Université de Montréal

Département des sciences biologiques | Institut de recherche en sciences biologiques
Faculté des arts et des sciences

Effets de l'irrigation par des eaux usées sur le développement, la morphologie et la composition du bois de saule

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Cette thèse intitulée :

Effets de l'irrigation par des eaux usées sur le développement, la morphologie et la composition du bois de saule

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Résumé

Dans un contexte de transition énergétique, l'utilisation de la biomasse lignocellulosique comme source d'énergie renouvelable pour la production de biocarburants a le potentiel de diminuer notre dépendance vis-à-vis des énergies fossiles polluantes et de réduire de façon radicale notre empreinte carbone. Autre que les résidus forestiers et agricoles; la biomasse lignocellulosique peut provenir des cultures énergétiques dédiées à base de plantes à croissance rapide comme les saules (*Salix* spp.). En effet, ces plantes ligneuses sont faciles à mettre en place et produisent de hauts rendements pendant plusieurs années. L'eau et les nutriments (essentiellement l'azote) étant cependant les principaux facteurs limitants à leur développement. L'utilisation des effluents municipaux constituent une alternative intéressante à l'irrigation conventionnelle et à la fertilisation par des engrais chimiques dont la synthèse même contribue à l'augmentation des gaz à effet de serre. En effet, les eaux usées qu'elles soient partiellement et /ou totalement traitées contiennent encore de grandes quantités d'azote et de phosphore qui peuvent être captées par les saules améliorant d'autant leur croissance et leur productivité.

La phytofiltration; c.-à-d. l'utilisation de filtre végétal est donc un concept séduisant; permettant d'une part de solutionner des problématiques sociétales et environnementales telles que la gestion des grands volumes d'effluents organiques et la préservation des milieux naturels et d'autre part de valoriser les contaminants qui sont en solution dans les effluents pour promouvoir le développement des plantes et en augmenter les rendements de la biomasse. Cette biomasse lignocellulosique pourrait aussi servir de matière première pour la production de biocarburant, néanmoins, il est important que l'emphase soit mise non seulement sur les quantités des bois produits, mais aussi sur leur qualité. En effet, lors de la production de biocarburant lignocellulosique, l'efficacité de la conversion biochimique d'une biomasse donnée est étroitement liée à la composition chimique de cette dernière.

L'objectif de cette thèse est de déterminer l'effet de l'irrigation par des effluents organiques sur la structure anatomique et la composition chimique du bois de divers génotypes de saules et de comprendre les répercussions d'une altération du bois, si elles existent, sur la récalcitrance du bois à l'hydrolyse enzymatique.

Une première étude a été conduite sur une plantation de saules *Salix miyabeana* 'SX67'. Le but de cette étude était de déterminer l'effet à moyen terme de l'irrigation par des eaux usées avec

traitement primaire (et donc ayant gardé une forte portion de sa charge organique et azotée) en premier lieu sur la physiologie et ensuite sur les caractéristiques intrinsèques du bois telles que sa composition chimique ou encore la structure hydraulique et mécanique. Dans un premier temps, les résultats ont montré que la fertigation a altéré la morphologie des feuilles ainsi que les stratégies d'utilisation du carbone ce qui a permis de maximiser la surface photosynthétique et d'améliorer le rendement quantique par unité de surface foliaire. Aussi, des analyses histologiques ont permis de déceler une élongation de la longueur des stomates chez les plantes fertiguées, engendrant une augmentation de la conductance stomatique et inévitablement celle du taux de carbone assimilé (la photosynthèse) permettant ainsi de promouvoir la croissance et le développement des plantes irriguées par les eaux usées et d'en améliorer la productivité. Dans un second temps, les analyses chimiques du bois du cultivar *Salix miyabeana* 'SX67' ont montré que pour les plantes qui ont été irriguées par des eaux usées, il y a eu une diminution de la fraction des extractibles ainsi qu'une augmentation de la fraction cellulose sans toutefois que cela soit accompagné d'une diminution de la lignine. Les analyses histologiques ont aussi dévoilé une différence de la densité des vaisseaux et de leur diamètre entre les plantes fertiguées et les témoins, toutefois, il ne peut être exclu que cette différence soit en partie occasionnée et/ ou accentuée par la méthode d'échantillonnage qui cible des régions différentes de la plante.

Dans une deuxième étude, la biomasse issue de trois expériences distinctes, réalisées au Canada (Beaverlodge et Whitecourt) et en Grande-Bretagne (Hillsborough) et dans lesquelles différents cultivars de saules ont été irrigués durant trois années de croissance par différents effluents organiques (des eaux usées avec traitement secondaire et des effluents de laiterie non traités), a été analysée afin d'étudier les conséquences de la fertigation sur la structure anatomique et la composition chimique du bois ainsi que sur sa récalcitrance à la saccharification enzymatique. Pour tous les sites, les résultats n'ont pas montré de différence pour les variables anatomiques et la densité du bois entre les plantes témoins et celles fertiguées , suggérant que ces cultivars étaient résilients à l'application d'effluents organiques. Cependant la fertigation a eu un effet sur la composition chimique et la récalcitrance du bois. La réponse des plantes a varié en fonction du site avec pas d'effets pour les cultivars de Beaverlodge, une diminution du taux d'extractibles et une augmentation des rendements en sucre pour certains cultivars de Whitecourt et une augmentation du contenu en xylose et une diminution des sucres libérées pour la plupart des génotypes de Hillsborough. Ces différences dans la réponse peuvent suggérer que des facteurs spécifiques aux

sites tels que les conditions environnementales et la composition des eaux usées pourraient avoir influencé la composition chimique du bois ainsi que sa récalcitrance à l'hydrolyse enzymatique. Suite à l'hydrolyse enzymatique, nous avons constaté que la récalcitrance d'une biomasse donnée n'est pas exclusivement liée à son contenu en cellulose étant donné que pour plus d'un cultivar, le rendement en glucose a été plus élevé que pour d'autres génotypes qui avaient une plus grande teneur en cellulose. Les analyses chimiques des différents cultivars ont permis de déceler une grande variabilité dans la teneur en cellulose (glucose) ainsi que dans l'indice de la récalcitrance entre les génotypes d'un même site, toutefois, la différence était plus prononcée entre les génotypes de sites différents. Aucun génotype n'étant commun aux trois sites nous ne pouvons cependant conclure clairement sur ce point qui mériterait donc des études subséquentes afin d'affiner plus encore la sélection des variétés au niveau local.

Cette thèse a permis de démontrer le potentiel des saules pour filtrer de grandes quantités d'effluents tout en apportant des informations nouvelles quant aux impacts sur la composition chimique et la récalcitrance de la biomasse produite dans ces circonstances. Les travaux ici présentés pouvant être l'assise nécessaire pour une optimisation efficace de cette phytotechnologie, tant du point de vue du choix variétal que de la modulation de la fertigation en vue de la double utilisation de la plantation productrice de biomasse pour les biocarburants et en tant que filtre végétal.

Mots-clés : phytoremédiation, traitement des eaux usées, saule, saccharification enzymatique, bioéthanol, xylème secondaire, conductance hydraulique.

Abstract

In the current context of transition to more sustainable energies, the use of lignocellulosic biomass as a renewable energy for biofuels production has the potential to decrease our dependence on polluting fossil fuels and drastically reduce our carbon footprint. Other than forestry and agricultural residues; lignocellulosic biomass can come from dedicated energy crops based on fast growing plants such as willows (*Salix spp*.). Indeed, these woody plants are easy to establish and produce high yields for several years, water and nutrients (mainly nitrogen) yet being the main plant development limiting factors. However, the use of municipal effluents is an interesting alternative to traditional irrigation and fertilization with chemical fertilizers, the very synthesis of which contributes to the increase in greenhouse gases. Indeed, wastewater, whether partially and /or fully treated, still contains large amounts of nitrogen and phosphorus which can be captured by willows, further improving their growth and productivity.

Phytofiltration; i.e. the use of plant-based filters is therefore an attractive concept; allowing on one hand to solve societal and environmental problems such as the management of large volumes of organic effluents and the preservation of natural environments; on the other hand to recover contaminants to promote plant development and increase biomass yields. Then, this lignocellulosic biomass could be used for the production of biofuel. But it is now important to focus research work on wood quality rather than on quantities produced. Indeed, during the production of lignocellulosic biofuel, biochemical conversion efficiency of a given biomass is closely linked to its chemical composition.

Therefore, the objective of this thesis is to determine the effect of irrigation by organic effluents on the anatomical structure and chemical composition of wood of various genotypes of willows and to understand the latter consequences on its recalcitrance to enzymatic hydrolysis.

A first study was carried out on a plantation of *Salix miyabeana* ‘SX67’ willows. The aim of this study was to determine the medium-term effect of irrigation by wastewater with primary treatment (with still a large portion of its organic and nitrogen load) first on plant physiology and then on intrinsic characteristics of wood such as its chemical composition or its hydraulic and mechanical structure. First, the results showed that fertigation altered leaf morphology as well as carbon utilization strategies with an increased photosynthetic area and improved quantum yield per unit

leaf area. Also, histological analyzes have made it possible to detect in fertigated plants stomata length elongation and therefore an increase in stomatal conductance and of the rate of assimilated carbon (photosynthesis) that promoted plant growth and development. In a second step, the chemical analyzes of the wood of the same *Salix miyabeana* 'SX67' plants showed that wastewater irrigation decreased the fraction of extractables as well as increased cellulose content, no change was observed for lignin fraction. Histological analyzes also revealed a difference in the vessel density and diameter between fertigated plants and controls, however it cannot be excluded that this difference is partly caused and /or accentuated by the sampling method chosen (region of the plant where the samples were taken).

In a second study, the biomass resulting from three separate experiments carried out in Canada (Beaverlodge and Whitecourt) and in Great Britain (Hillsborough) where different cultivars of willows were irrigated for three years by different organic effluents (wastewater with secondary treatment and primary dairy farm wastewater) was analyzed in order to study the consequences of fertigation on the anatomical structure and the chemical composition of wood as well as its recalcitrance to enzymatic saccharification. For all sites, the results showed no difference between control and fertigated irrigated plants neither for anatomical variables nor for wood density, suggesting that these cultivars would have been resilient to the application of organic effluent. Regarding the effect of fertigation on wood chemical composition and recalcitrance, the plants response varied between sites. No effects was observed for Beaverlodge cultivars but we measured a decrease in extractables content and an increase of sugar yields for some cultivars of Whitecourt and finally, an increase in xylose content and a decrease of the rate of sugar yields for most of Hillsborough genotypes. This difference in response between the three sites may suggest that site-specific factors such as wastewater composition but also distinct environmental conditions may have influenced the chemical composition of the wood as well as its recalcitrance to enzymatic deconstruction. Following enzymatic hydrolysis, we found that the recalcitrance of a given biomass is not exclusively related to its cellulose content since for more than one cultivar glucose yield was higher than for others which had higher cellulose content. Finally, chemical analyzes of the different cultivars made it possible to detect a great variability in the content of cellulose (glucose) as well as in the index of recalcitrance between genotypes planted on the same site, but the differences were much more pronounced between different sites. However, no genotype being

common to the three sites, we cannot clearly conclude on this point, which would therefore merit subsequent studies in order to further refine the selection of varieties at the local level.

This thesis allows to demonstrate the potential of willows to filter large quantities of effluents while providing new information on the impacts on the chemical composition and the recalcitrance of the biomass produced under these circumstances. The work presented here may be the necessary basis for an effective optimization of this phytotechnology, both from the point of view of varietal choice and of the modulation of fertigation in the perspective of both the plantation producing biomass for biofuels and as a cost-effective planted filter.

Key words : phytoremediation, wastewater treatment, willow, enzymatic saccharification, bioethanol, secondary xylem, hydraulic conductance

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Liste des symboles et des abréviations

Kg m^{-1} Mpa $^{-1}$ s^{-1} :	Kilogrammes par mètre par mégapascal par seconde
A :	Le taux de la photosynthèse
A :	Surface de la lumière du vaisseau
$\bar{A}$:	Surface moyenne du vaisseau
ABA :	Acide abscissique
AIL :	La lignine insoluble par hydrolyse acide
ASE :	Extraction par liquide accélérée
ASL :	La lignine soluble par hydrolyse acide
C:N :	Ratio Carbone Azote
CCME :	Conseil canadien des ministres de l'environnement
CEC :	Capacité d’échange cationique
Chla :	Chlorophylle a
Chlb :	Chlorophylle b
CICR :	Culture intensive en courtes rotations
cmol kg^{-1} :	Centimoles par kilogramme
COD :	Demande chimique en oxygène
Cx :	Concentration des caroténoïdes
D :	Diamètre de la zone lumière du vaisseau
$\bar{D}$:	Diamètre moyen de la zone lumière du vaisseau
D_H :	Diamètre hydraulique moyen
DMSO :	Diméthylsulfoxyde
dS m^{-1} :	Deci Siemens par mètre
EC :	Conductivité électrique
ECCC :	Environnement et changement climatique Canada
EDG % :	Taux du glucane qui provient de l’hydrolyse enzymatique
EtOH :	Éthanol

F :	Fraction du xylème secondaire que représente la zone lumière des vaisseaux
FAA:	Abréviation pour formaldéhyde, acide acétique et alcool
FPU :	Unité de papier filtre de cellulose
g glucose g^{-1} ODW:	Gramme de glucose par gramme de biomasse anhydre
Gb:	Gigabyte
G-fiber :	Fibres gélatineuses
G-layer :	Couche la plus interne de la paroi cellulaire des fibres gélatineuses
gs :	Conductance stomatique à la vapeur d’eau
HPLC :	Chromatographie en phase liquide à haute performance
IRGA :	Analyseur de gaz infrarouge
Kg ha^{-1} yr^{-1} :	Kilogrammes à l’hectare par an
K_h :	Conductivité hydraulique théorique
K_S :	Conductivité hydraulique spécifique théorique
LA :	Surface foliaire
LAI :	Indice de surface foliaire
LMA :	Indice de la masse foliaire par la surface foliaire
Mc % :	Teneur en humidité
MELCC :	Ministère de l’Environnement et de la Lutte contre les changements climatiques
ML ha^{-1} yr^{-1} :	Million de litres par hectare par an
mol m^{-2} s^{-1} :	Mole par mètre carré par seconde
N/Area :	Concentration d’azote par unité de surface foliaire
NF :	Fraction du xylème secondaire autre que celle de la zone lumière des vaisseaux
NO_X :	Azote minérale
NREL :	Laboratoire national de l’énergie renouvelable
ODW :	Masse sèche anhydre
o-P04 :	Orthophosphate

pH :	Potentiel d’hydrogène
P_{Length} :	Longueur du pore du côté abaxiale de la feuille
PNUE :	Efficacité instantanée de l'utilisation de l'azote photosynthétique
PW :	Eau potable
P_{Width} :	Largueur du pore du côté abaxiale de la feuille
Ratio S/G :	Proportion des unités Syringyle et Guaïacyle de la lignine
S :	Index de la composition des vaisseaux dans le xylème
S_{Area} :	Surface du stomate du côté abaxiale de la feuille
SLA :	Surface foliaire spécifique
S_{Length} :	Longueur du stomate du côté abaxiale de la feuille
SPI :	Index du pore stomatique
SRWC :	Cultures de saules en courtes rotations
S_{Width} :	Largeur du stomate du côté abaxiale de la feuille
TN :	Azote total
UI :	Non irrigué
Unité G :	Guaïacyle
Unité H :	Hydroxyphényle
Unité S :	Syringyle
VI :	Index de vulnérabilité du vaisseau
Wt% :	Proportion par masse
WW :	Eaux usées
WWD :	Dose d’eaux usées
WWD1 :	Dose une d’eaux usées
WWD2 :	Dose deux d’eaux usées

بسم الله الرحمن الرحيم

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Chapitre 1 | Introduction générale

1.1 Avant-propos

L’approvisionnement en biomasse pour soutenir la transition vers l’utilisation d’énergies renouvelables constitue un des défis auquel l’humanité aura à faire face au courant des prochaines décennies (Volk *et al.*, 2004). Dans un contexte d’utilisation de la matière lignocellulosique pour la production de bioénergie (essentiellement les biocarburants), la biomasse peut provenir de plusieurs sources différentes telles que les forêts, les cultures agricoles, les produits du bois et les cultures énergétiques dédiées à base de plantes herbacées ou ligneuses (Volk *et al.*, 2004; Gomez, Steele-King and McQueen-Mason, 2008; Christersson, 2010). En effet, les cultures de plantes ligneuses à croissance rapide telles que les saules (*Salix* sp.) sont faciles à mettre en place et produisent de hauts rendements en biomasse pendant plusieurs années (Volk *et al.*, 2004; Guidi, Pitre and Labrecque, 2013; Sevel *et al.*, 2014). Toutefois, ces rendements sont fortement influencés par la disponibilité de l’eau et des nutriments, surtout l’azote (Labrecque and Teodorescu, 2003; Weih *et al.*, 2011; Guidi, Pitre and Labrecque, 2013; Sevel *et al.*, 2014). L’azote est souvent considéré comme le principal élément limitant pour le développement des cultures intensives en courtes rotations (CICR) de saules et son exportation en grande quantité lors des fréquents recépages peut diminuer la fertilité des sols et compromettre la durabilité des plantations (Labrecque, Teodorescu and Daigle, 1998; Adegbidi and Briggs, 2003; Guidi, Pitre and Labrecque, 2013). Une alternative à la fertilisation par les engrais chimiques, dont l’usage parfois abusif contribue à l’acidification des sols et l’eutrophisation des milieux (Gilbert, Thornley and Riche, 2011), est l’utilisation des effluents issus de l’activité agricole et municipale (Labrecque and Teodorescu, 2001; Larsson *et al.*, 2003; Guidi, Piccioni and Bonari, 2008; Cavanagh, Gasser and Labrecque, 2011; Jerbi *et al.*, 2015). En effet, les eaux usées contiennent de grandes quantités d’azote et de phosphore qui peuvent être problématiques si elles sont déversées dans l’environnement, mais bénéfiques si elles sont utilisées comme nutriments pour la production de saules (Guidi, Pitre and Labrecque, 2013; Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015). Pour le développement des bioénergies, outre le souci d’accroître les rendements en biomasse, il est également important de s’intéresser à la qualité du bois produit. En effet, la composition chimique d’une biomasse donnée influence directement l’efficacité de sa conversion biochimique lors de la production de biocarburant lignocellulosique (Serapiglia, Cameron, *et al.*, 2013). Ainsi, la détermination de la composition chimique du bois des saules suite à leur irrigation par des eaux usées est essentielle pour permettre, d'une part de déceler si un tel traitement occasionne une altération chimique de la macrostructure du bois et, ultimement, de comprendre les répercussions sur les rendements en biocarburant. Aussi, l'étude de plusieurs cultivars de saule permettrait de vérifier si les réponses varient selon les génotypes et d'identifier ceux dont la composition chimique est plus optimale pour la conversion en biocarburant ou en autres bioproduits d'intérêts.

1.2 Les cultures intensives en courte rotation de saules

1.2.1 Définition des cultures intensives des saules en courte rotation (CICR)

La CICR constitue un mode de production de biomasse ligneuse utilisant des plantes à croissance rapide telles que les peupliers hybrides et les saules (Wickham *et al.*, 2010). La mise en place des cultures se fait conventionnellement avec des boutures (20-25 cm de longueur) prélevées à partir de jeunes plantes (habituellement un an de croissance) qui sont plantées à une profondeur d' environ 18 cm (Guidi, Pitre and Labrecque, 2013) avec une densité qui généralement varie entre 16 000 et 20 000 plants/ha (Labrecque and Teodorescu, 2003, 2005). En CICR, les tiges sont récoltées par recépage et d'une façon répétitive à chaque deux, trois ou quatre ans d'intervalle, permettant une exploitation qui peut durer plusieurs années (20 à 25 ans) (Dimitriou and Aronsson, 2005).

