UNIVERSITÉ DU QUÉBEC À MONTRÉAL

RÔLE DU BIOFILM EPIPHYTIQUE DANS L'ACCUMULATION, LA MÉTHYLATION ET LA DÉMÉTHYLATION DU MERCURE

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STÉPHANIE HAMELIN

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AVANT-PROPOS

Cette thèse de doctorat regroupe trois chapitres sous la forme d'articles destinés à être publiés dans des journaux scientifiques. Afin d'en faciliter la publication, ces articles ont été rédigés en anglais, et sont précédés d'une introduction et suivis d'une conclusion générales, toutes deux écrites en français. Le premier chapitre porte sur les variations spatio-temporelles de la biomasse et des concentrations en mercure et méthylmercure des épiphytes et des macrophytes, qui sera soumis à la revue Environmental Pollution. Le deuxième chapitre, quant à lui traite des taux de méthylation et de déméthylation du mercure par les biofilms épiphytiques et sera soumis à la revue Science of the Total Environment. Finalement, dans le troisième chapitre, il a été question d'identifier les principaux groupes de micro-organismes responsables de la méthylation du mercure au sein du biofilm épiphytique. Ce dernier article a été publié en 2011 dans la revue Environmental Science and Technology.

En ce qui concerne la contribution relative des différents auteurs de ces articles, personnellement en tant qu'auteur principal, j'ai rédigé le projet de recherche avec l'encadrement de ma directrice Dolors Planas et de mon co-directeur Marc Amyot. J'ai ensuite préparé et effectué l'échantillonnage au lac St-Pierre avec l'aide d'assistants de recherche. J'ai réalisé l'ensemble des analyses de laboratoire (à l'exception des analyses d'isotopes de mercure qui ont été faites au laboratoire d'Holger Hintelmann, à l'université de Trent), des analyses de données et de la rédaction de la thèse. Pour le dernier chapitre, j'ai bénéficié de l'aide de Tamar Barkay et de Yanping Wang qui m'ont formée, puis encadrée afin que je puisse faire moi-même les analyses microbiologiques menant au séquençage d'ADN. Ma directrice Dolors Planas et mon co-directeur Marc Amyot ont collaboré tous deux à l'écriture des trois articles en commentant et en corrigeant chacun de mes manuscripts.

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RÉSUMÉ

Au sein des écosystèmes aquatiques, la contamination des organismes vivants par le mercure (Hg) continue à être un sujet de préoccupation pour la santé humaine et les écosystèmes dans de nombreux pays. Il est généralement admit que les milieux humides et les herbiers de plantes aquatiques jouent un rôle important dans les processus de filtration et de stockage des contaminants. Cependant, en ce qui concerne le Hg, le rôle des communautés littorales, particulièrement des épiphytes (biofilms périphytiques recouvrant les plantes aquatiques) et des macrophytes, dans l'accumulation et la méthylation du Hg (transformation du Hg en méthylmercure, MeHg) est mal connu. En effet, il existe très peu de données sur leurs concentrations en Hg et en MeHg en zone tempérée froide ainsi que sur les variables environnementales influençant les variations spatio-temporelles de ces concentrations, ainsi que sur la production nette de MeHg dans les épiphytes et les macrophytes. Considérant le fait que ces organismes se trouvent à la base de la chaîne alimentaire et qu'ils peuvent potentiellement transférer le Hg et le MeHg accumulés aux autres organismes des maillons trophiques supérieurs, le but global de cette étude était de vérifier la contribution des complexes épiphytes/macrophytes dans les processus de méthylation, déméthylation et d'accumulation du Hg et du MeHg afin d'assurer une meilleure gestion du de ces polluants dans les écosystèmes aquatiques. Les objectifs étaient: 1) mesurer les concentrations de Hg et MeHg dans les épiphytes et les macrophytes; 2) évaluer l'effet de la saisonnalité (température et lumière), de la proportion d'organismes autotrophes au sein du biofilm, de l'espèce de macrophytes et des carctéristiques chimiques du site sur les concentrations de Hg et MeHg dans les épiphytes et les macrophytes; 3) mesurer *in situ* les taux de méthylation et de déméthylation du Hg des complexes épiphytes/macrophytes; 4) évaluer l'influence de la lumière, de la température et de l'espèce de macrophyte sur les taux de méthylation, déméthylation et de production nette de MeHg des complexes épiphytes/macrophytes; 5) identifier les principaux microorganismes méthylateurs au sein du biofilm épiphytique par l'ajout d'inhibiteurs métaboliques spécifiques et par la charactérisation des microorganismes actifs (séquençage de gènes 16S rRNA), le tout simultanément aux mesures de méthylation/déméthylation. Cette étude a été réalisée dans les herbiers aquatiques du lac St-Pierre, un élargissement du fleuve St-Laurent (Québec, Canada). Lors du suivi saisonnier des concentrations de Hg et de MeHg, celles-ci étaient généralement un ordre de grandeur plus élevées dans les épiphytes que dans les macrophytes. Les concentrations en Hg dans les épiphytes diminuaient linéairement en fonction de l'indice d'autotrophie du biofilm épiphytique, et les proportions de MeHg/THg dans les épiphytes ont atteint des valeurs aussi élevées que 74%. Les variables ayant une plus grande influence sur les variations spatio-temporelles de concentrations en Hg et MeHg observées dans les épiphytes et les macrophytes étaient la température de l'eau, le % de lumière disponible, l'espèce de macrophyte, le niveau d'eau, ainsi que le carbone organique. Les mesures des taux de méthylation et de déméthylation avec ajout d'isotopes stables de ^{199}HgO et de $Me^{200}Hg$ comme traceurs ont démontré que les épiphytes du lac St-Pierre sont un

site important de méthylation du mercure, à des taux semblables à ce qui a été mesuré en milieu tropical et subtropical. Par contre, la méthylation n'a pas été détectée dans les macrophytes. La production nette de MeHg (normalisée par unite de masse d'épiphytes) varie au cours de la saison de croissance et semble être influencée par la lumière disponible, la température, la productivité du milieu ainsi que par la structure des communautés. Finalement, les mesures de taux de methylation et déméthylation du Hg effectuées avec ajout d'inhibiteurs ont révélé que que différents microorganismes au sein des épiphytes ont un rôle à jouer dans les processus de méthylation. Les résultats suggèrent que les méthanogènes seraient les principaux organismes méthylateurs des biofilms épiphytiques du lac St-Pierre, conclusion qui est supportée par la détection de séquences de gènes 16S rRNA propres au méthanogènes.

Mots clés: périphyton, macrophytes, mercure, methylmercure, méthylation.

INTRODUCTION GÉNÉRALE

Le mercure (Hg) est naturellement présent dans les écosystèmes aquatiques à de faibles concentrations. L'usage industriel de ce métal, la combustion du charbon et du pétrole, l'épandage de produits organomercuriels en agriculture, ainsi que la propriété du Hg à être transporté par l'atmosphère à grande échelle ont contribué à augmenter mondialement les concentrations de Hg dans les systèmes d'eau douce, les sédiments et les organismes vivants, et ce, même dans les régions éloignées de toute influence anthropique (Rada, *et al.*, 1989; Lindqvist, *et al.* 1991). La toxicité du Hg en milieu aquatique dépend des espèces chimiques présentes et de leur solubilité dans l'eau (Clarkson, 1998). Le méthylmercure (MeHg), une des formes les plus toxiques (produit par la méthylation du Hg inorganique), a la capacité de se bioaccumuler et d'être bioamplifié au long du réseau trophique (Bloom, 1992; Boening, 2000).

Dans les environnements aquatiques où les sources ponctuelles de Hg sont inexistantes, les dépositions atmosphériques, le ruissellement, et la percolation sont les principales voies d'entrée du Hg (Andersson, 1979; Rada, *et al.*, 1989; Mierle, 1990; Wiener, *et al.*, 1990; Aastrup, *et al.*, 1991). Le Hg atmosphérique est principalement inorganique, le Hg élémentaire (Hg^0) comptant pour 95 % de sa composition. Il voyage dans l'atmosphère jusqu'à ce qu'il soit oxydé, puis il se dépose, loin des sources, dans l'écosystème terrestre ou aquatique sous forme de précipitations sèches ou humides (Poissant et Casimir, 1998). En présence d'acides humiques dissous, le Hg s'y lie (Meili, *et al.*, 1991; Mierle et Ingram, 1991), ce qui contribue à augmenter sa persistance dans les sols et dans l'eau (Sorensen, *et al.*, 1990). Les concentrations de Hg retrouvées dans les organismes vivants sont le résultat de processus de méthylation du Hg s'effectuant au sein du milieu aquatique. En effet, le Hg qui rentre dans le système aquatique par voie atmosphérique ou terrestre est majoritairement sous forme inorganique (Fitzgerald et Clarkson, 1991). Alors, la question suivante se pose : Où et comment cette transformation de Hg à MeHg se produit-elle ?

Il est généralement admit que les milieux humides et les herbiers de plantes aquatiques jouent un rôle important dans les processus de filtration et de stockage des contaminants (Westling, 1991). Les plantes aquatiques agissent comme support pour le périphyton (Godward, 1937; Burkholder, 1996) et constituent une source d'alimentation pour les organismes herbivores (Sheldon, 1987; Elger et Lemoine, 2005). Les lits de macrophytes sont des sites d'alimentation ou de refuge pour le zooplancton, les macroinvertébrés et les poissons (Lodge et Lorman, 1987; Burks, *et al.*, 2001). Pour quelques espèces de poissons, (e.g. brochet, esturgeon), les herbiers sont l'habitat choisi pour frayer. Il a été démontré que les macrophytes réduisent la vitesse du courant, ce qui a pour effet d'augmenter la sédimentation des particules auxquelles les métaux sont associés. Les macrophytes agissent aussi sur le cycle diurne de l'oxygénation de l'eau, ce qui influence la biodisponibilité des métaux dissous. En effet, les conditions oxiques ont tendance à favoriser l'adsorption du Hg et du MeHg par les sédiments, alors que les conditions anoxiques en favorisent le relargage vers la colonne d'eau (Regnell et Tunlid, 1991). Les macrophytes sont souvent utilisées comme organismes indicateurs afin de mesurer la contamination en métaux dans les milieux aquatiques (Campbell, *et al.*, 1985; Coquery et Welbourn, 1994; Hudon, 1998). La capacité des complexes épiphytes/macrophytes à accumuler les métaux et leur position à la base de la chaîne alimentaire suggèrent qu'ils jouent un rôle important dans le cycle des métaux au sein des lacs, en les transférant aux organismes qui les consomment, comme c'est le cas avec le Hg (Cremona, *et al.*, 2009), en les excrétant dans la colonne d'eau via leurs stomates (Kraus, *et al.*, 1986), ou ou encore en les relargant lors de leur décomposition (Larsen et Schierup, 1981). Les concentrations en métaux des plantes aquatiques varient en fonction des espèces, de leur stade de croissance et de leur activité métabolique (Mudroch et Capobianco, 1979; Hudon, 1998). Ainsi elles devraient varier tout au long de la succession estivale.

Dans la zone littorale des lacs, on retrouve le périphyton. La communauté périphytique est une matrice complexe composée d'algues, de bactéries, de

champignons, de micro-invertébrés, de détritus et dépôts inorganiques (Young, 1945; Sand-Jensen et Wetzel, 1983). Ce biofilm constitue un microenvironnement où l'on peut trouver des variations spatiales (selon les différentes couches d'épaisseur) et temporelles du potentiel d'oxydo-réduction (Jørgensen, *et al.*, 1983; Revsbech, *et al.*, 1983). Le gradient de concentration d'oxygène crée des niches spécifiques permettant à différents groupes de microorganismes (autotrophes et hétérotrophes) de co-exister au sein du consortium périphytique. Le périphyton peut être attaché à une variété de substrats (sédiments, roches, macrophytes, etc.). Celui qui croît sur des plantes aquatiques est appelé épiphyton (ou épiphytes). Les épiphytes sont les contributeurs principaux de la production primaire des milieux humides, des rivières et des lacs (Cattaneo et Kalff, 1980; Loeb, *et al.*, 1983; Goldsborough et Robinson, 1996) et sont à la base de la chaîne alimentaire littorale (Vadeboncoeur, *et al.*, 2002), les organismes s'alimentant dans les herbiers les consommant préférentiellement (Cremona, *et al.*, 2009). Les variables ayant un effet sur la biomasse des épiphytes sont la température, les éléments nutritifs et l'abondance des organismes brouteurs (Kairesalo, 1983; Wetzel, 1983b; Lowe, 1996). La quantité de lumière est tout aussi importante puisqu'elle régule la photosynthèse et la croissance (Hill, 1996), la composition spécifique des algues (Steinman et McIntire, 1986) au sein du biofilm ainsi que leur succession saisonnière (Tuji, 2000). L'architecture de la plante elle-même va aussi influencer la biomasse des épiphytes, soit par l'altération du régime lumineux en créant de l'ombrage, soit par sa structure même: les macrophytes ayant des feuilles finement découpées tendent à développer une plus grande biomasse épiphytique, due à leur plus grande surface de colonisation disponible (Gosselain, *et al.*, 2005)

Le périphyton est situé à l'interface des milieux aquatiques et terrestres et est capable de retenir les éléments chimiques, incluant le Hg inorganique, transporté par les eaux de ruissellement et de percolation. En raison de sa position à la base du réseau trophique, il peut influencer l'ensemble des maillons supérieurs du réseau (Hill, *et al.*, 1996). L'accumulation de Hg par le périphyton a été mesurée dans quelques études. Cependant, la plupart d'entre elles ont été réalisées en région

tropicale et subtropicale (Cleckner, *et al.*, 1998; Guimarães, *et al.*, 2000; Roulet, *et al.*, 2000; Mauro, *et al.*, 2002; Huguet, *et al.*, 2010), alors que celles faites en milieu tempéré sont rares (Rask, *et al.*, 1994; Desrosiers, *et al.*, 2006b). De plus, il n'y a présentement que peu d'informations disponibles sur les variations saisonnières des concentrations de Hg et de MeHg des épiphytes et macrophytes sous nos latitudes. Considérant l'importance des milieux humides dans les écosystèmes aquatiques de l'hémisphère nord (Keddy, *et al.*, 2009), et en particulier au Canada où ils couvrent 14 % de la surface terrestre (Mike Arts, communication personnelle), il apparaît crucial d'obtenir davantage de données sur les concentrations en Hg et en MeHg des épiphytes et des macrophytes en région tempérée froide, ainsi que sur les facteurs environnementaux influençant ces concentrations, afin de pouvoir mieux prédire la contamination potentielle des poissons, puis des êtres humains.

La méthylation des métaux, incluant le Hg, a été proposée comme un mécanisme de détoxification par les microorganismes (Gadd et Griffiths, 1978). Il semblerait que le MeHg soit synthétisé à l'intérieur de la cellule via la voie de synthèse de l'acétyl-CoA par un transfert du groupe méthyl de la méthylcobalamine (un corrinoïde) à un ion Hg (Choi et Bartha, 1993; Choi, *et al.*, 1994). La méthylcobalamine est utilisée par les microorganismes lors de la synthèse des acides aminés tandis que l'acétyl-CoA intervient dans la formation de glucose (cycle du glyoxylate), des acides gras et dans le cycle de Krebs lors de la respiration (Horton, *et al.*, 1994). Jusqu'à présent, les recherches sur la méthylation du Hg se sont surtout concentrées sur les sédiments, et en particulier sur les bactéries sulfato-réductrices, identifiées comme les principaux microorganismes méthylateurs (Compeau et Bartha, 1985; Callister et Winfrey, 1986; Matilainen, *et al.*, 1991). Il y a peu de données sur les organismes, autres que les bactéries, qui peuvent méthyler le Hg (Kerry, *et al.*, 1991; Matilainen, 1995). La production de MeHg a aussi été mesurée dans les sols terrestres (Rogers, 1977), la colonne d'eau (Furutani et Rudd, 1980; Xun, *et al.*, 1987), dans les sédiments (Compeau et Bartha, 1985; Gilmour et Riedel, 1995) et dans le périphyton. Les études effectuées sur l'importance du périphyton dans les processus de méthylation du Hg sont très récentes et peu

nombreuses (Cleckner, *et al.*, 1999; Guimarães, *et al.*, 2000; Mauro, *et al.*, 2002; Achá, *et al.*, 2005; Desrosiers, *et al.*, 2006a) et les incubations sont généralement réalisées en conditions contrôlées en laboratoire plutôt qu'*in situ*. Mais, lorsque les compartiments épiphytes et sédiments sont comparés, il semble que les épiphytes soient plus importantes que les sédiments comme site de méthylation du Hg (Cleckner, *et al.*, 1999; Guimarães, *et al.*, 2000). En région tempérée, les marécages sont reconnus pour être des sites de production de MeHg pour les écosystèmes aquatiques (Hurley, *et al.*, 1995; St. Louis, *et al.*, 1996; Waldron, *et al.*, 2000), la méthylation du Hg par les épiphytes de ces écosystèmes doit être quantifiée.

La déméthylation du Hg est le processus inverse de la méthylation et elle se produit simultanément, d'où l'importance de mesurer ensemble la méthylation et la déméthylation du Hg afin d'évaluer la production nette de MeHg. La déméthylation vise à séparer le groupement CH_3 de l'ion Hg qui peut ensuite être excrété à l'extérieur de la cellule. Il existe deux voies métaboliques possibles à la déméthylation, la voie de l'enzyme organomercurielle MerB (Robinson et Tuovinen, 1984) et la voie oxydative (Oremland, *et al.*, 1991). Plusieurs groupes de bactéries sont capables de méthyler et déméthyler le Hg, mais la contribution de ces différents groupes n'est pas encore bien définie. Jusqu'à présent, les seules études effectuées simultanément sur la méthylation et la déméthylation du Hg par les épiphytes n'ont été faites qu'en milieu sub-tropical (Achá, *et al.*, 2005).

Au sein d'un même lac, les taux de méthylation du Hg peuvent varier de façon spatiale et saisonnière. La méthylation ne dépend pas seulement des concentrations initiales de Hg biodisponible, mais aussi de certaines variables du milieu: l'activité biologique microbienne, la température de l'eau (Wright et Hamilton, 1982) (Callister et Winfrey, 1986; Bodaly, *et al.*, 1993), le pH (Miskimmin, *et al.*, 1992), le potentiel redox (Matilainen, *et al.*, 1991), les formes de soufre présentes, et les concentrations de matière organique (Ullrich, *et al.*, 2001).

Le but général de cette thèse de doctorat était de vérifier l'importance des communautés littorales en tant que sites de méthylation du Hg et source potentielle de Hg pour les réseaux trophiques aquatiques. Les objectifs de cette étude étaient de: 1) mesurer les concentrations de Hg total (HgT) et MeHg dans les épiphytes et les macrophytes submergés et émergentes ; 2) évaluer l'effet de la saisonnalité (température et lumière), de la proportion d'organismes autotrophes au sein du biofilm épiphytique, de l'espèce de macrophytes et des caractéristiques chimiques du site sur les concentrations de HgT et de MeHg dans les épiphytes et les macrophytes; 3) mesurer *in situ* les taux de méthylation et de déméthylation du Hg des complexes épiphytes/macrophytes; 4) évaluer l'influence de la lumière, de la température et de l'espèce de plante hôte sur les taux de méthylation, déméthylation et de production nette de MeHg des complexes épiphytes/macrophytes; 5) identifier les principaux microorganismes méthylateurs au sein du biofilm épiphytique par l'ajout d'inhibiteurs métaboliques spécifiques et par la charactérisation des microorganismes actifs (séquençage de gènes 16S rRNA), le tout simultanément aux mesures de méthylation/déméthylation.

L'une des hypothèses de ce projet était d'observer un effet de la lumière et la température sur les concentrations en HgT, MeHg, ainsi que sur les taux de production nette de MeHg. En effet, comme ci-haut mentionné, la quantité de lumière disponible régule la photosynthèse et la croissance des épiphytes (Hill, 1996), la composition spécifique des algues (Steinman et McIntire, 1986) au sein du biofilm ainsi que leur succession saisonnière (Tuji, 2000). La lumière pourrait donc influencer l'accumulation de Hg ainsi que les taux de méthylation et de déméthylation du Hg, soit en stimulant le métabolisme des algues (et conséquemment aussi des autres microorganismes inclus dans dans le périphyton), soit en induisant des changements d'espèces présentes au sein du biofilm épiphytique. La lumière a aussi un effet sur la disponibilité du Hg et du MeHg de l'eau pour les processus de méthylation et de déméthylation, soit en photodégradant le MeHg (Sellers, *et al.*, 1996) ou en photoréduisant le Hg(II) (Amyot, *et al.*, 1997).

En ce qui concerne la température, il a été démontré en laboratoire, avec les sédiments, que les taux de méthylation du Hg sont directement proportionnels à la température, jusqu'à un seuil de ≈ 35°C (Callister et Winfrey, 1986) (Korthals et Winfrey, 1987). Par exemple, Wright et Hamilton (1982) ont observé que la quantité de MeHg relarguée par les sédiments à 4°C n'était que 50-70% de celle mesurée à 20°C. Cependant, les effets synergiques et antagonismes des autres variables reliées à la température ne doivent pas être écartés. Or, il est admit que les faibles températures ralentissent généralement la productivité microbienne (Bisogni et Lawrence, 1975). Puisque les concentrations de MeHg sont le résultat net du bilan de méthylation/déméthylation, une augmentation de la méthylation nette peut être aussi partiellement due à une baisse des taux de déméthylation plutôt qu'à une réelle augmentation des taux de méthylation. En effet, les taux de déméthylation semblent être inversement proportionnels à la température, alors que c'est le contraire pour les taux de méthylation, ce qui peut mener à une plus large production nette de MeHg durant les périodes chaudes (Ramlal, *et al.*, 1986; Bodaly, *et al.*, 1993).

Une autre hypothèse était d'observer des concentrations de HgT et de MeHg différentes dans les épiphytes et les macrophytes en fonction de l'espèce de macrophyte. Les macrophytes enracinées peuvent absorber le Hg de l'eau, et aussi des sédiments (Campbell, *et al.*, 1985; Crowder, 1991). Tout dépendant de leur morphologie (i.e. une plus grande surface de contact au niveau racinaire par rapport à la tige et aux feuilles), les macrophytes absorberont davantage le Hg de l'une ou l'autre des 2 sources (sédiments ou colonne d'eau), ceci ayant pour effet d'induire des différences de concentrations en Hg selon les espèces. Bien que le sujet soit controversé, les macrophytes ne sont pas un support inerte pour les épiphytes, et des échanges d'éléments chimiques peuvent avoir lieu entre l'hôte et le biofilm (Wetzel, 1983a). Ces échanges ont été démontrés, notamment, avec les éléments nutritifs (Allen, 1971; Moeller, *et al.*, 1988), et ceux-ci diffèrent selon le stade de croissance de la plante. L'espèce de macrophyte, de par sa morphologie, a aussi un impact sur la biomasse et l'activité métabolique des épiphytes (Gosselain, *et al.*,

2005) ainsi que sur leur composition spécifique (Pip et Robinson, 1984), notamment en interférant dans le régime lumineux disponible pour les épiphytes, toutes ces conditions pouvant influencer leurs concentrations en Hg et MeHg ainsi que leur activité de méthylation et déméthylation du Hg.

La productivité du milieu devrait aussi influencer positivement les taux de méthylation du Hg puisqu'il augmente l'activité métabolique des microorganismes (Cleckner, *et al.*, 1999). En effet, Cleckner *et al.* (1999) ont mesuré des taux de méthylation plus élevés dans le périphyton situé dans les zones les plus eutrophes des Everglades par rapport aux autres stations moins productives. L'état trophique du milieu a une influence, non seulement sur l'activité métabolique, mais sur la structure de la communauté. En effet, le type de microorganismes présents dans la communauté (espèces de bactéries et/ou algues) peut influencer, par différentes voies métaboliques impliquées (aérobie/anaérobie; autotrophes/hétérotrophes), les taux de méthylation et de déméthylation du Hg (Macalady, *et al.*, 2000).

