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LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

ADN	Acide désoxyribonucléique
ADP	Adénosine diphosphate
ARN	Acide ribonucléique
ATP	Adénosine triphosphate
CO_2	Dioxyde de carbone
Fd	Ferrédoxine
Glu	Glutamate
HCO_3^-	Bicarbonate
NADH	Nicotinamide adénine dinucléotide

CHAPITRE I

INTRODUCTION

1.1 Les microalgues

Les microalgues sont des organismes photosynthétiques eucaryotes qui se développent dans une large gamme de milieux aquatiques (Chinnasamy et al. 2010; Khan et al. 2018). Les principales exigences de croissance des microalgues sont un milieu aqueux, de l'énergie lumineuse, une température adéquate, une source de carbone organique ou inorganique et des nutriments. La lumière représente l'un des principaux facteurs limitant la culture des microalgues (Khan et al. 2018). Ces dernières ont besoin de lumière comme source d'énergie pour convertir l'eau et le dioxyde de carbone (CO_2) absorbés en biomasse, grâce au processus de la photosynthèse (Ozkurt 2009). Les produits issus de la photosynthèse, tels que des composants cellulaires ou des matériaux de stockage, s'accumulent et peuvent représenter 20 à 50 % de la biomasse totale (Chisti 2007). Lorsque les intensités lumineuses sont trop faibles (photolimitation) ou trop élevées (photoinhibition), la croissance des microalgues est affectée (Mata et al. 2010; Sforza et al. 2012; Yeh et Chang 2012). Chaque espèce de microalgue a une intensité lumineuse optimale lui permettant d'atteindre une croissance maximale (Khan et al. 2018). Certaines espèces de chlorelles, par exemple, peuvent se développer sous un large intervalle d'intensités lumineuses, se situant entre 25 à 600 $\mu mol/m^2/s$ (Chiu et al. 2011; Fu et al. 2012; Han et al. 2012; Hulatt et Thomas 2011; Lv et al. 2010; Takeshita et al. 2014; Safonova et al. 2004; Sydney et al. 2010). La durée des périodes claires et sombres, la pénétration de la lumière et sa distribution uniforme dans le milieu sont également des éléments qui doivent être pris en considération afin d'optimiser les besoins en lumière des microalgues (Khan et al. 2018).

Les sources de carbone ajoutées dans les cultures de microalgues peuvent se faire sous une forme organique (glucose, glycérol, acétate) ou inorganique (CO_2).

La forme choisie dépend du mode trophique utilisé par les microalgues. L'ajout de CO_2 ou de composés organiques, comme le glucose, a été appliqué pour améliorer la production de biomasse et augmenter la densité cellulaire des microalgues (Dubey et al. 2015; Lam et Lee 2013). La température est un autre facteur important, car elle influence également la photosynthèse (Khan et al. 2018). De fait, à basses températures, l'assimilation du carbone est réduite alors qu'à des températures trop élevées, les protéines photosynthétiques sont inactivées, ce qui perturbe l'équilibre de l'énergie dans la cellule. Chaque espèce a sa propre température de croissance optimale se situant, généralement, entre 20 à 30 °C (Singh et Singh 2015).

Plusieurs nutriments sont essentiels pour assurer la croissance des microalgues et la littérature scientifique abonde de travaux sur les impacts de la présence et de la carence en azote (N) et en phosphore (P), deux macronutriments requis en grande quantité pour assurer la synthèse des protéines, des acides nucléiques et des phospholipides (Kumar et al. 2010; Rao et al. 2011). La carence en azote dans les milieux de culture affecterait les taux de croissance des microalgues (Battah et al. 2013; Rodolfi et al. 2009; Widjaja et al. 2009; Yeh et Chang 2011), mais sa limitation favorisait la production de lipides (Battah et al. 2013; Converti et al. 2009; Illman et al. 2000; Rodolfi et al. 2009; Widjadja et al. 2009). Le même phénomène a également été rapporté pour le phosphore dans une étude de Fan et al. (2014). Les microalgues cultivées dans un milieu déficitaire en phosphore avaient une faible densité cellulaire par rapport à celles cultivées dans les milieux supplémentés en nutriments, mais une production en lipides totaux plus élevée. D'autres macronutriments tels que le magnésium (Mg), le calcium (Ca), le potassium (K) et le sodium (Na) sont également nécessaires pour la croissance des microalgues et une carence affecte généralement la production de cellules et de lipides (Khan et al. 2018). Enfin, l'apport en micronutriments, comme le fer (Fe) et le manganèse (Mn), par exemple, est requis en très faible quantité, mais est essentiel pour assurer de nombreuses activités métaboliques. De fait, il a été démontré dans une étude de Liu et al. (2008), que la présence de fer dans le milieu de culture permet à *Chlorella vulgaris* d'accumuler une forte concentration de lipides tout en augmentant sa croissance.

Finalement, pour avoir une productivité élevée en biomasse et en lipides lors de la culture de microalgues, il est essentiel de combler les exigences requises par ces dernières. De plus, d'autres facteurs tels que le mélange, l'aération et le pH du milieu doivent aussi être pris en considération afin d'augmenter le taux de réussite (Khan et al. 2018). Le mélange et l'aération des milieux de culture permettent une distribution uniforme des nutriments, de l'air, de la lumière et du CO_2 tout en évitant l'agrégation et la sédimentation de la biomasse (Khan et al. 2018; Show et al. 2015). Pour les exigences au niveau du pH du milieu, celles-ci diffèrent selon les espèces, mais la majorité se développe bien lorsque les valeurs se situent entre 6 à 8,8 (Khan et al. 2018). Il a été rapporté que certaines espèces sont plus tolérantes à la variation du pH dont *Chlorella vulgaris* qui a un taux de croissance et une productivité en biomasse maximaux à des valeurs se situant entre 9 et 10 (Daliry et al 2017; Lam et Lee 2012).

1.1.1 Mode trophique

Les microalgues peuvent être cultivées selon différents modes trophiques qui se caractérisent par les sources d'énergie et de carbone utilisées. Le choix du mode trophique varie en fonction des espèces et influence significativement la croissance et l'accumulation des lipides (D'Alessandro et Antoniosi Filho 2016; Yeh et Chang 2012). Les trois principaux modes trophiques sont l'autotrophie, l'hétérotrophie et la mixotrophie.

L'autotrophie est le mode de croissance le plus commun pour la culture des microalgues. La source d'énergie utilisée est la lumière et la source de carbone est inorganique, provenant généralement du CO_2 atmosphérique. La croissance des microalgues dans ces conditions est directement influencée par l'intensité lumineuse et la concentration en CO_2 (Carvhalo et al. 2006). En revanche, comme la disponibilité de la lumière et du CO_2 peut limiter la croissance, il est plus difficile d'atteindre de fortes productivités en biomasse et lipides. En autotrophie, les microalgues peuvent être cultivées dans un système ouvert en bassins extérieurs ou dans un système fermé en photo-bioréacteur. Lorsqu'elles sont cultivées en système ouvert, les risques de

contamination de la culture sont élevés et la réussite dépend des conditions climatiques (Zhan et al. 2017). Le photo-bioréacteur permet de contrer, en majeure partie, les risques de contamination provenant de l'extérieur, mais les coûts associés à ce système sont significativement plus élevés que ceux en bassin ouvert (Zhan et al. 2017).

En hétérotrophie, les composés organiques servent de source de carbone et aucune lumière n'est nécessaire pour la croissance des microalgues. Il a été rapporté, que dans ces conditions, les microalgues sont capables d'atteindre de fortes productivités en biomasse tout en ayant de hautes teneurs en huile (Liang et al. 2009; Xu et al. 2006). Même si la culture des microalgues en hétérotrophie est indépendante des conditions climatiques, le principal inconvénient de ce mode trophique réside dans les coûts associés à l'ajout de carbone organique dans le milieu de culture. Le glucose est généralement utilisé, car il se métabolise rapidement et fournit de l'énergie instantanée à la cellule (Dubey et al. 2015). D'autres sources de carbone bon marché comme le glycérol, un sous-produit de l'industrie du biodiésel, ou de l'acétate, présent dans les digestats de la biométhanisation, peuvent remplacer le glucose (Heredia-Arroyo et al. 2011; Liang et al. 2009). Toutefois, il a été rapporté que ces sources alternatives de carbone organique doivent être converties en glucose avant d'être utilisées par les microalgues, affectant les rendements en biomasse et en lipides (Dubey et al. 2015; Heredia-Arroyo et al. 2011; Liang et al. 2009). Par ailleurs, même si les microalgues soumises à des conditions hétérotrophes sont cultivées en milieu fermé, habituellement en bioréacteur, il peut arriver qu'elles soient contaminées par des bactéries indésirables en raison de l'ajout du carbone organique dans le milieu qui favorisent leur prolifération (Chen et al. 2011).

Finalement, le mode mixotrophe combine les deux conditions précédentes en utilisant à la fois la lumière comme source d'énergie et les composés organiques comme source de carbone (Sun et al. 2008). Dans ces conditions, les microalgues se développent en utilisant deux voies trophiques (Khan et al. 2018). En premier lieu, c'est le mode hétérotrophe qui sera priorisé dû à l'ajout initial de carbone organique dans le milieu (Zhan et al. 2017). Puis, une fois le carbone organique consommé, c'est le mode

autotrophe qui débute en induisant la photosynthèse et l'assimilation du CO_2 (Zhan et al. 2017). La mixotrophie permet de surmonter, au moins partiellement, certains des problèmes rencontrés dans les deux autres modes, tels que la dépendance à la lumière et les coûts associés à l'ajout de carbone organique dans les milieux de culture. Il a été démontré que la synergie de ces deux modes trophiques améliore la croissance des microalgues ainsi que la productivité en lipides (Bhatnagar et al. 2011, Heredia-Arroyo et al. 2011; Liang et al. 2009).

1.1.2 Source potentielle d'énergie renouvelable

Les microalgues sont actuellement considérées comme une source potentielle de matière renouvelable. De fait, leur biomasse contient, entre autres, des lipides, de l'amidon, de la cellulose et des protéines qui peuvent être utilisés pour la production de carburants renouvelables tels que le biodiésel et le bioéthanol (Carioca 2010). Les microalgues ont des stratégies de conversion de l'énergie solaire et d'acquisition de nutriments plus efficaces par rapport aux cultures agricoles traditionnelles (McGinn et al. 2011). Elles ont également des taux de croissance très élevés et peuvent produire des quantités significatives d'huiles qui peuvent être extraites et transestérifiées pour la production de biodiésel (D'Alessandro et Antonisi Filho; Grifftihs et Harrison 2009; McGinn et al. 2011; Rawat et al. 2011; Sharma et al. 2012).

Contrairement au maïs et au soja, les microalgues ne requièrent pas de terres agricoles pour leur culture (McGinn et al. 2011; Rawat et al. 2011). Elles ont la capacité de se développer dans un large éventail de milieux aqueux, allant de l'eau douce des lacs et des rivières à des milieux extrêmes tels que des eaux usées municipales et industrielles, permettant d'assainir et de réutiliser des déchets pour d'autres usages comme l'agriculture (Bhatnagar et al. 2011; Chinnasamy et al. 2010; Khan et al. 2018; McGinn et al. 2011; Rawat et al. 2011). Finalement, divers coproduits à valeur ajoutée peuvent être obtenus à partir de la biomasse algale résiduelle tels que des additifs pour l'alimentation animale, des produits thérapeutiques et des biosurfactants (McGinn et al. 2011; Rao et al. 2011; Rawat et al. 2011).

1.1.3 Inconvénients économiques des technologies à base de microalgues

Malgré les nombreux avantages que fournissent les microalgues, il existe quelques inconvénients rendant certaines technologies à base d'algues non viables à long terme. Les deux principaux inconvénients économiques sont le coût de la production d'algues et le coût du traitement de la biomasse pour la production de biocarburants. En effet, même si les microalgues requièrent peu d'exigences pour croître, certains besoins de base doivent être comblés. Lorsque le milieu de culture ne possède pas assez de nutriments, un supplément est nécessaire et le coût qui y est associé est relativement élevé (Chisti 2007).

Par ailleurs, le coût du traitement de la biomasse algale est très élevé, car pour chaque étape (ex. déshydratation, extraction de l'huile) un coût y est relié, rendant la production de biocarburants de troisième génération non-viable économiquement (Singh et Gu 2010; McGinn et al. 2011). Même si la production de biogaz est intéressante, d'autres limites ont été rencontrées lors de la digestion anaérobique de la biomasse (Sialve et al. 2009). Le problème majeur réside dans la difficulté à digérer la paroi cellulaire algale. Pour contrer ce problème, il existe divers prétraitements qui ont été développés pour enlever ou briser les parois cellulaires des algues (Alzate et al. 2012). Par contre, ces traitements supplémentaires augmentent, une fois de plus, les coûts de production.

Les algues sont généralement unicellulaires et se retrouvent en suspension dans leur milieu de culture (Moreno-Garrido 2008). Des floculants doivent être ajoutés afin de faciliter la récolte de ces dernières. Bien qu'il existe quelques stratégies pour éviter les coûts actuellement associés à la récolte, comme l'immobilisation des cellules, il y a encore des limitations au niveau industriel. Finalement, les procédures d'extraction des lipides sont en développement, complexes et très coûteuses (Rawat et al. 2011).

1.1.4 Alternatives proposées

Les technologies relatives aux microalgues sont confrontées à certaines limitations ne permettant pas une utilisation généralisée de ces dernières. En revanche, des alternatives ont été proposées pour contrer ces inconvénients. Premièrement, le coût de production de la biomasse peut être réduit si des eaux usées sont utilisées comme source de nutriments et s'ils sont présents en quantité suffisante (Prajapati et al. 2013). Deuxièmement, le coût d'exploitation de la biomasse peut être compensé si la biomasse générée est utilisée pour obtenir des produits à valeur ajoutée ou peut être rentable si la technologie des microalgues remplace une technologie plus coûteuse dans le même but (Chisti 2007; Sturm et Lamer 2011).

L'utilisation de microalgues est également limitée par le fait qu'une seule souche peut être inefficace ou simplement mourir lorsque les conditions changent. L'utilisation de souches algales qui possèdent des caractéristiques particulières peut être une approche permettant de réduire le coût d'exploitation de la biomasse. Ces caractéristiques peuvent être, par exemple, la tolérance à des températures extrêmes, une composition chimique prédominante pour les produits à haute valeur ajoutée, la propriété de sédimenter rapidement et la capacité de croître dans des milieux mixotrophes (Olguin 2003). L'utilisation d'un consortium (souches multiples comprenant généralement des bactéries) permet également de contrer cette limitation. Il confère aux organismes une robustesse face aux fluctuations environnementales, une certaine stabilité des espèces présentes, qui ont la capacité de partager des métabolites, et permet de résister à l'invasion d'espèces indésirables (Subashchandrabose et al. 2011). Il a été démontré que le consortium augmente l'efficacité de l'enlèvement des nutriments dans les eaux usées tout en générant une biomasse de microalgues utilisable pour la production de coproduits (Bhatnargar et al. 2011; Bourdeau et al. 2017; Chinnasamy et al. 2010; Gélinas et al. 2015).

Enfin, il a été proposé que l'implantation d'une installation de microalgues en cohabitation avec un émetteur industriel de CO_2 pourrait représenter une stratégie rentable et potentielle pour produire de grandes quantités de biomasse algale et recycler

le CO_2 (McGinn et al. 2011). Le CO_2 des effluents gazeux industriels représente une option intéressante pour alimenter les cultures algales et ainsi favoriser une croissance rapide en plus d'une diminution non négligeable des émissions de gaz à effet de serre provenant de ces industries (Kumar et al. 2010; Rao et al. 2011).

1.2 Les lipides des microalgues

Les microalgues ont la capacité de produire de bonnes quantités de lipides, pouvant aller de 1,5 à 75 % par poids sec (D'Alessandro et Antoniosi Filho 2016). La production et l'accumulation de lipides dépendent des espèces, du milieu de culture et du mode trophique employé. Plusieurs espèces d'algues vertes ont été étudiées quant à leur capacité à produire des lipides utilisables pour la production de biodiésel. La littérature abonde d'information sur ce produit par rapport à d'autres bioproduits issus des lipides algaux. Il a été rapporté que *Auxenochlorella prothothecoides*, *Chlorella vulgaris*, *Chlamydomonas rheinhardii* et *Dunaliella salina* sont de bons candidats potentiels pour cette production (D'Alessandro et Antoniosi Filho 2016). Pour qu'une espèce soit considérée comme étant un bon candidat pour son utilisation dans la production de biocarburants, certains critères doivent être rencontrés (D'Alessandro et Antoniosi Filho 2016). Tout d'abord, il a été mentionné que les espèces d'eau douce étaient préférées aux espèces marines en raison de la contamination possible du biodiésel avec le sodium. Par la suite, les microalgues doivent être robustes afin de pouvoir s'adapter à différents milieux complexes comme les eaux usées et de résister à l'invasion de microorganismes indésirables. Les taux de croissance doivent être rapides à court terme. La productivité des lipides doit être élevée et le profil lipidique doit contenir un taux adéquat d'acides gras saturés et insaturés. Finalement, afin de réduire les coûts de production et de traitement de la biomasse, les microalgues choisies devraient idéalement avoir une membrane cellulaire avec une faible adhérence au système de culture et elle devrait permettre la perméabilité de certains solvants pour faciliter l'extraction des acides gras ou autres composants. La présence de pigments de haute valeur est également attendue.

1.2.1 Catégories de lipides

Les microalgues produisent une grande variété de lipides tels que les lipides neutres, les lipides polaires, les esters de cire, les hydrocarbures, les dérivés prénylés (ex. caroténoïdes, terpènes) et les dérivés pyrroliques (ex. chlorophylles) (Sharma et al. 2012). En général, les lipides produits par les microalgues peuvent être classés en deux grandes catégories. La première représente les lipides polaires, aussi nommés lipides structuraux, et regroupe, entre autres, les phospholipides et les glycolipides (D'Alessandro et Antonisi Filho 2016; Sharma et al. 2012). Les lipides de cette catégorie possèdent une teneur élevée en acides gras polyinsaturés et représentent des nutriments essentiels pour les animaux aquatiques et les humains (Sharma et al. 2012). Les lipides polaires et les stérols jouent un rôle important dans les composants structuraux des membranes cellulaires (D'Alessandro et Antoniosi Filho 2016; Sharma et al. 2012). Tout d'abord, ils agissent comme barrière perméable sélective pour les cellules et les organites. De plus, certains de ces lipides fournissent une matrice pour opérer de nombreux processus métaboliques et participent au mécanisme de la fusion membranaire. Enfin, en plus d'avoir une fonction structurelle, certains lipides polaires agissent comme intermédiaires clés (ou précurseurs d'intermédiaires) dans les voies de signalisation cellulaire et interviennent dans la réponse aux changements environnementaux (Sharma et al. 2012).

La seconde catégorie est représentée par les lipides neutres, ou de stockage, qui se composent, entre autres, des acylglicérides (tri, di- et monoglycérides) et des acides gras libres (D'Alessandro et Antonisi Filho 2016; Sharma et al. 2012). Ces derniers, principalement présents sous forme de triacylglycérol (TAG), peuvent être transestérifés pour produire du biodiesel ou autres produits à valeur ajoutée (Sharma et al. 2012). La plupart des microalgues produisent des TAGs qui contiennent en majorité des acides gras saturés et monoinsaturés, mais certaines espèces ont la capacité d'accumuler de grande quantité d'acides gras polyinsaturés à longue chaîne (Bigogno et al. 2002; Sharma et al. 2012). En plus de jouer un rôle de stockage d'énergie, il a été rapporté que les acides gras polyinsaturés à longue chaîne servent également de réservoir d'acides gras spécifiques qui peuvent être utilisés lors d'un changement

soudain de conditions environnementales permettant une réorganisation membranaire adaptative rapide (Bigogno et al. 2002; Khozin-Goldberg et Cohen 2006; Makewicz et al. 1997; Sharma et al. 2012).

1.2.2 Les acides gras

Les acides gras présents dans les lipides des microalgues sont des acides carboxyliques possédant des chaînes de 4 à 6 carbones (D'Alessandro et Antoniosi Filho 2016). Trois types d'acides gras sont rencontrés. Il y a les acides gras saturés, qui ne possèdent pas d'insaturation, les monoinsaturés, qui possèdent une seule liaison insaturée et finalement, les di-tri et polyinsaturés, qui possèdent deux, trois et plus de trois liaisons insaturées (D'Alessandro et Antoniosi Filho 2016). La composition des acides gras influence la qualité du biodiésel produit. Lorsque de grandes quantités d'acides gras polyinsaturés sont produites, le biodiésel obtenu possède une bonne propriété d'écoulement à froid, mais une faible stabilité d'oxydation (Knothe 2005). À l'inverse, lorsqu'il y a de grandes quantités d'acides saturés et monoinsaturés produites, le biodiésel possède une bonne stabilité, mais peut avoir des problèmes d'écoulement à froid (Jeong et al. 2008; Ramos et al. 2009; Yeh et Chang 2012).

1.2.3 Induction des lipides

Dans des conditions de croissance optimales, les microalgues sont en mesure de produire de grande quantité de biomasse. En revanche, la teneur en lipides produits est assez faible, représentant seulement 5 à 20 % de leur poids sec (Sharma et al. 2012). Lors de conditions défavorables, les microalgues ont la capacité de modifier leur métabolisme lipidique pour répondre aux changements environnementaux (Guschina et Harwood 2006; Sato et al. 2000; Schuhmann et al. 2011). Elles modifient leurs voies de biosynthèse des lipides vers la formation et l'accumulation de lipides neutres, principalement sous forme de TAG, permettant d'augmenter la teneur en lipides représentant 20 à 50 % de leur poids sec (Sharma et al. 2012). Bien que l'accumulation et la production de TAG dépendent également de la génétique des individus, il a été

rapporté que les nutriments (ex. azote et phosphore), la salinité, la température, l'intensité lumineuse et le pH et sont les facteurs qui influencent le plus l'accumulation de lipides induite par le stress (D'Alessandro et Antoniosi Filho 2016; Sharma et al. 2012).

La disponibilité des nutriments a un impact significatif sur le développement des microalgues et la composition des lipides (Sharma et al. 2012). Lors de carence en nutriments, la croissance des microalgues diminue, mais il peut arriver, chez certaines espèces, que la synthèse des acides gras soit activée et qu'une plus grande production de TAG soit obtenue (Sharma et al. 2012). Le nutriment qui affecte le plus le métabolisme des lipides et qui est l'un des premiers à être appauvri pendant la culture des microalgues est l'azote (Sharma et al. 2012). Dans des conditions de carence en azote, la majorité des espèces de microalgues, y compris *Chlorella sp.*, ont une production de TAG plus élevée (Battah et al. 2013; Converti et al. 2009; Illman et al. 2000; Widjaja et al. 2009; Yeh et Chang 2011). La carence en phosphore peut également entraîner une hausse de la production de TAG chez les microalgues. Fan et al. (2014) ont observé une légère augmentation de la teneur en lipides chez les microalgues cultivées dans un milieu limité en phosphore par rapport à celle obtenue dans un milieu contrôle. La limitation d'autres nutriments dans les milieux de culture, tels que le fer (Liu et al. 2008) et le soufre (Matthew et al. 2009), peut également induire une augmentation de la teneur des lipides totaux chez certaines espèces de chlorelles.

Le stress salin permet d'augmenter la teneur en lipides chez certaines espèces de microalgues, tout en améliorant la composition des acides gras pour la production de biodiésel (Church et al. 2017; Heredia-Arroyo et al. 2011; Pandit et al. 2017; Rai et al. 2015; Shen et al. 2015; Wang et al. 2016). De fait, sous stress salin, les microalgues produisent une plus grande quantité d'acides gras saturés et une quantité moindre d'acides gras polyinsaturés. Church et al. (2017) ont montré que l'ajout de sel dans les milieux de culture a permis à *Chlorella vulgaris* d'accumuler une plus haute teneur en lipides totaux et d'augmenter la quantité d'acides gras saturés. L'intensité lumineuse affecte également le profil lipidique des microalgues (Sharma et al. 2012).

Khotimchenko et al. (2005) ont rapporté que sous faible intensité lumineuse, les microalgues produisaient davantage de lipides polaires, alors que sous forte intensité lumineuse, une plus grande quantité de lipides de stockage, sous forme de TAG, était produite.

La température est un stress environnemental important pour les microalgues, car en plus d'influencer leur croissance, la composition des lipides cellulaires, les besoins nutritionnels et la fixation du carbone varieront grandement en fonction de ce facteur (Juneja et al. 2013; Subhash et al. 2014). Il a été observé chez certaines espèces de microalgues que le changement de température avait influencé le profil lipidique (Sushchik et al. 2003; Converti et al. 2009; Subhash et al. 2014). Ces études ont montré que, lorsque la température diminue, il y a une augmentation des acides gras insaturés et inversement, une augmentation des acides gras saturés est observée lorsque la température augmente. Les microalgues produisent davantage d'acides gras insaturés pour maintenir la fluidité de la membrane et, par conséquent, s'adapter aux changements physiques de l'environnement (Paliwal et al. 2017).

L'état du pH du milieu influence directement les paramètres physiologiques des microalgues tels que la perméabilité de la membrane et la morphologie cellulaire (Liang et al. 2011). Un changement de pH dans le milieu de culture affectera donc l'osmose membranaire de certains ions et l'absorption de substances affectant ainsi la croissance cellulaire et la synthèse des lipides (Liang et al. 2011). Dans la littérature, il a été proposé qu'à pH élevé dans le milieu de culture, il y aurait une plus grande accumulation de TAG (Gardner et al. 2011; Guckert et Cooksey 1990). Ceci pourrait s'expliquer par le fait qu'à pH élevé, la croissance des microalgues serait inhibée et l'énergie de la cellule serait utilisée pour la production de TAG (Guckert et Cooksey 1990).

Les stress environnementaux permettent l'induction de la production de lipides chez les microalgues. En revanche, des taux de croissance plus faibles sont observés, ce qui, globalement, affecte la productivité totale de la biomasse et des lipides (Converti et al. 2009; Widjadja et al. 2009).

1.2.4 Les coproduits

Les lipides produits par les microalgues suscitent beaucoup d'intérêt quant à la production de biocarburants. En revanche, depuis quelques années les chercheurs s'intéressent davantage aux autres substances que peuvent produire les microalgues pour rentabiliser leurs procédés de production (Khan et al. 2018). La biomasse algale peut contenir des composés carbonés, des pigments, des composés bioactifs et des antioxydants qui peuvent être utilisés pour des applications nutritionnelles, médicales, pharmaceutiques et cosmétiques (Brennan et Owende 2010; Das et al. 2011; Moreno-Garcia et al. 2017). Plusieurs substances produites par les microalgues comme les acides gras polyinsaturés ont des effets thérapeutiques pour de nombreux troubles, notamment les maladies optiques et cardiaques, l'asthme et l'arthrite (Adame-Vega et al. 2011; Khan et al. 2018; Vijayavel et al. 2007). D'autres composés, tels que certains pigments (ex. astaxanthine), protègent contre les radicaux libres pour prévenir le stress oxydatif et sont utilisés comme antioxydants dans les nutraceutiques et les aliments (Khan et al. 2018; Moreno-Garcia et al. 2017). Comme les microalgues sont riches en protéines, en acides aminés essentiels et en glucides, elles représentent une bonne source de nourriture humaine et animale (Guil-Guerrero et al. 2004; Khan et al. 2018). Divers bioproduits peuvent être obtenus à partir de la biomasse algale et leur valeur ajoutée diminue les coûts associés à la production des microalgues.