1.2.2 Besoins et statut nutritionnel des saules en CICR

Plusieurs recherches ont montré que la fertilisation des CICR de saules lors de l'année d'établissement (la mise en terre des boutures) était inutile, et qu'elle pouvait même être à l'avantage des mauvaises herbes (Adegbidi and Briggs, 2003; Labrecque and Teodorescu, 2003; Guidi, Pitre and Labrecque, 2013). À l'opposé, certaines études, dont celle de Ledin (1986) rapportait qu'une quantité de 60 kg N ha^{-1} devrait être appliquée au cours de l'année d'établissement d'une plantation de saules en CICR et que des quantités annuelles de 80-120 kg N ha^{-1}, 30 kg P ha^{-1} et de 80 kg K ha^{-1} étaient nécessaires pour répondre aux besoins nutritionnels de la culture lors des années subséquentes. Généralement, dans les CICR, l'azote est considéré comme l'élément limitant pour la croissance des plantes et le principal facteur à considérer pour maintenir de hauts rendements de biomasse (Labrecque, Teodorescu and Daigle, 1998; Adegbidi and Briggs, 2003). Par ailleurs, plusieurs travaux ont montré que les saules pouvaient assimiler annuellement de grandes quantités d'azote, jusqu'à 600 kg N ha^{-1} (Nielsen, 1994; Adegbidi and Briggs, 2003;

Cavanagh, Gasser and Labrecque, 2011; Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015; Fabio and Smart, 2018b).

1.2.3 La croissance et les rendements des saules en CICR

Les rendements en biomasse des CICR influencent grandement la rentabilité de ces systèmes de cultures. En effet, la survie même d'une CICR exige qu'à travers les rotations, les rendements soient assez importants pour supporter le coût relié à leur exploitation (Heller, Keoleian and Volk, 2003; Volk *et al.*, 2006). Des études faites en Grande-Bretagne et ailleurs en Europe montrent que les rendements annuels moyens de matière sèche pour les cultures de saules en CICR se situaient entre 15-20 t ha^{-1}, alors que des recherches faites aux É.U et au Canada ont rapporté des rendements annuels aussi élevés que 24-30 t ha^{-1} (Adegbidi *et al.*, 2001; Labrecque and Teodorescu, 2003; Guidi, Pitre and Labrecque, 2013; Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015). Cela dit, la grande productivité de ces plantes entraîne aussi que d'importantes quantités de nutriments seront retirées du sol lors des recépages successifs. En effet, Adegbidi *et al.* (2001) rapportent que pour une biomasse sèche annuelle de 15-22 t ha^{-1} , les quantités d'azote, de phosphore et de potassium qui sont retirées lors de la récolte des tiges sont respectivement de 75-86, 10-11 et 27-32 kg ha^{-1}. Ainsi, il est important de fertiliser les cultures de saules suivant chaque recépage afin de compenser pour la perte des grandes quantités de nutriments retirées du système et garder le sol fertile.

1.3 Valorisation des effluents (eaux usées) pour la fertilisation des cultures de saules

La purification des eaux usées par des cultures de saules en CICR est une pratique qui a vu le jour en Suède, mais qui s'est largement répandue par la suite dans divers pays en raison du faible coût de cette pratique et des avantages environnementaux que celle-ci occasionne en comparaison aux méthodes traditionnelles de traitement des effluents (Perttu, 1994; Truu, Truu and Heinsoo, 2009; Guidi Nissim *et al.*, 2015).

Les saules possèdent plusieurs caractéristiques qui les rendent propices à l'utilisation en tant que filtre végétatif : une grande capacité de transpiration, de hauts rendements en biomasse, tolérance à divers contaminants et absorption sélective des métaux lourds (Elowson, 1999; Mitchell, Stevens and Watters, 1999; Aronsson and Perttu, 2001; Truu, Truu and Heinsoo, 2009). Outre le fait que les eaux usées municipales peuvent subvenir aux demandes hydriques des plantations de saules en CICR, leur composition présente une source de nutriments assez bien balancée qui peut combler les besoins de ces plantes sans que ce cela ne demande d'ajout additionnel d'engrais (Dimitriou and Aronsson, 2003, 2004; Guidi, Pitre and Labrecque, 2013). Mirck *et al.* (2005) ont trouvé que 60% de l'azote en solution dans l'eau usée utilisée pour l'irrigation de saules avait été assimilée dans la biomasse, alors qu'une grande partie de la fraction restante se retrouvait dans la composition minérale du sol et/ou perdue par dénitrification. Dimitriou (2005) estime, pour sa part, qu'un tel système permet de retirer plus de 200 kg N ha^{-1} annuellement.

1.4 Les propriétés du bois

1.4.1 La structure anatomique et hydraulique du bois (la densité, la taille et la conductance hydraulique des vaisseaux).

Le système vasculaire des plantes se divise en xylème et phloème qui transportent respectivement la sève brute et la sève élaborée entre les différentes parties de la plante. Pour les angiospermes, le xylème est formé principalement par l'agencement de trois types de cellules que sont les vaisseaux c.-à-d. les cellules conductrices non vivantes qui permettent de transporter l'eau et des nutriments longitudinalement depuis les racines jusqu'aux feuilles; les fibres c.-à-d. les cellules entourant les vaisseaux qui vivent plus longtemps que les vaisseaux, mais une fois mortes agissent comme éléments de soutien et les cellules parenchymateuses qui servent au stockage de divers composés et de l'eau ainsi qu'au transport entre les différentes cellules situées dans un l'axe radial (Evert, 2006a, 2006b; Poorter *et al.*, 2010; Oda and Fukuda, 2012; Lautner, 2013; Plavcová *et al.*, 2013). Toutefois, les fibres et les vaisseaux constituent la plus grande fraction du tissu conducteur qui peut représenter jusqu'à 85–90% (volume /volume) du bois mature chez certains peupliers (Mellerowicz *et al.*, 2001). La structure physique ainsi que l'abondance de ces deux types de cellules définissent majoritairement les propriétés mécaniques et hydrauliques du bois (Mellerowicz *et al.*, 2001; Plavcová *et al.*, 2013). Le diamètre des éléments conducteurs du bois aura des conséquences sur la capacité de conduction hydraulique d'une plante (Plavcová and Hacke, 2012; Plavcová *et al.*, 2013; Hacke *et al.*, 2017). Ainsi, une tige avec moins de vaisseaux, mais avec de larges diamètres sera plus avantageux pour le transport de l'eau qu'une tige avec de nombreux vaisseaux, mais avec une lumière plus étroite (Sperry, Hacke and Pittermann, 2006; Sperry, Meinzer and McCulloh, 2008; Martínez-Cabrera *et al.*, 2011). Une meilleur conductance hydraulique entrainera un plus haut taux de transpiration (conductance stomatique), et de photosynthèse et conséquemment permettra une meilleure croissance (Poorter *et al.*, 2010; Martínez-Cabrera *et al.*, 2011). Toutefois, des vaisseaux plus larges sont aussi plus à risque à l'embolie (occurrence de l’air dans un vaisseau ou un élément du vaisseau qui empêcherait la conduction de l’eau) en cas de sécheresse et/ou de gel (Plavcová and Hacke, 2012; Hacke *et al.*, 2017). Un système de transport avec des vaisseaux ayant un diamètre réduit rend ces derniers moins vulnérables à la cavitation et améliore la structure mécanique de la tige (c.-à-d. augmentation de la densité du bois). Cependant ceci est au coût d'une plus faible efficacité dans le transport de l'eau et subséquemment d’une moins grande productivité (Poorter *et al.*, 2010; Plavcová and Hacke, 2012; Ziemińska *et al.*, 2013; Hacke *et al.*, 2017).

1.4.2 La composition chimique du bois

Le bois résulte de la croissance successive et cumulative du xylème secondaire suivant les saisons de croissance (Pitre *et al.*, 2010). La composition chimique du bois fait généralement référence à celle des parois secondaires des cellules qui constituent le xylème. Ces parois se constituent de trois composés principaux, la lignine, les hémicelluloses et la cellulose (Lautner, 2013; Mikshina and Chernova, 2013).

La cellulose est un polymère linéaire simple (sans embranchement) de chaines de glucanes, formés par des résidus successifs de β -1,4-glucose avec une inversion de 180º entre deux résidus, formant l’unité de répétition qui est le cellobiose (disaccharide). Dans le bois, le taux de cellulose (qui est de 40–50 % en masse sèche) ainsi que l’angle de ces microfibrilles sont les principaux déterminants de la rigidité et du module d’élasticité (Plomion, Leprovost and Stokes, 2001; Lautner, 2013). Généralement, les microfibrilles de cellulose sont composées par 36 chaines de glucose et chaque chaine peut comporter entre 500-14 000 unités de D-glucose (Mohnen, Bar-Peled and Somerville, 2009; Zhao, Zhang and Liu, 2012).

Les hémicelluloses constituent un groupe de polysaccharides complexes avec un faible poids moléculaire et une grande solubilité dans l’eau et dans les solutions alcalines. Représentant 20 à 40% de la biomasse ligneuse, les hémicelluloses contribuent au renforcement de la paroi cellulaire en interagissant respectivement avec les microfibrilles de cellulose et la lignine par des liaisons hydrogène et covalente (Scheller and Ulvskov, 2010). Structurellement, les hémicelluloses sont formées essentiellement par un squelette composé de résidus β-(1,4)-D-pyranose avec de courtes chaines latérales (Ebringerová, 2005; Ochoa-Villarreal, Aispuro-Hernández, Emmanuel Vargas-Arispuro and Martínez-Téllez, 2012) et peuvent contenir cinq atomes de carbone (sucres en C5) tels que le xylose (le deuxième sucre le plus abondant dans la biosphère après le glucose (Lee *et* *al.*, 2011)) et l'arabinose ou six atomes de carbone (sucres en C6) tels que le glucose, le mannose, le galactose, l'acide galacturonique et l'acide glucuronique (Scheller and Ulvskov, 2010). Dépendamment de l'agencement moléculaire de ces différents monomères de sucres, les hémicelluloses sont classées en quatre grands groupes : les xyloglucanes, les xylanes (incluant les glucuronoxylanes, les arabinoxylanes, les glucuronoarabinoxylanes et les homoxylanes), les mannanes/glucomannanes (incluant les galactomannanes et les galactoglucomannanes) et les *β*-1,3;1,4-glucanes.

La lignine représente un vaste groupe de polymères aromatiques de composés phénoliques qui représente 25–35 % de la biomasse ligneuse. Ces polymères sont déposés principalement dans les parois secondaires des cellules lors des dernières étapes de la phase de différenciation cellulaire en conférant à ces parois la rigidité, l'imperméabilité et la résistance contre la dégradation microbienne (Boudet, 2000; Donaldson, 2001). Chimiquement, la lignine se constitue de polymères à squelette aromatique phénolique formé par l'agencement de trois alcools hydroxycinnamiques (monolignols) : l'alcool p-coumarylique, l'alcool coniférylique et l'alcool sinapylique, donnant naissance aux unités H, G et S, qui diffèrent entre elles que par leur degré de méthoxylation (Boerjan, Ralph and Baucher, 2003). Le taux de lignine et sa composition varient largement en fonction de la couche de la paroi secondaire, des types de cellules du xylème, du type de tissu, des individus et de l'espèce végétale. La lignine des angiospermes (bois dur) est davantage composée de monolignols coniférylique et sinapylique avec des traces d'alcool p-coumarylique, alors que pour les gymnospermes (bois tendre) la lignine est essentiellement formée par l'alcool coniférylique (Boudet, 2000; Lautner, 2013).

Outre les polysaccharides (cellulose et hémicellulose) et les phénols (lignine) qui représentent respectivement 65-75 % et 20-30 % de la biomasse ligneuse, le bois est aussi constitué d'autres composées mineurs qui se présentent sous forme d'extractibles organiques et inorganiques (c.-à-d. les cendres qui sont essentiellement du calcium, du magnésium, du manganèse et de la silice) qui représentent 4-10 % de la fraction du bois (Nascimento *et al.*, 2013). Généralement, les extractibles sont en lien avec des activités biologiques importantes dans le bois et lui donne sa couleur et son parfum et en assurant sa durabilité (Yang and Jaakkola, 2011). La nature et la composition des extractibles peuvent varier entre les différentes espèces de bois ainsi que les différents tissus d'un même individu (Yang and Jaakkola, 2011). Les extractibles organiques sont généralement classés en quatre grands groupes qui sont (i) les composés aliphatiques et alicycliques (les terpènes, les terpénoïdes, les esters d'acides gras, les acides gras et les alcools), (ii) les composés phénoliques (tels que les simples phénols, les stilbènes, les lignanes, les isoflavones, les tannins condensés, les flavonoïdes et les tannins hydrolysables), (iii) les gommes et (iv) autres divers composés (tel que les sucres, les cyclitoles, les tropolones, les acides aminés, les alcaloïdes, les coumarines, les quinones) (Roffael, 2016). Toutefois, le groupe des phénoliques se dégage du reste des extractibles par leurs importances physiologiques du fait que les phénols constituent une barrière mécanique (la lignine) et participent vivement à sa défense contre des attaques biologiques causant la biodégradation (bactéries, champignons, insectes) (Nascimento *et al.*, 2013; Berthod, 2015).

1.5 Le bois (biomasse lignocellulosique) dans un contexte de production de biocarburant (bioéthanol)

1.5.1 La biomasse lignocellulosique en tant que matière première

La production de biocarburant et d'autres produits d'intérêts à partir de la biomasse lignocellulosique est une alternative attrayante à l'utilisation des carburants fossiles de par leur caractère renouvelable et leur faible coût d'acquisition (Sassner, Galbe and Zacchi, 2008; Zoghlami and Paës, 2019). Plusieurs sources de biomasse cellulosique peuvent être utilisées pour la production de carburant : les résidus forestiers (copeaux et sciure de bois) et agronomiques (paille de céréales et de blé, tiges de maïs et bagasse de canne à sucre) ainsi que les cultures énergétiques dédiées de plantes à croissance rapide tel que le panic érigé, les peupliers et les saules (Sassner *et al.*, 2008; Sassner, Galbe and Zacchi, 2008; Zheng, Pan and Zhang, 2009; Serapiglia, Humiston, *et al.*, 2013; Volynets, Ein-Mozaffari and Dahman, 2017; Zoghlami and Paës, 2019).

1.5.2 Les cultures de saules en CICR dans un contexte de la production bioénergétique

L'utilisation de CICR de saules en tant que cultures énergétiques dédiées pour la production de biocarburant lignocellulosique constitue une alternative intéressante tant pour des questions environnementales qu'économiques. De plus les CICR peuvent être établies sur des sols marginaux pour l'agriculture tout en permettant l'obtention de rendements élevés (Brereton *et al.*, 2012; Ray *et al.*, 2012; Serapiglia, Humiston, *et al.*, 2013).

Le choix judicieux des espèces ainsi que l'amélioration des pratiques de cultures (essentiellement l'irrigation et la fertilisation) permettent d'améliorer la productivité de ces cultures et de produire davantage de biocarburant. Toutefois, la qualité de la biomasse (c.-à-d. sa composition chimique) et la facilité avec laquelle celle-ci peut être transformée en biocarburant constituent des éléments déterminants pour la rentabilité du processus (Brereton *et al.*, 2012; Ray *et al.*, 2012; Serapiglia, Cameron, *et al.*, 2013; Wan *et al.*, 2014). Au cours des dernières années, plusieurs études ont mis en évidence la riche variation des composantes chimiques du bois selon les espèces ou les cultivars de saule considérés (Serapiglia *et al.*, 2009, 2012; Brereton *et al.*, 2011; Ray *et al.*, 2012; Serapiglia, Cameron, *et al.*, 2013; Serapiglia, Humiston, *et al.*, 2013). Cependant, la structure et la composition chimique des parois cellulaires sont aussi responsables de la récalcitrance du bois à l'hydrolyse enzymatique et constituent le principal obstacle dans le processus de production du biocarburant (Wei *et al.*, 2009; Brereton *et al.*, 2010, 2012; Gilbert, 2010; Studer *et al.*, 2011; Serapiglia *et al.*, 2012; de Souza, 2013).

1.5.3 Les facteurs affectant la récalcitrance de la biomasse lignocellulosique

Généralement la récalcitrance de la biomasse à l'hydrolyse enzymatique est due à des facteurs chimique et/ou physique qui peuvent être liés directement ou indirectement à la structure de la paroi cellulaire et/ou à sa composition chimique (Studer *et al.*, 2011; Zhao, Zhang and Liu, 2012; Serapiglia, Humiston, *et al.*, 2013; Bichot *et al.*, 2018; Zoghlami and Paës, 2019). On considère que le degré de polymérisation des chaines de la cellulose, l'occurrence de groupements acétyle dans l'hémicellulose, la présence de groupes hydroxyle et des liaisons hydrogène de la lignine et le ratio S/G (syringyle/ guaïacyle) dans la lignine sont les principaux facteurs chimiques qui déterminent cette récalcitrance (Pan, Gilkes and Saddler, 2006; Mohnen, Bar-Peled and Somerville, 2009; Brereton *et al.*, 2010; Zhao, Zhang and Liu, 2012; Meng *et al.*, 2017; Yoo *et al.*, 2018; Zoghlami and Paës, 2019). Les facteurs physiques sont essentiellement le taux de la cristallinité de la cellulose (l'occurrence des zones cristallines par rapport aux zones amorphes dans les fibres de cellulose) et la taille des particules de bois (Pan, Gilkes and Saddler, 2006; Mohnen, Bar-Peled and Somerville, 2009; Zhao, Zhang and Liu, 2012; Zoghlami and Paës, 2019).

1.6 Effet de l'irrigation, de la fertilisation et de l'environnement sur les propriétés du bois des espèces ligneuses tel que les saules ou le peuplier

Le processus de formation du bois (la xylogenèse) est grandement affecté par les conditions environnementales telles que la disponibilité de l'eau, de la lumière et des nutriments qui peuvent altérer le développement des plantes ainsi que les caractéristiques anatomiques et chimiques du bois (Arend and Fromm, 2007; Hacke *et al.*, 2010; Pitre *et al.*, 2010; Plavcová and Hacke, 2012; Anfodillo, Petit and Crivellaro, 2013; Plavcová *et al.*, 2013).

1.6.1 L'effet sur la physiologie, le développement et la production

Un déficit en eau et/ou en azote peut affecter grandement le développement et la productivité des plantes. Toutefois, la réponse peut varier en fonction des différentes espèces ainsi que de l'intensité et de la durée du stress en question (Munns, 2002; Shrivastava and Kumar, 2015). Généralement, le manque de ressources hydriques et/ou de l'azote résulte en une diminution de la taille des feuilles, un accroissement de l'épaisseur des feuilles, une diminution du contenu en chlorophylle et une diminution de la conductance stomatique (Lambers, Chapin and Pons, 2008). En effet, la conductance stomatique est essentiellement contrôlée par le degré d'ouverture des stomates qui, à leur tour, sont sensibles au signal chimique provenant des racines. Lorsque l'eau devient limitante, les racines déshydratées vont synthétiser de l'acide abscissique (ABA) dont le transport, jusqu'au niveau des feuilles et la captation par les récepteurs à ABA localisés au niveau des cellules de garde, va engendrer une fermeture partielle ou totale des stomates réduisant ainsi la conductance stomatique et inévitablement la quantité de carbone atmosphérique assimilée (Long and Hällgren, 1993; Liu *et al.*, 2001; Chaves, 2002; Davies, Wilkinson and Loveys, 2002; Holbrook, 2002; Bacon, 2009).

La fertilisation des plantes par l'azote induit généralement une augmentation de la surface foliaire, de la concentration d'azote foliaire et de la concentration des pigments photosynthétiques. Ceci se traduit par un accroissement du taux de carbone assimilé et conséquemment une meilleure croissance de la plante (Merilo *et al.*, 2006; Ennajeh *et al.*, 2010; Hacke *et al.*, 2010; Park *et al.*, 2016).

1.6.2 L'effet sur les caractéristiques anatomique, hydraulique et mécanique du bois

La formation du bois est un processus dynamique qui est largement affecté par les conditions environnementales (Arend and Fromm, 2007; Hacke *et al.*, 2010; Anfodillo, Petit and Crivellaro, 2013; Plavcová *et al.*, 2013). Lors de la xylogenèse, le bois peut être affecté par divers facteurs environnementaux. Toutefois ces variations restent difficiles à interpréter, du fait de l'implication d'autres facteurs dans le développement du bois tel que la balance hormonale, la fixation et l'allocation du carbone, le statut hydrique, etc. (Drew *et al.*, 2009; Budzinski *et al.*, 2016).

