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TOXICITY OF PERFLUORINATED PHOSPHONIC ACIDS IN *CHLAMYDOMONAS REINHARDTII*

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TABLE OF CONTENT

LIST OF FIGURES

Figures Pages

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
EPA	Environnemental Protection Agency (U.S)
PFC	Perfluorinated compound
PFPA	Perfluorinated phosphonic acid
C8-PFPA	Perfluorooctylphosphonic acid
C10-PFPA	Perfluorodecylphosphonic acid
PFOS	Perfluorooctane sulfonate
PFOA	Perfluorooctanoic acid
ROS	Reactive oxygen species
GPX	Glutathione peroxidase
CAT	Catalase
SOD-1	Mn-superoxide dismutase
GST	Glutathione S-transferase
APX I	Ascorbate peroxidase
AOt1/2	Atmospheric oxidation half-life
PB&T	Persistent, bioaccumulative and toxic substance
POP	Persistent organic pollutant
BCF	bioconcentration factor
log Kow	Log Octanol–water partitioning coefficient
YAC	Yeast artificial chromosome
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
LC	Liquid chromatography
H_2DCFDA	2-7-dichlorodihydrofluorescein diacetate
FDA	Fluorescein diacetate
TCA	Trichloroacetic acid
TBARS	Thiobarbituric acid reactive substance

RÉSUMÉ

Ce projet de recherche porte sur les composés perfluorés (PFCs), produits chimiques synthétiques utilisés depuis 60 ans dans une variété de produits commerciaux et résidentiels. Les PFCs contiennent des liaisons carbone-fluore (C-F) qui confèrent à ces composés une grande stabilité physique et chimique. Ces substances peuvent se propager et s'accumuler dans l'environnement. Plusieurs congénères des PFCs, tels que l'acide perfluorooctanesulfonique (PFOS) et l'acide perfluorooctanoïque (PFOA), détectés à travers le monde dans le sang des animaux et des humains, peuvent être toxiques et persistants.

Ces dernières années une nouvelle classe de PFC est apparue: les acides perfluoro phosphoniques (PFPAs). Ces composés ont été détectés dans les eaux de surface aux Pays-Bas, en Allemagne et au Canada ainsi que dans six des sept centres d'épuration des eaux étudiés au Canada. Jusqu'à présent aucune donnée toxicologique n'est disponible pour les PFPAs chez les plantes. Cette recherche utilise les micro-algues vertes *Chlamydomonas reinhardtii* pour étudier les effets des PFPAs sur les organismes aquatiques en utilisant une approche combinant la physiologie, la biochimie et la génétique. La recherche se concentre plus spécifiquement sur l'acide perfluorooctylphosphonique (C8-PFPA) listée par l'agence américaine de protection de l'environnement (US-EPA) comme un agent chimique produit en grande quantité, et l'acide perfluorodecylphosphonique (C10-PFPA).

Les objectifs de l'étude sont d'étudier les effets de ces deux composés sur la viabilité cellulaire, les espèces réactives de l'oxygène (ROS), la peroxydation lipidique et la transcription de plusieurs gènes (ex : GPX, CAT, SOD, GST et APX) chez *Chlamydomonas reinhardtii* afin de mieux comprendre les impacts de l'exposition du phytoplancton au C8-PFPA et au C10-PFPA. Après 72 heures d'exposition aucune différence n'a été observée au niveau de la viabilité cellulaire entre les organismes exposés et non exposés aux deux produits chimiques. La concentration de ROS a augmenté significativement après 72 heures (36% et 25,6% avec une concentration de 125 et 250 µg/L pour le C10-PFPA). Une augmentation de la peroxydation des lipides a été observée pour les groupes exposés à une concentration de 125 et 250 µg/L pour le C10-PFPA. Une qRT-PCR a été effectuée dans le but d'analyser l'abondance transcriptionnelle de *SOD I, CAT, APX I, GPX* et *GST*. Les analyses ont révélé que *CAT* et *APX I* sont surexprimés lors de l'exposition au C10-PFPA pour toutes les concentrations. De plus, les PFPAs ont été détectés sur trois sites dans des échantillons d'eaux de surface du fleuve Saint-Laurent au Canada avec une concentration de C8-PFPA variant de 250 à 850 pg/L et de 380 à 650 pg/L pour le C10-PFPA. Ces données indiquent que les PFPAs sont des contaminants environnementaux répandus dans l'écosystème aquatique, et qu'ils peuvent provoquer des effets au niveau du système de défense des antioxidants chez *C. reinhardtii*. Cette recherche contribue à la compréhension des impacts et modes d'action toxique du C8-PFPA et du C10-PFPA sur le phytoplancton

Mots clés: *Chlamydomonas reinhardtii,* transcription des gènes, composés perfluoroalkyles, ROS, peroxydation des lipides.

ABSTRACT

Perfluorinated compounds (PFC) are synthetic chemicals used over the past 60 years in a variety of commercial and residential products. The strong carbon-fluor bond (C-F) confers great physical and chemical stability to these substances with high environmental persistence and bioaccumulation/biomagnification potential. Many of these compounds such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) can be toxic and have been detected in humans and animals worldwide.

In recent years a new class of PFC, the perfluorinated phosphonic acids (PFPAs), was reported for the first time in effluents from six of seven wastewater treatment plants investigated in Canada as well as in surface water from the Netherlands, Canada, and Germany. Toxicological effects of PFPAs have never been investigated in plants. Herein, using a green microalga *Chlamydomonas reinhardtii* at the logarithmic growth stage, we studied the toxic effects of PFPAs. The research was focused more specifically on perfluorooctylphosphonic acid (C8-PFPA), listed by the US-EPA as a high production volume chemical, as well as perfluorodecylphosphonic acid (C10-PFPA). The goal of this study was to evaluate the effects of C8-PFPA and C10-PFPA exposure to *Chlamydomonas reinhardtii* using genomic tools (qRT-PCR), physiological (cellular viability), and biochemical indicators (ROS production and lipid peroxidation). After 72 hr of exposure, no differences were observed in cellular viability for any of the two chemicals. ROS concentrations were significantly increased at 72 hr (36 % and 25.6 % at 125 and 250 µg/L of C10-PFPA, respectively). Lipid peroxidation level (measured as malondialdehyde, MDA) was observed only in the C10-PFPA exposure groups, whereas lipid peroxidation remained unchanged in the C8-PFPA exposure groups. To get insights into the molecular response, a qRT-PCR-based assay was performed to analyze the transcript abundance of *SOD-1, CAT, APX I, GPX* and *GST*. Our analysis revealed that expression of the *CAT* and *APX I* was up-regulated for all the concentrations of C10-PFPA. In addition, PFPAs were detected in surface water at three different sites in the St. Lawrence River, Canada with concentrations of C8-PFPA ranging from 250 to 850 pg/L and 380 to 650 pg/L for C10-PFPA. These data indicate that PFPAs are prevalent contaminants in the aquatic ecosystem and may induce disturbance in the antioxidant defensive system of *C. reinhardtii*. This multi-level ecotoxicological approach, integrating physiological, biochemical and genetic markers, will contribute to the understanding of impacts and modes of action of C8-PFPA and C10-PFPA in phytoplankton.

Keywords: *Chlamydomonas reinhardtii*, gene transcription, perfluoroalkyl phosphonic acids, reactive oxygen species, lipid peroxidation.

INTRODUCTION

Water is the most abundant element on the planet; it dilutes, disperses or degrades by physicochemical or biological action processes vast amounts of wastewater from commercial, industrial, and residential activities. Industrial development throughout history has developed and used generations of chemicals for several purposes and hundreds of new chemicals are produced annually. The process pertaining to the evaluation of the impacts of these chemicals on the health of humans and ecosystems has led to the development of risk assessment and regulation. The impact assessment of these new chemicals in the environment is critical to our sustainable development.

In recent years, new classes of pollutants have received attention from the scientific community due to their introduction into the aquatic environment, unforeseen toxic effects associated with their exposure and their bioaccumulation in the aquatic food chain. Many of these emerging contaminants are poorly studied, making predictions regarding their toxicity to aquatic organisms or environmental fate difficult. Perfluorinated compounds (PFCs) have been used in industries for over 60 years because of their unique chemical properties such as thermostability, chemical stability and oleophobicity (refers to the physical property of a molecule that is repelling oil). The carbon-fluorine (C-F) bond is very strong (110 kcal/mol), which confers the thermal and chemical stability to many PFCs. Indeed, PFCs are extremely persistent, therefore, resistant to hydrolysis, photolysis, biodegradation, and metabolism. These properties account for their high environmental persistence and bioaccumulation propensity.

The two most commonly studied PFCs, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have been detected globally in a variety of biotic and abiotic matrices, being bioaccumulative and toxic in a range of animal species (Kennedy and Butenhoff, 2004). However, in recent years, a new class of perfluorinated compounds has emerged that contain a functional group with phosphonic acid as hydrophilic group: the perfluorinated phosphonic acids (PFPAs).

Perfluorinated phosphonic acids have been used mostly in the industrial sector as leveling and wetting agents and defoaming additives in pesticides (Dinglasan et al., 2004). PFPAs have chain lengths of 6, 8 or 10 carbon atoms, and are saturated in fluorine.