1.3 Traitement des eaux usées par les microalgues

1.3.1 Phycoremédiation

L'une des premières descriptions de la phycoremédiation a été réalisée dans les années 1950 par Oswald et Gotaas (1957). La phycoremédiation est décrite comme étant l'utilisation de macro ou microalgues pour la suppression ou la biotransformation de polluants, y compris les nutriments et les xénobiotiques (ex. pesticides et médicaments), dans les eaux usées tout en générant une biomasse utilisable pour la fabrication de biocarburants et autres coproduits utiles (Moreno-Garrido 2008; Mulbry et al. 2008; Olguin 2003; Olguin et al. 2004; Rawat et al. 2011). Le but de la phycoremédiation est

de faire profiter les microalgues par la consommation de nutriments (ex. phosphore et azote) ou d'autres polluants qui se trouvent dans les environnements aquatiques, ce qui diminue les risques d'eutrophisation des cours d'eau (Rawat et al. 2011).

De nombreuses applications sont reliées à la phycoremédiation (Olguin 2003; Rawat et al. 2011). Il y a tout d'abord l'enlèvement des nutriments dans les eaux usées et les effluents riches en matières organiques municipales, l'enlèvement des éléments nutritifs et des composés xénobiotiques à l'aide de bioabsorbants à base d'algues et le traitement des eaux usées qui contiennent des acides et des métaux lourds. La phycoremédiation peut aussi être utilisée pour la détection de composés toxiques à l'aide de biocapteurs à base d'algues. De plus, comme les microalgues ont la capacité de fixer le CO_2 atmosphérique, une diminution des gaz à effets de serre peut être observée (Rao et al. 2011). Finalement, la phycoremédiation est un processus respectueux de l'environnement et permet un recyclage efficace des éléments nutritifs (Mulbry et al. 2008; Muñoz et Guieysse 2006; Pizarro et al. 2006).

1.3.2 Enlèvement des nutriments et des métaux

À ce jour, il existe diverses techniques qui sont utilisées pour réduire la charge en éléments nutritifs, métaux et autres polluants dans les eaux usées. Ces différentes techniques peuvent être classées en deux grandes catégories, soit les méthodes physicochimiques et les traitements biologiques (Eccles 1999; Mehta et Gaur 2005; Wang et al. 2006; Singh et Thomas 2012; Renuka et al. 2013). L'utilisation des microalgues est une technique qui se classe parmi les traitements biologiques et peut parfois être utilisée comme traitement tertiaire des eaux usées.

Les eaux usées sont principalement composées de protéines, d'hydrate de carbone, de lipides, d'huile, d'urée, de produits chimiques organiques synthétiques et de composés inorganiques (p. ex. azote, phosphore, fer) (Muttamara 1996; Rawat et al. 2011). Comme les microalgues requièrent certains de ces éléments pour assurer leur croissance, il a été rapporté que les eaux usées pouvaient servir de milieu de croissance

pour leur culture. De nombreuses études ont démontré le potentiel des microalgues à traiter les eaux usées en accumulant les différents nutriments et métaux qui s'y trouvent (Bhatnagar et al. 2011; Chinnasamy et al. 2010; Mulbry et al. 2008; Olguin 2003; Pittman et al. 2011; Prajapati et al. 2013; Rao et al. 2011; Rawat et al. 2011; Sydney et al. 2011; Woertz et al. 2009). Les microalgues sont reconnues pour jouer un rôle au niveau du traitement des eaux usées tout en générant une biomasse utilisable pour la fabrication de produits à valeur ajoutée (Mulbry et al. 2008; Olguin et al. 2003; Rawat et al. 2011). Woertz et al. (2009) ont montré l'efficacité du traitement des eaux usées par un consortium d'algues vertes cultivées dans des eaux usées municipales et industrielles. Après 12 jours de culture, 96 % d'azote et 99 % de phosphate ont été retirés du milieu par les algues. La biomasse générée, contenant divers lipides algaux, avait également le potentiel d'être utilisée comme matière première pour la fabrication de biocarburants.

La culture des microalgues dans les eaux usées, municipales et industrielles, représente une méthode alternative viable et efficace par rapport aux méthodes physicochimiques déjà existantes (Chinnasamy et al. 2010; Metha et Gaur 2005; Rawat et al. 2011; Wang et al. 2010b). L'utilisation d'eaux usées riches en nutriments organiques et inorganiques pour cultiver les microalgues permet de remplacer l'eau douce, de réduire le coût élevé des nutriments qui sont, en général, ajoutés dans les milieux de culture en plus d'assainir le milieu ciblé (Bhatnagar et al. 2011; Chinnasamy et al. 2010).

1.3.3 Mécanismes impliqués

L'acquisition de nutriments par les microalgues est essentielle au développement de ces dernières. En plus des macronutriments importants (ex. azote, phosphore, carbone), elles ont également besoin de certains métaux (ex. cuivre, fer, manganèse) en plus petites quantités. C'est, entre autres, grâce à ces exigences que les microalgues sont capables d'assimiler et d'éliminer de grandes quantités de nutriments dans les eaux usées.

1.3.3.1 Carbone

Le carbone peut être assimilé sous différentes formes en fonction, principalement, du mode trophique employé par les microalgues. Les autotrophes vont fixer le carbone inorganique, sous forme de CO_2, à partir de l'atmosphère et des émissions de gaz à combustion, grâce à leur activité photosynthétique (Cai et al. 2013; Gonçalves et al. 2017). Les hétérotrophes vont, quant à elles, utiliser exclusivement des formes organiques de carbone comme l'acétate, le glucose, le glycérol et l'éthanol (Cai et al. 2013; Gonçalves et al. 2017). Les microalgues mixotrophes qui combinent simultanément les modes trophiques autotrophe et hétérotrophe, vont assimiler à la fois les formes inorganiques et organiques du carbone (Cai et al. 2013; Leite et al. 2013; Sun et al. 2008). Finalement, il existe une autre forme de carbone couramment utilisée par les microalgues. Il s'agit des carbonates solubles qui jouent un rôle dans la croissance cellulaire (Cai et al. 2013). L'absorption des carbonates peut se faire de deux façons. De fait, lorsque le pH du milieu est faible (entre 5-7), l'absorption des carbonates solubles en tant que CO_2 s'effectue de manière directe par diffusion (Cai et al. 2013; Gonçalves et al. 2017). En revanche, si le pH est supérieur à 7, le bicarbonate (HCO_3^-), une forme très courante de carbone inorganique en solution, devra être converti en CO_2 libre à l'aide de l'enzyme anhydrase carbonique qui permet le transport actif de cette source de carbone dans les cellules de microalgues (Cai et al. 2013; Gonçalves et al. 2017; Sayre 2010).

1.3.3.2 Azote

L'azote est un nutriment essentiel impliqué dans la croissance de tous les organismes (Cai et al. 2013). L'azote organique est retrouvé, entre autres, dans les acides nucléiques (ADN, ARN) et dans les protéines (Kumar et al. 2010; Rao et al. 2011). Cette forme d'azote est dérivée de sources inorganiques comme le nitrate, le nitrite, l'acide nitrique, l'ammonium, l'ammoniac et l'azote gazeux (Cai et al. 2013). Les microalgues sont en mesure de convertir l'azote inorganique en forme organique grâce au processus d'assimilation (Cai et al. 2013; Gonçalves et al. 2017). Ce processus s'effectue seulement en présence de trois sources d'azote inorganique soit le nitrate,

le nitrite ou l'ammonium. Ces dernières vont pénétrer dans les cellules des microalgues par transport actif au niveau de la membrane plasmique (Gonçalves et al. 2017).

Il a été rapporté que la forme d'azote préférée serait l'ammonium, car son assimilation serait directe, ne nécessitant aucune réaction de réduction et donc moins d'énergie (Cai et al. 2013; Gonçalves et al. 2017). Le nitrate et le nitrite doivent subir une réaction de réduction en ammonium, en deux étapes, à l'aide des enzymes nitrate réductase et nitrite réductase (Cai et al. 2013; Crofcheck et al. 2012; Gonçalves et al. 2017). Dans la première étape, la conversion du nitrate en nitrite se fait via la nitrate réductase qui utilise la forme réduite du nicotinamide adénine dinucléotide (NADH) comme agent réducteur. Par la suite, le nitrite est réduit en ammonium par le nitrite réductase et la ferrédoxine (Fd). Finalement, l'ammonium résultant de ces réactions peut être incorporé dans les cellules de microalgues et converti en acides aminés. La glutamine synthétase facilite l'incorporation d'ammonium dans l'acide aminé glutamine à l'aide du glutamate (Glu) et de l'adénosine trisphosphate (ATP).

1.3.3.3 Phosphore

Tout comme l'azote, le phosphore est un élément essentiel dans le métabolisme énergétique des microalgues (Cai et al. 2013; Gonçalves et al. 2017). Son apport énergétique peut être à l'origine de trois différents processus. Il y a l'oxydation des substrats respiratoires ou du système de transport d'électrons des mitochondries (phosphorylation au niveau du substrat et phosphorylation oxydative) et la transformation de l'énergie lumineuse (photophosphorylation) (Cai et al. 2013; Gonçalves et al. 2017). L'ATP est produite à partir de l'adénosine diphosphate (ADP) et de l'apport énergétique provenant d'un de ces trois processus (Gonçalves et al. 2017). Le phosphore est également requis en grande quantité afin d'assurer la synthèse des protéines, des acides nucléiques, des lipides et des intermédiaires du métabolisme des glucides (Cai et al. 2013; Gonçalves et al. 2017; Kumar et al. 2010; Rao et al. 2011). Pour que cet élément puisse être assimilé par les microalgues, il doit se trouver sous forme de phosphates. Lorsque le phosphore se retrouve sous d'autres formes,

il se combine avec des ions métalliques et précipite, rendant l'assimilation par les microalgues impossible (Kumar et al. 2010). L'absorption des phosphates dans les cellules de microalgues se produit par transport actif à travers la membrane plasmique (Cai et al. 2013; Gonçalves et al. 2017).

1.3.3.4 Métaux

Les microalgues libres en suspension dans les eaux usées peuvent assimiler directement certains métaux. Elles ont également la capacité d'absorber les ions toxiques des métaux sur leurs parois cellulaires (Tel-Or et Forni 2011). La capacité d'adsorption sur les cellules dépend de plusieurs facteurs, dont l'espèce choisie, le pH du milieu et la concentration de la biomasse algale (Mehta et Gaur 2005; Romera et al. 2007). Ce processus est intéressant pour le traitement des eaux usées et la récupération de métaux précieux, car il permet de réutiliser la biomasse qui peut être séparée après le traitement de l'effluent permettant la récupération de métaux précieux (Paperi et al. 2006; Tel-Or et Forni 2011).

1.3.4 Facteurs influençant l'élimination des éléments nutritifs

Plusieurs facteurs tels que la concentration initiale en nutriments, l'intensité lumineuse, le pH extracellulaire et la température peuvent influencer l'élimination des éléments nutritifs par les microalgues (Cai et al. 2013). Une concentration initiale, particulière selon les espèces, en nutriments est nécessaire afin de favoriser la croissance cellulaire et l'élimination des nutriments (Cai et al. 2013). L'intensité lumineuse affecte l'absorption des nutriments, car elle fournit de l'énergie aux microalgues (Cai et al. 2013). Une augmentation de l'apport en lumière pourrait permettre d'augmenter l'élimination des nutriments (Gonçalves et al. 2017).

Le pH du milieu représente l'un des facteurs les plus importants influençant l'élimination des nutriments par les microalgues (Cai et al. 2013; Gonçalves et al. 2017). L'élimination de l'azote et du phosphore peut fortement être affectée par le pH.

À valeur élevée et en présence d'une concentration élevée en oxygène dissous, le phosphate précipitera, car il ne peut exister à l'état gazeux. L'ammonium peut également être éliminé plus rapidement, car une augmentation du pH et de la température du milieu favorisent la volatilisation de grandes quantités d'ammoniac. Enfin, l'absorption du carbone par les microalgues dépend du pH, puisque la solubilité du CO_2 dans le milieu de culture ne sera pas la même.

La température est un autre paramètre pouvant affecter l'élimination des éléments nutritifs dans les eaux usées, car elle affecte les réactions chimiques et biologiques des organismes (Rawat et al. 2011). Ce facteur détermine divers paramètres comme le pH, la conductivité et le niveau de saturation des gaz (Rawat et al. 2011). De plus, comme les eaux usées contiennent de grandes quantités de microorganismes, une augmentation anormale de la température peut avoir l'effet d'augmenter la présence d'espèces indésirables et de certains champignons (Rawat et al. 2011).

1.4 Associations algues-bactéries

Les relations qui existent entre les microalgues et les bactéries sont complexes et encore mal comprises. En revanche, il a été rapporté que les bactéries hétérotrophes pouvaient jouer un rôle dans la croissance et la survie des algues par des mécanismes de communication complexe et des échanges de nutriments (Amin et al. 2015; Gonzalez et Bashan 2000; Kim et al. 2014; Philippot et al. 2013). Même s'il existe plus d'un type d'interaction entre les microorganismes, le mutualisme et le parasitisme sont ceux qui sont les mieux démontrés à ce jour. La relation de mutualisme se définit principalement par l'échange de nutriments et de vitamines qui se produit entre les algues et les bactéries. De nombreuses études ont montré que les bactéries ont la capacité de fournir aux algues certaines vitamines, comme la B_{12}, et des nutriments pouvant être limitants comme le fer (Amin et al. 2009; Croft et al. 2005; Grant et al. 2014). En retour, les algues vont fournir aux bactéries de l'oxygène et d'autres molécules organiques issus de la photosynthèse.

Quant à la relation de parasitisme, elle se base principalement sur la compétition pour les nutriments. Il a été rapporté que certaines bactéries peuvent affecter négativement les algues en lysant leurs cellules (Wang et al. 2010a). Le mécanisme impliqué serait le même que celui observé dans l'interaction plante-pathogène. Des enzymes comme les glucosidases, les chitinases et les cellulases serviraient à dégrader la paroi cellulaire des algues (Arora et al. 2012; Wang et al. 2010a). Dans certains cas, il peut arriver que les bactéries prennent le dessus sur les algues et que ces dernières soient disséminées du milieu après plusieurs générations. Malgré cet aspect négatif qui peut exister entre ces deux types de microorganismes, les bactéries et les algues évoluent ensemble dans les milieux aquatiques depuis très longtemps. Il a été suggéré que l'utilisation de consortium d'algues-bactéries devait être intégrée dans les biotechnologies algales et que plusieurs avantages seraient rencontrés (Ramanan et al. 2016). Ces avantages sont décrits dans la prochaine sous-section.

1.4.1 Avantages pour la culture et la récole

Dans le but d'améliorer la culture de masse des microalgues, il a été mentionné qu'il est souhaitable d'avoir une communauté bactérienne associée aux microalgues afin d'atteindre des taux de croissance plus élevés (Cho et al. 2015). Comme les bactéries jouent un rôle important dans la croissance et la survie des algues, il a été rapporté que leur présence permettrait d'améliorer la productivité totale de la culture en termes de cellules algales. Par contre, afin d'éviter que certaines bactéries indésirables prennent le dessus sur les algues, il est important de suivre la diversité microbienne pour maintenir la communauté algale-bactérienne souhaitable (Cho et al. 2015, Park et al. 2013). D'ailleurs, Bourdeau et al. (2017) ont développé une méthode pour faire ce suivi qui consiste à mesurer un facteur d'intégrité. Le niveau de bactéries et les contaminants biologiques (ex. rotifères, protistes), autres organismes que la souche d'algue utilisée, ont été évalués. Le suivi du facteur d'intégrité assure la stabilité des membres du consortium utilisé.

La récolte de la biomasse algale en fin de culture représente des coûts et certains défis non négligeables, car les algues sont généralement unicellulaires et se retrouvent en suspension dans leur milieu de culture (Moreno-Garrido 2008). Par contre, il a été rapporté que la présence de bactéries dans les cultures de microalgues pourrait permettre d'augmenter la floculation. De fait, les bactéries aident à la floculation des algues en augmentant la taille des flocons, ce qui facilite la récolte de la biomasse algale-bactérienne, permettant également de réduire certains coûts associés à l'ajout de floculant (Gardes et al. 2011; Kim et al. 2014; Lee et al. 2013).

1.4.2 Utilisation de consortia d'algues-bactéries

Depuis quelques années, des consortia d'algues-bactéries ont été utilisés pour dégrader des nutriments et des métaux (Bahr et al. 2011; Boivin et al. 2007; Borde et al. 2003 ; Muñoz et Guieysse 2006; Subashchandrabose et al. 2011; Tang et al. 2010). La mise en place de ces consortia pour la biodégradation de la matière organique se base notamment sur la production d'oxygène par les microalgues. Par la suite, cet oxygène est utilisé par des bactéries hétérotrophes qui vont s'en servir comme accepteur d'électrons dans le but ultime de dégrader les polluants organiques. Une relation de mutualisme s'opère entre les organismes de cette communauté. Le CO_2, libéré lors de la minéralisation des polluants par les bactéries, est alors utilisé par les algues qui en ont besoin comme source de carbone afin de compléter efficacement le processus photosynthétique (Bahr et al. 2011).

L'efficacité de la biodégradation des polluants réalisée par une association entre les algues et les bactéries a été mise en évidence par Borde et al. (2003). Dans cette étude, la capacité d'un consortium composé de bactéries aérobies et d'une algue verte d'eau douce, *Chlorella sorokiniana*, à biodégrader des polluants tels que du salicylate, du phénol et du phénanthrène a été étudiée. Les résultats ont montré que ces trois contaminants ont été éliminés (85 % d'enlèvement) en présence du consortium d'algues-bactéries, sous un éclairage continu. De plus, la biodégradation des polluants a seulement été efficace dans des conditions aérobies. Aucun des trois contaminants

n'a été dégradé dans des conditions anaérobies. Cette observation permet de confirmer que l'oxygène agit bel et bien à titre d'accepteur d'électrons et est utilisé par les bactéries. Enfin, les résultats ont également montré que dans l'obscurité, aucun polluant n'a été éliminé. La photosynthèse, effectuée par les algues en présence de lumière, représente donc la source d'oxygène qui est utilisée par les bactéries dans les cultures. À leur tour, les bactéries fournissent du CO_2 par minéralisation des polluants aux algues qui le fixent lors de la photosynthèse. Cette étude a permis de démontrer que la photosynthèse favorise la biodégradation des polluants toxiques à l'aide d'un consortium d'algues-bactéries.

Par ailleurs, il a été démontré que l'utilisation d'un consortium d'algues-bactéries pour le traitement des eaux usées s'avère plus efficace que l'utilisation d'une culture d'algue pure (Chinnasamy et al. 2010; Sniffen et al. 2016). Chinnasamy et ses collaborateurs (2010) ont démontré qu'il était possible de traiter des eaux usées, provenant d'une usine de fabrication de tapis, à l'aide d'un consortium d'algues-bactéries. Les résultats de cette étude ont montré que le consortium utilisé s'est révélé efficace quant à l'élimination des éléments nutritifs. Plus de 96 % de ces éléments ont été retirés des eaux usées, et ce, dans un temps relativement court de 72 heures. L'efficacité d'élimination des éléments nutritifs dans les eaux usées et les déchets riches en nutriments organiques et inorganiques par un consortium d'algues-bactéries a également été démontrée dans une étude réalisée par Sniffen et al. (2016). Ces chercheurs ont mis en place un système d'épuration d'eaux usées dans un bassin contenant comme source de nutriments des lixiviats de sites d'enfouissement. Ces derniers contiennent une grande quantité d'azote et d'ammoniac. Les résultats de cette étude ont montré que le consortium étudié avait une grande efficacité à éliminer l'ammoniac et l'azote présents dans les lixiviats traités.

L'utilisation d'un consortium algues-bactéries permet d'obtenir une production de biomasse plus résistante et efficace pour éliminer des polluants inorganiques, organiques et métalliques (Muñoz et Guieysse 2006; Subashchandrabose et al. 2011). De fait, les consortia d'algues-bactéries sont plus robustes lors de conditions défavorables,

car les membres ont la capacité de partager des métabolites, des nutriments ou autres composés désirés. De plus, les consortia sont plus résistants à l'invasion d'espèces indésirables. Ceci n'est pas le cas dans les monocultures d'algues. Si les conditions du milieu changent et que les éléments nutritifs deviennent limités, elles ne peuvent compter que sur elles-mêmes, ce qui mène généralement à une baisse de croissance et globalement de production de biomasse. Finalement, la biomasse résultante de l'utilisation d'un consortium d'algues-bactéries pour le traitement des eaux usées peut également être utilisée pour la production de biocarburant et autres coproduits utiles.

1.5 Problématique et objectifs de recherches

Depuis plusieurs années, le réchauffement climatique, l'augmentation du CO_2 et l'épuisement des ressources en énergie représentent des problématiques environnementales préoccupantes (Khan et al. 2018 Prajapati et al. 2013). Par ailleurs, le développement rapide des industries et l'augmentation mondiale de la population causent également de sérieux défis environnementaux en raison des grandes quantités d'eaux usées qui sont libérées (Arora et Saxena 2005; de-Bashan et Bashan 2010; Fu et Wang 2011). Plusieurs s'entendent que l'utilisation des microalgues pourrait résoudre en partie ces problèmes de façon durable et rentable (Rawat et al. 2011).

Les recherches sur la production de microalgues ne datent pas d'hier et le besoin important en nutriments, souvent coûteux, incite à utiliser des sources de nutriments bon marché ou à coût négatif. C'est le cas des eaux usées municipales qui sont perçues comme une source de nutriments à coût négatif pour la culture de microalgues. En effet, les microalgues ont longtemps été utilisées pour le traitement tertiaire des eaux usées visant entre autres à réduire leur teneur en phosphore lorsque les traitements physico-chimiques ou biologiques ne sont pas suffisants. Le Groupe de Recherche en Aquaculture et Recyclage Biologique (GREREBA) de l'Université Laval à Québec (Qc, Canada) avait d'ailleurs mené divers projets sur le traitement tertiaire d'eaux usées municipales ou domestiques par des microalgues et la récolte de la biomasse microalgale pour l'alimentation porcine (Van Coillie et al. 1990). Bien qu'ils existent encore très peu

de systèmes opérationnels de traitement tertiaire d'eaux usées avec des microalgues, quelques villes dans le monde, dont la Ville de Vancouver au Canada, travaillent à intégrer la production de microalgues à leur station d'épuration des eaux usées. C'est également le cas de la Ville de Victoriaville (Qc, Canada) et ses entreprises locales qui souhaitent aller de l'avant avec la production de microalgues dans leurs eaux usées pour les traiter tout en obtenant des produits biosourcés issus de la biomasse produite. Bien que l'épuration des eaux usées soit un des objectifs, la Ville de Victoriaville et les entreprises participantes voient avant tout une façon de créer de nouvelles synergies industrielles en valorisant les eaux usées de plusieurs entreprises pour l'obtention de bioproduits utilisables par ces mêmes entreprises.

En association avec l'Université du Québec à Trois-Rivières et ses collaborateurs, un projet intitulé « Projet VERTECH I : Intégration de culture de microalgues à Victoriaville pour l'obtention de produits biosourcés à usage local » a été démarré en 2014 (Figure I.I). Dans ce projet, les eaux usées de trois industries situées sur le site du parc industriel de Victoriaville, soit Parmalat-Lactantia, un fabricant de produits laitiers, Abbott Laboratories – Groupe Canlac, une division d'une grande compagnie pharmaceutique qui fabrique du lactulose et Groupe Sani Marc, un fabricant de produits nettoyants à usage domestique et industriel, sont utilisées comme milieu de culture de base pour les microalgues. Les lixiviats du lieu d'enfouissement technique de Gesterra à St-Rosaire (Qc, Canada), une compagnie de gestion des matières résiduelles municipales, située non loin du parc industriel de Victoriaville, étaient également utilisés pour enrichir le milieu en nutriments.

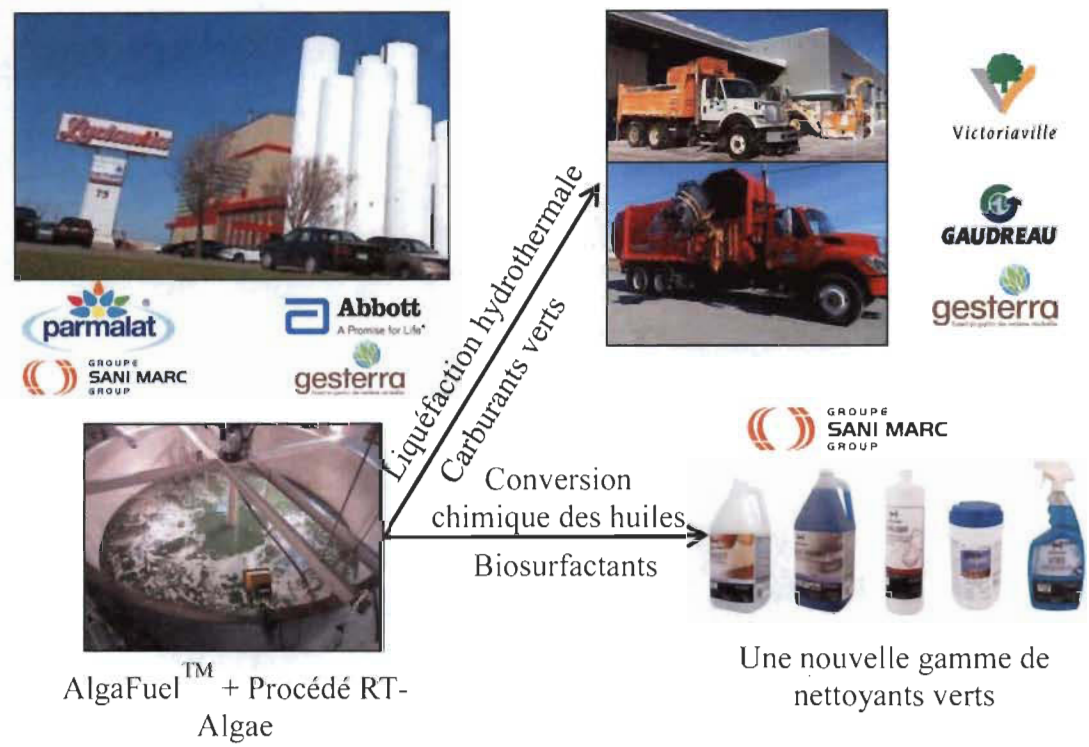

Figure 1.1 Projet VERTECH I : Intégration d'une culture de microalgues à Victoriaville pour l'obtention de produits biosourcés à usage local.

Diverses applications peuvent être ciblées dans ce projet, par exemple, la production de biodiésel pour subvenir aux besoins de la flotte des véhicules lourds de la ville de Victoriaville et de Gaudreau Environnement (une autre entreprise locale) ou l'obtention de biosurfactants, de biolubrifiants ou autres ingrédients biosourcés pour les produits nettoyants du Groupe Sani Marc. Les partenaires du projet se sont entendus pour focaliser sur l'obtention de biosurfactants pour la compagnie Sani-Marc tout en considérant la possibilité de réduire les coûts de gestion des eaux usées des différentes compagnies impliquées.

Le Groupe Sani Marc est le plus grand fabricant de produits nettoyants à usages domestiques et industriels au Canada. Pour fabriquer ses produits nettoyants, la compagnie utilise des surfactants d'origine pétrochimique obtenus à partir de ressources non renouvelables. Afin de réduire leur empreinte environnementale, Sani Marc est à la recherche d'ingrédients biosourcés. Les molécules requises pour la fabrication de leurs surfactants sont les acides gras C12 :0 (acide laurique) et C14 :0

(acide myristique) qui doivent par la suite être transformés en amines-oxydes. Ces deux acides gras peuvent être retrouvés dans des huiles naturelles produites notamment par les microalgues. Le Groupe Sani Marc souhaite utiliser les acides gras C12 :0 et C14 :0 provenant de la biomasse générée par les microalgues cultivées dans leurs eaux usées, mélangées à celles des autres compagnies impliquées dans le projet VERTECH I. Dans ce cas précis, les microalgues joueraient un double rôle, soit de traiter les eaux usées avec leur capacité épuratoire et de fabriquer avec la biomasse générée des coproduits, en l'occurrence des biosurfactants utilisables par Sani-Marc. Le projet VERTECH I comportait plusieurs activités de recherche, dont certaines requérant des recherches fondamentales. Un projet de doctorat sur l'utilisation des eaux usées pour cultiver des microalgues et obtenir des biosurfactants a donc été proposé et cette thèse en est le résultat.

