s})/(F_M - F_{50\mu s})$ $V_J = (F_{2ms} - F_{50\mu s})/(F_M - F_{50\mu s})$	Force et al., 2003
ET_0/RC	Electron transport in an active RC	$ET_0/RC = (TR_0/RC) \times (ET_0/TR_0)$ $ET_0/TR_0 = (1 - V_J)$	Force et al., 2003
DI_0/RC	Effective dissipation in an active RC	$DI_0/RC = (ABS/RC) - (TR_0/RC)$	Force et al., 2003

F_M, dark-adapted maximal fluorescence level; F'_M, light-adapted maximal fluorescence level; F_O, initial fluorescence; F_S, light-adapted constant fluorescence level, $F_{300\mu s}$, fluorescence at 300 μs; M_0, approximated initial slope of the fluorescence transient; RC, reaction center; V_J, relative variable fluorescence at the J-step

2.4.4. Pigment determination

After atrazine exposure, aliquots of cell suspensions were gently filtered onto Millipore membrane filters (0.2 μm) for pigment determination. The chlorophylls (Chl) and carotenoids (Car) were extracted in 80% methanol over night at -80°C then filtered through a 0.2 μm filter (Millipore). The absorbance spectra (400 – 750nm) for each extracted sample was obtained using a Cary 300 WinUV spectrophotometer (Varian, USA). The Chl *a*, Chl *b* and Car concentrations (μg/ml) were calculated using the equations cited in Wellburn (1994). For the determination of the phycocyanin (PC) and allophycocyanin (APC), sample filters were frozen in liquid N_2 and thawed at 4°C three times in the presence of phosphate buffer (mixing equal volumes of 0.1 mol/L KH_2PO_4 with 0.1 mol/L K_2HPO_4 solutions, pH 6.8), then vortexed. The solutions were centrifuged at 5000 x g for 15 min and the absorbance spectra (400 – 750 nm) of the supernatants were measured. The contents of PC and APC were calculated using the equations given in Bennett and Bogorad (1973) .

2.4.5. EC_{50} calculation

Dose-response curves for the Φ'_M parameter were obtained from non-linear least-square fits of the data by using a four-parameter logistic equation (Nyholm *et al.*, 1992): $p = a/[(1 + e^{b(z-c)}) + d]$, where a is the response range, b the slope coefficient, z the log (concentration) μM, c the inflection point of curve and d is the minimum response. The effective concentration causing 50% inhibition (EC_{50}) was also calculated from the non-linear least-square fits by using the regression curve inversely: $\log(EC50) = \ln[a/0.5 - d) - 1]/b + c$. Regression analyses were carried out using Origin software version 6.0 (Microcal software Inc., USA).

2.4.6. Data analysis

The statistical significance of any differences between treatments was analysed with a one-way analysis of variance (ANOVA) using JMP 5.1 statistical software (SAS institute, USA). Significant differences were accepted if $p < 0.05$. When significant differences were detected with the ANOVA, means were compared with post hoc Tukey-Kramer HSD test ($p < 0.05$).

2.5. RESULTS

2.5.1. Growth and PAM parameters

Growth rate, cell volume, maximum (Φ_M) and operational (Φ'_M) PSII quantum yields and non-photochemical quenching (NPQ) for the control and treated algae (72h - 0.1 µM atrazine) at different temperatures are shown in Table 2.2. It should be noted that lower temperatures had a stronger effect on growth than atrazine for all the studied species. The growth rates of the two *M. aeruginosa* strains (CPCC632 and CPCC299) were drastically affected by low temperature, since they decreased by 7 and 8.2-folds respectively when they were acclimated at 15°C compared to 25°C. No growth was obtained at 10°C for these cyanobacteria. Among the studied species, growth of *N. pelliculosa* was the least inhibited by low temperature, while this species was the only one affected by 0.1 µM atrazine when grown at 25°C and 15°C. The maximum PSII quantum yield (Φ_M) was affected by temperature only for the two cyanobacterial strains, where it decreased by approximately 20% at 15°C compared to 25°C. For the different growth temperatures, atrazine had little effect on the maximum PSII quantum yield. On the other hand, the operational PSII quantum yield (Φ'_M) was affected by both temperature and atrazine for all studied species. A dose-response curve for Φ'_M of phytoplankton exposed to atrazine at different temperatures can be obtained (data not shown) and the 72h-effective concentration (EC_{50}) can be calculated, i.e. the concentration of atrazine required to induce a 50% decrease in the operational PSII quantum yield (Table 2.2). We found that cells were more sensitive to atrazine at lower temperature, particularly for *M. aeruginosa* CPCC299, which was more than 2 times more sensitive to atrazine when acclimated to 15°C compared to 25°C. The diatom was the most tolerant species, while the non-toxic strain of *M. aeruginosa* (CPCC632) was slightly (but not

Table 2.2. Growth and photosynthesis parameters of *S. obliquus*, *N. pelliculosa* and *M. aeruginosa* CPCC632 and CPCC299 acclimated to three different temperatures and exposed or not to 0.1 µM atrazine for 72h.

		10°C		15°C		25°C		Interaction
Specie	Parameter	Control	Atz 0.1µM	Control	Atz 0.1µM	Control	Atz 0.1µM	Temp x Atz
S. obliquus	Growth rate (d^{-1})	**0.14** (0.02)^{b}	**0.14** (0.02)^{b}	**0.34** (0.03)^{b}	**0.29** (0.03)^{b}	**0.48** (0.02)	**0.42** (0.04)	n.s.
	Cell volume (μm^3)	**140** (20)^{b}	**105** (19)	**75** (14)	**73** (9)	**86** (25)	**69** (7)	n.s.
	Φ_M	**0.75** (0.01)^{b}	**0.68** (0.01)^{a,t}	**0.73** (0.001)	**0.70** (0.01)^{a,b}	**0.72** (0.02)	**0.76** (0.01)^{a}	**
	Φ'_M	**0.56** (0.01)^{b}	**0.31** (0.01)^{a,t}	**0.58** (0.02)^{b}	**0.36** (0.01)^{a,b}	**0.62** (0.002)	**0.43** (0.003)^{a}	**
	NPQ	**0.56** (0.07)^{b}	**0.15** (0.02)^{a}	**0.34** (0.03)^{b}	**0.09** (0.006)^{a}	**0.07** (0.03)	**0.06** (0.01)	**
	EC_{50}	**0.128** (0.003)^{b}		**0.123** (0.002)^{b}		**0.228** (0.009)		
N. pelliculosa	Growth rate (d^{-1})	**0.36** (0.04)^{b}	**0.37** (0.03)	**0.39** (0.05)^{b}	**0.29** (0.02)^{a,b}	**0.51** (0.03)	**0.40** (0.04)^{a}	n.s.
	Cell volume (μm^3)	**61.5** (0.6)^{b}	**61.3** (0.6)^{b}	**50** (2)	**53.1** (0.6)	**51** (1)	**54** (3)	n.s.
	Φ_M	**0.68** (0.01)	**0.67** (0.01)^{b}	**0.71** (0.001)^{b}	**0.70** (0.01)^{b}	**0.66** (0.004)	**0.65** (0.01)	n.s.
	Φ'_M	**0.60** (0.02)	**0.48** (0.01)^{a}	**0.64** (0.01)	**0.53** (0.01)^{a}	**0.62** (0.02)	**0.51** (0.01)^{a}	n.s.
	NPQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EC_{50}	**0.36** (0.03)		**0.36** (0.05)		**0.39** (0.06)		
M. aeruginosa CPCC632	Growth rate (d^{-1})	n.d.	n.d.	**0.07** (0.01)^{b}	**0.08** (0.01)^{b}	**0.49** (0.04)	**0.46** (0.02)	n.s.
	Cell volume (μm^3)	n.d.	n.d.	**45** (1)^{b}	**41.1** (0.1)^{a,b}	**25.5** (0.1)	**25.7** (0.5)	**
	Φ_M	n.d.	n.d.	**0.48** (0.02)^{b}	**0.50** (0.003)^{b}	**0.56** (0.004)	**0.57** (0.004)	n.s.
	Φ'_M	n.d.	n.d.	**0.33** (0.01)^{b}	**0.22** (0.01)^{a,b}	**0.40** (0.01)	**0.29** (0.01)^{a}	n.s.
	NPQ	n.d.	n.d.	**0.21** (0.03)	**0.16** (0.01)	**0.25** (0.02)	**0.17** (0.01)^{a}	n.s.
	EC_{50}	n.d.		**0.19** (0.02)^{b}		**0.27** (0.03)		
M. aeruginosa CPCC299	Growth rate (d^{-1})	n.d.	n.d.	**0.05** (0.01)^{b}	**0.04** (0.01)^{b}	**0.41** (0.03)	**0.37** (0.03)	n.s.
	Cell volume (μm^3)	n.d.	n.d.	**45.8** (0.3)^{b}	**43.5** (0.5)^{a}	**47.6** (0.5)	**44.3** (0.6)^{a}	n.s.
	Φ_M	n.d.	n.d.	**0.48** (0.004)^{b}	**0.46** (0.02)^{b}	**0.59** (0.02)	**0.62** (0.01)	*
	Φ'_M	n.d.	n.d.	**0.28** (0.01)^{b}	**0.15** (0.02)^{a,b}	**0.39** (0.03)	**0.29** (0.004)^{a}	n.s.
	NPQ	n.d.	n.d.	**0.21** (0.01)^{b}	**0.13** (0.01)^{a,b}	**0.47** (0.01)	**0.21** (0.01)^{a}	**
	EC_{50}	n.d.		**0.10** (0.01)^{b}		**0.23** (0.04)		

n.d. : not determined. Means are indicated with standard error in parenthesis. The letter a following the standard error represent a significant difference with control treatment, and the letter b, a significant difference with 25°C acclimated cells. The interaction between temperature and atrazine 0.1µM can be not significant (n.s.), significant at $p<0.05$ (*), or significant at $p<0.001$ (**).

significantly; $p > 0.05$) more tolerant than *S. obliquus* and *M. aeruginosa* CPCC299 (toxic strain). However, if temperature and atrazine both had a significant ($p < 0.05$) effect on Φ'_M of these microorganisms, the interaction between the two factors was significant ($p < 0.001$) only for *S. obliquus*. We might also consider an additive effect of temperature and atrazine on *M. aeruginosa* although this hypothesis could not be tested statistically. Non-photochemical quenching (NPQ) was 8 times higher at 10°C relative to 25°C for *S. obliquus* (Table 2.2). This increase was lower when cells were exposed to atrazine. For cyanobacteria, NPQ was higher at 25°C than at 15°C, particularly for *M. aeruginosa* CPCC299 where it was 2.2 times higher.

S. obliquus had larger cells than *N. pelliculosa*, and *M. aeruginosa* CPCC299 cells were slightly larger than CPCC632 (Table 2.2). Cell volume increased significantly ($p < 0.05$) when cells of *S. obliquus* and *N. pelliculosa* were grown at 10°C and cells of *M. aeruginosa* CPCC632 were grown at 15°C, compared to 25°C. Only *M. aeruginosa* CPCC299 showed a slight decrease in biovolume at 15°C compared to 25°C. The only groups with significantly ($p < 0.05$) reduced cell volumes in the presence of atrazine were the cyanobacteria.

2.5.2. Pigment content

Temperature and herbicide had different effects on pigment content of the microorganisms studied. For *S. obliquus*, an increase in Chl *a*/μm^3, Chl *b*/μm^3 and Car/μm^3 was measured with decreased acclimation temperature, while low acclimation temperature did not affect the pigment composition of *N. pelliculosa* (Table 2.3). The ratio Car/Chl *a* was 1.5 time higher at 10°C than at 25°C for *S. obliquus* and *N. pelliculosa*. When *M. aeruginosa* CPCC299 was grown at 15°C, instead of 25°C, Chl *a* and Chl *b* contents were reduced by 45% and 37%, respectively. The reduction of photosynthetic pigment content for *M. aeruginosa* CPCC632 was similar. However, carotenoid content was not affected by temperature

in *M. aeruginosa* CPCC632 while it increased by 48% in *M. aeruginosa* CPCC299. For the cultures acclimated to 15°C, the toxic strain of *M. aeruginosa* (CPCC299) had a distinctive yellow color caused by its high Car/Chl *a* ratio. Indeed, a Car/Chl *a* ratio of 2.1 and 2.7 times higher at 15°C than at 25°C was observed for *M. aeruginosa*

Table 2.3. Chlorophyll *a* and *b*, total carotenoids, phycocyanin and allophycocyanin (fg/μm^3) of *S. obliquus*, *N. pelliculosa* and *M. aeruginosa* CPCC632 and CPCC299 acclimated to three different temperatures and exposed or not to 0.1 μM atrazine for 72h.

		10°C		15°C		25°C		Interaction
Specie	Pigment	Control	Atz 0.1μM	Control	Atz 0.1μM	Control	Atz 0.1μM	Temp x Atz
S. obliquus	Chl *a*	**3.82** (0.37)^{b}	**5.75** (0.86)^{a,b}	**3.62** (0.57)^{b}	**3.60** (0.66)^{b}	**1.37** (0.3)	**2.04** (0.21)	n.s.
	Chl *b*	**1.48** (0.17)^{b}	**2.32** (0.35)^{a,b}	**1.53** (0.22)^{b}	**1.62** (0.35)^{b}	**0.55** (0.11)	**0.08** (0.10)	n.s.
	Car	**1.00** (0.11)^{b}	**1.22** (0.19)^{b}	**0.58** (0.16)	**0.57** (0.11)	**0.24** (0.07)	**0.33** (0.05)	n.s.
N. pelliculosa	Chl *a*	**2.64** (0.24)	**2.92** (0.06)	**2.78** (0.12)	**3.00** (0.19)	**2.79** (0.22)	**3.45** (0.36)^{a}	n.s.
	Chl *b*	**0.58** (0.04)^{b}	**0.79** (0.18)	**0.89** (0.04)	**0.95** (0.11)	**0.99** (0.04)	**1.03** (0.04)	n.s.
	Car	**0.69** (0.13)	**0.70** (0.03)	**0.48** (0.05)	**0.56** (0.05)	**0.50** (0.20)	**0.75** (0.25)	n.s.
M. aeruginosa CPCC632	Chl *a*	n.d.	n.d.	**2.90** (0.14)^{b}	**3.16** (0.18)^{b}	**5.42** (0.22)	**5.14** (0.03)	*
	Chl *b*	n.d.	n.d.	**0.47** (0.04)^{b}	**0.84** (0.09)^{a,b}	**1.32** (0.06)	**1.27** (0.01)	**
	Car	n.d.	n.d.	**0.96** (0.07)	**0.95** (0.08)	**0.86** (0.06)	**1.08** (0.03)^{a}	*
	PC	n.d.	n.d.	**10.45** (0.29)^{b}	**13.11** (0.34)^{b}	**31.08** (2.38)	**30.46** (1.10)	*
	APC	n.d.	n.d.	**1.36** (0.68)^{b}	**2.53** (0.73)^{b}	**8.77** (1.69)	**9.50** (1.69)	n.s.
M. aeruginosa CPCC299	Chl *a*	n.d.	n.d.	**2.53** (0.29)^{b}	**3.03** (0.07)^{b}	**4.55** (0.30)	**4.88** (0.27)	n.s.
	Chl *b*	n.d.	n.d.	**0.46** (0.44)	**0.54** (0.25)	**0.73** (0.05)	**0.79** (0.04)	n.s.
	Car	n.d.	n.d.	**2.04** (0.42)	**2.38** (0.25)^{b}	**1.38** (0.09)	**1.13** (0.16)	n.s.
	PC	n.d.	n.d.	**6.89** (1.63)	**6.22** (1.12)	**10.97** (2.57)	**4.77** (0.91)^{a}	*
	APC	n.d.	n.d.	**8.54** (2.18)	**7.68** (1.60)	**13.13** (2.89)	**3.94** (3.87)	*

n.d. : not determined. Means are indicated with standard error in parenthesis. The letter a following the standard error represent a significant difference with control treatment, and the letter b, a significant difference with 25°C acclimated cells. The interaction between temperature and atrazine 0.1 μM can be not significant (n.s.), significant at $p < 0.05$ (*), or significant at $p < 0.001$ (**).

CPCC632 and CPCC299 respectively. Although there was a decrease in the phycobilisomes at low temperature for the two *M. aeruginosa* strains, this decrease was only significant ($p < 0.001$) for CPCC632 where a decrease of 66% and 85% was measured for PC and APC, respectively.

The pigment content tended to increase in presence of 0.1μM atrazine when compared to control (especially at 10°C and 15°C), but the interaction between herbicide and temperature was significant only for *M. aeruginosa* CPCC632 (Chl *a*, *b*, Car and PC) and *M. aeruginosa* CPCC299 (PC and APC) ($p < 0.05$; see Table 2.3).

2.5.3. Rapid fluorescence kinetics and energy fluxes within photosystem II (PSII)

Figure 2.1 presents the rapid Chl *a* fluorescence polyphasic kinetics of *S. obliquus, N. pelliculosa* and the two strains of *M. aeruginosa* acclimated to different growth temperatures, in presence or absence of atrazine 0.1 μM. We note that the acclimation temperature did not affect the fast fluorescence rise of the studied algal species. However, fluorescence intensity of the J-I steps, representing the electron transport between Q_A and Q_B, was higher when growth temperature was lower for both strains of cyanobacteria. Atrazine increased the J level of all phytoplankton independently of the growth temperature, demonstrating the partial blockage of electron flow between Q_A and Q_B.

These rapid Chl *a* fluorescence kinetics allow the calculation of different parameters which facilitate an understanding of how PSII energy fluxes are distributed between absorption, trapping, electron transport and dissipation (Force *et al.*, 2003). Therefore, a closer look at the energy dissipation parameters provides a useful indication of how temperature acclimation and herbicide treatment affect energy fluxes within PSII (Table 2.4). There was a significant interaction between both factors for the absorption flux per reaction center (ABS/RC), which is an

indication of the PSII antenna size. A 20% decrease in the antenna size (ABS/RC) was found for *S. obliquus* acclimated to 10°C, while no effect was observed for *N.*

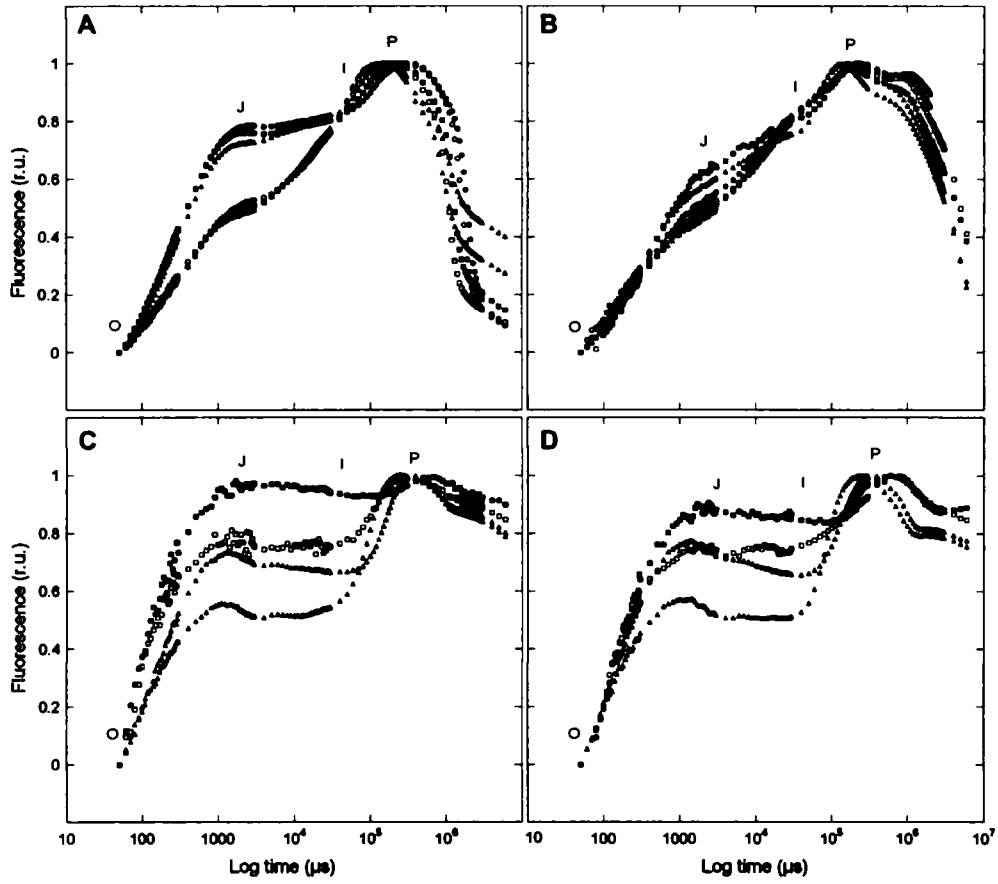

Figure 2.1. Fast fluorescence kinetics of *S. obliquus* (A), *N. pelliculosa* (B), and *M. aeruginosa* CPCC632 (C) and CPCC299 (D), acclimated to 10°C (circles), 15°C (squares) or 25°C (triangles), control (empty symbols) or exposed to atrazine 0.1 µM for 72h (filled symbols).

pelliculosa in the absence of atrazine at this temperature when compared to 25°C. However, we noticed that atrazine had a similar effect on both algae, by increasing the PSII antenna size at 10°C, and decreasing it at higher growth temperatures (15°C and 25°C). No significant interaction of temperature and atrazine on ABS/RC was found for the two strains of cyanobacteria ($p > 0.05$), but these two strains responded differently to temperature. A 44% decrease was observed for *M. aeruginosa* CPCC299 acclimated to 15°C, while *M. aeruginosa* CPCC632 had a 1.5 X greater antenna size at lower temperature. The electron transport rate per active reaction center (ET_0/RC) decreased by 15% for *S. obliquus* grown at 10°C and by 50% for the two strains of cyanobacteria grown at 15°C relative to 25°C, however, *N. pelliculosa* ET_0/RC was not affected by temperature. It was also observed that atrazine exposure decreased the electron transport rate (ET_0/RC) by blocking electron flow between Q_A and Q_B for all species and temperatures. The maximal rate by which an exciton is trapped by active reaction centers (TR_0/RC) was only affected by temperature and atrazine in *S. obliquus* acclimated to 10 and 15°C, resulting in a significant interaction ($p < 0.05$) of both factors for this green alga. Finally, the effective dissipation per reaction center (DI_0/RC) was modified in accordance to the change in the absorption flux per reaction centre (ABS/RC). However, contrary to what was observed for the antenna size, the interaction between temperature and atrazine on the effective dissipation per reaction center was significant for all the studied species ($p < 0.05$). This indicates that lower acclimation temperature modified the dissipation of excess energy when PSII is partially inhibited by atrazine.

Table 2.4. Energy fluxes within PSII for *S. obliquus*, *N. pelliculosa* and *M. aeruginosa* CPCC632 and CPCC299 acclimated to three different temperatures and exposed or not to 0.1 µM atrazine for 72h.

		10°C		15°C		25°C		Interaction
Specie	Parameter	Control	Atz 0.1µM	Control	Atz 0.1µM	Control	Atz 0.1µM	Temp x Atz
S. obliquus	ABS/RC	**2.57** (0.03)^{b}	**3.08** (0.10)^{a}	**2.85** (0.07)	**3.25** (0.11)^{a}	**3.05** (0.11)	**3.08** (0.03)	*
	TR_0/RC	**1.98** (0.01)	**2.16** (0.05)^{a,b}	**2.11** (0.01)	**2.26** (0.04)^{a,b}	**2.15** (0.02)	**2.17** (0.02)	*
	ET_0/RC	**0.97** (0.06)^{b}	**0.48** (0.005)^{a,b}	**1.06** (0.04)	**0.55** (0.02)^{a}	**1.12** (0.02)	**0.60** (0.005)^{a}	n.s.
	DI_0/RC	**0.59** (0.03)^{b}	**0.92** (0.06)^{a}	**0.73** (0.06)	**0.99** (0.08)^{a}	**0.90** (0.12)	**0.90** (0.01)	*
N. pelliculosa	ABS/RC	**2.70** (0.07)	**3.97** (0.29)^{a}	**2.97** (0.11)	**2.15** (0.27)^{a}	**2.88** (0.04)	**2.54** (0.04)	*
	TR_0/RC	**2.16** (0.07)	**2.01** (0.08)	**2.43** (0.14)	**1.84** (0.29)^{a}	**2.21** (0.07)	**2.05** (0.03)	n.s.
	ET_0/RC	**1.11** (0.02)	**0.67** (0.10)^{a}	**1.27** (0.07)	**0.69** (0.08)^{a}	**1.19** (0.06)	**0.87** (0.07)^{a}	n.s.
	DI_0/RC	**0.55** (0.01)	**1.96** (0.21)^{a,b}	**0.54** (0.03)	**0.32** (0.04)	**0.68** (0.03)	**0.45** (0.07)	*
M. aeruginosa CPCC632	ABS/RC	n.d.	n.d.	**11.36** (0.41)^{b}	**10.05** (0.42)^{b}	**7.57** (0.09)	**6.78** (0.16)	n.s.
	TR_0/RC	n.d.	n.d.	**3.27** (0.20)	**3.09** (0.10)	**3.20** (0.05)	**2.92** (0.07)	n.s.
	ET_0/RC	n.d.	n.d.	**0.68** (0.26)^{b}	**0.16** (0.03)^{a}	**1.49** (0.05)	**0.82** (0.05)^{a}	n.s.
	DI_0/RC	n.d.	n.d.	**8.09** (0.29)^{b}	**6.96** (0.33)^{a,b}	**4.37** (0.04)	**3.86** (0.12)	*
M. aeruginosa CPCC299	ABS/RC	n.d.	n.d.	**5.19** (0.58)^{b}	**5.04** (0.24)^{b}	**9.20** (0.55)	**7.12** (0.26)	n.s.
	TR_0/RC	n.d.	n.d.	**2.98** (0.27)	**2.94** (0.25)	**3.31** (0.13)	**3.00** (0.02)	n.s.
	ET_0/RC	n.d.	n.d.	**0.75** (0.03)^{b}	**0.39** (0.11)^{a}	**1.49** (0.08)	**0.73** (0.03)^{a}	*
	DI_0/RC	n.d.	n.d.	**2.21** (0.31)^{b}	**2.10** (0.02)^{b}	**5.89** (0.43)	**4.12** (0.28)	*

n.d. : not determined. Means are indicated with standard error in parenthesis. The letter a following the standard error represent a significant difference with control treatment, and the letter b, a significant difference with 25°C acclimated cells. The interaction between temperature and atrazine 0.1 µM can be not significant (n.s.), significant at $p<0.05$ (*), or significant at $p < 0.001$ (**).