L'échantillonnage pour ce projet a été réalisé dans les herbiers aquatiques du lac St-Pierre, un élargissement du fleuve St-Laurent (Québec, Canada). Les travaux de recherche qui en découlent ont fait partie des activités d'un réseau de recherche pan-canadien supporté par le CRSNG, le Réseau Collaboratif de Recherche sur le Mercure (COMERN), plus précisément de l'étude de cas du fleuve St-Laurent.

Le premier chapitre de cette thèse est consacré essentiellement aux objectifs 1 et 2. Les concentrations de HgT et de MeHg des épiphytes et des macrophytes ont été mesurées mensuellement dans quatre stations ayant des caractéristiques physico-chimiques distinctes. Des échantillons prélevés sur différentes espèces de macrophytes à trois profondeurs ont été comparés (article à soumettre à la revue Environmental Pollution).

Dans le deuxième chapitre il a été question de mesurer les processus de méthylation et de déméthylation du Hg dans les épiphytes et les macrophytes afin

d'établir ce qui contrôle la production nette de MeHg (objectifs 3 et 4). Des complexes macrophytes/épiphytes ont été incubés *in situ* pendant 48h dans les herbiers du lac St-Pierre avec ajout d'isotopes stables de ^{199}HgO et de $Me^{200}Hg$ comme traceurs. Les mesures ont été effectuées dans 2 stations ayant des caractéristiques physico-chimiques distinctes (article à soumettre à la revue Science of the Total Environment).

Finalement dans le troisième chapitre (objectifs 3 et 5), l'emphase a été mise sur les consortiums de microorganismes hétérotrophes et autotrophes peuplant le biofilm épiphytique afin de déterminer lesquels parmis eux sont actifs dans les processus de méthylation et de déméthylation du Hg. Des complexes macrophytes/épiphytes ont été incubés *in situ* pendant 48h avec ajout d'isotopes stables de ^{199}HgO et de $Me^{200}Hg$ comme traceurs en absence et en présence d'inhibiteurs métaboliques. Le chloramphenicol a été utilisé comme bactériostatique, le molybdate comme inhibiteur de la réduction des sulfates, le BESA (2-bromoethane sulfonic acide) pour inhiber la méthnogénèse et enfin le DCMU (3-(3,4-dichlorophenyl)-1,1 dimethyl urée) afin d'inhiber la photosynthèse. Des sous-échantillons de biofilm épiphytiques ont été prélevés à la fin des incubation afin de caractériser la communauté des microorganismes actifs lors des mesures de méthylation et déméthylation du Hg à l'aide du séquençage des gènes du 16S rRNA (article publié, (Hamelin, *et al.*, 2011)).

CHAPITRE I

SPATIO-TEMPORAL VARIATIONS IN BIOMASS AND MERCURY CONCENTRATIONS OF EPIPHYTIC BIOFILMS AND THEIR HOST IN A LARGE RIVER WETLAND (LAKE ST. PIERRE, QC, CANADA)

Stéphanie Hamelin, Dolors Planas et Marc Amyot

À soumettre pour publication à Environmental Pollution

1.1 Abstract

Within wetlands, epiphytes and macrophytes play an important role in storage and transfer of metals, such as Hg, through the food web. However, there is a lack of information about spatial and temporal changes in their mercury levels, a key priority contaminant of aquatic systems. The objectives of our study were to assess total mercury (THg) and methylmercury (MeHg) concentrations of epiphyte/macrophyte complexes in Lake St.Pierre, a large fluvial lake of the St. Lawrence River (Québec, Canada). THg and MeHg concentrations were ten fold higher in epiphytes than in macrophytes. THg concentrations in epiphytes linearly decreased as a function of the autotrophic index and % of MeHg in epiphytes reached values as high as 74%. Spatio-temporal variability in THg and MeHg concentrations in epiphytes and macrophytes were influenced by water temperature, available light, host species, water level, dissolved organic carbon and dissolved oxygen.

Keywords: periphyton, epiphytes, macrophytes, mercury, methylmercury.

1.2 Introduction

Many lakes are affected by mercury (Hg) contamination which can alter human health, mainly through exposure by fish consumption. Wetlands are often seen as sinks for nutrients and metals, such as Hg, but sources for methylmercury (St. Louis, *et al.*, 1996). Furthermore, methylmercury (MeHg) concentrations in lakes and rivers have been shown to be correlated to the percentage of wetland within the drainage basin (Mierle and Ingram, 1991). A perturbation of these ecosystems (*e.g.,* dredging or water level fluctuations) can have dramatic consequences on the release of these stored materials in the environment (Westling, 1991).

Although aquatic plants in wetlands can accumulate Hg, the periphytic biofilm that covers them (epiphytes) has a more rapid tournover rate than its host, and is the main food sources for macroinvertebrate herbivors (Cleckner, *et al.*, 1998; Cremona, *et al.*, 2009; Molina, *et al.*, 2010). Furthermore, it is known that epiphytes can methylate Hg (Cleckner, *et al.*, 1999; Mauro, *et al.*, 2001; Hamelin, *et al.*, 2011) and that MeHg concentrations within biofilm matrix can reach high levels (e.g., 3 to 55 ng gDW^{-1} for periphyton growing on rocks, (Desrosiers, *et al.*, 2006c)). Hg and MeHg accumulation in aquatic primary producers could be an important pathway of Hg transfer from the watershed to the aquatic foodweb (Rask, *et al.*, 1994; Hill, *et al.*, 1996; Cremona, *et al.*, 2009).

Few studies have focused on periphytic biofilms as a potential source of Hg to food webs. Most of them were conducted in tropical and subtropical regions (Cleckner, *et al.*, 1998; Guimarães, *et al.*, 2000; Roulet, *et al.*, 2000; Mauro, *et al.*, 2002; Huguet, *et al.*, 2010), whereas those in temperate regions are sparse (Rask, *et al.*, 1994; Desrosiers, *et al.*, 2006b in boreal lakes). Considering the extensive presence of wetlands in northern hemisphere (Keddy, *et al.*, 2009), it is crucial to investigate the importance of Hg and MeHg concentrations in epiphytes and macrophytes in temperate regions, in order to better predict fish contamination and human exposure.

Epiphyte biomass is not only related to nutrients and light availability, but is also closely related to its macrophyte host as the latter will determine the available surface for colonisation (Cattaneo and Kalff, 1980; Engelhardt and Ritchie, 2001), and specific composition of epiphyte biofilms (Pip and Robinson, 1984). Functional differences in community composition of microorganisms are known to influence Hg methylation and demethylation rates, as well as Hg and MeHg concentrations in biofilms (Macalady, *et al.*, 2000; Hamelin, *et al.*, 2011). Thus, indicators of biofilm composition, such as the proportion of autotrophes *vs* heteotrophes, known as the autotrophic index (see section 1.3.3), should be linked to epiphytes Hg contamination.

Physico-chemical characteristics of the water (pH, dissolved oxygen concentrations, nutrients, temperature) have been shown to affect Hg bioavailability and methylation in biota (Callister and Winfrey, 1986; Mauro, *et al.*, 1999; Ullrich, *et al.*, 2001; Le Faucheur, *et al.*, 2011). These physico-chemical variables may vary through the growing season of primary producers, as well as the dominant macrophyte species that will change by following seasonnal succession. Thus, fluctuations in epiphyte and macrophyte Hg and MeHg concentrations are expected from the beginning to the end of the summer. There are currently no published seasonal trends of THg and MeHg concentrations in epiphytes or macrophytes of the northern hemisphere.

The aim of the present study was to measure THg and MeHg concentrations in epiphytes/macrophytes complexes, evaluating the effect of: *(i)* host specificity (macrophytes species and habitat), *(ii)* temperature and light (related to depth and season), *(iii)* the proportion of autotroph organisms in biofilms (autotrophic index), and *(iv)* chemical characteristics of sites (stations).

The study has been conducted in, a large fluvial lake (Lake St. Pierre), characterized by high spatial variability in water physico-chemical proprieties, as it is formed by three distinct water masses that flow side by side without mixing (Centre Saint-Laurent, 1996).

1.3 Materials and methods

1.3.1 Study site

Lake St. Pierre (46°12'N, 72°49'W) is an enlargement of the St. Lawrence River downstream of Montreal, Québec, Canada (figure 1.1). This long (30 km) lake is relatively shallow (mean depth ~3 m, excluding the navigation channel) and supports large emergent marshes and extensive beds of submerged aquatic vegetation (Hudon, 1997). From mid- to late summer, submerged aquatic plants cover 260 km^2 (85%) of the lake's area (mean biomass 54 g dry mass.m^{-2}) and represent an annual production of 8700 tonnes of carbon (Vis, *et al.*, 2007). This lake is one of the most valuable sites in Canada for freshwater sport and commercial fisheries, with over 1,400 tons of fish caught annually (Caron and Lucotte, 2008). Water residence time is 20 to 120 days, and inter-annual water level fluctuations can reach up to 2 m (Hudon and Carignan, 2008). Large spatial variations in physical and chemical water characteristics can be found in Lake St. Pierre, arising from the presence of distinct water masses, which may flow side by side without mixing (Centre Saint-Laurent, 1996). The central water mass (which includes the navigation channel) is composed of relatively clear and hardwaters, with low dissolved organic carbon (DOC) and suspended matter concentrations coming from the Great Lakes (Cossa, *et al.*, 1998; Vis, *et al.*, 2007). These waters represent 80% of discharge, and are restricted to the central portion of the lake (Vis, *et al.*, 2003). The north shore water mass comes from the Ottawa River and north shore tributaries (L'Assomption, Chicot, Yamachiche, Maskinongé and Du Loup rivers). These humic-brown waters are relatively rich in nutrients and suspended particles and have comparatively high DOC concentrations

derived from humic and fulvic acids (Frenette, *et al.*, 2003). Tributaries (Richelieu, Yamaska, Nicolet and St. François rivers) draining farmlands discharge in the south shore and bring turbid brown-clay and nutrient rich waters (Vis, *et al.*, 2003). The upstream section of Lake St. Pierre, around the Sorel archipelago, is heavily impacted by nearby industries from Sorel-Tracy (metallurgy and chemistry) and agricultural loadings (nutrients and pesticides (La Violette, *et al.*, 2003)).

Over 60 aquatic plants species have been recorded in Lake St. Pierre during the 3-year study (2002-2004). Dominant macrophytes species in our sampling sites were *Scirpus fluviatilis* and *Typha angustifolia Linné* (emergent); *Nymphea tuberosa Paine (floating leaves)*; *Ceratophyllum demersum Linné, Potamogeton perfoliatus Linné, Vallisneria americana Michaux* and *Elodea canadensis Michaux* (submerged). Floating macroalgae form a significant part of the biomass in the middle of the summer, particularly when water levels were low.

1.3.2 Sampling

Four stations were chosen for their contrasting physico-chemical characteristics, but also for their interest as commercial fishing sites (figure 1.1). Two were located on the north shore, one close to the Girodeau Island (GIR, 46° 09' 82" N; 72° 59' 10" W) and the other in the Maskinongé Bay (MASK, 46° 11' 56" N; 72° 56' 63" W). The two other stations were situated on the south shore, one in the Anse-du-Fort (ADF, 46° 08' 24" N; 72° 54' 87" W) and the other in a large eutrophic bay, St. François Bay, (BSF, 46° 06' 97" N; 72° 55' 87" W), which receives agricultural inputs from the Yamaska and St. François Rivers.

Sampling was carried out once a month for 3 years (in July and August 2002, from June to August 2003 and from May to September 2004), and at 3 different depths from the surface (0 cm, 30 cm and 60 cm). At each depth and station, three field replicates of macrophyte/epiphyte complexes per dominant macrophyte species were sampled by scuba divers using 0.68 L Pac-man boxes, (a smaller cylindrical

version of the 6 L Downing box (Downing, 1986), modified by C. Vis (Parks Canada, Cornwall). Great care was taken during sampling by scuba divers to keep the integrity of the biofilm.

In the laboratory, epiphytes were separated from macrophytes by mechanical shaking (9 min in a Red Devil® paint shaker), a method previously tested in our laboratory for removing periphyton without destroying algal cells (Hamelin *et al.*, unpublished data). 100 ml of epiphyte suspension was subsampled for each measurement: chlorophyll-α (chl, x2), dry weight (DW, x2), ash free dry weight (AFDW, x2), THg (x3) and MeHg (x3). Aliquots for chl, DW and AFDW were filtrated on GF/C filters that were pre-combusted (90 minutes at 500°C) and pre-weighed for DW and AFDW. Filters were kept frozen (-80°C) until analysis.

Physicochemical characteristics of the water within macrophytes beds were measured with specific probes: pH (Chekmite 4 pH meter), light (Biospherical QSL-101 quantum meter), temperature and dissolved oxygen (WTW Oxi340 oximeter). Water grab samples were also taken in each macrophyte bed for DOC, THg_{water}, $MeHg_{water}$ and major ions. Water chemistry data for these last samples are presented in the Supporting Information (Annexes A and C).

1.3.3 Biomass (chlorophyll-α, dry weight and ash free dry weight)

Chlorophyll-α (chl) was extracted with hot ethanol (Nusch, 1980) and measured by spectrophotometry (Spectronic Unicam UV300) with correction for phaeopigments (Sartory and Grobbelaar, 1984). Dry weight (DW) was measured after drying the filters at 45°C to constant weight, and ash free dry weight (AFDW) was measured after combustion at 500° F for 2 hours, (APHA, 1989). Macrophyte biomass was estimated with 10 quadrats of 1m x 1m, randomly sampled in each station, in which all the macrophyte species were identified and weighed (wet and dry). The autotrophic index (AI) of the epiphyte community was calculated as the ratio of AFDW by chl (Biggs and Close, 1989).

1.3.4 Total mercury and methylmercury analysis

All sampling materials for Hg measurements were carefully acid-washed and rinsed with nanopure water. Prior to THg and MeHg analysis, periphyton samples were freeze-dried and weighed. THg concentrations were measured by thermal decomposition using a direct mercury analyzer (DMA 80; Milestone, MLS). 0.05 to 0.10 g of samples were dried, combusted and further decomposed on a catalytic column at 750°C. Hg vapors were collected on a gold amalgamation trap and subsequently desorbed by heat and then measured by atomic absorption spectrometry at 253.7 nm (Boylan and Kingston, 1988; Cizdziel, *et al.*, 2000). MeHg samples were extracted in KOH/methanol (25%) and analyzed by Cold Vapour Atomic Fluorescence (Bloom and Fitzgerald, 1988; Bloom, 1989). The working detection limit was 0.01 ng g^{-1} of Hg for DMA 80 and 0.1 ng g^{-1} for CVFAS (three times the standard deviation of 10 procedural blanks). Blanks and certified reference materials (Tort-2, SO-2 and IAEA, CNRC) were analyzed every 10 samples to ensure the reproducibility of the analysis and to assess quality assurance/quality control. Hg concentrations in reference material varied slightly over time (CVFAS: TORT-2 CV=1.9%; DMA: TORT-2 CV=2.3%, SO-2 CV=1.6% and IAEA CV=2.1%) but were not significantly ($p > 0.05$) different from certified values. No substantial Hg contamination was detected in the blanks.

Estimates of THg and MeHg burdens in epiphytes and macrophytes for the whole lake were been calculated by multiplying minimum and maximum [THg] and [MeHg] measured throught the growing season by minimum and maximum epiphyte biomass by seasonal maximum macrophyte biomass (all species) calculated for the whole lake (Vis, *et al.*, 2007).

1.3.5 Statistical analyses

Results of biomass, THg and MeHg concentrations between stations, depths, and macrophytes species were compared with one-way or two-way ANOVA (previously Log_{10} transformed to meet normality requirements as verified by Shapiro-Wilk test). When significant differences were found, Tukey-Kramer HSD (honestly significant difference) test for multiple mean comparisons was applied. When testing for seasonality effect, comparisons between different sampling periods were performed with repeated measures analyses of variance. A principal components analysis (PCA) on environmental descriptors was used to identify the variables that are correlated to the variance of [THg] and [MeHg] in epiphytes and macrophytes. Statistical analyses were performed with JMP 7 statistical package (SAS Institute Inc. 1991, α set at 5%) and Canoco for Windows 4.54 (ter Braak and Smilauer 1998).

1.4 Results

1.4.1 Biomass

Through the season, epiphyte DW varied from 8.2 to 3188.3 mg g DW macrophyte^{-1} (data not shown). Similar range of variability was observed with autotroph biomass as estimated by chl, going from 27.3 to 2432.3 µgchl gDWmacrophyte^{-1} (figure 1.2 A). Epiphyte biomass differed between macrophyte species (table 1.1), and was significantly higher in submerged macrophytes than in emergent ones (one-way ANOVA, p=0.0049, N=36).

Macrophytes biomass also differed between species and sites, ranging from 43.7 to 1458.2 gDW m^{-2} (figure 1.2 B). Highest values for both epiphytes and macrophytes were reached during the summer months (i.e. between June and August). While macrophytes had higher densities in BSF (south shore), epiphytes

biomass per gDW macrophytes was more abundant in Girodeau (north shore; figure 1.2 A and B).

1.4.2 THg and MeHg concentrations

Within sites, THg and MeHg in epiphytes and macrophytes varied as a function of macrophyte species and seasonality (table 1.2 and figure 1.2 C, D, E, F). Higher THg concentrations were found in epiphytes (from 2 to 284 ng gDW^{-1}) than in macrophytes (from 1 to 54 ng gDW^{-1}; table 1.1). THg in epiphytes were approximately 10 fold higher than in macrophytes (One-Way ANOVA, $p<0.0001$, N=63).

MeHg concentrations were 2 to 17 fold higher in epiphytes (from 0.1 to 24.0 ng gDW^{-1}; table 1.1 and 1.2) than in macrophytes (from 0.2 to 6.3 ng gDW^{-1}; One-Way anova, $p<0.0001$, N=63). The highest [MeHg] was measured in epiphytes growing on floating filamentous macroalgae (table 1.2).

No significant differences in [THg] or [MeHg] in epiphytes were observed as a function of substrate type (emergent vs submerged macrophytes). Higher [THg] in epiphytes were found in June on the north shore and in May on the south shore. Greater [MeHg] in epiphytes were found in May for MASK and ADF, and from June to July in BSF and GIR. The station with the highest [MeHg] was BSF, while the one with the maximum [THg] was GIR (figure 1.2 C, E).

Whole lake THg burden estimates for the maximum seasonal macrophyte biomass represent 25 to 1400 g in macrophytes and can reach higher values in epiphytes, from 1 to 23 000 g. MeHg burdens varied from 0.02 to 1950 g and from 5 to 160 g, for epiphytes and macrophytes respectively. The huge variation between minimum and maximum estimates reflects the differences in [THg] or [MeHg] measured on different species of macrophytes through the growing season.

Inter-annual variations were observed for epiphytes, with biomass 5 fold higher (one-way ANOVA, $p<0.0001$, N=54) in 2003 than in 2002 or 2004 (figure 1.3 A). No corresponding inter-annual change was observed for [THg] (emergent and submerged) or [THg] in water, suggesting that biodilution does not occur in these biofilms (figure 1.3 B). [MeHg] remained stable for epiphytes on emergent macrophytes, but they were higher in 2003 than in 2002 or 2004 for epiphytes on submerged macrophytes (figure 1.3 C).

1.4.3 Factors influencing THg and MeHg in epiphytes and macrophytes

No significant relationship was observed between THg and chl ($r^2=0.02$; $p=0.1794$, N=39), THg and DW ($r^2=0.04$; $p=0.0973$, N=39) or between THg and AFDW ($r^2=0.04$; $p=0.0717$, N=39) measured in epiphytes. However, THg in epiphytes linearly decreased as a function of the autotrophic index (figure 1.4, $r^2= 0.53$; $p < 0.0001$, N=39). This implies that a higher proportion of autotroph organisms in a biofilm may lead to higher THg, suggesting a role of algae in epiphyte Hg accumulation.

In the PCA of THg and MeHg in epiphytes (figure 1.5 A and B), 67% of the total variance was explained by the 3 first principal components. [THg] in epiphytes was positively correlated with Axis I and Axis III while [MeHg] in epiphytes was negatively correlated to Axis I and Axis II. Axis I, which explained 27% of the variance, was positively correlated to water level , and to a lesser extent, negatively correlated with temperature and DOC. Axis II, which explained 22% of the variance, was highly correlated with [O2], and to a lesser extend to [SO4], and $[THg]_{water}$. Axis III explained 18% of the variance and was correlated with % light, and to a lesser extend, negatively correlated to DOC.

In the PCA of THg and MeHg in macrophytes, the three principal components explained 69% of the total variance (figure 1.5 C and D). [THg] in macrophytes was negatively correlated with Axis II and III while [MeHg] in macrophytes was negatively

correlated with Axis II. Axis I explained 28% of the variance and was positively correlated with water level, and negatively correlated with [O2], temperature, and to a lesser extend to $[THg]_{water}$. Axis II explained 22% and was slightly correlated with [O2] and water level. Axis III explained 19% of the variance, was highly correlated with % light, but negatively correlated with DOC.

1.5 Discussion

1.5.1 Spatial variability

Macrophyte biomass was highest in the south shore whereas epiphytes biomass reached its maximum in the north shore. This contrast can be explained by the physico-chemical proprieties of the water in the two sections of the lake. Stations on the south shore receive important loads of suspended particles carried from tributaries which decrease water transparency (Hudon and Carignan, 2008). Also, large amounts of nutrients are found in water and sediments of this area, supporting high macrophyte growth, further decreasing light penetration by shading. As a result, epiphyte growth is light-limited on the south shore compared to the north shore.

THg concentrations of epiphytes are the highest in the North-East section of the lake, close to the Girodeau station. This area is under the influence of the plume from Sorel-Tracy industrial area and the discharge of waste water from the Montreal Urban Community (Langlois and Sloterdijk, 1989; Laviolette, *et al.*, 2003). These waters have a more acidic pH, lower DOC concentrations, and as mentioned before, are characterized by deeper light penetration when compared to south stations. Light may increase Hg bioavailability by photodegrading organic matter in waters (Sellers, *et al.*, 1996). All these factors are known to increase Hg bioavailability in microorganisms and should therefore favour Hg absorption by epiphytic biofilms. On the contrary, total mercury in macrophytes was the highest in BSF station on the south shore. Slow-growing plants collected in deeper or turbid waters, and thus

exposed to low light intensity, would accumulate more metals from sediments, than fast-growing plants with high light intensity (Hudon, 1998).

In contrast, MeHg concentrations tend to be higher on the south shore, particularly in BSF. Higher DOC found in BSF may act as a substrate for methylating organisms (Gill and Bruland 1990) and provide binding sites, thus simply increasing the total Hg dissolved in water (Lee and Hultberg 1990). This station is the most productive area of Lake St. Pierre, receiving large inputs of nutrients from agricultural lands drained by the Yamaska and St-François rivers. It is known that local productivity can increase microbial metabolic activity, and changes community composition (Cleckner, *et al.*, 1999; Macalady, *et al.*, 2000), leading to higher Hg methylation rates and higher accumulation in biofilms when Hg biodilution does not occur.

1.5.2 Hg and MeHg concentrations

THg in epiphytes from Lake St.Pierre were in the same range than levels observed in periphyton from different substrata and climatic regions (table 1.3): glass plates from a boreal Finland lake (Rask, *et al.*, 1994); epilithon from boreal lakes in northern Quebec (Desrosiers, *et al.*, 2006c), in epiphytes from Amazonia, Brazil (Roulet, *et al.*, 2000; Coelho-Souza, *et al.*, 2011), epiphytes from macrophyte rhizospheres in Bolivia (Achá, *et al.,* 2005) and free-floating mats of periphyton from the Everglades, USA (Cleckner, *et al.*, 1998). This suggests that the global range of [THg] in algal biofilms is similar in northern than in tropical or subtropical regions, and that only spatio-temporal variations within that range can be predicted by physico-chemical variables in a given site. Higher values were reported in highly Hg contaminated sites such as periphyton from the East Fork Poplar Creek in Tennessee, that reached Hg(II) concentrations up to 50 000 ng g DW^{-1}, caused by historical spills of elemental Hg (Hill, *et al.*, 1996).