Le but du projet de doctorat est d'utiliser les effluents riches en nutriments et les résidus organiques des entreprises du parc industriel de Victoriaville et de ses environs pour produire une biomasse algale riche en lipides, spécialement en acides gras C12 :0 et C14 :0. Ces deux acides gras pourraient être convertis en intermédiaires de synthèse chimique par des techniques de chimie verte et d'oléochimie. Ces intermédiaires de synthèse chimique « biosourcés » seraient alors utilisés par l'entreprise Sani Marc pour fabriquer des biosurfactants et formuler des produits nettoyants plus respectueux de l'environnement.

Pour atteindre ce but, plusieurs objectifs de recherche sont proposés. Chaque objectif de recherche fait l'objet d'un article scientifique. Les objectifs de recherche ainsi que les hypothèses à confirmer ou infirmer sont décrits dans les prochaines sous-sections.

1.5.1 Objectif 1 : Déterminer les conditions de croissance optimales du consortium de microalgues-bactéries

Afin d'identifier les conditions de culture permettant aux microalgues d'atteindre une biomasse élevée et une forte teneur en lipides (par poids sec), le consortium de

microalgues-bactéries a été cultivé dans un mélange contenant les effluents des quatre entreprises impliquées dans le projet comme milieux de croissance. Trois modes trophiques (autotrophie, mixotrophie, hétérotrophie), différentes concentrations de CO_2 injecté (1, 2 et 5 %) et différentes intensités lumineuses (30, 200 et 550 µmol/m^2/s) ont été testés. Le mode mixotrophique est une combinaison des modes autotrophique et hétérotrophique qui utilise à la fois la lumière comme source d'énergie en plus du CO_2 et des substrats de carbone organique comme source de carbone. Dans la littérature scientifique, il a été montré que ces conditions améliorent la croissance des microalgues ainsi que la productivité lipidique (Bhatnagar et al. 2011, Heredia-Arroyo et al. 2011; Liang et al. 2009). Il a également été montré que certaines espèces de chlorelles peuvent tolérer des intensités lumineuses pouvant aller jusqu'à 600 µmol/m^2/s (Takeshita et al. 2014). En revanche, il a été démontré qu'au-delà d'une certaine intensité lumineuse (généralement autour de 200 µmol/m^2/s), il y aurait photoinhibition limitant la photosynthèse et la croissance des microalgues (Vandenhecke et al. 2015). Concernant l'injection du CO_2 dans le milieu de culture, il a été rapporté par Lam et Lee (2013) que les meilleures productivités en termes de biomasse et de lipides ont été obtenues lorsque 5 % de CO_2 était injecté dans une culture contenant *Chlorella vulgaris*, par rapport à d'autres concentrations qui avaient été testées (CO_2 atmosphérique, 0,5, 1 et 2 %). Les rendements les plus élevés au niveau de la biomasse algale et des lipides totaux seraient probablement atteints en mixotrophie, avec une injection de 5 % de CO_2 combinée à une intensité lumineuse de 200 µmol/m^2/s. Cette hypothèse doit être vérifiée.

1.5.2 Objectif 2 : Identifier les conditions environnementales permettant aux microalgues de produire une forte teneur en lipides et plus spécifiquement en acides gras C12 :0 et C14 :0

Pour identifier les conditions environnementales permettant d'induire une forte production de lipides, et plus spécifiquement, des deux acides gras recherchés (C12 :0 et C14 :0), différentes carences concernant les nutriments du milieu et divers stress (milieu de culture, modes trophiques, salinité et pH) ont été appliqués sur le consortium de microalgues-bactéries. Il a été rapporté que de nombreux stress environnementaux

peuvent induire la production de lipides chez les microalgues (D'Alessandro et Antoniosi 2016; Sharma et al. 2012). Parmi ces stress, la carence en nutriments, plus particulièrement en azote, a été montrée pour augmenter la production des lipides (Battah et al. 2013; Converti et al. 2009; Illman et al. 2000; Widjaja et al. 2009; Yeh et Chang 2011). Les différents modes trophiques, variant selon les espèces, influencent également de manière significative l'accumulation des lipides (Yeh et Chang 2012). Par ailleurs, certaines études ont rapporté que le stress salin peut améliorer la production de lipides (Church et al. 2017; Heredia-Arroyo et al. 2011; Pandit et al. 2017; Rai et al. 2015; Shen et al. 2015; Wang et al. 2016). Finalement, comme le pH du milieu influence les paramètres physiologiques des microalgues, un changement de valeur dans la culture pourrait affecter la synthèse des lipides (Gardner et al. 2011; Guckert et Cooksey 1990; Liang et al. 2011). Une carence en nutriments, des conditions de croissance mixotrophiques, une concentration de sel élevée dans le milieu et un pH élevé permettront possiblement d'induire significativement la production de lipides par les microalgues. Des travaux de recherche sont requis pour valider cette hypothèse dans le cadre du projet VERTECH I.

1.5.3 Objectif 3 : Évaluer la performance de la culture en continu par rapport à la culture en deux étapes

Des cultures en une seule étape ont été comparées à des cultures en deux étapes basées sur la densité cellulaire et la teneur en lipides. Dans la première étape, toutes les cultures étaient soumises à des conditions mixotrophiques. Pour les cultures en deux étapes, après 3 jours, une bonne quantité de biomasse algale a été transférée dans un nouveau milieu de culture où différents stress (pH, salinité, mode hétérotrophique, type d'eaux usées) ont été induits. Lorsque les conditions de croissance sont optimales, les microalgues ont la capacité de produire une biomasse élevée. En revanche, dans ces mêmes conditions, la teneur en lipide produite est relativement faible, représentant entre 5 à 20 % du poids sec des microalgues (Sharma et al. 2012). Certains stress comme la carence en nutriments, la salinité, le changement du pH ont été rapportés pour augmenter la teneur en lipides. Les stress augmentent l'accumulation de lipides, mais il a été rapporté que le taux de croissance est affecté, ce qui réduit globalement la

productivité lipidique (Converti et al. 2009). Une stratégie de culture en deux étapes pourrait représenter une solution à cette limitation. Une première étape, représentée par des conditions de croissance prédéterminées, pourrait permettre de produire une biomasse élevée et la seconde étape, représentée par l'induction de stress, pourrait permettre la production de lipides. Un équilibre entre la production de biomasse et de lipides pourrait être atteint. Cette hypothèse de recherche doit également être vérifiée dans le cadre du projet VERTECH I.

1.5.4 Objectif 4 : Évaluer la capacité du consortium de microalgues-bactéries à éliminer les nutriments contenus dans les eaux usées industrielles

Différentes conditions ont été testées, y compris l'injection de CO_2, l'ajout de glucose et de nutriments dans les milieux de culture pour évaluer quelles conditions permettent la meilleure efficacité d'enlèvement de nutriments et des métaux contenus dans les eaux usées industrielles. Il a été rapporté, dans la littérature, que les microalgues requièrent certains éléments qui se trouvent dans les eaux usées pour leur croissance, ce qui leur confère une capacité à traiter ces dernières en accumulant les différents nutriments et métaux qui s'y trouvent (Bhatnagar et al. 2011; Chinnasamy et al. 2010; Mulbry et al. 2008; Olguin 2003; Pittman et al. 2011; Prajapati et al. 2013; Rao et al. 2011; Rawat et al. 2011; Sydney et al. 2011; Woertz et al. 2009). Les microalgues cultivées dans un mélange d'eaux usées industrielles sans ajout de nutriment, de glucose et de CO_2 devraient se montrer plus efficaces à traiter les eaux usées en accumulant les différents nutriments et métaux qui s'y trouvent. Cette hypothèse mérite d'être validée dans le cadre du projet VERTECH I.

1.5.5 Objectif 5 : Identifier les populations bactériennes et algales composant le consortium qui, en équilibre, bénéficient à la fois au traitement des eaux usées et à la production de bioproduits

Pour identifier la flore microbienne qui compose le consortium utilisé dans ce projet de recherche, le séquençage des algues et des bactéries a été réalisé dans différents milieux de culture. Un milieu contrôle, composé d'eau déminéralisée et de nutriments

BBM, un milieu contenant le mélange d'eaux usées industrielles et de nutriments BBM, un milieu contaminé par des espèces indésirables et un bassin de 400 litres ont été testés afin d'identifier les changements dans les populations bactériennes et algales. Il a été rapporté que l'utilisation d'un consortium composé d'algues et de bactéries est efficace pour le traitement des eaux usées (Bahr et al. 2011; Borde et al. 2003; Chinnasamy et al. 2010; Muñoz et Guieysse 2006; Sniffen et al. 2016). L'échange de nutriments et autres composés entre ces deux types d'organismes permet d'obtenir une meilleure productivité de la biomasse. L'identification de la flore microbienne qui compose le consortium utilisé dans ce projet permettra de mieux contrôler ou augmenter les rendements de production. Un équilibre entre les populations de bactéries et d'algues est attendu au sein du consortium utilisé dans le mélange d'eaux usées industrielles. Des changements dans les populations bactériennes et algales sont aussi attendus entre les différents milieux de culture. Des travaux de recherche ont été proposés pour valider ces deux hypothèses dans le cadre de ce projet de doctorat.

CHAPITRE II

CULTIVATION OF AN ALGAE-BACTERIA CONSORTIUM UNDER DIFFERENT TROPHIC CONDITIONS IN A MIXTURE OF WASTEWATERS FROM AN INDUSTRIAL PARK TO OBTAIN VALUABLE PRODUCTS USABLE LOCALLY

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Abstract

Profitability of biofuel production from microalgae is difficult to achieve but co-locating production with wastewater treatment plants is a possible avenue. A microalgae culturing project was conducted using wastewater from an industrial park to obtain valuable metabolites from the biomass to eventually use them locally. Different growth conditions were tested with a mixture of wastewater as the culture medium and a native microalgae-bacteria consortium isolated from the same wastewater. The results showed that this consortium grows well in wastewater and that different fatty acids profiles are produced under the different growth conditions. The highest percentages of the two desired fatty acids (C12: 0 and C14: 0) were obtained under heterotrophic conditions. High amount of other fatty acids was also produce and could potentially be used to make other co-products. In addition to reducing wastewater treatment costs, the manufacture of biosurfactants could bring additional income to the overall microalgae production process.

Keywords: microalgae-bacteria consortium, trophic conditions, wastewater, industrial park, biosurfactant.

Introduction

The use of microalgae for biomass production has brought strong interest due to their growth characteristics and the multiple functions during growth they are can fulfill. Microalgae have relatively high growth rates, short generation times and only few requirements for growth (Rawat et al., 2011). They are eukaryotic photosynthetic organisms that can grow in a wide range of aquatic environments from freshwater lakes and oceans to more extreme environments such as municipal and industrial wastewater (Chinnasamy et al., 2010; Khan et al., 2018). Since light is required for photosynthesis, it's availability and quantity affect the physiology and growth of microalgae (Khan et al., 2018; Khoeyi et al., 2012; Krzeminska et al., 2014). In addition to carbon dioxide (CO_2) used as a source of carbon, microalgae also require nutrients to grow. Nitrogen and phosphorus are required in relatively large quantities (increasing the cost of production) for the synthesis of proteins, nucleic acids and phospholipids (Kumar et al., 2010; Prajapati et al., 2013; Rao et al. 2011) while trace elements (e.g. Na, Mg, Ca, Mn, Cu, Fe and Mo) and vitamins are required in smaller amounts.

Microalgae can be grown under different trophic modes that are characterized by the energy and carbon sources. These modes will significantly influence growth rate and lipid accumulation (Yeh and Chang, 2012). The three main modes are autotrophic, heterotrophic and mixotrophic. Under autotrophic conditions, microalgae absorb light energy and assimilate atmospheric CO_2 (Carvalho et al., 2006). This mode does not generally lead to the highest growth rates because of the availability of light and CO_2 can become limiting especially at high biomass. Under heterotrophic conditions, organic compounds (e.g. glucose) are used, as a source of carbon and no light is required for growth. These conditions have been shown to lead to high growth rates and biomass (Liang et al., 2009, Xu et al., 2006). However, in industrial settings the costs of adding organic carbon to the culture medium represent a considerable disadvantage. Finally, under mixotrophic conditions, light provides part of the energy while organic compounds provide carbon and an energy complement (Sun et al., 2008). This mode overcomes some of the limitations encountered in the other modes and it has been

shown to improve the growth of microalgae (Bhatnagar et al., 2011, Heredia-Arroyo et al., 2011).

The use of municipal or industrial wastewater to cultivate microalgae is an interesting option because: 1) there is no extra need for freshwater; 2) the cost of nutrients addition is reduced or eliminated depending on their presence in the wastewater; and 3) wastewater treatment is carried out at the same time as the production of biomass (Bhatnagar et al., 2011). In addition, it reduces the cost of wastewater management. Microalgae cultivated in wastewater assimilate and, thereby, remove nutrients such as nitrogen and phosphorus required for growth. For example, Woertz et al. (2009) showed that a consortium of green algae introduced into municipal wastewater containing dairy farm waste removed 96% of the nitrogen and 99% of the phosphate while a lipid-rich microalgae biomass was obtained for further valorization. In addition, this medium increased the growth of algae and the lipids production. Microalgae production co-located with industrial plants can benefit from waste nutrient (present in wastewater) and energy (e.g. excess heat in colder climates). Furthermore, for industries producing CO_2, it can be recycled by injection in the media and captured through algal photosynthesis (McGinn et al., 2011). Therefore, in addition to promoting growth and lipid productivity, a reduction of the industrial greenhouse gas emissions can theoretically be achieved (Kumar et al., 2010; McGinn et al., 2011; Rao et al., 2011).

Despite the many potential benefits of growing microalgae in industrial settings, major improvement to production are required to make it viable. For example, it has been reported that, depending on the species and growth conditions, the percentage of lipids by dry weight produced by microalgae varies from 1.5 to 75% (D'Alessandro and Antoniosi Filho, 2016). It has also been shown that, under optimal growth conditions, microalgae can produce large quantities of biomass, but only 5 to 20% by dry weight of lipids (Sharma et al., 2012). With these yields, the production of biofuels from microalgae is not profitable (McGinn et al., 2011). To have a profitable co-located microalgal production, the co-products utilization must be targeted. The fatty acids produced by microalgae can be used in many products such as animal feed,

biosurfactants, fertilizers, etc. Finally, the use of a microbial consortium, containing several organisms, but with a dominance of beneficial microalgae species, could be advantageous because it provides culture robustness during environmental fluctuations and prevents the invasion of undesirable species (Subashchandrabose et al., 2011). When operating in open ponds and using wastewaters as culture media, such robustness is essential.

We report here on a case study carried out in the industrial park of the city of Victoriaville (Quebec, Canada) where a manufacturer of household and industrial cleaning products is looking to reduce its environmental impact. Formulation of household and cleaning products is generally based on petrochemical surfactants obtained from non-renewable resources. This manufacturer is looking to replace petroleum-based surfactants with a renewable source. In this case, the targeted molecules are lauric (C12: 0) and myristic (C14: 0) fatty acids, which are converted into amine-oxides and added in the cleaning product formulations. These fatty acids can be found in oils naturally produced by microalgae. This company would thus like to obtain these two fatty acids using microalgae grown in its own wastewater and as well as other local wastewaters that would provide enough waste nutrients for the culture. This would simultaneously reduce the wastewater treatment costs through nutrient removal by microalgae and benefit from greener products.

Herein, a microalgae-bacteria consortium was grown in a mixtures containing the effluents of 4 companies. The main objective of this study was to identify the culture conditions allowing high biomass and lipid production, and more particularly, large quantities of the two desired fatty acids (C12: 0 and C14). To do this, three trophic modes (autotrophic, mixotrophic, heterotrophic) and different growth conditions (CO_2 and light intensity) were tested.

Materials and methods

Wastewaters and leachates

The wastewaters were collected from industries located in the Victoriaville (Quebec, Canada) industrial park: a dairy industry, a pharmaceutical industry (lactulose production) and a household and cleaning product manufacturer. The leachate comes from a municipal solid waste landfill site in the area of the industrial park. The wastewaters and leachate were stored in plastic containers at 4 °C until they were used for the experiments within 2 days.

Consortium and inoculum preparation

The consortium used in this work was a native microalgae-bacteria consortium isolated from a sample taken on the site of a wastewater stabilization pond from a dairy wastewater treatment station in the industrial park of Victoriaville city (Quebec, Canada). This consortium was mainly composed of *Chlorella* spp. For inoculum preparation, algal biomass was harvested from a seed culture in exponential growth phase and resuspended into Erlenmeyer flasks containing a mixture of the four collected wastewaters (45% pharmaceutical, 41% dairy, 10% chemical cleaners and 4% leachate, v/v), supplemented with Bold's basal medium (BBM) minerals (K_2HPO_4, 10 mL/L; $MgSO_4$, 10 mL/L; KH_2PO_4, 10 mL/L; major stock solution, 10 mL/L; trace metal stock solution, 1mL/L; Boron stock solution 1 mL/L; EDTA stock solution, 1 mL/L and acidified iron stock solution, 1 mL/L). The proportion of each wastewater in the blend was determined according to the relative volume available in the industrial park. The inoculum was maintained in Erlenmeyer flasks on an orbital shaker set at 110 rpm at 25 °C, under a photosynthetically available irradiance of 20 μmol m^{-2} s^{-1} on a 12 h/12 h light/dark cycle until algal cell concentration reached 1×10^8 cell mL^{-1} for further experiments.

Erlenmeyer flask experiments

The nine experiments, corresponding to the rows in Table 1, were carried out to evaluate which trophic modes and culture conditions led to the highest biomass, extracted lipids, and in particular the C12: 0 and C14: 0 fatty acids percentage. They were performed over a period of five days. For all treatments, 25% (v/v) of inoculum, containing the microalgae-bacteria consortium, was cultured in a one-liter flask containing five hundred milliliters of the same wastewater mixture use for maintenance. Erlenmeyer flasks were placed on an orbital shaker set at 110 rpm, at 25° C and under 12 h/12 h light/dark cycle. The growth conditions for each experiment differed by the combination of CO_2 concentration, light intensity and glucose addition (Table 1). CO_2 was supplied as a mixture with N_2 and O_2, for autotrophy and mixotrophy, at rate of 0.42 L min^{-1} through a porous sparing stone. Concentration of CO_2 in the gas was adjusted to 1%, 2% or 5% with 21% O_2 and nitrogen as the remaining portion. It was bubbled in the culture media for 15 minutes every hour during the light phase only. Otherwise, atmospheric air was injected into the media. Cool-white fluorescent provided 30, 200 or 550 μmol m^{-2} s^{-1}, except for heterotrophic condition where darkness was achieved by wrapping the cultures in aluminum foil. Glucose (0.5 g L^{-1} d^{-1}) was added the first four days as organic carbon source to supply microalgae under heterotrophy and mixotrophy. These experiments are referred by a four letters system (Table 1). The second and fourth letters refer to light intensity (L) and CO_2 (C) concentration. The first and third letters refer to Low (L), Medium (M) or high (H) conditions for light and CO_2. For example, the 550 μmol m^{-2} s^{-1} and 1% CO_2 experimentation is referred as HL-LC. In addition, for each experiment, three trophic modes (autotrophic, mixotrophic and heterotrophic) were compared in triplicate, giving a total of nine flasks per experiment (Table 1).

Analysis

Biological and physicochemical parameters

Biomass and pH monitoring were performed on a daily basis. Biomass (dry weight per liter) culture was followed by filtering, using a Buchner and vacuum, 10 ml of the

medium on Whatman™ 934 AH™ glass microfiber filters (effective pore size of 1.5 µm). A sample was taken and observed daily and counted with a Neubauer chamber (hemacytometer) using a phase-contrast microscope. This monitoring also helped to ensure that the culture medium was not contaminated by undesirable species such as rotifers (Bourdeau et al. 2017).

Lipid extraction and fatty acid methyl esters (FAME) profile

Lipids were quantified as described in Bélanger-Lépine et al. (2018), based on Bligh and Dyer (1959) method. Briefly, vacuum dried algae were extracted using methanol and chloroform and weighted after evaporation.

Fatty acids were analyzed by GC-MS after the transesterification of extracted lipids as described in Bélanger-Lépine et al. (2018) according to the method of Li et al. (2013).

Statistical analysis

Variance analyzes (ANOVA) were used to compare the treatments for each data set using the JMP Pro 11 Software. We considered p value smaller than 0.05 statistically significant.

Results and discussion

Influence of trophic modes on biomass

Culture conditions can significantly affect microalgal biomass production (D'Alessandro and Antoniosi, 2016; Yeh and Chang, 2012). Because initial biomass differed slightly between treatments (but not for different trophic mode within a treatment), comparison of biomass between treatments is not possible. In the low light condition treatment microalgae grown in mixotrophic and heterotrophic conditions achieved significantly higher biomasses than those grown in autotrophic conditions (Fig. 1) independently of

the carbon addition. In the medium light treatment, all modes produced similar biomass in the low carbon condition, but the medium and high carbon conditions led to higher growth in the heterotrophic and mixotrophic modes. In the high light conditions, the different modes were not significantly different except in the low carbon condition the heterotrophic mode allowed to reach higher biomasses. This result could be explained by the presence of 'contamination' in heterotrophic cultures in this treatment. A higher amount of bacteria and protozoa was observed in these cultures compared to those grown in autotrophic and mixotrophic modes. Finally, it should be noted that, although it is not always significantly different, in all treatment the mixotrophic cultures had higher biomass that the autotrophic one.

These results are consistent with other studies (Liang et al., 2009; Heredia-Arroyo et al., 2011; Abreu et al., 2012; Dubey et al., 2015). Abreu et al. (2012) compared the growths of *Chlorella vulgaris* under autotrophic and mixotrophic conditions. The biomasses obtained at the end of the culture were higher in mixotrophy compared to those obtained in autotrophy. Liang et al. (2009) compared the biomass (mg L^{-1}) obtained from *Chlorella vulgaris* under different growth conditions. Microalgae grown under autotrophic conditions reached lower biomass values than those grown in mixotrophic and heterotrophic mode. Dubey et al. (2015) compared the biomass reached for *Chlorella minutissima* under autotrophic, heterotrophic and mixotrophic growth conditions and found that the maximum biomass was reached in heterotrophic mode. The lowest biomass was observed in autotrophy. In mixotrophy, the biomass values obtained were higher than those obtained in autotrophy, but lower than those obtained in heterotrophy. Heredia-Arroyo et al. (2011) showed that the highest biomass was obtained when *Chlorella vulgaris* were grown under mixotrophic conditions. The biomass values obtained in mixotrophy and heterotrophy were similar and superior to those obtained under autotrophic conditions.

The presence of glucose in the mixotrophic and heterotrophic cultures has generally led to the higher biomass compared to autotrophic cultures. Glucose is a source of organic carbon that is metabolized rapidly and provides instantaneous energy to the cell

(Dubey et al., 2015). It can be used directly by the cell, unlike other carbon sources that must be converted to glucose before they can be used by microalgae (Dubey et al., 2015).

Influence of trophic modes on lipids production

Once normalized to biomass comparison across treatments are possible. In the low light condition treatment, all modes produced similar extracted lipids by dry weight (Table 2) independently of the carbon addition. In the medium light treatment, the low carbon condition produced higher extracted lipids in autotrophic mode unlike in the medium carbon condition where it's the mixotrophic mode that reaches higher extracted lipids. Under high light conditions, the different modes were not significantly different except under the low carbon conditions the mixotrophic showed higher extracted lipids. In the heterotrophic mode (which is not influence by the light level for a treatment), differences of a few percent were observed but often these were generally not significantly different from another treatment.

Extracted lipid values in the microalgae biomass were relatively good under all three growing conditions after only five days of cultivation, ranging from approximately 13-20% (Table 2). The conditions tested were first established in order to obtain optimal growth conditions and in a second time to see if these conditions also influenced the production of lipids. In a study by Sharma et al. (2012), it has been reported that under optimal growth conditions, high biomass but low lipid contents of 5 to 20% of their dry weight are achieved. Our results are consistent with what was reported in this study. High biomasses have been obtained, but the highest lipid content does not significantly exceed 20%. Overall, no strong pattern emerged from the light and trophic mode with respect to fractional biomass in lipids with similar percentage in all but a few treatments.

The culture conditions tested may therefore be optimal for growth, but other factors must be taken into account to increase the value of total lipids. Numerous studies have shown the positive effects of nutritional stress on lipid production (Fan et al., 2014;

Praveenkumar et al., 2012; Widjaja et al., 2009; Yeh and Chang, 2011). Various external factors such as salinity (Church et al., 2017; Rai et al., 2015; Shen et al., 2015; Wang et al., 2016), pH (Gardner et al., 2011; Guckert and Cooksey, 1990) and temperature (Sushchick et al., 2003; Converti et al., 2009; Subhash et al., 2014) have also been reported to induce lipid production. Combining the best growing condition, found in this study, with one of these stresses may lead to better total lipid production while producing high biomass.

Fatty acids production

The fractional concentration of the various fatty methyl esters (FAMEs) measured span almost 4 orders of magnitude (Fig. 2) with the longer chain fraction generally more abundant (C16 and longer) with percentages in the tens of percent. The other (C12 to C15 and C17) are around 1% or below of the total fatty acids mass or slightly higher (up to 4.3%) for C14: 0. Although the amount of lipids produced is important, as mentioned above we are also particularly interested in the potential for the production of C12: 0 and C14: 0 fatty acids. Therefore, their concentration relative to total fatty acids (w/w), is also of interest. The fatty acids profiles also show some significant changes between the treatments.

Under the low light condition treatment, all modes produced similar amounts of C12: 0 and C14: 0. Except in the low carbon conditions the heterotrophic mode led to lower C14: 0 (Fig. 2a-b-c). In the medium light treatment, the low and high carbon conditions produce higher amounts of the two desired fatty acids in the heterotrophic mode (Fig. 2d-f). Under the low carbon conditions, the production of C12: 0 (0.16 ± 0.02%) and C14: 0 (2.1 ± 0.2%) in heterotrophic mode is significantly higher than in autotrophic (0.03 ± 0.03% and 0.99 ± 0.09%, respectively) (Fig. 2d). In this same light treatment, in high carbon conditions, the heterotrophic mode produces a significantly higher amount of C12: 0 (0.46 ± 0.08%) and C14: 0 (3.6 ± 0.5%) than autotrophic (0.2 ± 0.2% and 1.6 ± 0.7%, respectively) and mixotrophic (0.09 ± 0.07% and 1.10 ± 0.04%) (Fig. 2f). In the medium carbon conditions, it is the mixotrophic cultures that produced a

smaller amount of the two desired fatty acids (Fig. 2e). Finally, in the high light condition treatment, the highest amounts of C12: 0 and C14: 0 were obtained in the heterotrophic mode (Fig. 2g-h-i) in the majority of carbon conditions. In this light treatment, under low and medium carbon conditions, the amounts of lauric and myristic acids are significantly higher in heterotrophic cultures compared to those in autotrophic and mixotrophic modes (Fig. 2g-h). Under the high carbon conditions, there is only C14: 0 production in heterotrophic mode that is significantly higher than in mixotrophic (Fig. 2i). The highest values of the two desired fatty acids were obtained in low carbon conditions. In fact, the amount of C12: 0 (1.1. ± 0.2%) produced in heterotrophic mode is about 2 times higher than in mixotrophic (0.5 ± 0.2%) and autotrophic (0.41 ± 0.07%) modes (Fig. 2g) and the amount of C14: 0 (4.3 ± 0.6%) produced in heterotrophic mode is about 2 times higher than in mixotrophic (2.3 ± 0.5%) and autotrophic (1.8 ± 0.2%) modes (Fig. 2g).