2.5.4. Multiple time point measurements

Table 2.5 shows that *M. aeruginosa* CPCC299 cell density increased over a period of 72h in presence of 0.4µM atrazine by 36% and 100% at 15 and 25°C, respectively. However, Φ'_M was significantly affected by atrazine after 6h of exposure (compared to time 0; $p < 0.001$), but this inhibition did not change significantly over 72h of exposure ($p > 0.05$). Also, the concentration of atrazine in the media did not decrease significantly (data not shown).

Table 2.5. Effect of atrazine 0.4 µM on cell density and operational quantum yield (Φ'_M) of *M. aeruginosa* CPCC299 acclimated to 15 or 25°C, with their standard errors in parenthesis.

Temperature	Parameter	0h	6h	24h	48h	72h
15°C	Cell density ($x10^4.ml^{-1}$)	**20.6** (0.4)^a	**21.7** (0.3)^{ab}	**24.0** (1.6)^{ab}	**25.9** (2.7)^{ab}	**28.1** (5.2)^b
	Φ'_M	**0.27** (0.04)^a	**0.073** (0.003)^b	**0.072** (0.004)^b	**0.077** (0.003)^b	**0.085** (0.003)^b
25°C	Cell density ($x10^4.ml^{-1}$)	**20.2** (0.1)^a	**22.2** (0.4)^a	**25.3** (0.6)^b	**27.4** (0.4)^b	**40.8** (2.2)^c
	Φ'_M	**0.44** (0.02)^a	**0.12** (0.01)^b	**0.13** (0.01)^b	**0.12** (0.004)^b	**0.10** (0.2)^b

For every measurement, standard errors followed by different letters are significantly different according to Tukey HSD test.

2.6. DISCUSSION

2.6.1. Effect of atrazine

Atrazine is considered to be one of the most widely used herbicides in the world (Graymore *et al.*, 2001). Although the maximum concentration for the protection of aquatic life is 1.5 µg/L in U.S.A. and 1.8 µg/L in Canada (CCME, 1999; US EPA, 2003), the concentration detected in watersheds of agricultural areas often exceeds 30 µg/L (0.14 µM) (Solomon *et al.*, 1996; Giroux, 2010) and even reach concentrations as high as 1000 µg/L (deNoyelles *et al.*, 1982). Our study demonstrated that an atrazine concentration of 0.1 µM (21.6 µg/L) had no effect on algal and cyanobacterial growth at 25°C, except for *N. pelliculosa* (Table 2.2). On the other hand, the operational photosystem II (PSII) quantum yield (Φ'_M) and the electron transport rate in the active reaction center (ET_0/RC) were significantly reduced in presence of 0.1µM atrazine for all studied phytoplankton species, which is in agreement with previous studies showing that electron transport was blocked between the primary and the secondary electron acceptors (Q_A and Q_B) (Ralph, 2000; Macinnis-Ng & Ralph, 2003). Our findings are in accordance with previous work showing that ET_0/RC was altered in the presence of PSII-herbicides but not the other PSII energy fluxes (Chalifour *et al.*, 2009). The non modification of the other energy fluxes together with the very little changes observed in pigment content tend to demonstrate that at this atrazine concentration, other protective mechanisms (such as antioxidant enzymatic system) are involved in the scavenging of reactive oxygen species, and thus prevent damage to the photosynthetic apparatus (Rutherford & Krieger-Liszkay, 2001). DeLorenzo *et al.* (2004) have shown that the sensitivity of different algal species to atrazine could be related to cell biovolume; i.e. smaller cells being more sensitive to this herbicide than larger ones. They also found a positive correlation between growth rate-EC_{50} and Chl *a* concentration per cell, arguing that a

higher Chl *a* content was related to a higher amount of photosystems II and therefore more available molecular targets for atrazine. *Navicula pelliculosa* was by far the least sensitive to atrazine in our study but did not have a higher cell volume or Chl *a* concentration than *S. obliquus* or *M. aeruginosa* (CPCC299). This indicates that the tolerance of this species to PSII inhibiting herbicides might be due to other physiological characteristics such as the high PSII/PSI ratio observed in some diatoms (Strzepek & Harrison, 2004), their specific pigment composition (presence of fucoxanthin) protecting against photo-oxidation (Wetzel, 2001) or the presence of partial heterotropic behaviour providing an alternate energy source (Tuchman *et al.*, 2006). Further research is needed to elucidate the exact mechanism responsible of the greater tolerance found in *N. pelliculosa*.

2.6.2. Effect of temperature

Amongst all environmental factors, temperature variation, like light, may have important effects on algal and cyanobacterial photosynthesis and physiology. Indeed, as we have demonstrated here (Tables 2.2 and 2.4), growth and photosynthetic electron transport rates have been shown to be reduced when phytoplankton were grown at low temperature (Raven & Geider, 1988; Butterwick *et al.*, 2005; Staehr & Brikeland, 2006).

In the case of *S. obliquus*, our results indicated that growth rate was 3.4 times lower at 10°C compared to 25°C while the overall photosynthesis (Φ'_M) was decreased by about only 10%. This indicates that growth at low temperature is influenced also by other biochemical processes (see review by Raven and Geider (1988)). We also noted that non-photochemical quenching (NPQ) increased by 8 fold when *S. obliquus* was grown at 10°C rather than 25°C (Table 2.2). This increase in NPQ could be due to the dissipation of light energy by heat through the xanthophyll cycle (Wilson *et al.*, 2003). The observed changes in energy dissipation processes

may be explained by pigment content. For *S. obliquus*, a 2.5 to 4 fold increase in pigments per unit biovolume was observed at 10°C compared to 25°C (Table 2.3). This finding contradicts previous studies demonstrating that algal cultures grown at low temperature (2-5°C) had lower amounts of chlorophylls and carotenoids (Steeman Nielsen & Jorgensen, 1968; Maxwell *et al.*, 1995; Huner *et al.*, 2003; Wilson *et al.*, 2003). However, some other authors have shown an increase in Chl *a* with decreasing temperature in green algae (Morris & Glover, 1974; Rhee & Gotham, 1981; El-Sabaawi & Harrison, 2006), indicating that the effect of temperature on Chl content may vary depending on the species, the growth temperature or the light intensity. According to Wilson and Huner (2000), the redox state of the PQ pool and the trans-thylakoid ΔpH regulates the accumulation of Chl and LHCII polypeptides. Considering that the photosynthetic activity of *S. obliquus* (assessed by lower Φ'_M and ET_0/RC, Table 2.2 and 2.4) slowed down during growth at low temperature, as per previous observations (Maxwell *et al.*, 1994; Necchi Jnr, 2004), the inhibition in electron transport would therefore increase Chl accumulation. The over-excitation pressure created by low temperature and exacerbated by Chl accumulation would have to be quenched and may explain the concomitant increase in Car content (Table 2.3). The reduction of light absorption by reaction center (ABS/RC) of control *Scenedesmus obliquus* and effective dissipation in active reaction centers (DI_0/RC) at lower temperature compared to their optimal growth temperature (25°C; see table 2.4) could be due to a higher amount of chlorophylls at the PSI reaction center or a proportionally higher amount of PSII reaction center (and by consequence, lower ratio of Chl/RC; (Greenbaum & Mauzerall, 1991).

As we observed for *S. obliquus*, the absorption of light per reaction center (ABS/RC) of control *M. aeruginosa* (CPCC299) decreased at lower temperature compared to their optimal growth temperature (25°C). For *M. aeruginosa* CPCC632 however, the antenna size (ABS/RC) was higher even when pigment content was lower at 15°C compared to 25°C. The concomitant increase in DI_0/RC for this strain

indicates that the extra energy received is dissipated through heat as opposed to use in electron transport. Both strains of *M. aeruginosa* showed a significant decrease in chlorophylls and an increase in carotenoids per unit biovolume at low temperature (Table 2.3). This is consistent with previous studies, and reflects the need to protect PSII against over-excitation pressure (Tang & Vincent, 1999; Miskiewicz *et al.*, 2000). However, even if protective modification in pigmentation was observed for cyanobacteria, maximal and operational PSII quantum yields were found to decrease at lower temperature. This decrease could be due to 1) a depressed activity of rubilose-1,5-bisphosphate carboxylase oxygenase (Rubisco), the main enzyme involved in carbon fixation (Li *et al.*, 1984; Raven & Geider, 1988); 2) the inability to up-regulate their non-photochemical energy dissipation processes (NPQ) due to their lack of xanthophyll cycle (Hirschberg & Chamovitz, 2004) and 3) a lower membrane fluidity and therefore slower state transition between PSII and PSI (Sarcina *et al.*, 2001). It is interesting to note that both strains of *M. aeruginosa* showed an increase in the J and P levels of the rapid Chl *a* fluorescence and that the shape of the fluorescence kinetic at low temperature was very similar to the one obtained in presence of atrazine at 25°C (Fig. 2.1). These responses indicate an increase in the excitation pressure on PSII. Moreover, the differences in the energy fluxes between the two strains of *M. aeruginosa* are in agreement with a previous report by Vonshak and Novoplansky (2008) showing a decreased PSII activity (expressed as O_2 evolution•mg Chl a^{-1}•h^{-1}) of 45 and 26% with two strains of the cyanobacteria *Arthrospira platensis* when acclimated to low (15°C) relative to an optimal growth temperature (30°C). The strain that was more sensitive to low temperature appeared to have an impairment at the acceptor side of PSII while the more tolerant strain decreased the concentration of active PSII reaction centers and maintained a higher capacity of Q_A^- reoxidation (Vonshak & Novoplansky, 2008). The difference in the energy dissipation processes might be due to the toxicity of the two *M. aeruginosa* strains (CPCC632 is non-toxic, while CPCC299 produces microcystin), since toxin

content of this species was previously shown to be linked to photosynthesis (Deblois & Juneau, 2010).

Contrary to the other test species, the diatom *N. pelliculosa* was well acclimated to colder temperatures, proving its "cool-season metabolism" reported by many authors (Watermann *et al.*, 1999; van der Grinten *et al.*, 2005). No major changes were observed in the pigment content nor in the energy fluxes within PSII, resulting in a fairly constant growth rate and photosynthetic efficiency at all studied temperatures. This response was previously observed for the marine diatom *Thalassiosira pseudonana* over the 15 to 25°C range (Sobrino & Neale, 2007). At 10°C, a 44% greater Car/Chl *a* ratio was measured, probably exerting a photoprotective effect on the PSII. We can not exclude, as proposed by Davison et al. (1991) , that the effective acclimation of brown algae growth at low temperature could be due to the up-regulation of Rubisco and other Calvin cycle enzymes (Mortain-Bertrand *et al.*, 1988; Mock & Hoch, 2005).

2.6.3. Effect of temperature on atrazine toxicity

Our results showing that the diatom *N. pelliculosa* was the most tolerant species are in agreement with a previous study, in which *Navicula* sp. had an atrazine Φ_M-based EC_{50} 3.5 times higher than the green alga *Nephroselmis pyriformis* (0.46 µM and 0.13 µM respectively; (Magnusson *et al.*, 2008). In field studies, Dorigo *et al.*, (2004) showed that atrazine contamination in Ozanne river has shifted the composition of the community towards diatom dominance. Moreover, *N. pelliculosa* had comparable Φ'_M-EC_{50} after 72 hours treatment (0.36-0.40 µM). Bérard *et al.*, (1999) found that spring blooms of diatoms and cryptophytes were not affected by atrazine contamination. We may explain the high tolerance of *N. pelliculosa* when exposed to atrazine at low temperature (10°C) by its higher thermal energy dissipation (See Table 2.4). On the other hand, for the green alga and cyanobacteria,

our data clearly indicated that atrazine had a stronger inhibitory effect on photosynthesis at lower water temperatures. In presence of atrazine, the electron transport toward PSI was reduced and the PQ pool stayed in an oxidized state. This could send a signal of light limitation to the PSII (Plumley & Davis, 1980) and, according to Wilson and Huner (2000), will up-regulate LHCII polypeptide and Chl *a* accumulation. Yet, this greening process will increase light absorption, which will cause more photo-oxidative damage due to the blockage in the electron transport, and as shown in Table 2.2, NPQ is 3.7 times lower in presence of atrazine than in control when *S. obliquus* is grown at 10 and 15°C resulting in the inability of the cell to efficiently dissipate the excess energy. This could be due to the fact that the xanthophyll-cycle-dependant non-photochemical quenching also requires a high trans-thylakoid ΔpH, again not obtained in presence of PSII inhibiting herbicide (Gilmore, 1997).

According to Millie *et al.* (1992), cells of *Anabaena circinalis* were more sensitive to simazine, another triazine herbicide, when they were acclimated to high light than to low light conditions, an acclimation similar to low temperature in terms of light excitation pressure (Maxwell *et al.*, 1994). The authors attributed this difference in sensitivity to the pigment content of acclimated cells (Millie *et al.*, 1992). Bérard and collaborators (1999) have shown that monocultures of the cyanobacterium *Oscillatoria limnetica* had an atrazine EC_{50} based on growth of 24.2 μg/L when cultivated at 13°C and 52.3 μg/L when cultivated at 20°C. This difference was ascribed to the rate of repair of PSII which is affected by temperature, probably by inhibiting the transcription and translation of psbA genes and the *de novo* synthesis of the D1 protein.

It is also important to recognize that the two strains of *M. aeruginosa* responded differently to atrazine when temperature was decreased. At 25°C both strains had a similar Φ'_M-based EC_{50}, while at 15°C, the toxic *M. aeruginosa* strain (CPCC299) was twice as sensitive as the non-toxic *M. aeruginosa* CPCC632 (Table

2.2). This sensitivity difference seems to be related to the acclimation of both strains to low temperature. *M. aeruginosa* CPCC299 had a greater reduction in NPQ and DI_0/RC at 15°C compared to CPCC632, suggesting that these energy dissipation pathways are less efficient to protect against atrazine effect at 15°C in *M. aeruginosa* CPCC299 compared to *M. aeruginosa* CPCC632 (Table 2.2).

One could argue that the increased tolerance to atrazine at higher temperatures for the different phytoplankton species was due to the higher growth rate of these organisms at 25°C (Table 2.2) which resulted in a higher number of cells at the end of the 72h exposure and/or to a degradation of atrazine during the experiment. We tested the possibility of a lower atrazine to cell ratio by measuring (for *M. aeruginosa* CPCC299) growth and photosynthesis at 0h, 6h, 24h, 48h and 72h of exposure to 0.4 µM atrazine (Table 2.5) and by monitoring the concentration of atrazine in the media at each time point. This species was chosen for this experiment because of its important temperature-dependant sensitivity to atrazine and its stronger physiological response to low temperature, assessed by growth rate, photosynthetic efficiency and pigmentation (Table 2.2 and 2.3). The results indicated that the effect of atrazine on *M. aeruginosa* CPCC299 operational quantum yield did not change between the beginning and end of the experiment (time 72h) although we did measure increased cell densities (36% and 100% at 15°C and 25°C respectively) during that period (Table 2.5). Moreover, the concentration of atrazine measured in the media remained constant (0.38-0.40 µM) during the time course at both temperatures (data not shown), indicating that there was no degradation of atrazine by this species or by growth conditions (light/temperature) over this period of time.

2.7. CONCLUSIONS

We can conclude that the temperature-dependent sensitivity of autotrophic microorganisms to atrazine, a PSII-inhibiting herbicide, is based on their physiological acclimation to growth temperature. Some algae, like the diatom used in this study, conserved efficient metabolism over a large range of temperatures, which seemed afford protection against some chemical stressors like PSII-inhibiting herbicides. For other species, however, atrazine will have more deleterious effects on an already depressed growth and photosynthesis.

Considering the results obtained in this study, a revision of guidelines for the protection of aquatic life with more realistic environmental conditions is needed. Other factors not included in this work, such as nutrients, irradiance, pH or biotic factors should also be considered when establishing water quality guidelines. Finally, we can now hypothesize, based on our findings, that cyanobacterial blooms will be more promoted by higher water temperature, even in herbicide contaminated watersheds.

2.8. REFERENCES

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CHAPITRE III

COMBINED EFFECT OF TEMPERATURE AND HERBICIDES ON *CHLAMYDOMONAS REINHARDTII*. 1. EFFECT OF LOW TEMPERATURE ON GROWTH, PHOTOSYNTHESIS, OXIDATIVE STRESS, PIGMENT AND FATTY ACID COMPOSITION

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Contribution des auteurs

J'ai effectué toutes les expériences, analysé les résultats et rédigé l'article. Michael T. Arts a participé à l'extraction des lipides et l'identification des acides gras dans les différents traitements. Martin J. Kainz m'a permis d'utiliser le matériel de son laboratoire et d'y effectuer des expériences. Philippe Juneau a supervisé mes travaux, et participé à l'interprétation des résultats. Michael T. Arts, Martin J. Kainz et Philippe Juneau ont apporté leurs commentaires et leurs corrections lors de la rédaction et de la soumission de l'article.

3.1. RÉSUMÉ

La température est un important facteur environnemental pouvant affecter l'activité des enzymes, la fluidité des membranes et l'efficacité de la photosynthèse. Nous avons mesuré l'effet de deux herbicides blanchisseurs (c.-à-d. les inhibiteurs de la phytoène désaturase norflurazon et fluridone) sur l'efficacité photosynthétique d'une algue verte (*Chlamydomonas reinhardtii*) acclimatée à 8, 15 et 25°C. Cette algue était plus sensible au norflurazon à 15 qu'à 25°C, mais plus sensible au fluridone à 25 qu'à 15°C. *C. reinhardtii* était très tolérante aux deux herbicides à 8°C. Nous avons quantifié les changements dans le taux de division cellulaire, l'efficacité photosynthétique, l'activité des enzymes antioxydantes, le stress oxydatif, le contenu en pigments et la composition en acides gras dans le but de mieux comprendre l'interaction entre la toxicité des herbicides et la température. En absence d'herbicide, le taux de croissance et l'efficacité photosynthétique étaient similaires à 15 et 25°C, mais une diminution de 36% dans la photosynthèse et une division cellulaire très minime a été observée à 8°C. Les espèces réactives oxygénées de même que l'activité de la catalase et de la superoxyde dismutase diminuaient à 8°C, comparativement à 25°C. Aucune différence significative n'a été mesurée dans l'activité de l'ascorbate peroxydase, mais la peroxydation des lipides était plus élevée à 15°C comparativement aux deux autres températures. Bien que le contenu total en pigment ait diminué, une augmentation dans les caroténoïdes photoprotecteurs, relativement au contenu en chlorophylles, a été observée à 8 et 15°C. De plus, la composition en acides gras a été modifiée avec la température et le niveau d'insaturation était clairement plus élevé à 15°C comparativement à 25°C. À 8°C, toutefois, et malgré une diminution du contenu en acide gras de 2,4 fois, le niveau d'insaturation était similaire aux cellules acclimatées à 25°C. *C. reinhardtii* a ajusté ses réponses biochimiques pour maintenir une croissance similaire à 15°C comparativement à 25°C. Par contre, la croissance à 8°C était caractérisée par un temps de latence substantiel dans la division cellulaire, permettant à la cellule d'accumuler des protéines et des caroténoïdes, ce qui l'aida à être mieux protégée contre la toxicité des herbicides blanchisseurs.

Mots clés : algues, ascorbate peroxydase, catalase, caroténoïdes, flurorescence de la chlorophylle *a*, espèces réactives oxygénées, superoxyde dismutase, niveau d'insaturation

3.2. ABSTRACT

Temperature is an important environmental factor affecting the enzymatic activity, membrane fluidity and efficiency of photosynthesis. We measured the effect of two bleaching herbicides (i.e. the phytoene desaturase inhibitors norflurazon and fluridone) on the photosynthetic efficiency of a green alga (*Chlamydomonas reinhardtii*) acclimated to 8, 15 and 25°C. The alga was more sensitive to norflurazon at 15 than at 25°C, but more sensitive to fluridone at 25 than at 15°C. *C. reinhardtii* tolerated both herbicides at 8°C. We quantified changes in rates of cell division, photosynthetic efficiency, antioxidant enzyme activity, oxidative stress, pigment content, and fatty acid composition in order to better understand the interaction between herbicides toxicity and temperature. In absence of herbicide, growth rate and photosynthetic efficiency were similar at 15 and 25°C, but a 36% decrease in photosynthesis and marginal cell division was observed at 8°C. Reactive oxygen species as well as catalase and superoxide dismutase activity decreased at 8 compared to 25°C. No significant change in ascorbate peroxidase activity was measured, but the lipid peroxidation level was higher at 15°C compared to the other two temperatures. Although the overall pigment content decreased, there was an increase in photo-protective carotenoids relative to chlorophylls at both 15 and 8°C. Moreover, the fatty acid composition was modified and the unsaturation level was clearly increased at 15 compared to 25°C. At 8°C, however, despite a 2.4X lower fatty acid content, the unsaturation level was similar to 25°C acclimated cells. *C. reinhardtii* adjusted its biochemical response to maintain a similar growth at 15 compared to 25°C. Growth at 8°C, however, was characterised by a substantial lag phase in the cell division and where accumulated proteins and carotenoids helped protect the cells against the toxicity of bleaching herbicides.

Keywords: algae, ascorbate peroxidase, catalase, carotenoids, chlorophyll a fluorescence, reactive oxygen species, superoxide dismutase, unsaturation index

3.3. INTRODUCTION

Temperature is an environmental factor that can significantly modulate the toxicity of xenobiotics. Most toxicity bioassays are done at room temperature (U.S. EPA, 1996, Van Coillie, 1983) however the water temperature at which some xenobiotics (e.g. pesticides) reach non-target species can be much lower than 20°C, especially during springtime in temperate climates (Cantin *et al.*, 2011). Some studies have found a higher toxicity of xenobiotics, such as the photosynthesis-inhibiting herbicide atrazine, on microalgae or cyanobacteria at low temperature (Chalifour *et al.*, 2011, Bérard *et al.*, 1999). Alternatively, a higher toxicity of cadmium or polycyclic aromatic hydrocarbons at higher temperature has been observed in marine algae (Wang *et al.*, 2008; Vieira & Guilhermino, 2012). Modifications in the photosynthetic components, membrane fluidity or activity of some enzymes may all be responsible for the differential toxicity observed at different temperatures.