Perfluorooctylphosphonic acid (C8-PFPA; eight carbon atoms) was listed by the US-EPA as a high production volume chemical in 2007 with an annual production ranging between 4,5 and 230 tons (Howard and Meylan, 2007). PFPAs are strong acids, resulting in a high migration capability into water. D'eon et al. (2009) were the first to demonstrate the presence of PFPAs in surface water and wastewater treatment plant effluent in Canada. These authors reported the presence of PFPAs in 80% of the samples with (C8-PFPA) as dominant compound as well as perfluorodecylphosphonic acid (C10-PFPA; ten carbon atoms). The presence of PFPAs was also confirmed in the European environment in a study from the Netherlands in which 1 ng/L of C8-PFPA was measured in surface water collected from Amsterdam (Esparza et al., 2011).

Current analytical techniques enable the detection and quantification of a large number of organofluorinated compounds. However, the lack of information on the effect of those chemicals in wildlife, fish and other aquatic organisms is a problem nowadays. There are to our knowledge no reports on the toxic effects of C8-PFPA and C10-PFPA on phytoplankton. Green algae are primary producers in aquatic/marine ecosystems; they are in fact an essential part of the seas, rivers and lakes. These organisms are often the first organisms affected by contaminants discharged in aquatic environments (Carrera-Martinez et al., 2011) because of their direct contact with the contaminated medium. The use of microalgae in the toxicity assessment of chemical compounds is therefore instrumental for measuring the impact of xenobiotics within an ecosystem. *Chlamydomonas reinhardtii,* a unicellular green alga, is an aquatic model that has been used in research for more than 50 years. This "green yeast" has the advantages of having a short generation cycle and a fully sequenced genome allowing the assessment of effects at genomic and physiological level, and thus, enabling a better understanding of the modes of action and risks associated with the exposure of PFPAs in aquatic organisms. This study will help address critical knowledge gaps by investigating the effects of C8-PFPA and C10-PFPA in *Chlamydomonas reinhardtii* under controlled conditions.

CHAPTER I

LITERATURE REVIEW

1.1 Perfluorinated compounds (PFCs)

Perfluorinated compounds have been manufactured since the 1950s. The efficient surface tension, surfactant proprieties and thermal stability of PFCs have led to their integration in a myriad of industrial and commercial applications and products (Hansen et al., 2001) such as paints, hydraulic fluids, firefighting foams, polishes, packaging materials, cookware, lubricants, and water repellents for textiles, paper and leather (Kissa, 2001).

Perfluorinated compounds consist of a fluorinated carbon chain of varying length (typically C_4 to C_{16}) and an acidic functional group such as carboxylic and sulfonic acids. PFOA and PFOS have been the most used by the industry due to the high stability of the carbon-fluorine bond, making them resistant to hydrolysis, photolysis, biodegradation and metabolism, and therefore persist in the environment. PFCs have been detected globally in a variety of matrices such as air (Barber et al., 2007), sea water (Yamashita et al., 2005), freshwater (Vierke et al., 2012), sludge (Bradley and Smith, 2011), as well as wildlife and humans (Houde et al., 2006). Main producers worldwide of PFOS phased out the production of this compound in 2002; the manufacture, importation, use and sale of PFOS are prohibited in Canada since 2013. The same implementation is planned for long-chain perfluorocarboxylic acids and PFOA (expected to be applied in 2016). However, developing countries have taken up the production of materials that have been restricted in other parts of the world, becoming a variable to consider in the global PFC emissions. Moreover, the utility of perfluorinated chemistry makes it highly likely that industries will continue to develop and use these chemicals for the foreseeable future.

Recently, a new class of PFCs, constituted of phosphonic acid as hydrophilic group, has emerged: the perfluorinated phosphonic acids (PFPAs). These novel compounds have recently been detected for the first time in surface water and wastewater effluent in Canada (D'eon et al., 2009) and the Netherlands (Esparza et al., 2011).

1.1.1 Perfluorinated Phosphonic Acids (PFPAs).

Perfluorinated phosphonic acids are characterized by a fully fluorinated alkyl chain with three congeners that differ in the number of carbons (C_6, C_8 and C_{10}) and a hydrophilic and phosphate group, PO_3 (molecular formula: $C_nH_2F_{2n+1}PO_3$; Fig. 1.1).

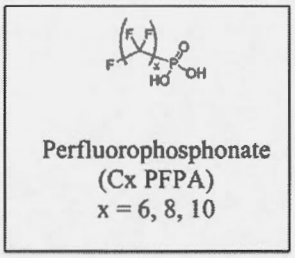

Figure 1.1 General chemical structure of perfluorinated phosphonic acids (PFPAs).

The perfluorinated phosphonic acids are prevalent commercial surfactants that are used as anti-foaming agents in the textile industry, lubricants, leveling and wetting agents, as well as cleaning products and aqueous coatings and defoaming additives in pesticides (Dinglasan, et al., 2004; Buck et al., 2011). Perfluorinated phosphonic acids are constituted of hydrogen atoms and carbon-fluorine bond with similar carbon chain lengths, suggesting they may be similarly resistant to degradation, and present the same patterns of migration of PFOS that once were used commercially. Production and use of PFPAs may be of importance; C8-PFPA (CAS number: 40143-78-0) was indeed listed by the US-EPA as high production volume chemical in 2007 with an estimated annual production volume ranging between 4.5 and 230 tons (Howard and Meylan, 2007).

Recently, the environmental occurrence of PFPAs was shown for the first time in surface water from rivers and creeks from Ontario, Alberta and Saskatchewan, Canada, with specific emphasis on agriculture and urban inputs, as well as effluents of six wastewater treatment plants (WWTPs) (D'eon et al., 2009). Levels of C8-PFPA in wastewater ranged from 760 to 2500 pg L^{-1} and from 88 to 3400 pg L^{-1} in surface water. In this study, PFPAs were detected at 24 of the 30 sites investigated with C8-PFPA as the most abundant congener at all sites as well as C10-PFPA (CAS number: 52299-26-0) with levels ranging from 380 to 460 pg L^{-1}.

The presence of PFPAs in the European environment was also confirmed in a study from the Netherlands where 1000 pg/L of C8-PFPA was found in surface water collected from Amsterdam (Esparza et al., 2011). Furthermore, PFPAs have been proposed as potential persistent, bioaccumulative and toxic substance (PB&T) candidates due to their high predicted bioconcentration factors (BCF=19510) and atmospheric oxidation half-life (AOt1/2 = 38.2 days) (Howard and Meylan, 2007). These values are relatively high compared to criteria for persistent organic pollutants (POPs); i.e., BCF >5000 and AOt1/2 >2 day, which were adopted under North American Agreement for Environmental Cooperation (NAAEC, 1997). Despite the wide commercial application of PFPAs, very little is known about the fate and effects of these compounds in wildlife and aquatic organisms. Recently, studies have demonstrated that PFPAs induced liver enlargement in mouse with potential adverse developmental effects (Das et al., 2011; Tatum et al., 2011). To our knowledge, no information is presently available on the toxicity of PFPAs to aquatic species, including algae.

1.2 Algae as bioindicators of chemical exposure

Algae are photosynthetic organisms able to convert solar energy into chemical energy. As primary producers in aquatic ecosystems, green algae are keystone species (Smith et al., 1999) in oceans, rivers and lakes. The cellular biomass generated through the photosynthetic process becomes the basis of the food chain of invertebrates, fish and other aquatic animals. The oxygen production by these organisms is influenced by the light conditions as well as temperature, oxygen, nutrients, pH, and external environmental factors like the presence of environmental pollutants (Hanelt and Nultsch, 1995). These factors may affect the growth and development of algae, as well as the overall ecosystem productivity (Hamilton and Lewis, 1992).

Microalgae are the first organisms affected by contaminants discharged into aquatic environments because they are directly in contact with the contaminated medium, separated only by the cell wall and the cytoplasmic membrane. The cellular membrane is a dynamic and selective barrier that plays an essential role in regulating biochemical and physiological events, so any alteration in the environment is likely to provoke changes in the population of microalgae. Therefore, the sensibility of microalgae to environmental changes makes these

organisms ideal sentinel species of ecological changes and chemical stress in water bodies (Rost et al., 2018).

A large amount of toxic and bioaccumulative contaminants are found in trace amounts in aquatic ecosystems. An assessment based solely on data derived from the analysis of water samples can therefore be misleading. Contaminants absorption by phytoplankton is the first step in the bioaccumulation in the aquatic food chain. The contaminants with low octanol–water partition coefficients (log Kow<5) can be spread in water and accumulated by aquatic organisms while more hydrophobic pollutants (log Kow>5) may partition into lipid membranes of cells, leading to their biomagnification along the food chain (Binelli and Provini, 2003). Microalgae can cause an adverse effect to organisms that use microalgae as a food source, thus increasing the possibility of toxicity (Okay et al., 2000).

Photosynthetic organisms have increasingly been used as sentinel organisms for the assessment of xenobiotics in marine environments in a variety of research programs (Delahaye et al., 2005; Yokoyama and Ishihi, 2010; Fabricius et al., 2012). Because of their natural and widespread occurrence along seashores worldwide, these photosynthetic organisms can be readily used in the health assessment of ecosystems exposed to toxic compounds (Twist et al., 1997; Ma et al., 2006; Choi et al., 2012).

1.2.1 *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas reinhardtii is a unicellular green microalga of about 10 micrometers in diameter that swims using two flagella. It has a cell wall made of hydroxyproline-rich glycoproteins, a large cup-shaped chloroplast, a large pyrenoid, and an "eyespot" that senses light. It has been widely used as a model organism; important molecular tools have been developed with *Chlamydomonas* awarding this species the title of "green yeast" (Goodenough, 1992). One of the most important developments in genetic engineering is the creation of selectable markers by introduction of DNA into cells. Cosmid (Zhang et al., 2004), yeast artificial chromosome (YAC) (Vashishtha et al., 1996) and heat shock protein (HSP70B) mRNA (Schroda et al., 1999) were also developed using this species.