Effet de l'eau — Lors de la xylogénèse, le processus d'expansion des cellules précurseures des fibres et des vaisseaux est en fonction de la turgescence cellulaire et donc lié à l'absorption et l'accumulation de l'eau et des solutés (Langer *et al.*, 2002; Kim, Funada and Singh, 2015). La carence de ces ressources, surtout hydriques, affecte l'activité cambiale ainsi que le processus d'expansion cellulaire affectant de facto la densité du bois (Drew *et al.*, 2009; Kim, Funada and Singh, 2015). Un stress hydrique entraine des répercussions anatomiques (une réduction de la longueur et du diamètre des fibres, une augmentation de la densité des éléments vaisseaux, une réduction du diamètre des vaisseaux et une augmentation de l'épaisseur de leur paroi) et mécaniques qui permettent de minimiser les risques d'embolie (Harvey and van den Driessche, 1999; Hacke *et al.*, 2001, 2010, 2017; Wimmer, Downes and Evans, 2002; Bouriaud *et al.*, 2005; Arend and Fromm, 2007; Drew *et al.*, 2009; Lautner, 2013; Tausz and Grulke, 2014).

Effet de la salinité — La salinité réduit la capacité des cellules à assimiler et à retenir l'eau et contribue grandement à la variation des propriétés du bois avec des traits semblables à ceux occasionnés par une carence en eau. Ces altérations incluent une augmentation de la densité des vaisseaux, une diminution de leur aire, un épaississement de leur paroi et une augmentation de la densité du bois. Toutefois, le degré d'altération est spécifique à l'espèce ainsi qu'au type et à l'ampleur du stress (Junghans *et al.*, 2006; Janz *et al.*, 2012; Lautner, 2013; Bhattacharjee and Saha, 2014; Shrivastava and Kumar, 2015).

Effet de la fertilisation (azote) — La fertilisation (essentiellement en azote) affecte grandement les phases de la division et de la différenciation cellulaire ce qui peut engendrer des changements dans l'anatomie du bois et en affecter les capacités hydrauliques et les propriétés mécaniques (Luo *et al.*, 2005). En effet, plusieurs études ont montré que la fertilisation azotée occasionnait des altérations an niveau des fibres et des vaisseaux avec, d'une part, une augmentation du diamètre des fibres et une diminution de l'épaisseur de leur paroi et, d'autre part une augmentation du diamètre des vaisseaux, ce qui entrainait une augmentation considérable la conductivité hydraulique théorique spécifique K_S (Harvey and van den Driessche, 1999; Luo *et al.*, 2005; Bucci *et al.*, 2006; Hacke *et al.*, 2006, 2010; Pitre *et al.*, 2007, 2010; Pitre, Cooke and Mackay, 2007; Poorter *et al.*, 2010; Serapiglia, Cameron, *et al.*, 2013). Étant donné que la fertilisation azotée est généralement associée à une augmentation de la surface foliaire ainsi qu'une augmentation de l'indice de surface foliaire (LAI) (Bollmark and Sennerby-Forsse, 1999; Labrecque and

Teodorescu, 2001; Kinoshita *et al.*, 2014), l'augmentation de l'efficacité du transport de l'eau est alors nécessaire afin de supporter une plus large surface évaporative.

1.6.3 L'effet sur la composition chimique du bois

La composition du bois est fortement influencée par les conditions de croissance surtout en ce qui concerne la disponibilité des ressources hydriques et des nutriments. En effet, une étude sur deux espèces de peupliers a montré qu'une déficience en eau a induit une réduction du taux de lignine en comparaison aux plantes qui ont été irriguées jusqu'à capacité au champ (Cocozza *et al.*, 2011). D'autres recherches, essentiellement sur le peuplier, ont rapporté que la fertilisation par l'azote affectait la composition chimique du bois avec une augmentation du taux de cellulose et une diminution de la lignine (Harvey and van den Driessche, 1999; Luo *et al.*, 2005; Pitre *et al.*, 2007, 2010; Pitre, Cooke and Mackay, 2007; Serapiglia, Cameron, *et al.*, 2013).

1.7 Objectifs de la thèse

L'objectif général de cette thèse est d'investiguer l'effet de l'irrigation par des effluents municipaux sur les propriétés physiologiques, morphologiques, hydrauliques, mécaniques et chimiques du bois des saules. Les résultats d'une première expérience ont servi à la rédaction du deuxième et du troisième chapitre de cette thèse et portent essentiellement sur l'effet de la fertilisation par des eaux usées chargées en azote sur la physiologie et les caractéristiques du bois d'un cultivar de saule (*Salix miyabeana* 'SX67'). Les résultats de trois autres expériences distinctes ont servi à la rédaction du quatrième chapitre et vont permettre de déterminer s'il y a une variation dans la composition chimique et la structure anatomique du bois de divers génotypes de saules en réponse à la fertilisation par les effluents organiques et explorer l'implication d'une telle variation sur le rendement en sucres suite à l'hydrolyse enzymatique.

Chapitre 2 (premier chapitre des résultats)

Objectif : Comprendre les effets de l'application d'effluents municipaux avec traitement primaire (fortement chargé en azote) sur la physiologie et la morphologie des parties aériennes des saules (essentiellement les feuilles) ainsi que sur la productivité de ces cultures.

Hypothèse 1: L'irrigation par les eaux usées affectera positivement le développement des saules. Cela se vérifiera par une augmentation de(s) :

i) la surface foliaire,

ii) la densité stomatique par unité de surface,

iii) la concentration des nutriments dans les feuilles

et iv) échanges gazeux (c.-à-d. une augmentation de la transpiration et la photosynthèse).

Chapitre 3 (deuxième chapitre des résultats)

***Objectif** :* Déceler et identifier les altérations anatomiques et chimiques dans le bois du cultivar *Salix miyabeana* 'SX67' suite à l'irrigation par un effluent municipal fortement chargé en azote.
***Hypothèse 1**:* L'irrigation par des eaux usées va favoriser la formation d'une structure anatomique contribuant davantage à une efficacité hydraulique qu'à une rigidité mécanique.

***Hypothèse 2**:* L'irrigation par des eaux usées va stimuler la formation du bois de tension.

***Hypothèse 3**:* L'irrigation par les eaux usées va altérer la composition chimique du bois de saules avec une augmentation de la fraction cellulosique et une diminution de la lignine.

Chapitre 4 (troisième chapitre des résultats)

***Objectif** :* Investiguer l'effet de l'irrigation par des effluents sur la composition chimique et la structure anatomique du bois de différents génotypes de saules ainsi que sur leur rendement en glucose suite à l'hydrolyse enzymatique.
***Hypothèse 1**:* L'irrigation par des effluents va altérer la composition chimique et la structure anatomique des saules d'une manière spécifique (c.-à-d. différemment en fonction du génotype).
***Hypothèse 2**:* Les rendements en sucres suite à la saccharification de la paroi cellulaire seront différents en fonction du génotype.

***Hypothèse 3**:* Les rendements en sucres suite à la saccharification de la paroi cellulaire seront plus élevés pour les plantes qui ont été irriguées par les effluents.

Contribution des auteurs

Tous les articles ont été entièrement élaborés, produits et écrits par l'étudiant/ postulant au doctorat **Ahmed Jerbi**. La pertinence des résultats présentés dans chacun des trois articles résulte de l'originalité des expériences qui ont été conduites dans le cadre des recherches affiliées à ces articles. Pour l'ensemble des manuscrits scientifiques présentés, les directeurs de recherche **Michel Labrecque et Frédéric Pitre** ont fourni le support financier, matériel et logistique nécessaire au déroulement des expériences et aux analyses reliées à ces études. Ci-dessus est présentée d'une manière plus détaillée la contribution des auteurs pour chacun des trois articles qui sont joints à la présente thèse. Outre le premier auteur, l'ordre de présentation des auteurs pour chaque article ne reflète pas l'implication globale de chaque auteur à ces articles. Tous les auteurs ont lu et approuvé la version finale de chaque article et, ont donné leur accord à l'inclusion des articles dans la présente thèse.

Premier article

Le premier auteur **Ahmed Jerbi** a contribué à la conceptualisation de l'étude, la collecte des données et l'analyse des échantillons (investigation), l'analyse statistique des données, l'interprétation des résultats, la rédaction de la version originale du manuscrit, sa révision et son édition. **Yves Comeau, Michel Labrecque et Frédéric Pitre** ont fourni le support matériel, logistique et financier et ont collaboré à la conceptualisation, la rédaction et à la révision du manuscrit. **Nicholas .J.B. Brereton et Eszter Sas** ont contribué à la conceptualisation, la rédaction de la version originale, la révision et l'édition du manuscrit. **Simon Amiot** a contribué à la collecte des données et l'analyse des échantillons (investigation) ainsi qu'à la révision du manuscrit. **Xavier T. Lachapelle** a participé à la révision du manuscrit.

Deuxième article

Le premier auteur **Ahmed Jerbi** a contribué à la conceptualisation de l'étude, la collecte des données et l'analyse des échantillons (investigation), l'analyse statistique des données, l'interprétation des résultats, la rédaction de la version originale du manuscrit, sa révision et son édition. **Simon Barnabé, Michel Labrecque et Frédéric Pitre** ont fourni le support matériel, logistique et financier et ont aussi participé à la révision du manuscrit. **Joan Laur** a contribué à la révision et à l'édition du manuscrit. **Mike Kalwahali Muissa** a collaboré à la collecte des données et à l'analyse des échantillons (investigation) ainsi qu'à la révision du manuscrit. **Kevin Lajoie** a collaboré à la collecte des données et à l'analyse des échantillons (investigation) ainsi qu'à la révision du manuscrit. **Pierre-Paul Gallant** a collaboré à la conception des algorithmes (codage) pour les analyses numériques des coupes histologiques ainsi qu'à la révision du manuscrit.

Troisième article

Le premier auteur **Ahmed Jerbi** a contribué à l'analyse des échantillons (investigation), l'analyse statistique des données, l'interprétation des résultats, la rédaction de la version originale du manuscrit, sa révision et son édition. **Michel Labrecque et Frédéric Pitre** ont fourni le support logistique et financier et ont participé à la révision du manuscrit. **Richard Krygier, Chris Johnston et Martin Blank** ont fourni le support matériel (les plantes récoltées au niveau des trois sites expérimentaux) et ont participé à la révision du manuscrit. **Joan Laur**: a contribué à la révision et à l'édition du manuscrit. **Simon Barnabé** a contribué à la révision du manuscrit. **Mike Kalwahali Muissa** a collaboré à l'analyse des échantillons (investigation) ainsi qu'à la révision du manuscrit. **Pierre-Paul Gallant** a collaboré à la conception des algorithmes (codage) pour les analyses numériques des coupes histologiques ainsi qu'à la révision du manuscrit. **M. Sarrazin**: a contribué à l'analyse des échantillons et à la révision du manuscrit. **Nicholas .J.B. Brereton** a contribué à la rédaction de la version originale du manuscrit, sa révision et son édition.

Chapitre 2 | Effet de l'irrigation par des eaux usées sur les paramètres physiologiques et le développement d'une culture de saules *Salix miyabeana* 'SX67'

Bien que des études antérieures aient montré la capacité des saules en culture à filtrer les effluents et à produire de hauts rendements en biomasse, on n'en connait très peu quant aux mécanismes physiologiques et morphologiques associés à ces performances. Dans cette perspective, une culture de *Salix miyabeana* 'SX67' a été irriguée par des eaux usées fortement chargées en azote afin d'examiner les effets sur les propriétés physiologiques (p. ex. la conductance stomatique et la photosynthèse) et les altérations morphologiques (essentiellement au niveau des feuilles) induits par les eaux usées.

High biomass yield increases in a primary effluent wastewater phytofiltration are associated to altered leaf morphology and stomatal size in *Salix miyabeana*

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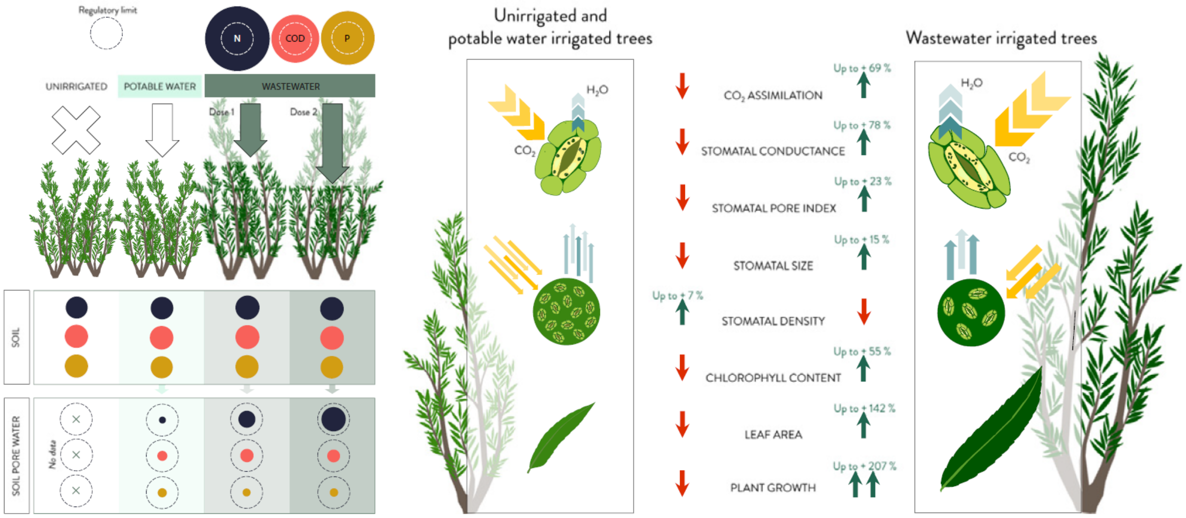
Graphical abstract



2.1 Abstract

Municipal wastewater treatment using willow 'phyto'-filtration has the potential for reduced environmental impact compared to conventional treatment practices. However, the physiological adaptations underpinning tolerance to high wastewater irrigation in willow are unknown. A one-hectare phytofiltration plantation established using the *Salix miyabeana* cultivar 'SX67' in Saint-Roch-de-l'Achigan, Quebec, Canada, tested the impact of unirrigated, potable water or two loads of primary effluent wastewater 19 and 30 ML ha^{-1} yr^{-1}. A nitrogen load of 817 kg N ha^{-1} from wastewater did not increase soil pore water nitrogen concentrations beyond Quebec drinking water standards. The willow phytofiltration phenotype had increased leaf area (+106–142%) and leaf nitrogen (+94%) which were accompanied by significant increases in chlorophyll a + b content. Wastewater irrigated trees had higher stomatal sizes and a higher stomatal pore index, despite lower stomatal density, resulting in increased stomatal conductance (+42–78%). These developmental responses led to substantial increases in biomass yields of 56–207% and potable water controls revealed the nitrogen load to be necessary for the high productivity of 28–40 t ha^{-1} yr^{-1} in wastewater irrigated trees. Collectively, this study suggests phytofiltration plantations could treat primary effluent municipal wastewater at volumes of at least 19 million litres per hectare and benefit from increased yields of sustainable biomass over a two-year coppice cycle. Added-value cultivation practices, such as phytofiltration, have the potential to mitigate negative local and global environmental impact of wastewater treatment while providing valuable services and sustainable bioproducts.

Keywords: Willow, Phytofiltration, Leaf morphology, Stomata, Wastewater, Sustainable biomass

2.2 Introduction

More than 5.9 trillion litres per year of municipal wastewater is discharged into surface water in Canada (Statistics Canada, 2017), requiring extensive treatment to limit the significant damage to the environment caused by ammonia. Standard treatment strategies are usually employed, but have a high financial cost which leads to the release of untreated or undertreated wastewater that can devastate the Canadian environment (Holeton, Chambers and Grace, 2011) . The Canadian government estimates that over 150 billion litres of the wastewater are released into Canadian waterways annually is either untreated or undertreated, exemplified by events such as the release of ~5 billion litres of untreated wastewater into the St Lawrence River in November, 2015 (ECCC, 2017).

In parallel to this need for environmental and economically viable municipal wastewater treatment, the biomass yield of fast-growing willow can be limited due to their high water demands. In Sweden, high biomass yielding *Salix viminalis* and *S. viminalis* x *S. caprea* varieties required 140 mm of water irrigation to maintain 20 t ha^{-1} yr^{-1} yields (Grip, Halldin and Lindroth, 1989) . Sustainable biomass cultivation, of crops such as willow, is often seen as the most substantial hurdle for economically feasible renewable bioenergy and bioproduct generation (Hamelinck, Hooijdonk and Faaij, 2005; Gnansounou and Dauriat, 2010) . Willow phytofiltration plantations could represent a solution for environmental wastewater treatment by combining high biomass yield, water demand and transpiration rates with contamination tolerance (Grip, Halldin and Lindroth, 1989; Börjesson and Berndes, 2006; McCracken and Johnston, 2015; Brereton *et al.*, 2016; Gonzalez *et al.*, 2018) as well as improving local biodiversity and urban greening (Haughton *et al.*, 2009; Dzhambov and Dimitrova, 2015; Van den Berg *et al.*, 2015) in contrast to conventional water treatment solutions.

The high water demand and transpiration rates associated with increased biomass yields are driven by the positive association between photosynthesis rate (A) and stomatal conductance (gs) (Hetherington and Woodward, 2003; Lawson and Blatt, 2014). The relationships between these foliar traits and biomass yield have been well-studied under drought conditions, with plants limiting water loss while maximising carbon assimilation by modifying gs (Farquhar, Schulze and Kuppers, 1980). The long-term modification of gs is determined by developmental alterations to stomatal size and stomatal density, with smaller stomata and higher stomatal density traditionally associated with increased gs (Drake, Froend and Franks, 2013). Franks *et al.*, (2009) observed a negative correlation between stomatal size and density in fossilised leaves over the Phanerozoic era, hypothesising that this plasticity arose to adapt photoassimilation and water-use efficiency during extreme changes in atmospheric CO_2 and resulting in a general rise in stomatal conductance. When water limited (at atmospheric CO_2 levels), Arabidopsis regulates gas exchange to minimise water loss through transpiration by developing leaves with smaller stomatal size, as opposed to altering stomatal density (Doheny-Adams *et al.*, 2012). Similarly, in six deciduous tree species, stomatal length was negatively associated with the response to limit water loss during water limitation, while stomatal frequency only varied slightly (Aasamaa, Sõber and Rahi, 2001).

In *Salix*, Chen *et al.*, (2008) observed substantial variation in stomatal size and density between 29 species, ranging from average stomatal lengths of 18.0–51.7 µm and densities 42.2–842.2 stomata per mm^2. Although few studies in *Salix* have assessed the developmental plasticity of stomatal traits in response to water availability, Wikberg *et al.*, (2007) observed a reduction of between 24 and 57% in stomatal conductance in four hybrid *Salix* species pot-grown over four months under moderate drought stress. Similarly, Pereira *et al.*, (1977) reported stomatal conductance increased during flooding of *Salix nigra* over 37 day pot trials and that flooding did not induce the leaf senescence (observed in Populus deltoides) but did induce fast hypertrophy of lenticels (enlargement of stem pores). However, Fontana *et al.*, (2017) found that quite large changes in rainfall were insufficient to drive substantial stomatal change in a field trial of *Salix miyabeana*. The stomatal pore index (SPI) (Sack *et al.*, 2003; Sack, Tyree and Holbrook, 2005) uses both stomatal density and (either guard cell or pore) length to assess the impact of stomatal variation on potential leaf hydraulic conductance. Two studies have observed a negative relationship between SPI and water availability in the same 13 *Salix* species cultivated in 40 common gardens (Wei *et al.*, 2017) or three replicated field sites in America (Savage and Cavender-Bares, 2012) in across natural water gradients.

Willow phytofiltration has the potential to treat municipal wastewater in an environmental manner; however, the adaptations which allow for tolerance to high wastewater volumes are still unclear. The primary effluent wastewater phytofiltration field trial used in this study was planted with *Salix miyabeana* ‘SX67’ at a field site in Saint-Roch-de- l'Achigan, Quebec, Canada. The first year of the trial's growth within a two-year coppice cycle demonstrated that increases in biomass yield were likely to be observed at harvest (Lachapelle-T., Labrecque and Comeau, 2019). In addition to determining the treatment efficacy wastewater phytofiltration system at field-scale and accompanying two-year coppice cycle biomass yields, this study aimed at revealing the physiological adaptions underpinning high-load primary effluent wastewater irrigation tolerance in willow.