Some polyunsaturated fatty acids produced by microalgae are important because they play a role in tissue integrity and can confer beneficial effects on health (Khan et al., 2018). This is particularly the case of omega-3 and omega-6 fatty acids that are essential for humans who cannot synthesize these molecules (Khan et al., 2018). Our results showed that, in addition to the two sought fatty acids (C12: 0 and C14: 0) for the manufacture of biosurfactants, microalgae produced other interesting molecules. In fact, linolenate (C18: 3 (3) cis-9,12,15) fatty acid, an omega-3 polyunsaturated fatty acid, were produced in large quantities in the majority of experiments performed and regardless of the trophic mode used (Fig. 2). Additional income could come from producing this omega-3 polyunsaturated fatty acid, making the co-location of a microalgae culture on the site of an industrial park even more profitable.

One confounding aspect of our experimental set-up is that all treatments were not carried out at the same time. This is a result of having many different treatments that require relatively large amounts of material. In a consortium, this can lead to more variability in results as the consortium composition are invariably changing with time. This is clearly observed in the heterotrophic mode where all treatments for the same carbon

concentration should be identical irrespective of the light level of that treatment (since light is not provided to the heterotrophic mode). However, large variations are observed (e.g. compare 0.75% with 4.3% for C14: 0 production). Therefore, a key result from this study is that while consortium are more resistant to environmental changes (Muñoz et Guieysse 2006; Subashchandrabose et al. 2011), the consistency in the production of a given fatty acid will likely vary with time as the inoculum is invariably changing even if production conditions remain the same (as in this experiment). This may not be amenable for a production strategy for industries where constant production of a given fatty acids is required. However, the sturdiness to environmental changes will be a desirable feature to industries where the overall biomass or energy content of the consortium is more important.

Finally, although it was not the main objective of this study, we have measured the profile of lipids produced by a consortium which is important in the context of biodiesel production. The quality of biodiesel is determined by the amounts of each fatty acid present (Ramos et al., 2009). It has been reported that lipids composed of fatty acids with 16 to 18 carbons atoms would have good properties for biodiesel (Huang et al., 2010). On the other hand, a greater amount of saturated and monounsaturated fatty acids would provide adequate biodiesel properties, such as improved stability (Ramos et al., 2009 ; Yeh and Chang, 2012). In most of the experiments, the lipid profile analysis showed that a interesting amount of saturated and monounsaturated fatty acids is produced compared to polyunsaturated fatty acids. It suggests that oils produced by microalgae grown in industrial wastewater may be used as a biofuel source.

Conclusion

The objective of this study was to cultivate a microalgae-bacteria consortium in a mixture of industrial wastewater to generate a biomass containing two fatty acids (C12: 0-C14: 0) sought by one of the companies involved. The results showed that the consortium develops well in wastewater and that the microalgae produced small amounts of the two desired fatty acids in addition to high amount of other lipids that

could potentially be used to make other co-products. The possibility to cultivate microalgae in wastewater while generating value-added products has been demonstrated. Future work will explore other interesting bio-molecules for local uses.

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Tables

Table 1. Trophic modes and other conditions tested (irradiance, CO_2, glucose) in shake flask experiments.

Treatments	Autotrophic	Mixotrophic	Heterotrophic
LL-LC	30 μmol m^{-2} s^{-1} 1% CO_2	30 μmol m^{-2} s^{-1} 1% CO_2 Glucose	Darkness Glucose
LL-MC	30 μmol m^{-2} s^{-1} 2% CO_2	30 μmol m^{-2} s^{-1} 2% CO_2 Glucose	Darkness Glucose
LL-HC	30 μmol m^{-2} s^{-1} 5% CO_2	30 μmol m^{-2} s^{-1} 5% CO_2 Glucose	Darkness Glucose
ML-LC	200 μmol m^{-2} s^{-1} 1% CO_2	200 μmol m^{-2} s^{-1} 1% CO_2 Glucose	Darkness Glucose
ML-MC	200 μmol m^{-2} s^{-1} 2% CO_2	200 μmol m^{-2} s^{-1} 2% CO_2 Glucose	Darkness Glucose
ML-HC	200 μmol m^{-2} s^{-1} 5% CO_2	200 μmol m^{-2} s^{-1} 5% CO_2 Glucose	Darkness Glucose
HL-LC	550 μmol m^{-2} s^{-1} 1% CO_2	550 μmol m^{-2} s^{-1} 1% CO_2 Glucose	Darkness Glucose
HL-MC	550 μmol m^{-2} s^{-1} 2% CO_2	550 μmol m^{-2} s^{-1} 2% CO_2 Glucose	Darkness Glucose
HL-HC	550 μmol m^{-2} s^{-1} 5% CO_2	550 μmol m^{-2} s^{-1} 5% CO_2 Glucose	Darkness Glucose

Table 2. Extracted lipids (%) of *Chlorella sp.* under autotrophic, mixotrophic and heterotrophic conditions, after five days of cultivation.

Treatments	Extracted lipids (%)		
	Autotrophic	Mixotrophic	Heterotrophic
LL-LC	14.3 ± 3.0	13.7 ± 1.3	15.2 ± 1.2
LL-MC	12.8 ± 1.3	12.8 ± 1.4	13.5 ± 1.4
LL-HC	12.7 ± 2.7	13.0 ± 0.73	12.2 ± 1.9
ML-LC	20.3 ± 1.3	19.6 ± 3.4	15.2 ± 1.7
ML-MC	13.3 ± 2.8	19.3 ± 1.2	16.2 ± 0.63
ML-HC	17.1 ± 2.8	15.8 ± 0.29	15.6 ± 1.5
HL-LC	14.0 ± 1.8	19.0 ± 2.0	18.0 ± 2.7
HL-MC	13.9 ± 2.5	16.4 ± 1.7	14.7 ± 2.0
HL-HC	18.4 ± 1.6	17.0 ± 1.9	17.8 ± 1.7

Figure legends

Figure. 1. Biomass (g L^{-1}) of *Chlorella sp.* under autotrophic, mixotrophic and heterotrophic conditions, after five days of cultivation.

Figure 2. Fatty acid profiles of *Chlorella sp.* for the nine experiments subjected to various trophic modes: (a-b-c) low light (30 µmol m^{-2} s^{-1}), (d-e-f) medium light (200 µmol m^{-2} s^{-1}) and (g-h-i) high light (550 µmol m^{-2} s^{-1}) with different CO_2 concentrations (1, 2 and 5%), glucose addition and dark conditions, measured after 5 days of incubation. Values are expressed as % relative to total fatty acids (w/w). Letters placed in superscript mean that the treatments are significantly different from each other (a = mixotrophic or heterotrophic are different from autotrophic and b = mixotrophic and heterotrophic conditions are significantly different from each other).

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Figures

Figure 1.

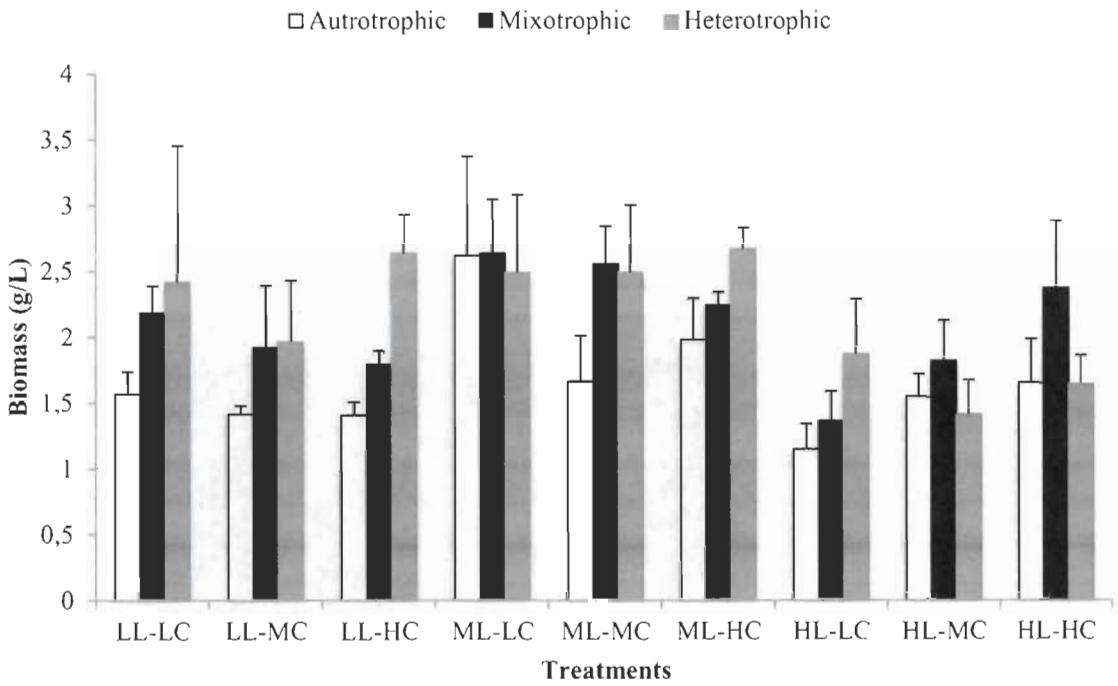


Figure 2. (a)-(b)-(c)

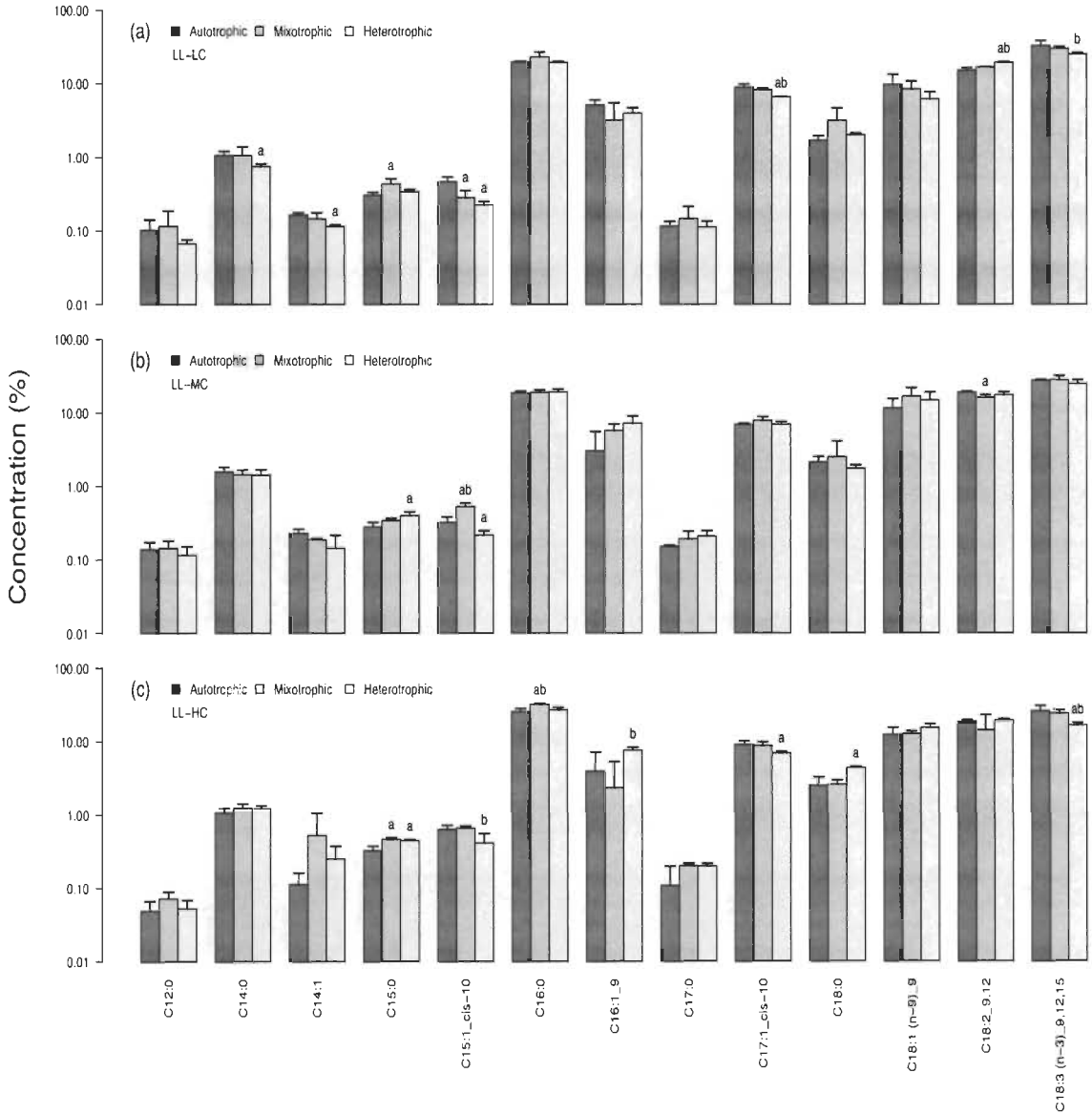

(a)
Autotrophic
Mixotrophic
Heterotrophic
LL-LC
(b)
LL-MC
(c)
LL-HC
Concentration (%)
100.00
10.00
1.00
0.10
0.01
C12:0
C14:0
C14:1
C15:0
C15:1_cis-10
C16:0
C16:1_9
C17:0
C17:1_cis-10
C18:0
C18:1 (n-9)_9
C18:2_9,12
C18:3 (n-3)_9,12,15

Figure 2. (d)-(e)-(f)

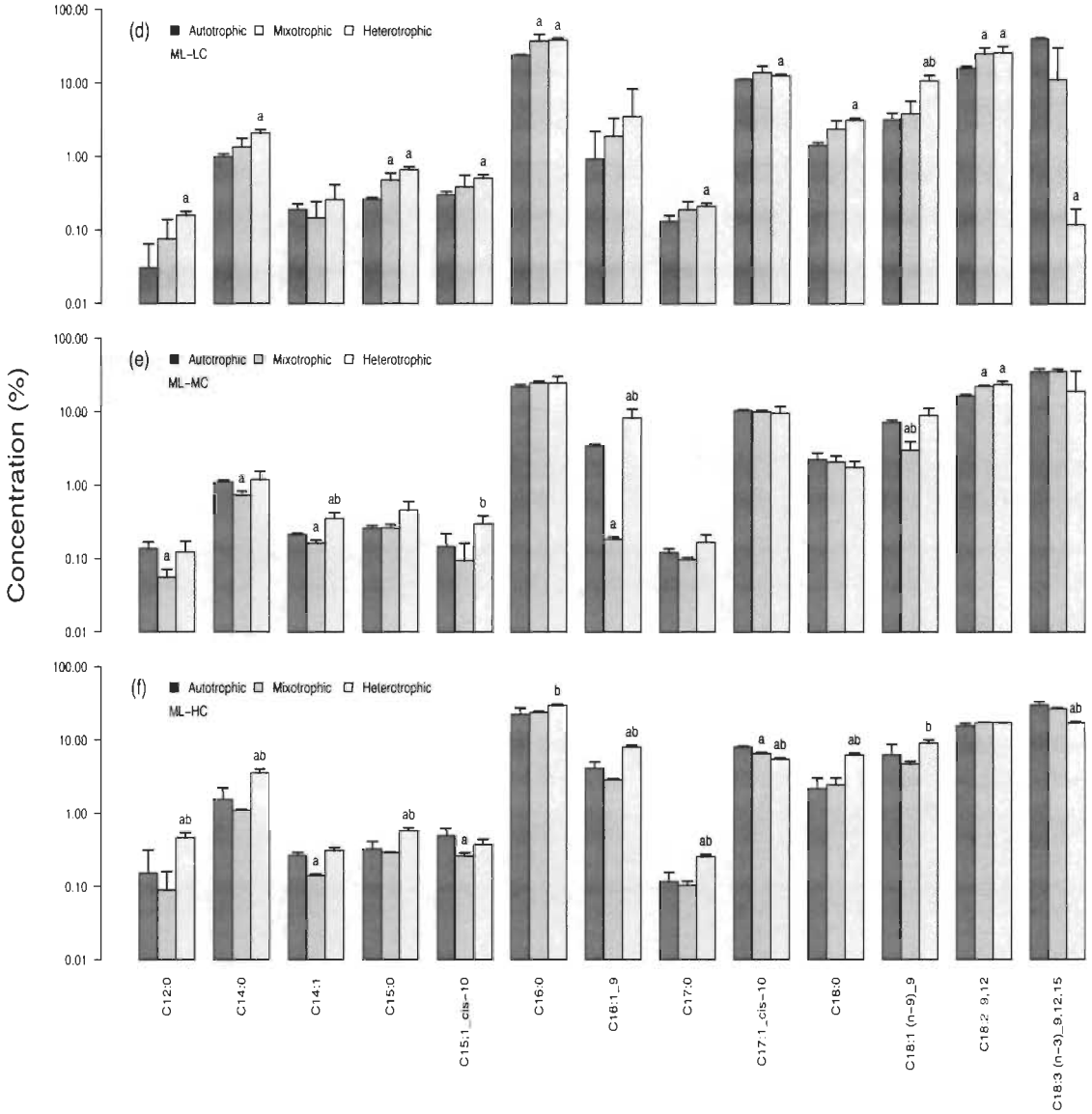
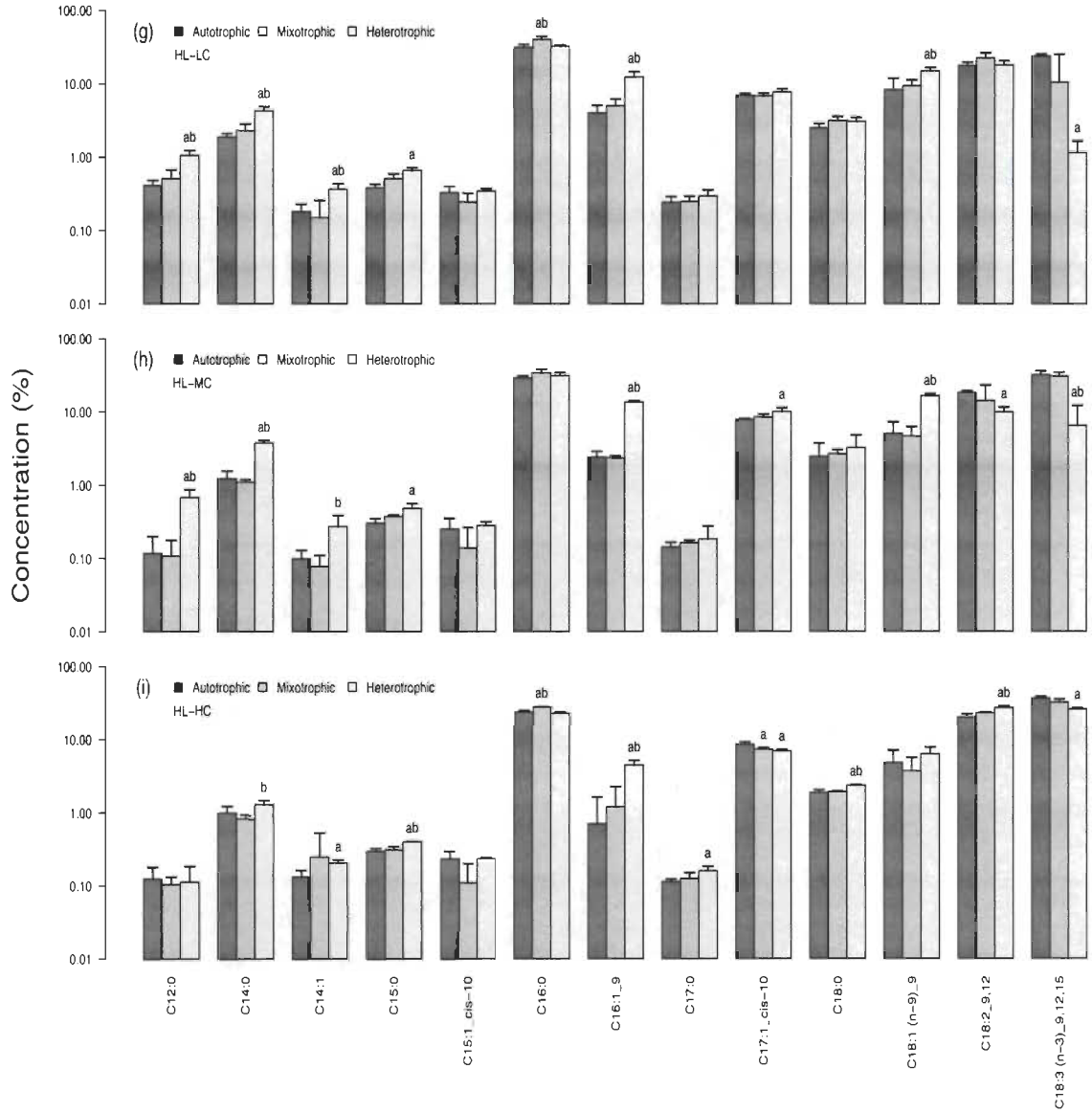

(d)
Autotrophic
Mixotrophic
Heterotrophic
ML-LC
(e)
ML-MC
(f)
ML-HC
Concentration (%)
100.00
10.00
1.00
0.10
0.01
C12:0
C14:0
C14:1
C15:0
C15:1_cis-10
C16:0
C16:1_9
C17:0
C17:1_cis-10
C18:0
C18:1 (n-9)_9
C18:2 9,12
C18:3 (n-3)_9,12,15

Figure 2. (g)-(h)-(i)


(g)
Autotrophic
Mixotrophic
Heterotrophic
HL-LC
(h)
HL-MC
(i)
HL-HC
Concentration (%)
100.00
10.00
1.00
0.10
0.01
C12:0
C14:0
C14:1
C15:0
C15:1_cis-10
C16:0
C16:1_9
C17:0
C17:1_cis-10
C18:0
C18:1 (n-9)_9
C18:2_9,12
C18:3 (n-3)_9,12,15

CHAPITRE III

CULTIVATION OF AN ALGAE-BACTERIA CONSORTIUM IN WASTEWATER FROM AN INDUSTRIAL PARK: EFFECT OF ENVIRONMENTAL STRESS AND NUTRIENT DEFICIENCY ON LIPID PRODUCTION

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Abstract

Adoption of microalgae-sourced products depends on the economic feasibility. In the case of fatty acids, it is crucial to obtain high lipid yield, especially in the form of storage lipids (TAGs). However, the production of these lipids often comes into competition with the microalgae biomass, resulting in a decrease in growth. A microalgae culture integration project was conducted in an industrial park in Canada in order to cultivate microalgae from park's wastewaters and then obtain products from the biomass. Different deficiencies and stresses were tested to evaluate what condition allowed the induction of the highest lipids accumulation without compromising the growth of microalgae. The results showed that the medium controlled to pH 7.0 allowed reaching the largest amount of extracted lipids (28 ± 4.3%). Companies involved in this project could be able to make significant savings by the reduced wastewater treatment costs and by not adding expensive nutrients in culture.

Keywords: industrial wastewaters; microalgae-bacteria consortium; induction; lipids; co-products.

Introduction

Harnessing the full potential of microalgae could make them an important biological resource as in addition to performing several functions (e.g. bioremediation of wastewater, carbon capture), some species are capable of producing significant amounts of extractable oil that can be converted to biodiesel and other co-products (McGinn et al., 2011; Rawat et al., 2011). Indeed, because of their more efficient solar energy conversion and nutrient acquisition strategies, microalgae are among the most effective photosynthetic organisms in terms of biomass and lipid productivity (Fan et al., 2014; McGinn et al., 2011). Among the different species, Chlorella sp. has been shown to be an excellent candidate for commercial oil production. It is fast growing, relatively easy to grow, and produces large amounts of protein, vitamins and minerals, pigments, fatty acids and growth factors (Fan et al., 2014; Lv et al., 2010). For several years, intensive research has been conducted to produce biofuels from microalgae oil. So far, third generation biofuel production has not proven to be profitable on a large scale.

In contrast, many of the fatty acids produced by microalgae can be used to make other products (e.g. biosurfactants, animal feeds). In addition, if the microalgae culture is integrated into a wastewater treatment system, the energy and financial inputs can be optimized while production costs will be reduced to make in overall the process more profitable (Sharma et al., 2012). Moreover, it has been shown that the use of several species (e.g. microalgae and bacteria) would confer many advantages such as greater robustness during environmental fluctuations, the exchange of certain metabolites between members of the consortium and resistance to invasion of undesirable species (Subashchandrabose et al., 2011). The use of a consortium could lead to better growth in wastewater and therefore a higher biomass could be generated for the production of co-products.

Lipids can be classified into two groups. First, the polar lipids, which consist of phospholipids and glycolipids, used to form cell membranes and, second, the neutral lipids, which include acylglycerides (di-tri and monoglycerides) and free fatty acids, used by microalgae as energy source (D'Alessandro and Antoniosi Filho, 2016).

Despite the potential benefits of lipid production by microalgae, there is a significant challenge with lipid production. Under optimal growing conditions, microalgae are capable of producing large quantities of biomass, however, under these same conditions, lipid contents are generally low, representing 5 to 20% of their dry weight (Sharma et al., 2012). This can be explained by the fact that photosynthesis products are used for the growth of microalgae rather than for the production of storage lipids (TAGs) (Sharma et al., 2012). It is essential to find a solution, stimulate the biosynthesis of lipids and make possible the commercial production of microalgae oil.

Although the production and composition of fatty acids depends on the species chosen, it has been shown that physical, chemical or environmental stress can enhance the production of these lipids (McGinn et al., 2011). In the face of adverse conditions, microalgae modify their lipid biosynthetic pathways and are able to accumulate 20 to 70% of cellular lipids per mass, mostly in the form of TAG (Illman et al., 2000; McGinn et al., 2011). Different stresses have been proposed as a solution to induce lipid production in microalgae. Temperature, pH, salinity change and nutrients reduction in the culture medium can influence lipid accumulation. Numerous studies have shown the positive impact of nutritional (Fan et al., 2014; Praveenkumar et al., 2012; Widjaja et al., 2009; Yeh and Chang, 2011) and environmental stresses (Converti et al., 2009; Subhash et al., 2014; Sushchik et al., 2003) on lipid production. Unfortunately, often, a compromise must to be made between lipid content and biomass production; important lipid content usually happen when microalgal growth is slow. Nutrient deficiency to induce lipid production by microalgae is one of the most documented strategies. There are few studies on other stress induced mostly in industrial wastewater using *Chlorella sp.* as a strain.

To reduce the costs related to the production of microalgae, it has been proposed to colocate production with an industry that could provide nutrients or a carbon source. For example, McGinn et al. (2011) proposed to install a microalgae production system near an industrial CO_2 emitter in order to capture it and feed the microalgae, which can enhance the microalgae growth and reduce industrial CO_2 emission.

Industrial wastewaters rich in nutrients (ammonia, nitrate, organic carbons, phosphorus) may also be an efficient and low cost culture media. Industries that pay significant amount each year to treat their effluents may see algae production as an opportunity to simultaneously reduce these cost and obtain valuable products (McGinn et al., 2011).

This paper is a case study in the industrial park of Victoriaville city (PQ, Canada) where a manufacturer of household and industrial cleaning products is looking into this approach to reduce its environmental impact and produce value added products for its own use. Production of household and cleaning products requires petrochemical surfactants obtained from non-renewable resources. To reduce their environmental footprint, this chemical industry is looking for bio-based molecules that could replace petroleum-based surfactants. In this particular case, the molecules required for the surfactant production are lauric (C12: 0) and myristic (C14: 0) fatty acids, which have to be converted into amine-oxides for use. These fatty acids can be found in oils naturally produced by microalgae. This company would like to obtain these two fatty acids using microalgae grown in their own wastewater. This would, potentially at the same time reduce the wastewater treatment costs with nutrient removal through algae growth and benefit from greener biosurfactants. As nutrients in their wastewater are not enough to sustain a high production of algae biomass, other local sources of cheap nutrients are required. These could be elsewhere in an industrial park waste.

This paper reports a study on microalgae growth using the wastewater from a household and cleaning product company and other wastewaters generated in its industrial park and its surrounding area. Mixtures containing the effluents of 4 companies were used as culture media to grow a microalgae-bacteria consortium. The main objective of this study was to identify environmental conditions allowing microalgae to produce a high lipid content, and more specifically, large quantities of the two desired fatty acids (C12: 0 and C14: 0). To do so, different deficiencies (nutrients) and stress (wastewater, trophic modes, salinity and pH) were tested.