[THg] measured in macrophytes were in the same range of concentrations than cultivated macrophytes in Sweden (Göthberg and Greger, 2006), macrophytes close to mines in BC, Canada (Siegel, *et al.*, 1985) and macrophytes from uncontaminated lakes of northern Qc, Canada (Grondin, 1994). They were slightly lower than those sampled in reservoirs from Brazil (Molisani, *et al.*, 2006) and those measured in the same macrophyte species (*Potamogeton sp.*, *Myriophyllum sp.* and *Elodea sp.*), upstream in the St. Lawrence River near Cornwall (Thompson-Roberts, *et al.*, 1999). This section of the river, distant from the study site by 200 km, has historically received important loads of mercury from the surrounding industries that persist in the sediments. Moreover, differences in Hg concentrations could also be explained by methodological differences: macrophytes from Cornwall have been cleaned from epiphytes with deionized water, without brushing or shaking, which may have leave some periphyton on the plants that might have slightly increased macrophytes concentrations.

Epiphytes from Lake St. Pierre appear slightly more contaminated in MeHg (1 to 2 fold) than epiphytes from the Tapajos, in Amazon Brasil (Roulet, *et al.*, 2000). They are in the same range than periphyton from the Everglades, Florida (Cleckner, *et al.*, 1998), epiphytes from free floating macrophyte rhisospheres in Bolivia (Achá, *et al.*, 2005) and epiphytes from Tapajos Amazonia, Brazil (Coelho-Souza, *et al.*, 2011) and slightly less contaminated (1 to 2 fold) than periphyton on rocks (epilithon) from Boreal lakes (Desrosiers, *et al.*, 2006c) or periphyton from the contaminated East Fork Poplar Creek in Tennessee (Hill, *et al.*, 1996).

Usually the average proportion of MeHg over total Hg increases from about 10% in the water column to 15% in phytoplankton, 30% in zooplankton, and 95% in fish (Watras and Bloom, 1992). In the studies previously cited for [THg] and [MeHg] in epiphytes, %MeHg over THg vary from 0.1 to 36% (table 1.3). But in Lake St-Pierre, ratios up to 74% MeHg/THg were measured. These high ratios were found in BSF during July, when the water temperature and the nutrient inputs were utmost, enhancing bacterial productivity, and thus, mercury methylation. These

exceptionnally high %MeHg must have a direct punctual effect on contamination of organisms from higher trophic levels.

The maximum Hg burdens calculated for epiphytes and macrophytes (23 kg and 1,4 kg, respectively) are slightly lower than the seasonnal storage of cadmium, estimated in macrophytes of Lake St.Pierre (30 kg Cd, (St-Cyr, *et al.*, 1994)) and definetly lower than gross mercury export from the St. Lawrence River, calculated as 1180 kg yr^{-1} (Quémerais, *et al.*, 1999). However, we know that exported material include not only decay of macrophytes and epiphytes, but also organic (plankton) and inorganic particles. Macrophytes and epiphytes estimates are more conservative as they were done with punctual concentrations and do not take into account turnover rates of epiphytes and macrophytes, which have not been considered either in other studies, but that would certainly increase the calculated Hg burdens .

Considering that mean [THg] and [MeHg] in water were relatively low (0.4-4.1 ng L^{-1} and 0.05-0.3 ng L^{-1} respectively) in Lake St.Pierre, and that THg accumulation in epiphytes and macrophytes reached high concentrations, comparable and often higher than the concentration in sediments sampled at the site (Caron and Lucotte, 2008), it is likely that epiphytes/macrophytes complexes are an important compartment for Hg accumulation. Furthermore, MeHg levels appear to be from 1 to 2 orders of magnitude higher in epiphytes than in sediments (Goulet, *et al.*, 2007; Caron and Lucotte, 2008), suggesting that Hg methylation could be important in epiphytes. As epiphytes are the main food source for macroinvertebrates in lakes, including Lake St. Pierre (Cremona, *et al.*, 2009; Molina, *et al.*, 2010), epiphytic Hg could be easily transferred through the food web and should be included in environmental assessments. These data demonstrate that epiphyte biofilms are an important site for MeHg accumulation and production (chap. II) and represent significant storage compartments of Hg and MeHg that can be transferred to the foodweb.

1.5.3 Factors affecting THg and MeHg concentrations in epiphytes and macrophytes

In relation to seasonality, differences in [THg] and [MeHg] between July and August may be explained by a 10 fold decrease in light penetration in August due to an increase in macrophyte density and biomass, and changes in the dominant species. It has been demonstrated that the architecture of the community influences light penetration (Gosselain, *et al.*, 2005). Light intensity may also modify Hg^{2+} or carbon substrata availability in two different way: 1) solar radiation may degrade dissolved organic matter and lead to increased bioavailability of [Hg^{2+}] (Ravichandran, 2004); 2) deeper light penetration may stimulate primary production (as indicated by a lower epiphyte biomass in august, except in ADF), but also heterotrophic biofilm metabolic activity and microbial interactions, which may have a direct effect on Hg accumulation and demethylation rates in biofilms (Ullrich, *et al.*, 2001). Furthermore, from May to July, all stations were dominated by the submerged macrophyte *Potamogeton perfoliatus*, but in August, there was a successional change in the dominant macrophyte species. *Potamogeton perfoliatus* was dying and was replaced by *Ceratophyllum demersum* in BSF, *Elodea canadensis* in Girodeau and *Vallisneria americana* in ADF. This new substrate was then colonised by biofilms at an early successional stage, characterised by higher metabolic activity than older ones (Acs, *et al.*, 2007). This observation reinforces our previous hypothesis about higher [MeHg] in relation to an increase in metabolic activity in biofilms.

Higher [MeHg] were found in epiphytes when water temperature was warmer (figure 1.5). It is known that Hg methylation rates in sediments are proportional to temperature, up to a threshold of around 35°C, and that this process is linked to microbial metabolic activity (Bisogni and Lawrence, 1975; Callister and Winfrey, 1986; Korthals and Winfrey, 1987; Winfrey and Rudd, 1990; Matilainen and Verta, 1995; Watras, *et al.*, 1995). For instance, Wright and Hamilton (1982) (Wright and Hamilton, 1982) have shown that the amount of MeHg released from sediments at 4°C was only 50-70% of the one measured at 20°C. Furthermore, experiments with

periphyton from artificial substrates have demonstrated higher methylation rates at a temperature of 20-25°C than at 15°C (Desrosiers, *et al.*, 2006a). In addition, high temperatures in the middle of the summer are often coupled with low dissolved oxygen levels, which are known to enhance bacterial mercury methylation (Henry, *et al.*, 1995). The increase in temperature coupled to a decrease in oxygen concentrations through the growing season has probably increased [MeHg] in epiphytes. Higher THg concentrations were found in biofilms containing higher proportion of autotrophic organisms. This is important information, as algae represent a large proportion of epiphytic biofilms, and that most of the studies about Hg and periphyton, particularly the ones about methylation and demethylation processes, focus on limited amount of bacterial groups (mainly sulfate reducers) and often neglect phototrophic organisms. Algae, while using the glyoxylate cycle during photorespiration, may participate in Hg methylation by transferring a methyl group from methycobalamin to Hg. Furtermore, algae are known to reduce Hg also by means of reducing compounds contained in photosystem II (Ben-Bassat and Meyer 1978). As algae may play a role in both Hg methylation and reduction, they would influence THg concentrations measured in autotrophic biofilms.

A 5-fold increase in epiphyte biomass was measured in 2003, year at which the water level was lower than in 2002 or 2004 (average water level year, figure 1.3). Low water level years lead to lower Hg concentrations in emergent macrophytes and allow 10 times more light penetration, thus stimulating epiphyte productivity. This increase in epiphyton biomass did not lead to a decrease in THg or MeHg by unit of epiphytes DW, suggesting that biodilution did not occur in the epiphytic community, at least in our system. Therefore, it is likely that lower climate-induced water levels will significantly increase Hg burden at the ecosystem scale by raising epiphytes and macrophytes biomass. Water level fluctuations are important in Lake St.Pierre. The cumulative effect of, warmer climate and water level regulation of the Great Lakes by the International Joint Commission have a drastic effect on water level decrease (up to 2 m) observed in Lake St.Pierre (Hudon and Carignan, 2008). Labile organic matter from flooded areas is known to promote bacterial metabolism and/or Hg

availability (Ullrich, *et al.*, 2001) and could favor MeHg formation (Roulet, *et al.*, 2001; Coelho-Souza, *et al.*, 2011), particularly when coupled with warmer temperatures. Wetlands are usually greater sources of MeHg during years of high water yields (St. Louis *et al.*, 1996). It is known that intermittent flooding, as seen in Lake St.Pierre, can increase MeHg bioavailability to organisms since these fluctuations in water levels cause release of nutrients and mercury from sediment, stimulating bacterial methylation (George and Batzer, 2008). For example, mercury levels were higher in fish collected from South Carolina wetlands that experienced frequent fluctuations in water levels than those that were deep and permanent (Snodgrass, *et al.*, 2000).

1.6 Conclusion

Epiphytes and macrophytes both represent significant sites of Hg and MeHg accumulation, epiphytic biofilms being ten fold more contaminated than their macrophyte host. Higher [THg] were found in biofilms containing higher proportion of autotrophic organisms. Percentages of [MeHg] over [THg] in epiphytes reached values as high as 74%, pointing out the importance of including epiphytes in foodweb Hg contamination modelling. These concentrations are mainly driven by available light, dissolved organic carbon, dissolved oxygen concentrations, water temperature and water level fluctuations.

1.7 Acknowledgments

This project was part of the Collaborative Mercury Research Network (COMERN). The study was made possible through funding grant to D.P and M.A. by NSERC-COMERN, and scholarships from UQÀM (faculté des sciences), Groupe de recherche interuniversitaire en limnologie (GRIL) and Centre de recherche en géochimie et géodynamique (GEOTOP) to S.H. We are grateful to Dominic Bélanger for his advice during lab work, and to Serge Paquet for his help with statistical

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Table 1.1: Mean Biomass (in g DW m^{-2}), [THg] and [MeHg] (both in ng Hg g DW^{-1}) ± standard error in epiphytes and their host at the BSF in August 2002 (nd = non-detectable).

		Biomass		THg		MeHg	
		Epiphytes	Macrophytes	Epiphytes	Macrophytes	Epiphytes	Macrophytes
Emergent	*Scirpus fluviatilis*	83.3 ± 10.4	1031.9 ± 112.4	60 ± 6	8 ± 1	5.1 ± 0.7	0.3 ± 0.1
	Thypha angustifolia	31.4 ± 4.6	624.8 ± 84.2	46 ± 7	5 ± 1	6.3 ± 0.9	nd
Floating leaves	*Nymphea tuberosa*	43.1 ± 9.5	786.9 ± 108.1	61 ± 2	6 ± 1	5.4 ± 1.6	nd
	Hydrocharis morsus-ranae	16.9 ± 1.9	106.9 ± 34.3	71 ± 4	10 ± 1	4.1 ± 0.3	nd
Submerged	*Filamentous macroalgae*	10.8 ± 0.8	139.4 ± 32.0	93 ± 7	18 ± 5	23.1 ± 1.3	1.9 ± 0.1
	Ceratophyllum demersum	85.7 ± 7.0	213.9 ± 22.1	82 ± 4	19 ± 6	2.7 ± 0.2	0.3 ± 0.1
	Potamogeton perfoliatus	43.2 ± 3.1	195.5 ± 57.8	130 ± 7	25 ± 3	2.7 ± 1.1	1.5 ± 0.1

Table 1.2: Means and ranges of [THg] and [MeHg] in epiphytes and macrophytes. Data are pooled from 2002 to 2004, and separated by stations and dominant macrophyte species (nd = non detectable).

		[THg] (ng/gDW)		[MeHg] (ng/gDW)		% [MeHg]/[THg]
Station	Macrophyte species	Epiphytes	Macrophytes	Epiphytes	Macrophytes	Epiphytes
BSF	*Potamogeton perfoliatus*	49 (N=27) 22-77	14 (N=29) 6-37	3 (N=21) 0.1-6	2 (N=12) 1-2	6 (N= 21) 0.3-10
	Thypha angustifolia	51 (N=180) 18-284	3 (N=180) 1-16	6 (N=123) 0.4-11	1 (N=33) 0.2-3	16 (N=123) 1-50
	Ceratophyllum demersum	47 (N=74) 10-108	14 (N=36) 6-26	7 (N=47) 3-16	2 (N=30) 0.2-6	19 (N=47) 3-70
	Myriophyllum spicatum	48 (N=18) 35-63	17 (N=18) 8-23	10 (N=18) 7-14	3 (N=18) 2-4	22 (N=18) 13-32
	Scirpus fluviatilis	11 (N=6) 6-19	8 (N=9) 4-11	5 (N=7) 4-6	0.3 (N=3) 0.2-0.4	53 (N=6) 27-74
	Filamentous macroalgae	68 (N=15) 34-114	25 (N=12) 13-31	23 (N=3) 22-24	2 (N=3) 1.8-2	24 (N=3) 22-26
	Vallisneria americana	44 (N=9) 22-97	7 (N=9) 4-9	6 (N=3) 5-7	Nd	16 (N=3) 9-23
	Nymphea tuberosa	43 (N=15) 11-73	6 (N=18) 3-14	5 (N=7) 3-6	Nd	8 (N=6) 7-9
ADF	*Potamogeton perfoliatus*	65 (N=135) 2-158	10 (N=126) 2-36	4 (N=81) 1-9	1 (N=31) 0.5-2	5 (N=81) 2-10
	Scirpus fluviatilis	52 (N=33) 22-80	3 (N=33) 2-5	7 (N=27) 4-11	Nd	13 (N=27) 6-25
	Thypha angustifolia	62 (N=9) 55-74	2 (N=9) 2-3	5 (N=9) 4-6	1 (N=9) 0.8-1	8 (N=9) 7-11
	Vallisneria americana	39 (N=27) 28-51	10 (N=27) 7-12	4 (N=27) 3-5	1 (N=9) 1.2-2	10 (N=27) 7-14
GIR	*Scirpus fluviatilis*	128 (N=144) 24-267	5 (N=144) 2-54	5 (N=94) 0.3-13	1 (N=33) 0.2-2	4 (N=94) 0.2-10
	Potamogeton perfoliatus	112 (N=45) 79-153	11 (N=45) 5-23	3 (N=45) 2-6	1 (N=27) 1-3	3 (N=45) 1-6
	Elodea canadensis	64 (N=21) 44-101	10 (N=21) 7-14	3 (N=18) 2-4	2 (N=18) 1-3	5 (N=18) 3-10
	Thypha angustifolia	113 (N=9) 99-144	2 (N=9) 1-2	2 (N=9) 1.6-2	0.4 (N=9) 0.4-0.5	2 (N=9) 1.5-2
MASK	*Potamogeton perfoliatus*	56 (N=135) 20-109	8 (N=135) 2-26	3 (N=99) 0.1-6	2 (N=36) 0.5-4	7 (N=99) 0.2-16
	Scirpus fluviatilis	78 (N=27) 50-132	2 (N=27) 2-3	5 (N=27) 3-6	1 (N=9) 0.5-2	6 (N=27) 4-9
	Thypha angustifolia	30 (N=12) 24-42	2 (N=12) 1-3	7 (N=9) 5-9	1 (N=9) 0.6-1	23 (N=9) 19-31
	Filamentous macroalgae	27 (N=3) 24-34	14 (N=3) 13-16	5 (N=3) 4-5	2 (N=3) 0.5-2	17 (N=3) 12-20

Table 1.3: Minimum and maximum concentrations of THg and MeHg (in ng g DW^{-1}, or in ng g wet $weight^{-1}$ when WW is specified) measured in periphyton, macrophytes, phytoplankton and sediments from different ecosystems

Authors	Sample	Site	THg	MeHg	% MeHg/THg
Watras and Bloom 1992	phytoplankton	Little Rock lake, WI, USA	30 *(in WW)	3-5*(in WW)	10-17
Roulet *et al* 2000	phytoplankton	Tapajós River, Brazil	17-125	2-25	7-24
This study	periphyton (macrophytes)	Lake St-Pierre, Qc, Canada	6-284 0.3-12* (in WW)	1.3-24 0.05-1.0* (in WW)	0.2-74
Desrosiers *et al* 2006	periphyton (teflon artificial substrate)	Boreal lakes, Qc, Canada	42-271	3-55	2-36
Rask *et al* 1994	periphyton (glass plates)	Boreal lake, Finland	60-90	-	-
Hill *et al* 1996	periphyton (cobbles)	East Fork Poplar Creek, TN, USA	60-50000	5-60	0.1-8
Cleckner *et al* 1998	periphyton (free floating mats)	Everglades, FL, USA	1.38-4.02 * (in WW)	0.03-0.44* (in WW)	1-28
Coelho-Souza *et al* 2011	periphyton (macrophyte rhisospheres)	Tapajós River, Brazil	67-198	1-6	2-16
Roulet *et al* 2000	periphyton (macrophytes)	Tapajós River, Brazil	96-254	2-8.5	1.5-8.3
Achà *et al* 2005	periphyton (macrophyte rhisospheres)	Floodplain lake, Bolivia	64-101	3-10	4-10
This study	macrophytes	Lake St-Pierre, Qc, Canada	1-54	0.2-6.3	12-20
Thompson-Robert *et al* 1999	macrophytes	St-Lawrence River, Qc, Canada	6.4-240	-	-
Grondin 1994	macrophytes	Lakes of northern Quebec, Canada	24-56	-	-
Siegel *et al* 1985	macrophytes	Lakes close to mines, BC, Canada	13-198	-	-
Göthberg and Greger 2006	macrophytes	Cultivated macrophytes, Sweden	23-64	0.5-2.7	2-4
Molisani *et al* 2006	macrophytes	Reservoirs, Brazil	46-246	-	-
Caron and Lucotte 2008	sediments	Lake St-Pierre, Qc, Canada	4-150	0.1-3.8	0.3-4.5

1.8 Figure captions

Figure 1.1: Maps of Canada and Lake St. Pierre (St. Lawrence River, Québec, Canada) with the four sampling stations Girodeau Island (GIR), Maskinongé Bay (MASK), St. François Bay (BSF) and Anse-du-Fort (ADF).

Figure 1.2: Mean (±standard error) of seasonnal variations observed in four stations of Lake St.Pierre. A) epiphyte biomass (chlorophyll-α) B) macrophyte biomass, C) [THg] in epiphytes, D) [THg] in macrophytes, E) [MeHg] in epiphytes, F) [MeHg] in macrophytes. Repeated time analysis of variance indicate very high significant differences between month ($p < 0.0001$) and stations ($p < 0.0001$), * marks significant difference between stations in a given month. Open symbols represent south shore stations, black symbols north shore stations, dash line indicate a successional change in august in the dominant macrophyte specie for 3 out of 4 stations (from *Potamogeton perfoliatus* in all stations at the beginning of the summer to *Elodea canadensis* in GIR, *Vallisneria americana* in Mask and *Ceratophyllum demersum* in BSF). N=9 for each point in the graph.

Figure 1.3: Interannual mean (±standard error) variations in epiphytes from BSF between 2002 and 2004 (August data) a) Chlorophyll-α; b) [THg] and c) [MeHg]. One-Way ANOVA found significant ($p < 0.0001$) differences between years. Significant differences between years (Tukey-Kramer test, $\alpha < 0.05$) are marked by capital letters. For each bar, N=9.

Fig 1.4: Relationship between [THg] in epiphytes from different macrophyte species and the Autotrophic Index at the Baie St. François in 2002 (N=39).

Figure 1.5: Principal component analysis of environmental descriptors and A) and B) [THg] and [MeHg] in epiphytes, the first 3 principal axes explained 68% of the variance; C) and D) [THg] and [MeHg] in macrophytes, the first 3 principal axes explained 69% of the variance (N=41).