2.3 Materials and methods

2.3.1 Field trial location

The plantation was located in Saint-Roch-de-l'Achigan (45° 50′ 50″ N–73° 38′ 27″ W), 55 km northeast of Montreal (Quebec), Canada. The average annual precipitation was 1102 mm for 2005–2015. Four hectares of *Salix miyabeana* ‘SX67’ were established in 2008 at a density of 16,000 trees ha^{-1} (Supplementary file 1). Spacing between rows was 1.8 m and 35 cm between cuttings within each row. In 2009, a drip irrigation system was installed at a depth of 30 cm (Jerbi *et al.*, 2015). The plantation was coppiced in the autumn 2015 and 12 experimental square plots of 100 m^2 were designed, each containing six rows of willow with the four central rows irrigated. Four treatments (x three plots) comprised: unirrigated control (UI), potable water (PW), and primary effluent wastewater dose one (WWD1) and primary effluent wastewater dose two (WWD2) irrigated (Figure 2.1). Plots were designed in a non- randomised manner to minimise groundwater contamination as described in Lachapelle-T. *et al.* (2019). Wastewater was pumped directly from the local municipal wastewater treatment facility. In the facility, the wastewater was allowed to rest for at least 24 h in a conventional septic tank prior to irrigation but was otherwise untreated. Irritrol® IBOC® Plus controllers (12 stations) were installed to regulate mean daily irrigation of 14 (PW), 10 (WWD1) and 16 (WWD2) mm over 2016 and 13 (PW), 12 (WWD1) and 18 (WWD2) mm for 2017.

2.3.2 Soil and water sampling and characterisation

At the beginning of the trial (spring 2016), five soil samples per plot were taken for soil chemical characterisation at a depth of 0–30 cm prior to treatment (Supplementary file 1). Five soil samples per plot were then taken at the end of the two-year harvest cycle (winter 2017) to assess treatment effects on soil chemical proprieties. Primary effluent wastewater (prior to application) and the soil pore water beneath experimental plots were sampled every 2 weeks during the growth seasons

(June–October). Soil pore water was sampled only for PW or wastewater irrigated plots through 27 (three per plot) lysimeters (Model 1900 Soil moisture Equipment Corp.) which were equipped with porous ceramic cartridges (1.3 µm maximum pore size) and installed at 60 cm depth (beyond the willow root zone which rarely exceeds 40 cm (Jerbi *et al.*, 2015)).

Soil and water analyses were conducted as described by Lachapelle-T. *et al.* (2019). Briefly, phosphorus and micronutrients were extracted from soil using Mehlich 3 method (Mehlich, 1984) and phosphorus was measured using ascorbic acid molybdate blue method for phosphorus (Murphy and Riley, 1962). Organic matter was estimated thermogravimetrically (ASTM, 2007) and electrical conductance was assessed using 1:1 soil-to-water method (Warncke and Brown, 1998). Chemical oxygen demand (COD) of water was measured according to (Baird, Eaton and Rice, 2017) and electrical conductance was measured using a conductivity meter (SevenCompact, Mettler Toledo). For both soil and water samples, nitrogen and phosphorus compounds were measured with a flow injection analysis (Quickchem 8500, (Lachat Instruments, 2003)) and atomic absorption spectroscopy (AAnalyst 200, Perkin Elmer) for calcium, magnesium, potassium and sodium while chloride was measured with chloride test strips (Quantab CAT 27449-40, HACH).

2.3.3 Leaf gas exchange, composition and morphology

Three sets of leaves were sampled to assess composition and morphology from multiple trees of each plot in each treatment. CO_2 assimilation rate (A) and water vapour stomatal conductance (gs) were measured during August and September using four trees for each of three plots per treatment, with one fully expanded leaf below the largest stem mid-point selected for each of the 48 trees (a total of 48 leaves). Measurements were performed on a sunny day within a time window 10:00 and 14:00 using a portable open infrared gas exchange analyser (IRGA) fitted to a LED light source equipped leaf chamber (Li-6400, Li-Cor Inc., Lincoln, Nebraska, USA) covering an area of 6 cm^2. Leaf chamber conditions were a photosynthetic photon flux density of 1500 µmol photons m^{-2} s^{-1}, a CO_2 concentration of 400 µmol CO_2 mol^{-1} air, air flow of 500 µmol s^{-1} and a temperature of 26 °C ± 0.5. Immediately after the gas-exchange measurements, leaves were destructively harvested and scanned using WinFOLIA software (WinFOLIA™ Pro Version, Regent Instruments, Quebec, Canada) to determine leaf area (LA) before being oven-dried (70 °C for 72 h) and weighed. Carbon and nitrogen content per dry mass were measured using an elemental analyser (vario MICRO cube, Elementar, USA). Specific leaf area (SLA) was calculated per leaf as area per dry mass (cm^2 g^{-1}) while leaf mass per area (LMA) as dry mass per area (g DM cm^{-2}). LMA and nitrogen content are used to calculate leaf nitrogen content per unit area (g N m^{-2} or mmol N m^{-2}) and instantaneous photosynthetic nitrogen-use efficiency (PNUE) was determined as the ratio of the CO_2 assimilation to the leaf nitrogen content per unit area (μmol CO_2 m^{-2} mol^{-1} s^{-1}) (Rijkers, Pons and Bongers, 2000; Escudero and Mediavilla, 2003).

A second set of leaf sample included three fully expanded leaves per tree (four trees per plot, for a total of 144 leaves) destructively harvested for chlorophyll and carotenoids content determination and stored in the dark at −80 °C. Extraction followed (Hiscox and Israelstam, 1979); briefly, three circular disks were punched using a cork borer from each leaf (0.6 cm – 1 cm diameter depending on the size of the leaf) and incubated with 7 mL of dimethyl sulfoxide solvent (DMSO) at 65 °C for 2 h, until the tissue became colourless. Extraction liquid was made up to a total volume of 10 mL with DMSO, before measuring absorbance at 480, 649 and 665 nm using a NanoPhotometer P300 (Implen, Germany). Concentrations were calculated following the equations (Wellburn, 1994): chlorophyll a = (12.47 x A665) – (3.62 x A649), chlorophyll b = (25.06x A649) – (6.50 x A665) and total carotenoids = (1000 x A480–1.29 Chla –53.78 Chlb)/220).

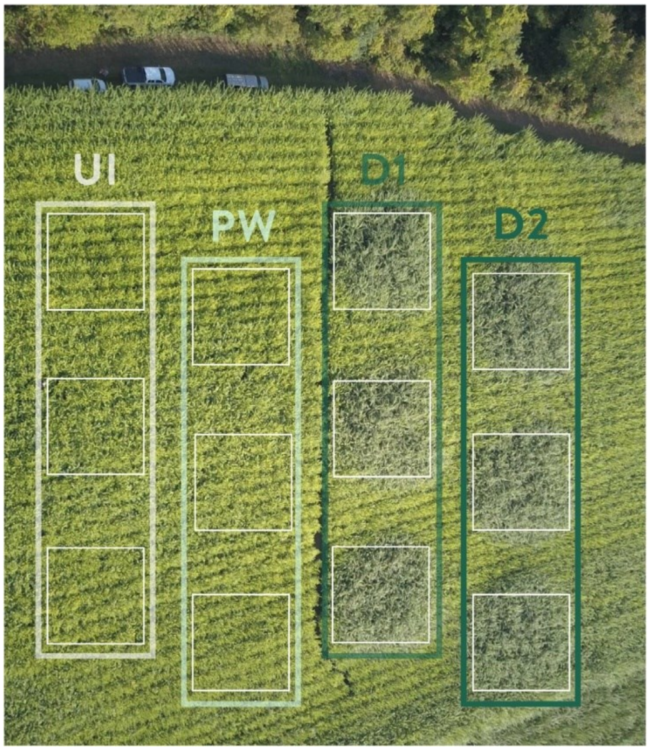
A third set of leaves were sampled comprising two fully expanded leaves per tree (four trees per plot, for a total of 96 leaves) for stomatal measurements, assessed using the silicon rubber impression technique (Weyers and Johansen, 1985). Stomatal counts and dimensions were assessed on the abaxial epidermal surface from midway between the leaf tip and base while avoiding leaf veins. Slides were scanned with the ScanScope CS2 digital slide scanner (Leica Biosystems, Buffalo Grove, IL, USA) and viewed with Aperio Image Scope software (Leica Biosystems Imaging, Vista, CA, USA). Stomatal density was measured with counts over an area of 106 μm^2. A Zeiss AxioImager Z1 microscope was used to measure stomata lengths and widths, and stomatal pore lengths and widths of 10 stomata chosen randomly on abaxial surface of each leaf. Stomatal density and stomatal pore length were used to calculate the total stomatal pore area index (SPI); a theoretical index of maximum stomatal conductance (mean total stomatal density x mean stomatal pore length^2) (Sack *et al.*, 2003; Sack, Tyree and Holbrook, 2005).

2.3.4 Biomass yield

At the end of the two-year harvest cycle, all above-ground biomass from four trees were randomly harvested from each plot. Immediately after harvest, all 48 trees were weighed fresh and subsampled to assess moisture content by oven drying at 105 °C for 72 h. Biomass yields are estimated based on dry matter yields at the planting density of 16,000 trees ha^{-1}.

2.3.5 Statistics

Analysis of variance testing was followed by multiple comparisons of means according to Tukey's Honestly Significant Difference (HSD) ($\alpha = 0.05$) using JMP statistical software version 9.0 (SAS Institute, Cary, NC), unless otherwise stated.



Treatment		Irrigation rate (mm, 2017)
Unirrigated	UI	0
Potable water irrigated	PW	2200
Wastewater irrigated dose 1	WWD1	1900
Wastewater irrigated dose 2	WWD2	3000

Figure 2. 1 : Saint-Roch-de-l'Achigan phytofiltration field site and experimental design. The *Salix miyabeana* 'SX67' plantation was established at a density of 16,000 trees ha^{-1} across four hectares northeast of Montreal, Canada. Twelve experimental square plots of 100 m^2 (10 m × 10 m), each containing six rows of trees, were treated with one of four treatments (three plots per treatment): unirrigated control (UI), potable water (PW), primary effluent wastewater dose one (WWD1) and primary effluent wastewater dose two (WWD2) irrigated. Left: Arial photograph taken by drone of the plantation (treatment plots are highlighted). Top right: Representative PW plot (left) and WWD2 plot (right) taken before harvest in 2017. Bottom right: Irrigation loads.

2.4 Results

2.4.1 Wastewater loads, soil pore water and soil characterisation

The wastewater loads applied during year two of the willow coppice growth cycle were 1944 and 2961 mm for WWD1 and WWD2 treatment doses, respectively; an equivalent of 19 and 30 ML ha^{-1} yr^{-1} (Figure 2.1). The chemical oxygen demand (COD) of the applied wastewater was an average of 290 mg COD L^{-1} (±29) (Table 2.1; Figure 2.2). The subsequent COD of soil pore water increased significantly beneath wastewater treated trees to 19–21 mg COD L^{-1} when compared to 12 mg COD L^{-1} in potable water (PW) treated trees. However, soil organic matter did not differ significantly between unirrigated (UI), PW or wastewater treated trees, which ranged between 3.48 and 3.64% of dry weight.

The average nitrogen concentration of wastewater throughout the season was 42 mg N L^{-1} equivalent to nitrogen loads of 817 and 1245 kg ha^{-1} yr^{-1} for WWD1 and WWD2 treatments, and the prevalent nitrogen forms were ammonia and organic nitrogen in a 1:1 ratio. The impact on soil pore water and soil of this nitrogen load from wastewater was assessed after two years of treatment. The nitrogen concentrations in soil pore water significantly increased in plots receiving wastewater irrigation, from 0.5 mg N L^{-1} in PW irrigated plots to 2.8 mg N L^{-1} for WWD1 and 5.8 mg N L^{-1} for WWD2 (Table 2.1, Figure 2.2). Within the nitrogen observed in soil pore water, ammonia concentrations ranged from 0.06–0.34 mg N L^{-1} and did not significantly differ between treatments. However, nitrite and nitrate concentrations were significantly higher in soil pore water from wastewater plots, increasing substantially for the higher wastewater dose from 0.1 mg N L^{-1} in PW to 5.3 mg N L^{-1} in WWD2. Similarly, the ammonia concentrations in soil of did not significantly vary between treatments, ranging between 36.93 and 38.97 mg N kg^{-1}, but soil nitrites and nitrates concentrations increased to 12.4 (± 1.9) and 14.6 (±2.7) mg N kg^{-1} for WWD1 and WWD2 from 2.6 (±0.8) and 3.8 (±1.0) mg N kg^{-1} in UI and PW treated control plots, respectively.

The average concentration of the total phosphorus in applied waste- water was 4.1 mg P L^{-1} throughout the season, the majority of which was present in the form of orthophosphates (2.7 mg P L^{-1}) (Table 2.1). This corresponds to total phosphorus loads of 79 and 121 kg ha^{-1} yr^{-1} for WWD1 and WWD2 treated trees, respectively. When the impact of this phosphate load on soil pore water and soil was measured, neither total phosphorus, orthophosphate, or the available phosphorus were significantly different between any treatments. The average concentrations of sodium and chloride in the applied wastewater were 145 mg Na L^{-1}, leading to loads of 2.83 and 4.31 t Na ha^{-1} yr^{-1}, and 190 mg Cl L^{-1}, leading to loads of 3.70 and 5.63 t Cl ha^{-1} yr^{-1} for WWD1 and WWD2 treated trees, respectively (Figure 2.2; Supplementary file 1). There were no significant differences observed in the pH of soil pore water between treatments; however, electrical conductivity (EC) differed significantly, increasing from 0.3 dS m^{-1} under PW irrigated trees to 1.4 dS m^{-1} under both WWD1 and WWD2 irrigated trees (Supplementary file 1). Soil pH varied between 5.6 and 6.0

although only UI trees (pH 5.6) varied significantly from WWD1 (pH 6.0) treated trees. Similarly, EC significantly increased from 0.05–0.06 dS m^{-1} in UI and PW irrigated plots to 0.11–0.12 dS m^{-1} in WWD1 and WWD2 irrigated plots, although only UI and WWD2 treated trees were significantly different.

Concentrations of the macronutrient elements calcium, magnesium and potassium in wastewater (Figure 2.2) led to loads of 1.90 and 2.90 t Ca ha^{-1} yr^{-1}, 0.55 and 0.84 t Mg ha^{-1} yr^{-1}, and 0.20 and 0.30 t K ha^{-1} yr^{-1} in WWD1 and WWD2 treated trees, respectively (Figure 2. 2). The impact of these loads upon soil pore water was not measured but concentrations of magnesium and potassium in soil did not significantly vary between treatments, ranging from 20 to 38 mg Mg kg^{-1} and 73–114 mg K kg^{-1}, while calcium significantly differed between controls only, 819 (± 13) in UI and 658 (± 21) mg Ca kg^{-1} in PW treated trees (Supplementary file 1).

	COD mg L^{-1}	N mg N L^{-1}	NH_4 mg N L^{-1}	NO_x mg N L^{-1}	P mg P L^{-1}	pH	EC dS m^{-1}
Wastewater	290 (29)	42.06 (6.2)	20.08 (1.8)	0.06 (0.01)	4.09 (0.65)	7.59 (0.07)	1.49 (0.13)
Soil	Organic matter %	N mg N kg^{-1}	NH_4 mg N kg^{-1}	NO_x mg N kg^{-1}	P mg P kg^{-1}	pH	EC dS m^{-1}
UI	3.64 (0.03)	1113 (43)	38.97 (0.33)	**2.56** **(0.83)**^{b}	1230 (87.87)	**5.55** **(0.07)**^{b}	**0.05** **(0.005)**^{b}
PW	3.48 (0.22)	1083 (88)	38.01 (0.38)	**3.85** **(1.02)**^{b}	1027.5 (85.94)	**5.66** **(0.12)**^{ab}	**0.06** **(0.002)**^{ab}
WWD1	3.59 (0.04)	1224 (136)	36.93 (0.37)	**12.14** **(1.87)**^{a}	1134 (106.81)	**6.01** **(0.05)**^{a}	**0.11** **(0.018)**^{ab}
WWD2	3.49 (0.32)	1168 (130)	38.26 (0.20)	**15.01** **(2.75)**^{a}	1065 (135.78)	**5.90** **(0.18)**^{ab}	**0.12** **(0.018)**^{a}
soil pore water	COD mg L^{-1}	N mg N L^{-1}	NH_{-4} mg N L^{-1}	NO_x mg N L^{-1}	P mg P L^{-1}	pH	EC dS m^{-1}
PW	**12.03** **(1.47)**^{b}	**0.46** **(0.10)**^{c}	0.06 (0.01)	**0.06** **(0.01)**^{c}	0.08 (0.02)	6.65 (0.04)	**0.34** **(0.02)**^{b}
WWD1	**21.16** **(1.67)**^{a}	**2.82** **(0.41)**^{b}	0.34 (0.26)	**2.15** **(0.42)**^{b}	0.06 (0.01)	6.71 (0.10)	**1.37** **(0.11)**^{a}
WWD2	**19.46** **(0.69)**^{a}	**5.78** **(0.91)**^{a}	0.14 (0.06)	**5.32** **(0.84)**^{a}	0.07 (0.01)	6.71 (0.10)	**1.40** **(0.11)**^{a}

Table 2. 1 : Wastewater, soil and soil pore water characterisation. Chemical oxygen demand (or organic matter), nitrogen, ammonia, nitrates/nitrites, phosphorus, pH and electrical conductance measured in wastewater, soil and soil pore water. Mean values for wastewater comprise 8–12 samples taken regularly over six months of the growing season. Soil means each comprise three plot values from five samples per plot taken prior to harvest. Soil pore water means each comprise three plot values from three lysimeters per plot sampled regularly over six months of the growing season. UI: unirrigated, PW: potable water irrigated, WWD1: wastewater irrigated dose 1 and WWD2: wastewater irrigated dose 2. Standard error is shown in parentheses and Tukey's Honestly Significant Difference is indicated using different letters (α = 0.05) with the values highlighted in bold. See Supplementary file 1 for more extensive data.

2.4.2 Leaf physiology and biomass yield

Leaf elemental analysis revealed that both nitrogen and carbon fractions in WWD1 and WWD2 treated trees were significantly higher than leaves of UI and PW irrigated trees (Figure 2.3). Leaf nitrogen concentration was 94% higher in both WWD1 and WWD2 treated trees than in UI and PW irrigated trees, increasing to 32.88 and 33.01 mg g^{-1} from 16.77 and 17.28 mg g^{-1}, respectively.

Leaf carbon concentration was significantly higher in leaves of wastewater treated trees that found in controls but increased in small absolute amounts from 446.39 (± 0.15) and 448.13 (± 0.03) mg g^{-1} in UI and PW irrigated trees to 456.16 (± 0.15) and 455.17 (± 0.14) mg g^{-1} in WWD1 and WWD2 trees. Leaf carbon to nitrogen ratios (C:N) were therefore significantly higher for the UI and PW irrigated trees, 26.9 and 26.5C:N respectively, than for both wastewater treatments at 14.0C:N. The concentrations of chlorophyll a and chlorophyll b were 38.2 and 18.2 μg cm^{-2} in WWD1 trees, and 40.9 and 19.8 μg cm^{-2} WWD2 trees, both significantly higher than unirrigated trees at 25.3 and 13.8 μg cm^{-2} and PW irrigated trees at 30.2 and 15 μg cm^{-2} (Figure 2.3). The concentrations of carotenoids were also significantly higher in wastewater treated trees than either control treatment, with WWD2 having the highest concentration of 9.4 μg cm^{-2} compared to the lowest, 7.4 μg cm^{-2} for UI treatment.

Average leaf area (LA) was significantly different for trees between treatments. Trees receiving wastewater had a higher average LA of 30.1 cm^2 for WWD1 and 34 cm^2 for WWD2, while LA for the UI and the PW irrigated trees was 14.6 cm^2 and 14.1 cm^2, respectively (Figure 2.4). The specific leaf area (the ratio of leaf area to leaf dry mass) was also significantly increased for the trees receiving wastewater treatment, from 123 ± 10 and 113 ± 3 cm^2 g^{-1} in UI and the PW irrigated trees to 168 ± 11 and 159 ± 15 cm2 g^{-1} in WWD1 and WWD2 irrigated trees, respectively. In addition to altered leaf shape, trees receiving wastewater had observationally delayed senescence at the end of the growth season (Figure 2.1).