Materials and methods

Wastewaters and leachates

The different wastewaters and leachates used in this study were collected from 3 industries, either a dairy industry, a pharmaceutical industry (lactulose producer) and a household and cleaning product manufacturer, that are located in the targeted industrial park. Also, leachate from a municipal solid waste landfill site was used. The samples were stored in plastic containers at 4 °C until they were used for the experiments within 2 days.

Consortium and inoculum preparation

The consortium used in this work was a native microalgae-bacteria consortium isolated from a sample taken on the site of a wastewater stabilization pond from a dairy wastewater treatment station in the industrial park of Victoriaville city (PQ, Canada). This consortium was mainly composed of *Chlorella* spp. For inoculum preparation, algal biomass was harvested from a seed culture in exponential growth phase and resuspended into Erlenmeyer flasks containing a mixture of the four collected wastewaters (45% pharmaceutical, 41% dairy, 10% chemical cleaners and 4% leachate, v/v), supplemented with minerals to mimic Bold's basal medium (BBM) (K_2HPO_4, 10 mL/L; $MgSO_4$, 10 mL/L; KH_2PO_4, 10 mL/L; major stock solution, 10 mL/L; trace metal stock solution, 1 mL/L; Boron stock solution 1 mL/L; EDTA stock solution, 1 mL/L and acidified iron stock solution, 1 mL/L). The proportion of each wastewater in the blend was determined as a function to the relative volume available in the industrial park. The inoculum was maintained in Erlenmeyer flasks on a stirring plate set at 110 rpm at 25 °C, under a photosynthetically available irradiance of 200 μmol m^{-2} s^{-1} on a 12 h/12 h light/dark cycle until algal cell concentration reached 8×10^7 algae cell mL^{-1} for further experiments.

Erlenmeyer flask experiments

Various nutrient deficiencies and environmental stress were explored (Table 1). Experiments were carried out with 10% (v/v) of the inoculum in 1L shake flasks containing 500 mL of the wastewater blend (same as described above) over a period of five days. Each experiment was performed in triplicate and had one control. Each Erlenmeyer flask was placed on a stirring plate set at 110 rpm, at 25 °C and under 200 µmol m^{-2} s^{-1}, 12 h/12 h light/dark cycle.

Analysis

Biological and physicochemical parameters

Daily pH monitoring was performed using a Symphony™ SB70P pH meter (SB70P model VWR, Radnor, USA). To follow the growth of microalgae and bacteria cells, 10 µl sample was taken and observed daily and counted with a Neubauer chamber (hemacytometer) using a phase-contrast microscope (Axio Scope A1 from ZEISS, Toronto, Canada). This monitoring also helped to ensure that the culture medium was not contaminated by undesirable species such as rotifers (Bourdeau et al., 2017).

Lipid extraction

Extraction and quantification of total lipids were performed, according to a method adapted from Bligh and Dyer (1959), gravimetrically from vacuum dried biomass with a Savant Speedvac System (Thermo Savant, model SS21, Waltham, USA) and harvested on the last day of the experiment. In summary, 50 mg of the microalgae biomass was mixed with 3 mL of methanol:chloroform (2:1 v/v). The samples then spent 24 hours in a water bath at 65 °C with continuous stirring. In order to remove cell debris, each sample was filtered (0.45 µm) using a glass syringe and rinsed twice with methanol. Finally, the extracted lipids were obtained after the solvents were evaporated under a nitrogen flow and then weighed for quantification.

Fatty acid methyl esters (FAME) profile

Fatty acids analysis was done by GC-MS after the transesterification of extracted lipids according to the method of Li et al. (2013). Briefly, 2.5 mL of BF_3-methanol (14% v/v) was added to each tube containing the extracted lipids and then placed in a water bath at 65 °C for 20 minutes. Subsequently, 2 mL of a saturated NaCl solution and 1 mL of hexane were added to each tube of samples that were stirred for 1 minute and then centrifuged for 5 minutes at 2000 *g*. The supernatant was collected for analysis of fatty acid methyl esters (FAME) performed using an Agilent (Santa Clara, USA) 7820A gas chromatograph with a DB-WAX capillary GC column (30 m, I.D. 0.25 mm, film thickness 0.25 mm) connected to a mass spectrometer (Agilent, model 5977E, Santa Clara, USA). During mass spectra registration, the injection temperature was 90 °C and helium was used as a carrier (10.5 PSI, 90 °C). Electron ionization was 70eV, the frequency 2.5 readings per second and the range 50-650 m/z. The initial temperature of 100 °C has been increased by 10 °C min^{-1} for 2 minutes and by 5 °C min^{-1} until it reached 250 °C. FAME identification and quantification were performed using the Supelco® 37 Component FAME Mix (Bellafonte, USA) mass spectrum standard (Bourdeau et al., 2017).

Statistical analysis

Variance analyzes (ANOVA) were used to compare the treatments for each data set using the JMP Pro 11 Software. We considered p value smaller than 0.05 statistically significant.

Results and discussion

To determine what conditions allow microalgae to produce a high lipid content, and more specifically, large amounts of the two desired fatty acids (C12: 0 and C14: 0), without adverse effect on growth, different deficiencies (nutrients) and stress (wastewater, trophic modes, salinity and pH) were tested. The fractional concentration

of the various fatty methyl esters (FAMEs) measured span almost 4 orders of magnitude (Fig. 1) with the longer chain fraction generally more abundant (C16 and longer).

Effect of nutrient starvation

Nitrogen

The majority of the microalgae species studied, including *Chlorella sp.*, have a higher production of TAG under nitrogen deficiency conditions (Battah et al., 2013; Converti et al., 2009; Illman et al., 2000; Widjaja et al., 2009; Yeh and Chang, 2011). We did not observe this effect in our study as there was no significant difference in the amount of lipids extracted and in the concentrations of the different fatty acids when compared to the control medium (Table 2 and 3, Fig. 1a) when we did not supplement the medium with nitrogen. There was even a decrease in total lipids extracted in the treatment with nitrogen deprivation (11.86 ± 0.94%) compared to the control treatment (12.2 ± 1.7%) (Table 2 and 3). This is likely because the nitrogen concentration initially present in the wastewater mixture was sufficient to support the balanced growth of microalgae over 5 days without increased lipids accumulation.

It has been reported that higher lipid production through nitrogen deficiency requires several days, generally more than 7 (Battah et al., 2013; Widjadja et al., 2009; Yeh and Chang, 2011), of culture and a decrease in microalgae growth rate would also result from this stress and consequently decrease the total lipids production. In our study, microalgae were subjected to nitrogen limitation conditions for only 96 hours. Over this period, the growth rate was not different from the control (Fig. 2), which suggest that nitrogen did not become limiting enough to cause stress and induce higher lipid accumulation.

Phosphorus

Phosphorus is an essential element in the energy metabolism of microalgae in addition to being required in large quantities to ensure the synthesis of proteins, nucleic acids,

lipids and intermediates of carbohydrate metabolism (Cai et al., 2013; Gonçalves et al., 2017). In the treatment were phosphorus was not added, a decrease in growth is expected as well as an increase in lipids caused by this stress. As for the nitrogen exclusion, over the five days, the microalgae growth that have been subjected to phosphorus exclusion have not had significantly lower growth and therefore, phosphorus did not become strongly limiting for growth. The cell density obtained on the last day of culture is slightly lower (0.65 ± 0.01 cell $L^{-1} \times 10^{10}$) compared to the control (0.76 ± 0.06 cell $L^{-1} \times 10^{10}$), but growth is observed from day 2 to day 5 (Fig. 3) albeit slower on the last two days.

On the other hand, this stress had a positive impact on lipid production. Indeed, the microalgae subjected to this deficiency had a higher amount of extracted lipids ($14.72 \pm 0.73\%$) compared to the microalgae of the control medium ($12.2 \pm 1.7\%$) (Table 2 and 3). Finally, two polyunsatured fatty acids were produced in significantly greater amounts than control. These are oleic (C18:1 (9) cis-9) and linolenate (C18:3 (3) cis-9,12,15) fatty acids (Fig. 1a). These results are similar to those obtained by Praveenkumar et al. (2012). In their study, there was no significant increase in lipid content for microalgae grown without added phosphorus treatment compared to those grown in control medium while oleic acid (C18:1 (9) cis-9) was found to be dominant when phosphorus deprivation occurred. A slight increase in lipid content was also observed in a study by Fan et al. (2014) in a culture of microalgae deficient in phosphorus compared to a control culture medium.

Iron

Iron is a micronutrient essential for the growth of microalgae. Although there are few studies on iron concentration in the culture medium and lipid accumulation, it has been shown in a study by Liu et al. (2008) that a high concentration of iron induced a high lipid accumulation while increasing the growth of the microalgae *Chlorella vulgaris*. To evaluate the impact of iron concentration in the culture medium on microalgae growth and lipid production, a treatment containing no iron (no additions at the

beginning of culture) was compared with controls containing iron. The results showed that no significant difference was observed in terms of the lipid profile, the amount of lipids extracted and the two fatty acids lauric and myristic produced (Fig. 1c, Table 3). The same thing is observed for growth. No significant difference was observed between the cell densities of microalgae control (1.18 ± 0.23 cell $L^{-1} \times 10^{10}$) compared to those who did not receive iron at the beginning of culture (1.15 ± 0.15 cell $L^{-1} \times 10^{10}$). Therefore, iron was not limiting to growth, as there was likely plenty in the wastewater media and it did not affect lipid content.

Other nutrients

The results obtained by comparing the microalgae in the control treatments compared to those which were not supplemented with nutrients at the beginning of the experiments, showed that there were only small differences in the lipid profile (Fig. 1a). On the other hand, no significant difference is observed in the amount of lipids extracted, C12: 0 and C14: 0 and the cell density compared to the control (Table 2 and 3). This indicates that the microalgae-bacteria consortium used can grow well and produce lipids without the addition of nutrients in the culture medium. This would potentially represents savings in the process for the industries involved, as the addition of nutrients to the culture media represents sizeable amounts in the microalgae production process. As no significant difference is observed both in terms of growth and lipid production, this indicates that the necessary nutrients for microalgae are already in the wastewater mixture.

Treatment for exclusion of groups of nutrients did not show any significant differences with controls either in the for the accumulation of lipids (Fig. 1b). There is, however, a significant greater amount of fatty acids relative to biomass in the treatment relative to the control when macronutrients where excluded (Table 2 and 3). For the rest, no significant difference is observed.

Finally, for all other nutrients that were not added at the beginning of culture and tested one by one, no significant difference was observed in the lipid profile, the lipids

extracted and the amount of the two desired fatty acids C12: 0 and C14: 0 products (Fig. 1a-b-c-d-e and Table 3). These results highlight, again, that the addition of BBM nutrients in the culture medium would not be necessary to maintain productivity of microalgae in terms of growth and lipids over 5 days.

Effect of trophic mode

Microalgae can be grown according to different growth conditions that are characterized by the energy and carbon sources used whether autotrophy, heterotrophy and mixotrophy. These modes will vary among different species and will significantly influence growth rate and lipid accumulation (Yeh and Chang, 2012). The growth of microalgae in autotrophic conditions will be directly influenced by the irradiance and CO_2 concentration (Carvalho et al., 2006). In this condition, it is generally more difficult to achieve the highest growth rates and lipid productivity at high densities because of the availability of light and CO_2, which is often become limiting to growth. Microalgae grown in the heterotrophic mode will use organic compounds (e.g. glucose) as a carbon source and do not require light for growth. Under these conditions, high productivity in algal biomass as well as high oil content have been reported (Liang et al., 2009; Xu et al., 2006). The costs associated with adding carbon to the culture medium, however, represent a considerable disadvantage. Finally, the mixotrophic mode combines the two previous conditions by using both light as a source of energy and organic compounds as a source of carbon (Sun et al., 2008). This trophic mode allows to at least partially overcome some of the problems found in the two other modes and it has been shown that the synergy of these two processes improves the growth of microalgae (Bhatnagar et al., 2011; Heredia-Arroyo et al., 2011).

The three different trophic modes were tested in order to know which one achieves the highest cell density and the best lipid production, including our two desired fatty acids. The microalgae grown under mixotrophic conditions reached the highest cell density ($6.8 \pm 1.2 \times 10^{10}$ cell L^{-1}). This value was significantly higher than those obtained in autotrophy and heterotrophy (Table 2 and 3). The results showed that the mixotrophic

microalgae cells also reached significantly higher lipid contents (16.09 ± 0.53%) compared to the autotrophic (13.5 ± 1.0%) and heterotrophic (13.0 ± 1.4%) conditions (Table 2 and 3). In addition, no significant difference is observed between autotrophic and heterotrophic cells. Regarding the relative amount of lauric (C12: 0) and myristic (C14: 0) acids produced, there is no significant difference between the three conditions (Fig. 1f). These results concerning growth are similar to other studies (Heredia-Arroyo et al., 2011; Liang et al., 2009) that showed that the highest productivities were obtained in mixotrophy for *Chlorella vulgaris*. Liang et al. (2009) also achieved the best lipid productivity with mixotrophic microalgae cells.

Effect of salt

Many studies have shown that salt stress can improve lipid production (Church et al., 2017; Heredia-Arroyo et al., 2011; Pandit et al., 2017; Rai et al., 2015; Shen et al., 2015; Wang et al., 2016). In our study, the addition of NaCl to the culture medium did not increase the extracted lipids content (9.88 ± 0.56%) compared to control (12.28 ± 0.83%) (Table 2 and 3), but several interesting results were found.

Firstly, the production of lauric acid (C12: 0) is significantly higher with microalgae subjected to salt stress (1.39 ± 0.60%) compared to control (0.08 ± 0.05%). It is also the same thing that is observed for the myristic acid (C14: 0) that is produced in a significantly higher quantity in saline medium (3.4 ± 1.1%) compared to the control (0.80 ± 0.21%). These results are very important because these molecules will be extracted and converted into amine-oxide for the production of co-products, biosurfactants, which will be used by the company involved in this project.

Finally, in terms of lipid profile, our results are similar to those obtained in other studies concerning *Chlorella vulgaris* (Church et al., 2017; Pandit et al., 2017). Indeed, under salt stress, microalgae produced a greater amount of saturated fatty acids and a smaller amount of polyunsaturated fatty acids. The saturated fatty acids; lauric (C12: 0), myristic (C14: 0), pentadecylic (C15: 0), palmitic (C16: 0), margaric (C17: 0) and stearic

(C18: 0) were produced in larger quantities by microalgae subjected to stress than those in a controlled medium. In contrast, polyunsaturated fatty acids; pentadecenoic methyl ester (C15:1 cis-10), heptadecenoic methyl ester (C17:1 cis-10), linoleate (C18:2-cis 9,12) and linolenate (C18: 3 (3) cis-9,12,15) were produced in smaller amounts compared to control (Fig. 1d).

Effect of pH

Many nutritional factors have been recognized to significantly affect lipid induction, but there are also physical factors such as temperature and pH that significantly influence cell growth and lipid synthesis. The pH status of the medium directly influences the physiological parameters of microalgae such as membrane permeability and cell morphology (Liang et al., 2011). A change in pH in the culture medium will therefore affect the membrane osmosis of certain ions and the absorption of substances thus affecting cell growth and lipid synthesis (Liang et al., 2011). In some studies (Gardner et al., 2011; Guckert et Cooksey, 1990), it has been proposed that at high pH in the culture medium, there would be a greater accumulation of TAG.

To test the influence of pH on microalgae growth and lipid accumulation, two different pH values (7 and 10) were tested in order to compare the data with those of the control medium (in which the pH increased from 8 to 10 over 5 days). The growth and lipid production was highest at pH 7. In fact, the cell density (cell L^{-1}), the lipids extracted (%) and the production of C12: 0 and C14: 0 (%) are significantly higher in microalgae grown at pH 7 compared to the control (Table 2 and 3). Regarding the results of the control compared to treatment at pH 10, there is no significant difference, except that the cell density was significantly higher in the control.

The results obtained do not agree with those of the two studies mentioned. This could be explained by the fact that the observed effect of a high pH that allows the accumulation of TAG would be amplified when there is nitrogen deficiency. Indeed, Gardner et al. (2011) showed that the effect of TAG accumulation at high pH was even more

pronounced when there was a drop of nitrates in the culture medium. They observed that TAG accumulation remained constant throughout the culture and once there was nitrogen depletion there was an accumulation of TAGs that was observed. Since the only stress induced in culture media is pH control, the expected effect may not be observable. Nutrient stress of nitrogen combined with controlled pH at a value of 10 may have shown the expected effects. Once again, if the cultivation had been carried out for more than 5 days, there would have been nitrogen depletion at some point and perhaps a large accumulation of TAG would have been observed.

Finally, the addition of carbon in acetic acid could have positively affected the growth of microalgae and the production of lipids if it contributed to alleviate a possible carbon limitation (assuming it is available for growth).

Effect of wastewaters

As the microalgae-bacteria consortium of microalgae bacteria used is well adapted to the wastewater blend, an experiment was carried out by cultivating it in a BBM medium, i.e. in demineralized water with nutrients, in order to know what would be the impacts on the microalgae growth and lipid production. The results showed that there is no significant difference between the growths of microalgae in control medium, i.e. wastewater blend and nutrients compared to those grown in BBM. For the amount of lipids extracted, BBM microalgae cells produced a greater proportion (16.80 ± 0.15%) than cells from those grown in wastewater (12.02 ± 0.56%) (Table 2 and 3). With regard to the composition of the lipids, especially the two desired fatty acids, no significant difference was observed for the production of lauric acid (C12: 0), but a there was an almost 4 times increase in myristic acid (C14: 0) was produced in microalgae cells cultured in wastewater (1.40 ± 0.42%) compared to those grown in BBM medium (0.36 ± 0.14%) (Fig. 1e and Table 2 and 3).

The results obtained are interesting because they show that the yield of microalgae grown in wastewater for growth and lipid production can be the same, or even better,

than those obtained with the standard medium. These results support what has been demonstrated in several studies over the last few years regarding the use of microalgae technologies in wastewater (Bhatnagar et al., 2011; Chinnasamy et al., 2010; Prajapati et al., 2013; Rao et al., 2011; Rawat et al., 2011; Woertz et al., 2009). Indeed, the microalgae used in this project could play a role of bioremediation of wastewater in addition to generating a biomass that will be used to produce a high value-added product. As shown, the microalgae are very successful in growing in these wastewaters in addition to producing the two targeted fatty acids, C12: 0 and C14: 0, that will be used by the household and cleaning product manufacturer to manufacture biosurfactants.

FAME profile

The structure of the fatty acid chains that make up TAGs is important. For example, it determines the type and quality of biodiesel produced during transesterification (Ramos et al., 2009). When there is a large amount of saturated and monounsaturated fatty acids, the expected biodiesel will have good stability, but low cold flow properties. On the other hand, if the polyunsaturated fatty acid content is high, the biofuel produced will have better cold flow properties but low stability.

Our results of fatty acid profiles (Fig. 1) showed that a large proportion of saturated and monounsaturated fatty acids are produced compared to polyunsaturated acids. This suggests that oils produced by microalgae grown in industrial wastewater could potentially be used as a biofuel source with good stability properties but poorer cold flow properties. Although few C12: 0 and C14: 0 fatty acids are produced by our consortium, they can be used for the production of biosurfactants and other fatty acid molecules can be used for biodiesel production.

Conclusion

Among all the stress conditions that were tested, the pH control at 7 during cultivation allows the highest amount of lipids extracted was reached (28 ± 4.3%). As for the largest

quantities of the two desired fatty acids, C12: 0 and C14: 0, they were obtained in the treatment which had no nutrient supplement (2.22 ± 0.78% and 6.42 ± 2.3%, respectively). These results show that the fatty acids sought can be produced without requiring the addition of nutrients, which will make it possible significant savings on the cost of wastewater treatment and the addition of nutrients in culture media.

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Tables

Table 1. Erlenmeyer flask experiments. Conditions of each experiment carried out.

Treatments	Conditions
Nutrient starvation	• Omitting one of the added nutrients at a time; • Omitting groups block; all nutrients, micro and macronutrients, trace metal solution, boron stock solution and alkaline EDTA solution.
Trophic modes	• Autotrophic, exposed to light only; • Heterotrophic, no light with addition of 2 g L^{-1} of glucose; • Mixotrophic, exposed to light with addition of 2 g L^{-1} of glucose.
Wastewater	• Media free of wastewater (demineralized water and BBM nutrients only) was compared to those grown in the wastewater blend.
Salinity	• 35 g L^{-1} of NaCl was added to the culture medium.
pH	• pH 7, controlled with acetic acid (CH_3COOH, 5% v/v); • pH 10, controlled with NaOH (1 M).

Table 2. Percentage of extracted lipids relative to dry weight, percentage of C12: 0 and C14: 0 (%) relative to total fatty acids and cell density (cell L^{-1}) for the controls after five days of culture (n = 3; mean ± standard deviation) in the seven experiments.

Experiment	Extracted lipids (%)	C12: 0 (%)	C14: 0 (%)	Cell density (cell $L^{-1} \times 10^{10}$)
1	12.2 ± 1.7	1.74 ± 0.10	4.85 ± 0.20	0.76 ± 0.06
2	12.3 ± 1.1	0.36 ± 0.05	2.61 ± 0.73	1.18 ± 0.23
3	12.02 ± 0.56	0.17 ± 0.17	1.40 ± 0.42	1.09 ± 0.17
4	14.0 ± 1.4	0.07 ± 0.04	1.68 ± 0.72	1.24 ± 0.23
5	12.28 ± 0.83	0.08 ± 0.05	0.80 ± 0.21	1.09 ± 0.16
6	13.5 ± 1.0	0.07 ± 0.4	1.04 ± 0.57	1.50 ± 0.36
7	14.36 ± 0.13	0.06 ± 0.04	0.67 ± 0.12	0.99 ± 0.11

Table 3. Percentage of extracted lipids relative to dry weight, percentage of C12: 0 and C14: 0 (%) relative to total fatty acids and cell density (cell L^{-1}) after five days of culture for each treatment (n = 3; mean ± standard deviation) for the seven experiments. Numbers in parenthesis next to each treatment refer to the associated control experiment from Table 2.

Treatments	Extracted lipids (%)	C12: 0 (%)	C14: 0 (%)	Cell density (cell $L^{-1} \times 10^{10}$)
Deficiency:				
Nutrient (1)	14.1 ± 1.1	2.22 ± 0.78	6.4 ± 2.3	0.67 ± 0.08
Phosphorus (1)	14.72 ± 0.73	1.58 ± 0.21	4.74 ± 0.61	0.65 ± 0.01
Nitrogen (1)	11.86 ± 0.94	1.73 ± 0.79	4.7 ± 1.8	0.68 ± 0.05
Magnesium (2)	11.11 ± 0.50	0.22 ± 0.05	2.28 ± 0.87	1.28 ± 0.03
Iron (2)	10.1 ± 1.1	0.33 ± 0.15	2.06 ± 0.50	1.15 ± 0.15
Potassium (2)	12.96 ± 0.50	0.25 ± 0.06	1.75 ± 0.41	0.99 ± 0.05
Calcium (3)	9.78 ± 0.15	0.40 ± 0.66	1.44 ± 0.88	1.02 ± 0.05
Alkaline EDTA (3)	9.3 ± 1.2	0.28 ± 0.16	0.91 ± 0.32	1.01 ± 0.10
Micronutrients (4)	12.13 ± 0.13	0.07 ± 0.05	0.88 ± 0.36	1.14 ± 0.11
Macronutrients (4)	16.0 ± 1.3	0.03 ± 0.04	0.60 ± 0.05	1.33 ± 0.10
Sodium (4)	12.00 ± 0.38	0.11 ± 0.02	0.76 ± 0.07	1.16 ± 0.04
Trace metal (5)	12.36 ± 0.28	0.06 ± 0.02	0.76 ± 0.33	1.00 ± 0.07
Boron stock (5)	12.21 ± 0.61	0.07 ± 0.03	0.67 ± 0.19	1.02 ± 0.05
Stress:				
BBM (3)	16.80 ± 0.15	0.04 ± 0.03	0.36 ± 0.14	1.35 ± 0.07
Mixotrophy (6)	16.09 ± 0.53	0.11 ± 0.04	1.05 ± 0.28	6.8 ± 1.2
Heterotrophy (6)	13.0 ± 1.4	0.20 ± 0.09	1.52 ± 0.30	3.97 ± 0.61
Salinity (5)	9.88 ± 0.56	1.39 ± 0.60	3.4 ± 1.1	0.84 ± 0.08
pH 7 (7)	28.0 ± 4.3	0.55 ± 0.08	2.39 ± 0.63	3.86 ± 0.12
pH 10 (7)	12.03 ± 0.95	0.10 ± 0.09	1.3 ± 1.1	0.78 ± 0.17

Figure legends

Figure 1. Fatty acid profiles of *Chlorella sp.* for the seven experiments subjected to various stresses: nutrients starvation (a-b-c-d-e), salinity (d), BBM medium (e), trophic modes (f) and pH variation (g), measured after 5 days of incubation. Values are expressed as % relative to total fatty acids (w/w). Asterisk means that the value is significantly different from the control ($p < 0.05$).

Figure 2. Cell density (cell L^{-1}) of *Chlorella sp.* under control and nitrogen limitation over five days of culture.

Figure 3. Cell density (cell L^{-1}) of *Chlorella sp.* under control and phosphorus limitation over five days of culture.