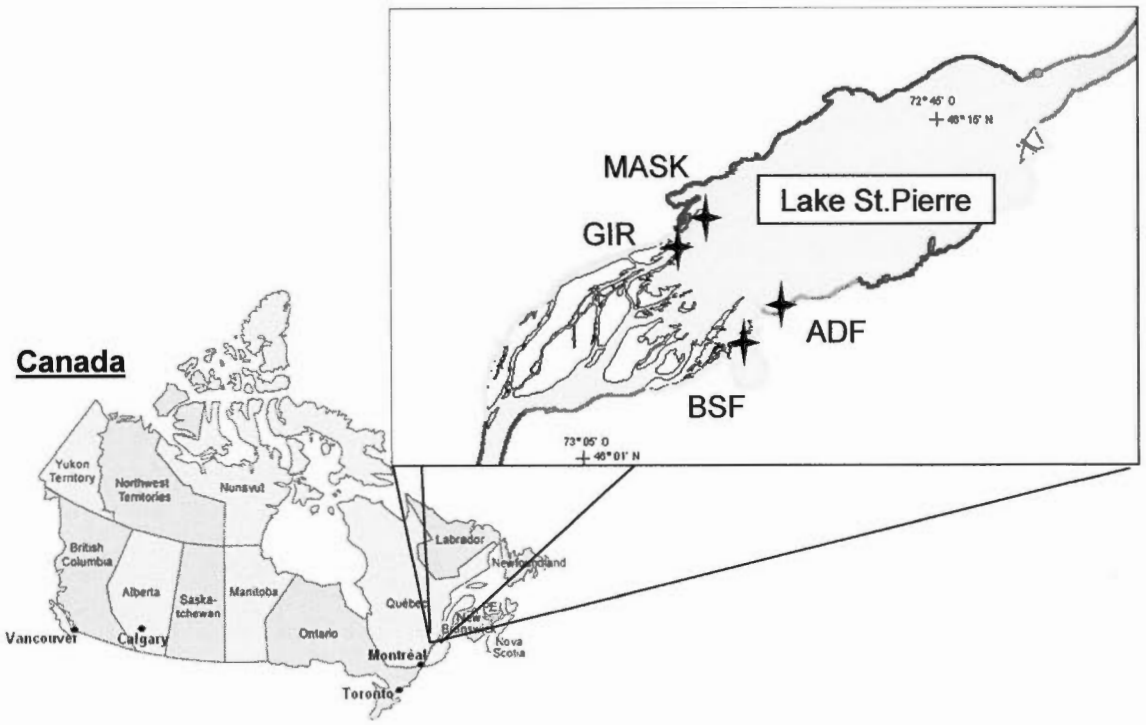

MASK
Lake St.Pierre
GIR
ADF
BSF
72° 45' O
46° 15' N
73° 05' O
46° 01' N
Canada
Yukon Territory
Northwest Territories
Nunavut
British Columbia
Alberta
Saska-tchewan
Manitoba
Ontario
Québec
Labrador
Newfoundland
Nova Scotia
Vancouver
Calgary
Montréal
Toronto

Figure 1.1

Epiphytes : **Macrophytes :**

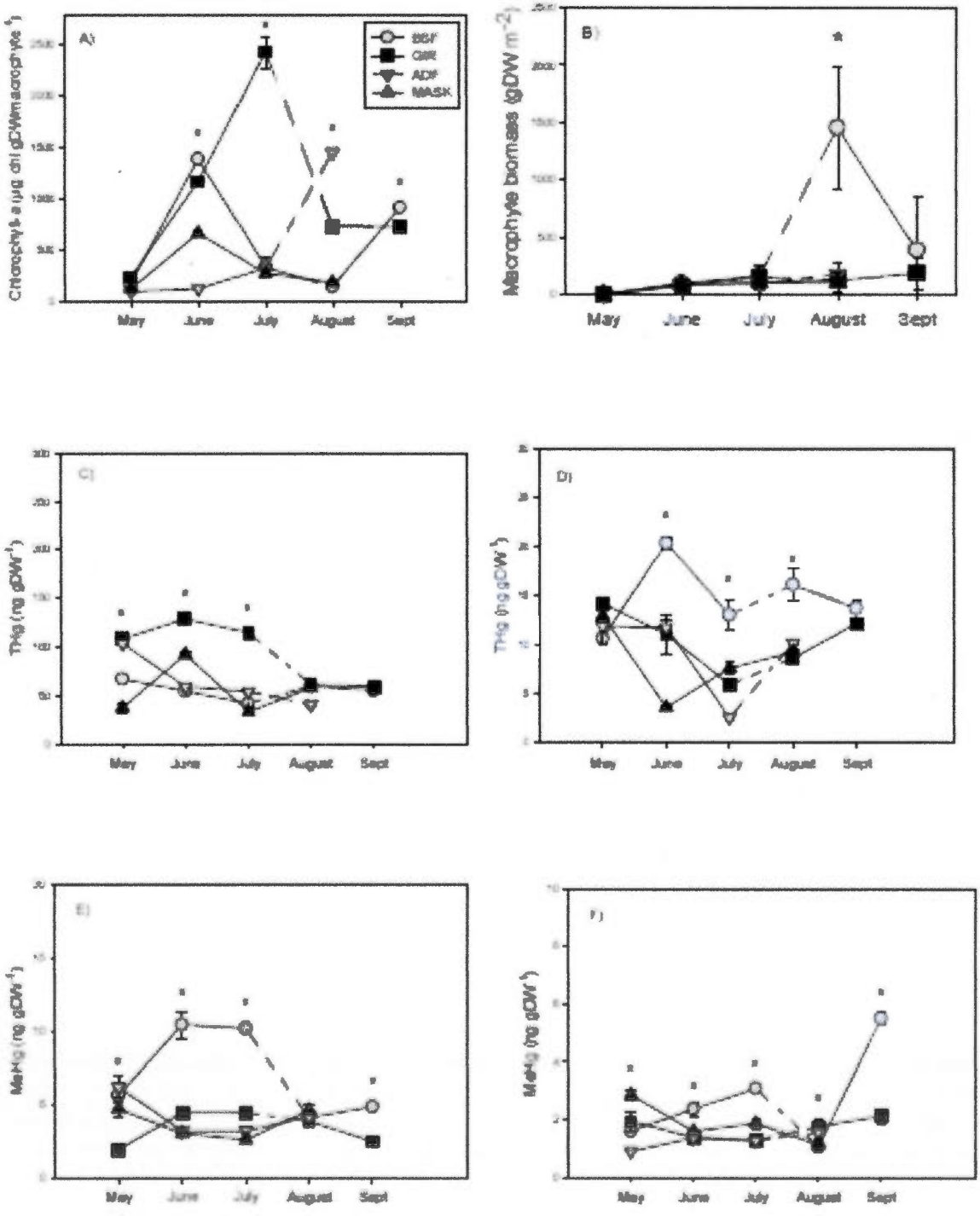


Figure 1.2

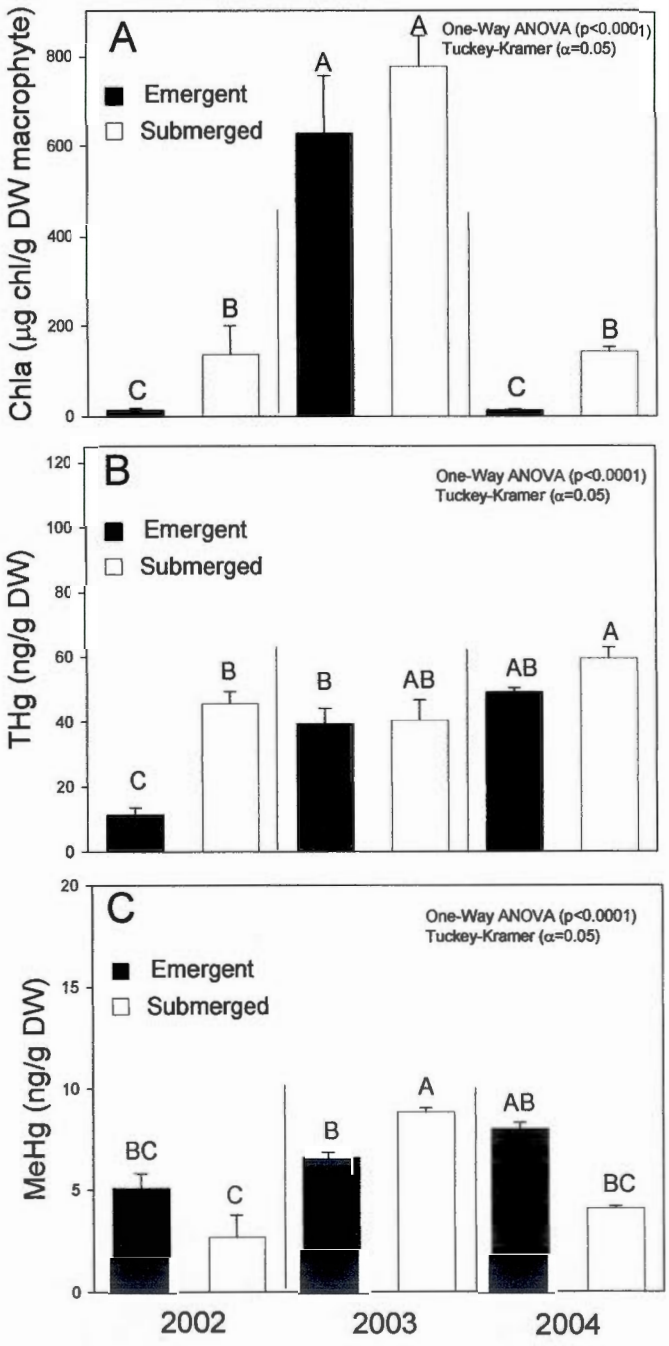

A
One-Way ANOVA (p<0.0001)
Tuckey-Kramer (α=0.05)
Emergent
Submerged
Chla (µg chl/g DW macrophyte)
0
200
400
600
800
C
B
A
A
C
B
B
One-Way ANOVA (p<0.0001)
Tuckey-Kramer (α=0.05)
Emergent
Submerged
THg (ng/g DW)
0
20
40
60
80
100
120
C
B
B
AB
AB
A
C
One-Way ANOVA (p<0.0001)
Tuckey-Kramer (α=0.05)
Emergent
Submerged
MeHg (ng/g DW)
0
5
10
15
20
BC
C
B
A
AB
BC
2002
2003
2004

Figure 1.3

Baie St. François

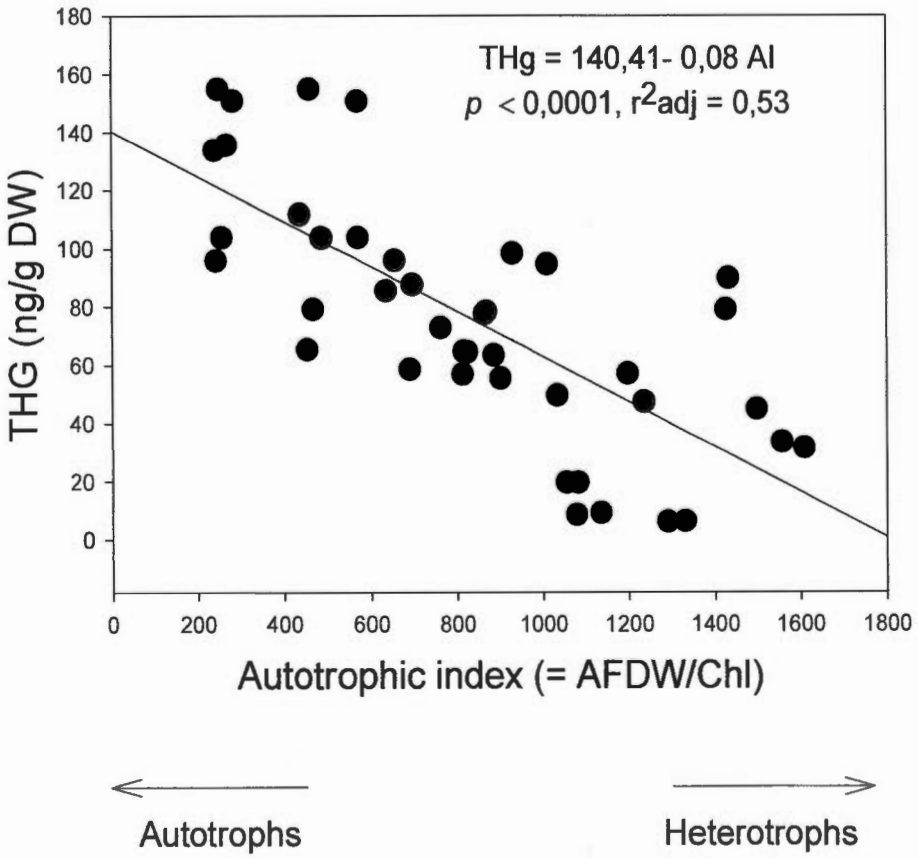
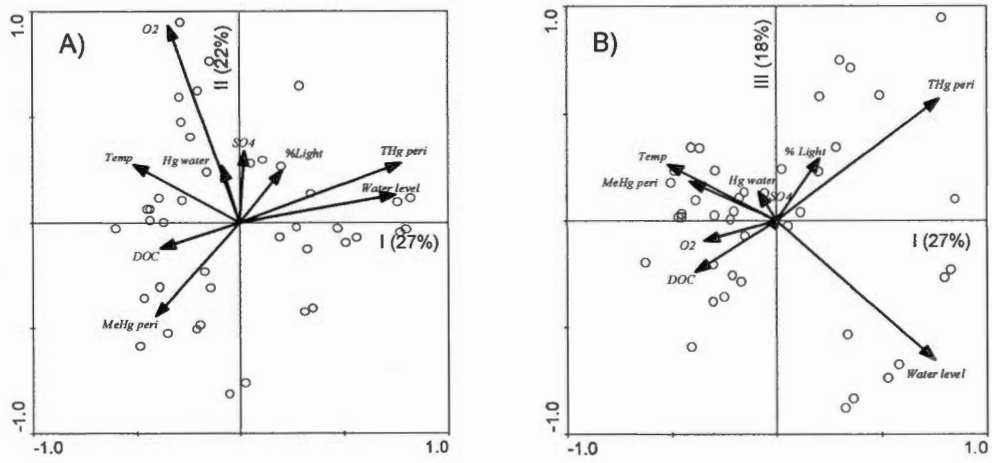


Figure 1.4

THg **MeHg**

Periphyton



Macrophytes

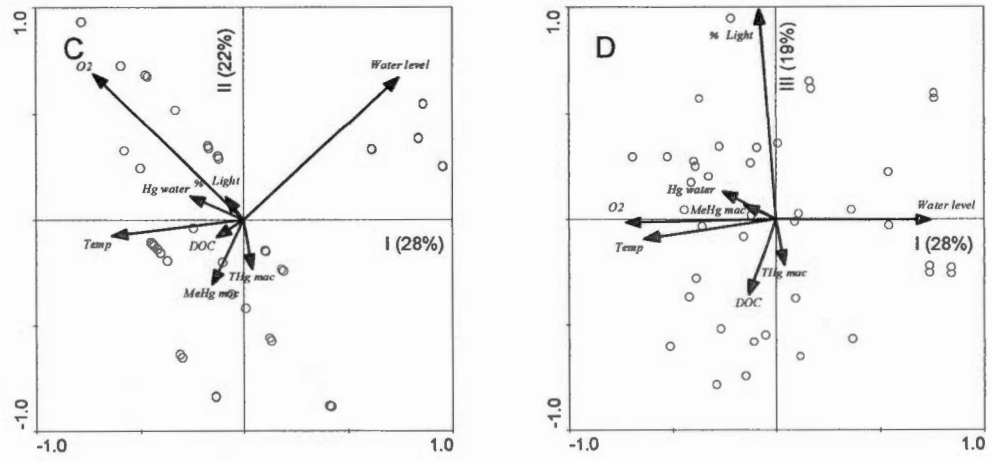


Figure 1.5

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CHAPITRE II

MERCURY METHYLATION AND DEMETHYLATION BY EPIPHYTIC BIOFILMS AND THEIR HOST IN A FLUVIAL WETLAND OF THE ST. LAWRENCE RIVER (QC, CANADA)

Stéphanie Hamelin, Dolors Planas et Marc Amyot

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2.1 Abstract

In aquatic ecosystems, contamination of biota by methylmercury is a subject of concern. But until now, it is unclear how methylmercury is produced, or how it enters the foodweb, or variables that influence these processes. Considering the fact that wetlands are known to be a production site of methylmercury, and that they occupy a large surface of aquatic ecosystems, questions are rising about the significance of that production. Information is lacking about mercury methylation and demethylation rates by primary producers in the northern hemisphere, as well as about which variables control the net MeHg production, that will therafter be accumulated in these organisms. To answer some of these questions, mercury methylation and demethylation rates were measured in periphyton biofilms growing on submerged plants (epiphytes) from a shallow fluvial lake located in a temperate cold region (St. Lawrence River, Quebec, Canada). Incubations were performed *in situ* within macrophytes beds using low-level spikes of ^{199}HgO and $Me^{200}Hg$ stable isotopes as tracers. Epiphytes of Lake St. Pierre are a significant site of net MeHg production. This production varies through the plants growing season and is mainly driven by environmental variables such as available light, dissolved oxygen, temperature, plant community structure and productivity of the habitat.

Keywords : epiphytes, mercury, methylation, demethylation, wetland.

2.2 Introduction

Bioaccumulation of mercury (Hg) in aquatic organisms is a subject of concern. Not only the Hg concentrations, but also its chemical form is important. Effectively, most of the Hg accumulated in biota is in its organic form, methylmercury (MeHg), which is by far the most toxic form of Hg. No clear relationship has been found between aqueous Hg, mainly inorganic, and MeHg measured in freshwater ecosystems (Kelly, *et al.*, 1995). It is important to identify the key sites of Hg methylation in aquatic systems and the drivers controlling the net MeHg production rates.

It is generally accepted that Hg methylation takes place in sediments or overlying hypolimnetic waters (Furutani and Rudd, 1980; Matilainen, *et al.*, 1991; Zang and Planas, 1994; Krabbenhoft, *et al.*, 1998). Therefore, most of studies on Hg methylation have focused on sediments, and mainly on sulfate-reducing bacteria (SRB) as principal Hg methylators (Compeau and Bartha, 1985; Kerry *et al.*, 1991; Gilmour *et al.*, 1992). However, there are other sites in the aquatic environment where Hg methylation occurs. Recently, some studies have demonstrated the ability of periphyton to methylate Hg (Mauro, *et al.*, 2002; Achá, *et al.*, 2005; Desrosiers, *et al.*, 2006a; Huguet, *et al.*, 2010; Hamelin, *et al.*, 2011), and, in some instances, at higher rates than sediments (Cleckner, *et al.*, 1999; Guimarães, *et al.*, 2000).

All previous studies, except for Desrosiers, *et al* (2006a) and Hamelin, *et al* (2011) were done in tropical or subtropical regions. There is a lack of data about periphyton Hg methylation in the northern hemisphere, where lower temperatures may have an important role in controlling Hg methylation rates (Desrosiers, *et al.*, 2006a). Net MeHg production is regulated by the concurrent processes of MeHg formation (Hg methylation) and MeHg decomposition (MeHg demethylation). Rates of both MeHg formation and MeHg degradation are seldom measured simultaneously, and there is still great uncertainty regarding factors and mechanisms controlling each of these processes (Kelly, *et al.*, 1995; Ullrich, *et al.*, 2001; Avramescu, *et al.*, 2011). It has

been shown that periphyton can accumulate significant concentrations of MeHg, as high as 55 ng/gDW, (Hamelin, *et al.*; Roulet, *et al.*, 2000; Desrosiers, *et al.*, 2006c), which can be higher than sediments of the same site (chap.I). Periphyton is also a direct food source for primary comsumers in littoral habitats (Hecky and Hesslein, 1995; Vadeboncoeur, *et al.*, 2002; McIntyre, *et al.*, 2006; Cremona, *et al.*, 2009).

Changes in light penetration, as a result of macrophytes growth and species sucession through the growing season (Burkholder and Wetzel, 1989), is an important factor determining epiphyton biomass (Gosselain, *et al.*, 2005). Within macrophyte stands, the physical vertical structure created by the plants influences light and substratum availability and thus the biomass and productivity of epiphyte communities attached to macrophyte leaves and stems (Vis, *et al.*, 2006). Epiphyte biomass and their specific composition are closely related to the structure of the host plant (Cattaneo and Kalff, 1980; Pip and Robinson, 1984; Engelhardt and Ritchie, 2001). It is likely that differences in microbial biofilms species composition can influence Hg methylation and demethylation rates, through a combination of contrasting metabolic pathways (Macalady, *et al.*, 2000).

In this study, our objectives were: 1) to measure *in situ* Hg methylation and MeHg demethylation rates in epiphytes (periphyton growing on aquatic plants) in the wetland area of a large shallow fluvial lake; 2) to assess seasonal variations of methylation and demethylation rates, and hence of net MeHg production, as a function of light and temperature; 3) to determine the influence of macrophytes species on Hg methylation and MeHg demethylation rates.

2.3 Methodology

2.3.1 Study site

Lake St. Pierre (46°12'N, 72°49'W), is a shallow fluvial lake, formed by an enlargement of the St. Lawrence River, between Sorel and Trois-Rivières (Québec, Canada). With a total surface area of 375 km^2, this large lake has 85% of its total surface covered with macrophyte beds and wetlands at the peak of the growing season. One important particularity of Lake St. Pierre, is the spatial heterogeneity due to the presence of distinct water masses, which may flow side by side without mixing, thus creating large spatial variations in physical and chemical water characteristics (Centre Saint-Laurent, 1996; Vis, *et al* 2007). The two stations sampled for this study were chosen for their contrasting physico-chemical proprieties. One station was located on the north shore, close to the island of Girodeau (GIR, 46° 09' 82" N; 72° 59' 10" W). In this section of the river, the bulk of the water mass comes from the Ottawa River and north shore tributaries (L'Assomption, Chicot, Yamachiche, Maskinongé and Du Loup rivers). In these colored humic waters, nutrients and suspended particles are highly concentrated as well as dissolved organic carbon (DOC) concentrations, derived from humic and fulvic acids (Frenette, *et al.*, 2003). The second station was situated on the south shore, in a large hyper-eutrophic bay, St. François Bay, (BSF, 46° 06' 97" N; 72° 55' 87" W), which receives agricultural inputs from the south shore tributaries (Richelieu, Yamaska, Nicolet and St. François rivers) draining farmlands and bringing suspended brown-clay and nutrient rich waters (Vis, *et al.*, 2003).

2.3.2. Sampling

Before collecting any samples, all sampling devices and tools for Hg measurements were carefully acid-washed and rinsed with nanopure water. Sampling was carried out in September 2002, July-September 2003 and September 2004 in station BSF, and every month from May to September 2004 in station GIR. Sampling of dominant macrophytes and their associated epiphytes was done by scuba divers using 0.68 L Pac-man boxes, (a smaller cylindrical version of the 6L Downing box (Downing, 1986), modified by C. Vis, Parks Canada). Great care was

taken during sampling to minimize losses and disturbance of the biofilms. Samples were collected in triplicates for each macrophyte species and each depth.

Physicochemical characteristics of the water within macrophytes beds were measured *in situ* using specific probes: pH, (Chekmite 4 pH meter), light (Biospherical QSL-101 quantum meter), temperature and dissolved oxygen (WTW Oxi340 oximeter). Water grab samples for DOC, THg_{water}, $MeHg_{water}$ and major ions were also taken simultaneously to epiphytes/macrophytes sampling (at mean sampling depth of 30 cm). Water chemistry data comparing GIR and BSF stations and methods are presented in the Supporting Information (Annexe B and D).

2.3.3 Methylation/demethylation assays

Filtered lake water (0.20 µm porosity) was spiked with 3 $ng.L^{-1}$ (final concentration) of ^{199}HgO and $Me^{200}Hg$ (Oak Ridge National Laboratory), and pre-incubated for 1 h to allow equilibration with dissolved ligands. Thereafter, entire macrophytes with their associated epiphytic biofilms (7-10g dry weight (DW)), were added to the spiked filtered water and were incubated *in situ* in clear polycarbonate bottles (total incubation volume of 2L) within macrophyte beds. Polycarbonate was chosen because this material has optimal solar radiation transmittance in the visible waveband with minimal Hg and MeHg sorption to container walls (Cleckner, *et al.*, 1999; Gorski, *et al.*, 2006). Total incubation time was 48h, but every 12 hours (at dawn and twilight), three replicate bottles were collected and amended with 8 ml of 4N HCl; samples were then stored in the dark at *in situ* water temperature. Once in the laboratory, periphyton was separated from macrophytes by mechanical shaking (9 min in a Red Devil® paint shaker), a method we had previously tested for removing periphyton efficiently without destroying algal cells. The periphyton suspension was then split in 3×100 ml aliquots for each measurement (DW, THg, MeHg, and Hg stable isotopes). Aliquots for DW were filtered on GF/C filters that were previously combusted (90 minutes at 500°C) and pre-weighed. Dry weight was measured after drying the filters at 45°C to constant weight (APHA, 1989). Filters for

DW were kept frozen (-80°C) until analysis and the other aliquots (THg, MeHg, and Hg stable isotopes) were kept frozen (-80°C), and then freeze-dried.

Hg stable isotopes samples were analysed at Trent University (Dr. Hintelmann's Laboratory), Ontario, Canada. The formation and degradation of MeHg were determined by monitoring the concentrations of the respective isotopes $Me^{199}Hg$ and $Me^{200}Hg$ by gas chromatography-inductively coupled plasma mass spectrometry, following protocols in Hintelmann and Ogrinc (Hintelmann and Ogrinc, 2003). To calculate methylation and demethylation rate constants, we used the following equations (Hintelmann, *et al.*, 2000):

$$\text{Net MeHg production} = K_m [Hg^{2+}] - K_d [MeHg^{+}] \quad (1)$$

where K_m is the specific methylation rate constant (in d^{-1}) and K_d is the specific demethylation rate constant (in d^{-1}). K_m and K_d were calculated from the initial part of the slope of the relation (first 12h) between isotopic $[MeHg^{+}]$ and time; when $[Me^{199}Hg^{+}]$ and $[^{200}Hg^{2+}]$ were low enough that K_m and K_d can be calculated from:

$$K_m = [Me^{199}Hg^{+}]/(t\,[^{199}Hg^{2+}]_0) \quad (2)$$

$$[Me^{200}Hg+] = [Me^{200}Hg^{+}]_0\ e^{-Kd\ t} \quad (3)$$

where $[Me^{200}Hg^{+}]_0$ and $[^{199}Hg^{2+}]_0$ are the initial substrate concentrations added to the sample in ng g^{-1}. K_d was obtained by linear regression of $\ln[Me^{200}Hg^{+}]$ versus time (t) in days. Since the bioavailability of stable isotopes added is probably higher than Hg found in the natural waters, these rates are considered maximum potential rates. Net MeHg production by epiphytes with *in situ* concentrations was calculated as follows:

$$\text{Net MeHg production} = (K_m \,.\, [\text{inorganic Hg}]_{\text{in epiphytes}}) - (K_d \,.\, [\text{MeHg}]_{\text{in epiphytes}}) \quad (4)$$

The estimation of epiphyte net MeHg production per m^2 was obtained by multiplying the calculated net MeHg production by the epiphyte biomass by unit area (DW m^{-2}).