The nitrogen leaf content per unit area also increased significantly in wastewater irrigated trees, whereas no difference (ANOVA, p N 0.05) was identified between any treatments for the photosynthetic nitrogen use efficiency (PNUE), which ranged between 9.2 and 10.8 μmol CO_2 g^{-1} N s^{-1} (Figure 2.5). The net rate of CO_2 assimilation was significantly and substantially higher in leaves of trees irrigated with both doses of wastewater when compared to UI or PW irrigated trees, increasing to 20.3 and 22.9 μmol CO_2 m^{-2} s^{-1} in WWD1 and WWD2 from 13.6 μmol CO_2 m^{-2} s^{-1} in both UI and PW treated trees, respectively. Accordingly, stomatal conductance was significantly higher in WWD1 and WWD2 at 0.45 and 0.52 mol H_20 m^{-2} s^{-1} respectively, compared to 0.32 and 0.29 mol H_20 m^{-2} s^{-1} in UI and PW treated trees. However, abaxial stomatal density was significantly higher in UI and PW irrigated trees at 648 ± 10 and 646 ± 10 stomata per mm^2 compared to 604 ± 10 and 604 ± 15 stomata per mm^2 in WWD1 and WWD2 treated trees, respectively (Figure 2.4). Conversely, stomatal length and width were higher in the leaves of wastewater irrigated trees. Stomatal length in UI and PW irrigated trees were 14.4 and 13.9 µm, respectively, whereas WWD1 and WWD2 were 15.2 and 15.1 µm, although waste- water treatment was only identified as having significantly higher stomatal length than PW (9% increase).

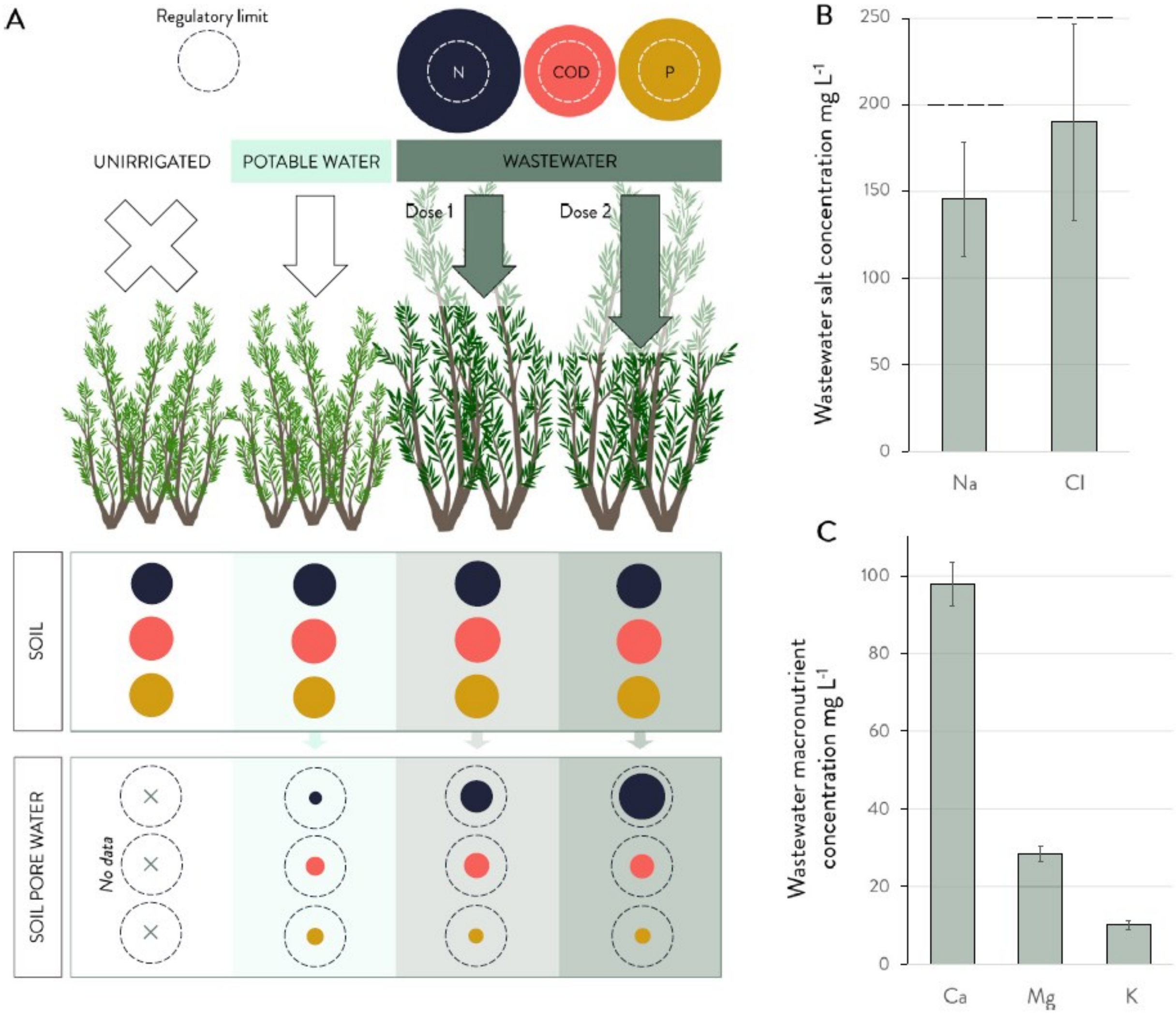


Figure 2. 2 : Phytofiltration reduction of wastewater contaminants below regulatory limits. A) Schematic illustrating nitrogen (N), chemical oxygen demand (COD), and phosphorus (P) concentrations relative to regulatory limits before and after phytofiltration using willow. Water discharge regulations are inconsistent for different compounds. The regulatory concentration limit for nitrates and nitrites in drinking water in Quebec is 10 mg N L^{-1} (CCME, 2002; MELCC, 2019), limits for COD set by the European commission are 125 mg COD L^{-1} (Miguel Ad *et al.*, 2014; Union, 1991), and the limit for phosphorus release into surface waters in Quebec is 1 mg P L^{-1} (CCME, 2002; MELCC, 2015). Wastewater concentrations of B) the most abundant salt concentrations and C) the most abundant macronutrients are means of 8–12 wastewater samples taken over 6 months prior to field application with standard error. Quebec regulatory limits for discharge of sodium and chloride into surface water is 200 mg Na L^{-1} and 250 mg Cl L^{-1} (CCME, 2002; MELCC, 2019) but have not yet been established for calcium, magnesium or potassium discharge. See Supplementary file 1 for more extensive composition data.

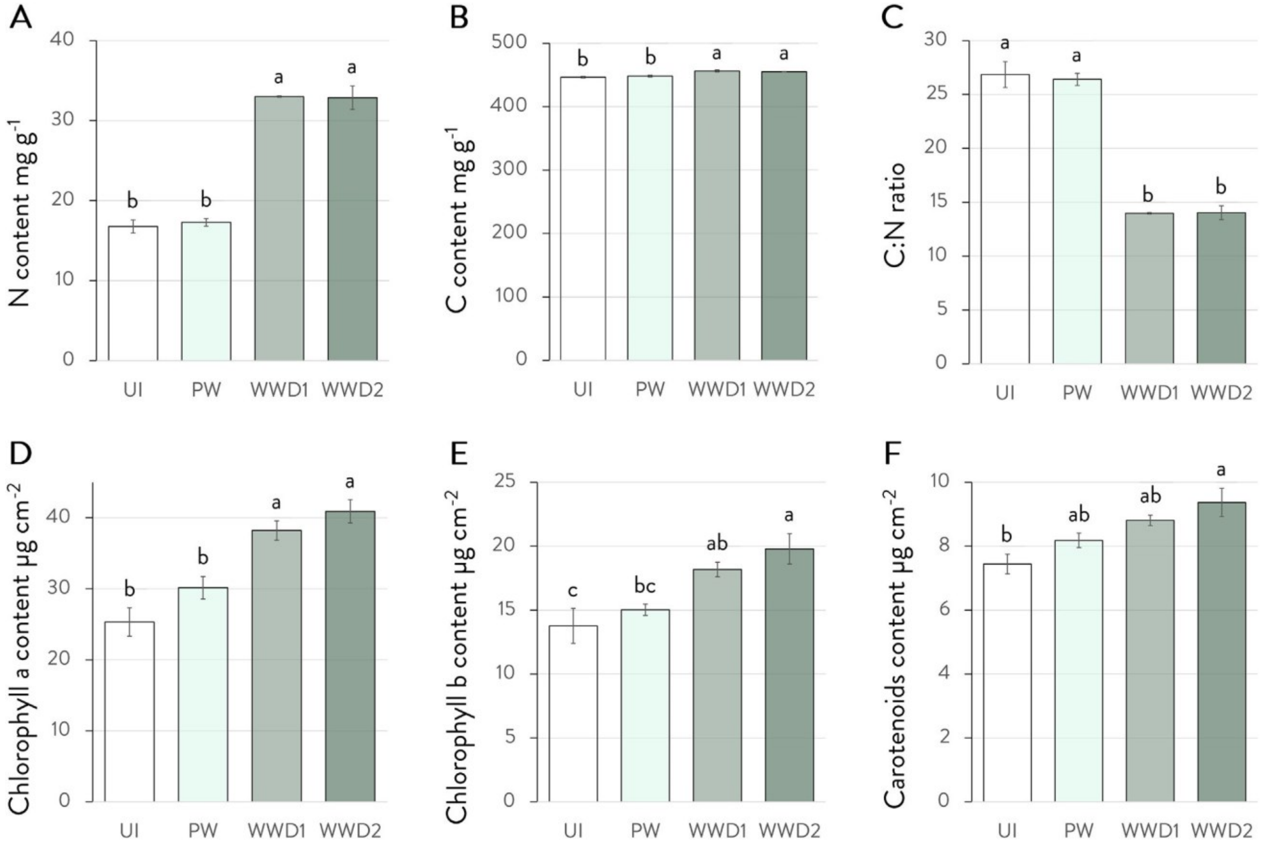


Figure 2. 3 : Leaf composition varies in response to wastewater treatment. A) Leaf nitrogen content, B) leaf carbon content, C) carbon-nitrogen ratio, D) chlorophyll a concentration, E) Chlorophyll b and F) carotenoids concentration. Mean values for unirrigated (UI), potable water (PW), wastewater dose 1 (WWD1) and 2 (WWD2) irrigated trees (n = 3 plots; standard error is shown). Tukey's Honestly Significant Difference is indicated using different letters (α = 0.05).

Stomatal width and pore length showed a similar pattern but with the only significant difference observed between the higher stomatal width of 8.4 µm and pore length of 11.0 µm in WWD2 compared to a stomatal width of 7.5 µm and pore length of 9.8 µm in PW treated trees. This change in stomatal morphology was reflected in the stomatal pore area index (SPI), which was significantly higher for wastewater treated trees, to 7.3×10^{-2} in WWD1 and 7.7×10^{-2} in WWD2, when compared to both UI and PW treated trees, which averaged 6.3×10^{-2} and 6.2×10^{-2}, respectively (Figure 2.5). As the SPI is calculated as stomatal pore length^2 x stomatal density, the calculated difference between treatments was due to an increase in stomatal pore length, which correlated with SPI ($r = 0.89$; $p < 0.001$) and gs ($r = 0.56$; $p < 0.001$), unlike stomatal density ($p > 0.05$) (Supplementary file 1). The total harvested biomass yields were higher for both doses of wastewater irrigated trees compared to UI and PW irrigated trees after two years of growth. Wastewater irrigation increased dry matter biomass yields from 18.3 ± 3.5 t ha^{-1} yr^{-1} and 13.1±

1.6t ha^{-1} yr^{-1} in unirrigated and PW irrigated trees to 28.8 ± 6.3 t ha^{-1} yr^{-1} and 40.4 ± 4.9 t ha^{-1} yr^{-1} in WWD1 and WWD2, respectively (Figure 2.5).

2.5 Discussion

2.5.1 Primary effluent wastewater phytofiltration had minimal impact on soil pore water and soil

The average COD of the Saint-Roch-de-l'Achigan wastewater, used to give an indication of organic matter concentration, was relatively low for untreated municipal wastewater (Feigin, Ravina and Shalhevet, 1991; Levy, Fine and Bar-tal, 2011). However, due to the very high loads of wastewater applied to the plantation, rates of 19 and 30 ML ha^{-1} yr^{-1}, absolute COD loads reached 4 and 6 t COD ha^{-1} yr^{-1} for WWD1 and WWD2, respectively. Surprisingly, given these high COD loads, no differences between the wastewater and either control treatments (i.e. UI and PW) were detected for organic matter in soil (Table 2.1; Figure 2.2). The additional organic matter load in the WW plots could undergo mineralisation, often high in the type of well-aerated loamy sand soil present here (Hassink *et al.*, 1993; Li *et al.*, 2009), and therefore be available for plant uptake.

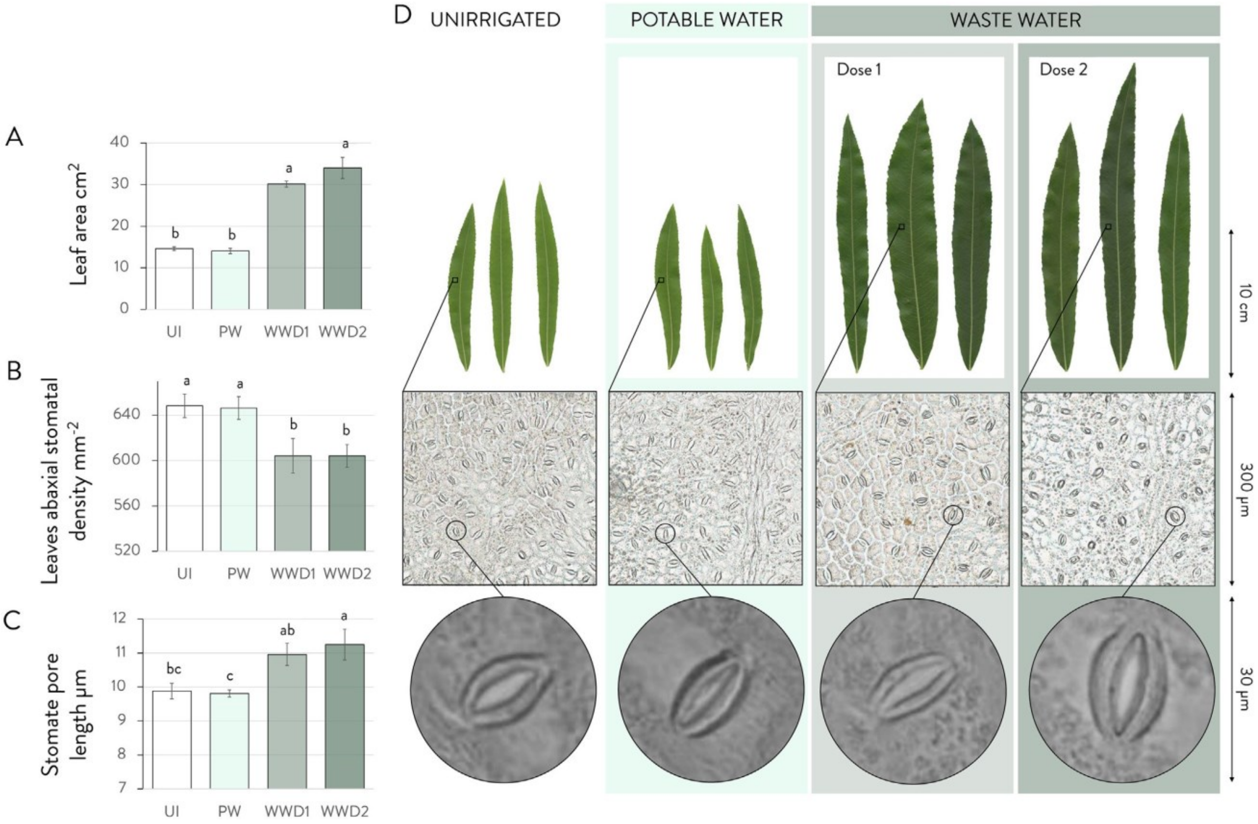


Figure 2. 4 : Leaf area, stomatal density and stomatal size vary due to wastewater treatment. A) Leaf area, B) leaf stomatal density, C) leaf stomatal size and D) representative images of unirrigated (UI), potable water (PW) and wastewater dose 1 (WWD1) and 2 (WWD2) irrigated trees. A-C illustrate mean values of three plots per treatment (± standard error). Tukey's Honestly Significant Difference is indicated using different letters ($\alpha = 0.05$).

Another fate of organic matter which could be of potential environmental concern would be a corresponding increase in the groundwater. While soil pore water organic matter in wastewater treated plots was significantly higher than the PW irrigated plots (increasing by 75% compared to PW), the increase was small in absolute terms and did not account for the substantial organic matter load applied through wastewater. In the province of Quebec, Canada, there is currently no established limit for the COD of discharged wastewater from treatment facilities; however, the soil pore water COD concentrations from all treatments (which varied between 12 and 21 mg COD L^{-1}) were well below the limit of 125 mg COD L^{-1} (Figure 2.2) established by the European Council (Council of the European Union, 1991; Miguel *et al.*, 2014). The willow plots can therefore be considered as likely to have treated primary effluent wastewater without contaminating groundwater beyond environmental standards in terms of COD. Conventional wastewater treatment methods remove substantial amounts of the organic matter from primary effluent wastewater, although to a lesser degree than observed here using willow phytofiltration; typically reducing COD levels by 35–50% (Feigin, Ravina and Shalhevet, 1991; Levy, Fine and Bar-tal, 2011), similar to total nitrogen reduction levels of 30–60% in conventional treatment systems (Levy, Fine and Bar-tal, 2011; Nourmohammadi *et al.*, 2013; Tazkiaturrizki, Soewondo and Handajani, 2018). The nitrogen concentrations of the wastewater applied here were similar to those commonly reported for untreated sewage/municipal wastewater (FAO, 1992; Asano, 1994; Levy, Fine and Bar-tal, 2011; Sedlak, 2018). Although positive yield increases have been observed when fertilizing willow at rates as high as 590 kg N ha^{-1} (Cavanagh, Gasser and Labrecque, 2011), the total nitrogen load applied here via wastewater is substantially greater than the 0–100 kg N ha^{-1} yr^{-1} commonly reported (Fabio and Smart, 2018b), suggesting that nitrogen could be very much in excess (8–12 times higher) in WW treated trees at a potential detriment to the environment. Soil pore water ammonia concentrations under WW irrigated trees were, however, similar to the UI and PW irrigated trees (Table 2.1), suggesting either ammonia retention/adsorption within soil upper layers and/or nitrification. No significant differences in soil ammonia were observed between treatments but high nitrification during WW treatment was indeed indicated, as nitrate proportions increased from <0.16% of total nitrogen in primary effluent wastewater to 67–91% within soil pore water of WW treated plots. When compared to PW, the WW plots had soil pore water nitrate concentrations increased by 3500% and 8800% for WWD1 and WWD2. While this represents a potentially substantial release of nitrogen into the environment, average concentrations were below the Quebec drinking water standards limits values of 10 mg N L^{-1} (CCME, 2002; MELCC, 2019) and total nitrogen concentration in the soil pore water was reduced to 5–13% of that in wastewater (Figure 2.2). This suggests the willow crop filtration system efficiently reduced nitrogen leaching but that the higher nitrogen dose of WWD2 is likely close or beyond the system limit, if dangerous levels of nitrogen leaching into the environment is to be prevented. While the total soil nitrogen concentrations remained similar between treatments, wastewater also increased soil nitrate concentrations compared to UI and PW treatment, by 220–390% and 280–470% for WWD1 and WWD2 respectively. As nitrate is the preferred uptake form of nitrogen for plants (Tischner, 2000), this leads to the potential for wastewater irrigation to increase biomass yields of willow phytofiltration plantations in addition to reducing the common practice of environmentally damaging release of untreated or undertreated effluent into waterways.

Phosphorus input through wastewater irrigation was higher than estimated willow crops requirements (i.e. 15–40 kg P ha^{-1} yr^{-1} (Caslin, Finnan and McCracken, 2010; Mccracken and Johnston, 2015). Similar to nitrogen, these high loads could lead to leaching if the phosphorus is not assimilated by plants or retained in the soil matrix. Soil pore water concentrations of phosphorus were well below the Quebec municipal river water discharge limit of 1 mg P L^{-1} (CCME, 2002; MELCC, 2015) and soil analysis revealed no significant differences in phosphorus between different treatments (Table 2.1, Figure 2.2). Taken together, this suggested complete removal of the high load from municipal sewage by tree assimilation and/or sorption to the soil matrix, indicating the potential of willow phytofiltration to prevent phosphorus associated environmental damage, such as eutrophication. The predominant phosphorus form, orthophosphate, could also drive secondary benefits in having the potential to enhance biomass yield in field- grown willow (Fillion *et al.*, 2011; Guidi Nissim *et al.*, 2015).