Temperature is one of the most important factors affecting the metabolism of photosynthetic organisms. Growth at low, but non-freezing, temperatures is experienced as a high photosystem II (PSII) excitation pressure by those organisms (Maxwell *et al.*, 1995; Huner *et al.*, 1998; Miskiewicz *et al.*, 2000). Indeed, since the absorption of light by PSII is independent of temperature (Raven & Geider, 1988) algae need to reduce their light-harvesting capacity to compensate for decreases in enzymatic activities involved with oxygen evolution and/or carbon fixation at lower temperatures. The balance between energy absorption and utilisation through metabolism needs to be tightly regulated; otherwise the excess energy not used for photochemistry will induce the production of damaging reactive oxygen species (ROS) (Suzuki & Mittler, 2006). Plants have therefore evolved a series of mechanisms to balance the effect of low temperature on growth, photosynthesis, and enzymatic activity (Raven & Geider, 1988; Davison, 1991; Dai *et al.*, 2009;

Theocharis *et al.*, 2012). For example, decrease in chlorophyll content and increase in xanthophyll pool may be observed in cold acclimated autotrophs to decrease the antennae size and increase the dissipation of excess energy through heat (Huner *et al.*, 2003). Moreover, the activity of different ROS-scavenging enzymes may also be up-regulated at low temperature to protect proteins and membranes from increased oxidative stress (Suzuki & Mittler, 2006). The unsaturated fatty acid content also increases to compensate for decreases in membrane fluidity (Harwood, 1998a), thereby maintaining the stability of PSII (Hölzl & Dörmann, 2007; Mizusawa & Wada, 2012) and the movement of plastoquinones involved in the electron transporter system (Horváth *et al.*, 1986; Sarcina *et al.*, 2001). Acclimation to low temperature also increases resistance to other biotic and abiotic stresses like pathogens (Plazek & Zur, 2003), freezing (Morris, 1976), and high light (Öquist & Huner, 1991; Maxwell *et al.*, 1995). Therefore, understanding the acclimation processes to temperature is essential to better interpret the combined effects of other environmental factors, such as herbicides. Indeed, the combination of temperature and herbicides is even more relevant since most herbicides inhibit processes also affected by temperature, such as photosynthesis (diuron, atrazine), protein synthesis (imazapyr, glyphosate) or lipid synthesis (diclofop-methyl, metolachlor) (Fedtke & Duke, 2004).

Norflurazon and fluridone are both considered bleaching herbicides because they inhibit phytoene desaturase; an enzyme involved in the synthesis of carotenoids (Bartels & Watson, 1978). The depletion of these photoprotective carotenoids leads to the loss of chlorophylls, chloroplast ribosomes and grana structures, and consequent inhibition of photosynthesis (Bartels 1978, Della Vecchia 2001). While norflurazon and fluridone decrease carotenoid content of cells, low temperature increases the proportion of those photoprotective pigments, as mentioned previously. Therefore, we thus expect an antagonistic effect of both factors on photosynthesis of *C. reinhardtii*. Since to our knowledge no study has been conducted on this interaction, the aim of this study was to examine how acclimation to different

temperatures affects the physiology of *C. reinhardtii*, leading to the differences in bleaching herbicide toxicity.

3.4. MATERIAL AND METHODS

3.4.1. Algal culture and growth conditions

Chlamydomonas reinhardtii (G.M. Smith) wild type strain (CC125) was obtained from the *Chlamydomonas* Resource Center, University of Minnesota, USA. Algae were cultivated in semi-continuous culture in Erlenmeyer flasks containing 300 mL of High Salt Medium (HSM) (Sueoka *et al.*, 1967) at a pH of 6.8. The cultures were acclimated to three different temperatures (8, 15 and 25°C) for at least three weeks to achieve constant growth rates. The light intensity in the growth chambers was kept at an irradiance of 100 µmol photons m^{-2} s^{-1} provided by a combination of incandescent bulbs (Philips 60W) and white fluorescent lamps (Philips F72T8/TL841/HO, USA) with a 14:10 h light:dark cycle.

Cells were collected during the exponential growth phase and transferred, at an initial density of $2x10^5$ cells mL^{-1}, into 500 mL Erlenmeyer flasks containing 300 mL HSM medium. They were exposed to two concentrations of norflurazon (Nf) (1.25 and 2.5 µM) and one concentration of fluridone (Fd) (1.25 µM) dissolved in dimethyl sulfoxide (DMSO). The control flasks were exposed to DMSO 0.25%. Herbicides (Nf : PS-1044; Fd : Fluka 45511) and DMSO (472301) were purchased at Sigma-Aldrich (Oakville, Canada). Three replicates were done for each treatment at each temperature.

3.4.2. Cell growth and photosynthesis

After dilution, cell density and volume were measured over a period of 96 h using a Coulter Counter particle analyser (Multisizer 3, Beckman Coulter Inc., Fullerton, USA), and all physiological measurements were done 96 h after the

transfer in the new medium. Culture growth rates μ_{cell} and $\mu_{\mu m^3}$ were calculated according to the equation $\mu = \ln(X_t/X_0)/t$ where X is the cell density (cell ml^{-1}) or cell biovolume (μm^3 ml^{-1}), respectively, at a specific time point (t = 4 days).

The maximum (Φ_M) and operational (Φ'_M) PSII quantum yields were evaluated from the fluorescence induction curves obtained with a Water-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) using these equations: $\Phi_M = (F_M-F_0)/F_M$ (Kitajima & Butler, 1975) and $\Phi'_M = (F'_M-F_0)/F'_M$ (Genty *et al.*, 1989). All measurements were done at the growth temperature using the temperature controller provided with the fluorometer. Prior to each fluorescence measurement, samples were kept in the dark for 15 min at their specific growth temperature to completely re-oxidise the PSII primary electron acceptors.

3.4.3. Reactive oxygen species content

The effect of temperature acclimation on the ROS level and esterase activity was measured by flow cytometric analysis using 2',7'-dichlorofluorescein diacetate (H_2DCFDA; cat no. D399) and fluorescein diacetate (FDA; cat no. F1303) (both from Invitrogen, Burlington, Canada), respectively (Peperzak 2011). Solutions of H_2DCFDA 10 mM and FDA 250 µM were prepared in DMSO. One mL of algal culture was incubated with 20 µL H_2DCFDA or FDA for 25 min, after which the sample was analysed. The measurements were performed on a flow cytometer (FACScan, Beckton Dickinson, Mississauga, Canada) at the FL-1 parameter. The green fluorescence emitted by DCF and fluorescein (emission wavelength = 530 nm) was excited with Argon ion laser (excitation wavelength = 488 nm).

3.4.4. Antioxidant enzymatic activity

Algae were harvested by centrifugation at 15,000 g for 15 min and re-suspended in potassium phosphate buffer 50 mM containing 0.1 mM EDTA, 1% PVP and protease cocktail inhibitor (Cat no. P9599, Sigma-Aldrich, Oakville, Canada), with an addition of 0.5 mM of ascorbic acid for the ascorbate peroxidase (APX) assay. The buffer was adjusted to pH 7.0 for catalase (CAT) and APX and pH 7.8 for superoxide dismutase (SOD) assays. Cell density was determined using a particle analyser. Cells were disrupted by 3 bursts of ultrasonication, 30 sec each (Sonic dismembrator Model 100, output power of 3 watts, Fisher Scientific, Pittsburgh, USA). Samples were kept on ice between sonication sessions. This sonication regime disrupted 95-99% of the cells in the solution, as confirmed by the particle analyser and by observation on the microscope (data not shown). Cell debris and unbroken cells were removed by centrifugation (15,000 g for 25 min at 4°C). The supernatant was collected and used as the crude cell extract. Protein concentration was determined using bovine albumin serum as a standard (Bradford, 1976b). CAT activity was determined by measuring the consumption of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 2 min according to Aebi (1984), using 50 µL crude extract and 10 mM H_2O_2 in a 3 mL potassium phosphate buffer 50 mM, pH 7.0. APX activity was determined by measuring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM cm^{-1}) for 3 min according to Nakano and Asada (1981), using 500 µL crude extract and 10 µL H_2O_2 30 mM, in a 3 mL potassium phosphate buffer 50mM, pH 7.0 volume containing 0.1 mM EDTA and 0.5 mM ascorbic acid. SOD activity was determined in microplate by measuring the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) (Beauchamp & Fridovich, 1971). The crude extract was diluted four times in the extraction buffer in order to add 50 µL, 25 µL, 12.5 µL and 6.25 µL of sample to the reaction mixture. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 0.9 mM riboflavin, 0.025% TritonX-100 and 50 µL of the extract

dilutions in a total volume of 200 µL. The plate was illuminated under a white fluorescent tube for 20min (~200µmol photons m^{-2} s^{-1}), after which the absorbance was read at 560 nm. One unit of SOD was defined as the amount of crude extract that caused a 50% decrease of NBT reduction under the assay conditions.

3.4.5. Lipid peroxidation

Lipid peroxidation level was determined in terms of thiobarbituric acid reactive substances (TBARS) and expressed as equivalent of malondialdehyde (MDA) according to the method of Heath and Packer (1968). Algae were harvested by centrifugation (15,000 g for 15 min) and resuspended in potassium phosphate buffer (50 mM containing 0.67% trichloroacetic acid; TCA). Cell density was determined using the particle analyser before the addition of TCA. Cells were then broken up by ultrasonication as described above. A standard curve was prepared using the tetramethoxypropane (TMP; cat no. 156812, MP Biomedical, Solon, USA). The homogenate or standard solutions (300 µL) were mixed with 300 µL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was heated at 95°C for 30 min, cooled to room temperature then centrifuged at 15,000 g for 20 min. The absorbance of the supernatant was measured at 532 nm and corrected with the non-specific absorbance at 600 nm. The concentration of MDA was calculated using the TMP standard curve and expressed on the dry weight of the sample.

3.4.6. Pigments

Algal cultures (10 mL) were filtered on a 0.8 µm membrane filter (Whatman nucleopore, Piscataway, USA), frozen in liquid nitrogen and stored in the dark at -

80°C. The extraction was done under green, dim light. The filtered cell pigments were extracted by adding 1.5 mL of acetone (90%) and sonicating for 30 sec with an output power of 3 watts. The membrane filters were kept in acetone at 4°C overnight, after which the extract in acetone was filtered through a 0.22 μm syringe filter (Millex-GV, Millipore, Billerica, USA). Extracts were stored at -20°C until analysis. Chromatographic analyses were performed using a Waters reverse-phase HPLC system comprised of a Millenium 32 program, a model 510 pump, a Waters 2487 absorbance detector (Dual λ) set at 445 nm and a model 7725i Rheodyne injector. Chlorophylls and carotenoids were separated on a Inertsil ODS C18 column (4.6 x 150 mm, 5 μm particle size) protected by a Upchurch Scientific guard column (Oak Harbor, WA, USA) packed with C18 material and using 0.5 μm frits. All pigments were analysed according to the method of García-Plazaola (1999). The injection volume was 30 μL for the 25 and 15°C samples and 60 μL for the 8°C samples. Pigments were identified and quantified by comparison of retention times and peak area with those of standards of chlorophylls (PPS-CHLA and PPS-CHLB, DHI, Horsholm, Denmark), xanthophylls (PPS-ANTH, PPS-LUTE, PPS-NEOX, PPS-VIOL and PPS-ZEAX, DHI, Horsholm, Denmark) and β-carotene (Cat no. C4582, Sigma-Aldrich, Oakville, Canada).

3.4.7. Total lipids and fatty acids

For the total lipids and fatty acid (FA) analysis, 100 mL of algae culture were filtered on a precombusted, pre-weighted GF/F filter (Whatman, Piscataway, USA) and stored in the dark at -80°C. Filters were then freeze-dried and weighed. The dry weight (DW) of cells was thus determined. Lipids were extracted and analyzed as fatty acid methyl esters as in McMeans *et al.* (2012). Briefly, lipids were extracted by homogenizing samples, three times, in 2 mL of 2:1 (v/v) chloroform:methanol (Folch

et al., 1957). Fatty acid methyl esters were generated using the sulphuric-methanol (1:100) method, and were subsequently analyzed on a Hewlett Packard 6890 GC using splitless injection on a Supelco SP-2560 column and identified using known FA standards. "∑SAFA" was used to indicate the sum of all FA with no double bonds (i.e. saturated FA), "∑MUFA" indicates the sum of all FA with one double bond, and "∑PUFA" indicates the sum of all FA with ≥ 2 double bonds. The unsaturation index (UI) was calculated as the sum of the percentages of individual unsaturated fatty acids multiplied by the number of their double bonds.

3.4.8. Statistical analyses

Comparison of parameter values among temperatures were done with ANOVA and Tukey-Kramer post-hoc analysis using JMP 6.0 software (SAS Institute, Cary, NC, USA).

3.5. RESULTS

3.5.1. Combined effect of temperature and herbicides on photosynthesis

The operational quantum yield of photosynthesis (Φ'_M), a measure of photosynthetic efficiency, revealed that *C. reinhardtii* was significantly more inhibited by Nf (at 2.5 μM) at 15 than at 25°C (Fig 3.1). Contrary to Nf, Fd (1.25μM) inhibited photosynthetic efficiency significantly more at 25 than at 15°C. Both herbicides did not affect photosynthetic efficiency at 8°C.

3.5.2. Effect of temperature on growth and photosynthesis

Growth rate (μ) of *C. reinhardtii* increased with temperature (Fig. 3.2). Daily μ_{cell} were 0.04, 0.35, and 0.43 at 8, 15 and 25°C, respectively. However, cells acclimated to 8°C had a significantly higher cell biovolume resulting in daily $\mu_{\mu m^3}$ of 0.13, 0.33 and 0.41 for cultures acclimated to 8, 15 and 25°C, respectively. Furthermore, the 8°C cultures had ~5% of their cells in a tetrad formation, i.e. still enclosed in the mother cells. The presence of tetrads was negligible at 15 and 25°C (data not shown). The PSII operational quantum yield (Φ'_M) was significantly reduced (36% lower) at 8 when compared to 25°C (Fig. 3.1). However, the PSII maximum quantum yield (Φ_M) was not affected by temperature (Fig. 3.2).

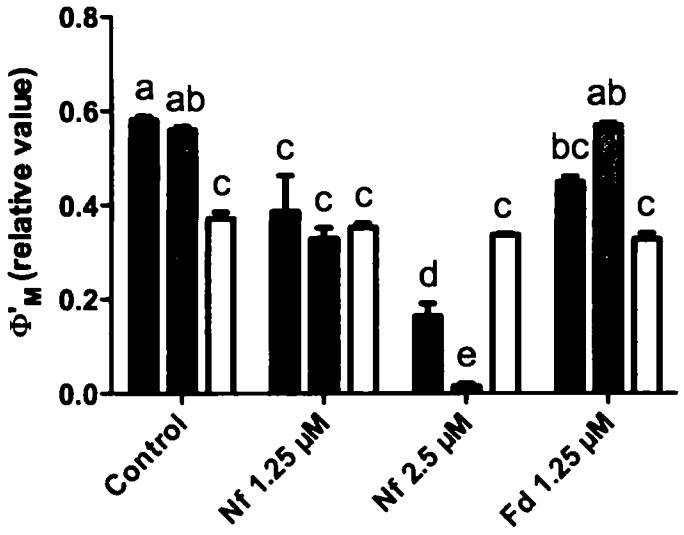

Figure 3.1. Operational quantum yield of photosynthesis of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf) or fluridone (Fd) for 96h. Letters above bars represent the significant difference between treatments at $p < 0.05$.

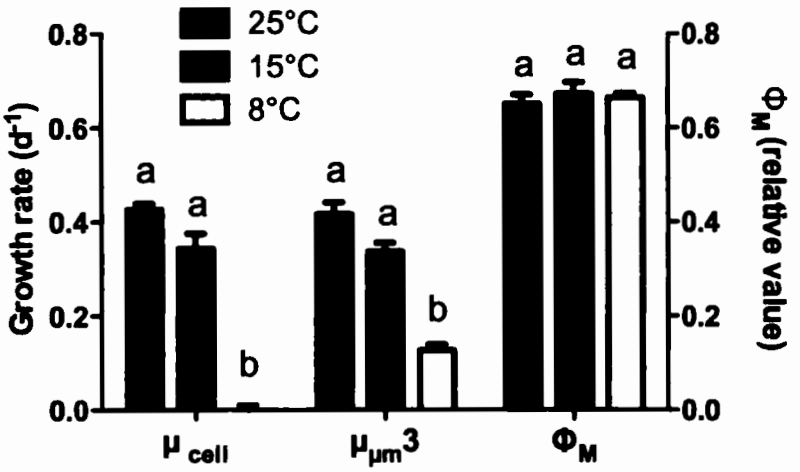

Figure 3.2. Growth rates based on cell density (μ_{cell}) or biovolume ($\mu_{\mu m3}$), and maximal quantum yield of photosynthesis (Φ_M) of *Chlamydomonas reinhardtii* acclimated to three different temperatures. Letters above bars represent the significant difference between treatments at $p < 0.05$.

3.5.3. Effect of temperature on oxidative stress

H_2DCFDA is a non-polar, non-fluorescent, molecule that diffuses across membranes. Upon entering cells it is de-acetylated by esterase enzymes to give the polar compound H_2DCF. H_2DCF can then be oxidized by ROS to produce the fluorescent compound dichlorofluorescein (DCF). Because the entry of H_2DCFDA into cells, and its subsequent esterification, can be modulated by temperature we also used the fluorescein diacetate (FDA) probe as a proxy for esterase activity. This cell-permeable molecule is esterified to fluorescein upon cell entry. FDA is also used as a vitality and cell membrane integrity probe (Peperzak & Brussaard, 2011). The H_2DCFDA:FDA ratio was used to correct for the effect of the esterase activity on the fluorescence level emitted by the DCF (Oukarroum *et al.*, 2012). The fluorescence

emitted by the DCF and the fluorescein was 2.5 and 3.6X higher at 8 than at 25°C, respectively, resulting in a H_2DCFDA:FDA ratio that was significantly lower at 8 compared to 25°C (Table 3.1). The ROS content was consequently also lower at 8°C. The lipid peroxide level (measured as the MDA content in the culture extract) was highest at 15°C.

Table 3.1. Reactive oxygen species (ROS) fluorescence level, protein content, activity of antioxidant enzymes and lipid peroxidation of *Chlamydomonas reinhardtii* acclimated to three different temperatures.

Parameter	**25°C**		**15°C**		**8°C**	
H_2DCFDA	**4.8** (0.3)	a	**5.0** (0.0)	a	**11.9** (3.1)	b
FDA	**347.1** (12.0)	a	**378.9** (65.7)	a	**1245.0** (164.5)	b
H_2DCFDA:FDA ($x10^{-3}$)	**13.9** (1.5)	a	**13.2** (0.3)	ab	**9.6** (1.8)	b
Protein (µg:mg DW)	**27.6** (2.2)	a	**34.6** (10.6)	ab	**55.7** (15.8)	b
Catalase (U:µg DW)	**5.7** (0.9)	ab	**6.8** (0.4)	a	**4.2** (0.9)	b
Ascorbate peroxidase (U:µg DW)	**28.8** (4.8)	a	**22.9** (1.6)	a	**38.3** (8.1)	a
Superoxide dismutase (U:µg DW)	**40.0** (5.8)	a	**17.8** (4.1)	b	**22.3** (1.2)	b
Lipid peroxidation (MDA:µg DW)	**20.8** (2.9)	a	**32.5** (5.4)	b	**16.7** (5.4)	a

Data shown are means (n=3) with standard deviation in parenthesis. The letters represent the significant difference between the acclimation temperatures at $p < 0.05$.

3.5.4. Effect of temperature on protein content and antioxidant enzymatic activity

The protein content per dry weight of *C. reinhardtii* was up to 2X higher at 8 than at 25°C (Table 3.1). Thus, to more equitably compare physiological parameters we normalized the antioxidant and enzymatic measurements to dry weight. The trends in antioxidant and enzyme activities were similar when expressed on a dry weight or a protein basis (data not shown). CAT activity was higher at 25 and 15°C than at 8°C, but the difference was only significant for the cells grown at 15°C. APX activity was not significantly different among the three temperatures. SOD was ~2X more active at 25 than at 15°C and 8°C.

3.5.5. Effect of temperature on pigment composition

Cold acclimation decreased the content of most pigments, except the photoprotective lutein and zeaxanthin (Table 3.2). Indeed, the content of these two xanthophylls did not decrease at 8°C compared to 25°C and increased by 50% at 15°C compared to 25°C. There was 3.5X less Chl *a*, 3X less Chl *b*, and 2.6X less β-carotene at 8 compared to 25°C, resulting in lower Chl *a*:*b* and higher β-carotene:Chl *a* ratios at 8°C. Moreover, the lut+zea:Chl *a* ratio was 1.8 and 3.4 X higher at 15 and 8°C, respectively, than at 25°C.

Table 3.2. Pigment content (μg:mg DW) and ratios of pigments of *Chalmydomonas reinhardtii* acclimated to three different temperatures.

Parameter	**25°C**		**15°C**		**8°C**	
Chlorophyll a	**393.12** (43.13)	a	**330.41** (36.31)	a	**113.73** (3.87)	b
Chlorophyll b	**142.14** (12.77)	a	**115.89** (5.96)	b	**46.71** (1.12)	c
β-carotene	**25.71** (3.14)	a	**20.81** (2.83)	a	**10.05** (2.27)	b
Violaxanthin	**1.84** (0.37)	a	**0.72** (0.20)	b	**0.60** (0.07)	b
Antheraxanthin	**0.49** (0.14)	a	**0.55** (0.01)	a	**0.22** (0.02)	b
Lutein + zeaxanthin	**2.25** (0.09)	a	**3.36** (0.17)	b	**2.21** (0.11)	a
Neoxanthin	**3.68** (0.24)	a	**1.95** (0.13)	b	**0.57** (0.01)	c
Chl a:Chl b	**2.76** (0.11)	ab	**2.84** (0.20)	a	**2.44** (0.13)	b
β-car:Chl a	**0.065** (0.005)	a	**0.062** (0.004)	a	**0.088** (0.017)	b
Lut+zea:Chl a	**0.0058** (0.0008)	a	**0.0102** (0.0007)	b	**0.0193** (0.0003)	c

Data shown are means (n=3) with standard deviation in parenthesis. The letters represent the significant difference between the acclimation temperatures at $p < 0.05$.

3.5.6. Effect of temperature on total lipids and fatty acid composition

The total lipids per unit dry weight were about 10% lower at 15 than at 25°C, but 2.4X lower at 8°C (Supplementary table 1). There were significantly more n-3 FAs and less n-6 at 15°C than at 25°C, the 8°C-grown cells having only lower amount of n-6 FAs when compared to 25°C (Fig. 3.3). The cells acclimated to 8°C also had higher contents of SAFA and MUFA per unit biomass, but less PUFA than cells grown at 15 or 25°C. Cells acclimated to 15°C had significantly higher contents of PUFA, and higher UI values at 15°C than at the other two temperatures.

Cold acclimation decreased the mass fractions (μg mg^{-1} dry weight) of most individual FAs (Supplementary table 1). Some FAs (e.g. 16:1n-7, 20:0, 20:1n-9 and 20:2n-6) had similar mass fractions between the different temperatures. We also measured traces of 22:1n-9, 20:3n-3, 26:0 in *C. reinhardtii* grown at 8°C, FAs that were not present when algal cells were acclimated to higher temperatures.

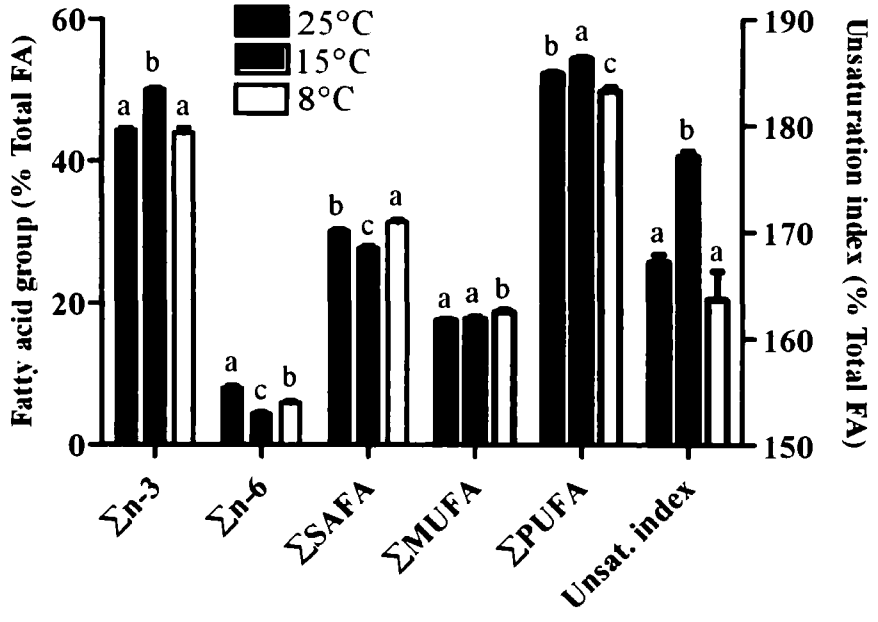

Figure 3.3. Relative amount (% of total measured fatty acids) of n-6, n-3, saturated fatty acid (SAFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and unsaturation index (UI) of *C. reinhardtii* acclimated to three different temperatures. Letters above bars represent the significant difference between treatments at $p < 0.05$.

3.6. DISCUSSION

The sensitivity of *C. reinhardtii* to the bleaching herbicides norflurazon (Nf) and fluridone (Fd) depended on acclimation temperature. At 2.5 μM, Nf completely inhibited the operational quantum yield of photosynthesis at 15°C, while decreasing it by 72% at 25°C. In contrast, Fd only inhibited the photosynthetic efficiency of algae acclimated to 25°C. Moreover, the absence of inhibition of photosynthetic efficiency (i.e. lack of toxicity) at 8°C for both herbicides was unexpected. This suggests that some physiological modifications at low temperature, perhaps related to the maintenance of adequate levels of photo-protective carotenoids, protected the cells from the bleaching actions of Nf and Fd thereby preserving their photosynthetic efficiency. We therefore investigated physiological differences in cells acclimated to the three temperatures at the endpoint (96 h after dilution) of the experiment.