2.3.4 THg and MeHg Analyses

Prior to THg and MeHg analysis, periphyton samples were freeze-dried and weighed. THg concentrations were measured by thermal decomposition using a direct mercury analyzer (DMA 80; Milestone, MLS; Boylan and Kingston, 1988; Cizdziel, *et al.*, 2000). Samples for MeHg analysis were freeze-dried, weighed, and extracted in KOH/methanol (25%), and extracts were analyzed by cold vapor atomic fluorescence (CVFAS: Bloom and Fitzgerald, 1988; Bloom, 1989). The working detection limit was 0.01 ng g^{-1} of Hg for DMA 80 and 0.1 ng g^{-1} for CVFAS (three times the standard deviation of 10 procedural blanks). Blanks and certified reference material (Tort-2, SO-2 and IAEA, CNRC) were analyzed every 10 samples to ensure the reproducibility of the analysis and to assess quality assurance/quality control. Hg concentrations in reference material varied slightly over time (CVFAS: TORT-2 CV=1.9%; DMA: TORT-2 CV=2.3%, SO-2 CV=1.6% and IAEA CV=2.1%) but were not significantly (Student T-test, $p>0.05$) different from certified values. No substantial Hg contamination was detected in the blanks.

2.3.5 Statistical analyses

Results of Hg methylation and demethylation between stations, depths, and macrophytes species were compared using one-way or two-way ANOVA (previously Log_{10} transformed to meet normality requirements as verified by Shapiro-Wilk test) for independent samples. When significant differences were found, Tukey-Kramer HSD (honestly significant difference) test for multiple mean comparisons was applied. For testing seasonality effect, comparisons between sampling periods of the same year were performed with repeated measures analysis of variance, since samples were dependent of each other. All statistical analyses were done using JMP 7 statistical packages (SAS Institute Inc. 1991, α set at 5%).

2.4 Results

2.4.1 Hg methylation and MeHg demethylation rates: effect of depth, substrate (macrophyte species) and station

Isotopes concentrations in macrophytes were below the detection limit (<0.001ng gDW^{-1}), resulting to non-detectable methylation or demethylation rates in macrophytes during the 48-h incubation. In epiphytes, from 2002 to 2004, wide ranges of methylation (K_m: 0.002 to 0.137 d^{-1}) and demethylation (K_d: 0.096 to 0.334 d^{-1}) rate constants were observed, related to macrophyte species, sampling depth and stations (GIR or BSF).

In BSF during July 2003 (figure 2.1 A and B), significant differences were found when comparing K_m and K_d measured in epiphytes from *Ceratophyllum demersum* (p=0.0498, N=18), K_m being 1.5 to 2.3 fold higher than K_d at 30 cm and 60 cm depth respectively. But, either for K_m or K_d, no significant differences were found between the 3 depths (0, 30 and 60cm) at which periphyton samples from *C. demersum* were collected. However, when different macrophytes species were sampled at their *in situ* growing depth (macroalgae at 0 cm, *C. demersum* at 30 cm and *Vallisneria americana* at 60 cm depth), significant differences were found between depths for K_m and K_d (p<0.0001, N=18). K_m were 3 fold higher at 0 and 30cm than at 60 cm depth while Kd were 2 fold higher at 0 cm than 30 cm, and 3 fold higher than at 60 cm depth. The mean net MeHg production reflect these differences as it was 3 fold lower in epiphytes growing on *V. americana* (0.74 ± 0.17 ng gDW^{-1} d^{-1}) than mean net MeHg production measured on *C. demersum* or macroalgae (2.64 ± 0.14 ng gDW^{-1} d^{-1}).

On the north shore, in station GIR during June 2004 (figure 2.1 C), significant differences were also found between K_m and K_d (p<0.0001, N=18) measured in *Potamogeton perfoliatus* epiphytes at all three depths (0, 30 and 60 cm). But this time, K_d were higher than K_m by 19 to 450 times (p<0.0001, N=18). No differences

were detected between depths with either K_m or K_d when they were all measured on the same macrophyte (*P. perfoliatus)*. Furthermore, when different macrophytes species were compared for the same depth (figure 2.1 D), *P. perfoliatus* and *Scirpus fluviatilis* at 30 cm, no differences were found between macrophytes species for either K_m or K_d, but K_d was still 19 to 59 fold higher than K_m (p=0.0015, N=12).

In September 2004, when both stations GIR and BSF were compared using epiphytes from the same macrophyte species, *Elodea canadensis* at 30 cm depth (figure 2.1 E), significant differences were detected between K_m and K_d of both stations ($p<0.0001$, N=12). K_m were 6 times higher in BSF than in GIR, but the inverse situation was found with K_d, that were 2 fold lower in BSF than in GIR. These higher K_m coupled with lower Kd in BSF, compared to GIR, resulted in a greater net MeHg production in BSF (3,73 ± 0,13 ng gDW^{-1} d^{-1}) than in GIR (0.001 ± 0.05 ng gDW^{-1} d^{-1}).

2.4.2 Seasonnal and inter-annual variations of methylation and demethylation rate constant.

From May to September 2004 in station GIR, K_m and K_d were measured in epiphytes from the dominant submerged macrophyte, P. perfoliatus at 30 cm depth (figure 2.2). Repeated time analysis of variance showed significant differences for K_m between months, with the lowest K_m measured in May and August, 4 fold lower than in June, July or September (p=0.0124, N=15). K_d was not significantly different from May to August, but it was 2 fold higher in September than in other months (p=0.0054, N=15). Net MeHg production significantly increased 20 times from May to July (p= 0.0118, N=15) up to 0.98 ± 0.18 ng gDW^{-1} d^{-1}, but dropped to - 0.61 ± 0.12 ng gDW^{-1} d^{-1} in August and slightly increased to 0.001 ± 0.05 ng gDW^{-1} d^{-1} in September. Once calculated by unit area, the net MeHg flux in epiphytes varied from -126 ng $m^{-2}d^{-1}$ to +200 ng $m^{-2}d^{-1}$.

K_m and K_d measured in this study were representative of the *in situ* [THg] and [MeHg] measured in epiphytes (figure 2.3). A linear correlation was found between [MeHg] in epiphytes and K_m (MeHg = 3.3 + 92.9 K_m; r^2=0.59, $p < 0.0001$, N=34) as well as a linear correlation between [inorganic Hg] in epiphytes and K_d (inorganic Hg = 5.2 + 707.4 K_d, r^2 = 0.60, $p < 0.0001$, N=34). [THg] and [MeHg] in epiphytes varied from 14 to 151 ng g DW^{-1} and from 1.9 to 15.7 ng g DW^{-1} respectively.

Inter-annual variations in K_m and K_d in station BSF with epiphytes growing on *C. demersum* showed no significant differences in K_m between 2002 to 2004, or in K_d between 2003 and 2004 (figure 2.4). In 2003 and 2004, K_d were 1.5 fold higher than K_m (p<0.0061).

2.5 Discussion

2.5.1 Hg methylation and MeHg demethylation

Methylation rates in Lake St. Pierre periphyton were similar to the ones measured in a northern temperate cold lake (0.096-1.224 ng MeHg d^{-1}; Desrosiers, *et al.*, 2006a), as well as to the ones obtained with epiphytes of subtropical and tropical regions like Florida wetlands (0.1-20%; Cleckner, *et al.*, 1999; Mauro, *et al.*, 2002) and Brazil wetlands (6.5-10.4%; Guimarães, *et al.*, 1998). But, they were higher than values measured in epiphytes from a Wisconsin oligotrophic lake (0.011-0.062%; Korthals and Winfrey, 1987).

As for demethylation rates measured in this study were in the same range than the ones found with epiphytes from Florida wetlands (6-20%; Mauro, *et al.*, 2002). However, our K_d were up to 35 times higher than the ones measured in periphyton of a northern temperate oligotrophic lake in Wisconsin (0.62-1.28%; Korthals and Winfrey, 1987). K_d or K_m in epiphytes seem to reach similar rates in different latitudinal ecosystems, except for the lower values observed in Wisconsin. Two

factors may explain the lower rates measured in Wisconsin, the productivity of the system and the integrity of the periphyton biofilm used in the measurements. The lake sampled by Kortals and Winfrey (1987) was a low productivity lake, while the other sites, except the study of Desrosiers *et al* (2006a), were all eutrophic. In a productive system, the metabolic activity of microorganisms is higher than in an unproductive system, consequently, K_m and K_d will be stimulated in the former (Cleckner, *et al.*, 1999).

Biofilm's integrity, could explain differences found between the two oligotrophic lakes. The multiple layers within periphyton matrix form a microcosm with strong redox gradient (Jørgensen, *et al.*, 1983), thus creating narrow niches through biofilm's depth for different microbial guilds participating in methylation and demethylation processes. In Desrosiers *et al.* study (Desrosiers, *et al.*, 2006a), periphyton grown on artificial teflon substrates were used, and incubations for methylation measurements were done with the entire community attached to the substrate. In Wisconsin, the periphyton was scraped from the substrate prior to incubation (Korthals and Winfrey, 1987). Scraping biofilms destroys the integrity of the mat structure, which may result in lower methylation and demethylation rates. Substrate integrity could also explain differences in the demethylation rates between productive systems, e.g. Mauro *et al.* (2002) and ours. In Mauro *et al.* study, macrophytes were chopped into pieces, whereas this study, epiphytes incubations were done on the whole plant without altering the biofilm structure.

When rates measured in this study are compared with a few measurements selected from other aquatic compartments where K_m and K_d were measured, periphyton from Lake St. Pierre methylate Hg at higher rates than runoff waters and aerobic waters from a Finland lake (<0.12%; Matilainen and Verta, 1995), water from an Ontario lake in Canada (0.05-0.14 %; Miskimmin, *et al.*, 1992) or hypolimnetic waters of lakes in northern Wisconsin (0.01 to 0.09 d^{-1} ; Eckley, *et al.*, 2005). But for the two first studies, similar rates of demethylation were found than in Lake St. Pierre, while higher rates were measured in epiphytes than in hypolimnetic waters

(0.03 to 0.05 d^{-1} ; Eckley, *et al.*, 2005). Sediments, the most studied aquatic compartment regarding Hg methylation, had lower or similar methylation rates than periphyton, either in temperate or subtropical systems. Periphyton methylate Hg at higher rates than sediments of Finland (0,0006 - 0,0104 %; Matilainen and Verta, 1995) or the Everglades in Florida (< 3%; Gilmour, *et al.*, 1998), and at similar rates as sediments from Wisconsin (1,6-7,4 %; Gilmour and Riedel, 1995) and Massachusetts (0,1 - 16,3 %; (Gilmour, *et al.*, 1992). Moreover, the net MeHg production rates estimated in this study were 2 orders of magnitude higher (in terms of loss as well as production) than net MeHg production measured within sediments of Lake St. Pierre, that ranged from -2.1 to + 5.5 ng m^{-2} d^{-1} (Goulet, *et al.*, 2007). These data demonstrate that epiphyte biofilms are an important site for MeHg production and accumulation. As periphyton is the main food source for macroinvertebrates (Cremona, *et al.*, 2009; Molina, *et al.*, 2010), Hg methylated by periphyton could be easily transferred through the food web and must be better integrated in environmental studies.

2.5.2 Factors controlling K_m and K_d in Lake St. Pierre

In BSF during July 2003 (figure 2.1 A and B), no significant differences were found in K_m or K_d between epiphytes sampled at three different depths and growing on the same host, *C. demersum*. But, when epiphytes measurements were done on different macrophytes species sampled at their *in situ* growing depth, significant differences were found. Macrophytes can influence epiphytes biomass and, ultimately, K_m and K_d, in two ways: 1) by providing a surface for colonisation, and 2) by interfering with light availability by creating a shadow. Both will vary as a function of the macrophyte's architecture. When the same macrophyte was sampled at three different depths, the % of available light in the macrophyte bed was the same for the three depths (3%). But when different macrophyte species were used, we had a gradient in the percentage of available light that went from 46 % in the macrolagae mat collected at 0 cm, to 0.5 % with *V. americana* living at 60 cm. As a consequence of that light gradient, periphyton biomass (chlorophyll-a) was two fold higher on

macroalgae at 0 cm than on *V. americana* at 60 cm. Macroalgae, as well as *C. demersum*, by their dense divided leaves provide much more surface for periphyton colonization than the ribbon-leaves *V. americana*. Confirming observations about light effect can be made with results from GIR station in 2004 (Figure 2.1 C and D). Unlike in BSF station, in GIR no differences were found in either K_m or K_d between the 2 species of macrophytes, both sampled at 30 cm depth (*P. perfoliatus* and *S. fluviatilis*). However this time, light availibility was almost the same between both macrophytes that were, 40% for *P. perfoliatus* and 38% for *S. Fluviatilis,* resulting in no differences in epiphytic biomass, neither in K_m or K_d. Light is then one important factor driving Hg methylation and MeHg demethylation in epiphytes. Visible light and U.V. degrade dissolved organic matter and lead to increased bioavailability of Hg^{2+} for methylation (Ravichandran, 2004). Organic matter degradation may stimulate metabolic activity and microbial interactions, which may have a direct effect on Hg methylation and demethylation rates in biofilms (Ullrich, *et al.*, 2001). Furthermore, with higher available light in GIR, MeHg may have also undergone more photodegradation, as it has been observed in lakes (Sellers, *et al.*, 1996). When GIR and BSF stations are compared, it seems that Hg methylation is more important in BSF and MeHg demethylation in GIR, resulting in higher Kd and [THg] in epiphytes from GIR and higher Km and [MeHg] in epiphytes from BSF. The most important difference regarding physico-chemical variables between both stations are the higher concentrations of nutrients and DOC, as well as a lower concentration in dissolved oxygen in BSF, than in GIR. It is known that Hg methylation is stimulated in anaerobic conditions when demethylation is higher under aerobic conditions (Compeau and Bartha, 1985; Ramlal, *et al.*, 1986), thus explains the differences observed in both processes in these two stations.

Generally, Hg methylation rates decrease with increasing [DOC] due to Hg complexation (Miskimmin, *et al.*, 1992). Here, it seems that the difference of 10 mg L^{-1} in [DOC] between GIR and BSF was not important enough to lower methylation rates in BSF. An alternative explanation could be that organic matter has stimulated metabolic activity (as discussed previously), or that other variables such as light, low

dissolved oxygen concentrations and high nutrient loads, have a stronger stimulating effect on methylation.

Seasonal trends of K_m, K_d and therefore net MeHg production in Lake St. Pierre seem to be linked with temperature and macrophyte community structure. Indeed, the highest net MeHg observed in GIR was in July, when temperatures were at their maximum, as K_m. A decrease in Km during August with a simultaneous increase in K_d resulted in a drastic decrease in net MeHg production. August is characterized by the succession change in the dominant macrophyte species. *P. perfoliatus* was dying and was then replaced by *E. canadensis*. This new substrate was colonised by biofilms at an early successional stage, characterised by higher metabolic activity than older ones (Acs, *et al.*, 2007), thus increasing metabolic activity in an aerobic milieu likely conducted to higher K_d that continued to increase in september, probably due to lower water temperatures. A similar 3 fold increase in K_d along with a decrease in temperature was also observed between July and September 2003 in BSF. Wright and Hamilton (1982) have shown that the amount of MeHg released from sediments at 4°C was only 50-70% of the one measured at 20°C (Wright and Hamilton, 1982). In Lake St. Pierre, a 2 fold increase in K_d was observed when temperature decreased to 7°C.

No interannual differences were observed in BSF station regarding MeHg concentrations, either with K_m or K_d, which is in agreement with MeHg measured in epiphytes (chap. I). The year of 2003 was characterised by low water levels when compared with 2002 or 2004. Seasonal decreases of water levels in the St. Lawrence River usually coincide with major increases in epiphyte biomass (Gosselain, *et al.*, 2005). Even if K_m and K_d remain stables, if periphyton biomass gets higher, it will likely increase Hg burden at the ecosystem scale.

2.6 Conclusions

Epiphytes of Lake St. Pierre represent a significant site of net MeHg production. This production can reach values as high as 200 ng m^{-2} d^{-1}. As periphyton is the main food source for macroinvertebrates in wetlands, this MeHg produced by epiphytes could be rapidly transferred through the food web. Such high methylation rates argue for the inclusion of epiphytes, along with sediments, when modeling MeHg food web dynamics. Environmental variables that drive net MeHg production include available light at the macrophyte growing depth, dissolved oxygen, temperature, productivity of the system and community structure.

2.7 Acknowledgements

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2.8 Figure captions

Figure 2.1: Mean Hg methylation and MeHg demethylation rates (± standard error) in epiphytes; A) on *Ceratophyllum demersum* at 3 different depths (0, 30 and 60 cm) in BSF, July 2003 B) on 3 different macrophytes at their *in situ* depth (*Macroalgae* at 0 cm, *Ceratophyllum demersum* at 30 cm and *Vallisneria americana* at 60 cm) in BSF, July 2003; C) on *Potamogeton perfoliatus* at 3 different depths (0, 30 and 60 cm) in GIR, June 2004 D) on *Potamogeton perfoliatus* and *Scirpus fluviatilis* at 30 cm depth in GIR, June 2004; E) on *Elodea canadensis* at 30 cm depth in BSF and GIR, September 2004. For each bar, N=3 replicates.

Figure 2.2: Mean (± standard error) Hg methylation rates (black dots), MeHg demethylation rates (white dots) and net MeHg production (grey bars) in epiphytes growing on dominant submerged macrophytes, in GIR from May to September 2004. Repeated time analysis of variance showed significant differences between months (K_m : p = 0.0124; K_d : p = 0.0054 and Net MeHg production : p = 0.0118). Dash line indicate a successional change in August in the dominant macrophyte species (from *P. perfoliatus* at the beginning of the summer to *E. canadensis* in August). For each point or bar, N= 3.

Figure 2.3: Linear regression of MeHg concentrations in epiphytes in function of Hg methylation rate and linear regression of inorganic Hg concentrations in epiphytes in function of MeHg demethylation rates.

Figure 2.4: Inter-annual variations of mean (± standard error) Hg methylation and MeHg demethylation in epiphytes from *C. demersum* in BSF, September 2002-2003-2004. For each bar, N= 3; MeHg demethylation was not measured in 2002.

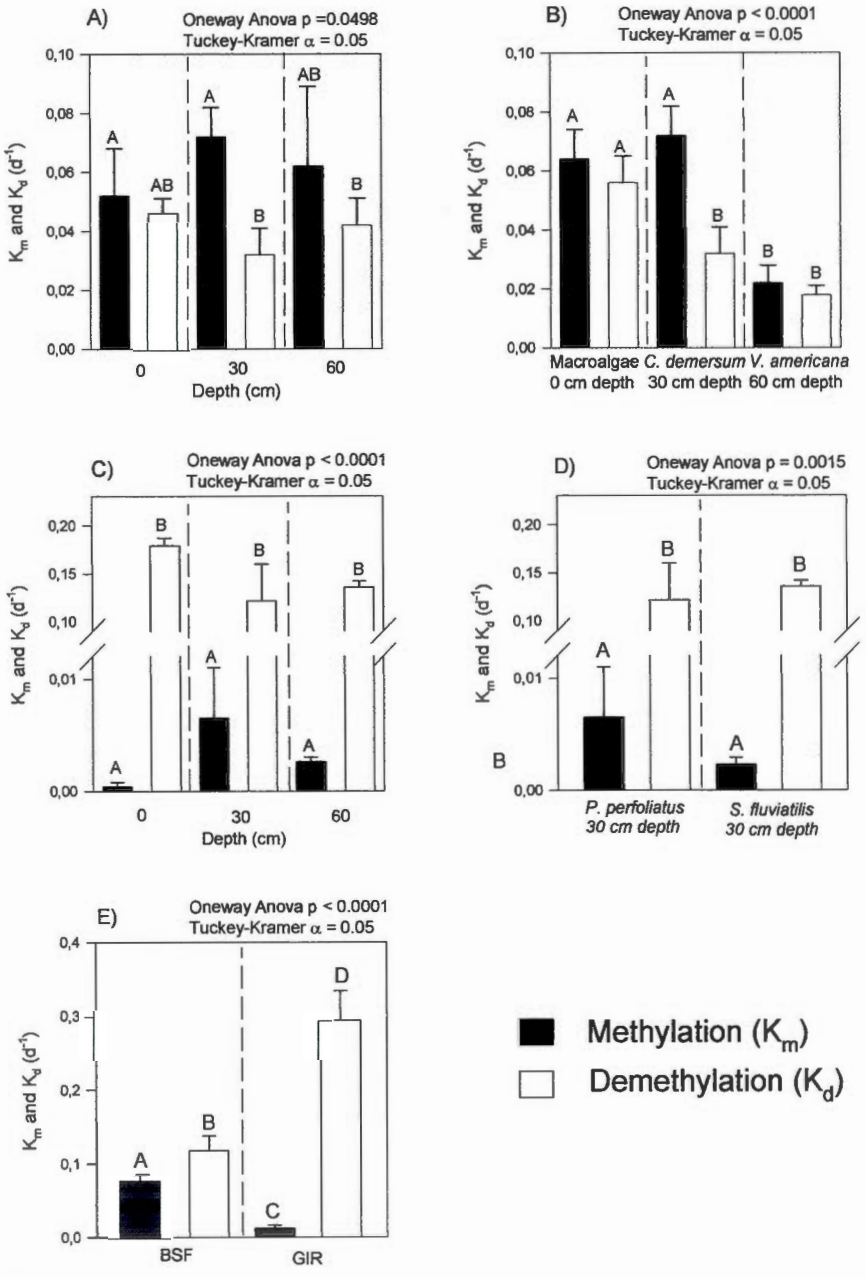
A)
Oneway Anova p =0.0498
Tuckey-Kramer α = 0.05
B)
Oneway Anova p < 0.0001
Tuckey-Kramer α = 0.05
C)
Oneway Anova p < 0.0001
Tuckey-Kramer α = 0.05
D)
Oneway Anova p = 0.0015
Tuckey-Kramer α = 0.05
E)
Oneway Anova p < 0.0001
Tuckey-Kramer α = 0.05
Km and Kd (d-1)
Depth (cm)
Macroalgae C. demersum V. americana
0 cm depth 30 cm depth 60 cm depth
P. perfoliatus
30 cm depth
S. fluviatilis
30 cm depth
BSF
GIR
Methylation (Km)
Demethylation (Kd)

Figure 2.1

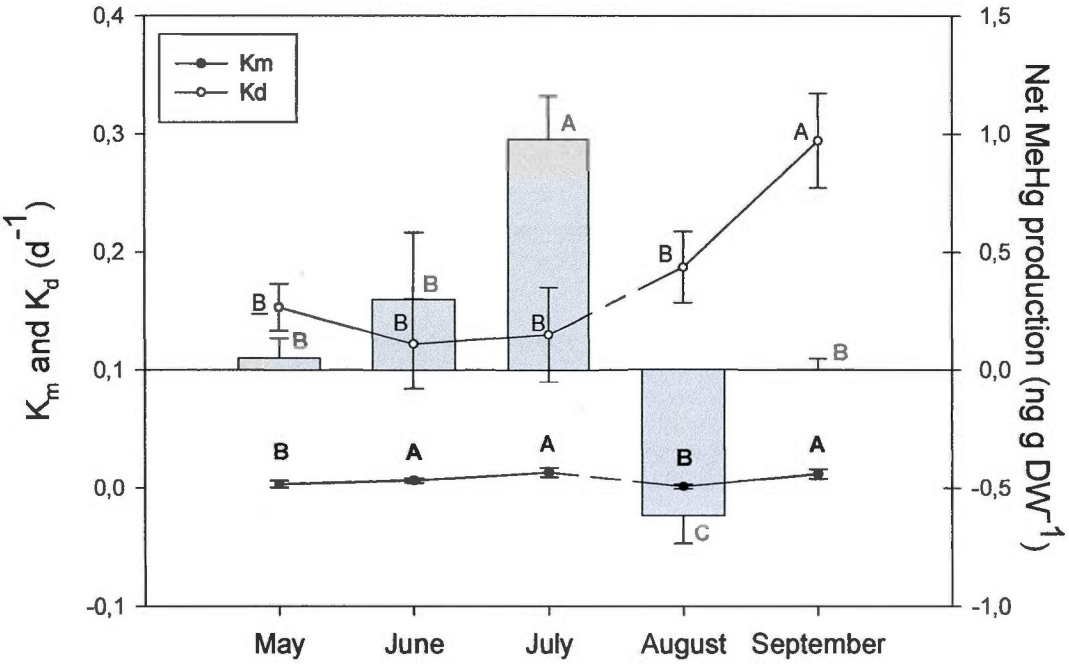
Km
Kd
0,4
0,3
0,2
0,1
0,0
-0,1
1,5
1,0
0,5
0,0
-0,5
-1,0
K_m and K_d (d^{-1})
Net MeHg production (ng g DW^{-1})
May
June
July
August
September
A
B
C