Furthermore, *Chlamydomonas* has served as a useful experimental system for genetic research on chloroplast biogenesis and function, flagellar assembly and motility, metabolic pathways (including lipid production), the circadian clock, and many other fundamental biological processes. Other studies have investigated the stress responses at transcriptional levels in *Chlamydomonas reinhardtii* related to the nutrient limitation, excess light, osmotic stress, responses to heat and contamination by metals and other xenobiotics. Goho et al. (2000) have reviewed a series of articles related to the genetic fitness and responses of *Chlamydomonas* cells to environmental changes.

In addition, *Chlamydomonas reinhardtii* has a fully known genome sequence, therefore offering the possibility to incorporate genomic analyses (i.e., qRT-PCR) to biological indicators (i.e., endpoints and biomarkers). This combined evaluation in ecotoxicological assessment can greatly help understanding the impacts and modes of action of chemical substances such as perfluorooctylphosphonic acid (C8-PFPA) and perfluorodecylphosphonic acid (C10-PFPA) at several biological levels. As an important food resource for secondary organism in the food chain, *Chlamydomonas reinhardtii* represents a great model organism for ecotoxicological assessment due to its key position in ecosystem. In addition to a wealth of genetic information already available, this alga has a short generation time, it is both a heterotroph and a facultative autotroph, and it can reproduce both sexually and asexually (Merchant et al., 2007).

Previous studies have demonstrated that perfluorinated compounds like PFOS and PFOA cause a significant reduction of algae growth and biomass (Latała et al., 2009; Mitchell et al., 2011; Xu et al., 2013). The reduced growth could be due to the excessive generation of reactive oxygen species, causing oxidative damage to cells (Arukwe and Mortensen, 2011; Boltes, et al., 2012). However, algae have multiple strategies to acclimate to toxic compound assault. These include immobilization, exclusion, chelation and gene regulation (Torres et al., 2008; Jamers et al., 2013). At the physiological level, establishment of an anti-oxidative system is one of the important strategies for algal adaptation to oxidative stress. Perfluorinated compounds induce oxidative stress, therefore, increase activities of antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST) and ascorbate peroxidase (APX) all of which reflect not only

the degree of toxicity, but also the ability to tolerate the oxidative stress in plants (Puckette et al., 2007; Zhou et al., 2009).

The evaluation of different endpoints like cellular viability, production of reactive oxygen species, lipid peroxidation and the gene transcription that constitute the first step of the gene expression in *Chlamydomonas reinhardtii* in response to exposure to PFPAs under controlled conditions represent a good tool to assess environmental impacts of PFPAs (i.e., C8-PFPA and C10-PFPA) in the aquatic environment.

1.3 Objectives and hypotheses

In view of the information gathered the overall objectives of this research are to determine the presence of PFPAs in St. Lawrence River and assess the toxicity of C8-PFPAs and C10-PFPA in *Chlamydomonas reinhardtii* at several biological levels including gene transcription and physiological parameters (e.g., cell growth and viability, accumulation of reactive oxygen species and lipid peroxidation). The specific objectives are:

1. Determine the environmental prevalence of C8-PFPA and C10-PFPA in surface water of the St. Lawrence River system.

   Hypothesis I: Perfluorinated phosphonic acids are found in the St. Lawrence River ecosystem.

2. Evaluate the effects of C8-PFPA and C10-PFPA on cellular viability, production of reactive oxygen species, lipid peroxidation and gene transcription in *Chlamydomonas reinhardtii* using *in vitro* assays.

   Hypothesis II: Perfluorinated phosphonic acids induce oxidative stress by an increase in the reactive oxygen species (ROS) and lipid peroxides. Thus, the antioxidant system increases the transcriptional abundance of all the antioxidative enzymes to response against the oxidative stress generated by C8-PFPA and C10-PFPA in *Chlamydomonas reinhardtii.*

CHAPTER II

Transcriptional and cellular responses of the green alga *Chlamydomonas reinhardtii* to perfluoroalkyl phosphonic acids

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2.1 Résumé

Les PFPAs font partie d'une nouvelle catégorie de perfluoroalkyl récemment détectée dans les eaux de surfaces et à la sortie des usines de traitements des eaux. Les PFPAs sont utilisés dans le secteur industriel comme tensioactifs. A notre connaissance, les effets toxicologiques des PFPAs sur les organismes du milieu aquatique n'ont à ce jour pas été étudiés. Les objectifs de cette recherche sont d'évaluer les effets du C8-PFPA et du C10-PFPA sur les *Chlamydomonas reinhardtii* en utilisant des outils génomiques (qPCR), physiologiques, et biochimiques (production de ROS, viabilité cellulaire et peroxydation des lipides). Après 72 heures d'exposition aucune différence n'a été observée au niveau de la viabilité cellulaire entre les organismes exposés et non exposés aux deux produits chimiques. La concentration de ROS a augmenté significativement après 72 heures (36% et 25,6% pour les concentrations de 125 et 250 µg/L de C10-PFPA). Une augmentation de la peroxydation des lipides a aussi été observée pour les groupes exposés à une concentration de 125 et 250 µg/L pour le C10-PFPA. Exposition à C8-PFPA n'a pas eu d'impact sur la production de ROS et la peroxydation des lipides dans les algues. Afin d'obtenir des résultats au niveau moléculaire, une RT-PCR a été effectuée dans le but d'analyser l'abondance transcriptionnelle de *SOD-1, CAT, APX I, GPX* et *GST.* Les analyses ont révélé que l'expression de *CAT* et *APX I* se régulaient à la hausse lors de l'exposition au C10-PFPA pour toutes les concentrations. De plus, les PFPAs ont été détectés dans des échantillons d'eaux de surfaces dans le fleuve Saint-Laurent avec une concentration de C8-PFPA allant de 250 à 850 pg/L et 380 à 650 pg/L pour le C10-PFPA. Les données indiquent que les PFPAs sont des contaminants environnementaux, et qu'ils peuvent provoquer des réponses au niveau du système de défense des antioxydants chez *C. reinhardtii.*

Mots clés: *Chlamydomonas reinhardtii,* transcription des gènes, perfluoroalkyl, production de ROS, peroxydation des lipides.

2.2 ABSTRACT

Perfluoroalkyl phosphonic acids (PFPAs), a new class of perfluoroalkyl substances, were recently detected in surface waters and wastewater treatment plant effluents. PFPAs have been used primarily in the industrial sector as surfactants. To our knowledge, toxicological effects of PFPAs in aquatic organisms have as yet not been investigated. The objective of the present study was to evaluate the effects of perfluorooctylphosphonic acid (C8-PFPA) and perfluorodecylphosphonic acid (C10-PFPA) exposure on *Chlamydomonas reinhardtii* using genomic tools, and physiological (cellular viability) and biochemical indicators (reactive oxygen species (ROS) production and lipid peroxidation). After 72 hr of exposure, no differences were observed in cellular viability for any of the two perfluorochemicals. ROS concentrations were significantly increased at 72 hr (36% and 25.6% at 125 and 250 μg/L of C10-PFPA, respectively) and lipid peroxidation (measured as malondialdehyde levels) was observed after 72 hr of exposure to C10-PFPA (35.5% and 35.7% at 125 and 250 μg/L, respectively). C8-PFPA exposure did not impact ROS production and lipid peroxidation in algae. To get insights into the molecular response and modes of action of PFPA toxicity, qRT-PCR-based assays were performed to analyze the transcription of genes related to antioxidant responses including superoxide dismutase *(SOD-1),* glutathione peroxidase *(GPX),* catalase *(CAT),* glutathione S-transferase *(GST)* and ascorbate peroxidase (*APX I).* Genomic analyses revealed that the transcription of *CAT* and *APX I* was up-regulated for all the C10-PFPA concentrations. In addition, PFPAs were detected in St. Lawrence River surface water samples with concentrations of C8-PFPA ranging from 250 to 850 pg/L and 380 to 650 pg/L of C10-PFPA. This study supports the increasing prevalence of PFPAs in the aquatic environment and suggests potential impacts of PFPA exposure on the antioxidant defensive system in *C. reinhardtii.*

Keywords: *Chlamydomonas reinhardtii*, gene transcription, perfluoroalkyl phosphonic acids, reactive oxygen species, lipid peroxidation.

2.3 Introduction

Perfluoroalkyl phosphonic acids (PFPAs) are chemicals widely used in the industrial sector as surfactants. Applications include leveling and wetting agents, as well as defoaming additives in several pesticide formulations (Dinglasan, et al. 2004). Chain lengths of fully fluorinated (perfluorinated) phosphonic acids vary between 6, 8, and 10 carbon atoms. Among these, perfluorooctylphosphonic acid (C8-PFPA) was listed by the US-EPA in 2007 as a high production volume chemical with an annual production ranging from 4.5 to 230 tons between 1998 and 2002 (Howard and Meylan, 2007). PFPAs can also be produced by degradation of perfluorophosphinates (PFPIAs). Lee et al. (2012) have done dietary bioaccumulation with PFPIAs that indicated production of PFPAs from the PFPIAs. PFPAs have been identified as persistent, bioaccumulative and toxic (PB&T) candidates due to their high-predicted bioconcentration factors and long-range atmospheric transport according to Howard and Meylan (2007). D'eon et al. (2009) were the first to report the presence of PFPAs in surface water from Canada (i.e., 3400 ± 900 pg/L for C8-PFPA and 660 ± 40 pg/L for perfluorodecylphosphonic acid (C10-PFPA). In that study, PFPAs were detected at 24 of the 30 sites investigated with C8-PFPA as the most abundant congener at all sites. The presence of PFPAs was also reported in the Netherlands where 1 ng/L of C8-PFPA was determined in surface water collected from Amsterdam (Esparza et al., 2011) as well as in tap water samples from Spain in which C10-PFPA was found at concentrations ranging from 8.2 to 23 ng/L (Llorca et al., 2012). The increasing detection of PFPAs in the environment and the structural characteristics that confer these contaminants bioaccumulative and persistent properties may represent risk to aquatic organisms (D'eon and Mabury, 2010). Recently, a series of studies have demonstrated that PFPAs induced liver enlargement on mice with potential adverse developmental effects (Das et al., 2011; Tatum et al., 2011). To our knowledge, no information is presently available on toxicity of PFPAs to plants, especially algae.