Figures

Figure 1. (a)-(b)-(c)

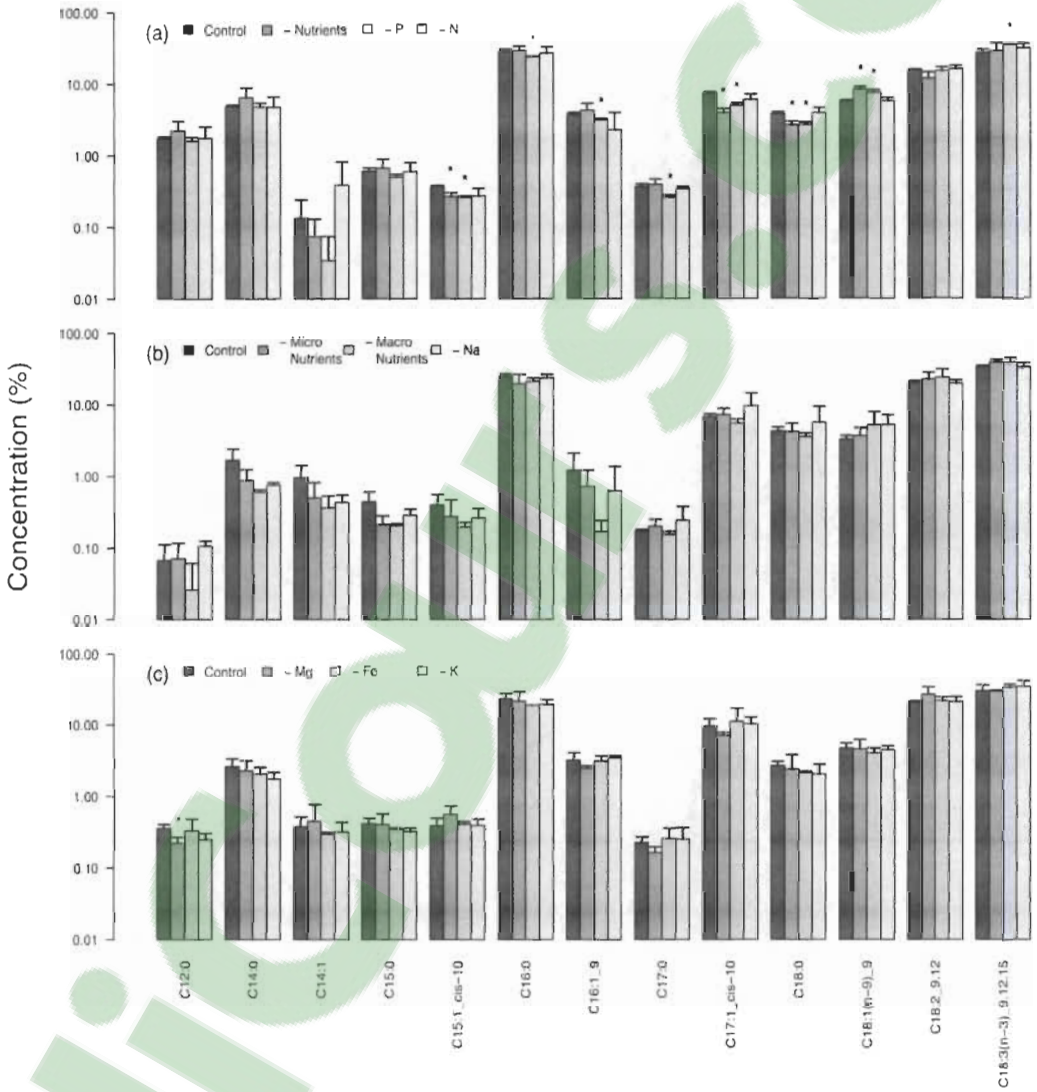

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Figure 1. (d)-(e)-(f)

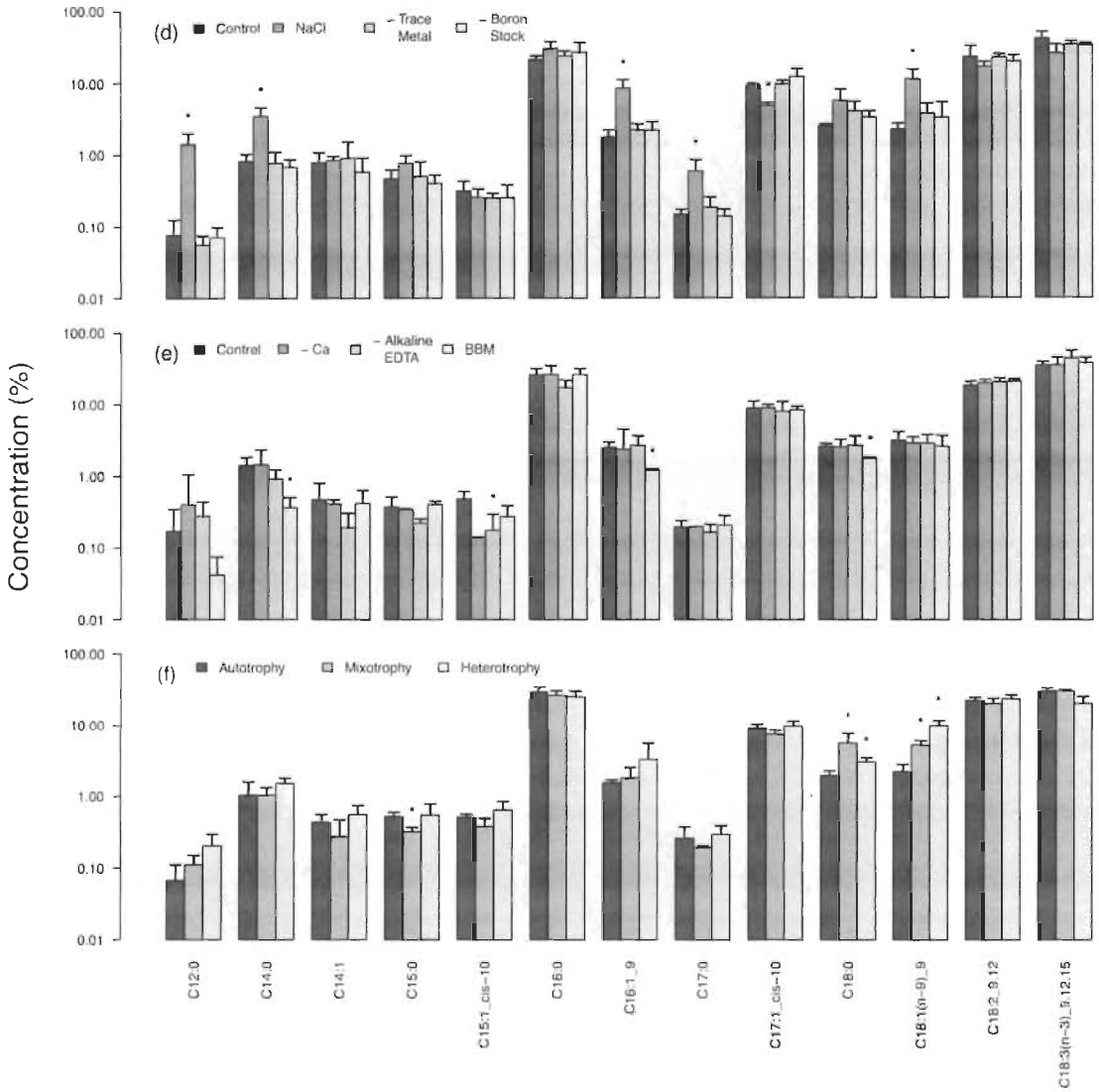

(d) Control NaCl - Trace Metal - Boron Stock
(e) Control - Ca - Alkaline EDTA BBM
(f) Autotrophy Mixotrophy Heterotrophy
Concentration (%)
100.00
10.00
1.00
0.10
0.01
C12:0
C14:0
C14:1
C15:0
C15:1_cis-10
C16:0
C16:1_9
C17:0
C17:1_cis-10
C18:0
C18:1(n-9)_9
C18:2_9,12
C18:3(n-3)_9,12,15

Figure 1. (g)

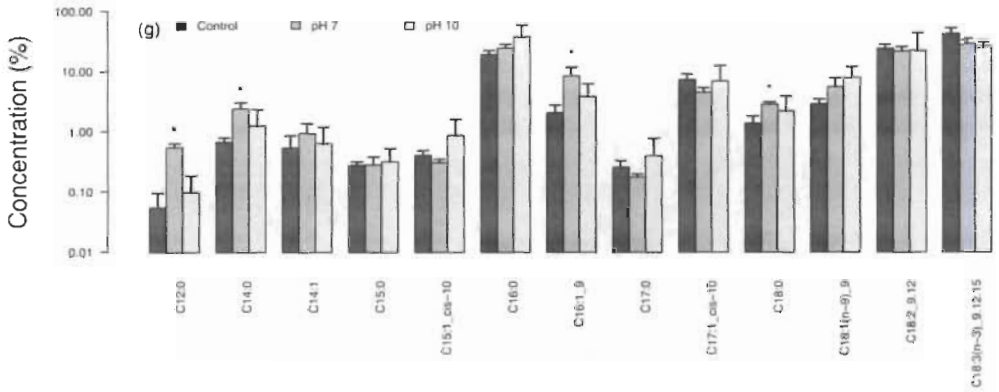

(g)
Control
pH 7
pH 10
Concentration (%)
100.00
10.00
1.00
0.10
0.01
C12:0
C14:0
C14:1
C15:0
C15:1_cis-10
C16:0
C16:1_9
C17:0
C17:1_cis-10
C18:0
C18:1(n-9)_9
C18:2_9,12
C18:3(n-3)_9,12,15

Figure 2.

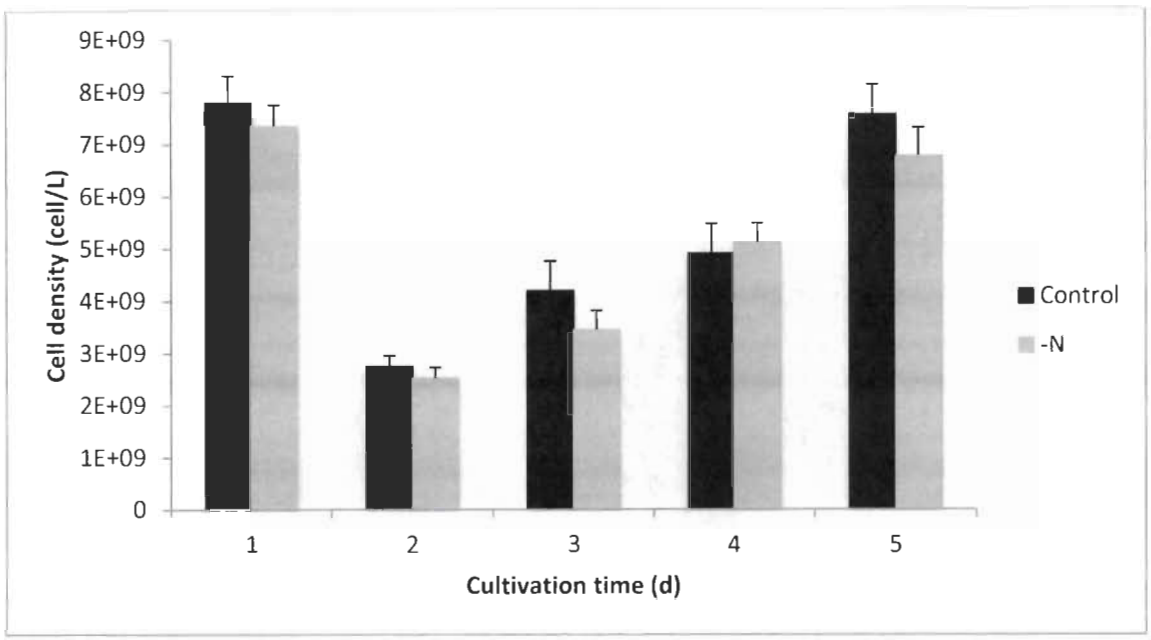
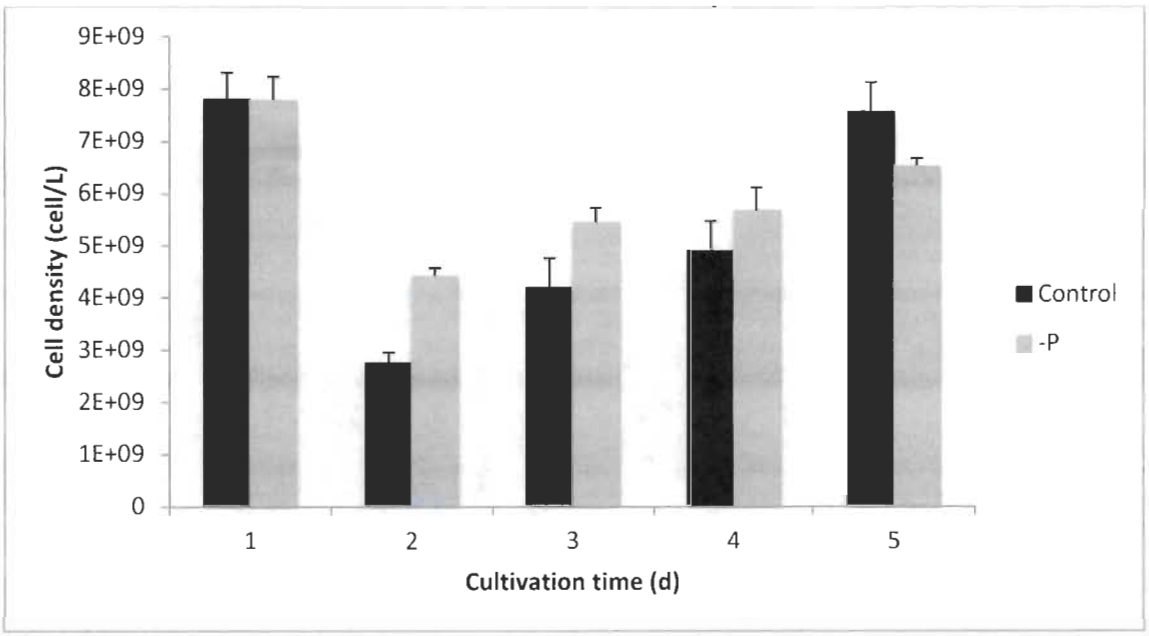

9E+09
8E+09
7E+09
6E+09
5E+09
4E+09
3E+09
2E+09
1E+09
0
Cell density (cell/L)
1
2
3
4
5
Cultivation time (d)
Control
-N

Figure 3.


9E+09
8E+09
7E+09
6E+09
5E+09
4E+09
3E+09
2E+09
1E+09
0
Cell density (cell/L)
1
2
3
4
5
Cultivation time (d)
Control
-P

CHAPITRE IV

EFFECT OF TWO-STAGE CULTURE AND STRESS ON CELL GROWTH AND LIPID PRODUCTION OF *CHLORELLA SP.* IN INDUSTRIAL WASTEWATER

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Abstract

Microalgae are considered as a potential renewable resource because of their rapid growth rate and the large amounts of oil they can produce. On the other hand, a compromise between the biomass and the quantity of lipid produced is often observed because of the competition between these two factors. A two-stage culture strategy represent an interesting solution for balancing the biomass obtained and the lipids produced. The cell density and the amount of lipids extracted from microalgae grown in single-stage cultures were compared to those grown in two-stage cultures. In the first stage, all the flasks are subjected to mixotrophic conditions and, in the second stage, the microalgae are transferred to an environment where stress and culture conditions (e.g. pH, salinity) were induced. Mixtures containing industrial wastewaters from four companies were used as culture media for the growth of microalgae. The results showed that the two-stage culture strategy was not the most effective for increasing lipid content and cell density. The one-stage cultivation, under mixotrophic conditions, reached better results in terms of cell density (cells L^{-1}) and total lipids produced (%). In addition, it has been observed that the induced stress by pH adjustment and salt addition resulted in higher lipid levels (22-25%).

Keywords: culture strategy, industrial wastewater, lipid production, salinity, pH.

Introduction

Microalgae are currently considered as a potential source of renewable material. They have more effective strategies for converting light energy and acquiring nutrients while having much higher biomass productivity rates than other agricultural crops (e.g. soy) (McGinn et al., 2011). They can produce significant amounts of extractable oil that can be transesterified and converted to biodiesel (McGinn et al., 2011). Microalgae can also grow in a wide range of aquatic environments from freshwater lakes and rivers to extreme environments such as municipal and industrial wastewater (Chinnasamy et al., 2010; Khan et al., 2018; McGinn et al 2011). Of particular interest, when grown in wastewater they remove nutrients and metals while producing biomass for the production of biofuels or other useful co-products such as carotenoids, feed additives and biomolecules (Chinnasamy et al., 2010; Khan et al. 2018; Mulbry et al., 2008; Rawat et al., 2011; Rao et al., 2011).

Despite the many benefits that microalgae provide, it is difficult to achieve high cell density and total lipid production at the same time. It has been reported that the percentage by dry weight of microalgae can vary between 1.5 to 75% depending on the environment and the culture strategy (D'Alessandro and Antoniosi Filho 2016). High lipid content can be obtained naturally, depending on the species cultivated, but it has been shown that under stress conditions, microalgae modify their lipid biosynthesis pathways towards the formation and accumulation of neutral lipids, mainly under triacylglyceride (TAG) form (Sharma et al., 2012). Nutrient deficiency, especially nitrogen (Battah et al., 2013; Converti et al., 2009; Illman et al., 2000; Widjaja et al., 2009; Yeh and Chang, 2011), salinity (Church et al., 2017; Heredia-Arroyo et al., 2011; Pandit et al., 2017; Rai et al., 2015; Shen et al., 2015; Wang et al., 2016), temperature (Sushchik et al. 2003; Converti et al. 2009; Subhash et al. 2014) and pH (Gardner et al., 2011; Guckert et Cooksey, 1990; Liang et al. 2011) are factors that influence stress-induced lipid accumulation.

Unfortunately, even if the stress induced in the culture increases the accumulation of lipids, the growth rate of the microalgae is reduced, which negatively affects the overall

productivity of the lipids (Converti et al., 2009; Yeh and Chang 2011). In contrast, under optimal growing conditions, microalgae produce high biomass, but only 5 to 20% of lipids per dry weight (Sharma et al., 2012). This can be explained by the fact that biomass and TAG production compete for the same photosynthetic energy (Sharma et al., 2012). A compromise must thus be made between the production of biomass and lipids. A two-stage culture strategy is therefore an interesting solution. In the first stage, growth conditions would promote biomass production and in the second stage, stress would increase the lipid content. Only few studies have been published on this approach, and none when growing microalgae in wastewater based culture media.

In a previous study, we identified the growth conditions that led to the highest lipid content and more particularly the largest quantities, relative to the total fatty acids, C12: 0 and C14: 0 (Bélanger-Lépine et al., 2018). The results of this study showed that among all conditions tested, pH control at 7 during culture yielded the largest amount of extracted lipids (28 ± 4.3%). While the addition of NaCl (35 g L^{-1}) to the culture yielded larger amounts of C12: 0 (1.39 ± 0.60%) and C14: 0 (3.4 ± 1.1%) compared to the control. This study is a follow-up this work on microalgae growth using the wastewater from a household and cleaning product company and other wastewaters generated in its industrial park and its surrounding area. In both studies, an algae-bacteria consortium is grown in a mixture of effluents from four companies. Here, the experiments aimed to identify a culture strategy to achieve a high lipid content and obtain large fractions, relative to total fatty acids, of C12: 0 and C14: 0 fatty acids, sought by one of the companies involved, while also producing high cellular densities. Cell density and lipid content obtained in single-stage cultures were compared to two-stage cultures. In parallel, different stresses and culture conditions applied on the microalgae culture (pH, salinity, heterotrophic mode, type of wastewater) were studied.

Materials and methods

Origin of wastewaters

Three industrial wastewaters and one landfill leachate were used in this study. The wastewaters were collected from industries located in the Victoriaville (Quebec, Canada) industrial park: a dairy industry, a pharmaceutical industry (lactulose production) and a household and cleaning product manufacturer. The leachate comes from a municipal solid waste landfill site in the area of the industrial park. The wastewaters and leachate were stored in plastic containers at 4 °C until they were used for the experiments within 2 days.

Consortium and inoculum preparation

The consortium used in this work was a native microalgae-bacteria consortium, mainly composed of *Chlorella* spp. isolated from a sample taken on the site of a wastewater stabilization pond from a dairy wastewater treatment station in the industrial park of Victoriaville city (Quebec, Canada). Briefly, algal biomass was harvested from a seed culture in exponential growth phase and resuspended into Erlenmeyer flasks containing a mixture of the four collected wastewaters (45% pharmaceutical, 41% dairy, 10% chemical cleaners and 4% leachate, v/v), supplemented with minerals to mimic Bold's basal medium (BBM) as described in Bélanger-Lépine et al. (2018). The inoculum was maintained in Erlenmeyer flasks on an orbital shaker set at 110 rpm at 25 °C, under a photosynthetically available irradiance of 200 μmol m^{-2} s^{-1} on a 12 h/12 h light/dark cycle until algal cell concentration reached 1 x 10^8 algae cell mL^{-1} for further experiments.

Erlenmeyer flask experiments

Different environmental stresses have been explored for a two-stage culture strategy (Table 1). In the first step all the treatments were placed under the same mixotrophic conditions to optimize the growth, either under cool-white fluorescent provided

200 µmol m^{-2} s^{-1}, 12 h/12 h light/dark cycle, placed on an orbital shaker set at 110 rpm, at 25 °C and with addition of glucose (2 g L^{-1}). After three days of culture, 50% of inoculum has been transferred to a second growth medium where the stress is induced (Table 1). The influence of CO_2 injection has also been tested for one and two-stage culture strategies. Concentration of CO_2 in the gas was adjusted to 5% with 21% O_2 and nitrogen as the remaining portion. It was bubbled, at rate of 0.42 L min^{-1}, through a porous sparing stone in the culture media for 15 minutes every hour during the light phase only. The experiments were performed with 10% (v/v) of the inoculum in 1-liter shake flasks containing 500 ml of the wastewater mixture (as described above) over a period of five days. Each experiment was performed in triplicate.

Analysis

Biological and physicochemical parameters

Cell density and pH monitoring were performed on a daily basis. A sample was taken and observed daily and counted with a Neubauer chamber (hemacytometer) using a phase-contrast microscope. This monitoring also helped to ensure that the culture medium was not contaminated by undesirable species such as rotifers (Bourdeau et al. 2017).

Lipid extraction and fatty acid methyl esters (FAME) profile

Lipids were quantified as described in Bélanger-Lépine et al. (2018), based on Bligh and Dyer (1959) method. Briefly, vacuum dried algae were extracted using methanol and chloroform and weighted after evaporation. Fatty acids were analyzed by GC-MS after the transesterification of extracted lipids as described in Bélanger-Lépine et al. (2018) according to the method of Li et al. (2013).

Statistical analysis

Variance analyzes (ANOVA) were used to compare the treatments for each data set using the JMP Pro 11 Software. We considered p value smaller than 0.05 statistically significant.

Results and discussion

Effect of pH

It has been reported that a change in pH in the culture medium would affect the membrane osmosis of some ions and the uptake of substances that would affect cell growth and lipid synthesis (Liang et al., 2011). In a previous study, the pH controlled at 7 made it possible to reach the highest lipid content in a one-stage culture (Bélanger-Lépine et al., 2018). The two-stage culture strategy was tested to determine whether this stress still achieves high lipid content in addition to high cell density compared to a single-stage culture where the pH has not been controlled. The results showed that the highest cell density ($9.6 \pm 1.4 \times 10^{10}$ cell L^{-1}) was obtained in the control culture that was supplemented with CO_2 (Table 2). On the other hand, no significant difference in cell density was observed between treatments.

With regard to lipid production, the highest lipid content ($21.5 \pm 1.8\%$) was achieved in the two-stage treatment, with CO_2 injection in the first step and a controlled pH of 7 in the second step (Fig. 1). No significant difference was observed between the lipids content of the two controls. In contrast, microalgae grown in two-stage cultures achieved significantly higher lipid levels than those grown in both controls. Finally, no significant difference is observed between the two treatments in two stages where the only difference comes from the CO_2 injected in the first three days of culture.

Regarding the amount of the two desired fatty acids, the results showed that the greatest yields relative to the total fatty acids of C12: 0 ($0.77 \pm 0.13\%$) and C14: 0 ($1.06 \pm 0.03\%$) were achieved in control media without CO_2 and with CO_2 injection

respectively (Table 3). Microalgae grown in controls with CO_2 injection produced significantly more C12: 0 and C14: 0 compared to two-step cultures with and without CO_2 injection. Control without CO_2 injection also allowed microalgae to produce significantly more C14: 0 compared to the CO_2-supplemented two-stage cultures.

Adjusting the pH to 7 in two-stage cultures yielded the highest lipid content without affecting the growth of microalgae. These results are consistent with those obtained in our previous study (Bélanger-Lépine et al., 2018), which was done to assess what growth conditions allowed the greatest amount of lipids to be produced. Contrariwise, in the previous study, the cell density was also higher compared to the control suggesting that the addition of acetic acid for pH adjustment (which can be used as a carbon source) could have positively influenced the growth of microalgae and lipid production if it helped to overcome a possible limitation of carbon (assuming it is available for growth). This was not the case in the two-step culture strategy suggesting that better results were obtained by cultivating microalgae in a single step and changing the culture conditions by controlling the pH to 7, at the beginning of culture over a period of five days.

Effect of salinity

Different studies have shown that salt stress increases lipid production (Church et al., 2017; Heredia-Arroyo et al., 2011; Pandit et al., 2017; Rai et al., 2015; Shen et al., 2015; Wang et al., 2016). In our previous study, the addition of NaCl in the culture medium did not increase the content of lipids extracted compared to those obtained in control media (Bélanger-Lépine et al., 2018). On the other hand, larger amounts of C12: 0 and C14: 0 were obtained compared to the controls. In that study, since the microalgae were grown in a medium supplemented salt for five days, the effect of stress may have been reduced. Herein, by adding NaCl in the medium after 3 days of culture, the effect of the stress could be further observed.

The results showed that the highest cell density (10.3 ± 1.5 cell $L^{-1} \times 10^{10}$) was obtained in the control that was supplemented with CO_2 (Table 2). The cell densities achieved by the microalgae grown in the two control media are significantly higher than those obtained in the two-stage treatments. These results are consistent with a reduced growth rate under stress conditions (Converti et al., 2009; Yeh and Chang 2011).

The highest value of extracted lipids (24.8 ± 6.3%) was reached in the 2-stage treatment, with addition of 35 g L^{-1} of NaCl in the second stage (Fig. 1). The microalgae grown in the 2-stage treatment supplemented with CO_2 obtained a significant amount of extracted lipids compared to those grown in the control without the addition of CO_2. Contrary to the results obtained in our previous study (Bélanger-Lépine et al., 2018), the addition of salt in the culture medium after 3 days of culture seems to have a positive effect on the lipid content of microalgae.

The largest productions of C12: 0 (2.4 ± 1.3%) and C14: 0 (3.2 ± 0.13%) relative to total fatty acids were achieved in the two-stage treatments with and without CO_2 respectively (Table 3). The amount of lauric acid produced by the microalgae grown in the 2-stage treatment without CO_2 was significantly higher than that produced in the control without CO_2. This is also what has been observed in the 2-stage treatment with CO_2 injection at the beginning of the culture compared to control with CO_2. For myristic acid, the microalgae grown in the two-stage treatments with and without CO_2 significantly obtained larger amounts compared to controls with and without CO_2 injection. The amount produced in the two-stage treatment without CO_2 was significantly higher than the amount produced in the two-stage treatment supplemented with CO_2.

The lipid content produced by the microalgae (24.8 ± 6.3%) when NaCl was added in their culture medium after 3 days of culture was also higher in comparison to the lipid produced by the microalgae (9.88 ± 0.56%) that have received NaCl during the first day of culture in our previous study (Bélanger-Lépine et al., 2018). On the other hand, the cell densities obtained under these conditions were significantly lower than those obtained in the control cultures. The balance between biomass and lipid production has therefore not been reached.

Effect of heterotrophic conditions

The trophic mode, which is characterized by the energy and carbon sources that are used, significantly influences the growth and accumulation of lipids (Bhatnagar et al., 2011; Heredia-Arroyo et al., 2011; Liang et al., 2009; Yeh and Chang 2012). In our previous study on environmental stress inducing lipid accumulation and production (Bélanger-Lépine et al., 2018), the mixotrophic mode allowed microalgae to reach the highest cell densities and the highest lipid contents compared to the autotrophic and heterotrophic modes. On the other hand, it has been reported that microalgae have the ability to switch from one trophic mode to another (when glucose is present in culture media Zheng et al., 2012). By subjecting microalgae to darkness after three days of cultivation, they could produce more lipids compared to biomass because of unfavourable growing conditions.

The highest cell density (8.6 ± 1.1 cell $L^{-1} \times 10^{10}$) was reached in control cultures without the addition of CO_2 (Table 2). Microalgae grown in controls with and without CO_2 achieved significantly higher cell densities compared to those grown in two-stage cultures. Control without the addition of CO_2 produced significantly greater cell density compared to the supplemented control. This is the same thing that is observed for both two-stage culture treatments. Treatment not supplemented with CO_2 produced significantly greater cell density compared to supplemented treatment.

The highest lipid content was achieved in the control medium without CO_2 (24 ± 6%) (Fig. 1). It is also in this medium that the highest amounts of C12: 0 (0.70 ± 0.04%) and C14: 0 (1.01 ± 0.02%) relative to the total fatty acid, compared to other treatments, have been reached (Table 3). Microalgae grown in controls with and without CO_2 produced significantly more C14: 0 compared to those grown in the CO_2-supplemented two-stage culture. No other significant difference was observed in lipid production and accumulation.

The results on microalgae growth are consistent with those obtained in our previous studies (Bélanger-Lépine et al., 2018) as well as those obtained in other studies

(Abreu et al., 2012; Heredia-Arroyo et al., 2011; Liang et al., 2009). Indeed, Heredia-Arroyo et al. (2011) and Liang et al. (2009) showed that microalgae grown under mixotrophic conditions had the highest growth compared to those grown under autotrophic and heterotrophic modes. Liang et al. (2009) also reported that the highest lipid productivity was achieved in mixotrophic mode.