Figure 2.2

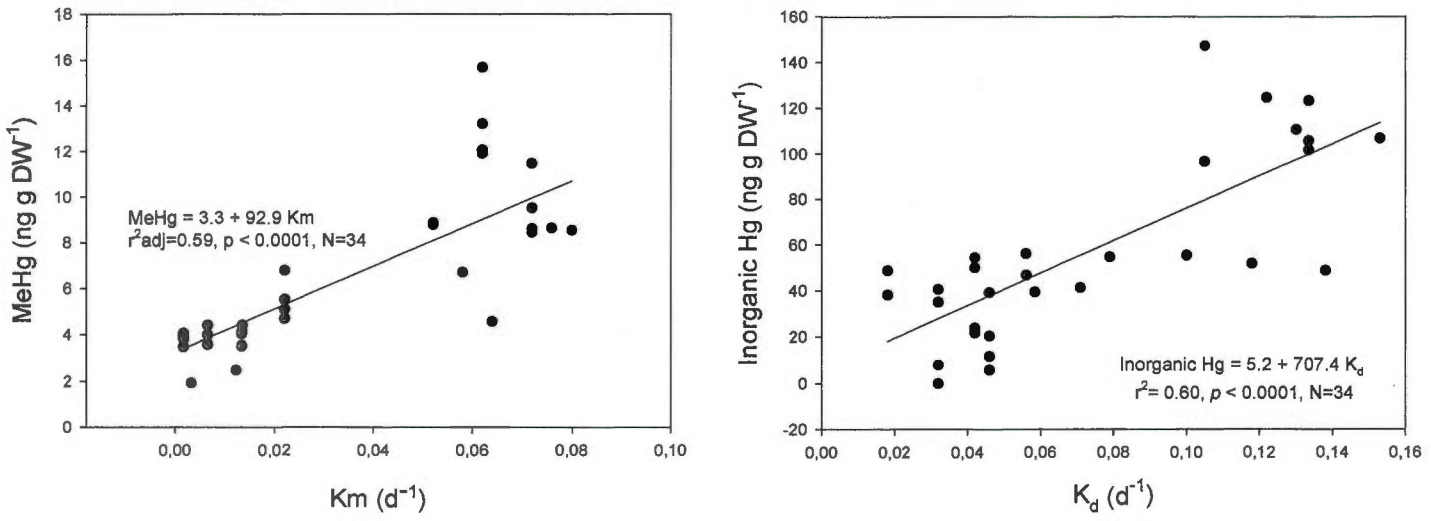
MeHg (ng g DW^{-1})
Km (d^{-1})
MeHg = 3.3 + 92.9 Km
r^2adj=0.59, p < 0.0001, N=34
Inorganic Hg (ng g DW^{-1})
K_d (d^{-1})
Inorganic Hg = 5.2 + 707.4 K_d
r^2= 0.60, p < 0.0001, N=34

Figure 2.3

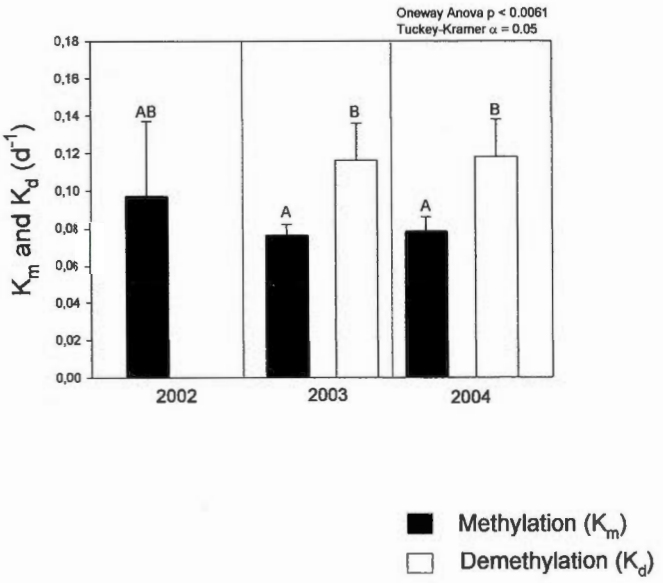
Oneway Anova p < 0.0061
Tuckey-Kramer α = 0.05
K_m and K_d (d^{-1})
0,18
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
AB
A
B
A
B
2002
2003
2004
Methylation (K_m)
Demethylation (K_d)

Figure 2.4

2.9 References

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CHAPITRE III

METHANOGENS: PRINCIPAL METHYLATORS OF MERCURY IN LAKE PERIPHYTON

Stéphanie Hamelin, Marc Amyot, Tamar Barkay, Yanping Wang et Dolors Planas

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3.1 Abstract

Mercury methylation and demethylation rates were measured in periphyton biofilms growing on submerged plants from a shallow fluvial lake located along the St. Lawrence River (Quebec, Canada). Incubations were performed *in situ* within macrophytes beds using low-level spikes of ^{199}HgO and $Me^{200}Hg$ stable isotopes as tracers. In order to determine which microbial guilds are playing a role in these processes, methylation/demethylation experiments were performed in the absence and presence of different metabolic inhibitors: chloramphenicol (general bacteriostatic inhibitor), molybdate (sodium molybdate, a sulfate reduction inhibitor), BESA (2-bromoethane sulfonic acid, a methanogenesis inhibitor) and DCMU (3-(3,4-dichlorophenyl)-1,1 dimethyl urea, a photosynthesis inhibitor). Active microbes of the periphytic consortium were also characterized using 16S rRNA gene sequencing. Methylation rates in the absence of inhibitors varied from 0.0015 to 0.0180 d^{-1} while demethylation rates ranged from 0.083 to 0.217 d^{-1}, which corresponds to a net methylmercury balance of -0.51 to 1.28 ng gDW periphyton^{-1} d^{-1}. Methylation rates were significantly decreased by half by DCMU and chloramphenicol, totally inhibited by BESA and were highly stimulated by molybdate. This suggests that methanogens rather than sulfate reducing bacteria were likely the primary methylators in the periphyton of a temperate fluvial lake, a conclusion supported by the detection of 16S rRNA gene sequences that were closely related to those of methanogens. This first clear demonstration of methanogens' role in mercury methylation in environmental periphyton samples expands the known diversity of microbial guilds that contribute to the formation of the neurotoxic substance methylmercury.

Keywords : epiphytes, mercury, methylation, demethylation, inhibitors, rRNA

3.2 Introduction

Mercury (Hg) methylation is a key process in the understanding of the Hg biogeochemical cycle. Once produced in an aquatic system, methylmercury (MeHg), the neurotoxic form of Hg, is bioaccumulated in organisms and bioamplified through food webs. In freshwater systems, it is generally accepted that Hg methylation takes place in deep and littoral sediments (Furutani and Rudd, 1980; Matilainen, *et al.*, 1991; Krabbenhoft, *et al.*, 1998), and in anoxic hypolimnions of lakes (Eckley, *et al.*, 2005). However, some studies have also demonstrated the ability of periphyton to methylate Hg (Cleckner, *et al.*, 1999; Mauro, *et al.*, 2002; Achá, *et al.*, 2005; Desrosiers, *et al.*, 2006a; Huguet, *et al.*, 2010). For instance, in the Everglades and Brazil, periphyton methylated Hg at higher rates than sediments (Cleckner, *et al.*, 1999; Guimarães, *et al.*, 2000), which can be explained by the higher microbial biomass per gDW in periphyton than in sediments. MeHg concentrations generally account for 3 to 50 % of total Hg (THg) measured in wetland periphyton (chap. II). High Hg methylation (MHg) rates and MeHg concentrations in periphyton could lead to high MeHg concentration in fish, since periphyton is a more direct food source than sediment microbes for benthic primary consumers (Hecky and Hesslein, 1995; McIntyre, *et al.*, 2006; Cremona, *et al.*, 2009). Considering the rapid turnover of the periphytic biofilm organisms (Lamberti, 1996; Robinson, *et al.*, 1997), the amount of Hg that can be transferred to the top of the food chain could be very high.

Differences between biofilm microbial composition and MeHg concentrations in the ingested food could partly explain the marked differences observed between mercury levels in grazers from different aquatic systems (Dominique, *et al.*, 2007). In aquatic environments, mercury methylation is generally ascribed to the activity of anaerobic bacteria, mainly to sulfate-reducing bacteria (SRB). This conclusion principally rests on the common observation that inorganic Hg methylation rates declined when sediments were treated with molybdate, a specific inhibitor of sulfate reduction (Compeau and Bartha, 1985; Kerry, *et al.*, 1991; Gilmour, *et al.*, 1992). Even in periphyton, SRB have been shown to play an important role in mercury

methylation (Cleckner, *et al.*, 1999; Achá, *et al.*, 2005; Desrosiers, *et al.*, 2006a). However, recent studies reported that other microorganisms, namely iron reducing bacteria, may methylate inorganic Hg (Fleming, *et al.*, 2006; Kerin, *et al.*, 2006).

In this study, our objectives were to: (1) measure Hg methylation and demethylation rates by periphyton in the wetland area of a large shallow fluvial lake, using stable isotope tracers; (2) identify the main methylators by the addition of metabolic inhibitors; (3) characterize the active microbes in the periphytic biofilms using 16S rRNA gene sequencing. In most studies where MHg or demethylation (DHg) rates were related to the composition of the microbial community, organisms previously known as Hg methylators, like SRB, were the only ones targeted by molecular probes (Achá, *et al.*, 2005). Here, the whole community RNA, rather than DNA, was used and thus, mostly microbes that were metabolically active were detected. Results of metabolic inhibitor experiments together with the molecular characterization of a large sprectrum of periphyton microorganisms suggests for the first time a role for methanogens in periphyton mercury methylation.

3.3 Methods

3.3.1 Study site

The study took place in aquatic meadows of Lake St. Pierre, a widening of the St. Lawrence River (46° 09' 82" N; 72° 59' 10" W) between Sorel and Trois-Rivières, Québec, Canada. More than half of this fluvial lake (total area of 375 km^2) is covered by macrophyte beds and wetlands. The dominant macrophyte species observed in our sampling site were *Potamogeton perfoliatus* (Perfoliate pondweed), *Elodea Canadensis* (Canadian waterweed), *Scirpus fluviatilis* (River bulrush) and *Thypha angustifolia* (Narrowleaf cattail).

3.3.2 Sampling

All sampling devices and tools for Hg measurements were carefully acid-washed and rinsed with nanopure water. Sampling was carried out at the end of summer, when macrophytes densities and water temperatures were high (mid-August 2004). A total of fifteen field replicates (3 per inhibitor treatment and control) of submerged macrophytes and their associated periphyton were sampled using 0.68 L Pac-man boxes (a smaller cylindrical version of the 6L Downing box (Downing, 1986), modified by C. Vis, Parks Canada) at 30-45cm depth. Care was taken during sampling to minimize losses and disturbance of periphyton. Physicochemical characteristics of the water (pH, light, temperature and dissolved oxygen) were measured *in situ* with specific probes (Chekmite 4 pH meter, Biospherical QSL-101 quantum meter, WTW Oxi340 oximeter), and water grab samples for DOC, THg_{water}, $MeHg_{water}$ and major ions were also taken once within macrophytes beds at the same depth and the same time than periphyton/macrophytes sampling was conducted. Water chemistry data and details on laboratory analyses for these last samples are presented in the Supporting Information (Annexe B and C).

3.3.3 Methylation/demethylation assays

Filtered lake water (0.20 µm porosity) was spiked with ^{199}HgO and $Me^{200}Hg$ (Oak Ridge National Laboratory) at 3 $ng.L^{-1}$ each, and preincubated for 1 h to allow equilibration with dissolved ligands. Thereafter inhibitors were added before the addition of periphyton-macrophyte complexes (7-10g dry weight, (DW)). Entire macrophytes with their associated biofilm were incubated *in situ* in clear polycarbonate bottles (total incubation volume of 2L) within macrophyte beds. Polycarbonate was chosen because this material has optimal light transmittance with minimal Hg and MeHg sorption to container walls (Cleckner, *et al.*, 1999; Gorski, *et al.*, 2006). All the treatments contained periphyton-macrophytes complexes and ^{199}HgO and $Me^{200}Hg$, and included: 1) Control (without any inhibitor); 2) BESA (with addition of 5 mM 2-bromoethane sulfonic acid, a methanogenesis inhibitor); 3)

DCMU (with addition of 10 µM 3-(3,4-dichlorophenyl)-1,1 dimethyl urea, a photosynthesis inhibitor); 4) molybdate (with addition of 20 mM sodium molybdate, a sulfate reduction inhibitor), and 5) the general bacteriostatic inhibitor chloramphenicol (0.2 mM) (Balch and Wolfe, 1979; Oremland and Capone, 1988; Desrosiers, *et al.*, 2006a). Total incubation time was 48h, but every 12 hours (at dawn and twilight), three replicate bottles were sacrificed by adding 8 ml of 4 N HCl and samples were stored in the dark at 21-23°C (depending on *in situ* water temperature). Once in the laboratory, periphyton was separated from macrophytes by mechanical shaking (9 min in a Red Devil® paint shaker), a method we had previously tested for removing periphyton efficiently without destroying algal cells. The periphyton suspension was then split in 3×100 ml aliquots for each measurement (biomass, THg, MeHg, MeHg stable isotopes, community characterization). MeHg stable isotopes samples were analysed at Trent University (Dr. Hintelmann's Laboratory), Ontario, Canada. The formation and degradation of MeHg were determined by monitoring the concentrations of the respective isotopes $Me^{199}Hg$ and $Me^{200}Hg$ by gas chromatography-inductively coupled plasma mass spectrometry, following protocols in Hintelmann and Ogrinc (2003). To calculate MHg and DHg rate constants, we used the following equations (Hintelmann, *et al.*, 2000):

$$\text{Net MeHg production} = K_m [Hg^{2+}] - K_d [MeHg^{+}] \quad (1)$$

Where K_m=specific MHg rate constant (in d^{-1}) and K_d=specific DHg rate constant (in d^{-1}). K_m and K_d were calculated from the initial part of the slope of the relation (first 12h) between isotopic $[MeHg^{+}]$ and time when $[Me^{199}Hg^{+}]$ and $[^{200}Hg^{2+}]$ were low enough that K_m and K_d can be calculated from:

$$K_m = [Me^{199}Hg^{+}]/(t\,[^{199}Hg^{2+}]_0) \quad (2)$$

$$[Me^{200}Hg+] = [Me^{200}Hg^{+}]_0\, e^{-Kd\ t} \quad (3)$$

where $[Me^{200}Hg^{+}]_0$ and $[^{199}Hg^{2+}]_0$ are the initial substrate concentrations added to the sample in ng g^{-1}. K_d was obtained by linear regression of $\ln[Me^{200}Hg^{+}]$ versus time (t) in days. As the bioavailability of stable isotopes added is probably higher than Hg found in the natural waters, these rates are considered maximum potential rates.

3.3.4 Biomass determinations

Four 100 ml aliquots of periphyton suspension were filtered on pre-combusted and pre-weighed GF/C and kept at –80°C until analysis. For chlorophyll-α, the extractions were done with hot ethanol (Nusch, 1980). Dry weight, was measured by drying filters (45°C) to constant weight (APHA, 1989). Identification of algae phototrophes to species level in Lugol's solution was done using an inverted microscope (Leica DMIRB), following Utermöhl's method (Lund, *et al.*, 1958).

3.3.5 THg and MeHg analyses

Prior to THg and MeHg analysis, periphyton samples were freeze-dried and weighed. THg concentrations were measured by thermal decomposition using a direct mercury analyzer (DMA 80; Milestone, MLS). From 0.05 to 0.10 g of samples were dried, combusted and further decomposed on a catalytic column at 750°C. Mercury vapors were collected on a gold amalgamation trap and subsequently desorbed by heat and then measured by atomic absorption spectrometry at 253.7 nm (Boylan and Kingston, 1988; Cizdziel, *et al.*, 2000). Samples for MeHg analysis were freeze-dried, weighed, extracted in KOH/methanol (25%) and extracts were analyzed by Cold Vapour Atomic Fluorescence (CVFAS) (Bloom and Fitzgerald, 1988; Bloom, 1989). The working detection limit was 0.01 ng g^{-1} for Hg and 0.1 ng g^{-1} for MeHg (three times the standard deviation of 10 procedural blanks). Blanks and certified reference material (Tort-2, SO-2 and IAEA, CNRC) were analyzed every 10 samples to ensure the reproducibility of the analysis and to assess quality assurance/quality control. Hg concentrations in reference material varied slightly over time (CVFAS: TORT-2 CV=1.7%; DMA: TORT-2 CV=2.2%, SO-2 CV=1.7% and

IAEA CV=2.2%) but were not significantly ($p > 0.05$) different from certified values. No substantial Hg contamination was detected in the blanks.

3.3.6 Algal and microbial production

In parallel to MHg/DHg measurements, determination of algal and microbial production was performed by incubating periphyton/macrophytes complexes during 48h (with sub-sampling every 12h) in order to assess the efficiency of inhibitors in controlling periphyton metabolic activity. Algal primary production was measured by oxygen (O_2) production directly in the bottles before and after MHg/DHg incubations (YSI model 59, precision of ± 0.01 mg $O_2.L^{-1}$). Net primary production was calculated by subtracting initial O_2 concentration measured at time zero from the O_2 concentration measured after incubation. Community respiration was measured by the O_2 consumption in dark bottles kept under the same conditions as clear ones. For gross primary production calculation, community respiration was added to the net O_2 production in the clear bottles (Vis, *et al.*, 2006).

Production by the entire microbial community was estimated from ^{3}H-thymidine incorporation (Wicks and Robarts, 1987; Stanley, *et al.*, 2003). Assumption was made that most of the bacteria and archaea were able to incorporate thymidine. One formaldehyde-sterilized control and three replicate subsamples from MHg/DHg bottles were spiked with 5 nM thymidine (specific activity = 85 µCi $nmol^{-1}$) and incubated for 30 min in glass vials. Incubations were conducted in a dark insulated box containing water from the sampled station. At the end of the incubation, thymidine incorporation was stopped using formaldehyde (2% v/v final concentration). Cells were collected on 0.2 µm Gelman polycarbonate filters, incubated for 10 min, and rinsed twice with 5 mL of cold 5% trichloroacetic acid. Filters were stored at 4°C and analyzed later by a liquid scintillation counter (Beckman LS1801, Beckman Instruments).

3.3.7 Microbial community characterization

Biofilm RNA was chosen rather than DNA in order to identify which microbes from the biofilm were actively metabolizing and thus could have played a role in MHg/DHg processes. The validity of this approach rests on the fact that active microbes contain more ribosomal RNA than inactive ones (Kemp, *et al.*, 1993). The methods for RNA extraction, DNAse treatment and end-point reverse transcription (RT)-PCR were used as described previously (Wang, *et al.*, 2009). Briefly, total RNA from the periphyton samples was extracted using the DNAeasy Kit (Qiagen). The extracted crude RNA samples were then diluted to 50 $\mu g.mL^{-1}$ for DNAse treatment with the TURBO DNA-free kit (Applied Biosystems) according to the manufacturer's instructions. The DNAse-treated RNA samples were then diluted to 10 $\mu g.mL^{-1}$ and 1 µl (10 ng) of each RNA sample was used as a template in each RT-PCR reaction, by following the protocol for the Access RT-PCR System kit (Promega life science). For bacterial sequences, PCR amplifications of the 16S rRNA gene were carried out with primers 27f and 519r (Lane, *et al.*, 1985), whereas for archaean sequences, amplifications were carried out with primers 344f and 907r (Lane, 1991; Raskin, *et al.*, 1994). Control reactions did not include RT. Reactions were incubated at 45 °C for 45 min for reverse transcription to produce the first-strand cDNA, followed by 94 °C for 2 min to denature the RT, and followed by 40 amplification cycles of 94 °C for 30 s, 53 °C for 20 s (for Bacteria) or 48 °C for 30 s (for Archaea), 68 °C for 1 min, and final extension at 68 °C for 10 min.

Six clone libraries for the three most interesting treatments: control, molybdate and BESA (33 clones in each library), were constructed with the PCR products of 16S rRNA genes of Bacteria and Archaea, individually. The 16S rRNA gene PCR product of each sample was extracted from 1% agarose gel by using the QIAquick Gel Extraction Kit (Qiagen Sciences, Maryland), and was then cloned into pGEMT-easy vector (pGEMT-easy Vector System, Promega, Madison, WI) according to the manufacturer's instructions. The ligation mixture was transformed into *Escherichia coli* DH10B competent cells (Invitrogen, USA), and transformants were selected on LB (Luria broth) agar plates supplemented with ampicillin (100 $\mu g.ml^{-1}$) and X-gal (40

$\mu g.ml^{-1}$). White colonies were picked and inoculated in LB containing ampicillin and plasmids were extracted from these cultures to screen for insert-containing clones. For Restriction Fragment Length Polymorphism (RFLP) analysis, the clones were digested with *Eco*RI and *Hae*III and run on 1% agarose gel. At least three clones of each RFLP pattern were chosen, in total 31 clones were sent to Genewiz (South Plainfield, NJ) for sequencing.

The resulting sequence from each clone was used as query in searches performed using BlastN (National Center for Biotechnology Information, Bethesda, MD) and myRDP (Ribosomal Database Project II, East Lansing, MI) to identify the most closely related sequences to the periphyton 16S rRNA genes. When more than one sequence was found as top hits for a single sequence, the highest common taxonomic rank shared by all hits was chosen as the most closely related to the environmental clone. In the case that a common rank did not exist among the hits, we eliminated those that based on their known characteristics (e.g., extreme halophiles, thermophiles or acidophiles) were not likely to be found in the studied periphyton. The sequences have been deposited in GenBank under accession numbers HQ848523-HQ848553.

3.4 Results

3.4.1 Periphyton biomass, total and methylmercury concentrations

The algal community was mainly dominated by diatoms (*Coconeis placentula, Navicula radiosa, Fragilaria capucina),* with few chlorophytes (*Stigeoclonium nanum, Protoderma viride, Spyrogyra sp.*) and cyanophytes (*Oscillatoria tenuis, Coelosphaerium kuetzingianum).*

Periphyton biomass varied from 1.5 to 4.8 mg DW gDW of macrophytes^{-1} and algal biomass represented 678 to 773 µg Chl-α gDW of macrophytes^{-1}. Mean THg and MeHg concentrations in periphyton were 88 ± 30 and 3.9 ± 0.3 ng gDW^{-1} respectively.

3.4.2 Mercury methylation and demethylation rates

In the absence of inhibitors, methylation rates (K_m) varied from 0.0015 to 0.0180 d^{-1} while demethylation rates (K_d) ranged from 0.083 to 0.217 d^{-1}. A mass balance between MHg and DHg was calculated considering *in situ* [THg] and [MeHg]; we obtained a net variation of MeHg from -0.51 to 1.28 ng gDW periphyton^{-1} d^{-1}. These theoritical variations of MeHg are consistent with the seasonnal variations in [MeHg] measured *in situ* (chap. I and II).