Green algae are primary producers in aquatic ecosystems and they are widely used key indicator organisms in ecotoxicological assessments (e.g., Delahaye et al., 2005; Yokoyama and Ishihi, 2010; Fabricius et al., 2012). The unicellular alga *Chlamydomonas reinhardtii* is a useful model to study stress responses.

Growth of this alga is rapid with a short generation time, attaining logarithmic growth phase in two days, and the entire genome of this organism has been sequenced as part of the *Chlamydomonas* Genome Project (http://www.chlamy.org/). This microalga is also highly sensitive to a wide range of metals, nanomaterials, chemicals and xenobiotic compounds with quantifiable responses at the genomic and cellular levels (Wang et al., 2008; Elbaz et al., 2010; Altenburger et al., 2012; Petit et al., 2012).

Previous studies have demonstrated that perfluoroalkyl substances (PFAS) such as perflurooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) cause significant reduction of algae growth and biomass (Latała et al., 2009; Mitchell et al., 2011; Xu et al., 2013). The reduced growth could be due to the excessive generation of reactive oxygen species (ROS), such as peroxide or super-peroxide ions, causing membrane lipid peroxidation and cell membrane damage (Arukwe and Mortensen, 2011; Boltes et al., 2012). Lipid peroxidation is one of the consequences of stress-induced cellular build-up of ROS. However, algae have multiple adaptive strategies against toxic compound assault; these include chemical immobilization, exclusion, sequestration by chelation (metals), and gene regulation (Torres et al., 2008; Jamers et al., 2013). At the physiological level, establishment of an anti-oxidative system represents one of the most important strategies for algae adaptation to oxidative stress. Oxidative stress influence expression of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST) and ascorbate peroxidase (APX), all of which reflect not only the degree of toxicity but also the ability to tolerate the oxidative stress (Puckette et al., 2007; Zhou et al., 2009). Currently, there is a growing usage of new shorter chain in replacement of the more persistent and toxic ones including PFOS and PFOA, although there is a lack of information about their occurrence, behavior in the aquatic environment, and fate in organisms. To our knowledge, no investigations have been conducted to evaluate the effects of PFPAs in aquatic organisms including algae.

The objectives of the present study were determine the presence of PFPAs in surface water samples collected from a densely-populated region of the St. Lawrence River, Canada and investigate the toxicity of C8-PFPA and C10-PFPA to *C. reinhardtii.*

Effects of these two PFPAs were evaluated on cell viability, ROS production, lipid peroxidation, and transcription of antioxidant-related genes (*GPX, CAT, SOD-1, GST,* and *APX I*). Considering that other PFC like PFOA and PFOS have shown to be highly toxic compounds to algae (Mitchell et al., 2011; Xu et al., 2013).

2.4 Materials and methods

2.4.1 Preparation of PFPA solution

Standards of C8-PFPA and C10-PFPA (chemical purity > 98 %; in methanol with final concentration 50 mg/L) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). One mL of PFPAs stock was diluted with 200 mL of grown medium to prepare a 250 µg/L solution. This solution was then diluted in aliquots of 100 mL of the algal culture to obtain a final concentration of 125, 62.5, 31.25 µg/L. All the culture media were constituted with 0.5% of methanol. Preliminary test were performed to verify that 0.5% of methanol did not affect the growth and viability of *C. reinhardtii*. Spiked culture media with PFPAs (250 µg/L) were analyzed twice during the course of the experiment to evaluate the stability of the chemicals.

2.4.2 Algal strain and culture conditions

Chlamydomonas reinhardtii cultures (strain CC-125) were obtained from the Canadian Phycological Culture Center (Toronto, Ontario, Canada). Cultures were grown in 1 L Erlenmeyer flasks containing 250 mL of sterile Sueoka's high salt medium also known as HSM (Harris, 1989). Flasks were maintained at 22 ± 1°C under continuous light (100 µmol m−2 s−1), provided by fluorescent lamps (Sylvania Gro Lux F15W, Germany), with continuous shaking (100 rpm). Exponentially growing cells were diluted with fresh medium to achieve a cell density of 1 x 10^6 cells/mL in 25 mL of growth medium as a final volume. Algae were exposed to PFPAs for three different periods of time (6, 24, and 72 hr) at the same condition. The genomic markers were evaluated at 6 and 24 hr because the transcription was assumed to change during the early stage of exposure, however, the physiological parameters were evaluated at 24 and 72 hr until maturation in the cellular response. All the experiments were carried out in triplicate.

2.4.3 Environmental sample collection

Surface water samples from the St. Lawrence River were collected at four sites (n = 3/site) in June 2013 (Fig. 2.1). Samples were collected in pre-cleaned (using successive methanol washes) polypropylene bottles, and kept at 4 °C until chemical analysis. Sampling sites included Lake Saint François and the southern end of Lake Saint-Louis (i.e., île Paix); these sampling sites are located in fluvial lakes that are influenced by the water masses from the Great Lakes. An additional site was located at the northern side of the Lake Saint-Louis (i.e., île Dowker), which receives water masses from the Ottawa River, and a last site located about 4 km downstream of the point of release of the Montreal's primary wastewater treatment plant effluent (WWTP; L'îlet Vert).

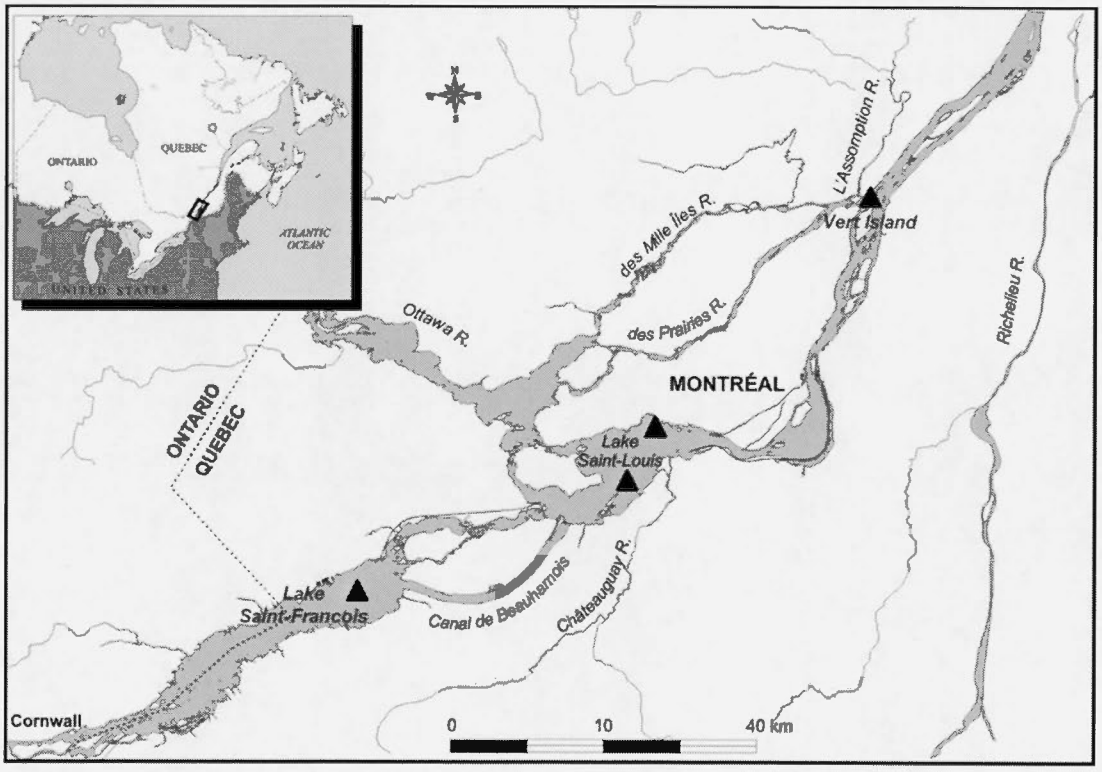

Figure 2.1 Surface water sampling sites (▲) (n = 4) in the St. Lawrence River (Quebec, Canada).