Effect of wastewater type

In this study, the strain (algae-bacteria consortium) maintenance is made in the wastewater mixture containing four effluents. So, the consortium is well adapted to the wastewater mixture, but a change in the composition of this mixture could affect the growth and lipid production of microalgae. The wastewater from the household and industrial cleaning industry was only used as a growing medium in the second cultivation stage. The highest cell density (5.73 ± 0.25 cell $L^{-1} \times 10^{10}$) was obtained in the control cultures with CO_2 addition (Table 2). Microalgae grown under these conditions had significantly higher cell densities compared to those grown in two-stage cultures with and without CO_2.

The highest lipid content ($18.98 \pm 0.22\%$) was also obtained in control cultures with CO_2 addition (Fig. 1). In this treatment, the microalgae produced significantly more lipids compared to those grown in two-stage CO_2 supplemented cultures. The largest amounts of C12: 0 ($1.02 \pm 0.06\%$) and C14: 0 (1.24 ± 0.07) produced relative to the total fatty acids were obtained in control cultures without the addition of CO_2 (Table 3). Microalgae grown in control media with and without CO_2 produced significantly more lauric acid compared to microalgae grown in two-stage cultures with and without CO_2. Control without adding CO_2 produced significantly more C12: 0 compared to control with added CO_2. The two-step culture without CO2 addition also produced significantly more C12: 0 compared to the two-step culture without the addition of CO_2. Finally, microalgae grown in CO_2-free control media produced significantly more C14: 0 compared to the two-step CO_2-free culture. The CO_2-free two-stage crop produced more myristic acid than the two-stage culture with added CO_2.

The expected effect of stress caused by the use of another culture medium to induce lipid production and accumulation in a two-step strategy was not observed. In this case, stress may not have been induced long enough and microalgae have not produced more lipids. Effects of this two-step culture strategy by changing the trophic mode conditions could perhaps be observed if the cultivation was longer than five days. The best results in terms of growth and lipid production were obtained in the control cultures, under mixotrophic conditions, in a single cultivation step.

Overall, our results showed that a single-step culture appears to be more effective in stimulating lipid production. The results obtained are similar to those obtained in a study by Yeh and Chang (2011). In this study, all two-stage cultures resulted in a decrease in biomass production, which also resulted in a decrease in lipid productivity. The results of this study also showed that a single-step culture appears to be more effective in stimulating lipid production. Nitrogen deficiency, which was their stress in the two-step culture, instead favoured lipid accumulation, as was shown in our experience with the addition of salt in two-step cultures.

Conclusion

The two-stage culture strategy did not achieve the desired compromise of increasing lipid production while having a high cell density. The addition of salt in culture media and pH adjustment had an impact on lipid production. The one-step culture, under mixotrophic conditions, has been shown to be more effective in terms of cell density and lipid production compared to the two-step culture where stress was induced in the second stage of the culture. Different culture strategies and various stresses have been applied to induce lipid production. High contents have been achieved in some cases without being able to balance the production of lipids and biomass. Future studies on the modification of the lipid synthesis pathway in microalgae could be done. It has been reported that one way to increase the TAG content is to block the metabolic pathways involved in the production of energetic compounds (Radakovits et al., 2010).

The genetic engineering of microalgae for improved lipid production, especially in the form of TAG, could be an interesting avenue.

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Tables

Table 1. Erlenmeyer flask experiments. Conditions of each stress and culture conditions tested.

Stress	Conditions
pH	pH 7, controlled with acetic acid (CH_3COOH, 5% v/v).
Salinity	35 g L^{-1} of NaCl was added to the culture medium.
Heterotrophic conditions	Placed in dark.
Single wastewater	Only one effluent was used compared to the usual wastewater mixture.

Table 2. Cell density (cell $L^{-1} \times 10^{10}$) of microalgae grown under different culture conditions and different stresses (pH, salinity, heterotrophic conditions, wastewater type) for each of the four experiments.

Treatments	Control	Control/CO_2	Two-stage	Two-stage/CO_2
	Cell density (cell $L^{-1} \times 10^{10}$)			
pH 7	8.4 ± 1.1	9.6 ± 1.4	7.0 ± 2.4	8.1 ± 3.0
Salinity	7.91 ± 0.88	10.3 ± 1.5	3.6 ± 1.1	3.7 ± 1.2
Heterotrophy	8.6 ± 1.1	6.34 ± 0.62	3.90 ± 0.62	2.85 ± 0.48
Wastewater type	4.93 ± 0.77	5.73 ± 0.25	4.08 ± 0.50	4.38 ± 0.46

Table 3. Percentage of C12: 0 (first line) and C14: 0 (second line) (%) relative to total fatty acids after five days of culture for each treatment (n = 3; mean ± standard deviation) for the four culture conditions and different stresses: pH, salinity, heterotrophic conditions and wastewater type.

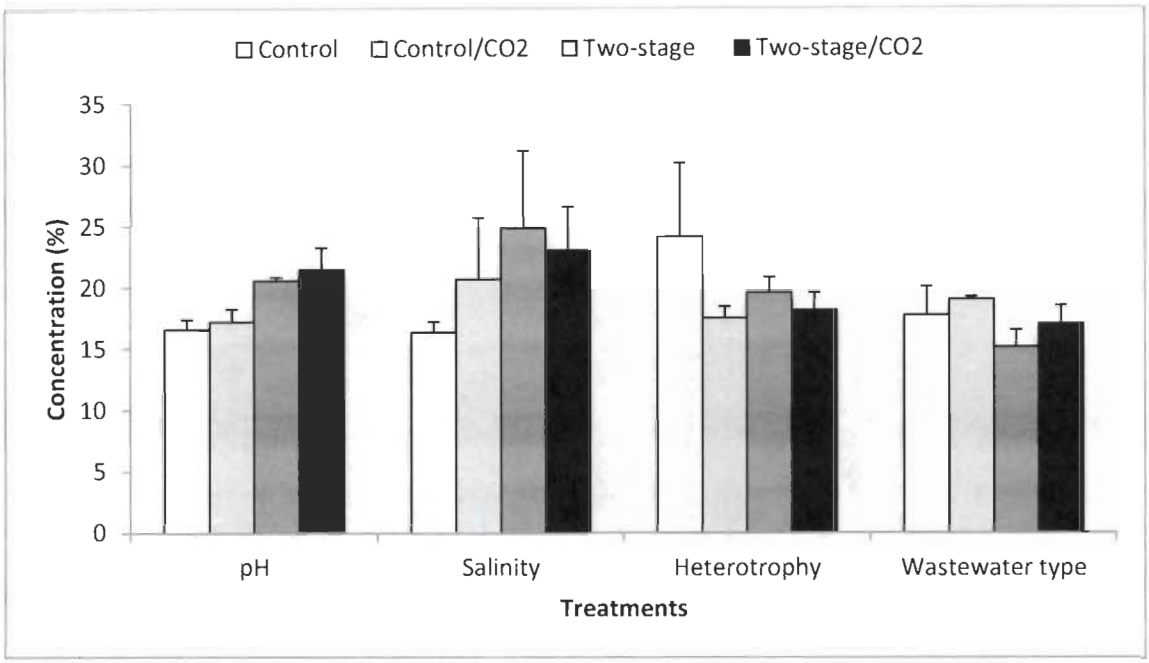
Treatments	Control	Control/CO_2	Two-stage	Two-stage/CO_2
pH 7	0.77 ± 0.13	0.73 ± 0.02	0.59 ± 0.06	0.55 ± 0.10
	1.05 ± 0.10	1.06 ± 0.03	0.81 ± 0.05	0.85 ± 0.10
Salinity	0.77 ± 0.09	0.71 ± 0.12	1.06 ± 0.08	2.4 ± 1.3
	1.43 ± 0.13	1.35 ± 0.15	3.20 ± 0.13	2.32 ± 0.06
Heterotrophy	0.70 ± 0.04	0.66 ± 0.07	0.61 ± 0.11	0.58 ± 0.05
	1.01 ± 0.02	1.01 ± 0.05	0.88 ± 0.07	0.90 ± 0.04
Wastewater type	1.02 ± 0.06	0.80 ± 0.02	0.90 ± 0.02	0.71 ± 0.04
	1.24 ± 0.07	1.08 ± 0.04	1.12 ± 0.01	1.00 ± 0.05

Figure legends

Figure 1. Percentage of extracted lipids relative to dry weight of microalgae grown under four culture conditions and different stresses: pH, salinity, heterotrophic conditions and wastewater type, after five days of culture.

Figures

Figure 1.



CHAPITRE V

NUTRIENT REMOVAL IN WASTEWATER OF AN INDUSTRIAL PARK BY A MICROALGAE-BACTERIA CONSORTIUM

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Abstract

Many wastewater and nutrient-rich wastes provide an excellent growth medium for microalgae. Production of microalgae can be then combined to waste treatment. The efficiency of a microalgae-bacteria consortium to remove nutrients in wastewater from an industrial park was studied. The consortium was grown in a mixture containing wastewater from a pharmaceutical company, a dairy industry and a manufacturer of cleaning products as well as the leachate from an integrated waste management company in the proportion expected from their effluent volumes. Different conditions were tested, including CO_2 injection, glucose and the addition of specific nutrients to the culture medium to determine the best removal efficiency for the nitrogen and phosphorus nutrients contained in industrial wastewater. Measurements of different micro and macronutrients at the start of the cultures and at the end, 5 days later, were made for each condition. The specific growth rate and the removal efficiency were also measured. The results showed removal efficiencies of up to 90% for nitrogen and 100% for phosphorus. This study suggests that microalgae production can be implanted in industrial parks to treat mixture of different wastewater and potentially obtain value products from the biomass produced.

Keywords: wastewater treatment, removal efficiency, biomass, industrial wastewater, industrial park.

Introduction

The world demand for energy has steadily increased and is mostly filled by conventional energy sources (Prajapati et al., 2013); worldwide only 14% of global primary energy consumption is satisfied by renewable energy sources (Converti et al., 2009). At the same time, the increasing global human population and the rapid industrial development are causing serious environmental challenges because of the large quantities of wastewater released into the environment (de-Bashan and Bashan 2010; Doria et al., 2012; Fu and Wang, 2011; Renuka et al, 2013). This wastewater generally contains an excess of nutrients, more particularly nitrogen and phosphorus, which disturb the environment in which they are discharged (Renuka et al., 2013). Eutrophication is the main result of the accumulation of these nutrients in aquatic environments. A reduction in biodiversity, an increase in water toxicity and a decrease in the life span of the affected streams, and lakes are the main consequences of eutrophication (Cai et al. 2013). In addition to having adverse effects on the environment, the wastewater discharged carries risks to human health, as besides containing organic contaminants, heavy metals, such as zinc, mercury and lead, may also be present. These are not biodegradable, they bioaccumulate in living organisms and are, in general, toxic or carcinogenic (Fu and Wang, 2011). It is then essential to carry out a wastewater treatment before discharge.

Faced with the problems of rationalizing the use of energy resources and the increasing quantities of wastewater released into the environment, a sustainable and profitable solution appears urgent. The solutions must be based on the development of new potential sources of renewable and sustainable energy, while considering the use of less expensive raw materials with a secure supply (Converti et al., 2009; Renuka et al., 2013). It has been proposed that these problems can be solved, in part, by the use of microalgae because they are capable of performing several functions (Moreno-Garcia et al., 2017; Rawat et al., 2011). They can play a role in the bioremediation of wastewater while generating biomass that can be used for many commercial applications such as third-generation biofuels, pharmaceuticals, food additives and high-value bioactive compounds (Rao et al., 2011; Rawat et al., 2011; Sydney et al., 2011).

In addition to a source of carbon, microalgae require mainly nitrogen and phosphorus to grow and synthesize proteins, nucleic acids and phospholipids, can therefore reduce these nutrients in wastewater used to grow them (Beuckels et al., 2015; Kumar et al., 2010; Pittman et al., 2011; Prajapati et al., 2013; Rao et al., 2011). Several studies have shown that microalgae are excellent candidates for bioremediation of wastewater (Bhatnagar et al., 2011; Chinnasamy et al., 2010; Mulbry et al., 2008; Olguin, 2003; Pittman et al., 2011; Rao et al., 2011; Rawat et al., 2011; Sydney et al., 2011; Woertz et al., 2009).

Microalgae technologies, however, face a key limitation to widespread use as the costs of producing and processing biomass can be high. Solutions can be applied to counteract these economic downsides. First, the cost of producing biomass can be reduced if nutrients are present in sufficient quantities in the wastewater (Prajapati et al., 2013). Secondly, the cost of exploiting biomass can be offset if the biomass generated is used to produce value-added products (Sturm and Lamer, 2011) or can be profitable if microalgae technology replaces a more expensive technology for the same purpose. Another limitation faced by the use of microalgae is that a single strain can be inefficient or simply die when conditions change. The use of a consortium (multiple strains generally including bacteria) allows robustness against environmental fluctuations, stability of the species present, ability to share metabolites and allows to resist to the invasion of undesirable species (Subashchandrabose et al., 2011). It has been shown to increase the efficiency of nutrient removal in wastewater while generating a useful microalgae biomass (Bhatnagar et al., 2011; Boudreau et al., 2017; Chinnasamy et al., 2010; Gélinas et al., 2015).

Therefore, if costs can be managed, the treatment of wastewater using microalgae represents a viable, sustainable and environmentally friendly alternative to physicochemical methods. Indeed, in addition to eliminating nutrients, there can be a mitigation of greenhouse gases depending on the use of the resulting biomass, reduction of additional pollutants (e.g. sludge), low energy requirements and biomass production that can be converted into energy or other useful products (Beuckels et al., 2015;

Chinnasamy et al., 2010; Mehta and Gaur, 2005; Pittman et al., 2011; Rawat et al., 2011; Sydney et al., 2011; Wang et al., 2010).

Some industries are currently actively seeking processes to effectively treat their wastewater (Rao et al., 2011), while decreasing their environmental footprints. A few companies located in an industrial park in Canada decided to move towards an integrated solution that would combine their different wastewaters for biological treatment and simultaneous value-added production. A microalgae culture integration project was then conducted in this industrial park, in order to treat wastewater with a native microalgae-bacteria consortium to develop biobased products usable by local companies from the biomass generated. A wastewater mixture containing the effluents of four industries were used as the main culture medium. The microalgae-bacteria consortium used in this project was isolated on the site of a wastewater stabilization pond in the industrial park.

This paper reports on an experiment conducted as part of this project pertaining to the efficiency of a microalgae-bacteria consortium to remove nutrients in the wastewater mixture and the amount of biomass obtained in the process. Different conditions were tested, including CO_2 injection, glucose and nutrient additions to the culture medium to assess which conditions provided the best removal efficiency for the nutrients contained in these industrial wastewater.

Materials and methods

Wastewaters and leachates

We used 3 wastewaters and 1 leachate in this study. The wastewaters were collected from industries located in the Victoriaville (PQ, Canada) industrial park: a dairy industry, a pharmaceutical industry (lactulose production) and a household and cleaning product manufacturer. The leachate comes from a municipal solid waste landfill site in

the area of the industrial park. The wastewaters and leachate were stored in plastic containers at 4 °C until they were used for the experiments within 2 days.

Consortium and inoculum preparation

A native microalgae-bacteria consortium was obtained from a sample taken on the site of a wastewater stabilization pond from a dairy wastewater treatment station in the Victoriaville industrial park from which a seed culture was made. The algal genus presents in the consortium were mainly *Chlorella*. The inoculum preparation was done as described in Bélanger-Lépine et al. (2018). Briefly, algal biomass was harvested from a seed culture in exponential growth phase and resuspended into Erlenmeyer flasks containing a mixture of the four collected wastewaters, supplemented with minerals to mimic Bold's basal medium (BBM). The inoculum was maintained in Erlenmeyer flasks on an orbital shaker set at 110 rpm at 25 °C, under a photosynthetically available irradiance of 200 μmol m^{-2} s^{-1} on a 12 h/12 h light/dark cycle until algal cell concentration reached 8×10^7 algae cell mL^{-1} for further experiments.

Experimental cultures and treatments

The experiment conducted to evaluate the effectiveness of wastewater treatment was performed over five days. Four treatments in triplicate were performed. For all treatments, 10% (v/v) of inoculum, containing the microalgae-bacteria consortium, was cultured in a 1 L flask containing 500 mL of the wastewater mixture (without added nutrients) of the four industries use for maintenance. Each Erlenmeyer flask was placed on an orbital shaker set at 110 rpm, at 25 °C and under an irradiance of 200 μmol m^{-2} s^{-1} on an 12 h/12 h light/dark cycle. The growth conditions for each treatment differed according to addition of nutrients and/or glucose whether or not CO_2 was injected into the culture medium (Table 1). For flasks with CO_2 injection it was done continuously for six hours each day at a rate of 0.42 L min^{-1} through a porous sparing stone. Otherwise, air was bubbled into the media. Concentration of CO_2 in the gas was adjusted to 5%

(21% O_2 and 74% N_2). For flasks with glucose addition, it was added to the culture medium on each of the first four days at a rate of 0.5 g L^{-1} d^{-1}.

Analysis

Biological and physicochemical parameters

Biomass and pH monitoring were performed on a daily basis. Culture biomass (dry weight per liter) was followed daily by filtering 10 ml of culture on Whatman™ 934 AH™ glass microfiber filters (effective pore size of 1.5 µm). The specific growth rate (SGR) µ was calculated using the initial (x_i, g L^{-1}) and final (x_f, g L^{-1}) biomass concentrations and the corresponding cultivation time (t_i and t_f, days):

$$\mu = \frac{ln\,(x_f/x_i)}{(t_f - t_i)}$$

A daily sample was observed and counted with a Neubauer chamber (hemacytometer) using a phase-contrast microscope. At the same time, the culture was checked for contamination by undesirable species such as rotifers (Boudreau et al., 2017).

Nutrients removal

For nutrient monitoring, filtrates from the first and last day of culture were collected and kept in the freezer until analysis. The analysis of total nitrogen was performed using a CHNS elemental analyzer (vario MACRO cube from Elementar). Total phosphorus and other nutrients were analyzed using microwave plasma atomic emission spectrometry (Agilent 4210 MP-AES). The removal efficiencies (%) of nutrients were calculated as follows:

$$\text{Removal efficiencies (\%)} = \frac{(Initial\ concentration) - (Final\ concentration)}{Initial\ concentration} \times 100$$

Statistical analysis

Variance analyzes (ANOVA) were used to compare the treatments for each data set using the JMP Pro 11 Software. We considered p values smaller than 0.05 to be statistically significant.

Results and discussion

Influence of culture medium and conditions on growth

Four treatments were tested to identify the conditions that led to the best removal efficiencies and biomass accumulation. The microalgae-bacteria consortium sustained higher growth rates and reached higher biomasses in the N/G and N/G/CO_2 treatments (Table 2). There were no significant differences in either biomass or growth rates. Lower biomasses were reached in the treatments with no additions and with CO_2 only and both showed identical biomass. Note, however, that the growth rate was significantly higher in the CO_2 treatment (#3) as the initial biomass (not shown) was slightly lower.

The addition of CO_2 therefore did not affect the biomass yield under our culture conditions but led to higher growth rates in the absence of added glucose. The biomass yield and growth rates were, however, higher with nutrients and glucose addition. Though our design does not allow us to verify which of the nutrients or glucose promoted growth. These results are, consistent with other studies that observed significantly higher biomass yields for Chlorella species grown under in mixotrophic conditions (addition of organic carbon to the culture in the presence of light) compared with those grown in autotrophy and heterotrophy (Bhatnagar et al., 2011; Heredia-Arroyo et al., 2011; Liang et al., 2009; Mitra et al., 2012). Bhatnagar et al. (2011) showed that *Chlorella minutissima* grown under mixotrophic conditions accumulated seven times more biomass than when grown in autotrophic conditions. This trophic mode allows to at least partially overcome the limitations in the two other modes and it has been shown that under these conditions the growth of microalgae is

improved (Bhatnagar et al., 2011; Heredia-Arroyo et al., 2011). Alternatively, the nutrients could have been limiting in the cultures without nutrients added and this could have limited the biomass yield.

Influence of nutrient addition on growth

The addition of BBM nutrients to culture media at the beginning of the experiment increases total nitrogen concentrations by more than a factor of two compared to the wastewater (Table 3). Since there is significant nitrogen at the end of the experiment in all the treatments it does not appear that nitrogen was limiting growth in the cultures.

In the nutrient-supplemented media the concentration of phosphorus is about eight times higher than those that did not receive nutrients (Table 3). There are indications that phosphorus might have become limiting for growth in the non-supplemented treatments, in particular for the no addition treatment (#2) where no phosphorus could be measured at the end of the experiment.

Finally, as microalgae also need trace elements such as metals (e.g. Na, Mg, Ca, Mn, Zn, Cu, Fe and Mo), their presence in culture media is necessary for algal growth. For magnesium (Mg), copper (Cu) and manganese (Mn), the initial concentrations found in the N/G and N/G/CO_2 treatments were all higher than those found in treatments without addition and CO_2 only. There is, however, significant concentrations left these three micronutrients and as such they were unlikely limiting.

Removal efficiencies

Several factors such as initial the nutrient concentration, the light intensity, media pH, temperature, and inoculation density can influence nutrient removal by microalgae (Cai et al., 2013). In our study, light intensity and temperature were the same for each treatment while slight differences occurred in the initial biomass. However, the initial concentration of nutrients varied greatly (Table 3). In addition, because the culture

conditions (e.g. CO_2, glucose, nutrients) were not the same between treatments, the extracellular pH varied from one treatment to another. Between 85 and 90% of the nitrogen is removed from the wastewater and no significant difference between treatments was found for removal efficiency (Table 4). The results obtained for the removal efficiency (%) of the nitrogen are consistent with those obtained in other studies (Qin et al., 2016; Woertz et al., 2009). This result is interesting because it shows that under different conditions, the microalgae-bacteria consortium used in this project has the ability to remove a large amount of nitrogen from the industrial wastewater mixture.

The highest removal efficiency of total phosphorus (100%) was observed in treatment with no addition, where the microalgae-bacteria consortium was grown only the wastewater mixture (Table 4). The removal efficiency (%) obtained in this treatment is significantly higher than those obtained in N/G and N/G/CO_2, which have been supplemented (Table 4). No significant difference is observed between treatments with no addition and with CO_2 only, where the only difference is the injection of CO_2 (Table 4). Two hypotheses could explain that the removal efficiency is 100% in treatment with no addition. First, since the initial concentration in treatment with no addition is lower, it is likely that all the phosphorus has been used by the microalgae and that this nutrient became limiting in this culture medium. Alternatively, it is also possible that the disappearance of phosphorus was influenced by an external factor; it has been shown that phosphate removal can be influenced by environmental factors such as pH and dissolved oxygen (Cai et al., 2013; Gonçalves et al., 2017). At high pH, typically above 8.0, and at high dissolved oxygen concentration, phosphate will precipitate from the medium. This may be what happened in treatment with no addition where the pH value (10.1 ± 0.28) is significantly higher than that of the other three treatments (Table 2). This could also explain the reduced biomass accumulation. The results obtained for the removal efficiency of the total phosphorus, between 54 and 100% depending on the treatment, are consistent with those obtained in other studies (Chinnasamy et al., 2011; Qin et al., 2016; Woertz et al., 2009).

Finally, the magnesium removal efficiency for treatment with CO_2 only is significantly lower compared to all other treatments. No differences were observed for copper removal efficiency. For manganese, treatment with no addition has a significantly higher removal percentage compared to all other treatments. N/G also has a higher removal percentage compared to treatment with CO_2 only. The removal efficiency of magnesium, copper and manganese are lower than what has been observed in other research (Safonova et al., 2004; Znad et al., 2018). This could be explained by the fact that the elimination efficiencies were measured over a longer period compared to our study. Znad et al. (2018) have tested the elimination efficiency over 13 days while in our case it is over 5 days of cultivation.

Overall, the results showed the ability of microalgae to effectively remove the macronutrients found in the industrial wastewater mixture. It is likely that optimization of growth conditions could lead to increased removal especially for nitrogen. The efficiency of removal of micronutrients was lower but nevertheless significant and was strongly influenced by the treatment.

Conclusion

This study shows that a microalgae-bacteria consortium can remediate wastewaters while the producing a biomass that could be used to obtain valuable products. This study suggests that microalgae production can be implanted in industrial parks to treat mixture of different wastewaters and potentially obtain value products from the biomass produced. While scaling up the approach will require significant efforts, it appears to be an interesting approach to reduce wastewater cost management while contributing to the industrial ecology of the park with potential biobased products usable on site and new and usual synergies between companies from different sectors. To examine further the treatment potential, future work should focus on the fate of micropollutants that could be found in the wastewater of the household and cleaning product manufacturer that was used in this study and see if microalgae-bacteria consortia can be used as a tertiary treatment for this type of company.

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Tables

Table 1. Growth conditions for each treatment.

Treatments[1]	Nutrients (BBM)	Glucose (0.5 g L^{-1} d^{-1})	CO_2 (5%)
1 (N/G)	+	+	-
2 (No addition)	-	-	-
3 ($N/G/CO_2$)	+	+	+
4 (CO_2)	-	-	+

[1] In the abbreviated name we use 'N' to represent 'Nutrients', 'G' for glucose' and 'CO_2' for CO_2.

Table 2. Final biomass (g L^{-1}), specific growth rate (μ, d^{-1}) and pH for the four treatments after five days of culture.

Treatment	Biomass (g L^{-1})	μ (d^{-1})	pH
1 (N/G)	1.88 ± 0.06	0.25 ± 0.02	8.81 ± 0.04
2 (No addition)	0.92 ± 0.13	0.09 ± 0.01	10.1 ± 0.28
3 (N/G/CO_2)	1.77 ± 0.24	0.24 ± 0.05	8.37 ± 0.2
4 (CO_2)	0.98 ± 0.06	0.17 ± 0.02	8.53 ± 0.03

Table 3. Concentration of nutrients (N, P, Mg, Cu, Mn) at the beginning (i) and at the end (f) of the five days of culture for the four different treatments.

Nutrient (mg L^{-1})	$T1_i$	$T1_f$	$T2_i$	$T2_f$	$T3_i$	$T3_f$	$T4_i$	$T4_f$
N	85.1 ± 3.5	8.14 ± 0.05	39.15 ± 0.51	5.98 ± 0.27	81.72 ± 0.12	8.93 ± 0.41	38.3 ± 0.73	4.5 ± 1.2
P	48.0 ± 3.8	15.1 ± 1.8	9.07 ± 0.31	0 ± 0	48.0 ± 1.7	22.1 ± 1.7	8.13 ± 0.81	1.0 ± 1.7
Mg	15.4 ± 1.1	9.93 ± 0.78	11.31 ± 0.04	8.38 ± 0.50	16.71 ± 0.59	12.1 ± 1.0	11.01 ± 0.34	10.43 ± 0.15
Cu	0.49 ± 0.01	0.360 ± 0.006	0.090 ± 0.006	0.08 ± 0.01	0.49 ± 0.01	0.38 ± 0.02	0.10 ± 0.02	0.08 ± 0.02
Mn	0.49 ± 0.01	0.34 ± 0.03	0.08 ± 0.00	0.040 ± 0.006	0.510 ± 0.006	0.43 ± 0.04	0.080 ± 0.006	0.070 ± 0.006

Table 4. Removal efficiencies (%) of the microalgae-bacteria consortium for six nutrients (N, P, Mg, Cu, Mn) under four different treatments after five days of cultivation.