The total wastewater irrigation load for Ca, Mg and K suggests a possible potassium shortfall and over-application of the calcium and the magnesium, which could negatively impact willow viability overtime (Fromm, 2010; Rokosa *et al.*, 2017). Similarly, wastewater concentrations of sodium and chloride were high compared to commonly reported values for raw municipal sewage, such as 120–460 mg Na L^{-1} (Feigin, Ravina and Shalhevet, 1991) and 90 mg Cl L^{-1} (Levy, Fine and Bar-tal, 2011). Although chloride can be growth-limiting, these high applications (cumulative waste- water loads where 11 t Na ha^{-1} and 17 t Cl ha^{-1} for WWD2) would cause toxicity to many plants by reducing water uptake (Raven, 2016). The high yields of wastewater treated trees demonstrate that these salt loads were not toxic and while soil salinity did increase to 0.12 dS m^{-1} in wastewater irrigated plots, levels were lower than the 5.0 dS m^{-1} previously reported as not impacting growth in common willow varieties and substantially lower than the 8 dS m^{-1} tolerated by extreme saline adapted varieties (Hangs *et al.*, 2011). Increases in soil or groundwater salinity could compromise the environmental sustainability of willow wastewater treatment (Halliwell, Barlow and Nash, 2001; Belaid *et al.*, 2010). Wastewater irrigation did not impact the cation exchange capacity of soil here, and so was unlikely to have driven substantial change to the soil environment in the short-term. However, these prolonged high salinity wastewater applications could represent a challenge to the long-term feasibility of wastewater phytofiltration, and further studies need to establish this long-term impact and potential risk of salt and other micronutrient imbalances upon such systems.

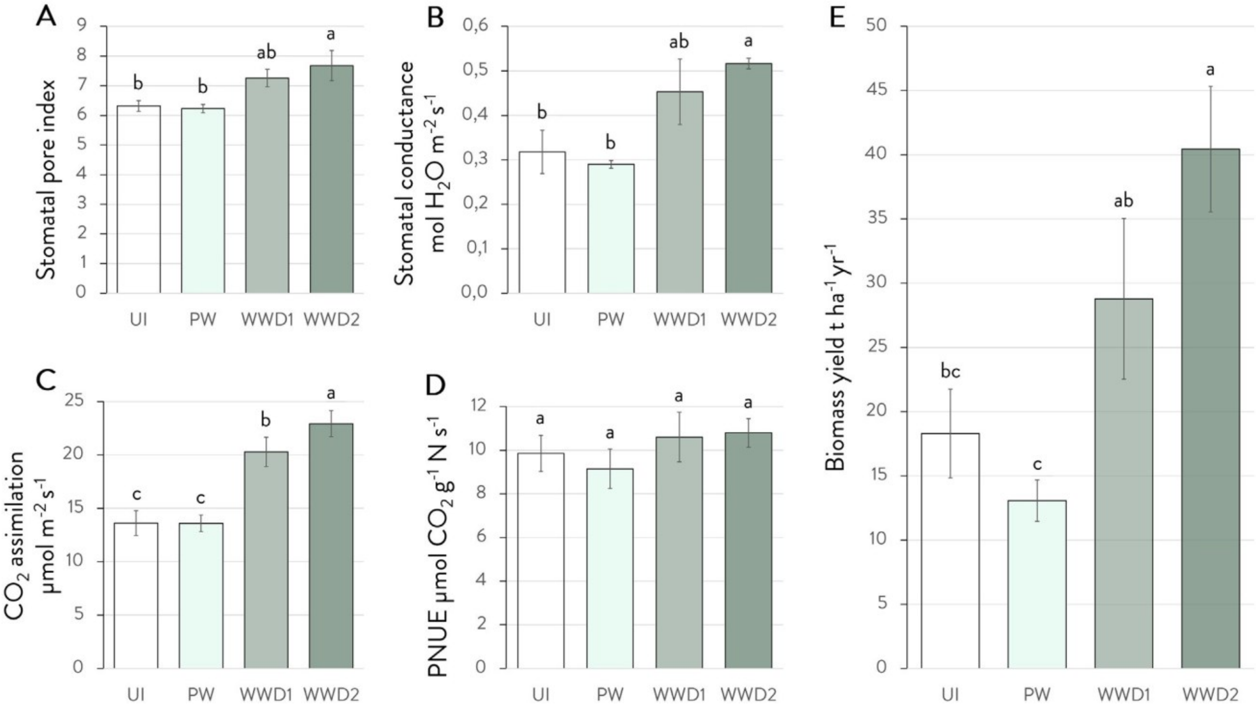


Figure 2. 5 : Wastewater treatment increases stomatal pore index, photosynthesis and biomass yield. A) stomatal pore index, B) stomata conductance (gs) C) net CO_2 assimilation rate (A), D) photosynthetic nitrogen-use efficiency (PNUE) and E) harvested biomass yields (dry matter). Mean values for unirrigated (UI), potable water (PW), wastewater dose 1 (WWD1) and 2 (WWD2) irrigated trees (n = 3 plots; standard error is shown). Tukey's Honestly Significant Difference is indicated using different letters (α = 0.05).

2.5.2 Wastewater irrigation modifies leaf morphology and stomatal traits

Irrigation with the different wastewater loads significantly increased the leaf area by 106−142% when compared to leaves of UI or PW treated trees. The corresponding increase in specific leaf area, the ratio of area over dry mass (Poorter *et al.*, 2009) (Figure 2.4), reveals a shift in leaf morphology of increased areas of photosynthetic activity and gas exchange which could enhance net carbon assimilation. This morphology would also inevitably lead to higher water loss through transpiration (Merilo *et al.*, 2006; Bacon, 2009; Hacke *et al.*, 2010), but is unlikely to have the accompanying physiological burden here due to the extreme excess water load.

The increased specific leaf area in wastewater irrigated trees was associated with an increase in the proportion of nitrogen investment per leaf area unit, and a corresponding decrease in the carbon to nitrogen ratio. This shift in C:N was driven by a +94% change in nitrogen content rather than the carbon investment, the latter being significantly higher for WWD1 and WWD2 leaves compared to UI and PW treated trees, but only by small absolute amounts (Figure 2.3). Increased investment of nitrogen into photosynthetic apparatus represents a change in the tree resource allocation strategy driven by wastewater irrigation, which has previously been observed in willow due to increased nitrogen availability (Bowman and Conant, 1994; Fabio and Smart, 2018a). This association of increased specific leaf area and higher leaf nitrogen allocation is also coherent with other reports of decreased specific leaf area found in low water and/or nutrient habitats, alongside higher allocation of nitrogen to stem biomass synthesis at the expense of photosynthetic machinery (Escudero and Mediavilla, 2003; Benomar, DesRochers and Larocque, 2012).

Nitrogen investment into leaves, as opposed to other organs, is often associated with a decrease in the rate of photosynthesis per unit of nitrogen (PNUE) (Guo *et al.*, 2011; Goodman *et al.*, 2014). Surprisingly, the PNUE did not decrease in leaves of WW irrigated trees and was similar between treatments, indicating that the high nitrogen-use-efficiency of willow commonly observed in nitrogen limiting environments (Isebrands *et al.*, 1996) is maintained in wastewater irrigated trees. A higher nitrogen investment into leaves by wastewater irrigated trees was associated with substantial increases in the concentrations of the major nitrogen containing pigments, 27–62% for chlorophyll a and 21–43% for chlorophyll b, when compared to leaves of either UI or PW treated trees (Figure 2.3). Similarly, the major non-nitrogen leaf pigments, the carotenoids, were also significantly increased within wastewater treated trees compared to UI and PW, increasing by between 8 and 26%. The chlorophyll content and, understandably, leaf nitrogen content were both highly correlated with photosynthesis and stomatal conductance (Supplementary file 1). The increase in carotenoids is also expected as they play an important role in protecting chlorophyll pigments from photo-oxidative damage (Iori *et al.*, 2015). It is not surprising then that, following the irrigation with wastewater, the rate of photosynthesis increased by ~49% in WWD1 and ~ 69% in WWD2 in trees compared to either control treatments (which were similar). Similarly, stomatal conductance increased by 42 and 56% in WWD1 and 62 and 78% in WWD1 when compared to UI or PW treated trees. The common response to water deficient conditions in trees is to decrease stomatal conductance to protect against water losses, inevitably leading to a decrease in CO_2 assimilation by the leaves (Mooney, Pearcy and Caldwell, 1997; Chaves, 2002; Bacon, 2009). Interestingly, while the UI plants may have been water limited, this was not the case for the PW irrigated trees, which received similar very high loads of water as wastewater treatments (Figure 2.1). This demonstrates that water availability did not drive the variation in stomatal conductance observed between the treatments and aligns with findings by Bowman *et al.* (1994) that stomatal conductance positively associates with nitrogen fertilisation in willow.

Natural variation in stomatal conductance is primarily determined by the density (Schluter, 2003; Franks and Beerling, 2009) and/ or the size of stomata (Lawson and Blatt, 2014; Bucher *et al.*, 2016). Both PW (high water load) and wastewater (high water and nitrogen load) irrigation could be expected to drive differences in stomatal conductance of leaves, when compared to unirrigated controls, due to modification of stomatal density or size. Surprisingly, as stomatal conductance was increased in wastewater irrigated trees, the density of stomata was significantly higher in trees of both UI and PW controls compared to trees of either wastewater treatment. This indicates that the stomatal density did not drive the increases in the stomatal conductance observed in wastewater irrigated trees. Interestingly, there was no difference between UI and PW controls, revealing that the very high water loads present in PW and wastewater irrigation treatment did not trigger modification of stomatal density here. The investigation of stomatal distribution patterns revealed similar densities (approximately 600–650 stomata mm^2) to that observed in previous studies in willow (Cooper and Cass, 2003).

When stomatal size was explored, both stomata and pore length significantly increased in wastewater irrigated trees when compared to UI and PW controls and stomatal pore length, in particular, was highly correlated with stomatal conductance (Figures 2.4, 2.5, Supplementary file

1). High correlation of the stomatal conductance and pore length has previously been observed in *Salix* (Aasamaa, Sõber and Rahi, 2001). Stomatal density was inversely correlated with stomatal size, suggesting that common factors determine stomatal density and size, and that the traits are antagonistic in terms of the high stomatal conductance phenotype induced here by wastewater irrigation. This inverse relationship has been notably reviewed (Hetherington and Woodward, 2003; Lawson and Blatt, 2014) in terms of natural variation and as a mechanism of developmental response to the environment, well demonstrated in Arabidopsis grown under different CO_2 and water regimes (Doheny-Adams *et al.*, 2012).

Although high stomatal density and smaller stomatal size is widely reported as a drought phenotype, with smaller stomata enhancing water-use-efficiency (Galmés *et al.*, 2007; Butler *et al.*, 2013), the variation observed here was not associated with water load. Wastewater induced increases in stomatal pore index (SPI), the pore area in a given leaf area (Sack, Tyree and Holbrook, 2005; Savage and Cavender-Bares, 2012), were governed predominantly by changes in stomatal size rather than the stomatal density. This is contrary to studies in many herbaceous species (Bucher *et al.*, 2016) and poplar (Fichot *et al.*, 2011), which report SPI variation as most influenced by the changes in stomatal densities, whereas stomatal size remains comparably constant or contributes only to a small degree. The disagreement potentially derives from the distinction between a low SPI drought phenotype minimising passive water loss (Savage and Cavender-Bares, 2012; Wei *et al.*, 2017) and the high SPI wastewater phenotype, observed here, likely produced by the combination of water and nitrogen in excess.

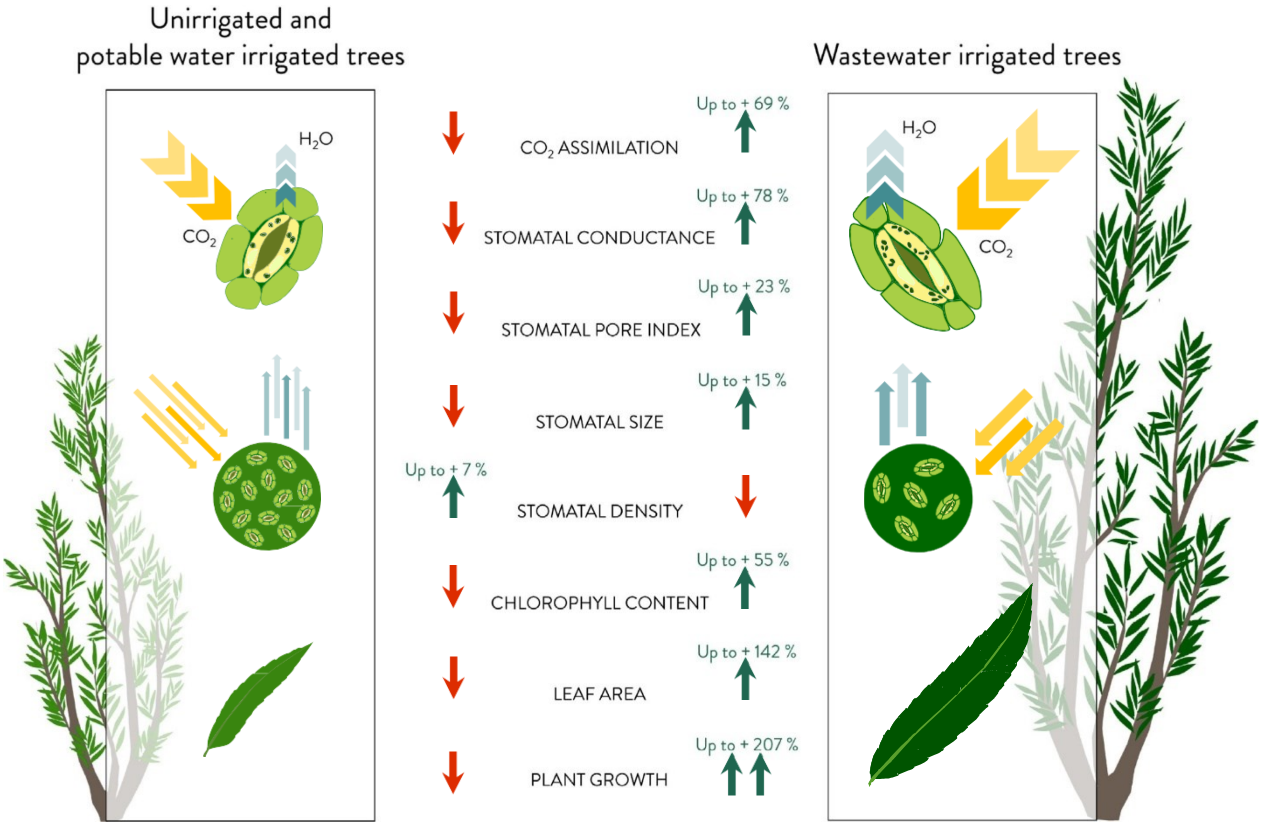


Figure 2. 6 : Willow wastewater phenotype. Summary schematic of tree modifications following primary effluent wastewater irrigation.

2.5.3 Wastewater modified physiology enhances biomass yield in willow

Trees which were irrigated with PW did not differ significantly from UI control trees for chlorophyll content, leaf area, stomatal size, stomatal density, stomatal conductance or CO_2 assimilation rate. It is therefore not surprising that the high potable water irrigated tress did not vary in biomass yield from unirrigated trees. By comparison, leaf area, nitrogen allocation, chlorophyll content, SPI, stomatal conductance and CO_2 assimilation rates were all significantly increased in wastewater irrigated trees compared to the unirrigated and the potable water irrigated trees, and biomass yields were correspondingly elevated (Figure 2.6).

Stomatal conductance is often considered to be most altered by water availability, unirrigated and high PW irrigated controls suggest an upper limit to growth in willow when water is in excess, which is reflected in limited stomatal conductance. Common thinking with *Salix* physiology is that the trees require and can respond positively to high volumes of water; these findings suggest that for very high loads of water, the trees become limited in nutrients essential for the photosynthesis. Importantly, the increased nitrogen and phosphorus loads, present as environmentally concerning contaminants in municipal wastewater, can serve to overcome these limitations to increase carbon uptake and transpiration by acting as de facto fertilisers. These substantial improvements in biomass yields ultimately lead to a high-volume capacity for phytofiltration, therefore reducing the need for prior conventional wastewater treatment.

2.6 Conclusions

These findings reveal that willow phytofiltration plantations can be used to efficiently treat high volumes of primary effluent municipal wastewater over a two-year short rotation coppice cycle, and that trees actually benefit from the treatment process, with stomatal adaptions accompanying an increased yield of sustainable biomass. While more extended trials are needed to establish the long-term viability of such phytofiltration systems, with salt accumulation being a focus of concern, these field-scale findings are promising. The displaced costs of annual conventional wastewater treatment in Canada can be as high as 50% of local municipal budgets. If the additional benefits of sustainable biomass can be integrated with added-value (or no-net-cost) cultivation practices, such as functioning phytofiltration, there is the potential to mitigate both negative local and global environmental impact of wastewater while generating sustainable and economically valuable services and bioproducts.

Credit authorship contribution statement

A. Jerbi: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **N.J.B. Brereton**: Conceptualization, Writing original draft, Writing - review & editing. **E. Sas**: Writing - original draft, Visualization, Writing - review & editing. **S. Amiot**: Investigation, Writing - review & editing. **T.X. Lachapelle**: Investigation, Writing - review & editing. **Y. Comeau**: Conceptualization, Writing - review & editing. **F.E. Pitre**: Conceptualization, Writing - original draft, Writing - review & editing. **M. Labrecque**: Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

2.7 Acknowledgments

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2.8 Supplementary material

Supplementary file 1 can be found online at https://doi.org/10.1016/j.scitotenv.2020.139728 and includes field site precipitation, soil properties prior to treatment, additional wastewater, soil and soil pore water composition, Pearson correlations.

	Precipitation		Irrigation	
Year	2016	2017	2016	2017
Treatment	mm	mm	mm	mm
UI	390	769	0	0
NW	390	769	1510	2177
WWD1	390	769	1160	1944
WWD2	390	769	1820	2951

Table S2. 1 : Precipitation, water and wastewater loads during the year’s growth trial (2016 and 2017).

Parameters	Units	Soil depth (cm)	
		0–30	30–70
Sand	wt%	79	88
Silt	wt%	17	8
Clay	wt%	4	4
Texture		Loamy sand	Sand
Organic matter	wt%	4.4 ± 0.7	1.2 ± 0.4
Total nitrogen	mg N kg-1	1510 ± 250	285 ± 145
Total ammonia	mg N kg-1	1.2 ± 0.5	0.8 ± 0.1
Nitrates and nitrites	mg N kg-1	2.4 ± 2.8	1.5 ± 0.5
Phosphorus	mg P kg-1	995 ± 140	667 ± 217
Extractable Calcium	mg Ca kg-1	762 ± 111	253 ± 92
Extractable Magnesium	mg Mg kg-1	87 ± 26	34 ± 15
Extractable Potassium	mg K kg-1	13 ± 10	24 ± 6
Cation-exchange capacity	cmolc kg-1	4.6 ± 0,7	1.7 ± 0,7

Table S2. 2 : Soil physical and chemical properties prior starting irrigation. The results represent the averages values (mean ± standard error) for each of the soil chemical properties.

Parameter	Unit	Primary wastewater mean
Chemical oxygen demand	COD mg L^{-1}	290 ± 29
Total nitrogen	N mg L^{-1}	42.1 ± 6.2
Total ammonia	NH4 mg N L^{-1}	20.1 ± 1.8
Nitrates and nitrites	NOx mg N L^{-1}	0.06 ± 0.01
Total Phosphorus	P mg L^{-1}	4.1 ± 0.6
Orthophosphates	o-PO4 mg P L^{-1}	2.7 ± 0.7
Calcium	Ca mg L^{-1}	98 ± 6
Magnesium	Mg mg L^{-1}	29 ± 2
Potassium	K mg L^{-1}	10.1 ± 1.2
Sodium	Na mg L^{-1}	145 ± 33
Chloride	Cl mg L^{-1}	190 ± 57
pH	pH	7.6 ± 0.1
Electroconductivity	dS m^{-1}	1.5 ± 0.1

Table S2. 3 : Chemical characterisation of the primary effluent applied during the second year of growth 2017 (From May 29th to November 16th 2017). The results represent the averages values (mean ± standard error) for each of the chemical components.