2.4.4 Chemical analyses

Surface water samples were extracted using a modified version of the solid-phase extraction (SPE) method using weak anion-exchange (WAX) SPE cartridges (D'eon et al., 2009). Briefly, 250 mL of surface water was weighed and extracted using Oasis® WAX SPE cartridges (6 mL, 150 mg, 30 µm; Waters, Milford, Mississauga, Ontario, Canada) on a vacuum manifold. Before extraction, samples were spiked with the internal standard ^{18}O-labeled perfluorohexane sulfonate (MPFHxS; Wellington Labs, Guelph, Ontario, Canada), and extracted. The samples were first acidified to pH 4 using formic acid. Cartridges were conditioned using 5 mL of methanol and water (pH 4). Water samples were then loaded onto the cartridge at approximately 5 ml/minute using a SPE vacuum manifold. After drying using vacuum, cartridges were eluted by gravity with 10 mL of 90:10 (volume ratio) MTBE:MeOH solution. The eluant was evaporated to dryness under a nitrogen flow, and then reconstituted in 0.5 mL methanol. Analyses were conducted using an Acquity ultra high performance liquid chromatograph system (UHPLC; Waters, Milford, Mississauga, Ontario, Canada) coupled to a triple quadrupole mass spectrometer (Xevo TQS; Waters). The system was equipped with a BEH C18 column with a particle size of 1.7 μm (50 mm×2.1 mm). The limits of detection (LODs) were defined as the concentration producing a signal-to-noise (S/N) ratio equal or greater than 10. The lowest calibration standard was used as a limit of quantification (LOQ). The LOQ was 70 pg/L for C8-PFPA and 68 pg/L for C10-PFPA. C8/10-PFPAs were detected in the method blanks in concentration an order of magnitude less than St. Lawrence River sample, and thus concentrations were blank-corrected.

2.4.5 Quantitative RT-PCR

Total RNA was isolated using the RNeasy® Plus Mini kit (Qiagen, Valencia, California, USA), according to the manufacturer's protocol. Reverse transcription was performed with 500 ng of RNA QuantiTect Reverse Transcription kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions. The first cDNA was used as a template for RT-PCR amplification to analyze transcript level. Primer pairs for *GPX, CAT, SOD-1, GST,* and *APX I* were designed based on the sequences of *C. reinhardtii* in the GenBANK database. Genes selected for this study, and respective primers, can be found in Table 2.1.

All primers were designed by the authors using Primer-BLAST from NCBI (Primer 3; Rozen and Skaletsky, 2000). The presence of secondary structures was evaluated using Netprimer (PRIMER Biosoft, California, USA). Real time PCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was run in duplicate and consisted of 5 µL cDNA (equivalent to 5 ng cDNA), 6.5 µL iQ™ SYBR® Green Supermix (50 mM KCl, 20 nM Tris-HCl, pH 8.4, 0.2 mM of each dNTP, 25 units/mL iTaq DNA polymerase, 3 mM $MgCl_2$, SYBR Green I, and 10 nM fluorescein), primers (300 nM each), and DEPC treated-water up to a total volume of 13 µL. Cycling parameters were: 95 °C for 2 minutes, 40 cycles at 95 °C for 15 s, and 60°C for 5 seconds. Amplification specificity was verified with a melting curve. No-template control (NTC) was included on each plate. Data acquisition and analysis were performed by CFX Manager™ Software (Bio-Rad). Baseline and threshold were set manually when amplification signal started to appear. Quantification cycle (Cq) values were imported into GenEx Enterprise software (MultiD Analyses AB) in order to select reference genes.

Relative gene expression was calculated using reference genes (CBLP and UBCX) and the Cq values were corrected with the estimated PCR efficiency. The comparative threshold method (ΔΔCt; Livak and Schmittgen, 2001) was used for relative quantification. The control samples (non-treated algae) were chosen to represent 1X transcription of the genes of interest. Treated samples were expressed relative to the corresponding control to determine the difference in transcription.

Table 2.1 Genes, abbreviation, and primers used for the qRT-PCR in *Chlamydomonas reinhardtii*.

Gene name	Abbreviation	Primer sequence	Efficiency %	Amplicon size (bp)
G protein beta subunit-like polypeptide	*CBLP*	Forward 5' GCC ACT CCC TGT AAA TGC C 3' Reverse 5' CTC CTC CTA AAC CCC TCC AG 3'	93	88
Ubiquitin-conjugating enzyme E2	*UBCX*	Forward 5' ATA CAC AAC CAC GAT GAC GAT 3' Reverse 5' TTG CGT GTG TCC TCT CAT CA 3'	94	95
Glutathione peroxidase	*GPX*	Forward 5' CTG TTG CGG TTG TCC TGC TC 3' Reverse 5' GCC AGC CCC TAC GAT ACA AG 3'	98	119
Catalase	*CAT*	Forward 5' CAG GAG GCT GCA GGA AAA CT 3' Reverse 5' ATG ACA ACA TGT ACA TTA CGC GG 3'	97	109
Mn-superoxide dismutase	*SOD-1*	Forward 5' TGT CAG TTT TGT TTC CCT CGT 3' Reverse 5' CTT TCA TGT TCC TCG CCA GC 3'	105	108
Glutathione S-transferase	*GST*	Forward 5' GCT AGC AAG GCA AAA CTC TTT 3' Reverse 5' GCG TCA CAA TGT CAA TCT GG 3'	100	106
Ascorbate peroxidase	*APX I*	Forward 5' AGT TGT GTA GTG GGT GGC AG 3' Reverse 5' CGG TAT GAT GAT AAG GGT TCG CA 3'	108	100

2.4.6 Flow cytometric analyses: cell viability and ROS production

Aliquots of microalgae cultures were collected at 24 and 72 hr following exposure to 31.25, 62.5, 125, and 250 µg/L of C8- and C10-PFPA. Cells were counted, and fluorescence of samples was measured using three-color Guava EasyCyte Plus System cytometer (Guava Technologies, Hayward, California, USA) with a laser emitting at 488 nm. To respect the maximum limit of cell density recommended by the manufacturer (500 cells/µL), algal solutions were diluted with growth medium. Cells were incubated during 15 minutes in the dark with fluorescein diacetate (FDA; final concentration: 13 µg/mL). FDA, a cell-permeant esterase substrates, was used as a viability probe to measure enzymatic activities, which is required to activate its fluorescence and cell-membrane integrity (Xu et al., 2013). Alteration of the esterase activity resulting from the exposure of algae to PFPAs was measured by changes in green fluorescence (fluorescein) to assess cell viability. Histograms were used to set levels and exclude particles without red fluorescence, which represent non-algal particles because of the red fluorescence of the chlorophyll. The cell viability of cultures was expressed as the percentage of viable cells vs. the total amount of cells analyzed by flow cytometer.

The dye 2-7-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma-Aldrich, Oakville, Ontario, Canada) was used as an indicator for ROS production (Ostrovidov et al., 2000; Eruslanov and Kusmartsev, 2010). H_2DCFDA was dissolved in methanol to obtain a 5 mM stock solution. This hydrophobic non-fluorescent chemical can get across the cell membrane and be oxidized by intracellular ROS into a green fluorescent form, 2-7-dichlorofluorescein (DCF), therefore detecting changes in the redox state of a cell (Petit et al., 2012). Data processing was carried out using data acquisition and analysis software from Cytosoft (Guava Technologies, Hayward, California, USA). The abundance of cells producing ROS after each treatment was recorded, and results were corrected for the density of viable cells. Alteration in the ROS levels resulting from exposure of algae to PFPAs was quantified as the mean DCF fluorescence.

2.4.7 Lipid peroxidation

Levels of lipid peroxidation products were measured as thiobarbituric acid reactive substances (TBARS) based on the method described by Heath and Packer (1968). The method is based on the malondialdehyde (MDA) production during the oxidation of polyunsaturated fatty acids. Algae were harvested (1 x 10^6 cells) by centrifugation and dissolved in 1 mL phosphate buffer (50 mM) containing (0.67% w/v) trichloro-acetic acid (TCA) followed by cell disruption using ultra-sonication at 400 mA for 120 seconds. The homogenate was mixed with 300 µL of 0.5% thiobarbituric acid (TBA) (w/v) in 20% TCA (w/v). The mixture was heated to 95°C for 30 minutes, cooled on ice for 10 minutes, and centrifuged at 15,000 x g for 20 minutes. The absorbance of the supernatant was measured with a spectrophotometer (Power Wave X, Bio-Tek, Burlington, Ontario, Canada) at 532 nm, and corrected for unspecific turbidity by subtracting the absorbance of the same sample at 600 nm. The absorbance of the blank solution (0.25% TBA and 10% TCA) was used as a zero reference. MDA concentration in samples was determined using a six-point standard curve (R^2= 0.99).

2.4.8 Statistical analyses

All means were expressed with standard deviation (± SD). The assumption of normality was tested by Shapiro-Wilk W test. Data were analyzed by one way analysis of variance (ANOVA) to establish the difference between the treatments. When significant differences were observed, means were compared using the Dunnett post-hoc test. The significant threshold was established at 0.05. Analyses were carried out using JMP 10.0.0 software (SAS Institute, Riverside, California, USA).

2.5 Results and discussion

2.5.1 PFPA in surface water

Perfluoroalkyl phosphonic acids were detected in surface water at three of the four sites, although concentrations were not significantly different between these sites with concentrations of C8-PFPA ranging from 250 to 850 pg/L and 380 to 650 pg/L for C10-PFPA (Table 2.2). To our knowledge, this is the first detection of PFPAs in the St. Lawrence River. These concentrations were comparable to those reported by D'eon et al. (2009) in surface water of creeks and rivers from Ontario, Alberta and Saskatchewan (Canada), as well as in surface water from the Netherlands (Esparza et al., 2011) and tap water from Spain (Llorca et al., 2012). Overall, these results suggest that C8-PFPA and C10-PFPA are highly prevalent compounds in the aquatic environment. In the present study, PFPA concentrations in surface water were found at lower levels compared to other perfluoroalkyl substances reported in the same area of the St. Lawrence River (range: 2,550-2,770 pg/L and 2,380-2,810 pg/L for PFOS and PFOA, respectively) (Houde et al., 2014).