3.6.1. A lag phase necessary for protein accumulation

C. reinhardtii can adjust its antioxidant enzyme activity, pigment content, and fatty acid unsaturation level to maintain a similar growth rate and photosynthetic efficiency at 15 and 25°C. The similar growth rate at those two temperatures was reached after 2 months of cultivation at each temperature, where a small amount of algal culture was transferred into new media every week, until the cells were completely acclimated. However, this green alga showed a lag phase in cell division at 8°C; over a four day time course, cells increased in size but did not complete a whole division cycle. Approximately 5% of the cells were still in a tetrad formation at 96 h after dilution, where four daughter cells were retained within a common mother cell wall. Cell division resumed after 96 h (data not shown).

It is known that the length of the cell cycle and the rate of cell division depend on temperature. The cell cycle of *Chlamydomonas* is characterised by a pre-commitment phase, where cell size increases, cellular structures are built and energy reserves are accumulated (Vítová *et al.*, 2011). The length of the pre-commitment period depends on the rate of photosynthesis and energy assimilation (Zachleder & Van Den Ende, 1992). Photosynthetic activity was lower at 8°C compared to 15°C and 25°C creating conditions conducive for a longer pre-commitment period and providing cells more time to accumulate proteins and enzymes. This might explain why the protein content per dry weight was 2X higher at 8 than at 25°C. A temperature-dependent increase in protein content has been observed in other algal species. For example, Jørgensen (1968) measured a 2-fold higher protein content in cells of *Skeletonema costatum* acclimated to 7 compared to 20°C, while Morris and Farrell (1971) measured twice as much carbon incorporated into protein in *Dunaliella tertiolecta* grown at 12 compared to 20°C. Low temperature promotes an increase in protein synthesis as a way to increase enzyme content, or replace warm adapted enzymes with isoforms exhibiting higher *in vivo* activities, in order to keep an adequate level of catalytic activity (Raven & Geider, 1988; Somero, 1995; Savitch *et al.*, 1996). This likely explains why, at different temperatures, we found similar activities for catalase and ascorbate peroxidase when expressed on a dry weight basis. Indeed, the higher protein content of cells acclimated to 8°C is correlated to a higher enzyme content. We expect the phytoene desaturase enzyme content to increase with low temperature, since more carotenoids are synthesised. This increase in phytoene desaturase content would result in more molecular targets for its inhibitors (Nf and Fd), which will likely decrease their effect at low temperature, relatively to 25°C cells.

3.6.2. A slower metabolism induces less oxidative stress

The H_2DCFDA probe has previously been used to measure the ROS content of algal cells (Knauert & Knauer, 2008; Elbaz *et al.*, 2010; Liu & Pang, 2010). Most authors report the fluorescence level of treated cells relative to control cells; however they usually do not correct for possible differences in penetration and/or esterase enzyme activity due to the toxicity of treatment. When comparing the effect of temperature acclimation on intracellular ROS level, it is important to consider the temperature dependence of the probe's diffusion rate, cleavage by esterase, and enzymatic oxidation of DCFH. For example, Collèn and Davison (1997) observed an increase in DCF formation with increasing temperature in the macroalga *Fucus evanescens*.

Both the H_2DCFDA and FDA probe showed a strong increase in fluorescence at 8 compared to 15 or 25°C, resulting in a decrease in the H_2DCFDA:FDA ratio at 8°C. A decrease in the ROS content could be related to a slower rate of active oxygen production in algae acclimated to low temperature (Collén & Davison, 1997; Zhang *et al.*, 2009) or to a higher level of antioxidant molecules (Pérez-Torres *et al.*, 2004; Zhang *et al.*, 2009). For instance, concentrations of lutein and zeaxanthin, which greatly increased at 8°C in our study, may prevent ROS production by quenching singlet Chl excited states (Niyogi *et al.*, 2001).

Our antioxidant enzyme activity results concur with the measured ROS contents. Lower superoxide dismutase activity at 8°C is consistent with lower general metabolic activity and might also correlate with a lower intracellular ROS content. Photosynthesis, assessed by the operational PSII quantum yield (Φ'_M), was reduced at 8°C, and Chl *a* and *b* content were 3.5 and 3X lower, respectively, than at 25°C. At the relatively low light intensity (100 µmol photons$\cdot m^{-2}\cdot s^{-1}$) used here, a slower metabolic rate, concomitant with a lower amount of photosystems and lower photorespiration rate at 8°C, would require a lower antioxidant enzyme activity.

Similarly, Janknegt *et al.* (2009) reported a lower ascorbate peroxidase and glutathione reductase activity in Antarctic compared to temperate microalgae, and Plazek and Zur (2003) found lower CAT activity in cold-acclimated barley. When grown at day:night temperatures of 12:5°C instead of 24:22°C, wheat seedlings showed no difference in superoxide dismutase activity, but a lower CAT activity and higher guaiacol peroxidase activity (Scebba *et al.*, 1998).

The level of lipid peroxidation can be related to the content of intracellular ROS, but also to the level of unsaturated fatty acid present in membranes, as singlet oxygen will react with the double bonds of PUFA to form lipid peroxides (Girotti & Kriska, 2004). Those lipid peroxides can then initiate a free radical-chain reaction that propagates through membranes unless a sufficient antioxidant level is present to terminate this process (Ledford & Niyogi, 2005; Fujisawa *et al.*, 2010). In our study, the higher level of lipid peroxidation found at 15°C could be related to the higher UI. At 8°C, it is possible that the relatively higher content in carotenoids prevented the formation of lipid peroxide. In fact, it was reported by Havaux (1998) that xanthophylls not only help to quench singlet oxygen and triplet chlorophylls, but they also increase membrane stability and decrease its susceptibility to lipid peroxidation. Moreover, when using a mutant of *Arabidopsis* deficient in β-carotene, Cazzaniga *et al.* (2012) observed a higher level of lipid peroxidation under high light and low temperature stress than in the wild type plant. Our higher β-car:Chl *a* and Lut+zea:Chl *a* ratios at 8°C agree with those findings. It was also shown that an increase in vitamin E (Fujisawa *et al.*, 2010) and proline content was related to a better protection against lipid peroxidation (Alia *et al.*, 1991; Alia *et al.*, 2001; Turan & Ekmekçi, 2011). The synthesis of both molecules was shown to be up-regulated at low temperatures (Xin & Li, 1993; Maeda *et al.*, 2006).

The lower ROS content and lipid peroxidation level in 8°C-acclimated cells, concomitant with high activities of catalase and ascorbate peroxidase are the evidences of a stronger protection against ROS effect in *C. reinhardtii* acclimated to

8°C. We may also suggest that this enhanced defence could protect the photosynthetic apparatus against the effect of Nf or Fd.

3.6.3. Accumulation of photoprotective pigments maintains PSII integrity

We observed an important decrease in pigment concentration, particularly the chlorophylls, when algae were grown at lower temperatures. Maxwell *et al.* (1994) noted a 5-fold decrease in the content of chlorophylls of *Chlorella* grown at 5°C compared to 27°C. It was reported that growing plants at low temperature is similar, in terms of excitation pressure on the photosynthetic apparatus, to growth under high light at normal temperature (Huner *et al.*, 1998) because in both growth conditions, the decrease in electron transport and rate of CO_2 assimilation creates a high excitation pressure on the PSII. To avoid detrimental effect of light on the photosystems, cells may reduce their content of light-harvesting Chl *a* and increase the content in photoprotective carotenoids (Maxwell *et al.*, 1995; Wilson & Huner, 2000; Huner *et al.*, 2003). In contrast to the results of Maxwell *et al.* (1994), we measured a lower Chl *a*:*b* ratio at 8 compared to 25°C. This was due to a higher decrease of Chl *a* content compared to Chl *b*. Absorption of light by Chl *b* primarily excites PSII over PSI, as a lower Chl *a*:*b* ratio is associated with the light-harvesting complex II (LHCII) (Falkowski & Raven, 2007). Moreover, Chl *b* is necessary for the assembly and stability of LHCII proteins (Preiss & Thornber, 1995; Tanaka & Tanaka, 2007), a stability that is probably compromised at 8°C. For cultures grown at low temperature, a lower epoxidation state, i.e. higher content in zeaxanthin+antheraxanthin relative to violaxanthin was reported previously (Huner *et al.*, 1996; Wilson & Huner, 2000). We were not able to calculate the epoxidation state of our cultures due to the overlapping peak of lutein and zeaxanthin on the HPLC chromatograph, but the decrease in violaxanthin relative to the Chl *a* content at 15

compared to 25°C tends to support the concept of a lower epoxidation state at this temperature.

The presence of a higher proportion of xanthophylls and β-carotene helps protect the photosynthetic apparatus against high excitation pressure (Maxwell *et al.*, 1994; Król *et al.*, 1999; Demmig-Adams & Adams III, 2006). At 8°C, the 1.4 higher ratio of β-car:Chl *a* and the 3.4X higher ratio of Lut+zea:Chl *a* indicate a stronger resistance to photoinhibition through heat dissipation by those two pigments. The similar maximal PSII quantum yield (Φ_M) obtained for the different acclimation temperatures indicates that the integrity of PSII was conserved at all temperatures. Xanthophylls also act as natural antioxidant to quench triplet chlorophyll and singlet oxygen (Niyogi *et al.*, 1997; Havaux, 1998; Müller *et al.*, 2001; Niyogi *et al.*, 2001; Passarini *et al.*, 2009). Even if these ROS were not directly measured in our study, the lower superoxide dismutase activity and lower DCF fluorescence measurements agree with the findings of the above-mentioned authors. We therefore suggest that these modifications in pigment composition help to maintain a reduced photosynthetic activity at 8°C without dramatically increasing oxidative stress and ensuing damage to chloroplast membranes. A higher carotenoid biosynthesis at 8°C is expected since their content is much higher at this temperature. The inhibition of phytoene desaturase by the same concentration of Nf and Fd would thus be relatively lower at 8 than at 25°C. This might also explain the absence of inhibition of photosynthesis by Nf and Fd herbicides at the lowest temperature.

3.6.4. Interdependence of photosynthesis and fatty acid desaturation

We measured a decrease in total lipid and most individual fatty acids (FA) at 8 compared to 25°C. A similar decrease in total lipids was recently observed with *Chlamydomonas globosa* acclimated to 10 compared to 25°C (Piepho *et al.*, 2012)

and these authors also found a similar FA composition at the two temperatures. The increase in protein per cell at low temperature resulted in a protein to lipid ratio 5X higher at 8 than at 25°C. We also observed very similar proportions among the different fatty acids among the three temperatures. Only the 15°C acclimated cells had a significantly higher and lower level of 18:3n-3 and 18:2n-6, respectively. This difference was not measured for the algae acclimated to 8°C, resulting in a UI that was similar between the 25°C and 8°C grown *Chlamydomonas*. Piepho *et al.* (2012) had also found a similar FA composition with *Chlamydomonas globosa* grown at 10°C and 25°C. The amount of 18:3n-3, the main FA found in chloroplast membranes, was also found in the same proportion of total FA between 6°C, 14°C, and 20°C, with a 15-17% increase at 9°C, in an Antarctic *Chlamydomonas* sp. (Teoh *et al.*, 2004). Those results are in contradiction with many studies that related the increase in membrane fluidity at low temperature to an increase in unsaturated fatty acids (Murata & Siegenthaler, 1998; Los & Murata, 2004; Guschina & Harwood, 2006). On the other hand, Arts and Kohler (2009) pointed out that the MUFA are the most important FA with respect to membrane fluidity. Yet, the MUFA relative content in 8°C acclimated cells were significantly higher than at 15 or 25°C in our study. The maintenance of membrane fluidity at all temperatures is particularly important for photosynthetic organisms, since the photosynthetic electron transport is dependent upon membrane-protein interaction (Morgan-Kiss & Dolhi, 2012). In our study, the inability of *C. reinhardtii* to substantially increase the degree of membrane fatty acid unsaturation may be responsible for the lower photosynthetic activity observed at low temperature. It has been found that PUFA help cells tolerate photoinhibition of photosynthesis at low temperature (Wada *et al.*, 1990; Gombos *et al.*, 1992). *Chlamydomonas* desaturase mutants which have decreased PUFA contents, demonstrate slower growth and reduced photosynthetic O_2 evolution (Sato *et al.*, 1995). Since the desaturation of fatty acids in plants and algae is an enzymatically-driven process (Moellering *et al.*, 2009), we suggest a decrease in their

activities when *C. reinhardtii* is grown at lower temperatures. Wada and Murata (1990) also showed that desaturation of 18-carbon fatty acids in the cyanobacteria *Synechocystis* required light and was inhibited by DCMU, a photosynthetic inhibiting herbicide, suggesting that photosynthetically-driven electron transport is necessary to the desaturation of fatty acids. Moreover, it was also shown that the electron transporter ferredoxin is required for the desaturation of fatty acids since it provides electrons to the desaturases (Schmidt & Heinz, 1990; Wada *et al.*, 1993). We demonstrated that the photosynthesis of *C. reinhardtii* was not photoinhibited at low temperature since its maximal quantum yield was not different among the three temperatures. However, the 36% lower operational quantum yield at 8 compared to 25°C, and the reduced activity of enzymes at low temperature, could reduce the fatty acid desaturase activities and explain the lower level of PUFA observed in *C. reinhardtii* grown at 8°C. Tuckey *et al.* (2002) discussed the importance of integrity, fluidity and permeability of membranes for the ability of cells to sequester lipophilic xenobiotics such as the fungicide propiconazole. In our study, it is possible that the membrane FA composition of 8°C grown *C. reinhardtii* might have decreased the penetration of Nf and Fd into the cells, thus preventing their inhibiting effect on photosynthesis.

3.7. CONCLUSIONS

We provide evidence that *C. reinhardtii* was able to grow at low temperatures after many physiological readjustments. At 15°C, the increase in photoprotective carotenoids and unsaturation of fatty acids helped to keep growth and photosynthesis rates comparable to those at 25°C. On the other hand, at 8°C, adjustments induced a lag phase of 96 to 120 hours where cells accumulated proteins and antioxidants to face the high light excitation pressure combined to slower enzyme activity.

Despite its lower growth and photosynthesis, *C. reinhardtii* showed a remarkable tolerance to phytoene desaturase inhibitors when acclimated to 8°C, a tolerance not found at higher temperature. It was demonstrated before that acclimation of barley to cold temperatures resulted in protecting it from pathogen infection (Plazek & Zur, 2003). This cross-resistance was attributable to a decreased membrane permeability and higher antioxidant activity. The acquired tolerance found in our study could be attributable to such factors induced by the acclimation to low temperature. The higher carotenoid content, resulting probably from a higher carotenoid biosynthesis, would mean more molecular targets for the phytoene desaturase inhibitors. Moreover, the uptake of Nf and Fd by the cells might have been different at the different temperature due the modifications in FA composition. Indeed, these hypotheses would require more investigations, such as measurement of the amount of herbicide incorporated into the cells or activity of phytoene desaturase enzyme. The measurements of the effect of norflurazon and fluridone on the oxidative stress, antioxidant enzyme activity, pigment and fatty acid content on 25°C, 15°C and 8°C acclimated *C. reihnardtii* will be further investigated in the companion paper and will permit to complete our understanding on the temperature-dependence of their toxicity.

Supplementary table 1. Mass fractions of fatty acids (μg·mg-1 DW) and total lipid (mg·mg-1 DW) in *Chlamydomonas reinhardtii* acclimated to three different temperatures.

Parameter	25°C			15°C			8°C		
14:0	**0.589**	(0.020)	b	**0.721**	(0.037)	a	**0.410**	(0.058)	b
15:0i	**0.153**	(0.022)	a	**0.024**	(0.041)	b	**0.073**	(0.027)	b
15:0ai	**0.201**	(0.015)	a	**0.185**	(0.033)	ab	**0.122**	(0.031)	b
15:0	**0.152**	(0.003)	a	**0.156**	(0.028)	a	**0.102**	(0.034)	a
16:0	**15.3**	(1.1)	a	**14.7**	(1.0)	a	**10.2**	(0.4)	b
16:1n-7	**0.513**	(0.076)	a	**0.563**	(0.036)	a	**0.456**	(0.105)	a
17:0	**0.468**	(0.037)	a	**0.490**	(0.048)	a	**0.289**	(0.027)	b
18:0	**1.735**	(0.106)	a	**1.755**	(0.162)	a	**1.171**	(0.064)	b
18:1n-9	**1.463**	(0.079)	a	**1.567**	(0.041)	a	**1.209**	(0.059)	b
18:1n-7	**9.4**	(0.7)	a	**9.9**	(0.5)	a	**5.9**	(0.3)	b
18:2n-6	**5.1**	(0.3)	a	**2.9**	(0.1)	b	**2.4**	(0.1)	c
18:3n-3	**28.8**	(1.5)	b	**34.3**	(2.1)	a	**18.2**	(0.6)	c
18:4n-3	**n.d**		a	**n.d**		a	**0.114**	(0.100)	a
20:0	**0.243**	(0.040)	a	**0.248**	(0.038)	a	**0.182**	(0.035)	a
20:1n-9	**0.149**	(0.027)	a	**0.159**	(0.004)	a	**0.152**	(0.017)	a
20:2n-6	**0.106**	(0.013)	a	**0.081**	(0.070)	a	**0.110**	(0.018)	a
20:3n-3	**n.d**		a	**n.d**		a	**0.096**	(0.010)	a
22:1n-9	**n.d**		a	**n.d**		ab	**0.115**	(0.031)	a
24:0	**n.d**		a	**n.d**		a	**0.209**	(0.027)	b
26:0	**0.052**	(0.045)	a	**0.030**	(0.052)	b	**0.164**	(0.012)	b
Total lipid	**1.723**	(0.083)	a	**1.54**	(0.07)	b	**0.717**	(0.029)	c

Data shown are means (n=3) with standard deviation in parenthesis. The letters represent the significant difference between the acclimation temperatures at $p < 0.05$.

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CHAPITRE IV

COMBINED EFFECT OF TEMPERATURE AND HERBICIDES ON *CHLAMYDOMONAS REINHARDTII*. 2. EFFECT OF BLEACHING HERBICIDES ON GROWTH, PHOTOSYNTHESIS, OXIDATIVE STRESS, PIGMENT AND FATTY ACID COMPOSITION

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Contribution des auteurs

Tout comme dans le chapitre précédent (les données du chapitre IV et du chapitre III proviennent des mêmes expériences), j'ai effectué toutes les expériences, analysé les résultats et rédigé l'article. Michael T. Arts a participé à l'extraction des lipides et l'identification des acides gras dans les différents traitements. Martin J. Kainz m'a permis d'utiliser le matériel de son laboratoire et d'y effectuer des expériences. Philippe Juneau a supervisé mes travaux, et participé à l'interprétation des résultats. Michael T. Arts, Martin J. Kainz et Philippe Juneau ont apporté leurs commentaires et leurs corrections lors de la rédaction et de la soumission de l'article.

4.1. RÉSUMÉ

Le norflurazon (Nf) et le fluridone (Fd) sont deux inhibiteurs de la phytoène désaturase qui sont abondamment utilisés comme herbicides pour le contrôle des herbes terrestres et des plantes aquatiques invasives. Ces herbicides se retrouvent dans les milieux aquatiques où ils peuvent affecter négativement les espèces non-ciblées, telles les algues. Leur toxicité envers les algues sera modifiée par des facteurs abiotiques comme l'intensité lumineuse, la température, le pH et les nutriments. L'effet de la basse température sur la toxicité du Nf et du Fd a une importance écotoxicologique parce que la température et ces herbicides vont affecter les mêmes processus physiologiques, c.-à-d. la biosynthèse des caroténoïdes. Nous avons démontré que le Nf diminuait la photosynthèse chez l'algue verte *Chlamydomonas reinhardtii* de façon plus importante à 15 qu'à 25°C, mais que le Fd avait un effet plus important à 25 qu'à 15°C. Les deux herbicides ne causaient aucune inhibition de la photosynthèse à 8°C. Cette étude démontre que la présence de 2,5 µM de Nf à 25°C a plus que doublé l'activité de la catalase, de l'ascorbate peroxydase et de la superoxyde dismutase comparativement au contrôle, mais, à 15°C, seule l'activité de la catalase a été augmentée. Cette augmentation a été accompagnée par une diminution de moitié des espèces réactives oxygénées. La peroxydation des lipides a été augmentée seulement lorsque les algues ont été exposées à 2,5 µM de Nf à 25°C. La plupart des pigments mesurés ont diminué de façon importante en présence de Nf et Fd à 15 et 25°C, en particulier la β-carotène qui tomba en dessous des limites de détection. Les acides gras mono-insaturés ont augmenté en même temps qu'une diminution dans les acides gras polyinsaturés était mesurée dans le traitement de 2.5 µM de Nf à 25°C. Les différences dans l'effet du Nf et du Fd sur la photosynthèse à 15 et 25°C peuvent être attribuables à la diminution marquée en caroténoïdes, qui, à leur tour, ont pu causer une diminution dans le contenu en acide gras polyinsaturés via l'inhibition du transport d'électrons photosynthétiques. À 8°C, l'absence d'effets apparents pourrait être due à une photoprotection accrue et/ou une diminution de l'absorption des herbicides par les algues.

Mots clés : algues, ascorbate peroxydase, catalase, caroténoïdes, fluorescence de la chlorophylle *a*, espèces réactives oxygénées, superoxyde dismutase, niveau d'insaturation

4.2. ABSTRACT

Norflurazon (Nf) and fluridone (Fd) are phytoene desaturase inhibitors and broadly used herbicides for the control of grasses and invasive aquatic weeds, respectively. These herbicides enter aquatic environments where they can negatively affect non-target plant species (e.g. algae). Their toxicity towards algae will be modified by abiotic factors such as light intensity, temperature, pH, and nutrients. The effect of low temperature on the toxicity of Nf and Fd is ecotoxicologically particularly important because both temperature and herbicides affect some of the same physiological process, i.e. carotenoid biosynthesis. We have demonstrated that Nf reduced photosynthesis in the green alga *Chlamydomonas reinhardtii* more strongly at 15 than at 25°C, while Fd showed stronger effects at 25 than at 15°C. Neither herbicide inhibited photosynthesis at 8°C. Here we show that the presence of 2.5 μM of Nf at 25°C increased the activity of catalase, ascorbate peroxidase and superoxide dismutase by > 2X when compared to the control, but, at 15°C, only catalase activity increased. This increase in catalase was accompanied by a 2X decrease in reactive oxygen species (ROS). Lipid peroxidation only increased when algae were exposed to 2.5 μM of Nf at 25°C. Most of the measured pigments decreased markedly in the presence of Nf and Fd at 15 and 25°C including β-carotene which fell below detection limits. Monounsaturated fatty acids (MUFA) increased concomitant with a decrease in polyunsaturated fatty acid (PUFA) in the 2.5 μM Nf treatment at 25°C. Differences in the effect of Nf and Fd on photosynthesis at 15 and 25°C can be associated with the marked decrease in carotenoids, which, in turn, caused a decrease in PUFA through the inhibition of photosynthetic electron transport. At 8°C the apparent lack of inhibitory effects could be due to enhanced photoprotection and/or decreased uptake of herbicides by the alga.

Keywords: algae, ascorbate peroxidase, catalase, carotenoids, chlorophyll *a* fluorescence, reactive oxygen species, superoxide dismutase, unsaturation index

4.3. INTRODUCTION

Norflurazon (Nf) and fluridone (Fd) are bleaching herbicides that are used worldwide. Their mode of action is the inhibition of the phytoene desaturase, an enzyme essential for the conversion of the colorless phytoene in phytofluene and ζ-carotene, both precursors of important carotenoids such as β-carotene and lutein (Bartels & Watson, 1978; Arias *et al.*, 2005). The resulting depletion in photoprotective carotenoids leads to chlorophyll oxidation and thus foliage bleaching in the affected plants (Dalla Vecchia *et al.*, 2001). Nf is used as a pre- or post-emergent herbicide for the control of grasses in crops such as cotton, peaches and cherries. Concentrations up to 360 $\mu g\ L^{-1}$ have been reported in surface water run-off downslope of a citrus plantation (Wilson *et al.*, 2007). Other monitoring studies in lakes and streams have reported a maximum concentration of 11.5 $\mu g\ L^{-1}$ (Senseman *et al.*, 1997). Fd has been used extensively in the U.S. for the treatment of water bodies invaded by weed species such as *Hydrilla* spp. or Eurasian watermilfoil. Fd is usually applied directly to water by surface spraying, underwater subsurface injection, or by broadcasting herbicide-laced pellets. Concentrations up to 150 $\mu g\ L^{-1}$ per annual growth cycle of the intended target plant are allowed in water bodies in the U.S. (HSDB, 2013b).