	Soil means										
	OM % DM	N mg L^{-1}	NH4 mg N L^{-1}	NOx mg N L^{-1}	P mg L^{-1}	o-PO4 mg P L^{-1}	K mg L^{-1}	Ca mg L^{-1}	Mg mg L^{-1}	pH	EC dS m^{-1}
UI	3.64	1113	38.97	2.56	1230	90.27	20.84	819	73.52	5.55	0.05
PW	3.48	1083	38.01	3.85	1028	73.23	38.47	658	80.04	5.66	0.06
WWD1	3.59	1224	36.93	12.14	1134	88.86	33.31	751	114.74	6.01	0.11
WWD2	3.49	1168	38.26	15.01	1065	95.78	37.61	727	101.74	5.90	0.12
	Std error										
UI	0.03	43	0.33	0.83	88	18.06	0.91	13	17.15	0.07	0.01
PW	0.22	88	0.38	1.02	86	5.86	3.55	21	20.91	0.12	0.00
WWD1	0.04	136	0.37	1.87	107	3.74	1.32	30	7.11	0.05	0.02
WWD2	0.32	130	0.20	2.75	136	22.20	9.03	65	23.14	0.18	0.02
	Tukey HSD (α = 0.05)										
UI	a	a	a	b	a	a	a	a	a	b	b
PW	a	a	a	b	a	a	a	b	a	ab	ab
WWD1	a	a	a	a	a	a	a	ab	a	a	ab
WWD2	a	a	a	a	a	a	a	ab	a	ab	a

Table S2. 4 : Soil chemical characterization following two years of irrigation. The results represent the averages values (mean ± standard error) for each of the soil chemical properties. Means with different letters were statistically different at $p < 0.05$.

	Groundwater means							
	COD mg L^{-1}	N mg L^{-1}	NH4 mg N L^{-1}	NOx mg N L^{-1}	P mg L^{-1}	o-PO4 mg P L^{-1}	pH	EC dS m^{-1}
PW	12.03	0.46	0.06	0.06	0.08	0.01	6.65	0.34
WWD1	21.16	2.82	0.34	2.15	0.06	0.01	6.71	1.37
WWD2	19.46	5.78	0.14	5.32	0.07	0.02	6.71	1.40
	Std error							
PW	1.47	0.10	0.01	0.01	0.02	0.00	0.04	0.02
WWD1	1.67	0.41	0.26	0.42	0.00	0.00	0.10	0.11
WWD2	0.69	0.91	0.06	0.84	0.01	0.01	0.10	0.11
	Tukey HSD (α = 0.05)							
PW	b	c	a	c	a	a	a	b
WWD1	a	b	a	b	a	a	a	a
WWD2	a	a	a	a	a	a	a	a

Table S2. 5 : Ground water chemical composition. The results represent the averages values (mean ± standard error) for each of the groundwater chemical properties. Means with different letters were statistically different at $p < 0.05$.

Pearson correlation coefficients $p < 0.05$

Parameter	Unit	*A*		*gs*		*LA*		*C/N*		*N/Area*		*PNUE*		*chla*		*chlb*		*Cx*		S_{Length}		S_{Width}		S_{Area}		$P_{Lenngth}$		*Stomata density*		*SPI*		*Yields* 2^{nd}	
CO2 assimilation	µmol CO2.m-2 .s-1	1																															
Stomatal conductance	mol H20.m-2.s-1	0.86	<0.001*	1																													
Leaf area	cm2	0.80	<0.001*	0.67	<0.001*	1																											
Carbon:Nitrogen	unitless	-0.82	<0.001*	-0.75	<0.001*	-0.88	<0.001*	1																									
Nitrogen concentration	mmol.m-2	0.67	<0.001*	0.54	<0.001*	0.79	<0.001*	-0.71	<0.001*	1																							
Photosynthetic nitrogen use efficiency	µmol CO2.m-2.mol-1 .s-1	0.35	0.016*	0.35	0.014*	-0.04	0.793	-0.11	0.467	-0.45	0.001*	1																					
Chlorophyll a	µg cm-2	0.75	<0.001*	0.74	<0.001*	0.71	<0.001*	-0.82	<0.001*	0.60	<0.001*	0.17	0.235	1																			
Chlorophyll b	µg cm-2	0.62	<0.001*	0.67	<0.001*	0.60	<0.001*	-0.74	<0.001*	0.47	<0.001*	0.18	0.232	0.92	<0.001*	1																	
Carotenoids	µg cm-2	0.52	<0.001*	0.55	<0.001*	0.48	<0.001*	-0.53	<0.001*	0.52	<0.001*	-0.02	0.887	0.77	<0.001*	0.63	<0.001*	1															
Stomatal length	µm	0.47	<0.001*	0.43	0.002*	0.48	<0.001*	-0.57	<0.001*	0.20	0.167	0.33	0.022*	0.55	<0.001*	0.55	<0.001*	0.19	0.187	1													
Stomatal width	µm	0.50	<0.001*	0.35	0.014*	0.57	<0.001*	-0.53	<0.001*	0.46	<0.001*	0.01	0.925	0.44	0.002*	0.39	0.007*	0.18	0.230	0.59	<0.001*	1											
Stomatal area	µm2	0.55	<0.001*	0.44	0.002*	0.60	<0.001*	-0.62	<0.001*	0.39	0.006*	0.17	0.242	0.55	<0.001*	0.51	<0.001*	0.20	0.166	0.85	<0.001*	0.92	<0.001*	1									
Pore length	µm	0.62	<0.001*	0.67	<0.001*	0.58	<0.001*	-0.69	<0.001*	0.30	0.041*	0.38	0.008*	0.63	<0.001*	0.62	<0.001*	0.37	0.010*	0.76	<0.001*	0.43	0.002*	0.65	<0.001*	1							
Stomata density	Unitless	-1.00	0.005*	-0.48	<0.001*	-0.37	0.010*	0.47	<0.001*	-0.12	0.418	-0.08	0.014*	-0.42	0.003*	-0.41	0.004*	-0.10	0.519	-0.56	<0.001*	-0.21	0.152	-0.40	0.005*	-0.53	<0.001*	1					
Stomatal pore index	Unitless	0.52	<0.001*	0.51	<0.001*	0.48	<0.001*	-0.56	<0.001*	0.30	0.038*	0.23	0.120	0.52	<0.001*	0.51	<0.001*	0.39	0.006*	0.61	<0.001*	0.41	0.004*	0.56	<0.001*	0.89	<0.001*	-0.09	0.553	1			
Biomass yield	t DM ha^{-1} yr^{-1}	0.66	<0.001*	0.52	<0.001*	0.56	<0.001*	-0.52	<0.001*	0.25	<0.001*	0.12	0.430	0.41	0.004*	0.39	0.007*	0.20	0.183	0.26	0.069	0.37	0.009*	0.38	0.008*	0.32	0.024*	-0.23	0.111	0.27	0.066	1	

Table S2. 6 : Pearson correlation coefficients (at $p < 0.05$) and P-values between Salix cultivar 'SX67' leaf physiological traits i.e. net CO_2 assimilation rate (A), stomata conductance (gs), leaf area (LA), carbon-nitrogen ratio (C/N), leaf N content per unit area (N/Area), photosynthetic nitrogen use efficiency (PNUE), chlorophyll a concentration (chla), Chlorophyll b concentration (chlb), carotenoids concentration (Cx), abaxial leaf stomata length (S_{Length}), abaxial leaf stomata width (S_{Width}), abaxial leaf stomata area (S_{Area}) abaxial leaf pore length (P_{Length}), abaxial leaf pore width (P_{Width}), abaxial leaf stomata density, abaxial leaf Stomatal pore area index (x 10^2) (SPI) and biomass yield (Yields 2^{nd}).

2.9 Synthèse du chapitre 2

Les résultats présentés dans ce chapitre ont montré que l'irrigation d'une culture de saules *Salix miyabeana* 'SX67' par des eaux usées avec traitement primaire (et donc riche en azote) avait affecté plusieurs paramètres morphologiques et physiologiques. En outre, nous avons observé des changements dans la morphologie des feuilles et les stratégies d'utilisation du carbone des plantes fertilisées. Notamment une augmentation du nombre de feuille par arbre (constaté visuellement), une augmentation de la surface foliaire spécifique, une augmentation de la concentration de l'azote foliaire et des pigments photosynthétiques ce qui a permis de maximiser la surface photosynthétique et d'améliorer le rendement quantique par unité de surface foliaire. D'autre part la fertilisation a induit un accroissement de la longueur des stomates ce qui a engendré une augmentation de la conductance stomatique et inévitablement du taux de carbone assimilé favorisant une meilleure croissance et une plus grande productivité.

Ces résultats ont par ailleurs permis de soulever un autre point important soit l'efficacité du système filtre pour traiter des fortes charges hydrauliques (allant jusqu'à 30 millions de litres d'effluents par année) et à capter de grandes quantités d'azote c.-à-d. 817 kg N ha^{-1} an^{-1} soit par accumulation dans la partie végétative, comme en témoigne l'augmentation de l'azote foliaire, et/ou au niveau du sol. Bien que la fertigation ait occasionné une augmentation du niveau de la demande chimique en oxygène ainsi que du nitrate dans les solutions de sol, les valeurs sont restées sous les standards recommandés par les instances environnementales au Québec.

Chapitre 3 | Effet de l'irrigation par les eaux usées sur le développement du xylème secondaire, la densité du bois et la composition du bois de saules *Salix miyabeana* 'SX67'

Au chapitre d'introduction, nous avons fait mention de résultats de recherches faites sur des espèces ligneuses (notamment des peupliers) qui avaient montré que la fertilisation azotée avait stimulé la formation de bois de tension et engendré une altération de la composition chimique du bois avec une augmentation de la fraction de la cellulose et une diminution de celle de la lignine. Dans cette perspective, et dans un contexte de filtre végétal, il est alors important d'investiguer l'effet de l'irrigation par de grandes quantités d'eaux usées riches en azote sur la composition chimique du bois ainsi que l'occurrence du bois de tension.

Au chapitre 2, les résultats ont montré que l'irrigation par les eaux usées riches en azote avait altéré la morphologie et la physiologie des parties aériennes du cultivar *Salix miyabeana* 'SX67', notamment en induisant un accroissement de la surface foliaire ainsi qu'un allongement des stomates ce qui a engendré une augmentation de la conductance stomatique et de la photosynthèse (et ainsi une amélioration de la productivité). Un accroissement de la surface foliaire et une augmentation de la conductance stomatique suppose qu'il y aurait une amélioration de l'efficacité de transport de l'eau pour compenser pour une plus large surface évaporative et une plus grande transpiration. Dans ce contexte, il est alors pertinent de mieux comprendre dans quelle mesure l'apport d'eau et/ou de nutriments ont altéré la structure anatomique des fibres et des vaisseaux et conséquemment les propriétés mécaniques du bois.

Field irrigation with primary wastewater alters wood composition in willow *Salix miyabeana* ‘SX67’

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3.1 Abstract

Traditional treatment of wastewaters is a burden for local governments. Using short rotation coppice willow (SRCW) as vegetal filter has several environmental and economic benefits. Here, we investigated the effect of primary wastewater irrigation on wood structure and chemical composition of willow cultivar *Salix miyabeana* 'SX67' following two years of growth. Compared to unirrigated plants (UI), stem sections of plants irrigated with primary wastewater (WWD) showed an unexpected decrease of hydraulic conductance (K_S) because of difference in vessel density but not vessel diameter. 86 % of vessels belonged to vessel diameter range groups [20-30[, [30-40[and [40-50[µm and contributed to > 75 % of theoretical K_S, while the group class [50-60[µm (less than 10 % of vessels) still accounted for > 20 % of total K_S regardless irrigation treatments. WWD significantly alters the chemical composition of wood with an increase of glucan content by 9 to 16.4 % and a decrease of extractives by 35.3 to 36.4 % when compared to UI plants or to plants irrigated with potable water (PW). The fertigation did also increase the proportion of the tension wood which highly correlates with the glucan content. In the context of energetic transition and mitigation of climatic change, such results are of high interest since WWD effectively permit the phytofiltration of large amounts of organic contaminated effluents without impairing SRCW physiology.

Keywords: Willow, wastewater, phytofiltration, cell wall composition, hydraulic conductance, sustainable biomass

3.2 Introduction

The use of short-rotation willow coppice (SRWC) as a vegetation filter has potential to both drastically enhance plantation productivity and improve the quality of pretreated wastewater prior to discharge into a water body (Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015). Since this approach fulfills willow water and nitrogen requirement, it allows shrubs to overcome their high evapotranspiration demand and thus improve carbon assimilation and biomass production (Dimitriou and Aronsson, 2004, 2011; Miguel *et al.*, 2014; Guidi Nissim *et al.*, 2015). Previous studies have reported the positive effects of wastewater fertigation on various physiological and morphological SRWC parameters: leaf area, leaf N content, chlorophyll content, stomatal conductance, photosynthesis and carbon assimilation, below ground biomass and above ground productivity (Dimitriou and Aronsson, 2011; Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015, 2020; Lachapelle-T., Labrecque and Comeau, 2019). Xylem development and the resulting wood characteristics are also strongly affected by environmental parameters, namely water or nitrogen availability (Arend *et al.*, 2007; Hacke *et al.*, 2010; Pitre *et al.*, 2010; Plavcová *et al.*, 2012, 2013; Anfodillo *et al.*, 2013, Wang *et al.*, 2016) which are not limited in the present context. Yet, very little is known about the effect of municipal effluent fertigation on the structural and chemical biomass composition even though its alterations could not only influence plant physiology (Jerbi *et al.*, 2020) but also biofuel production.

Therefore, one would expect to observe alterations of wood structure when fertigated with primary wastewater compared to traditional SRWC. At the anatomical level, the physical structure of vessel and fiber cells (i.e. number per unit area) within the xylem tissue have a great impact on plant function as a whole and on wood hydraulic and mechanical properties (Mellerowicz *et al.*, 2001; Pitre *et al.*, 2007a; Martínez-Cabrera *et al.*, 2011). Specific conductivity (theoretical K_S) and hydraulic conductance are functions of vessel density and stem (Tyree and Zimmermann, 2002). Although larger vessels are likely to improve hydraulic conductance, such conduits result in a lower fraction of supporting tissue and thus lead to a decrease in stem mechanical strength (Poorter *et al.*, 2010). They are also more vulnerable to embolism when exposed to environmental stresses like drought, heat waves and freeze-thaw events (Sperry *et al.*, 2008; Martínez-Cabrera *et al.*, 2011; Plavcová *et al.*, 2012; Hacke *et al.*, 2017), the latter being frequent in remote regions of northern

Canada where the effective deployment of this combined phytotechnology (SRWC + vegetation filter) has the greatest potential.

Wood composition as well as the form of major polymers, lignin, cellulose and hemicellulose, vary also considerably between plant species, genotypes and because of environmental factors (Pitre *et al.*, 2007a; de Souza, 2013). Conducted mostly on poplar, several studies have reported the effects of N fertilization on wood structure (mostly fiber lumen and cell walls) and chemical composition, especially the content of extractives, lignin, glucose, xylose and arabinose (Serapiglia *et al.*, 2009, 2013a; Ray *et al.*, 2012; Wan *et al.*, 2014). Nitrogen availability affects the development of secondary xylem during cell division and differentiation and leads to an alteration of either xylem anatomy and/or wood structural composition (Luo *et al.*, 2005; Pitre *et al.*, 2007a, 2010) with an increase in cellulose content, a reduced lignin fraction, an increase of the tension wood proportion, a decrease of fiber cell walls and a decline in wood density (Harvey *et al.*, 1999; Luo *et al.*, 2005; Pitre *et al.*, 2007a-b, 2010; Serapiglia *et al.*, 2013b). Albeit willow cultivation is of equal economic importance, less research has assessed the effect of high N fertilization on the compositional and morphological traits of willow cultivars.

The aim of our study was to investigate the short-term effects of nitrogen fertilization with primary municipal wastewater on the mechanical structure and wood composition of willow cultivar *Salix miyabeana* 'SX67'. We hypothesized that fertigation with high N wastewater effluent (WWD) would alter xylem structure as well as the composition of the wood lignocellulosic cell-wall of fiber because an increased conductive capacity and wider xylem vessels compared to plants either unirrigated or irrigated with potable water.

3.3 Materials and methods

3.3.1 Study site and plant material

The experimental plantation was located in Saint-Roch-de-l'Achigan (45° 50′ 50″ N–73° 38′ 27″ W), 57 m above sea level, 55 km northeast of Montreal (Quebec), Canada. The regional climate is humid continental with noticeable seasonal temperature variations, warm, humid summers and cold winters. According to the nearest weather station in Assomption (45° 48 34"N–73° 26 05"W), the annual average minimum and maximum temperatures for the period 2003-2017 are respectively 1 ± 12 °C and 11 ± 13° C. During the growing period (from May 1 to October 31, 2017), average minimum and maximum temperatures were recorded by the in-field meteorological station and corresponded to 9.8 ± 5.6° C and 22.1 ± 6.5° C respectively (Amiot *et al.*, 2020). The average annual precipitation was 1102 mm (2005-2015).

Four hectares of *Salix miyabeana* 'SX67' were established in 2008 at a density of 16,000 trees ha^{-1} with 1.8 m and 35 cm spacing between willow rows and between cuttings within each row respectively. A secondary municipal effluent experiment was conducted on the plantation between 2009 and 2012 (Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015). The plantation was last coppiced prior the present experiment when plants roots were seven-year-old in the autumn of 2015. In 2016, a randomized block design was set up. It comprised four irrigation treatments replicated three times, including a control without irrigation (UI), irrigation with potable water (PW), and irrigation with two different primary effluent wastewater doses (Lachapelle-T., Labrecque and Comeau, 2019). Only UI, PW and the lowest dose of wastewater (noted here as WWD) are considered in the present study. The nine experimental square plots (3 treatments x 3 plots) of 100 m^2 were delimited, each containing six rows of willow with the four central rows irrigated. Four randomly chosen plants in each of those latter four rows were identified and served for most of sampling and analysis.

Effluent obtained from the local municipal wastewater treatment facility was allowed to rest for at least 24 h in a conventional septic tank prior to irrigation, with no further chemical or biological treatment. The plantation was irrigated 111 and 163 days respectively for 2016 and 2017 with a daily dose of respectively 14 mm and 13 mm for PW, 10 mm and 12 mm for WWD. Characterization of primary wastewater, annual precipitation and loads of water and wastewater are presented in the supplementary material, further details on the experiment are described in Lachapelle-T. *et al.* (2019), Amiot *et al.* (2020) and Jerbi *et al.* (2020).

3.3.2 Plant sampling and biomass processing

At the end of the 2017 growing season, all above-ground biomass from the four labelled trees within each plot were harvested, for a total of 36 plants (3 treatments x 12 replicates). All trees were fresh-weighed and random subsamples of stems were oven dried at 105° C for 72 h to assess moisture content. The biomass yield was estimated based on dry matter yields at planting density of 16,000 trees ha^{-1}.

3.3.3 Wood specific gravity (Wood density)

In August 2017, two 20-25 cm stem sections were collected from the 36 labelled trees. They were immediately fixed in a formaldehyde-acetic acid-alcohol solution (FAA solution: 3.7 % formaldehyde, 5 % acetic acid and 47 % alcohol). 3-4 cm subsections (one per tree) were labelled, vacuum infiltrated with water for 48 h and used to determine stem volume by water displacement. Wood specific gravity was later calculated on the basis of green volume and oven-dry weight at 105° C (Brereton *et al.* 2015; Berthod *et al.* 2015).

3.3.4 Microscopy and image analysis

As fertigated trees were more developed than UI and PW trees, sections were collected from different stem regions depending on the irrigation treatment in order to obtain comparable samples (Pitre *et al.*, 2007a) (e.g. sections at breast height for UI and PW, but at a higher level for WWD). Stems were sectioned where wood had already transitioned to secondary growth before irrigation began, thus allowing analysis of the wood (secondary xylem) that was formed before and during the irrigation period to assess the effect of nitrogen fertilization on growth, development and histologic wood properties (Pitre *et al.*, 2007a). The diameter of the sections was between 9 and 10 mm.

3.3.4.1 Stem sectioning and staining

From the second stem section fixed in FAA, a transverse section of 25 μm thickness was made using a rotary microtome (Leica RM2235, Germany). To monitor changes in the relative proportion of lignin and cellulose, the 25 μm cross section was double-stained with 1 % aqueous Safranin O to stain lignified cell walls and with 1 % Chlorazol Black E in methoxyethanol to staining cellulose G-fiber. Sections were permanently mounted on glass slides with DPX medium (Brereton *et al.*, 2011, 2012).