Table 2.2 Arithmetic mean (± SD) of PFPA concentrations in surface water (n =3) at four sites in the St. Lawrence River. The limits of quantification (LOQ) were 70 pg/L for C8-PFPA and 68 pg/L for C10-PFPA.

Sampling site	Concentration (pg/L)	
	C8-PFPA	C10-PFPA
Lake Saint François	440 ± 120	650 ± 92
île Paix	<LOQ	<LOQ
île Dowker	850 ± 80	380 ± 40
L'île Vert	250 ± 51	<LOQ

2.5.2 Effects of PFPAs on cell viability and ROS levels

The effects of C8-PFPA and C10-PFPA exposure on the viability of *C. reinhardtii* were determined using esterase activity. Unexposed cells grown for 72 hr in Sueoka's high salt medium had viability ranging between 93% and 96%. No statistical difference was observed between treatments and control. Therefore, no dose-related response was observed on cell viability following exposure to C8- and C10-PFPAs (Fig. 2.2).

During normal cell metabolism, several ROS are generated as an inevitable consequence of redox cascades of aerobic metabolism (Vavilin et al., 1998). It is of interest to test whether PFPAs also induced intracellular ROS production in *C. reinhardtii.* Results indicated that ROS concentrations remained unchanged at all concentrations of C8-PFPA and at any time points of exposure (24 and 72 hr). In contrast, ROS production was significantly increased following 72 hr of exposure to C10-PFPA (increase of 36 % and 25.6 % at 125 and 250 μg/L, respectively) compared to the control (p= 0.002 and p= 0.008; Fig. 2.3).

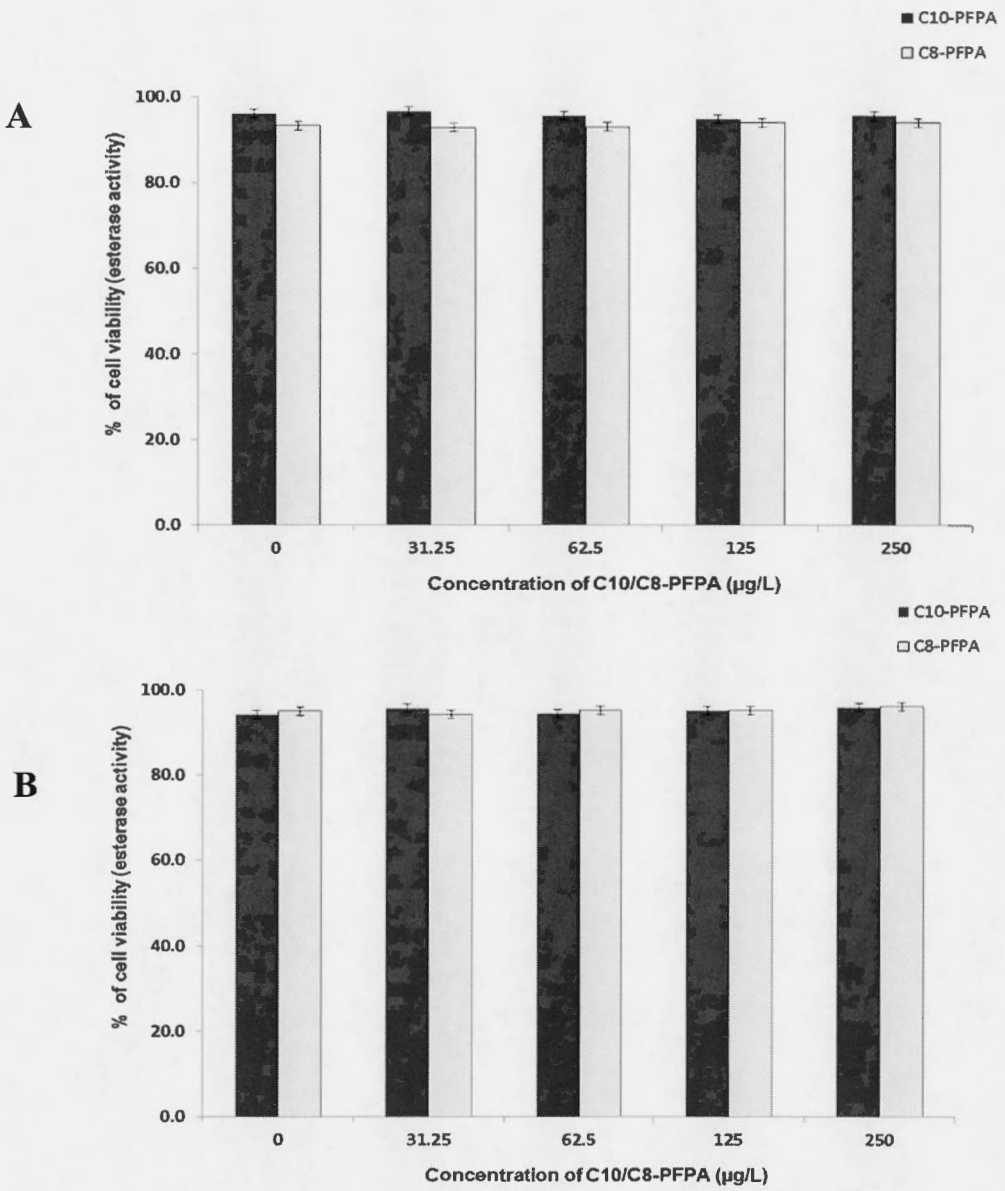

Figure 2.2 Percentage of cell viability (determined by esterase activity) in *Chlamydomonas reinhardtii* cultures after exposure to different concentrations (µg/L) of C8- and C10-PFPA at A) 24 hr and B) 72 hr. Data are arithmetic mean ± SD (n=3).

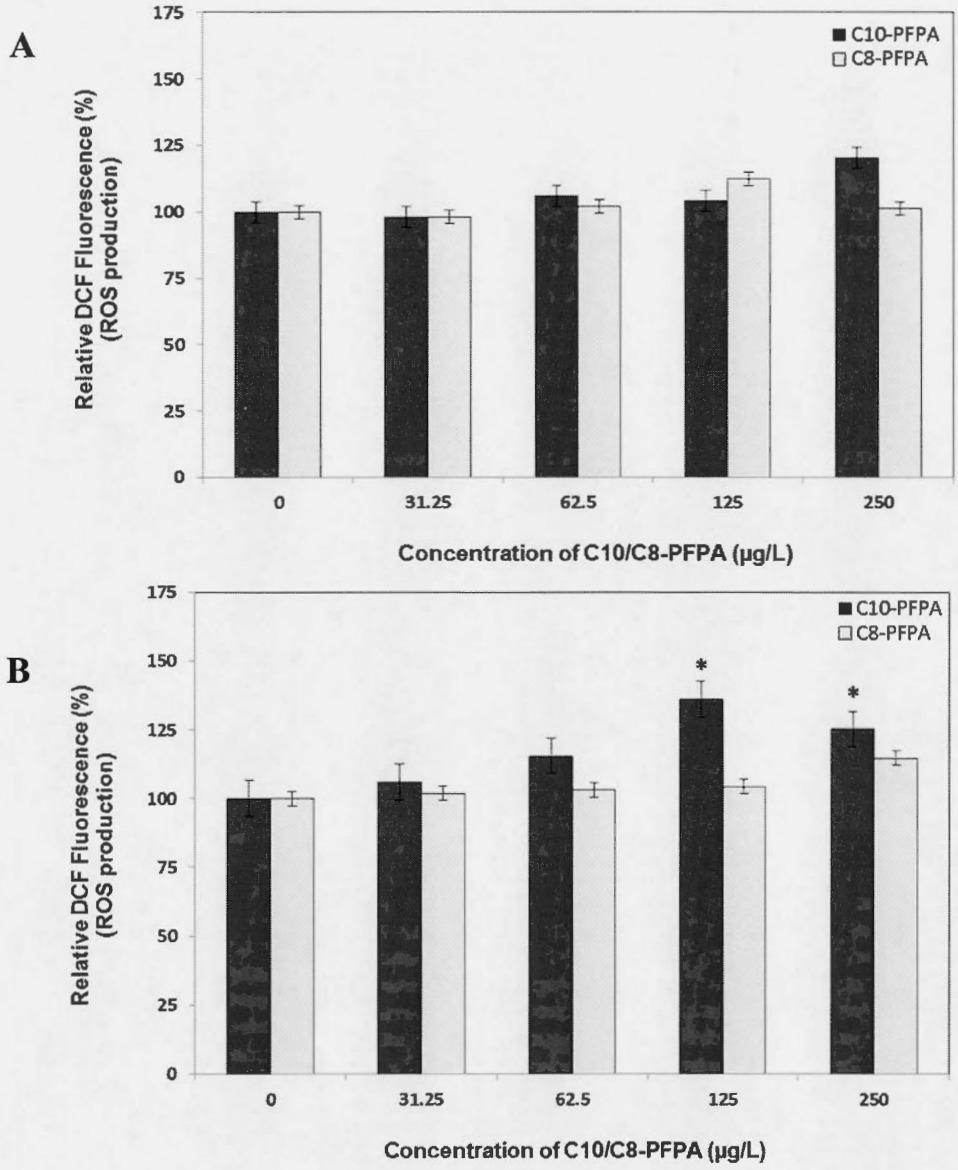

Figure 2.3 ROS production in *Chlamydomonas reinhardtii* cultures after exposure to different concentrations (µg/L) of C8- and C10-PFPA at A) 24 hr and B) 72 hr. Data are arithmetic mean ± SD *(n=3)*. The asterisk (*) indicates significant difference at α=0.05 between control and PFPA-treated cultures.