	Removal efficiencies (%)			
Nutrient	T1	T2	T3	T4
N	90.43 ± 0.45	84.71 ± 0.89	89.07 ± 0.51	88.2 ± 3.4
P	68.6 ± 1.8	100 ± 0	53.7 ± 4.0	87.5 ± 21.7
Mg	35.6 ± 3.2	25.9 ± 4.3	27.3 ± 4.7	5.1 ± 4.2
Cu	27.2 ± 2.6	14.1 ± 12.2	22.4 ± 3.9	23.1 ± 11.2
Mn	29.8 ± 8.0	54.2 ± 7.2	14.5 ± 6.5	4.2 ± 7.2

CHAPITRE VI

METAGENOMIC IDENTIFICATION OF AN ALGAE-BACTERIA CONSORTIUM FROM A DAIRY WASTEWATER TREATMENT STATION

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Abstract

Interactions between microalgae and bacteria are many and complex and our understanding of them remains limited. On the other hand, many benefits can be brought to both parts and these associations can be integrated into algal biotechnology. The objective of this study was to identify bacterial and algal populations that, in equilibrium, could benefit both the wastewater treatment process and the production of bioproducts. Different culture media (e.g. BBM medium, wastewater mixture, large volume), containing the native algae-bacteria consortium were tested in order to identify changes in the microbial flora. The dominant algal genus in the consortium growing in favourable growth conditions is *Chlorella*. Yeasts belonging to the genus *Candida* are also present and could be promising for the treatment of wastewater as well as the production of biosurfactants. *Proteobacteria* are the prokaryotes found in larger quantities in this consortium and culture media. The relative abundance of different groups of microbial flora varies with growing conditions, and changes are most commonly seen in eukaryotic genus. A better understanding of the species could lead to further research on other mechanisms for algal growth stimulation.

Keywords: industrial wastewater, algae, bacteria, wastewater treatment, biosurfactants.

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Introduction

Interactions between microalgae and bacteria are many and complex and our understanding of them remains limited. Among the types of interactions that may exist between these groups, mutualism and parasitism are those that are best demonstrated to date. The mutualism relationship is mostly defined by the exchange of nutrients and vitamins that occurs between algae and bacteria. Many studies have shown that bacteria have the ability to provide algae with certain vitamins, such as B_{12}, and limiting nutrients such as iron (Amin et al., 2009; Croft et al., 2005; Grant et al., 2014). In return, the algae will provide bacteria with oxygen and other organic molecules from photosynthesis. Parasitism is mainly based on competition for nutrients while it has been reported that some bacteria can negatively affect algae by lysing their cells (Wang et al., 2010). Microalgae have evolved in the presence of bacteria and it has been suggested that the use of algae-bacteria consortium in some algal biotechnologies would overall benefit from the interaction (Ramanan et al., 2016).

The use of algae-bacteria consortia has been shown to be effective in biodegrading organic pollutants and removing metals (Bahr et al., 2011; Borde et al., 2003; Muñoz and Guieysse 2006; Subashchandrabose et al., 2011). In these mutualistic consortia for organic matter biodegradation the production of oxygen by microalgae (Bahr et al., 2011) allow the heterotrophic bacteria to use it as an electron acceptor allowing the degradation of the organic pollutants (Bahr et al., 2011). The CO_2, released during the mineralization of pollutants by bacteria, is in turn used by the algae for photosynthesis (Bahr et al., 2011). It has also been shown that the use of an algae-bacteria consortium for the treatment of wastewater is more effective than the use of pure algae culture (Sniffen et al., 2016). The presence of a bacterial community associated with microalgae has been shown to lead to higher growth rates (Cho et al., 2015). However, to avoid undesirable bacteria gaining control over algae, it is important to monitor microbial diversity and maintain the desirable algal-bacterial community (Cho et al., 2015; Park et al., 2013).

The use of an algae-bacteria consortium results in more robust and efficient biomass production to remove inorganic, organic, and metallic pollutants (Muñoz and Guieysse 2006, Subashchandrabose et al., 2011). In fact, algae-bacteria consortia are more robust under adverse conditions because members have the ability to share metabolites, nutrients, or other desired compounds. In addition, consortia are more resistant to invasion of undesirable species. This is not the case in algal monocultures. If environmental conditions change and nutrients become limited, other species cannot outcompete the grown species and compensate the overall production reduction. Finally, the biomass resulting from the use of algae-bacteria consortia for wastewater treatment can also be used for the production of biofuel and other useful co-products.

A native algae-bacteria consortium was isolated from a sample taken from the site of a wastewater stabilization pond at a dairy wastewater treatment plant in the industrial park of City of Victoriaville (Québec, Canada) as described in Belanger-Lépine et al. (2018). Briefly, the wastewaters come from a dairy industry, a pharmaceutical industry (lactulose production), and a manufacturer of household and cleaning products, while the leachates come from a municipal solid waste landfill in the industrial park area. Different experiments have been carried out with this algae-bacteria consortium (Bélanger-Lépine et al., 2018). Results from Belanger-Lépine et al. (2018) showed that the use of this consortium in industrial wastewater has the potential not only to reduce the nutrient and metal load, but also to generate a biomass containing lipids for the production of co-products, such as biosurfactants.

The main objective of this study was to identify more in depth the bacterial and algal populations present in the consortium. The relative contribution of the different groups is expected to change with culture conditions. Different culture media, BBM medium, two wastewater mixtures, and a large volume (400 liters), containing the native algae-bacteria consortium were tested in order to identify changes in the microbial flora.

Materials and methods

Inoculum preparation

The algae-bacteria biomass was harvested from a seed culture in exponential growth phase and resuspended into Erlenmeyer flasks as described in Bélanger-Lépine et al. (2018). The inoculum was maintained in Erlenmeyer flasks on an orbital shaker set at 110 rpm at 25 °C, under a photosynthetically available irradiance of 200 µmol m^{-2} s^{-1} on a 12 h/12 h light/dark cycle.

Experimental culture conditions

The algae-bacteria consortium was inoculated in three different experimental conditions, defined as control, normal, and large-scale. The composition of each medium is detailed in Table 1. The control medium consisted of demineralized water and BBM nutrients. The medium used for normal conditions consisted of the industrial wastewater mixture (same as described above), supplemented with BBM nutrients. These two experimental conditions were carried out in 1 L shake flasks containing 500 mL of culture medium. Finally, the last condition was achieved in a 400 liter tank, with the industrial wastewater mixture and BBM nutrients.

The flasks were placed on a stirring plate set at 110 rpm and were maintained at 25 °C and under a 30 µmol m^{-2} s^{-1}, 12 h/12 h light/dark cycle. The 400 liters tank was constantly mixed with an agitator and an air stone, and maintained at 25 °C under a 30 µmol m^{-2} s^{-1}, 12 h/12 h light/dark cycle. After five days of culture, samples from each experimental condition were qualitatively assessed for their algae-bacteria ratios (Bourdeau et al. 2017). For further genetic identification, one sample was taken from each of the experimental conditions. Additionally, one sample was taken from a flask under normal conditions, but that resulted in an imbalance of the algae and bacteria ratio.

Total genomic DNA extraction

The bacterial DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's instructions for the "Pretreatment for Gram-Positive Bacteria" and the "Purification of Total DNA from Animal Tissues (Spin-Column)" protocols, with minor modifications. Briefly, the algae-bacteria cultures were centrifuged at 5000 *g* for 10 min then the bacterial pellets were resuspended in a solution made of 90 µL ATL buffer and 90 µL 10 mg/mL lysozyme solution. The subsequent extraction steps were as per the protocol. The algal DNA was extracted with the NucleoSpin Plant II (Machery-Nagel), following the manufacturer's instructions for the "Genomic DNA from plant" protocol.

PCR amplification and Sequencing

PCR amplifications and sequencing were performed at the McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada). The hypervariable region V6-V8 of the 16S rRNA gene and the hypervariable region V4 of the 18S rRNA gene were amplified for the bacteria and microalgae, respectively. Moreover, the Domain V of the 23S rRNA gene was targeted for cyanobacteria and plastid-bearing microalgae. All primers sequences are listed in Table 2.

High-throughput sequencing was carried out on an Illumina MiSeq platform. The raw sequencing reads were deposited in the NCBI Sequence Read Archive under accession number SRP156562. Bioinformatics analyses and taxonomy assessments for the 23S rRNA gene fragment were performed with a Qiime pipeline at the Canadian Centre for Computational Genomics (Montréal, Québec, Canada). Bioinformatics analyses of the 16S and 18S rRNA gene sequences were performed with the Mothur software (v. 1.35.1; Schloss et al., 2009) following the MiSeq SOP (Kozich et al., 2013). Taxonomic identification for the 16S rRNA gene sequences was performed against a modified 16S rRNA gene database based upon the GreenGenes97 reference file for pyrosequencing (Comeau et al., 2012). The 18S rRNA gene sequences taxonomy assessment was carried out against the SILVA Eukarya database

(http://www.arb-silva.de; Quast et al., 2013). The final reads were clustered into Operational Taxonomic Units (OTUs) at the 97% similarity level and all singletons were excluded.

Results and discussion

Eukaryotic population comparison

As expected, there are changes in the composition of eukaryotic organisms within the consortium depending on the culture medium tested (Fig. 1). Three main types of green algae have been identified. The *Chlorella* genus is mainly found in the BBM medium and its proportion decreases when the consortium is cultivated in wastewater. The same phenomenon is observed for the species that is in unclassified *Trebouxiophyceae*. In contrast, the genus *Sphaeropleales* increases in proportion when the environmental conditions change. Green algae species of this genus may be better adapted to this kind of conditions, which could explain their dominance over the species of the other two main genus. Overall, the consortium used in this study, comprised of several algal species mostly represented by *Chlorella*, unclassified *Trebouxiophyceae* and *Sphaeropleales*, is favorable to the elimination of nutrients contained in wastewater and lipid production.

The genus Glaucoma, a ciliate found in wastewater treatment systems (Madoni, 2011; Martín-Cereceda et al., 2001), is found in three of the four media tested. It is found in low concentration in the BBM medium, but in larger quantities in flasks containing wastewater – good and bad yield (Fig. 1). The results showed, in BBM medium, that Glaucoma could coexist with other microbial species without dominating the environment in a controlled medium. In the environment where there is bad yield, this genus has literally got the upper hand compared to the other eukaryotic species present. Ciliates such as Glaucoma sp. are well-adapted to live with high bacterial concentrations, with their main functions related to the channelling of organic matter through their predation of bacteria and to the remineralisation activity (Martín-Cereceda

et al., 2001). This correlates with the higher abundance of bacteria in flasks containing wastewater. The genus Halteria, another ciliate, is found in two of the four cultures tested (wastewater good yield and 400-liter tank) (Fig. 1). It has previously been shown to significantly remove organics from wastewater treatment processes (Papadimitriou et al., 2010).

The genus *Candida*, a yeast, is present in small quantities in all media in different concentrations (Fig. 1). It was reported by Daverey and Parkshirajan (2011) that the presence of *Candida bombicola*, belonging to the same genus as the yeasts found in our consortium, allowed the biological treatment of industrial wastewaters and was effective for dissolved organic carbon (DOC) removal of more than 90%. Moreover, this yeast makes it possible to produce biosurfactants by pretreating wastewater from the dairy industry containing oil and fats. *Candida utilis* was isolated and characterized in several natural environments and industries, especially for bioremediation and wastewater treatment (Environment and Climate Change Canada, Health Canada, 2014). Since this genus does not dominate algae, it could be interesting to use it effectively in order to increase the potential for nutrient removal by the consortium and to produce biosurfactants.

Changes in culture conditions have shown that the genera of Eukaryotes found are essentially the same, but differ as to the dominant genus. Under controlled conditions, the *Chlorella* genus dominates, which is not the case in other media. When ciliates are more abundant in the cultures, there appears to be a negative impact on the presence and growth of algae. This is a genus that will have to be controlled not to outbalance the presence of algae.

Prokaryotic population comparison

For the relative abundance of the phylum of the main prokaryotes, changes are also observed from one medium to another, but less important than that of eukaryotes (Fig. 2). The three main prokaryotic phylum found in the consortium under the

four different growth media are *Proteobacteria*, *Verrucomicrobia* and *Bacteroidetes*. Prokaryotes belonging to the phylum *Proteobacteria* are those that are found in greater quantity in all four culture conditions (Fig. 2). The medium that contains the least is the one that has demineralised water and nutrients, hence the most controlled environment.

In the phylum Verrucomicrobia, the most abundant genus is *Prosthecobacter* (not shown). They were identified in the four culture media in different proportions (Fig. 2). It has been reported in a study by Lee et al. (2014) that a bacterial strain belonging to the *Verrucomicrobia* genus was isolated from activated sludge. Since the algae-bacteria consortium is kept in a mixture of wastewater, it is possible that the origin of this genus comes from this place. These bacteria were present when the consortium was inoculated into the control medium and evolved with the dominant *Chlorella* species.

A change in relative abundance is observed within the four growth media. However, the main phylum's identified are found in the four environments. Changes in growth conditions appear to affect prokaryotes less than eukaryotes.

Conclusion

Chlorella is the dominant eukaryotic genus in the consortium when conditions are under control. Its proportion decreases when growth conditions vary. The presence of *Candida* in the consortium is promising for the biological treatment of industrial wastewater and the production of biosurfactants. *Proteobacteria* are the prokaryotes present in larger amounts in all culture media. Their presence does not seem to affect the development of the consortium. Ciliates, when in a larger proportion, seem to affect the normal development of the consortium. There is still very little information regarding the bacterial and algal genera involved in the consortium. A better understanding of these involved species may lead to further research on other mechanisms for algal growth stimulation. For example, bacteria could produce phycohormones and be added to stimulate algae production through plant growth promotors. The identification of species

by modern microbial characterization techniques would improve microbial diversity monitoring and predict that a production may potentially have yield losses if a species' population is absent or if its population decreases. The hope is that the characterization of the microbial flora that makes up the consortium used in this project will allow better control or increase production yields.

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Tables

Table 1. Microscopic characterization at 400X of algae and bacteria in samples from each experimental condition.

Scale	Sample and conditions	*Chlorella*-like algae (cell $L^{-1} \times 10^{10}$)	Other organisms[1] (cell $L^{-1} \times 10^{4}$)	Bacteria[2]
Small	BBM	1.3	0	L
	Wastewater-Good yield	0.14	0	M
	Wastewater-Low yield	0.6	2.2	M
Large	Wastewater-400 L tank	0.12	0.9	M

[1] This category excludes *Chlorella*-like algae and bacteria.

[2] Bacterial abundance was qualitatively assessed using a letter system that refers to the amount observed in the microscope: low (L), medium (M), and high (H).

Table 2. Primers for amplifying SSU rRNA genes 16S, 18S, and 23S.

Primer name	Amplified region	Specificity	Primer Sequence (5'-3')	Reference
B969F	16S V6-V8	Bacteria	ACGCGHNRAACCTTACC	Comeau et al., 2011
BA1406R	16S V6-V8	Bacteria	ACGGGCRGTGWGTRCAA	Comeau et al., 2011
E572F	18S V4	Microalgae	CYGCGGTAATTCCAGCTC	Comeau et al., 2011
E1009R	18S V4	Microalgae	AYGGTATCTRATCRTCTTYG	Comeau et al., 2011
p23SrV_f1	23S Domain V	Cyanobacteria & Microalgae plastids	GGACAGAAAGACCCTATGAA	Sherwood and Presting, 2007
p23SrV_r1	23S Domain V	Cyanobacteria & Microalgae plastids	TCAGCCTGTTATCCCTAGAG	Sherwood and Presting, 2007

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Figure legends

Figure 1. Relative abundance of the main eukaryotes genus identified in the consortium under four growing conditions (97% OTU).

Figure 2. Relative abundance of the main prokaryotes phylum identified in the consortium under four growing conditions (97% OTU).

Figures

Figure 1.

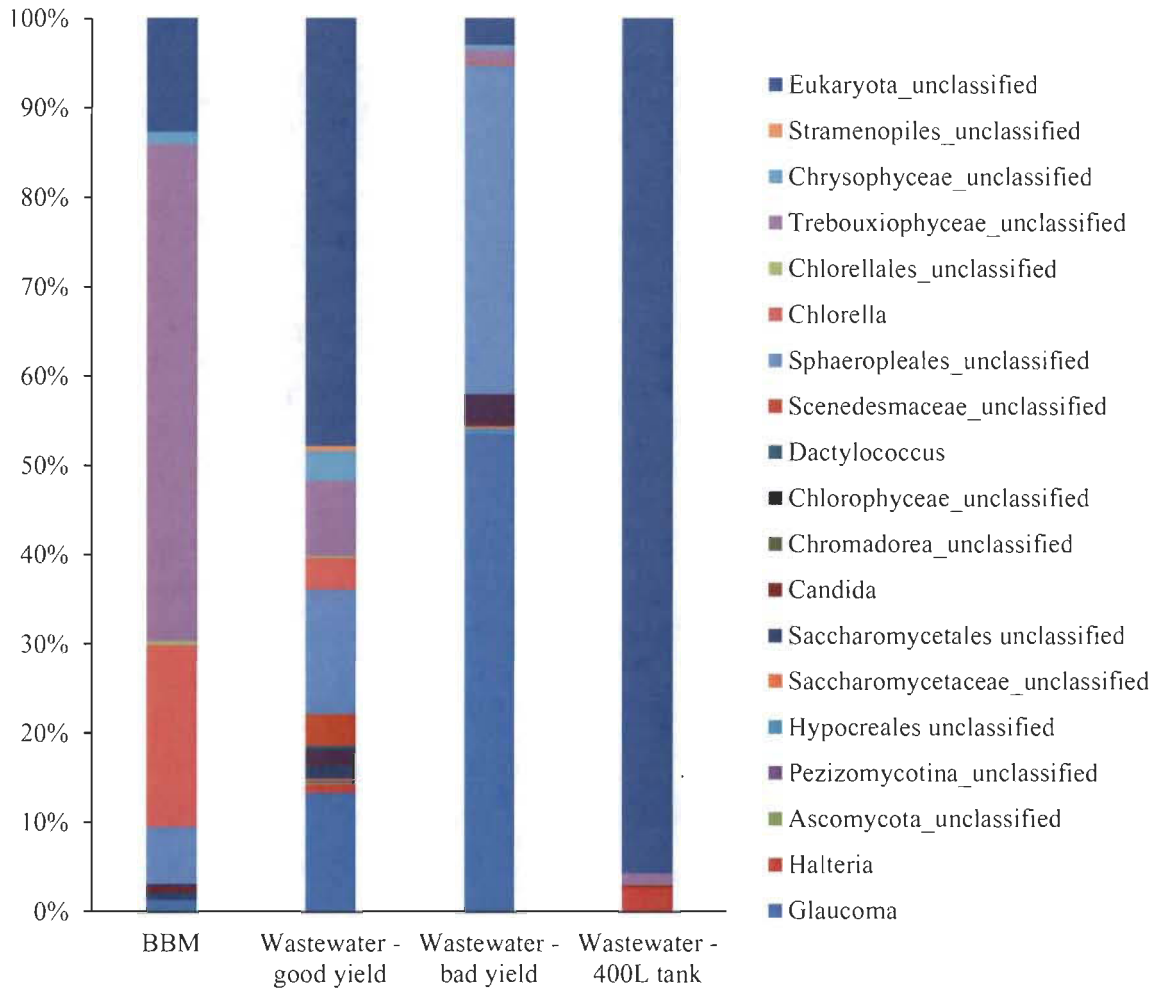
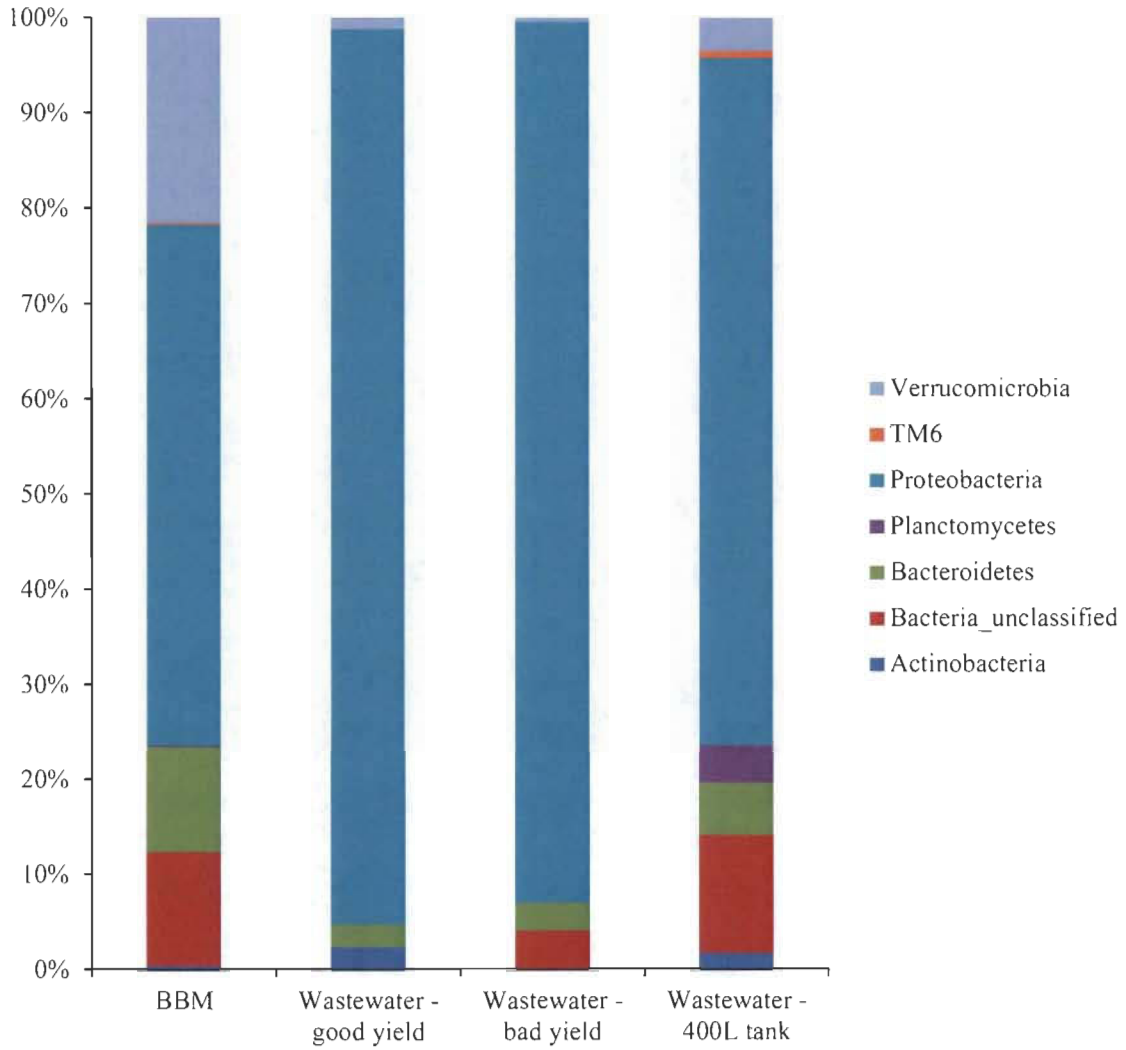


Figure 2.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
BBM
Wastewater - good yield
Wastewater - bad yield
Wastewater - 400L tank
Verrucomicrobia
TM6
Proteobacteria
Planctomycetes
Bacteroidetes
Bacteria_unclassified
Actinobacteria

CHAPITRE VII

CONCLUSION

Dans ce projet de doctorat, les conditions de croissances optimales du consortium d'algues-bactéries ont été déterminées. Les résultats ont montré que ce dernier a la capacité de se développer, dans le mélange d'eaux usées, dans différents modes trophiques. Les microalgues ont été en mesure de produire de faibles quantités des acides gras laurique (C12 :0) et myristique (C14 :0), recherchés, en plus d'une quantité élevée d'autres acides gras pouvant être utilisés pour fabriquer des coproduits. La mixotrophie et l'hétérotrophie sont les deux conditions de croissance qui permettent d'obtenir les plus hautes biomasses. Les plus grands pourcentages de lipides totaux extraits, par poids sec, ont été obtenus sous conditions autotrophiques et mixotrophiques. Enfin, les plus grandes quantités, relatives aux acides gras totaux, des deux acides gras recherchés (C12 :0 et C14 :0) ont été atteintes dans les cultures hétérotrophiques. Les résultats démontrent la possibilité de cultiver un consortium de microalgues-bactéries dans un mélange d'eaux résiduaires dans un parc industriel et ses environs tout en générant une biomasse utile à la production de produits à valeur ajoutée, en l'occurrence des biosurfactants utilisables par les entreprises locales. Des travaux futurs pourraient explorer la possibilité d'obtenir d'autres biomolécules intéressantes pour des utilisations locales.

Parmi toutes les conditions de stress testées, le contrôle du pH à 7 pendant la culture permet d'atteindre la plus grande quantité de lipides totaux extraits, par poids sec. Les plus grandes quantités, relatives aux acides gras totaux, des deux acides gras souhaités, C12: 0 et C14: 0, ont été obtenues dans le milieu de culture qui n'était pas supplémenté en nutriments. Ces résultats montrent que les acides gras recherchés peuvent être produits sans nécessiter l'addition d'éléments nutritifs, ce qui permettra des économies significatives sur le coût du traitement des eaux usées et l'ajout de nutriments dans les milieux de culture.

La stratégie de culture en deux étapes n'a pas permis d'obtenir le compromis souhaité entre la production de lipides et la densité cellulaire élevée. Des teneurs élevées en lipides totaux, par poids sec, ont été obtenues sous certains stress sans toutefois pouvoir équilibrer la production de lipides et de biomasse. La culture en une étape, dans des conditions mixotrophiques, s'est avérée plus efficace en termes de densité cellulaire et de production de lipides par rapport à la culture en deux étapes où le stress était induit au deuxième stade de la culture. L'addition de sel dans les milieux de culture et l'ajustement du pH sont deux stress environnementaux qui, une fois de plus, ont démontré leur impact sur la production de lipides. La culture en une étape avec stress induit semble être plus efficace pour la production de lipides. De futurs travaux pourraient porter sur la modification de la voie de synthèse des lipides dans les microalgues. Il a été rapporté que l'un des moyens d'augmenter le contenu en TAG consiste à bloquer les voies métaboliques impliquées dans la production de composés énergétiques (Radakovits et al. 2010). Le génie génétique des microalgues pour améliorer la production de lipides, en particulier sous la forme de TAG, pourrait constituer une piste intéressante.

Concernant l'efficacité du traitement des eaux usées par le consortium d'algues-bactéries utilisé dans ce projet, il a été démontré que ce dernier est efficace pour éliminer certains nutriments présents dans les eaux usées industrielles tout en produisant une biomasse pouvant être utilisée pour obtenir des produits à valeur ajoutée. Nos résultats suggèrent que la production de microalgues peut être implantée dans des parcs industriels pour traiter un mélange de différentes eaux usées et potentiellement obtenir des produits de valeur à partir de la biomasse produite. Bien que la mise à l'échelle de l'approche nécessite des efforts importants, elle semble constituer une approche intéressante pour réduire la gestion des coûts des eaux usées tout en contribuant à l'écologie industrielle du parc avec des produits potentiels d'origine biologique utilisables et des synergies nouvelles et habituelles. Pour examiner davantage le potentiel de traitement des eaux usées, des travaux futurs devraient porter sur le devenir des micropolluants présents dans les eaux usées des fabricants de produits ménagers et

de nettoyage utilisés dans cette étude et voir si les consortiums microalgues-bactéries peuvent être utilisés comme traitement tertiaire pour ce type d'entreprise.

Enfin, l'identification des populations bactériennes et algales qui composent le consortium permettra de mieux contrôler et d'augmenter la production des cultures. Comme attendu, les résultats ont montré des changements dans la composition des organismes eucaryotes et procaryotes au sein du consortium en fonction des milieux de culture testés. Les algues composant notre consortium semblent être plus affectées par la présence en masse de certains ciliés par rapport aux bactéries. Par ailleurs, l'identification d'une espèce potentiellement prometteuse, soit des espèces du genre *Candida*, est intéressante pour le traitement biologique des eaux usées industrielles et la production de biosurfactants. Comme il y a encore très peu d'informations concernant les genres bactériens et algaux impliqués dans le consortium, une meilleure compréhension de ces espèces impliquées pourrait mener à d'autres recherches sur des mécanismes de stimulation de la croissance des algues. Par exemple, les bactéries pourraient produire des phycohormones et être ajoutées pour stimuler la production d'algues par le biais de promoteurs de croissance. Une caractérisation physicochimique et biochimique des milieux de culture permettrait de mieux comprendre les interactions algues-bactéries afin de moduler l'approche de bioremédiation ou de production de coproduits.

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