3.4.3 Effect of inhibitors on primary production and microbial production

Net primary production without inhibition was 0.14±0.01 mg O_2 gDW^{-1} h^{-1}. DCMU totally suppressed periphytic photosynthesis while the other inhibitors did not affect algal metabolism (figure 3.1A). Microbial thymidine incorporation in control was 6.15±0.80 × 10^{-15} mole thymidine gDW^{-1} h^{-1}. BESA and chloramphenicol significantly decreased microbial heterotrophic production by 50% while molybdate stimulated it by 20% (figure 3.1B).

3.4.5 Effect of inhibitors on mercury methylation and demethylation rates

DCMU and chloramphenicol significantly decreased K_m by half in periphyton and the addition of BESA severely inhibited it by nearly 100% (figure 3.1C). In contrast, molybdate addition enhanced methylation rate up to 45 fold relative to unsupplemented controls (figure 3.1C). Molybdate, the only addition to affect demethylation rates, completely suppressed it (figure 3.1D).

3.4.6 Characterization of the active microbial community

Groups of active Bacteria and Archaea were identified at the end of the MHg/DHg experiments, for the treatments with BESA, molybdate, and for the control. From the 16S rRNA clone libraries, we obtained 9 to 12 sequences per treatment. Because of the low number of clones that were sequenced, these sequences represent highly dominant active taxa in the periphyton community (figure 3.2). Sequences representing taxa known to include Hg methylators such as those of the *Deltaproteobacteria*, were not detected in any of the libraries. On the other hand, sequences most similar to those of methanogens were present in the three archaean libraries. The control included 2 sequences most similar to those of methanogens (Methanococcales and Methanobacteriales) while BESA and molybdate treated peripyton included three such sequences (Methanobacteriales, Methanococcales and Methanosarcinales).

3.5. Discussion

The most important result reported here is the clear evidence for methylation by methanogens in environmental samples. This conclusion is based on the observation that methylation was totally inhibited by BESA (inhibitor of methanogenesis) and highly stimulated by molybdate (inhibitor of sulfate-reduction) and is strongly supported by the detection of transcripts of 16S rRNA genes most closely related to those of methanogenes in the active periphyton community. The evidence that methanogens methylate Hg in periphyton closes a circle in methylation research which started in the 1960's by showing that Hg was methylated by extracts of methanogens (Wood, *et al.*, 1968). Subsequently, many studies with pure cultures and environmental incubations contributed to the broadly accepted paradigm that SRB are the principle Hg methylators (Compeau and Bartha, 1985; King, *et al.*, 2001). Our report brings us back to the conclusion that methanogens in some

environments methylate Hg. Together with observations that Fe reducing bacteria methylate Hg (Fleming, *et al.*, 2006; Kerin, *et al.*, 2006), the findings reported here call for a change in our view of methylation from a process attributed to a single microbial guild, SRB, to a process that may be carried out by several guilds of anaerobic microbes. This change would necessitate studies on the microbiological and environmental factors that determine which group methylates under which conditions.

3.5.1 Mercury methylation and demethylation rates in periphyton

K_m rates in Lake St. Pierre periphyton were slightly lower than those measured by Desrosiers *et al.* (0.096-1.224 ngMeHg.d^{-1}, (Desrosiers, *et al.*, 2006a)) for epilithon of boreal shield lake, but they were similar to the ones obtained with epiphytes from Florida wetlands (0.2-20%, (Cleckner, *et al.*, 1999; Mauro, *et al.*, 2002)) and higher than values observed with epiphytes from a Wisconsin oligotrophic lake (0.011-0.062%, (Korthals and Winfrey, 1987)). Microbial community composition and biofilm's structure must be important factors controlling MHg. In Desrosiers *et al.* (Desrosiers, *et al.*, 2006a), periphyton growing on artificial teflon substrates were used, whereas in the Everglades (Cleckner, *et al.*, 1999; Mauro, *et al.*, 2002) macrophytes were chopped in pieces. In this study, epiphytes were collected on natural substrata (macrophytes) and incubations were done without altering the biofilm structure (e.g. without cutting macrophytes into pieces or seperating biofilms from their subsrate). In Wisconsin, the periphyton was scraped from the substrate prior to incubation (Korthals and Winfrey, 1987), which may have destroyed the integrity of the mat structure. The multiple layers within periphyton matrix must influence K_m and K_d rates as more layers in the mat may enable a redox gradient, thus creating more niches for different microbial groups to co-exist and participate in MHg processes.

The K_ds measured here were similar to the ones reported by Mauro *et al.* (nd-20%, (Mauro, *et al.*, 2002)) with dense macrophyte-associated periphyton from

highly eutrophied zones of the Everglades. However, our K_ds were up to 35 times higher than the ones measured by Korthals and Winfrey (0.62-1.28%, (Korthals and Winfrey, 1987)) with periphyton from an oligotrophic lake in Wisconsin. Productivity of the system must be a key factor controlling periphyton net DHg rates and MeHg accumulation (Wood, *et al.*, 1968).

3.5.2 Effect of metabolic inhibitors on mercury methylation and demethylation

Recent experiments using DCMU and chloramphenicol with periphyton have reported a partial decrease of MHg as compared to control samples (Cleckner, *et al.*, 1999; Desrosiers, *et al.*, 2006a). Here, these inhibitors decreased MHg rates by half, suggesting either direct or indirect contribution of prokaryotes and phototrophs to MHg processes. For instance, algae could directly methylate Hg (Pongratz and Heumann, 1998), or they could indirectly promote Hg methylation by releasing metabolites involved in redox reactions in the biofilm matrix thereby increasing bioavailable Hg concentrations for MHg (Price, *et al.*, 1990). They could also excrete more algal organic carbon that would fuel microbial metabolism and thus stimulate Hg methylation. As the inhibition of photosynthesis by DCMU did not affect microbial production, the hypotheses of either the direct effect and/or of controlling Hg bioavailability seem the more plausible.

Partial inhibition by chloramphenicol was also observed in other studies with similar inhibition experiments (Pak and Bartha, 1998b; Cleckner, *et al.*, 1999). Chloramphenicol is a broad-spectrum prokaryotic inhibitor of bacterial protein synthesis and is known to inhibit methanogens (Kates, *et al.*, 1993). However some bacteria, even some strains of SRB methylators, are resistant to this chemical (Henry, 1992).

By treating incubations with BESA (which inhibits co-enzyme M activity), we completely suppressed MHg and by using molybdate, we enhanced MHg 45 fold. As SRB are strong competitors for the same sources of energy, their inhibition by

molybdate would stimulate other microbial guilds, which suggest that methanogens played an important role in mercury methylation of the studied periphyton. Former studies that investigated methanogens and SRB involvement in MHg (Pak and Bartha, 1998a) found a slight inhibition after BESA addition. Compeau and Bartha found also a strong inhibition with molybdate, and they concluded that methanogens played an indirect role and/or that SRB were more efficient in the methylation processes (Compeau and Bartha, 1985).

In this study, we observed a large increase in mercury methylation following the addition of molybdate. This strongly suggests that SRB are not likely to be significant methylators in our periphyton biofilm even though the large increase in MHg may be partially due to the complete inhibition of DHg by this treatment. Indeed, as seen with the characterization of the active microbial community, none of the identified 16s rRNA gene sequences in the molybdate treatment was related to those of SRB. Most of the studies measuring periphyton MHg rates with molybdate addition found a substantial decrease in MHg rates, from 60 to 95% (Cleckner, *et al.*, 1999; Desrosiers, *et al.*, 2006a). As discussed before, maybe differences in trophic status of the systems, biofilm tickness and niche diversity enabled different groups of microbes to methylate. A few studies have reported an absence of inhibition, or a low level of stimulation of MHg, 1.5 to 1.9 fold, following molybdate addition to lake sediments or bacterial cultures (Matilainen, 1995; Fleming, *et al.*, 2006). Here, the observed 45 fold increase in MHg is markedly higher than what has previously been reported.

Our results indicate that microbes other than SRB may be important as methylators. In fact, molybdate increased microbial production (figure 3.1B), suggesting that molybdate addition changed the dynamics of microbial interactions in the periphyton consortium to favour the activity of the Hg methylators (figure 3.1C). Periphytic biofilms are complex communities with species responding differently to the presence of metabolic inhibitors. Competition and also syntrophy between methanogens and SRB is well documented. SRB inhibition by molybdate leads to an

accumulation of short-chain fatty acids and hydrogen, as well as acetate, propionate and butyrate. Some of these substrates can be used by methanogenic archaea in the absence of competition from SRB (Sørensen, et al., 1981). As they compete for some of the same nutrients and electron donors (Oremland and Taylor, 1978), inhibition of one of the 2 groups may channel the flow of energy toward the other group.

The absence of DHg under molybdate treatment suggest three possible hypotheses 1) SRB are the major players in DHg under the conditions present in our biofilm, 2) methanogens and other microbes are so efficient to methylate Hg that, even if DHg occurs, it was not detectable because of the fast turnover to MeHg, 3) given that a strong cooperation exists between methanogens and sulfidogens (Pak and Bartha, 1998a) whereby SRB provide metabolic products that are consumed by methanogens, then when SRB are inhibited, methanogens are not able to demethylate. Using a similar approach as in our study, Oremland *et al.* (Oremland, *et al.*, 1991) and Marvin-Dipasquale and Oremland (Marvin-Dipasquale and Oremland, 1998) investigated which microbes and processes were involved in oxidative DHg, the degradation of MeHg to inorganic Hg and CO_2, by sediment incubations and bacterial cultures. Based on experiments with specific inhibitors, they concluded that in anoxic freshwater sediments, both methanogens and sulfidogens contributed to this process. Here, BESA did not have an effect on DHg, suggesting that methanogens were not directly involved in mercury demethylation by periphyton in our samples. Together, this study suggests a clear distinction between the microbial guilds that methylate Hg (methanogens) and degrade MeHg (SRB) in the periphyton in Lake St. Pierre.

Most of the 16S rRNA gene sequences that were retrieved from control, BESA and molybdate treatments were most similar to those common in water and sediments from estuaries and freshwaters (Yoshida, *et al.*, 2008; Min and Rickard, 2009; Berrada and Telford, 2010; Li, *et al.*, 2011). Some of them are usually found under anaerobic conditions, but they may be found in anaerobic compartments

within the aerobic biofilm matrix (Teske, *et al.*, 1996; Ito, *et al.*, 2002). Sequences most closely related to those of methanogens were common in the three treatments. Moreover, the proportion of methanogens compared to the entire microbial community, ranged from 16.6% in BESA to 21.2% in control and to 33.3% in molybdate treatment (figure 3.2). Methanogens could be the ones responsible for the high MHg observed in this last treatment. These results provide an excellent starting point for future research into more specifically identifying the species of methanogens responsible for mercury methylation. We cannot exclude the possibility that the other active microbes in our samples, as indicated by similarity of 16S rRNA genes to those of common aerobes (*Neisseiriales, Pseudomonadales, Oscillatoriales, Thiotricales, Enterobacteriales, Actinomycetales, Burkholderiales,* and *Bacilliales)*, contributed directly or indirectly to MHg or DHg.

This study reports significant rates of MHg in periphytic biofilms of the largest fluvial lake of the St. Lawrence River. As macrophyte beds are covering more than half of this lake, the contribution of periphyton to the overall MeHg budget of this large river should not be ignored. On a larger scale, recent reports (Warner, *et al.*, 2003; Fleming, *et al.*, 2006) and this study, may lead to a paradigm shift regarding microbial methylation. Whereas in the past, SRB were seen as the main methylators, it is now clear that in some systems other microorganisms, such as methanogens and iron-reducers, methylate. The biotic complexity of periphytic biofilm and its ever-changing redox conditions probably create a highly competitive environment where populations and their activities change rapidly. More research is needed on the genetic identification of microbes in natural consortiums, synergies and competitions among them, and the *in situ* conditions determining who are the main Hg methylators in a given environment and under certain conditions.

3.6 Acknowledgements

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3.7 Supporting information Available

More information regarding physico-chemical proprieties of the water (Annexe C), details about physico-chemical analysis (Annexe D), clones identification (Annexe E), course of mercury methylation/demethylation during 48h incubation (Annexe F) and methylation/demethylation rates in all treatments (Annexe G) are accessible. This material is available free of charge via the Internet at http://pubs.acs.org.

3.8 Figure captions

Figure 3.1: Effect of inhibitor addition (mean ± standard error) on epiphytic community as compared to control treatment during 48h incubation A) Photosynthesis production, B) Microbial production, C) Mercury methylation rate constants (K_m d^{-1}) D) Mercury demethylation rate constants (K_d d^{-1}). * shows treatments that are significantly different from control (Dunnett's test, a =0,05).

Figure 3.2: Proportion of each taxa representing active Bacteria and Archaea in periphyton samples from MHg/DHg incubations with and without metabolic inhibitors. Clone identification is based on DNA sequences and groups established by RFLP patterns. The libraries obtained with primer sets targeting archaeal (344f/ 907r) and bacterial (27f/519r) were combined for presentation of each treatment results. The decaled portions and the associated percentage correspond to the methanogens. Archaeal orders are denoted by bold italic font and bacterial orders by italic font only.

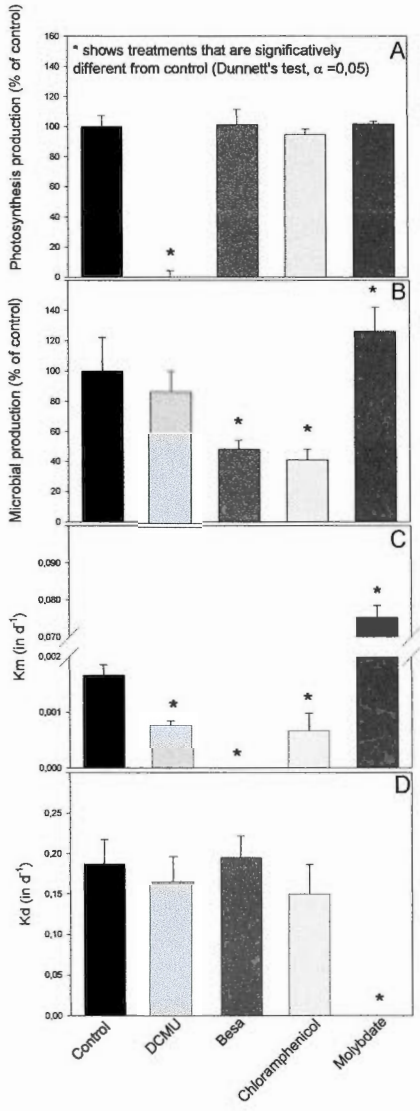
* shows treatments that are significatively different from control (Dunnett's test, α =0,05)
A
B
C
D
Photosynthesis production (% of control)
Microbial production (% of control)
Km (in d-1)
Kd (in d-1)
Control
DCMU
Besa
Chloramphenicol
Molybdate

Figure 3.1

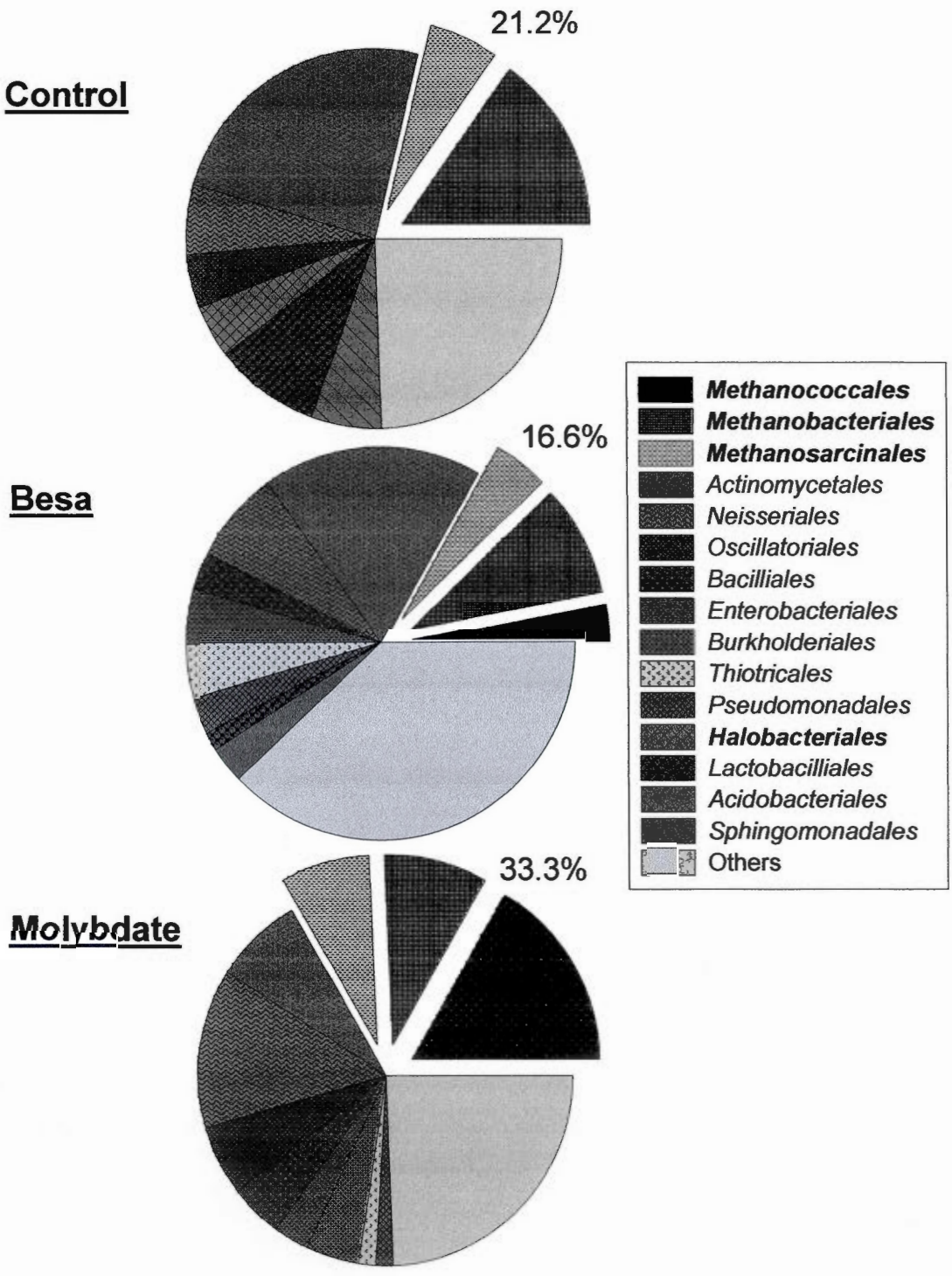
Control
21.2%
Besa
16.6%
Molybdate
33.3%
Methanococcales
Methanobacteriales
Methanosarcinales
Actinomycetales
Neisseriales
Oscillatoriales
Bacilliales
Enterobacteriales
Burkholderiales
Thiotricales
Pseudomonadales
Halobacteriales
Lactobacilliales
Acidobacteriales
Sphingomonadales
Others

Figure 3.2

3.9 References

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CONCLUSION GÉNÉRALE

Dans le cadre de cette thèse de doctorat, les épiphytes et les macrophytes des herbiers aquatiques du lac St-Pierre on été étudiés afin de mieux comprendre la dynamique du Hg (l'accumulation, la méthylation et la déméthylation) au sein des bancs de macrophytes submergés. Il n'y avait au départ que peu d'informations disponibles sur les variations saisonnières des concentrations de Hg et de MeHg des épiphytes et macrophytes, ni sur leurs taux de méthylation et déméthylation du Hg, en région tempérée froide. Considérant l'importance des milieux humides dans les écosystèmes aquatiques de l'hémisphère nord et le fait que les épiphytes et les macrophytes sont à la base de la chaîne alimentaire, il était crucial d'obtenir davantage de données sur leurs concentrations en Hg et en MeHg, leur contribution aux taux de méthylation et de déméthylation sous nos latitudes, ainsi que sur les facteurs environnementaux influençant ceux-ci, afin de pouvoir mieux prédire la contamination potentielle des poissons, puis des êtres humains.

Chapitre I

Les objectifs de ce chapitre étaient de mesurer les concentrations de Hg et MeHg dans les épiphytes et les macrophytes et d'évaluer l'effet de la saisonnalité (température et lumière), de la proportion d'organismes autotrophes au sein du biofilm, de l'espèce de plante hôte et des caractéristiques physico-chimiques du site sur les concentrations de Hg et MeHg dans les épiphytes et les macrophytes. Des concentrations importantes de Hg et MeHg ont été mesurées dans les épiphytes, allant de 2 à 284 ng gDW^{-1} pour le HgT et de 0.1 to 24.0 ng gDW^{-1} pour le MeHg. Les concentrations en HgT des épiphytes du lac St-Pierre sont comparables à celles mesurées en région tempérée froide dans le périphyton croissant sur substrat artificiel en Finlande et au Nord du Québec (Rask, *et al.*, 1994; Desrosiers, *et al.*, 2006c) ainsi qu'à celles mesurées sur des épiphytes en milieu tropical ou subtropical, que ce soit au Brésil, en Bolivie ou en Floride (Cleckner, *et al.*, 1998; Roulet, *et al.*, 2000; Achá, *et al.*, 2005; Coelho-Souza, *et al.*, 2011). En ce qui

concerne les concentrations des macrophytes, de 1 à 54 ng gDW^{-1} pour le HgT et 0.2-6.3 ng gDW^{-1} pour le MeHg ont été mesurés. Ces concentrations sont semblables à celles observées dans les macrophytes de Suède, de Colombie Britannique et du nord du Québec (Siegel, *et al.*, 1985; Grondin, 1994; Göthberg and Greger, 2006). Les épiphytes tendent à être environ dix fois plus contaminées que leurs hôtes, les macrophytes et la proportion de MeHg/HgT peut atteindre jusqu'à 74%, un pourcentage élevé pour un maillon à la base de la chaîne alimentaire. Il a été démontré que les concentrations en Hg dans les épiphytes diminuaient linéairement en fonction de l'indice d'autotrophie du biofilm épiphytique, ce qui suggère un rôle actif des algues dans l'accumulation du Hg. La station où les concentrations en HgT étaient les plus élevées était la station Girodeau sur la rive nord, alors que les concentrations en MeHg étaient plus hautes à la station Baie St-François sur la rive sud. Les variables ayant une plus grande influence sur les variations spatio-temporelles de concentrations en Hg et MeHg observées dans les épiphytes et macrophytes étaient la température de l'eau, le % de lumière disponible, l'espèce de macrophyte-hôte, le niveau d'eau, ainsi que le carbone organique dissous.

Chapitre II

Dans cette partie du projet de recherche, il a été question de mesurer *in situ* les taux de méthylation et de déméthylation du Hg des complexes épiphytes/macrophytes et d'évaluer l'influence de la lumière, de la température et de l'espèce de macrophyte-hôte sur les taux de méthylation, déméthylation et de production nette de MeHg des complexes épiphytes/macrophytes. Les épiphytes du lac St-Pierre se sont révélées être un site important de méthylation du mercure, les taux de méthylation variant de 0.002 à 0.137 d^{-1} alors que la déméthylation allait de 0.096 à 0.334 d^{-1}, la production nette de MeHg pouvant atteindre 200 ng m^{-2} d^{-1} ce qui correspond à 2 ordres de grandeur plus élevé que les taux de production nette de MeHg estimés à partir des sédiments du même site d'étude (Goulet, *et al.*, 2007). Les taux de méthylation des épiphytes du lac St-Pierre étaient semblables à ceux

mesurés avec le périphyton provenant d'un lac du Bouclier Canadien (Desrosiers, *et al.*, 2006a), et aussi à ceux obtenus avec les épiphytes des régions tropicales et subtropicales (Guimarães, *et al.*, 1998; Cleckner, *et al.*, 1999; Mauro, *et al.*, 2002). Par contre, aucune méthylation n'a été décelée dans les macrophytes. La production nette de MeHg varie au cours de la saison de croissance et semble être influencée positivement par la lumière disponible, la température, la productivité du milieu ainsi que par la structure des communautés des complexes épiphytes/macrophytes.