When applied to the environment, herbicides such as Nf and Fd can be in interaction with abiotic factors such as temperature, light intensity, pH, nutrients, and other contaminants (Di Baccio *et al.*, 2002; Puri *et al.*, 2009; Fischer *et al.*, 2010). Temperature, for example, affects many processes also targeted by herbicides including photosynthesis, pigment biosynthesis, enzyme activity, and protein and lipid metabolism (Raven & Geider, 1988; Huner *et al.*, 1998). Temperature can thus exert a strong influence on the toxicity of herbicides. Chalifour *et al.* (2013) showed that Nf reduced photosynthesis more strongly at 15 than at 25°C, while Fd showed

stronger effects at 25 than at 15°C. Surprisingly, both herbicides did not cause any inhibition of photosynthesis at 8°C. Here we examined the effect of Nf and Fd on growth, oxidative stress, pigment and lipid composition of *C. reinhardtii* acclimated to 8, 15 and 25°C to better understand the toxicity of these herbicides with respect to photosynthesis.

4.4. MATERIAL AND METHODS

4.4.1. Algal culture and growth conditions

Chlamydomonas reinhardtii (G.M. Smith) wild type strain (CC125) was obtained from the *Chlamydomonas* Resource Center, University of Minnesota, USA. Algae were cultivated semi-continuously in Erlenmeyer flasks containing 300 mL of High Salt Medium (HSM) (Sueoka *et al.*, 1967) at a pH of 6.8. The cultures were acclimated to three different temperatures (8, 15 and 25°C) for at least three weeks to achieve constant growth rates. The light intensity in the growth chambers was kept at an irradiance of 100 µmol photons m^{-2} s^{-1} provided by a combination of incandescent bulbs (Philips 60W) and white fluorescent lamps (Philips F72T8/TL841/HO, USA) with a 14:10 h light:dark cycle.

Algal cells were collected during the exponential growth phase and transferred, at an initial density of $2x10^5$ cells mL^{-1}, into 500 mL Erlenmeyer flasks containing 300 mL HSM medium. The cells were then exposed to 2 concentrations of Nf (1.25 and 2.5 µM) and one concentration of Fd (1.25 µM), both dissolved in dimethyl sulfoxide (DMSO). The control flasks were exposed to DMSO (0.25%). Herbicides (Cat no. Nf : PS-1044; Fd : Fluka 45511) and DMSO (Cat no. 472301) were purchased at Sigma-Aldrich (Oakville, Canada). Each treatment was replicated three times at each temperature.

4.4.2. Cell growth and photosynthesis

After dilution, growth rate ($\mu_{\mu m3}$) was measured over a period of 96 h using a Coulter Counter particle analyser (Multisizer 3, Beckman Coulter Inc., Fullerton, USA), and all physiological measurements were done 96 h after the transfer in the

new medium. Culture growth rates were estimated from the slopes of the linear regressions of the natural logarithm transformed cell biovolume (μm^3 ml^{-1}) versus time.

The maximum (Φ_M) and operational PSII quantum yields (Φ'_M) were evaluated from the fluorescence induction curves obtained with a Water-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as in Chalifour *et al.* (2014).

4.4.3. Cell size, complexity and reactive oxygen species (ROS) content

The effect of Nf and Fd on the cell size and complexity was analysed by flow cytometry (FACScan, Beckton Dickinson, Mississauga, Canada). According to Shapiro (2003), the forward light scatter (FSC) detected by the flow cytometer is indicative of the cell size while the side light scatter (SSC) is correlated with the granular structures in the cytoplasm, cellular intrusions and membrane ruffling, therefore to cell complexity. The ROS level was also measured by flow cytometric analysis using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA) (Cat no. D399, Invitrogen, Burlington, Canada) (Peperzak & Brussaard, 2011). A 10 mM H_2DCFDA solution was prepared in DMSO. Algal culture (1 mL) was incubated with 20 µL H_2DCFDA for 25 min, after which the sample was analysed. All measurements on the flow cytometer were performed at the FL-1 parameter. The green fluorescence emitted by DCF (emission wavelength = 530 nm) was excited with Argon ion laser (excitation wavelength = 488 nm).

4.4.4. Antioxidant enzymatic activity

Algae were harvested and proteins were extracted as in Chalifour *et al.* (2014). Protein concentration was determined using the Bradford (1976a) method using bovine albumin serum as a standard. Catalase (CAT) activity was determined by measuring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 2 min as in Aebi (1984), with modifications detailed in Chalifour *et al.* (2014). Ascorbate peroxidase (APX) activity was determined by measuring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 3 min (see Nakano and Asada (1981) and Chalifour *et al.* (2014)). Superoxide dismutase (SOD) activity was determined in microplates by measuring the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) as in Beauchamp and Fridovich (1971), with modifications detailed in Chalifour *et al.* (2014).

4.4.5. Lipid peroxidation

Lipid peroxidation level was determined in terms of thiobarbituric acid reactive substances (TBARS) and expressed as equivalent of malondialdehyde (MDA) according to the method of Heath and Packer (1968), and detailed in Chalifour *et al.* (2014).

4.4.6. Pigments

Algae cultures (10 mL) were filtered onto 0.8 µm membrane filters (Whatman nucleopore, Piscataway, USA), and extracted according to Chalifour *et al.* (2014). All pigments were analysed by HPLC following the method of García-Plazaola (1999). Pigments were identified and quantified by comparison of retention times and peak

areas with those of chlorophylls (PPS-CHLA and PPS-CHLB, DHI, Horsholm, Denmark), xanthophylls (PPS-ANTH, PPS-LUTE, PPS-NEOX, PPS-VIOL and PPS-ZEAX, DHI, Horsholm, Denmark) and β-carotene (Cat no. C4582, Sigma-Aldrich, Oakville, Canada).

4.4.7. Total lipids and fatty acids

For analysis of total lipids and fatty acids (FA), cultured algae (100 mL) were filtered on pre-combusted, pre-weighted GF/F filters (Whatman, Piscataway, USA), stored in the dark at -80°C and subsequently freeze-dried and weighed. Lipids were extracted and analyzed as in Chalifour *et al.* (2014). The unsaturation index (UI) was calculated as the sum of the percentage of each measured fatty acid multiplied by the number of double bonds present in each FA molecule.

4.4.8. Statistical analysis

For comparison of parameter values among the three test temperatures we used ANOVA and Tukey-Kramer post-hoc analysis using JMP ver. 6.0 software (SAS Institute, Cary, NC, USA). Differences were considered significant at $p < 0.05$.

4.5. RESULTS

Within 4 d, cells of *C. reinhardtii* acclimated to the three temperatures and exposed to Nf or Fd divided and increased in size, as shown by the FSC parameter of the flow cytometer (Table 4.1). We thus measured growth rate ($\mu_{\mu m^3}$) as the increase in total biovolume of cell cultures. *C. reinhardtii* $\mu_{\mu m^3}$ was not affected by herbicide treatment, except for cells exposed to 1.25 μM Nf at 8°C, which had a higher $\mu_{\mu m^3}$ than the control. Exposure to both herbicides also increased the proportion of tetrads at 8°C; up to 2X as many tetrads in the Fd (1.25 μM) treatment (data not shown). This likely explains the higher cell complexity (SSC parameter) detected by the flow cytometer.

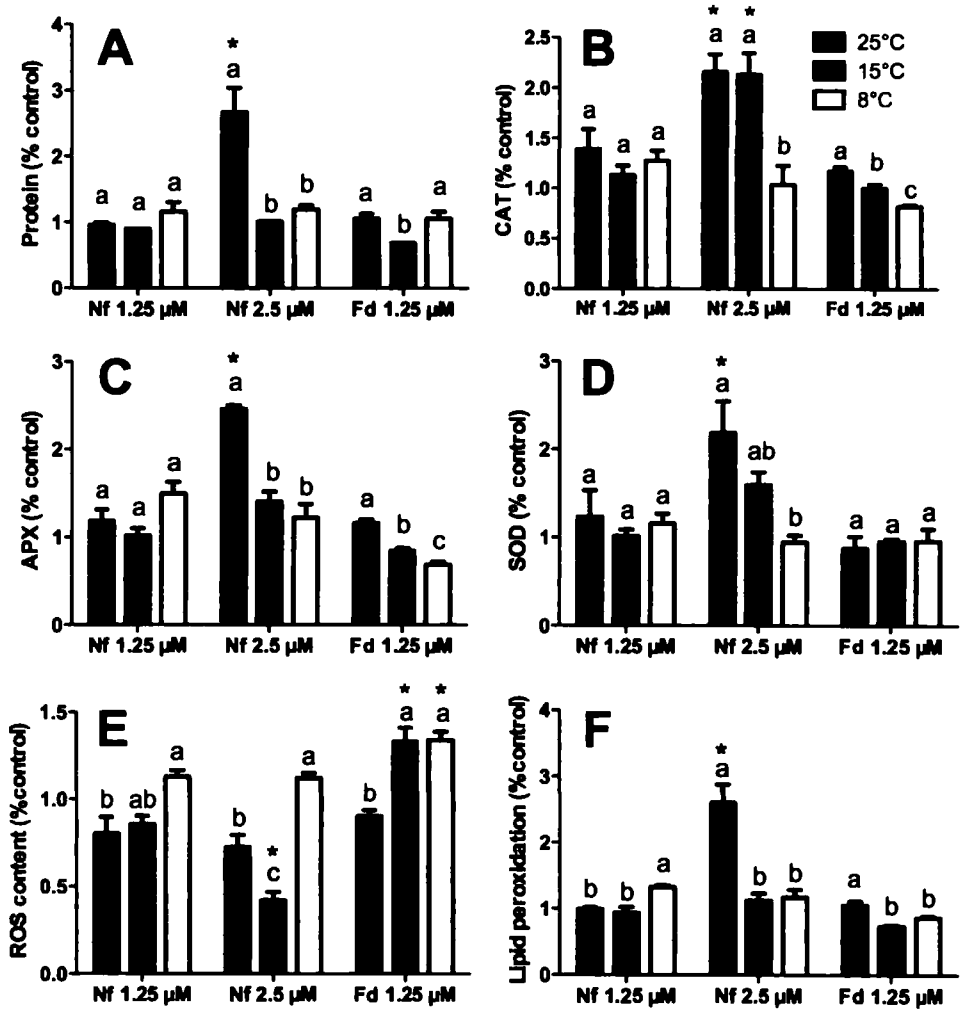
As for photosynthesis, the maximal PSII quantum yield (Φ_M) was significantly reduced by 57 and 98% in the 25 and 15°C acclimated cells, respectively, after exposure to 2.5 μM of Nf. The operational PSII quantum yield (Φ'_M) was even more decreased by Nf (2.5 μM), with a 72% lower Φ'_M in the 25°C cells and an almost complete inhibition in the 15°C cells. Φ_M and Φ'_M were only affected by fluridone at 25°C. Both herbicides did not significantly affect photosynthesis of *C. reinhardtii* acclimated at 8°C.

The different herbicide treatments increased the per unit dry weight protein content of the cells, but only in the 25°C treatment exposed to 2.5 μM Nf (Fig. 3.1.A). There was, however, > 2X more CAT activity with Nf (2.5 μM) in the 25 and 15°C acclimated cells, and ~ 2.5X more APX activity and 2.2 X more SOD activity in the 25°C cells. The APX and SOD activities did not differ between cells cultured at 8 and 15°C (Figs 4.1. C-D).

Table 4.1. Growth rate ($\mu_{\mu m3}$), cell size and granularity, and maximal (Φ_M) and operational (Φ'_M) quantum yields or photosynthesis (in % of control without herbicides) of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf; at 1.25 or 2.5 µM) or fluridone (Fd; at 1.25 µM) for 4 d.

Parameter	Treatment	25°C				15°C				8°C			
$\mu_{\mu m3}$	**Nf 1.25 µM**	**82%**	±	10%	c	**120%**	±	6%	b	**177%**	±	23%	*a
	Nf 2.5 µM	**95%**	±	2%	b	**94%**	±	4%	b	**162%**	±	29%	a
	Fd 1.25 µM	**88%**	±	11%	c	**115%**	±	5%	b	**161%**	±	8%	a
FSC (size)	**Nf 1.25 µM**	**125%**	±	5%	*a	**112%**	±	1%	*b	**101%**	±	2%	c
	Nf 2.5 µM	**134%**	±	2%	*a	**129%**	±	4%	*a	**107%**	±	1%	*b
	Fd 1.25 µM	**132%**	±	3%	*a	**104%**	±	1%	b	**109%**	±	1%	*b
SSC (granularity)	**Nf 1.25 µM**	**124%**	±	2%	*b	**136%**	±	4%	*a	**102%**	±	2%	c
	Nf 2.5 µM	**148%**	±	4%	*a	**142%**	±	2%	*a	**105%**	±	2%	b
	Fd 1.25 µM	**129%**	±	3%	*a	**113%**	±	4%	*b	**99%**	±	1%	c
Φ_M	**Nf 1.25 µM**	**80%**	±	13%	ab	**63%**	±	10%	*b	**100%**	±	3%	a
	Nf 2.5 µM	**43%**	±	6%	*b	**2%**	±	4%	*c	**99%**	±	2%	a
	Fd 1.25 µM	**84%**	±	11%	b	**103%**	±	4%	a	**96%**	±	5%	ab
Φ'_M	**Nf 1.25 µM**	**66%**	±	23%	*ab	**59%**	±	7%	*b	**95%**	±	4%	a
	Nf 2.5 µM	**28%**	±	8%	*b	**3%**	±	2%	*c	**91%**	±	1%	a
	Fd 1.25 µM	**77%**	±	4%	*c	**102%**	±	2%	a	**88%**	±	6%	b

Data shown are means (n=3) with standard deviation. The asterisk (*) represents the significant difference with control condition (no herbicide) and the letters represent the significant difference between the acclimation temperatures at $p < 0.05$.



Figures 4.1. Protein, antioxidant enzyme activity, reactive oxygen species and lipid peroxidation level, expressed in % of control without herbicide, of *C. reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf: at 1.25 and 2.5 µM) or fluridone (Fd; at 1.25 µM) for 96 h. Data shown are means (n=3) with standard deviation. The asterisk (*) represents the significant difference with control condition (no herbicide) and the letters the significant difference between the acclimation temperatures at $p < 0.05$.

When compared to control without herbicide, we observed 50% less ROS and 30% more ROS in the Nf (2.5 μM) and Fd (1.25 μM) treatments, respectively, at 15°C (Fig 4.1.E). The amount of ROS measured by the H_2DCFDA probe was also 34% higher in the Fd (1.25 μM) treatment at 8°C. The lipid peroxidation level was increased by 2.6X with Nf (2.5 μM) at 25°C, but was not different from the controls at the other temperature or herbicide treatments (Fig 4.1.F).

Most pigments decreased markedly with Nf and Fd at 15 and 25°C (Table 4.2). The β-carotene content was particularly affected, with a virtual disappearance of this carotenoid when algal cells were exposed to 2.5 μM Nf. Chlorophyll *a* decreased by 50 to 60% while chlorophyll *b* decreased by 30 to 40% in the highest Nf treatment. The xanthophylls also decreased as a result of herbicide exposure, with ~ 50% less neoxanthin and lutein+zeaxanthin with Nf (2.5 μM) exposure. Most pigments were more affected by fluridone at 25 than at 15°C, but pigments were largely unaffected by herbicide exposure at 8°C. There was, however, a small, but not significant, decrease in β-carotene at 8°C with both concentrations of Nf.

The herbicide treatments, at both 25 and 15°C, caused a decrease in *C. reinhardtii*'s total lipid content. This was particularly evident in the 2.5μM Nf treatment, where 45 and 37% less lipid (on a mg lipid·mg DW^{-1} basis) was recorded at 25 and 15°C, respectively (Supplementary table 2). There was also a 7X increase 15:0i FA in cells exposed to 2.5 μM of Nf at 15°C (Supplementary table 2). A concomitant decrease in MUFA and PUFA was also observed (Fig 4.2. D-E). The fatty acid 18:2n-6 was particularly decreased with the three herbicides treatments at 25°C and Nf (2.5 μM) at 15°C. We noticed a 2.7X and 6.7X increase in 26:0 at 25°C and 15°C, respectively, when algae were exposed to the highest concentration of Nf (Supplementary table 2). At 8°C, the FA composition of *C. reinhardtii* was the same as in the control treatment. The decrease in some unsaturated fatty acids resulted in lower total omega-3 and omega-6 FA contents, particularly at 25°C and when exposed to the higher Nf concentrations (2.5 μM) (Fig 4.2. A-B).

Table 4.2. Pigment content (in % of control) of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf; at 1.25 and 2.5 μM) or fluridone (Fd; at 1.25 μM) for 96 h.

Parameter	Treatment	25°C				15°C				8°C			
Chlorophyll *a*	**Nf 1.25 μM**	**64%**	±	10%	*b	**68%**	±	6%	*b	**102%**	±	10%	a
	Nf 2.5 μM	**42%**	±	4%	*b	**49%**	±	6%	*b	**106%**	±	14%	a
	Fd 1.25 μM	**55%**	±	8%	*c	**90%**	±	5%	b	**113%**	±	6%	a
Chlorophyll *b*	**Nf 1.25 μM**	**78%**	±	3%	*b	**85%**	±	5%	b	**103%**	±	3%	a
	Nf 2.5 μM	**61%**	±	4%	*b	**68%**	±	7%	*b	**108%**	±	3%	a
	Fd 1.25 μM	**70%**	±	3%	*c	**103%**	±	6%	b	**118%**	±	6%	a
β-carotene	**Nf 1.25 μM**	**41%**	±	10%	*b	**20%**	±	1%	*b	**88%**	±	16%	a
	Nf 2.5 μM	**0%**	±	0%	*b	**0%**	±	0%	*b	**77%**	±	12%	a
	Fd 1.25 μM	**27%**	±	5%	*b	**62%**	±	13%	*b	**99%**	±	20%	a
Violaxanthin	**Nf 1.25 μM**	**38%**	±	23%	*a	**45%**	±	27%	a	**127%**	±	53%	a
	Nf 2.5 μM	**16%**	±	9%	*b	**12%**	±	12%	b	**89%**	±	39%	a
	Fd 1.25 μM	**29%**	±	18%	*b	**107%**	±	18%	a	**41%**	±	3%	b
Antheraxanthin	**Nf 1.25 μM**	**92%**	±	24%	ab	**72%**	±	10%	b	**145%**	±	33%	a
	Nf 2.5 μM	**63%**	±	56%	a	**43%**	±	39%	a	**159%**	±	62%	a
	Fd 1.25 μM	**79%**	±	17%	b	**77%**	±	9%	b	**158%**	±	6%	a
Lutein+zeaxanthin	**Nf 1.25 μM**	**62%**	±	4%	*b	**51%**	±	3%	*b	**91%**	±	7%	a
	Nf 2.5 μM	**54%**	±	6%	*b	**45%**	±	4%	*b	**88%**	±	6%	a
	Fd 1.25 μM	**53%**	±	2%	*c	**76%**	±	4%	*b	**120%**	±	7%	*a
Neoxanthin	**Nf 1.25 μM**	**53%**	±	7%	*b	**51%**	±	5%	*b	**106%**	±	8%	a
	Nf 2.5 μM	**41%**	±	6%	*b	**47%**	±	9%	*b	**103%**	±	21%	a
	Fd 1.25 μM	**46%**	±	8%	*c	**84%**	±	6%	b	**118%**	±	4%	a

Data shown are means (n=3) with standard deviation. The asterisk (*) represents the significant difference with control condition (no herbicide) and the letters the significant difference between the acclimation temperatures at $p < 0.05$.

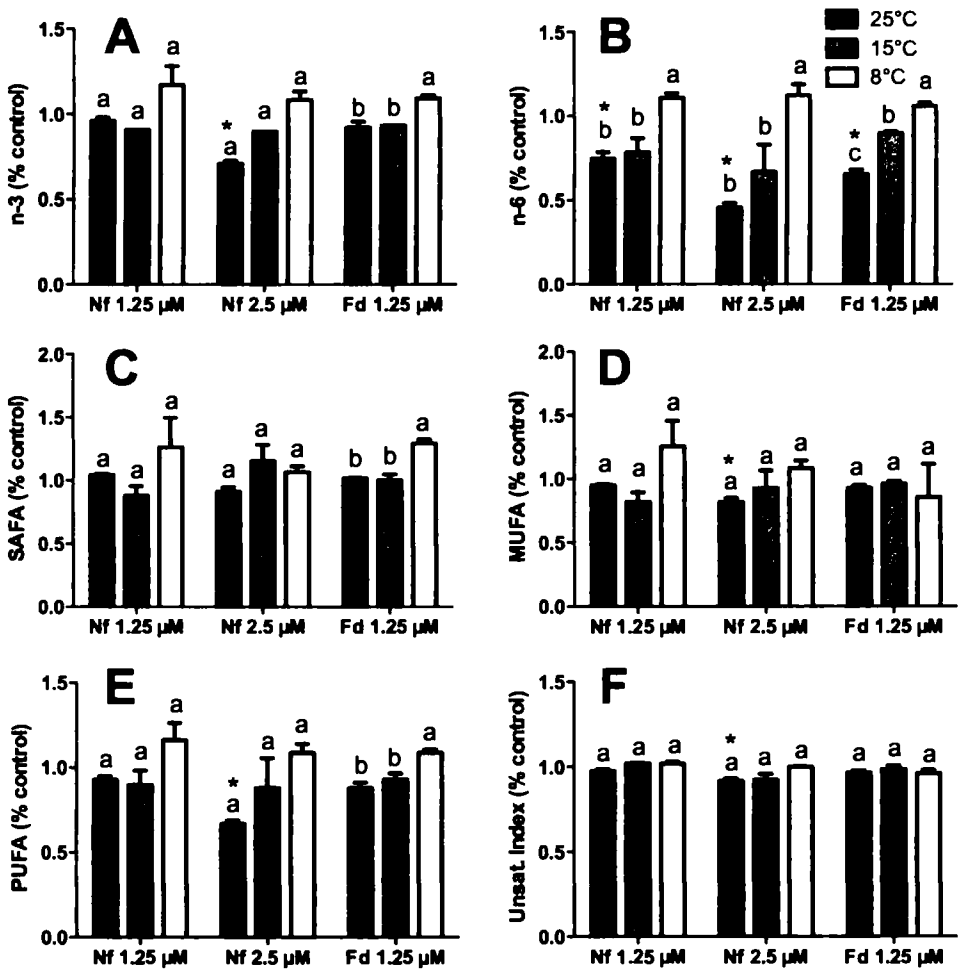


Figure 4.2. Sum of omega-3 and omega-6 fatty acids (A, B), saturated fatty acids (C), monounsaturated fatty acids (D), polyunsaturated fatty acids (E) and unsaturation index (F) (expressed in % of control without herbicides) of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (Nf: at 1.25 and 2.5 µM) or fluridone (Fd; at 1.25 µM) for 96 h. Data shown are means (n=3) with standard deviation. The asterisk (*) represents the significant difference with control condition (no herbicide) and the letters the significant difference between the acclimation temperatures at $p < 0.05$.

4.6. DISCUSSION

Norflurazon (Nf) and fluridone (Fd) are both phytoene desaturase inhibitors that interfere with carotenoids biosynthesis (Bartels & Watson, 1978). The depletion of carotenoids leads to chlorophyll oxidation and oxidative damage of photosynthetic components, which can explain the lower Φ_M and Φ'_M values we observed in algal cells exposed to these herbicides. *C. reinhardtii* acclimated to 25°C was less sensitive to Fd (1.25 µM) than to Nf at the same concentration, which might be due to differences in the location of the herbicide binding site on the enzyme phytoene desaturase (Chamovitz *et al.*, 1993). Nf has been shown to displace the cofactor plastoquinone on the binding site of phytoene desaturase (Breitenbach *et al.*, 2001), but the exact mode of action of Fd has not been found yet. Differences in the tolerance of plants to both herbicides have been measured previously. For example, Michel *et al.* (2004) found a growth-based EC_{50} of 0.458 µM for Nf and 0.096 µM for Fd in the duckweed *Lemna paucicostata*. Also, a tobacco (*Nicotiana* sp.) plant had a carotenoid content based EC_{50} of 58.1 nM for Nf and 6.6 nM for Fd. (Wagner *et al.*, 2002). The higher sensitivity of aquatic and terrestrial plants to Fd, compared to Nf, might be due to differences in the uptake of Fd, which is mainly absorbed from water by the shoots and roots of the plants. Fd is known to be less toxic to algae compared to higher plants (Arnold, 1979; Kegley *et al.*, 2011). The lower toxicity may also be related to the fact that weak acids are up to 1000 X more toxic to aquatic plants such as *Lemna minor* than algae such as *Pseudokirchneriella subcapitata* (Cedergreen & Streibig, 2005). Fd is considered as a very weak acid while Nf as a neutral molecule (PPDB, 2011).