In algae the excess ROS may affect many cellular functions through modification of nucleic acids, oxidation of proteins, and induction of lipid peroxidation (Okamoto et al., 2001; Mittler et al., 2004; Ledford and Niyogi, 2005). ROS are not only damaging to plant cells, but can also be important intracellular messengers. At sublethal levels, ROS have been shown to enhance the defence system through activation of pathways that reinforce defense responses and enhance survival to subsequent oxidative stress. More specifically, in a study by Xu et al. (2013), an increase in levels of ROS was observed in the green microalgae *Chlorella pyrenoidosa* and *Selenastrum carpricornutum* exposed to PFOA, resulting in membrane lipid peroxidation and oxidative damage to cells by the reduction of the cell membrane permeability. The authors of this study suggested that exposure to PFOA increases the level of stress in the algae, exceeding the capacity of their antioxidant system, which resulted in the accumulation of ROS and subsequent lipid peroxidation. In order to determine the mechanism involved in ROS generation in C10-PFPA-exposed *C. reinhardtii*, effects of the PFPAs on lipid peroxidation were examined.

2.5.3 Lipid peroxidation

Reactive oxygen species degrade polyunsaturated lipids to form malondialdehyde (MDA). Malondialdehyde is one of several low-molecular-weight end products formed by the decomposition of primary hydroperoxides and lipid polymers (Gill and Tuteja, 2010). Measurement of MDA level is routinely used as an index of lipid peroxidation in algae, and has been documented to vary in the algae *Chlorella pyrenoidosa* under many stressful conditions such as high temperatures (Vavilin et al., 1998) and exposure to wide range of trace elements in the alga *Pavlova viridis* (Li et al., 2006) and *Chlamydomonas reinhardtii* (Elbaz et al., 2010). In the present study, the levels of MDA remained unchanged at all doses after 72 hr of exposure to C8-PFPA. On the other hand, the levels of MDA in C10-PFPA-exposed cells were significantly increased after 72 hr of exposure (35.5% and 35.7% at 125 and 250 μg/L, respectively) (p= 0.0188 and p= 0.0183 Fig. 2.4).

ROS production must be carefully regulated to avoid unwanted cellular cytotoxicity and oxidative damage (Halliwell and Gutteridge, 1990). Here, results indicated lipid peroxidation in algal cell membranes after exposure to C10-PFPA, which could be induced by the increase in ROS production. These results are in agreement with studies of PFOA in *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* (Hu et al., 2014). These authors found an increase of MDA and subsequent cell-membrane damage at 20 mg/L of PFOA after 96 hr of exposure. This concentration was below the half maximal effective concentration (EC50) reported for this chemical, indicating defense responses against oxidative stress in the two algal species in the absence of growth inhibition.

ROS can also convert fatty acids into toxic lipid peroxides, thus damaging biological membranes. The accumulation of such reactive oxygen species can be counteracted by intrinsic antioxidant system including enzymatic scavengers such as GPX, CAT, SOD, GST, APX and other oxidative enzymes (Apel and Hirt, 2004).

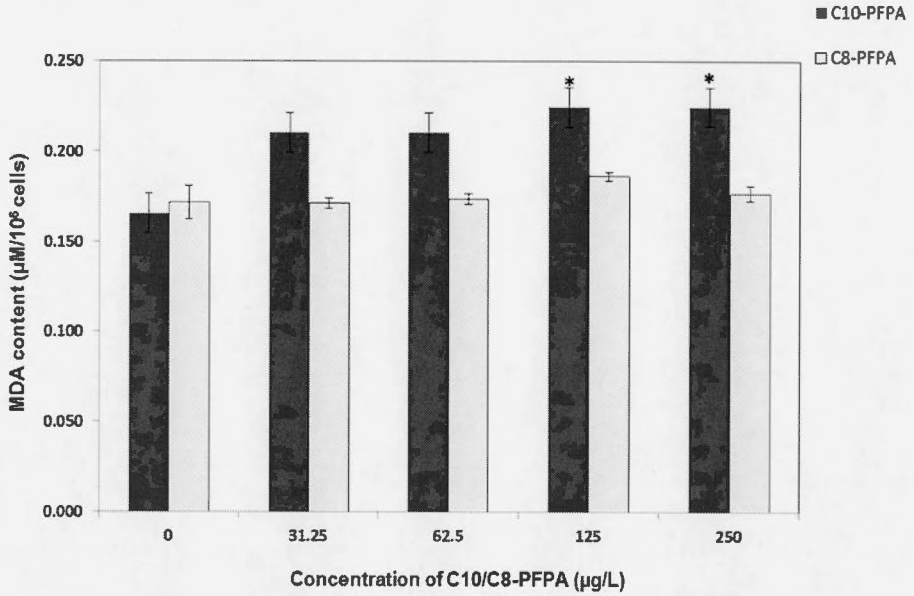

Figure 2.4 Lipid peroxidation (measured as MDA content) of *Chlamydomonas reinhardtii* cultures after 72 hr of exposure to different concentrations (μg/L) of C8-PFPA and C10-PFPA. Data are arithmetic mean ± SD *(n=3)* The asterisk (*) indicates significant difference at α=0.05 between control and PFPA-treated cultures.

2.5.4 Transcription of oxidative related genes

Genomic analyses were carried out for selected genes (Table 2.1). The relative gene transcription (fold change) in *C. reinhardtii* can be found in Table 2.3. Analyses indicated the transcription of the gene coding for *GST* was down-regulated at 45%, 57% and 36% at 62, 125 and 250 µg/L, respectively after 6 hr of exposure to C10-PFPA. In contrast, the relative transcript abundance of *GST, APX I* and *GPX* was up-regulated following exposure to C8-PFPA. After 6 hr of exposure, expression of *GST* was increased about 84% and 61% for 62 µg/L and 125 µg/L, respectively, while the expression of *GPX* was increased by about 46% at 125 µg/L. After 24 hr of exposure an up-regulation of the transcription of genes coding for *CAT, APX I,* and *GPX* following C10-PFPA exposure. More specifically, a two-fold increase in transcription was observed at all tested concentrations for *CAT* and *APX I* after 24 hr. For APX I, expression after 6 hr and 24 hr below 250 µg/L remained constant with an increase corresponding to 39 and 43%, respectively.

Table 2.3 Transcription of antioxidant enzyme-related *GPX, CAT, SOD-1, GST* and *APX I* in control and following treatment with different concentrations of (A) C8-PFPA and (B) C10-PFPA. Control samples were defined as 1 X expression level for each analyzed gene. Data are arithmetic mean ± SD (n=3). The asterisk (*) indicates significant difference at α=0.05 between control and PFPA-treated cultures.

A

6 hr of treatment (μg/L)	*CAT*	*SOD-1*	*GST*	*APX I*	*GPX*
0	100	100	100	100	100
31	103	99	124	120	119
62	111	122	184*	136	123
125	103	145	161*	138	146*
250	105	122	134	139*	106
24 hr of treatment (μg/L)	*CAT*	*SOD-1*	*GST*	*APX I*	*GPX*
0	100	100	100	100	100
31	95	64	68	114	114
62	95	66	83	131	110
125	112	83	78	138	105
250	102	107	77	143*	102

B

6 hr of treatment (μg/L)	*CAT*	*SOD-1*	*GST*	*APX I*	*GPX*
0	100	100	100	100	100
31	121	58	60	85	83*
62	93	54	45*	92	95
125	114	70	57*	136*	110
250	102	55	36*	109	87
24 hr of treatment (μg/L)	*CAT*	*SOD-1*	*GST*	*APX I*	*GPX*
0	100	100	100	100	100
31	172*	72*	111	186*	95
62	196*	79	121	231*	99
125	146*	74	99	179*	96
250	155*	106	127*	162*	98

In higher plants, a number of enzymes regulate intracellular hydrogen peroxide (H_2O_2) levels generated during the cellular metabolism. High intracellular levels of H_2O_2 were shown to induce DNA damage, cellular proliferation inhibition and cell death in organisms (Mittler et al., 2004). CAT and APX are considered the most important enzymes to catalyze the transformation of H_2O_2 to H_2O (Nakano and Asada, 1981; Livingstone, 2001). In the present study, transcription of genes coding for *CAT* and *APX I* were significantly increased at all concentrations of C10-PFPA and remained unchanged for C8-PFPA exposure. CAT is mainly located in the peroxisome and is responsible for the protection from the oxidation of unsaturated fatty acids in cell membrane. Therefore, the higher activity of *CAT* could be attributed to the increase of pro-oxidants such as H_2O_2. APX I is an enzyme involved in the ascorbate-glutathione cycle in the chloroplast, mitochondria as well as in peroxisomes. In chloroplast, APX I removes H_2O_2 using ascorbate as electron donor. Shigeoka et al. (2002) have demonstrated that a high level of endogenous ascorbate peroxidase is essential to maintain the antioxidant system that protects plants from oxidative damage from biotic and abiotic stresses. Our results indicate that transcription of *APX I* and *CAT* were up-regulated by the presence of C10-PFPA, suggesting that exposure to this chemical increase the levels of ROS, thus altering the antioxidant enzyme system used to neutralize free radicals and ROS in cells.