Chapitre III

Dans cette section du projet, l'emphase a été mise sur l'identification des principaux microorganismes méthylateurs au sein du biofilm épiphytique par l'ajout d'inhibiteurs métaboliques spécifiques et par la caractérisation des microorganismes actifs (séquençage de gènes 16S rRNA), le tout simultanément aux mesures de méthylation/déméthylation. Les taux de méthylation en présence de DCMU (inhibiteur de la photosynthèse) ont diminué de moitié par rapport aux contrôles (sans ajout d'inhibiteur); ils ont été nuls en présence de BESA (inhibiteur de la méthanogenèse) et fortement stimulés après l'ajout de molybdate (inhibiteur de la réduction des sulfates). Ceci démontre donc que différents microorganismes au sein des épiphytes interviennent dans les processus de méthylation. Ces résultats suggèrent que les méthanogènes seraient les principaux organismes méthylateurs des biofilms épiphytiques du lac St-Pierre, conclusion qui est supportée par la détection de séquences de gènes 16S rRNA propres aux méthanogènes. La mise en évidence de la méthylation du Hg par les méthanogènes au sein du périphyton supporte la recherche sur la méthylation du Hg qui a commencé dans les années soixante à l'aide d'extraits de méthanogènes (Wood, *et al.*, 1968). Par la suite, plusieurs études (par ex. (Compeau and Bartha, 1985; King, *et al.*, 2001), faites à partir de cultures pures de bactéries et d'échantillons environnementaux incubés avec un inhibiteur de la réduction des sulfates (le molybdate), ont ignoré l'importance potentielle de microorganismes autres que les bactéries sulfato-réductrices comme

méthylateurs du Hg. Les résultats obtenus dans cette thèse de doctorat indiquant que les méthanogènes du périphyton méthylent le Hg. Conjointement avec les observations faites par d'autres auteurs démontrant, dans certaines conditions, la méthylation du Hg par d'autres microorganismes que les bactéries sulfato-réductrices, notamment les bactéries réductrices du fer (Fleming, *et al.*, 2006; Kerin, *et al.*, 2006), ces résultats interpellent la communauté scientifique pour un changement de paradigme sur les processus de méthylation, qui n'étaient attribués qu'aux bactéries sulfato-réductrices, alors qu'ils semblent êtres effectués par plusieurs consortium de microorganismes utilisant des voies métaboliques différentes. Ce changement nécessitera des études supplémentaires sur l'identité des microorganismes méthylateurs de Hg ainsi que sur les variables environnementales qui déterminent quel groupe d'organismes méthyle le mercure et sous quelles conditions.

Les taux de méthylation et de déméthylation mesurés dans le chapitre II expliquent à eux seuls environ 60% de la variabilité des concentrations en MeHg et THg quantifiées dans le chapitre I. Pour l'avenir, il semble important de cibler : 1) quelles sont les autres variables qui influencent les concentrations de THg et de MeHg correspondant au 40% de la variabilité qui reste inexpliquée; 2) quelles sont les relations entre ces variables et celles qui influencent les K_m et K_d. Parmis les variables identifiées dans le cadre de ce projet, pour le MeHg nous avons : la diminution du % de lumière, la température, la diminution du niveau d'eau et le DOC,et ce sont les mêmes variables qui influencent les K_m. Les K_m et les concentrations de MeHg sont donc liés et amenés à fluctuer dans le même sens. En général, les milieux où le DOC, la pénétration de la lumière, et la température sont élevés accompagnés de faible niveaux d'eau et faible concentration d'oxygène dissous mèneront à de plus grandes concentrations en MeHg dans les épiphytes. Avec les activités anthropiques qui ont une tendance à faire évoluer les cours d'eau vers l'eutrophisation, il ne serait pas surprenant dans les années à venir de voir ces concentrations en MeHg augmenter.

Cependant, pour ce qui est du THg et du K_d, les variables identifiées sont différentes. Il y a donc une gestion différente à faire en ce qui concerne l'accumulation du Hg *vs* la déméthylation du MeHg ... qui mènent tous deux à de plus grandes concentrations de THg dans les épiphytes, mais par des voies métaboliques différentes. Il a été vu par exemple dans le chapitre I que l'indice d'autotrophie (AI) suggère un rôle actif des algues dans l'accumulation du THg et aussi que l'inhibition des algues par le DCMU dans le chapitre III mène à une inhibition de 50% des taux de méthylation, donc à la production de MeHg alors qu'elles ne semblent pas agir pas sur le Kd. Il sera donc crucial de creuser davantage pour comprendre quelles sont ces autres variables qui von avoir un rôle à jouer dans la déméthylation.

Comme perspectives futures, il serait intéressant de lier les concentrations de MeHg, de THg ainsi que les Kd et Km dans les épiphytes aux fluctuations en terme de proportions des différents sous-groupes d'intérêt dans la communauté (SRB, methanogènes, bactéries réductrices du fer, algues....) le tout en fonction de la saisonnalité (des différentes phases de colonisation du substrat). Ceci pourrait expliquer les changements saisonniers observés tant au niveau des changements de concentration (chapitre I) qu'au niveau des taux de méthylation et de déméthylation (chapitre II).

Il faudrait aussi mieux comprendre les voies métaboliques menant à l'accumulation du THg et à la méthylation. Beaucoup de recherches ont été faites sur les voies métaboliques menant à l'accumulation du Hg chez les bactéries, mais très peu sur les algues. Il faudrait aussi faire plus de recherches pour comprendre quelles sont les formes de Hg qui sont utilisées par les algues lors de l'accumulation et de la méthylation en testant différentes formes de Hg ayant une biodisponibilité différente (Hg-Matière organique, HgCl, HgO) pour mesurer l'accumulation et la méthylation...) et vérifier quelle est la vraie proportion du THg fourni qui est utilisable par ces oraganismes.

En résumé, les épiphytes et les macrophytes sont des sites importants d'accumulation du Hg et MeHg au lac St-Pierre. Les pourcentages élevés de MeHg/HgT ainsi que les taux de méthylation nette mesurés dans les épiphytes mettent en évidence l'importance d'inclure ces organismes dans les études de modélisation de la contamination en Hg des écosystèmes aquatiques. Ceci est particulièrement vrai pour des systèmes peu profonds où le littoral couvre en totalité ou en grand partie la superficie du plan d'eau.

De plus, les informations tirées des variations saisonnières des taux de méthylation et de déméthylation du Hg par les épiphytes ont montré que l'augmentation des taux de déméthylation contribuaient fortement à la baisse de la production nette de MeHg observée à la fin de l'été. Jusqu'à maintenant, la plupart des études sur la production de MeHg se sont limitées à mesurer uniquement la méthylation. À l'avenir, il faudrait donc davantage favoriser les études tenant compte aussi de la déméthylation par le périphyton, simultanément aux mesures de méthylation. Lors de la prise de ces mesures, il serait important de s'assurer que le biofilm périphytique conserve son intégrité afin de ne pas sous-estimer les taux de méthylation et de déméthylation. Aussi, puisque la déméthylation semble plus importante en eau froide qu'en eau chaude, il pourrait être intéressant d'effectuer des expériences de déméthylation en hiver dans les eaux courantes libres de glace, afin de voir si la production nette de MeHg continue de diminuer tout au long de l'hiver ou si elle atteint un état d'équilibre avec la méthylation jusqu'au retour du printemps.

Les résultats obtenus à partir de la caractérisation de la communauté d'épiphytes lors des mesures de méthylation et déméthylation sont un point de départ pour des recherches futures afin d'identifier plus précisément quelles sont les espèces de microorganismes responsables de la méthylation du Hg à l'aide des outils de microbiologie moléculaire. Depuis les débuts de la recherche sur la méthylation du Hg, la majorité des études portent soit sur des incubations *in vitro* pour lesquelles on modifie les conditions afin de vérifier l'effet de variables

environnementales tels le pH, le DOC, la température les sulfates, les sulfures, salinité; soit sur des comparaisons d'échantillons provenant de différents sites ayant des caractéristiques environnementales différentes, telles que celles précédemment mentionnées. Par contre, bien peu d'études se sont penchées sur les mécanismes de la méthylation via les multiples voies métaboliques utilisées par les microorganismes (Landner, 1971; Choi, *et al.*, 1994; Pak and Bartha, 1998b; Ekstrom, *et al.*, 2003). La méthode de microautoradiographie (MAR) couplée à la méthode de Fluorescence *in situ* Hybridisation (FISH), lors de mesures de méthylation et de déméthylation du Hg avec des épiphytes pourrait apporter de nouvelles informations sur les organismes méthylateur et les mécanismes employés. La méthode de microautoradiographie (MAR) est une technique qui permet la visualisation des cellules actives dans un milieu. Avec cette technique il est possible de prédire les fonctions écologiques de différentes cellules en énumérant la fraction de cellules actives, tout en faisant la distinction entre les différents substrats assimilés par les cellules. La méthode d'hybridation *in situ* en fluorescence (FISH), quant à elle, permet l'identification de microorganismes par l'hybridisation de segments d'ARN fluorescents (les sondes) avec l'ARN des organismes du milieu. Lorsque la méthode MAR est combinée à la méthode FISH, elle permet d'identifier quels membres de la communauté bactérienne sont présents, lesquels sont actifs et même quel substrat ils consomment, et ce, *in situ*. Certains substrats comme l'acétate et le H_2 sont partagés par compétition entre les SRB et les méthanogènes, alors que d'autres, comme les méthylamines, qui ne sont utilisés que par les méthanogènes (Oremland, *et al.*, 1982; Oremland and Polcin, 1982). L'utilisation de substrats enrichis en méthylamines permettrait de discriminer la méthylation faite par les bactéries sulfato-réductrices de celle faite par les méthanogènes (par exemple pour les épiphytes des milieux tropicaux et sub-tropicaux, où l'emphase a surtout été mise sur les bactéries surlafto-réductrices). Enfin, puisque qu'Ekstrom *et al.* (2003) ont proposé que la méthylation puisse se faire via la voie métabolique du propionate, ce substrat pourrait aussi être intéressant à utiliser afin de vérifier si cette voie métabolique est employée par les microorganismes des épiphytes. Davantage d'informations sont nécessaires afin de comprendre les interactions entre les

différents groupes de microorganismes vivant au sein du biofilm périphytique, leurs compétitions et leurs synergies, afin de pouvoir prédire quels seront les principaux organismes méthylateurs dans un milieu donné selon les conditions environnementales qui s'y trouvent.

ANNEXE A

PHYSICO-CHEMICAL PROPRIETIES OF THE WATER IN LAKE ST.PIERRE (2002-2004, DATA FROM 2002 ARE ADAPTED FROM CARON, *ET AL.* 2008).

	Mean	Min-Max
% light penetration	21.4	0.7 - 50.0
pH	7.8	6.4 - 10.8
DOC $mg.L^{-1}$	8.4	3.4 - 15.8
NO_3^- $mg.L^{-1}$	1.0	0.01 - 6.4
TP $mg.L^{-1}$	0.1	0.01 - 0.76
SO_4^{2-} $mg.L^{-1}$	15.2	4.7 - 33.8
THg $ng.L^{-1}$	1.7	0.4 - 4.1
MeHg $ng.L^{-1}$	0.012	0.05 - 0.30
T°C	20.6	10.8 - 26.2
Water level (m)	0.92	0.4 - 2.5
O_2 $mg.L^{-1}$	7.5	1.3 - 15.4

ANNEXE B

PHYSICO-CHEMICAL PROPRIETIES OF THE WATER MEASURED IN STATIONS GIRODEAU AND BSF WHEN METHYLATION AND DEMETHYLATION WERE COMPARED BETWEEN BOTH STATIONS (SEPTEMBER 2004, 30 CM DEPTH).

	Girodeau	BSF
% light penetration	5.3	1.25
pH	7.45	7.78
DOC $mg.L^{-1}$	5.4	15.0
NO_3^- $mg.L^{-1}$	0.9	6.4
TP $mg.L^{-1}$	0.05	0.05
SO_4^{2-} $mg.L^{-1}$	20.7	21.8
THg $ng.L^{-1}$	0.4	0.7
MeHg $ng.L^{-1}$	0.05	0.09
O_2 $mg.L^{-1}$	11.9	1.4

ANNEXE C

PHYSICO-CHEMICAL PROPRIETIES OF THE WATER AT GIRODEAU STATION DURING THE METHYLATION AND DEMETHYLATION MEASUREMENTS WITH AND WITHOUT INHIBITORS, AUGUST 2004.

Phytoplankton biomass $mg.L^{-1}$	3.98-13.22
% light penetration	5.26-50.00
pH	7.45-8.09
DOC $mg.L^{-1}$	4.89-5.82
NO_3^- $mg.L^{-1}$	0.15-0.94
TP $mg.L^{-1}$	0.04-0.06
SO_4^{2-} $mg.L^{-1}$	13.16-20.70
THg $ng.L^{-1}$	0.36-3.3
MeHg $ng.L^{-1}$	0.05-0.30
T°C	21.6-23.2
O_2 $mg.L^{-1}$	7.43-12.88

ANNEXE D

SUPPLEMENTARY INFORMATIONS FOR WATER SAMPLES COLLECTION AND ANALYSIS

Water samples for THg and MeHg were collected in duplicate and stored in Teflon bottles that had previously been acid-washed and thoroughly rinsed with ultrapure water. Preservation was assured by adding 0.4% ultraclean hydrochloric acid and samples were kept in the dark and refrigerated (4°C) until analysis. Analysis were carried out using an automated Tekran 2600 following U.S. Environmental Protection Agency method 1631.

Samples of all other analytes were collected in duplicate in Nalgene HDPE bottles. DOC, NO_3^- and SO_4^{2-} were determined in filtered samples using 0.45 μm polyethersulfone (PES) membranes. Colorimetric method following potassium persulfate oxidation at 120°C was used to measure TP (Astoria 2; MDL: 0.2 µg L-1). The DOC concentration was measured by high-temperature combustion on a platinum catalyst using a Shimadzu TOC-5000 Analyser (MDL: 0.10 mg L-1). NO_3^- was measured by an auto-sampler (Lachat, FIA) with respective MDLs of 5 and 1 µg L-1. SO_4^{2-} was analysed by ion chromatography using a DIONEX-DX500 (MDLs: 0.05 and 0.03 mg L-1 respectively).

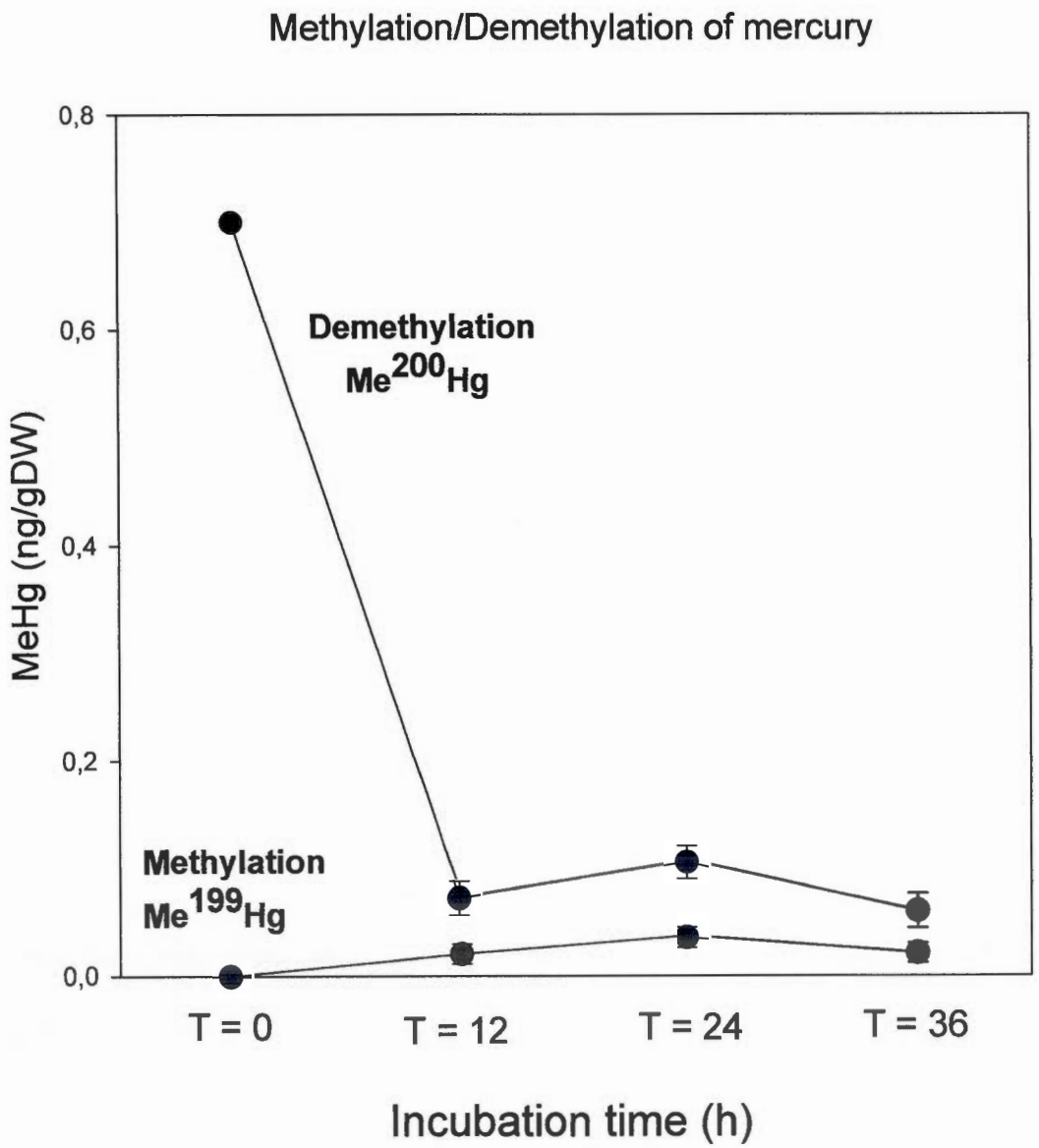
ANNEXE E

CLONES REPRESENTING ACTIVE BACTERIA AND ARCHAEA IN PERIPHYTON SAMPLES FROM MHG/DHG INCUBATIONS WITH AND WITHOUT METABOLIC INHIBITORS. BACTERIAL CLONES WERE OBTAINED FROM BACTERIAL LIBRARY WITH PRIMERS 27F/519R AND ARCHAEAL CLONES WERE OBTAINED FROM ARCHAEAL LIBRARY WITH PRIMERS 344F/ 907R.

Treatment	Clone	Most similar to the 16S rRNA gene of (% ID)	Lineage: kingdom, order, family
Control	LSP_periphyton_1	*Streptococcus sp.*, (98%)	Bacteria, Lactobacilliales, Streptococcaceae
	LSP_periphyton_2	*Neisseria sp.*, (99%)	Bacteria, Neisseriales, Neisseriaceae
	LSP_periphyton_3	*Halobacterium sp.*, (99%)	Archaea, Halobacteriales, Halobacteriaceae
	LSP_periphyton_4	*Streptococcus sp.*, (99%)	Bacteria, Lactobacilliales, Streptococcaceae
	LSP_periphyton_5	*Acidobacteria sp.*, (98%)	Bacteria, Acidobacteriales, Acidobacteriacea
	LSP_periphyton_6	*Actinomycetales* (99%)	Bacteria, Actinomycetales
	LSP_periphyton_7	*Oscillatoria sp.*, (99%)	Bacteria, Oscillatoriales, Oscillatoriaceae
	LSP_periphyton_8	*Oscillatoria sp.*, (99%)	Bacteria, Oscillatoriales, Oscillatoriaceae
	LSP_periphyton_9	*Actinomycetales* (99%)	Bacteria, Actinomycetales
BESA	LSP_periphyton_10	*Actinomycetales* (99%)	Bacteria, Actinomycetales
	LSP_periphyton_11	*Methanococcales* (98%)	Archaea, Methanococcales
	LSP_periphyton_12	*Francisella sp.*, (100%)	Bacteria, Thiotricales, Francisellaceae
	LSP_periphyton_13	*Actinomycetales* (98%)	Bacteria, Actinomycetales
	LSP_periphyton_14	*Alishewanella sp.*, (97%)	Bacteria, Enterobacteriales, Enterobacteriaceae
	LSP_periphyton_15	*Methanobacteriales* (98%)	Archaea, Methanobacteriales
	LSP_periphyton_16	*Methanosarcinales* (97%)	Archaea. Methanosarcinales
	LSP_periphyton_17	*Actinomycetales,* (98%)	Bacteria, Actinomycetales
	LSP_periphyton_18	*Neisseria sp.*, (99%)	Bacteria, Neisseriales, Neisseriaceae
	LSP_periphyton_19	*Sphingomonas sp.*, (99%)	Bacteria, Sphingomonadales, Sphingomonadaceae
Molybdate	LSP_periphyton_20	*Vogesella sp.* (98%)	Bacteria, Neisseriales, Neisseriaceae
	LSP_periphyton_21	*Ideonella sp.*, (98%)	Bacteria, Burkholderiales, Comamonadaceae
	LSP_periphyton_22	*Pseudomonas sp.*, (98%)	Bacteria, Pseudomonadales, Pseudomonadaceae
	LSP_periphyton_23	*Francisella sp.*, (100%)	Bacteria, Thiotricales, Francisellaceae
	LSP_periphyton_24	*Escherichia coli* ,(99%)	Bacteria, Enterobacteriales, Enterobacteriaceae
	LSP_periphyton_25	*Corynebacterium sp.*, (100%)	Bacteria, Actinomycetales, Corynebacteriaceae
	LSP_periphyton_26	*Exiguobacterium sp.*, (98%)	Bacteria, Bacilliales, Bacilliaceae
	LSP_periphyton_27	*Methanobacteriales* (98%)	Archaea, Methanobacteriales
	LSP_periphyton_28	*Methanococcales,* (99%)	Archaea, Methanococcales
	LSP_periphyton_29	*Neisseriaceae* (98%)	Bacteria, Neisseriales, Neisseriaceae
	LSP_periphyton_30	*Actinomycetales,* (100%)	Bacteria, Actinomycetales
	LSP_periphyton_31	*Neisseria sp.*, (98%)	Bacteria, Neisseriales, Neisseriaceae

ANNEXE F

COURSE OF ISOTOPIC MEHG (MEAN ± STANDARD ERROR) DURING 48H METHYLATION/DEMETHYLATION INCUBATION USING $ME^{200}HG$ AND $ME^{199}HG$.



ANNEXE G

MEAN MERCURY METHYLATION AND DEMETHYLATION RATES FOLLOWING 48H INCUBATION WITH AND WITHOUT ADDITION OF METABOLIC INHIBITORS.

Treatment	Mean Km ± Std error (in d^{-1})	Mean Kd ± Std error (in d^{-1})
Control	0.0017 ± 0.0002	0.1873 ± 0.0301
DCMU	0.0008 ± 0.0001	0.1650 ± 0.0310
BESA	Nd	0.1949 ± 0.0269
Chloramphenicol	0.0007 ± 0.0003	0.1500 ± 0.0363
Molybdate	0.0753 ± 0.0031	nd

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-L'expérience n'est pas le résultat de ce qui arrive à un homme, c'est ce que l'homme fait avec ce qui lui arrive-

Aldous Léonard Huxley

-In the middle of difficulty lies opportunity -

Albert Einstein