Although Nf strongly or completely inhibited photosynthesis at the two highest temperatures, no significant decrease in cell volume, used here as proxy for algal growth, was observed. This is in agreement with previous findings of only a

slight effect on cell growth when *C. reinhardtii* was exposed to Nf 10 μM, a concentration 4X higher than the highest concentration used in this study (2.5 μM) (Nestler *et al.*, 2012). Nestler *et al* (2012) also measured an increase in ATP production with increasing Nf concentrations. Considering the strong decrease in photosynthetic efficiency with this herbicide, we suggest that, over a course of 4 d, *C. reinhardtii* was using its energy reserves to maintain a similar growth rate in cultures exposed to Nf or Fd, compared to the controls. This hypothesis is corroborated by the observation that dry weight per unit cell biovolume decreased by 25-40% in cells exposed to 2.5 μM of Nf at 15 and 25°C, compared to control (data not shown), Jahnke and Mahlmann (2010) also observed a decrease in dry weight per unit biovolume when *Phormidium autumnale* was grown under low light compared to high light, a condition that also decreased the growth rate and photosynthesis of various phytoplankton species (Deblois *et al.*, 2013).

The higher cell volume and cell complexity of cells exposed to Nf and Fd is related to their lower cell division rates, and thus increased proportion of daughter cells enclosed in mother cells (tetrads) in both herbicide treatments. This lower division rate may be the consequence of the observed inhibition of photosynthesis by Nf and Fd. Due to the effects of both herbicides on photosynthesis, cells took more time to reach a size at which cell division normally occurs; thus the proportion of mother cells remained unusually high. A previous study demonstrated that low temperature, as the case for herbicide exposure, increases the length of the cell cycle resulting in a decreased rate of cell division (Chalifour *et al.* 2014). Similarly, the effect of herbicides increased the pre- or post-commitment period, resulting in additional commitment points and consequently in more daughter cells enclosed in a mother cell. This delay in cell division may be a defence strategy and/or reflect increased cellular requirements for protein synthesis related to detoxification and/or reparation of cell damage. However, the concomitant decrease in dry weight reflects the probable depletion of energy reserves in absence of photosynthesis, in addition to

the lower content of important pigments including chlorophylls and carotenoids. Similarly, when *Chlorella vulgaris* was exposed to the triazine herbicide terbutryn, Rioboo *et al.* (2002) observed a higher proportion of mother cells than daughter cells in treated cultures, compared to control ones. They attributed the increase in cell size and complexity in presence of terbutryn to the decrease in G1 phase progression of the cell cycle.

We suggest that the observed depletion of carotenoids and increase in ROS triggered the expression of anti-oxidative enzyme activity, especially with exposure to high concentrations of Nf (i.e. 2.5 μM) at 25°C. Nestler *et al.* (2012) also suggested that an anti-oxidative defence mechanism was induced by Nf, thus protecting *C. reinhardtii* from strong oxidative stress. The higher antioxidant enzyme activity probably helped the cells to decrease their ROS content, as shown by the lower DCFDA fluorescence. Alternatively, the lower (although not significantly) activity of CAT, APX and SOD with Fd at 8°C is probably associated with the increase in ROS content at low temperature.

As expected, carotenoid content of *C. reinhardtii* was strongly reduced in the presence of these two herbicides. It is noteworthy that, with a similar decrease in most pigments with the Nf treatment at 15 and 25°C, Nf had much more effect on Φ_M and Φ'_M at 15 than at 25°C. This observation is in line with the important role that carotenoid pigments play at low temperature in terms of protecting the photosynthetic apparatus (Huner *et al.*, 1998). The strong reduction in Φ_M at 15°C also confirmed the importance of β-carotene in maintaining the stability of PSII as suggested by Trebst and Depka (1997). Low temperature has a similar effect on photosynthesis as high light stress in terms of excitation pressure on the PSII (Huner *et al.*, 1998). Thus, the depletion in carotenoids at low temperature has a stronger effect on photosynthesis than at higher temperatures, resulting in stronger photoinhibition. Wagner *et al.* (2002) also showed a drastic decrease in D1 protein content - another sign of photoinhibition - of tobacco leaves treated with Nf.

Nf has also been shown to affect the lipid and FA composition of plants (Abrous *et al.*, 1998; Di Baccio *et al.*, 2002). These authors suggested that Nf could affect fatty acid desaturases in addition to phytoene desaturase resulting in a decrease in the 18:3/18:2 ratio (Abrous-Belbachir *et al.*, 2009). In our study, the 18:3/18:2 ratio increased because 18:2 decreased more than 18:3 in the presence of Nf. It is possible, as suggested by Abrous-Belbachir *et al.* (2009), that Nf inhibited some fatty acid desaturases. However, we propose that the effect of Nf exposure on fatty acid desaturation was an indirect consequence of the inhibition of photosynthesis because, in addition to ATP and NADPH, fatty acid desaturases also depend on ferredoxins as electron donors (Schmidt & Heinz, 1990). As measured in our study, the strong inhibition of Φ'_M indicates fewer electrons going through PSI to ferredoxin. Moreover, the lipid peroxidation level was increased for algae acclimated to 25°C and exposed to the higher Nf concentration (2.5 µM), where the highest decrease in FA was observed. The lower content in 18-carbon fatty acids could thus be due to the 2.7X higher peroxidation of lipids by ROS at 25°C. Whether mono- or polyunsaturated fatty acids were decreased by fatty acid desaturases or indirectly by decreased photosynthesis remains to be elucidated. However, considering the importance of MUFA and PUFA in the stability of the required photosynthetic components (Sato *et al.*, 1996), their decrease by Nf and Fd might also contribute to the observed inhibition of Φ_M and Φ'_M.

There was only a slight decrease of Φ'_M, β-carotene and lutein+zeaxanthin in *C. reinhardtii* when exposed to these herbicides at 8°C. The higher tolerance of 8°C-grown *C. reinhardtii* photosynthesis to Nf was also observed with higher concentration of this herbicide (5 µM; data not shown). The absence of inhibitory effects of bleaching herbicides at 8°C may be explained by increased phytoene desaturase content or activity. This increase has been shown in response to high light (Huang *et al.*, 2002) and it is also known that low temperature induce similar modifications in pigments (Maxwell *et al.*, 1994). For example, the mutant FD5 of

Synechococcus, that had a deletion mutation in the promoter region of the gene encoding for phytoene desaturase, the *pds* gene, contained more total carotenoids, and had a higher content in zeaxanthin than the wild type, and was also more resistant to Nf and Fd (Chamovitz *et al.*, 1993). It is also known that Nf is interacting with the cofactors NADP or plastoquinone for the phytoene desaturase enzyme (Breitenbach *et al.*, 2001). Low temperature has been associated with an important increase in plastoquinone (Griffith *et al.*, 1984; Gálvez-Valdivieso *et al.*, 2010). It is thus possible that at low temperature the effect of Nf is less important since there is a competition with the larger amount of plastoquinone available.

Maeda *et al.* (2005) provided experimental evidence that growth of *Synechocystis* sp. was inhibited by 5 μM of Nf only in a mutant that could not synthesise tocopherols. These authors concluded that tocopherol and carotenoids had an overlapping role in protection of the photosynthetic apparatus. Tocopherol was shown to be up-regulated at low temperature (Leipner *et al.*, 1997), which might explain why no significant increase in lipid peroxidation was detected in our experiments when *C. reinhardtii* was acclimated to 8 or 15°C.

Kent and Caux (1995) found a strong relationship between the bioaccumulation of the lipophilic insecticide fenitrothion and algal lipid content; *C. reinhardtii* with > 6X the total lipid content of *C. segnis* also accumulated 3X the amount of fenitrothion. In our study, *C. reinhardtii* acclimated to 15 and 25°C had ~ 2.4X more lipid per dry mass than cells acclimated to 8°C (Chalifour *et al.*, 2013). We also observed that Fd had more effect on photosynthesis at 8 and 25°C than at 15°C, while Nf had more effect at 15°C. This could also be linked to the octanol-water partition coefficient of both herbicides, and which is higher for Fd (3.16) than Nf (2.30) (Sabljic *et al.*, 1995). The octanol-water partition coefficient is known to influence the adsorption and penetration of herbicide inside cells (Dosnon-Olette *et al.*, 2011; Sun *et al.*, 2012). Moreover, the physical properties of the membrane can also influence the translocation and distribution of toxicants having various

lipophilicity (Baynes 2004). Thus, the proportion of proteins, the presence of unsaturated fatty acids and other antioxidants (such as tocopherols) will affect the movement of macromolecules across membranes. Under the same experimental conditions as in the present study, we demonstrated that 8°C-grown cells had a higher carotenoid content, lower unsaturation level and higher protein-to-lipid ratio (Chalifour *et al.*, 2013) compared to the ones growing at higher temperature. It is interesting to note that low temperature-induced protective systems (such as photoprotective carotenoids and ROS-quenching enzymes) could also enhance the tolerance of cells to a chemical stressor such as bleaching herbicides. In addition, carotenoids such as β-carotene, lutein and zeaxanthin have been shown to participate in the stabilization of thylakoid membranes (Havaux, 1998), therefore protecting against harmful action of environmental stressors. Our findings that some herbicides can have a greater effect at a higher temperature is a major concern for water quality issues around the world, especially considering the increase in water temperature expected with global warming.

Supplementary table 2. Fatty acid and total lipids (in % of control) of *Chlamydomonas reinhardtii* acclimated to three different temperatures and exposed to norflurazon (NF; at 1.25 and 2.5 µM) or fluridone (Fd; at 1.25 µM) for 96 h.

Parameter	Treatment	25°C				15°C				8°C			
14:0	**Nf 1.25 µM**	**133%**	±	11%	*a	**86%**	±	23%	a	**151%**	±	83%	a
	Nf 2.5 µM	**119%**	±	11%	a	**116%**	±	33%	a	**95%**	±	18%	a
	Fd 1.25 µM	**137%**	±	4%	*a	**100%**	±	19%	b	**104%**	±	5%	b
15:0i	**Nf 1.25 µM**	**32%**	±	29%	*a	**156%**	±	140%	a	**98%**	±	62%	a
	Nf 2.5 µM	**109%**	±	19%	b	**702%**	±	119%	*a	**72%**	±	27%	b
	Fd 1.25 µM	**58%**	±	19%	b	**378%**	±	66%	a	**140%**	±	32%	b
15:0ai	**Nf 1.25 µM**	**53%**	±	46%	a	**74%**	±	21%	a	**98%**	±	40%	a
	Nf 2.5 µM	**96%**	±	17%	a	**118%**	±	23%	a	**81%**	±	10%	a
	Fd 1.25 µM	**85%**	±	9%	b	**91%**	±	4%	b	**115%**	±	6%	a
15:0	**Nf 1.25 µM**	**130%**	±	27%	a	**102%**	±	14%	a	**122%**	±	117%	a
	Nf 2.5 µM	**99%**	±	19%	a	**157%**	±	58%	a	**0%**	±	0%	b
	Fd 1.25 µM	**40%**	±	69%	b	**97%**	±	19%	b	**209%**	±	16%	a
16:0	**Nf 1.25 µM**	**105%**	±	1%	a	**88%**	±	13%	a	**119%**	±	26%	a
	Nf 2.5 µM	**89%**	±	7%	a	**104%**	±	27%	a	**108%**	±	9%	a
	Fd 1.25 µM	**102%**	±	2%	b	**96%**	±	3%	b	**118%**	±	5%	a
16:1n-7	**Nf 1.25 µM**	**146%**	±	29%	a	**95%**	±	10%	a	**143%**	±	63%	a
	Nf 2.5 µM	**153%**	±	11%	*a	**183%**	±	64%	a	**109%**	±	0%	a
	Fd 1.25 µM	**140%**	±	10%	a	**113%**	±	19%	a	**144%**	±	8%	a
17:0	**Nf 1.25 µM**	**98%**	±	2%	a	**88%**	±	13%	a	**127%**	±	42%	a
	Nf 2.5 µM	**99%**	±	7%	a	**131%**	±	25%	a	**116%**	±	5%	a
	Fd 1.25 µM	**95%**	±	2%	b	**101%**	±	5%	b	**141%**	±	3%	a
18:0	**Nf 1.25 µM**	**112%**	±	4%	b	**89%**	±	13%	a	**195%**	±	152%	a
	Nf 2.5 µM	**106%**	±	7%	b	**187%**	±	24%	*a	**115%**	±	6%	b
	Fd 1.25 µM	**109%**	±	2%	b	**126%**	±	51%	b	**239%**	±	18%	a
18:1n-9	**Nf 1.25 µM**	**87%**	±	3%	ab	**81%**	±	14%	ab	**167%**	±	103%	a
	Nf 2.5 µM	**58%**	±	7%	*b	**110%**	±	18%	ab	**113%**	±	11%	ab
	Fd 1.25 µM	**77%**	±	5%	*ab	**103%**	±	32%	ab	**127%**	±	4%	ab
18:1n-7	**Nf 1.25 µM**	**93%**	±	2%	ab	**81%**	±	13%	b	**110%**	±	6%	a
	Nf 2.5 µM	**78%**	±	5%	*a	**79%**	±	27%	a	**107%**	±	10%	a
	Fd 1.25 µM	**91%**	±	3%	a	**91%**	±	4%	a	**69%**	±	60%	a
18:2n-6	**Nf 1.25 µM**	**74%**	±	7%	*b	**77%**	±	14%	b	**110%**	±	5%	a
	Nf 2.5 µM	**44%**	±	4%	*b	**60%**	±	27%	*b	**113%**	±	11%	a
	Fd 1.25 µM	**64%**	±	5%	*c	**86%**	±	3%	b	**105%**	±	3%	a
18:3n-3	**Nf 1.25 µM**	**95%**	±	4%	a	**90%**	±	15%	a	**107%**	±	4%	a

	Nf 2.5 µM	**69%**	±	6%	*a	**87%**	±	30%	a	**109%**	±	9%	a
	Fd 1.25 µM	**92%**	±	5%	b	**91%**	±	8%	b	**109%**	±	4%	a
18:4n-3	**Nf 1.25 µM**	**n.d.**				**n.d.**				**100%**	±	90%	a
	Nf 2.5 µM	**n.d.**				**n.d.**				**155%**	±	17%	a
	Fd 1.25 µM	**n.d.**				**n.d.**				**189%**	±	5%	a
20:0	**Nf 1.25 µM**	**116%**	±	6%	a	**93%**	±	17%	a	**114%**	±	28%	a
	Nf 2.5 µM	**111%**	±	18%	a	**131%**	±	70%	a	**111%**	±	7%	a
	Fd 1.25 µM	**117%**	±	3%	a	**74%**	±	14%	b	**131%**	±	1%	a
20:1n-9	**Nf 1.25 µM**	**146%**	±	6%	a	**110%**	±	22%	a	**158%**	±	96%	a
	Nf 2.5 µM	**146%**	±	24%	a	**199%**	±	67%	*a	**117%**	±	9%	a
	Fd 1.25 µM	**159%**	±	18%	*a	**96%**	±	8%	b	**132%**	±	4%	a
20:2n-6	**Nf 1.25 µM**	**148%**	±	6%	a	**147%**	±	28%	a	**106%**	±	21%	a
	Nf 2.5 µM	**161%**	±	40%	*a	**265%**	±	115%	a	**102%**	±	13%	a
	Fd 1.25 µM	**159%**	±	5%	*a	**133%**	±	10%	b	**125%**	±	5%	b
20:3n-3	**Nf 1.25 µM**	**197%**	±	15%		**n.d.**				**99%**	±	8%	
	Nf 2.5 µM	**157%**	±	11%		**n.d.**				**105%**	±	6%	
	Fd 1.25 µM	**212%**	±	4%		**n.d.**				**130%**	±	11%	*
22:1n-9	**Nf 1.25 µM**	**0%**	±	0%		**n.d.**				**88%**	±	23%	
	Nf 2.5 µM	**387%**	±	94%		**n.d.**				**93%**	±	16%	
	Fd 1.25 µM	**350%**	±	304%		**n.d.**				**107%**	±	1%	
24:0	**Nf 1.25 µM**	**n.d.**				**n.d.**				**100%**	±	22%	
	Nf 2.5 µM	**n.d.**				**n.d.**				**97%**	±	7%	
	Fd 1.25 µM	**n.d.**				**n.d.**				**115%**	±	8%	
26:0	**Nf 1.25 µM**	**131%**	±	114%	a	**284%**	±	61%	a	**98%**	±	5%	a
	Nf 2.5 µM	**272%**	±	74%	b	**675%**	±	68%	*a	**111%**	±	7%	c
	Fd 1.25 µM	**145%**	±	126%	a	**302%**	±	47%	a	**133%**	±	9%	*a
Total lipid	**Nf 1.25 µM**	**76%**	±	3%	*b	**94%**	±	3%	ab	**103%**	±	13%	a
	Nf 2.5 µM	**55%**	±	2%	*b	**63%**	±	4%	*b	**99%**	±	5%	a
	Fd 1.25 µM	**72%**	±	5%	*b	**104%**	±	4%	a	**102%**	±	5%	a

Data shown are means (n=3) with standard deviation. The asterisk (*) represents the significant difference with control condition (no herbicide) and the letters the significant difference between the acclimation temperatures at $p < 0.05$. N.d., not detected.

4.7. REFERENCES

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CHAPITRE V

SCENEDESMUS OBLIQUUS AND *MICROCYSTIS AERUGINOSA* EXPOSED TO ATRAZINE AND GLYPHOSATE : ACCLIMATION AND REMOVAL OF HERBICIDES

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Contribution des auteurs

J'ai élaboré le projet de recherche, effectué toutes les expériences et analysé les résultats obtenus. J'ai également rédigé l'article. Philippe Juneau a apporté ses commentaires lors de la correction de l'article.

5.1. RÉSUMÉ

Les algues et les cyanobactéries sont exposées à des arrivées fréquentes d'herbicides dans les cours d'eau en raison de l'utilisation massive de ces pesticides en agriculture. La présence des herbicides peut induire des modifications dans les communautés de phytoplancton en raison des différences dans la sensibilité des espèces aux herbicides. Cette étude a pour but de mesurer la capacité d'une algue verte (*Scenedesmus obliquus*) et d'une souche toxique et non-toxique d'une cyanobactérie (*Microcystis aeruginosa*) à s'acclimater à l'atrazine et au glyphosate, et ainsi augmenter leur tolérance à ces herbicides. Les résultats ont démontré que, après 30 jours, *S. obliquus* et *M. aeruginosa* ont réussi à s'acclimater à la présence d'atrazine et de glyphosate en augmentant leur taux de croissance jusqu'au niveau du contrôle. Par contre, cette acclimatation n'a pas résulté en une augmentation du EC_{50} du taux de croissance à aucun des herbicides chez les cellules acclimatées par rapport aux cultures non-acclimatées. La concentration d'atrazine est restée constante dans le milieu de culture après 6 jours d'exposition, mais une diminution du glyphosate a été mesurée en présence des deux cyanobactéries. Après 6 jours de croisances des organismes dans le milieu de culture contaminé à l'atrazine, le milieu filtré était plus toxique envers *S. obliquus* alors que le milieu filtré contenant le glyphosate aura stimulé la croissance de *S. obliquus*, comparé au milieu de croissance sans herbicides. Ces résultats confirment que le glyphosate est moins persistent et moins toxique que l'atrazine dans le milieu aquatique, et pourrait davantage être géré par les techniques de bioremédiation.

Mots clés : Taux de croissance, photosynthèse, φ_{Eo}, tolérance, EC_{50}

5.2. ABSTRACT

Algae and cyanobacteria are exposed to frequent inputs of herbicides in watercourses due to their extensive use in agriculture. The presence of herbicides can induce modifications in the communities of phytoplankton due to the differences in species sensitivity to the herbicides. This study aims to measure the ability of one green alga (*Scenedesmus obliquus*) and a toxic and non-toxic strains of cyanobacteria (*Microcystis aeruginosa*) to acclimate to atrazine and glyphosate and thus increase their tolerance to these herbicides. The results showed that, over 30 days, *S. obliquus* and *M. aeruginosa* were able to acclimate to the presence of atrazine and glyphosate by increasing their growth rate to the level of the control. However, this acclimation did not result in an increase in the growth rate EC_{50} to either herbicide in acclimated cells compared to non-acclimated cultures. Atrazine stayed relatively constant in the culture media over a 6 days time course, but a decrease in glyphosate was measured in the presence of both cyanobacteria. Filtered 6 days-old media contaminated with atrazine was more toxic to *S. obliquus* while 6 days-old media contaminated with glyphosate stimulated the growth of *S. obliquus*, when compared to normal growth media. Those results confirmed that glyphosate is less persistent and toxic than atrazine in the aquatic environment, and could be more easily managed by bioremediation techniques.

Keywords : Growth rate, photosynthesis, φ_{Eo}, tolerance, EC_{50}

5.3. INTRODUCTION

Atrazine and glyphosate are two herbicides frequently detected in water-pollution monitoring programs (Graymore *et al.*, 2001; Scribner *et al.*, 2007; Sullivan *et al.*, 2009). In 2010, atrazine was detected in 95% of samples and glyphosate in 86% of samples taken in four rivers located in agricultural areas of the province of Quebec (Giroux & Pelletier, 2012). In the United States, concentrations of glyphosate up to 99 µg/L (58.5 µM) have been detected in the Mississippi river, while concentrations up to 30 µg/L (0.14 µM) of atrazine have been detected in the Maumee river, although typical were in the range 0.1 to 10 µg/L for both herbicides (Scribner *et al.*, 2007; Sullivan *et al.*, 2009).

Atrazine and glyphosate have been shown to induce changes in phytoplankton community (Caux & Kent, 1995; Bérard *et al.*, 1999; Pérez *et al.*, 2007; Pannard *et al.*, 2009; Saxton *et al.*, 2011), which can have consequences on primary productivity and on the presence and physiology of other species feeding on phytoplankton (Alva-Martínez *et al.*, 2004; Schmitt-Jansen & Altenburger, 2005; Vera *et al.*, 2012). Atrazine is a photosynthesis-inhibiting herbicide blocking the electron flow between PSII and PSI by competing with the plastoquinone Q_B for the binding site on the D1 protein (Fedtke & Duke, 2004). Some species showed higher tolerance to atrazine, either by having enhanced photoprotection mechanisms, high PSI/PSII ratio, or smaller cell size (Tang *et al.*, 1998; Weiner *et al.*, 2004; Chalifour & Juneau, 2011). Moreover, pre-treatment with photosynthesis-inhibiting herbicides has been shown to increase the tolerance of microalgae to an additional exposure (Kasai, 1999; García-Villada & Reboud, 2007). On the other hand, glyphosate is an inhibitor of the enzyme 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase, an enzyme implied in the shikimate pathway for the synthesis of the aromatic amino acids tryptophan, phenylalanine and tyrosine (Duke & Powles, 2008). The detrimental effect of

glyphosate on plant cells could be due to the depletion in aromatic amino acids or deregulation of the carbon flow going to the shikimate pathway resulting in a reduction of carbon needed for other essential pathways such as carbon fixation (Siehl, 1997). Some species of algae and cyanobacteria could be particularly tolerant to glyphosate (Powell *et al.*, 1991; Forlani *et al.*, 2008; Vera *et al.*, 2010). The reason for this high tolerance could be the presence of an insensitive form of EPSP synthase or the capacity of cyanobacteria to degrade glyphosate (Forlani *et al.*, 2008).

Some microorganisms present in soils could participate to the degradation of atrazine by N-dealkylation, hydrolysis and dechlorination of the molecule (Struthers *et al.*, 1998). However, those degradation pathways were not very efficient in phytoplankton (Zablotowicz *et al.*, 1998). On the other hand, microalgae and cyanobacteria could participate to the decontamination of atrazine-contaminated media by removing the herbicide from its surrounding environment. In the study of Campo et al. (2013), the cyanobacterium *Microcystis novacekii* exhibited a high tolerance to atrazine, having a growth EC_{50} of 4.2 mg/L for a 96 hours exposure. *M. novacekii* was also able to decontaminate the media by accumulating atrazine, having an average level of atrazine removal of 27.2 % when exposed to 50, 250 or 500 µg/L of atrazine for 96 hours. Gonzales-Barreiro et al. (González-Barreiro *et al.*, 2006) showed, after 24 hours, a removal of 80% and 93% of atrazine in the medium by *Synechococcus elongatus* and *Chlorella vulgaris*, respectively.

Degradation of glyphosate by soil microorganisms could be done by two different mechanisms. The glyphosate oxidoreductase enzyme (GOX) could split the glyphosate C-N bond to produce aminomethylphosphonic acid (AMPA) and glyoxylate (Schuette, 1998). AMPA can be further cleaved by a C-P lyase to produced phosphate and methylamine (Van Herd *et al.*, 2003). On the other hand, the C-P lyase enzyme can also cleave the C-P bond of glyphosate to produce phosphate and sarcosine (Kishore & Jacob, 1987). Sarcosine is then degraded by a sarcosine oxidase to glycine and formaldehyde. Thus, both degradation pathways lead to the

production of inorganic phosphate that could be used for algal and cyanobacterial growth (Forlani *et al.*, 2008). However, the exact mechanism of degradation in cyanobacteria has not been elucidated yet.