GPX, SOD and GST are primary enzymes involved in the cell antioxidant defense system. Within cells, SOD constitutes the first line of defense that catalyzes the dismutation of superoxide (O_2^-) into O_2 and H_2O_2 (McCord and Fridovich, 1988). The biochemical function of GPX is to reduce lipid hydro-peroxides to their corresponding alcohols (OH-), and to reduce free H_2O_2 to H_2O (Epp et al., 1983). GST is best known for its ability to bind toxins and its function as transport proteins (Udomsinprasert et al., 2005) is important for the detoxification process inside the cells. In the present study, a significant increase in the relative transcript abundance of *GST, APX I* and *GPX* was observed following exposure to C8-PFPA. However, algae exposed to this chemical did not show an increase in ROS levels, suggesting that an up-regulation in the expression of these enzymes could be related with an

efficient antioxidative process inside the cells. On the other hand, a significant decrease in the transcription of the three genes coding for *GPX, GST* and *SOD-1* were found after exposure to C10-PFPA. This may suggest a failure in detoxification and occurrence of oxidative stress in the cells (Santos et al., 2004). In agreement with our observations, a study of primary hepatocyte cells in freshwater tilapia (*Oreochromis niloticus*) showed down-regulation of *GPX* and *GST* after exposure to PFOS and PFOA, and subsequent oxidative stress measured by lipid peroxidation and significant induction of ROS (Liu et al., 2007). These authors suggested that the oxidative system in tilapia was remodelled in response to this chemical stress. Indeed, in response to environmental changes, aquatic organisms and microalgae are able to remodel their antioxidative system in order to dissipate the excess of ROS that could generate membrane lipid peroxidation. Since ROS production and lipid peroxidation were increased following C10-PFPA exposure in *C. reinhardtii*, the modification of the expression of antioxidant related-genes, either increase *CAT* and *APX I* or a decrease for *GST*, may represent compensatory mechanisms to prevent an oxidative damage by an excess of ROS production.

2.6 Conclusion

The study confirms the presence of PFPAs in the St. Lawrence River system. Furthermore, we evaluated the effects of C8-PFPA and C10-PFPA exposure on *Chlamydomonas reinhardtii* using a multi-level biological approach. The regulation of transcription of antioxidant–related genes as well as ROS production and lipid peroxidation were impacted by exposure to C10-PFPA in *C. reinhardtii.* Gene regulation expression was the only parameter impacted by exposure to C8-PFPA in algae. These findings indicate that C10-PFPA induces oxidative stress in *C. reinhardtii* potentially through the activation of antioxidative enzymes including CAT, APX and GST to reduce the ROS. These results highlight the importance of assessing effects of exposure to chemical substances on multiple level of biological organisation. To our knowledge, this is the first study to demonstrate impacts of PFPAs on algae. Further research is needed to clarify long-term effects of PFPAs

on microalgae and the mechanism of ROS generation as well as the environmental fate and distribution of these chemicals in the environment.

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CONCLUSION

The versatile applications of PFPAs make them widely used as commercial surfactants. However, the large-scale use has led to their continuous release into the aquatic environment. Detection and quantification of PFCs have been the priority for the scientific community because of their global distribution, persistence and bioaccumulation (Houde et al., 2006; Dreyer et al., 2009; Houde et al., 2011). However, there is a lack of information concerning the toxicity of PFPAs in aquatic organisms, particularly in algae. Previous studies have demonstrated that other PFCs including PFOS and PFOA may cause adverse effects on physiological and biochemical components of the microalgae with a significant reduction of growth and a disturbance in the antioxidative system (Latała et al., 2009; Mitchell et al., 2011; Xu et al., 2013). Green algae are primary producers in the aquatic ecosystem and they are key indicator organisms for assessing water quality and toxicity of pollutants (Altenburger et al., 1990). The negative impacts of PFC affect the energy transfer in the aquatic food chain, which leads to the changes in the higher trophic level organisms and the ecosystem. In this study, we evaluated the effects of C8-PFPA and C10-PFPA on *Chlamydomonas reinhardtii* using a multi-level biological approach integrating physiological, biochemical and genetic levels. Indeed, the integration of the genomic tools in the ecotoxicological assessment contributes to the understanding of the impacts of contaminants in the aquatic system. Therefore, a change in the transcriptional abundance of oxidative related genes after exposure to PFPAs may constitute a sensitive tool that can help to understand the mode of action (toxicity) of these two compounds in the microalgae.

The first objective of this Master's project was to determine the environmental prevalence of C8-PFPA and C10-PFPA in the St. Lawrence River system. A second objective was to evaluate effects of C8-PFPA and C10-PFPA on cellular viability, production of reactive oxygen species, lipid peroxidation and gene transcription in *Chlamydomonas reinhardtii* using *in vitro* assays. In terms of toxicity response, other PFCs like PFOA were previously shown on algae to cause a significant reduction in the cell viability, which was associated with an increased in the ROS production (Xu et al., 2013).

Analysis of environmental samples confirmed the presence of PFPAs in surface water from St. Lawrence River at three of the four sites investigated (n=3 samples/site). At these sites, PFPAs were detected at concentrations of C8-PFPA ranging from 250 to 850 pg/L and 380 to 650 pg/L for C10-PFPA. To our knowledge, this is the first detection of PFPAs in the St. Lawrence River. These concentrations were comparable to those reported by D'eon et al. (2009) in surface water of creeks and rivers from Ontario, Alberta and Saskatchewan, Canada, as well as in surface water from the Netherlands (Esparza et al., 2011) and tap water from Spain (Llorca et al., 2012). Overall, these results suggest that C8-PFPA and C10-PFPA are prevalent compounds in the aquatic (freshwater) environment.

An *in vitro* assay was performed to establish the effect of PFPA in algae. We observed that C8/C10 PFPA did not reduce the cell viability; it nevertheless increased levels of ROS. Moreover, exposure of algae to C10-PFPA at 125 and 250 µg/L led to the highest production of ROS while viability was around 96% at these concentrations. Therefore, detection of elevated ROS levels should be considered as an early response to C10-PFPA exposure in *C. reinhardtii.* The increase of ROS production under environmental stress in microalgae can lead to different mechanisms. Reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl (OH^-) are constantly generated as by-products in chloroplast, mitochondria and peroxisome. Under controlled conditions, cells can control the ROS production by the antioxidative enzymes. However, the risks of cellular damages arise when ROS are overproduced under stress conditions. One of the expected consequences of ROS accumulation in the cells is the lipid peroxidation, which is a manifestation of free radical activity in the biological system (Vavilin et al., 1998). The present study showed a cellular lipid peroxidation induced by exposure to C10-PFPA that was significantly higher than control (35.5 % and 35.7% at 125 and 250 μg /L, respectively). Therefore, the lipid peroxidation measured in algae cell membranes after exposure to C10-PFPA can be a consequence of the ROS accumulation generated under these treatments. These results are in agreement with studies of PFOA exposure (96 hr) in *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* (Hu, et al., 2014). These authors found an increase of lipid

peroxidation at 20 mg/L of PFOA. This concentration was below the half maximal effective concentration (EC50), thus indicating defense responses against oxidative stress in the two algae species in the absence of algal growth inhibition. These results highlight the importance of assessing effects of exposure to chemical substances on multiple levels of biological organization.

On the other hand, ROS accumulation may also affect the expression of a number of genes including those coding for ROS scavengers such as GPX, CAT, SOD, GST and APX (Gill and Tuteja, 2010). The relative gene transcription (fold change) in *C. reinhardtii* indicate an up-regulation of the transcription of genes coding for *CAT, APX I* with a two-fold increase in transcription at all tested concentrations after 24 hr of C10-PFPA exposure, and *GST* was down-regulated at almost all concentrations of C10-PFPA after 6 hr of exposure. In addition, the relative transcript abundance of *GST, APX I* and *GPX* was up-regulated following exposure to C8-PFPA after 6 hr. Thus, the antioxidant enzyme system was modified in order to neutralize the ROS production. The lipid peroxidation and ROS accumulation likely caused by C10-PFPA stimulated the up-regulation of the genes known to be involved in antioxidant reactions. The modification of the expression for antioxidant-related genes, either a decrease of *GST* or an increase of *CAT, APX I* and *GPX,* in response to C8/C10-PFPA exposure, may be a compensatory mechanism to prevent oxidative damage generated by an excess of ROS production. These responses constitute a complex toxicological mechanism of action of PFPAs on algal cells and gene regulation at different concentrations of PFPAs. In this study we raised two hypotheses, the first one proposed that PFPAs were distributed in the St. Lawrence River based on studies for the same geographical area that showed presence for other perfluorocarbons (Houde et al., 2014). The results confirmed the presence of PFPAs; therefore, the first hypothesis is accepted. Regarding the second hypothesis, we suggested based on the literature that PFPAs generate oxidative stress on the cells with an increase in ROS production and lipid peroxidation as well as a disturbance in the transcriptional abundance of antioxidative enzymes. These findings show that both chemicals can modify the transcription of antioxidative enzymes in response to

oxidative stress related to an increase of ROS production and lipid peroxidation. However, ROS production and lipid peroxidation did not change after exposure to C8-PFPA. Therefore, we accepted the second hypothesis for C10-PFPA, but not for C8-PFPA. These results highlight the importance of assessing effects of exposure to chemical substances on multiple levels of biological organization. To our knowledge, this is the first study to demonstrate impacts of PFPAs on algae. Further research is needed to clarify long-term (chronic) effects of PFPAs on microalgae and the mechanisms of ROS generation. In addition, a measurement of the enzymatic activity for the genes evaluated will be necessary to better understand the mode of action of the PFPAs in algae as well as the environmental fate and distribution of these chemicals in the environment.

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