This study aims to measure the ability of the green alga *Scenedemus obliquus* and of a toxic and non-toxic strain of the cyanobacteria *Microcystis aeruginosa* to acclimate to the presence of atrazine and glyphosate in their culture media, and thus potentially develop a higher tolerance to both herbicides. It also aims to measure their ability to remove the herbicide in the media, and by doing so, decrease the toxicity of the culture media.

5.4. MATERIAL AND METHODS

5.4.1. Cultures, growth conditions and herbicide solutions

The green alga *Scenedesmus obliquus* (CPCC5) and two strains of the cyanobacteria *Microcystis aeruginosa* (CPCC299 and CPCC632) were cultivated in batch cultures in 250 mL Erlenmeyer's flask containing 100 mL of Bold's Basal Medium (BBM), pH 6.8 (Stein 1973). CPCC299 is a toxic strain of *M. aeruginosa*, known to produce microcystins, while CPCC632 is a non-toxic strain (Deblois & Juneau, 2012). Cultures were maintained at 25°C under an illumination of 100 µmol photon m^{-2} s^{-1} provided by a combination of incandescent bulbs (Philips 60W) and white fluorescent tubes (Philips F72T8/TL841/HO, USA) with a 14-h light/10-h dark cycle.

Stock solutions of atrazine, used in its pure form (Sigma-Aldrich) were prepared by dissolving the appropriate amount in distilled water. Glyphosate, used as the commercial herbicide Credit® Xtreme (540 g glyphosate/L, Nufarm Inc.) was diluted in distilled water.

5.4.2. Growth and photosynthesis measurements

Cell density was measured using a Coulter Counter particle analyzer (Multisizer 3, Beckman Coulter Inc., Fullerton, USA). Growth rate was calculated according to the equation $\mu = \ln(X_t/X_0)/t$ where X is the cell density (cell/mL) at a specific time point (t). At the same time, a two mL sample was kept in the dark for 15 minutes before assessing the fast polyphasic chlorophyll fluorescence kinetic using a Plant Efficiency Analyzer fluorometer (PEA, Hansatech Ltd., King's Lynn, Norfolk,

UK) with the liquid compartment attachment. The probability that an absorbed photon will move an electron into the electron transport chain (φ_{Eo}) was calculated as detailed in Strasser (2000), and used as an indicator of photosynthetic efficiency.

5.4.3. Dose-response curve and EC_{50} calculations

Cells of *S. obliquus* and *M. aeruginosa* were collected during their exponential growth phase and transferred, at a density of 5 x 10^5 cells/mL, in 50 mL glass tube containing 30 mL BBM and exposed to 7 concentrations of atrazine (0, 0.05, 0.1, 0.3, 0.5, 0.7 and 1.0 μM) or glyphosate (0, 60, 180, 300, 600, 1200 and 1800 μM) for 24 hours, in order to obtain a dose-response curve for both herbicides. Growth rate and photosynthesis were measured as endpoint, and EC_{50} for both parameters were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

After 30 days of acclimation in atrazine or glyphosate-contaminated media (see below), cultures were exposed again to 7 concentrations of atrazine (0, 0.05, 0.1, 0.3, 0.5, 0.7 and 1.0 μM) and 5 concentrations of glyphosate (0, 180, 300, 1200 and 1800 μM) for 24 hours. EC_{50} of acclimated cultures were calculated.

5.4.4. Acclimation to atrazine and glyphosate

Cultures of *S. obliquus* and *M. aeruginosa* were maintained in 250 mL Erlenmeyer's flask containing 100 mL normal BBM, BBM with 0.1 μM of atrazine or BBM with 180 μM of glyphosate. After 6, 15 and 22 days of growth in those conditions, 5 mL of the culture were transferred in 95 mL of fresh control or

contaminated BBM until the next transfer. Measurements of cell density and photosynthesis were made at day 1, 3, 6, 15, 22 and 30 in the culture that was then transferred.

5.4.5. Effect of cultures on atrazine and glyphosate toxicity in media

Non-acclimated and 30 days acclimated cultures of *S. obliquus* and *M. aeruginosa* were transferred, at a density of 5 x 10^5 cells/mL, in 50 mL glass tube containing 30 mL of BBM. Non-acclimated and acclimated cultures to atrazine were exposed to 0.01 or 0.1 μM of atrazine, and non-acclimated and acclimated cultures to glyphosate were exposed to 1 or 180 μM of glyphosate for 6 days. After inoculation of algae in medium, 2 mL of culture were taken and filtered on a 13 mm glass fiber filter (Type A/E, Pall Corporation, Michigan, USA). Filtrate was kept in -80°C for herbicide analysis. After 6 days, cultures were filtered on a pre-dried, pre-weighted GF/F filter (Whatman, Piscataway, USA), and 1 mL of filtrate was kept at -80°C for herbicide analysis. The remaining 27 mL of filtrate was used to inoculate fresh, non-exposed *S. obliquus*. Growth rate and photosynthesis of *S. obliquus* were then measured after 24 hours (see Fig. 5.1). Controls were obtained by doing the same experiments in absence of herbicides to test the effect of media depletion over 6 days on *S. obliquus*, and in the presence of herbicides but absence of algae to test the effect of spontaneous degradation of herbicide.

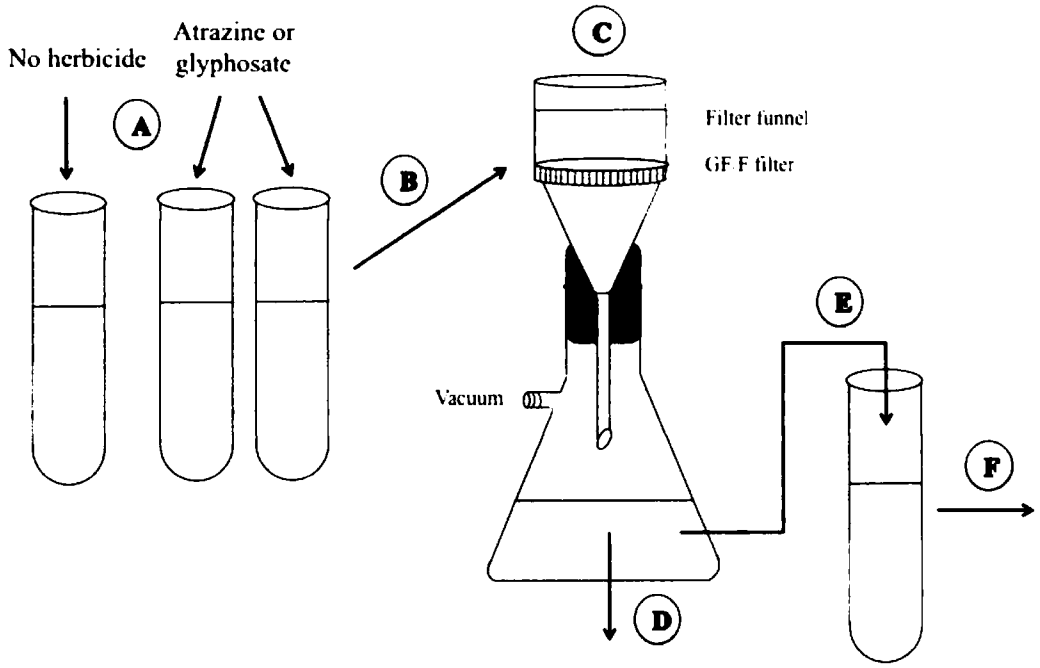

Figure 5.1. *S. obliquus* or *M. aeruginosa* were inoculated in 30 mL of fresh BBM or BBM containing either 0.1 µM of atrazine or 180 µM of glyphosate (A). A control of BBM with atrazine or glyphosate but without algae was also prepared. After 6 days of growth, the growth rate and photosynthetic efficiency were measured in all algae-inoculated tubes (B). All cultures and cell-free media were then filtered individually on a GF/F filter (C). 2 mL of the filtrate was used for herbicide determination (D; see "*Atrazine and glyphosate determination*" section). The rest of the filtrate was filtered a second time through a sterile 0.22 µm membrane filter and used to inoculate fresh, non-exposed *S. obliquus* (E). Growth rate and photosynthesis were measured after 24 hours (F; see "*Effect of cultures on atrazine and glyphosate toxicity in media*"), as for the dose-response curves.

5.4.6. Atrazine and glyphosate determination

Filtrated media containing atrazine were thawed then filtered trough a 0.22 µm syringe filter (Millex-GV, Millipore, Billireca, USA). Atrazine was analyzed by LC/MS according to LeBlanc and Sleno (2011).

Glyphosate was analysed according to the method of Moingt et al. (manuscript submitted). Briefly, filtrated media containing glyphosate were thawed and 1 ml was diluted in 20 mL of mili-Q water. The pH of the diluted solution was adjusted to 7.0 - 7.5. Media were then filtrated on a SPE column of chelex cation exchange resin Dowex® C-211 Na^+ form (J.T. Baker Chemical Co.) followed by a column packed with XAD-2® resin for purification of the sample. Glyphosate was then retained on a column of AG1-X8 formate form resins and eluted using HCl 0.6 N. Eluted samples were then evaporated to dryness using a rotary evaporator and gentle N_2 gas flow. Samples were derivatised using trifluoroethanol and trifluoroacetic anhydride (Fischer Scientific). Glyphosate was analyzed on a gas chromatograph Varian GC 3800, equipped with a capillary column Restek RXI-5SIL MS (30 m x 0.25 mm ID, 0.25 µm) coupled to an electron capture detector cell with ^{63}Ni foil model 02-001972-01. Standards of glyphosate (N-(phosphonomethyl)glycine) and AMPA ((aminomethyl)phosphonic acid) (both form Sigma-Aldrich, St.-Louis, USA) were used as quantification standards.

5.4.7. Statistical analysis

EC_{50} were compared by a one-way ANOVA, using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Multiple endpoint

measurements (acclimations) were compared using a two-way repeated measures ANOVA, design 6 x 2 (time x treatment), using SPSS Statistic 21.0 (IBM corporation, Armonk, NY, USA) software. All other results were compared using a one-way ANOVA followed by a Tukey-Kramer post-hoc analysis using JMP 6.0 statistical software (SAS Institute, Cary, NC, USA). Difference were considered significant at $p < 0.05$.

5.5. RESULTS

5.5.1. Effect of herbicides on growth and photosynthesis

EC_{50} calculated from the dose-response curves of clean, non-acclimated *Scenedesmus obliquus* and *Microcystis aeruginosa* exposed to 7 concentrations of atrazine or glyphosate are presented in Table 5.1. Growth rate (μ) and photosynthesis (φ_{Eo}) of *S. obliquus* and *M. aeruginosa* showed different sensitivities to atrazine and glyphosate. While φ_{Eo} was a parameter more sensitive to atrazine than μ, the latter was more sensitive to glyphosate. *S. obliquus* had a lower μ-based EC_{50} (0.32 μM) than *M. aeruginosa* CPCC299 and 632 (0.52 and 0.51 μM respectively) when exposed to atrazine, while φ_{Eo}-based EC_{50} of *S. obliquus* was higher than *M. aeruginosa* CPCC299 or CPCC632.

Table 5.1. EC_{50} (in μM) based on growth and photosynthesis of *S. obliquus* and *M. aeruginosa* CPCC299 and CPCC632 before and after 30 days of acclimation to atrazine or glyphosate.

Herbicide	*S. obliquus*		*M. aeruginosa* 299		*M. aeruginosa* 632	
	Non-acclimated	Acclimated	Non-acclimated	Acclimated	Non-acclimated	Acclimated
Atrazine						
Growth (μ)	0.324b	0.277b	0.520a	n/a	0.507a	0.399ab
Photosynthesis (φEo)	0.138b	0.188a	0.096c	n/a	0.084c	0.097c
Glyphosate						
Growth (μ)	494.4a	385.3a	290.7bc	n/a	120.3c	229.8bc
Photosynthesis (φEo)	21811a	34240a	1289b	n/a	1222b	1059b

The letters represent the significant difference between acclimation and species ($p < 0.05$)

In presence of glyphosate, *S. obliquus* μ-based EC_{50} was about 1.7 times higher than *M. aeruginosa* CPCC299, which was about 2.4 times higher than *M. aeruginosa* CPCC632. Photosynthesis of both algae and cyanobacteria was not much affected by glyphosate, although an EC_{50} of 21811 for *S. obliquus* and 1289 and 1222 μM for *M. aeruginosa* CPCC299 and CPCC632, respectively, was extrapolated by the dose-response curve fit.

5.5.2. Effect of acclimation on growth and photosynthesis based EC_{50}

Growth rate and photosynthetic efficiency was measured in *S. obliquus* and *M. aeruginosa* during 30 days of batch growth in atrazine or glyphosate-contaminated media (Fig. 5.2). In the presence of atrazine, growth was reduced during the 15 first days of exposure, then recovered to the level of the control at day 15 for *S. obliquus* and *M. aeruginosa*. In presence of glyphosate, growth rate was also significantly decreased during the 6 first days of exposure, but then increased up to the level of the control for *S. obliquus* and *M. aeruginosa* CPCC632 from day 15 to day 30. *M. aeruginosa* CPCC299, on the other hand, saw its growth rate decrease considerably after 6 days of exposure. The interaction between time and herbicides was significant for all conditions, with the exception of *S. obliquus* and *M. aeruginosa* CPCC632 exposed to atrazine. In this latter experiment, no effect of time or herbicide on the growth rate was measured.

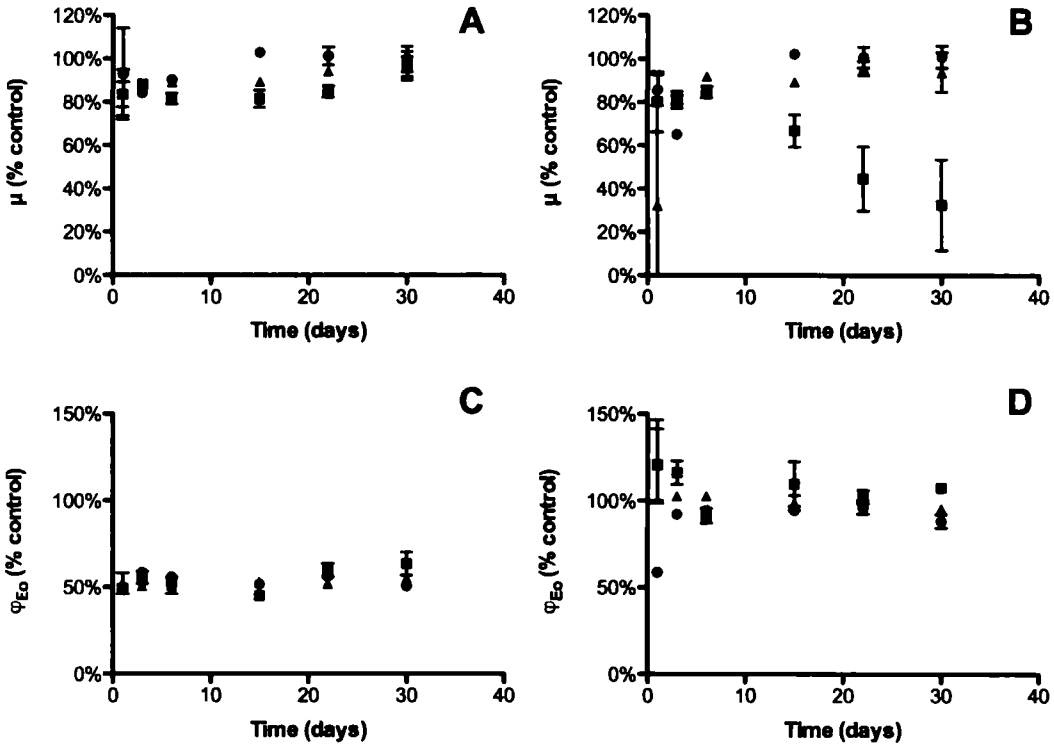

Figure 5.2. Growth rate (A, B) and photosynthesis (C, D) of *S. obliquus* (●), *M. aeruginosa* CPCC299 (■) and *M. aeruginosa* CPCC632 (▲) during acclimation to atrazine (A, C) or glyphosate (B, D).

Atrazine, at a concentration of 0.1 μM, induced a decrease of about 50 % of the photosynthesis of the three studied cultures. This inhibition stayed constant over the 30 days of acclimation. Glyphosate, at a concentration of 180 μM, did not inhibit significantly the photosynthesis of *S. obliquus* and *M. aeruginosa*. The interaction between time and herbicide was only significant for *S. obliquus* and *M. aeruginosa* CPCC299 acclimated to atrazine.

5.5.3. Tolerance to herbicides after 30 days of acclimation

M. aeruginosa CPCC299 did not reach, after 30 days of exposure to atrazine or glyphosate, a density large enough to conduct dose response curves. For *S. obliquus* and *M. aeruginosa* CPCC632, however, dose response curves of acclimated cells were measured, permitting to evaluate an EC_{50} (Table 5.1).

S. obliquus and *M. aeruginosa* CPCC632 had a lower, but not significant, μ-based EC_{50} after 30 days of acclimation to atrazine than non-acclimated cultures. *S. obliquus* had however a 36% higher φ_{Eo}-based EC_{50} after acclimation.

For glyphosate acclimated cultures, *S. obliquus* had a 1.2 X lower μ-based EC_{50} than non-acclimated cultures, while *M. aeruginosa* CPCC632 had a 1.9 X higher μ-based EC_{50}. When measuring the photosynthetic efficiency, cells of acclimated *S. obliquus* and *M. aeruginosa* CPCC632 had a similar EC_{50} than non-acclimated cultures.

5.5.4. Toxicity of medium after algal or cyanobacterial growth

Fig. 5.3 shows the 24h growth rate and photosynthesis of *S. obliquus* inoculated in filtered media in which *S. obliquus* or *M. aeruginosa* CPCC632 grew previously for 6 days, in absence or presence of atrazine or glyphosate. Results show that filtered growth media in absence of herbicide affected μ of *S. obliquus*, when compared to the cell-free medium. In the presence of atrazine-filtered media, growth of *S. obliquus* was also reduced by about 52% with *S. obliquus* and 45% with *M. aeruginosa* CPCC632. Growth in the presence of glyphosate was higher than for the control without herbicide or in presence of atrazine. Photosynthetic efficiency of *S. obliquus* was decreased when exposed to atrazine contaminated media, especially filtered medium in which *M. aeruginosa* CPCC632 had grown in for 6 days.

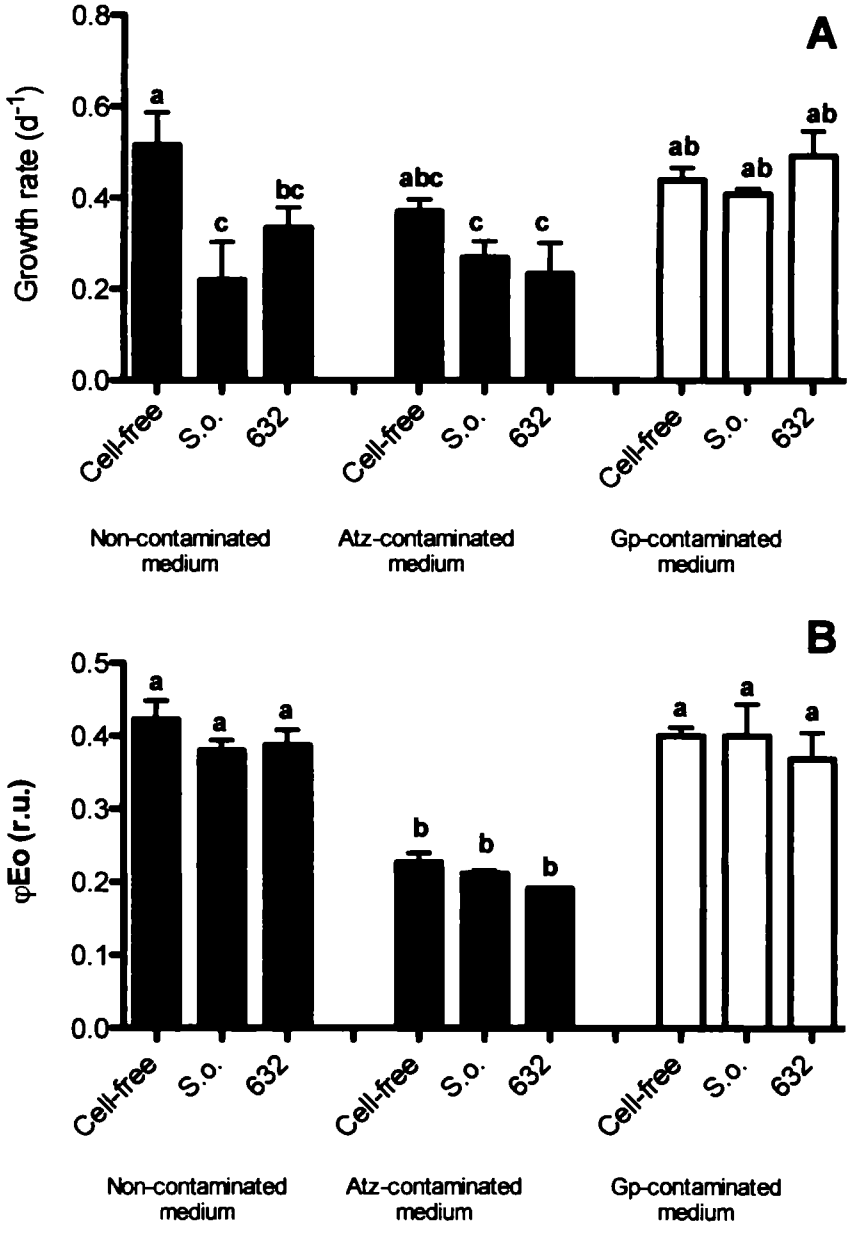

Figure 5.3. Effect non-contaminated, 0.1 μM of atrazine contaminated or 180 μM of glyphosate contaminated filtered media in which *S. obliquus* or *M. aeruginosa* CPCC632 have grown for 6 days, on the growth rate (A) and photosynthesis (B) of *S. obliquus*, compared to cell-free media. Letters above bars represent the significant differences between treatments ($p < 0.05$)

The ability of *S. obliquus* and the two strains of *M. aeruginosa* to decrease the concentration of atrazine or glyphosate in their media was assessed by measuring the concentration of the herbicides after inoculation of cells and after 6 days of growth in the contaminated media. In order to be more environmentally relevant and also to have concentrations not inducing toxic effect, concentrations used to measure degradation were much smaller than those used in acclimation and dose-response curve experiments. In addition, concentrations used for the acclimation experiments were too high to be able to measure any differences in concentration over 6 days of exposure (data not shown).

After 6 days of growth in presence of atrazine, no decrease in medium concentrations was observed for the three cultures (Table 5.2). In presence of glyphosate, a substantial decrease of about 57 % and 63% of initial concentration was measured in presence of *M. aeruginosa* CPCC299 and CPCC632, respectively. For *S. obliquus* and cell-free media, concentrations found after 6 days were not significantly different than concentrations measured just after the addition of glyphosate.

Table 5.2. Decrease in concentration of atrazine or glyphosate in media after *S. obliquus* or *M. aeruginosa* CPCC299 and CPCC632 have grown during 6 days, in % of initial concentration.

Herbicide	% removal
Atrazine	
S. obliquus	**2.78%** ± 6.55%
M. aeruginosa CPCC299	**0.92%** ± 1.30%
M. aeruginosa CPCC632	**5.48%** ± 3.46%
Cell-free media	**4.77%** ± 2.86%
Glyphosate	
S. obliquus	**35.79%** ± 25.70%
M. aeruginosa CPCC299	**63.16%** ± 2.91%
M. aeruginosa CPCC632	**57.62%** ± 8.32%
Cell-free media	**22.34%** ± 17.52%

5.6. DISCUSSION

This study aims to understand the ability of *Scenedesmus obliquus* and *Microcystis aeruginosa* to acclimate and remove the herbicides atrazine (in its pure form) and glyphosate (as a commercial herbicide) from the culture medium. Originally, the pure form of glyphosate (as salt of potassium) was tested, but no toxicity could be measured over 24 hours up to 1 mM (data not shown). This might be caused by the negligible uptake of glyphosate inside the cell in absence of adjuvants and surfactants such as isopropylamine or polyoxyethylene tallow amine. In the study of Forlani et al. (Forlani *et al.*, 2008), only a very low uptake of glyphosate was observed in some species of cyanobacteria. Glyphosate might enter the cell through a phosphate transporter having only a weak affinity with the herbicide (Denis & Delrot, 1993).

5.6.1. Tolerance to atrazine and glyphosate before acclimation

The green algae *S. obliquus* and the toxic and non-toxic strains of *M. aeruginosa* had different sensitivities to atrazine when growth or photosynthesis were evaluated. As the photosynthetic efficiency dose-response curves and EC_{50} showed, both strains of cyanobacteria were more sensitive to atrazine than *S. obliquus*. On the other hand, their growth rates were ~ 1.6 X less affected than the green algae, showing a higher growth tolerance to this herbicide. This suggests that, surprisingly, even with a lower photosynthetic activity, *M. aeruginosa* can support a higher cell division rate than *S. obliquus*. In cyanobacteria, due to the absence of compartments allowing the interconnection of the photosynthetic and respiration electron chain, the NADPH hydrogenase and succinate deshydrogenase of the respiratory chain can provide electrons to the plastoquinone pool (Vermaas, 2001). Because atrazine blocks

the electron flow at the Q_B site, i.e. before the plastoquinone pool, this electron deviation from the respiratory chain may provide electrons to PSI. In fact, nine alternative electron flow pathways have been elucidated in cyanobacteria, and may assist the linear electron flow in optimizing photosynthesis (Nogales *et al.*, 2012). It was also shown that cyanobacteria had a higher growth rate at low light intensities than other phytoplankton species (Deblois *et al.*, 2013). As observed in our growth conditions in the presence of atrazine (data not shown) and as discussed in our previous study (Chalifour & Juneau, 2011), photosynthetic-inhibiting herbicides such as atrazine act as a light limitation signal for the PSI, inducing responses such as pigment synthesis. This might explain why *M. aeruginosa* could outcompete *S. obliquus* when exposed to another triazine herbicide, metribuzin, in mixed culture (Lürling & Roessink, 2006).

In the presence of glyphosate however, *S. obliquus* was much more tolerant, when both growth and photosynthesis were evaluated, than both strains of cyanobacteria. Lipok et al. (2010) also reported that *M. aeruginosa* μ-based EC_{50} was 18 X more sensitive compared to the green algae *Chlorella vulgaris*, when exposed to the glyphosate-based herbicide Roundup®. According to the same authors, this higher sensitivity was attributable to isopropylamine found in the glyphosate-based herbicides, rather than to the pure glyphosate molecule. Few studies have reported the effect of isopropylamine on phytoplankton, but Haley (1989) measured a 96 hours μ-EC_{50} of 118 mg/L for *Pseudokirchneriella subcapitata*. In the commercial herbicide used in this study both potassium salt and isopropylamine salt of glyphosate were present (Nufarm Limited, 2013).

5.6.2. Acclimation to atrazine and glyphosate

In presence of 0.1 µM of atrazine over a period of 30 days, the initial decrease of about 20 % of the growth rate of *S. obliquus* and the two strains of *M. aeruginosa* was recovered to the level of the control after 15 and 30 days, respectively. During the whole 30 days period however, the inhibition of photosynthesis by atrazine stayed relatively constant, i.e. about 50% of the control cultures. According to Hatfield (Hatfield *et al.*, 1989), the acclimation of *Anacystis nidulans* to PSII-inhibiting herbicides induced changes similar to acclimation to low light condition, such as a higher content in phycocyanin. Our results suggested that some modifications in the physiology helped the cultures grow at a rate similar to the control condition, even with a 50% inhibition in photosynthesis. Cyclic electron flow (CEF) from PSI and ferredoxins to the plastoquinone pool and cytochrome b_6f complex may contribute to the maintenance of the proton gradient needed for ATP synthesis (Bendall & Manasse, 1995). It was shown previously that CEF was induced upon acclimation to low light conditions in *Chlamydomonas reinhardtii* (Bonente *et al.*, 2012). In addition, the exposure of *Chlorella pyrenoidosa* to cadmium significantly reduced the PSII activity and linear electron flow, but induced the CEF (Wang *et al.*, 2013).

It also took between 15 and 30 days, in presence of 180 µM of glyphosate, to get *S. obliquus* and *M. aeruginosa* CPCC632 growing at a rate similar to the control. In plants, detoxification of glyphosate proceeds through a glyphosate N-acetyltransferase enzyme (GAT), which, according to Castle et al. (Castle *et al.*, 2004) shows similarity with enzymes of the GCN5-related N-acetyltransferases (GNAT) superfamily (Dyda *et al.*, 2000). Although the GAT enzyme has not been isolated in phytoplankton yet, GNAT enzymes are found in algae and bacteria (Castle *et al.*, 2004). Therefore, the growth tolerance to glyphosate observed in phytoplankton could be due to the presence of those detoxifying enzymes or to a more active glyphosate degradation pathway (see below).

The toxic strain of *M. aeruginosa* was the slowest to recover a growth rate similar to the control in the presence of atrazine, and showed an important decrease in its growth rate after 6 days in presence of glyphosate. The two strains also showed a very different behavior when exposed to high light stress (Deblois & Juneau, 2012) and in presence of atrazine after acclimation to low temperature (Chalifour & Juneau, 2011). Further studies should be done to investigate the factors explaining this difference with the non-toxic strain, and to determine whether the microcystin had a role to play in this increased sensitivity.

5.6.3. Tolerance to atrazine and glyphosate after acclimation

After acclimation of *S. obliquus*, μ was not significantly affected by the atrazine concentrations. This is in agreement with other studies that measured a similar or increased sensitivity to atrazine after a prior exposure to this herbicide. Nelson et al. (1999) studied the effect of a chronic exposure (67 days) to a low concentration of atrazine (1 μg/L) on the growth rate of the diatom *Craticula cuspidata*. Although this concentration was not producing a toxic effect on the long term, it increased the sensitivity of the algae to high concentrations (83 – 750 μg/L) of atrazine.

On the other hand, *S. obliquus* φ_{Eo} was more tolerant to the same concentrations of atrazine after acclimation than before. Thus, it seems that the modifications induced by atrazine during acclimation, as discussed above, helped *S. obliquus* photosynthesis to be more tolerant to this herbicide. However, over only 24 hours, the increase of 36% in photosynthesis tolerance to atrazine compared to non-acclimated cultures, did not result in an increase in an increase in growth tolerance. Over 96 hours, Weiner et al. (2007) reported a decrease in protein content in atrazine-exposed algae. Atrazine could thus trigger protein deficiency due to the lack of

photosynthetates, which may be even more important after 30 days of acclimation to a 50% decrease in photosynthesis (see Fig 5.2) than without acclimation.

Although *M. aeruginosa* and *S. obliquus* μ-EC_{50} were almost 2 times higher and 22% lower, respectively, after 30 days of acclimation to, the differences measured were not significant. Nonetheless, and regardless of acclimation, both green algae and cyanobacteria were very tolerant to glyphosate, up to concentrations not found in the ecosystems (LOEC of ~180 μM, about 300 times higher than concentrations maximally found in the environment – see introduction). The trend observed also indicates that cyanobacteria could benefit from a long-term glyphosate exposure, which might explain why they outcompete other phytoplankton species in mesocosm studies (Pérez *et al.*, 2007; Vera *et al.*, 2010). To our knowledge, the effect of a pre- or chronic exposure to glyphosate on the sensitivity of phytoplankton to this herbicide has not been reported in the literature, probably because glyphosate is considered to have a short life-time and high biodegradation rate (Duke & Powles, 2008). However, frequent detections of glyphosate in streams indicate that phytoplankton could be exposed to chronic, low concentrations of glyphosate due to a constant input of this herbicide in aquatic ecosystems (Giroux & Pelletier, 2012).

Those results also prove that resistant cultures of algae or cyanobacteria to glyphosate were not obtained after 30 days of acclimation. The concentration of atrazine and glyphosate used in for the acclimation might not have been high enough to select mutants of microorganisms that would be resistant to either herbicide, as reported by previous authors with some pesticides (Huertas *et al.*, 2010; Marvá *et al.*, 2010; González *et al.*, 2012). In the study of López-Rodas et al. (2007), strains of *Microcystis aeruginosa* resistant to glyphosate were selected using very high concentration of glyphosate (710 μM). On the other hand, resistant cyanobacteria having a mutation on the gene (*psbA*) coding for the target site of atrazine on the D1 protein, have been arising in atrazine contaminated wastewater treatment systems (Sajjaphan *et al.*, 2002).

5.6.4. Toxicity of medium after algal or cyanobacterial growth

Table 5.2 shows that atrazine concentration, over a 6 days exposure, did not decrease significantly in the growth media. This is in accordance with previous studies where no degradation of atrazine was observed (Zablotowicz *et al.*, 1998; Chalifour & Juneau, 2011). It was also demonstrated that the uptake of atrazine was less than 2% of initial concentration in medium in five species of algae or cyanobacteria after 96 hours (Weiner *et al.*, 2004), indicating that a low uptake of atrazine by the cells may explain the unchanged atrazine concentration in the medium. Because spontaneous degradation was not important either in cell-free medium (Table 5.2), the atrazine concentration stayed constant enough to induce a similar toxicity when fresh *S. obliquus* was transferred in the 6 days old atrazine contaminated medium (Fig 5.2). On the other hand, some studies have reported an uptake of 27.2 % of atrazine over 96 hours using *Microcystis novacekii* (Campos *et al.*, 2013) while a removal of up to 93% of atrazine by *Chlorella vulgaris* over 24 hours have been measured (González-Barreiro *et al.*, 2006). The contradictory results found in the literature may be due to differences in phytoplankton cell size and growth conditions, and the lipid composition of algae, which may also be influenced by temperature (Siegenthaler & Trémolières, 1998).

When measuring the toxicity of 6 days old medium contaminated with glyphosate, it is interesting to note that the contaminated medium was promoting growth of *S. obliquus* better than herbicide-free 6-days old BBM medium. As measured with 1 µM of glyphosate with non-acclimated cyanobacteria, a decrease in glyphosate concentration was found after a 6 days exposure. When degraded, glyphosate may serve as a source of phosphorous for some strains of cyanobacteria (Vera *et al.*, 2010; Qiu *et al.*, 2013). An increase in total phosphorous in treated

microcosm or mesocosm with glyphosate was previously observed (Pérez *et al.*, 2007; Vera *et al.*, 2010; Vera *et al.*, 2012). Moreover, it was found that a low concentration of glyphosate promotes the growth of *Scenedesmus quadricauda* (Wong, 2000). Considering the absorption and possible degradation of glyphosate after 6 days in media, the residual glyphosate concentration may have stimulated growth of *S. obliquus* in our experiment.

5.6.5. Conclusions

Our results showed that there is an acclimation in the growth rate to both atrazine and glyphosate, but this acclimation did not resulted in an increased short-term tolerance to higher concentrations of herbicides. Atrazine concentration stayed constant in the culture media over 6 days and induced the same toxicity, even after the marginal uptake by *S. obliquus* or *M. aeruginosa*. On the other hand, uptake or degradation of glyphosate by those microorganisms could decrease its concentration and toxicity in culture media. Thus, microorganisms, particularly cyanobacteria, could be used in bioremediation systems for the treatment of glyphosate-contaminated waters. Whether a long-term exposure to those herbicides in the aquatic environment could induce changes in the community by selecting resistant species still remains an open question.

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CONCLUSION

« *Here again we are reminded that in nature nothing exists alone* » (Carson, 1962). Cette citation nous rappelle la complexité des écosystèmes dans lesquels se retrouve un nombre croissant de contaminants. Les herbicides, tout comme les métaux toxiques ou les perturbateurs endocriniens, ne peuvent être écologiquement considérés – et étudiés - comme des entités indépendantes. Ils interagissent non seulement avec les facteurs physiques auxquels tous les environnements sont soumis, mais également avec la matière organique, les organismes vivants et les autres contaminants présents autour d'eux.

Cette thèse a permis de démontrer qu'il y avait bien un effet de la température sur la toxicité des herbicides envers les algues et les cyanobactéries, mais que l'interaction entre ces deux facteurs dépendait de la nature de l'herbicide en question, de son mode d'action, de la température utilisée, ainsi que de l'espèce de phytoplancton étudiée. Il n'y a donc pas une règle précise qui s'applique à tous les herbicides, ou à toutes les espèces, concernant l'effet de la température. Chaque espèce d'algue et de cyanobactérie testée a par ailleurs une capacité d'acclimatation à la température ou aux herbicides qui sera propre à ses caractéristiques physiologiques particulières. Ainsi, deux souches d'une même espèce de cyanobactérie auront deux comportements différents face à un stress physique ou chimique.

Principaux résultats

Dans le chapitre II, il a été démontré que l'algue verte *Scenedesmus obliquus* et la cyanobactérie *Microcystis aeruginosa* étaient plus sensibles à l'atrazine lorsque leur température d'acclimatation était sous-optimale. Par contre, chez la diatomée *Navicula pelliculosa*, la température d'acclimatation avait peu d'effet sur l'inhibition de la photosynthèse par l'atrazine. Des caractéristiques physiologiques propres aux

diatomées, telles que la présence de la chlororespiration (Grouneva *et al.*, 2009), permettant le recyclage des électrons de la photosynthèse, des pigments photoprotecteurs supplémentaires comme les fucoxanthines et les diadinoxanthines (Brown, 1988), ou encore un haut ratio PSII/PSI (Strzepek et Harrison, 2004) pourraient être à l'origine de cette plus grande tolérance à l'atrazine à faible température. De plus, les études de Tang et Vincent (1999) ainsi que Weiner *et al.* (2004) ont démontré que les diatomées prélevaient beaucoup moins d'atrazine de leur milieu que plusieurs espèces d'algues vertes et de cyanobactéries. Ces différences pourraient être attribuables à la composition lipidique ou à la structure de la paroi cellulaire chez ces algues.

En outre, la température sous-optimale peut également avoir un effet bénéfique en augmentant la tolérance de certaines espèces aux herbicides. Ainsi, dans les chapitres III et IV, aucune toxicité n'a été détectée à 8°C chez *Chlamydomonas reinhardtii* exposée au norflurazon et au fluridone alors que la photosynthèse et la synthèse de β-carotène étaient complètement inhibés par la même concentration de norflurazon à 15°C. Il semblerait qu'il y ait eu une résistance croisée entre la température et les herbicides dans ce cas, c'est-à-dire que des modifications physiologiques engendrées par la basse température ont permis d'augmenter la tolérance aux herbicides. Serait-ce dû à une augmentation de l'activité ou de la quantité des enzymes de détoxification ? À une plus grande synthèse de pigments photoprotecteurs ? Ou à une modification de la composition lipidique des membranes de la cellule acclimatée à basse température ? Les chapitres III et IV ont de plus permis de démontrer que deux herbicides ayant le même mode d'action pouvaient avoir des effets très différents selon la température d'acclimatation. Ainsi, le norflurazon était plus toxique à 15°C que le fluridone, ce dernier ayant toutefois plus d'effet à 25°C que son congénère. L'importance de la solubilité et du caractère lipophile de ces deux molécules dans leur toxicité envers *C. reinhardtii* mériterait d'être étudiée.

Les chapitres II, III et IV ont également permis de mettre en évidence les modifications physiologiques engendrées par l'acclimatation à la basse température. En général, une diminution du taux de croissance et de l'efficacité opérationnelle de la photosynthèse est observée. Une augmentation de la quantité des caroténoïdes est néanmoins mesurée, cela permettant évidemment de protéger l'appareil photosynthétique contre l'excès d'énergie au PSII. Une augmentation dans la quantité de protéines est également mesurée, possiblement en lien avec un besoin plus grand en enzymes pour compenser la diminution de l'activité spécifique de celles-ci à basse température. Enfin, des modifications dans le niveau d'insaturation des acides gras permettent de maintenir une fluidité membranaire nécessaire au bon fonctionnement des complexes protéiques et transporteurs qui s'y trouvent.

Enfin, dans le chapitre V, il a été démontré que, tout comme les algues et les cyanobactéries pouvaient s'acclimater à la température, elles peuvent également s'acclimater à la présence d'herbicides. Une exposition à long terme (15 à 30 jours), et sur plusieurs générations, à l'atrazine et au glyphosate, a permis de retrouver un taux de croissance similaire au témoin. Malheureusement, cette acclimatation n'a pas permis de démontrer significativement une augmentation de la tolérance à un stress aiguë, sur 24 heures, de la croissance des organismes. Il semblerait que la durée d'acclimatation ou la concentration d'herbicide n'aient pas été suffisamment élevés pour engendrer des mutations chez *S. obliquus* ou *M. aeruginosa* leur permettant d'être résistantes aux herbicides atrazine et glyphosate. Des modifications au niveau de la chaîne de transport des électrons auraient toutefois permis une augmentation de la tolérance de la photosynthèse à l'atrazine chez *S. obliquus*.

Le chapitre V aura en outre permis de démontrer que l'incorporation d'atrazine dans les cellules est très faible, en deçà des limites détectables par l'appareil de mesure. Il n'y a donc ni diminution significative ni dégradation dans le milieu, ce qui signifie que l'atrazine reste présent dans l'eau et pourrait affecter les espèces aquatiques via leur consommation ou leur contact avec l'eau.

Pour le glyphosate, la situation est bien différente. Une diminution importante du glyphosate dans le milieu est observée, particulièrement avec les cyanobactéries. Il n'est pas clair si cette diminution est reliée seulement à l'incorporation du glyphosate par les cellules ou à une dégradation dans le milieu. Néanmoins, ces expériences démontrent que les microorganismes peuvent jouer un rôle important dans la bioremédiation des milieux contaminés par le glyphosate.

Limites de la thèse

Les chapitres III et IV ont permis de montrer des résultats intéressants sur les modifications physiologiques à basse température chez *Chlamydomonas reinhardtii*, tels qu'une activité des enzymes de détoxification élevée et un profil lipidique peu différent des cellules s'étant multipliées à 25°C. Toutefois, ces expériences n'ont pas permis d'apporter une explication claire sur l'absence d'effet des herbicides blanchisseurs à 8°C, alors qu'une disparition totale du contenu en β-carotène et du rendement photochimique était observée à 15°C. Des mesures supplémentaires mériteraient d'être effectuées afin de mieux comprendre le phénomène derrière cette absence d'effet. Il pourrait s'agir, comme il a été mentionné dans le chapitre IV, d'une non-pénétration des herbicides à 8°C, possiblement à cause de la composition lipidique particulière des cellules à cette température. Ainsi, une mesure de la quantité de norflurazon et de fluridone ayant pénétré à l'intérieur des algues aurait été très intéressante. Dans le cas d'une incorporation similaire des herbicides dans les cellules entre les différentes températures, une mesure de l'activité de l'enzyme cible, la phytoène désaturase, aurait pu permettre de vérifier si la même quantité d'herbicide aurait eu moins d'effets sur la synthèse totale de caroténoïdes. De plus, la mesure du contenu en plastoquinones, le cofacteur en compétition avec le norflurazon, aurait permis de connaître l'ampleur de la compétition entre les deux aux différentes températures.

Ces mesures n'ont pu être effectuées en raison de l'indisponibilité des équipements et des méthodes requises ; leur développement et leur optimisation auraient pris un temps considérable dépassant de loin les limites accordées à cette thèse.

Dans le chapitre V, l'incorporation de l'atrazine et du glyphosate dans les cellules, ainsi que la concentration en sous-produits de la dégradation des herbicides (le dééthyl-atrazine et l'AMPA) mériterait d'être effectuée afin de vérifier si une certaine dégradation de ces herbicides s'effectue à l'intérieur de la cellule. Les échantillons de phytoplancton étant disponibles et conservés à -80°C, il est fort probable qu'un étudiant ou un stagiaire poursuive ce projet avec les mesures manquantes dès l'hiver 2014.

Perspectives

Malgré les limites de cette thèse, les informations apportées par les différentes expériences mettent en lumière l'importance de l'interaction des différents facteurs en milieu naturel. Dans la littérature scientifique et les études gouvernementales, la plupart des contaminants, incluant les herbicides, ont été étudiés en laboratoire dans des conditions bien précises, souvent à température pièce ou à la température optimale des organismes étudiés (MDDEP, 2008 ; US EPA, 2004). Or, sur le terrain, un herbicide appliqué en pré-émergence, au printemps, aboutira dans des eaux beaucoup plus froides que 20 ou 25°C. L'intensité lumineuse à laquelle seront exposés les microorganismes aura également une influence sur la toxicité des herbicides (Deblois, Dufresne et Juneau, 2013). L'étude des contaminants et de leur interaction avec différents facteurs abiotiques, incluant les autres contaminants, devrait être de plus en plus facilitée par le développement de test miniaturisés et l'automatisation des mesures (Buonasera *et al.*, 2011 ; Choi, Berges et Young, 2012 ; Lefevre *et al.*, 2012 ; Schonbrun *et al.*, 2010). En ce sens, l'étude de la photosynthèse

par la fluorescence chlorophyllienne permet des lectures rapides et non-destructives (permettant ainsi différents temps de mesures), et représente également un indicateur sensible à plusieurs contaminants (Juneau, Qiu et Deblois, 2007 ; Perreault *et al.*, 2011 ; Perron et Juneau, 2011).

Si les expériences en laboratoire, avec des cultures isolées, telles qu'effectuées dans cette thèse, peuvent difficilement prédire le comportement des substances et des organismes en milieu naturel, elles apportent des tendances et des mécanismes d'action qui peuvent certainement aider à la compréhension des changements environnementaux. Il est intéressant de penser, suite aux résultats obtenus, que les cyanobactéries seront favorisées par un climat plus chaud, combiné à une contamination aux herbicides. Il est fort à parier que les floraisons de cyanobactéries seront encore plus fréquentes dans les prochaines années. Les études effectuées en micro- ou mésocosmes, semblent déjà confirmer ces prédictions (Bérard, Leboulanger et Pelte, 1999 ; De Senerpont Domis, Mooij et Huisman, 2007).

Selon Tilman (2001), si les pratiques actuelles d'agriculture persistent, une augmentation de 2,7 fois la quantité de pesticides utilisés en 2000 est à prévoir d'ici 2050. Cela correspondrait alors à une production de 10,1 millions de tonnes métriques par année. Or, les pesticides n'ont pas seulement des effets sur les écosystèmes ; ils peuvent également affecter la santé humaine, causant notamment des troubles de comportements et d'apprentissage chez les enfants (voir revue par Polańska *et al.* (2013)). Des approches alternatives à l'agriculture de masse, comme l'agriculture biologique et l'alimentation végétalienne, en plus des avancées effectuées en lutte biologique, pourraient certes contribuer à la diminution de la pression chimique imposée à notre environnement. Quant à la contamination déjà présente dans les sols et dans l'eau, les découvertes dans le domaine de la bioremédiation semblent prometteuses. L'utilisation d'arbustes du genre *Salix* et *Populus* pourrait permettre un meilleur contrôle du mouvement des pesticides dans le sol, bloquant ainsi leur lessivage vers les cours d'eau (Burken et Schnoor, 1997 ;

Warsaw *et al.*, 2012 ; Zalesny et Bauer, 2007) En milieu aquatique, les algues et les cyanobactéries pourraient également participer à diminuer la contamination. En plus des exemples mentionnés dans l'introduction de cette thèse concernant l'atrazine et le glyphosate, *Anabaena* et *Chlorella* pourraient dégrader 99% de l'insecticide fenamiphos (Cáceres, Megharaj et Naidu, 2008), alors que le 2,4-dinitrophénol serait complètement absorbé par *Chlamydomonas* et *Anabaena* (Hirooka *et al.*, 2006). Le génie génétique pourrait en outre créer ou sélectionner des mutants plus résistants ou plus efficaces à dégrader les contaminants, telles les cyanobactéries *Nostoc calcicola*, plus résistantes au cadmium (Raveender, Scaria et Verma, 2002) et *Anabaena*, possédant un gène lui permettant un taux de dégradation de lindane presque deux fois plus important (Kuritz et Wolk, 1995).

Bref, les algues et les cyanobactéries pourraient s'avérer être des outils extraordinaires pour le contrôle de la pollution et la remédiation des environnements aquatiques. Toutefois, plus de 15 000 nouvelles molécules chimiques sont produites à chaque jour, dont un grand nombre risque de se retrouver dans les cours d'eau (Kolvenbach *et al.*, 2014). En Europe, la contamination globale de ceux-ci par les contaminants organiques a été mesurée récemment, et plus de 223 molécules toxiques ont été rapportées, dont un grand nombre de pesticides risquant d'affecter les algues (Malaj *et al.*, 2014). Or, une biodiversité algale faible diminue la capacité de l'écosystème à être remédié (Cardinale, 2011). Les défis futurs seront donc d'arriver à diminuer l'exposition des milieux naturels aux contaminants d'origine anthropique, tout en tentant de mieux gérer la pollution déjà présente dans nos écosystèmes.

ANNEXE 1 : AUTRES CONTRIBUTIONS SCIENTIFIQUES

Articles scientifiques

Gomes, M.P., E. Smedbol, A. Chalifour, L. Hénault-Ethier, M. Labrecque, L. Lepage, M. Lucotte, et P. Juneau (2014). Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid : an overview, Journal of Experimental Botany, doi:10.1093/jxb/eru269

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Chalifour, A., M.H. Boily, P.A. Spear, C. Deblois, I. Giroux, N. Dassilva, D. Laliberté and P. Juneau (2007). Assessment of toxic effects of pesticide extracts on different green algal species by using chlorophyll a fluorescence, Toxicological & Environmental Chemistry, 91(7-8):1315-1329

Article de vulgarisation

Chalifour, A. (2013). Les cyanobactéries, des cols bleus microscopiques. ACFAS, concours de vulgarisation de la recherche, Le Soleil, dimanche 10 février 2013, p. 23

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