3.3.4.2 Image acquisition and analysis

All 36 slides were scanned digitally using a linear whole slide scanner (Aperio ScanScope CS2, Leica, Germany) at 40x objective magnification. Raw image data were stored in Aperio SVS file format, a multi-layered compressed JPEG (further information on the image format can be found in Shawki *et al.*, 2020). The slide images varied in size from 0.2 to 0.4 Gb and were first viewed using Leica Aperio Imagescope digital slide viewer version 12 (Leica Biosystems, Aperio) to examine each entire slide for any potential problems that could interfere with analysis, e.g. air/dust spots, partial staining, missing xylem parts. The SVS image slides were then analyzed by a script developed in-house in Fiji image-processing software (www.fiji.sc, ImageJ, (Schindelin *et al.*, 2012). More details on the script and the image analysis methods are presented in the supplementary material. The Fiji script was run on a super-computing platform so that the SVS file could be opened as a big tiff file (approximately 20 Gigabytes) and the whole image analyzed in smaller fragments to examine the various xylem features (which are described in the section below). The technique for distinguishing vessel lumen from the lumen of fiber and parenchyma ray, was first tested experimentally by assessing a threshold of the area, the circularity and the roundness. The proportion of tension wood (%) was measured based on the black and white method, by counting the black pixels in the monochrome images of the wood.

Average vessel area Ā is generally reported separately for each of these ring types (Zanne *et al.*, 2010), however, we did not separate vessels from springwood and summer wood growth rings due to the technical difficulty of distinguishing between them among different growth rings.

3.3.4.3 Histologic variables investigated

The variables assessed for each stem section were: the proportion (%) of xylem per stem section (xylem area was calculated by subtracting the pith area from the cross-sectional area), the proportion of tension wood (xylem black stained area divided by the xylem area), the proportion of vessels per secondary xylem, the proportion of fiber and ray cells per secondary xylem, the density of vessels per unit area (N), the density of fiber and parenchyma ray cells per unit area, the vessel to fiber density ratio (%) and the vessel to fiber area ratio.

Data on individual vessel lumen area from each stem section and for all sections were used to calculate average vessel lumen area Ā (μm^2), average vessel lumen diameter (D) (µm), theoretical hydraulic conductivity K_h within cross section area (calculated using the modified Hagen–Poiseuille law whereby conduit diameter corresponds to the vessel lumen diameter (D) (Tyree and Ewers, 1991)), and theoretical specific hydraulic conductivity (K_S) (kg m^{-1} Mpa^{-1} s^{-1}) calculated by normalizing K_h by stem section xylem area '$Xylem_{area}$' (i.e. scaling data such that the hydraulic conductance of stem section with different area can be compared on the base of their water transport efficiency) (Quintana-Pulido *et al.*, 2018) (Table 3.1).

Vessel density as well as theoretical specific hydraulic conductivity K_S were assessed per vessel lumen diameter class of 10 µm range and corresponded to a six-class group, i.e. 10-20 µm, 20-30 µm, 30-40 µm, 40-50 µm, 50-60 µm and 60-70 µm. Also calculated were: the relative frequency of the density of each diameter class (in relation to the density of all the vessels), the contribution of the K_S of each diameter class to the total K_S (i.e. to the total conductance within all the conduits of a stem section) and the accumulated K_S as a percentage of the total K_S. The range class was chosen based on image analysis data which show that the smallest and the largest vessel lumen area within and among all the samples were respectively 250,015 and 2999.9 μm^2 and correspond to a diameter D of 17.8 and 61.8 µm. Hydraulic parameters measured and calculated are presented in Table 3.1.

Additional xylem-vessel parameters calculated were the vessel lumen fraction (F) (Unitless), the non-lumen fraction NF (Unitless), the vessel vulnerability index (VI)($\mu m\ mm^{-2}$), the vessel composition index (S) (mm^4) and the mean hydraulic diameter (D_H) and are presented in the supplementary material.

3.3.5 Wood composition analysis

Prior to compositional analysis, 5 g of ODW milled and sieved biomass was extracted with 95 % ethanol according to the NREL protocol (Sluiter *et al.*, 2008), using a Dionex® Accelerated Solvent Extractor (ASE150) (the biomass in 33 ml cell size at 100° C under a pressure of 100 bar during 3 static cycles of 5 min for each extraction). The extracted biomass was then analyzed for structural carbohydrates and lignin in accordance with Sluiter *et al.* (2012). All sugars were quantified by high-performance liquid chromatography 'HPLC' system (Shimadzu Corporation, Kyoto, Japan) with a Bio-Rad Aminex HPX-87H column and refractive index detector. The HPLC data was corrected for the standard anhydro i.e. the contribution of water to the molecule weight of sugars between the monomer and the polysaccharide form (Serapiglia *et al.*, 2009).

3.3.6 Statistics

Analysis of variance testing was followed by multiple comparisons of means according to Tukey's Honestly Significant Difference (HSD) ($\alpha = 0.05$) using JMP statistical software version 9.0 (SAS Institute, Cary, NC), unless otherwise stated. Pearson correlations were calculated for all pairwise combinations of xylem properties and biomass composition.

3.4 Results

3.4.1 Biomass yield

After two years of growth, the total harvested biomass yields for the plants irrigated with the primary effluent WWD were higher than those of UI and the least productive PW irrigated trees with an annual yield per hectare of 18.3 ± 3.5, 13.1 ± 1.6 and 28.8 ± 6.3 Mg ha^{-1} yr^{-1} (Jerbi *et al.*, 2020) respectively for UI, PW and WWD (Figure 3.1A-D).

3.4.2 Secondary xylem

3.4.2.1 Wood density (specific gravity)

Wood density differed significantly between the different irrigation treatments, with the control UI and PW having a > 18 % higher specific density than WWD with respectively 0.37, 0.39 and 0.32 g cm^{-3} (Table 3.2 and Figure 3.1H).

Variable	**Acronyms**	**Formula**	**Units**	**Definition**	**References**
Vessel lumen area	A		μm^2		
Average vessel lumen area	Ā	$\sum_{i=1}^{n} \frac{A_i}{N}$	μm^2	Average area of all stem section vessels	
Vessel lumen diameter	D	$\sqrt{\frac{4A}{\pi}}$	μm	Vessel lumen diameter corresponding to circle with area A (vessel lumen area)	(Scholz *et al.*, 2013; Quintana-Pulido *et al.*, 2018)
Average vessel lumen diameter	D̄	$\sum_{i=1}^{n} \frac{D_i}{N}$	μm	Average vessel lumen diameter	
Vessel density	N	$N_{per\ Xylem\ area} / Xylem\ area$	Number mm^{-2}	Density per unit area	
Theoretical hydraulic conductivity	K_h	$\left(\frac{\pi\rho}{128\eta}\right)\sum_{i=1}^{n}(D_i^4)$	kg m Mpa^{-1} s^{-1}	Conductance per unit pressure gradient of all the vessels within the cross section area, where ρ is the density of water (998 kg m^{-3}), η is the dynamic viscosity of the water (10^{-9} MPa s^{-1} at 20 ºC; D is the diameter (m) of each vessel and N is the number of vessels within the cross section area	(Tyree and Ewers, 1991; Tombesi *et al.*, 2010; Scholz *et al.*, 2013; Kotowska *et al.*, 2015; Quintana-Pulido *et al.*, 2018)
Specific theoretical hydraulic conductivity	K_S	$\frac{\left(\frac{\pi\rho}{128\eta}\right)\sum_{i=1}^{n}(D_i^4)}{Xylem\ area}$	kg m^{-1} Mpa^{-1} s^{-1}	Theoretical hydraulic conductivity normalized by the stem section xylem area. It represents either a measure of stem segment porosity or measure of the 'efficiency' of stems in conducting water	(Bucci *et al.*, 2006; Hacke *et al.*, 2010; Scholz *et al.*, 2013; Kotowska *et al.*, 2015; Quintana-Pulido *et al.*, 2018)

Table 3. 1 : Hydraulic parameters measured and calculated with acronyms, units, main definition.

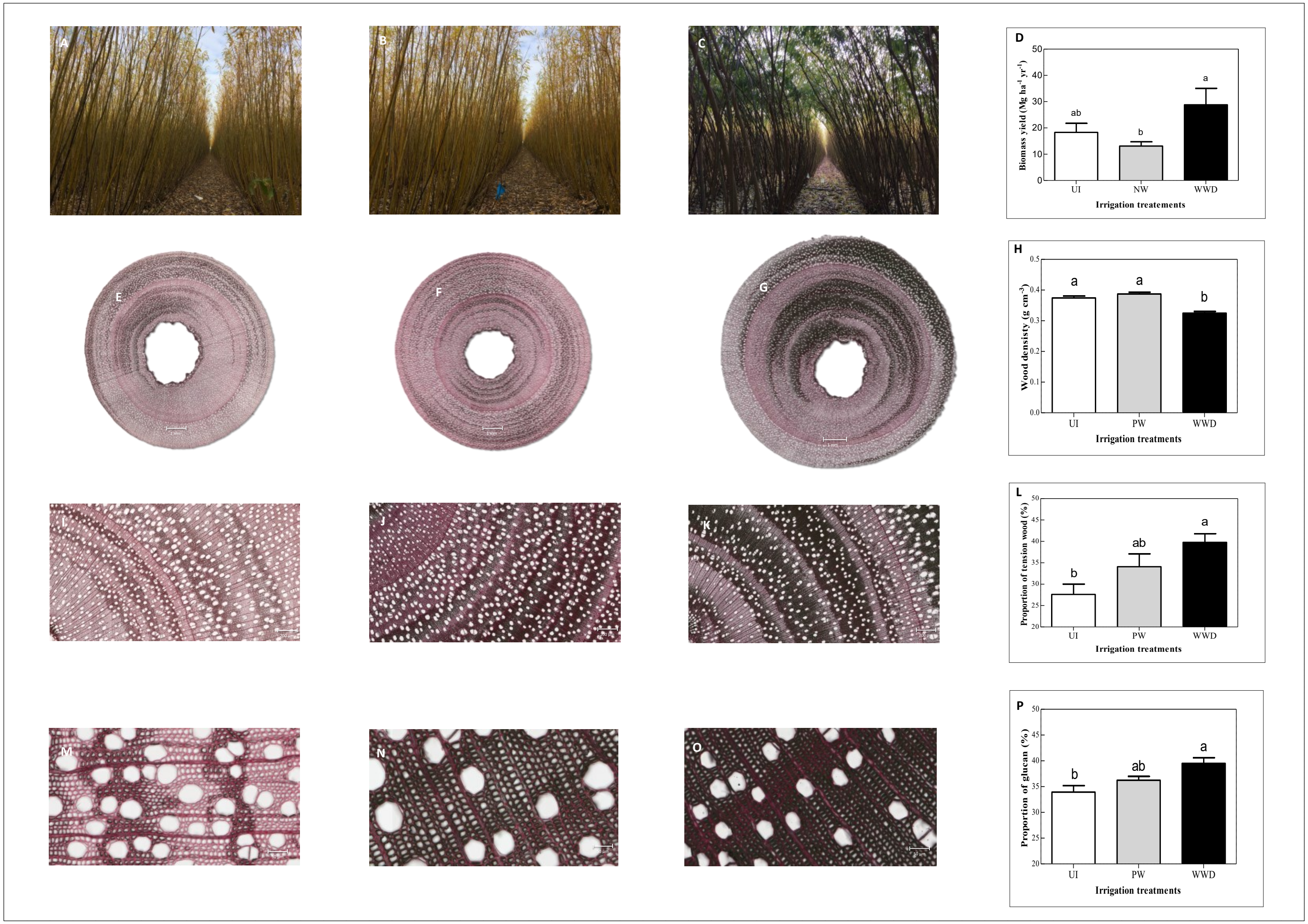

Figure 3. 1 : (A, B and C) Trees with irrigation treatments UI, PW and WWD respectively. (E, F and G) Stem section from trees with irrigation treatments UI, PW and WWD respectively. (I, J and K) 4x magnification of stem section region from trees with irrigation treatments UI, PW and WWD respectively. (M, N and O) 10x magnification of stem section region from trees with irrigation treatments UI, PW and WWD respectively. (D) Above ground biomass (Mg ha^{-1}) of irrigation treatments UI, PW and WWD respectively. (O) Wood density (g cm^{-3}) of treatments UI, PW and WWD respectively. (L) Proportion of tension wood (%) of irrigation treatments UI, PW and WWD respectively. (P) Wood proportion of glucan (%) of treatments UI, PW and WWD respectively. The results represent the average values (mean ± standard error) for each irrigation treatment. Different letters indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \leq 0.05$).

Treatment	Wood density (g cm^{-3})	Fibers + ray cells density (number mm^{-2})	Proportion of fibers + ray cell per secondary xylem (area %)	Proportion of tension wood per secondaty xylem (area %)	Average vessel lumen area (Ā) (µm^{2})	Average vessel lumen diameter D (µm)
UI	0.37 ± 0.01 a	5695 ± 90 a	81 ± 0.3 b	27.6 ± 2.4 b	1112 ± 16 a	36.2 ± 0.2 a
PW	0.39 ± 0.01 a	5756 ± 109 a	81 ± 0.4 b	34.1 ± 3.0 ab	1083 ± 15 a	36.0 ± 0.2 a
WWD	0.32 ± 0.01 b	6243 ± 70 a	84 ± 0.5 a	39.8 ± 2.0 a	1064 ± 18 a	35.5 ± 0.3 a
ANOVA *p* values	0.0126*	0.1219	0.0013*	0.0283*	0.0564	0.1143

Treatment	Vessels density (N) (N mm^{-2})	Theoretical sapwood area-specific hydraulic conductivity Ks (Kg. m^{-1}. Mpa $^{-1}$ s^{-1})	Proportion of secondary xylem per stem section (area %)	Proportion of vessels per secondary xylem (area %)	Vessels to fibers density ratio %	Vessel to fiber area ratio %
UI	172 ± 4 a	10.79 ± 0.2 a	92.4 ± 0.6 a	19 ± 0.3 a	3.0% ± 0.1% a	24% ± 0.5% a
PW	173 ± 3 a	9.99 ± 0.3 b	94.6 ± 0.4 a	19 ± 0.4 a	3.0% ± 0.1% a	23% ± 0.6% a
WWD	152 ± 4 b	8.85 ± 0.4 c	95.8 ± 0.6 a	16 ± 0.5 b	2.4% ± 0.1% b	19% ± 0.8% b
ANOVA *p* values	0.0021*	<.0001*	0.0808	0.0013*	0.0033*	0.0003*

Table 3. 2 : Xylem parameters and the vessels feature of *Salix miyabeana* 'SX67' plants under different irrigation treatments. The results represent the average values (mean ± standard error) for each irrigation treatment. For each variable, different letters indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \leq 0.05$).

3.4.2.2 Mechanical parameters: fibers

Although the density of fiber and ray cells did not vary significantly between UI, PW and WWD plants (Table 3.2), the proportion of fiber per secondary xylem area was higher in WWD plants compared to UI and PW plants, with respectively 84, 81 and 81 % while vessel to fiber area and density ratio were significantly lower (Table 3.2).

3.4.2.3 Tension wood

Under all irrigation treatments, wood reacted with both dyes. A higher proportion of the wood of the wastewater irrigated plants reacted more intensely with the Chlorazol black, resulting in a larger black staining region than the other treatments, especially the UI. Visual observations (Figure 3.1E-G) show that for all treatments, regions of the stem section displayed the presence of an additional layer in the inner part of the wall of some fiber cells (more pronounced for WWD), contributing to their thickness. Analysis of the proportion of wood presenting such formation (recognized as tension wood), showed a significant difference between irrigation treatments, especially between the UI control and the WWD plants, with the proportion respectively of 27.6, 34.1 and 39.8 % for UI, PW and WWD (Table 3.2 and Figure 3.1L).

3.4.3 Hydraulic parameters

The average vessel lumen area did not vary significantly between treatments, measuring 1112, 1083 and 1064 µm^2 respectively for UI, PW and WWD, nor did average vessel lumen diameter with quite similar values, i.e. 36.2, 36 and 35.5 µm (Table 3.2). Total vessel density (N mm^{-2}) did differ between treatments, with WWD showing lower density than those of UI and PW with respectively 152, 172 and 173 vessels mm^{-2}. As a result, the total theoretical specific hydraulic conductivity K_S (per stem section) did vary significantly between treatments, with UI the highest, PW intermediate and WWD significantly much lower with respectively 10.79, 9.99 and 8.85 Kg. m^{-1} Mpa^{-1} s^{-1}.

For all treatments, vessel density per vessel lumen diameter range varied between the different diameter classes, i.e. 10-20, 20-30, 30-40, 40-50, 50-60 and 60-70 µm and showed a unimodal distribution, with class diameters 30-40 µm showing the highest density (Figure 3.2A and Table S3.4 in the supplementary material section). Similarly, K_S per vessel diameter range varied between the different diameter classes, with the highest K_S value for classes 30-40 µm, 40-50 µm and 50-60 µm (Figure 3.2B and Table S3.5 in the supplementary material section).

The frequency of vessel density per lumen diameter varied between the different classes, with the highest contribution to total vessel density for the group 30-40 µm, with respectively 34.7, 39.4 and 37.6 % for UI, PW and WWD. Compared to PW and WWD, UI values for the 30-40 µm was significantly different. The groups 20-30 µm and 40-50 µm, which showed quite similar density frequencies, also contributed greatly to the total vessel density with 22.8, 21.4 and 25.0 % respectively for UI, PW and WWD for the former group and 26.5, 27.5 and 22.7 % for the latter group (Table 3.3). For K_S, the highest contribution was from the group 40-50 µm, with respectively 41.2, 45.1 and 38.1 % for UI, PW and WWD (Table 3.4; Figure 3.2C-D).

	The ratio of vessels with diameter D, where a µm < D <= b µm						
Treatment	$10 \leq D < 20$	$20 \leq D < 30$	$30 \leq D < 40$	$40 \leq D < 50$	$50 \leq D < 60$	$60 \leq D < 70$	$10 \leq D < 70$ All vessels
UI	6.1% ±0.5% a	22.8% ±0.6% a	34.7% ± 0.9% b	26.5% ±1.2% a	9.5% ± 1.1% a	0.5% ±0.1% a	100%
PW	5.1% ±0.2% a	21.4% ±0.8% a	39.4% ± 0.7% a	27.5% ±0.7% a	6.3% ± 0.6% a	0.2% ±0.0% a	100%
WWD	5.8% ±0.4% a	25.0% ±1.3% a	37.6% ± 0.9% a	22.7% ±1.5% a	8.4% ± 0.6% a	0.4% ±0.1% a	100%
ANOVA *p* values	0.1830	0.1111	0.0051*	0.3453	0.0771	0.3462	

Table 3. 3 : Vessel density frequency per vessel lumen diameter (D) range, i.e. the proportion of the density of each vessel class group per the density of all the vessels. Results represent the average values (mean ± standard error) for each irrigation treatment. Different letters in the same diameter class group indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \leq 0.05$).

Treatment	The ratio of the theoretical sapwood area-specific hydraulic conductivity Ks per vessel lumen diameter (D) range, where a µm < D <= b µm						
	10 ≤ D < 20	20 ≤ D <30	30 ≤ D < 40	40 ≤ D < 50	50 ≤ D < 60	60 ≤ D < 70	10 ≤ D <70 All vessels
UI	0.3% ± 0.02% a	3.9% ± 0.2% a	22.0% ± 1.2% c	41.2% ± 2.3% a	30.2% ± 2.8% a	2.3% ± 0.5% a	100%
PW	0.3% ± 0.01% a	4.1% ± 0.2% a	27.6% ± 1.1% a	45.1% ± 0.8% a	21.7% ± 1.6% a	1.2% ± 0.2% a	100%
WWD	0.3% ± 0.02% a	4.9% ± 0.4% a	24.8% ± 1.0% b	38.1% ± 2.1% a	29.6% ± 2.0% a	2.3% ± 0.4% a	100%
ANOVA *p* values	0.3397	0.0903	0.0044*	0.3717	0.1047	0.3523	

Table 3. 4 : The ratio of the theoretical sapwood area-specific hydraulic conductivity K_S (Kg m^{-1} pa $^{-1}$ s^{-1}) per vessel lumen diameter (D) range, i.e. the proportion of the K_S of a given diameter class group to the total K_S. Results represent the average values (mean ± standard error) for each irrigation treatment. Different letters in the same diameter class group indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \leq 0.05$).

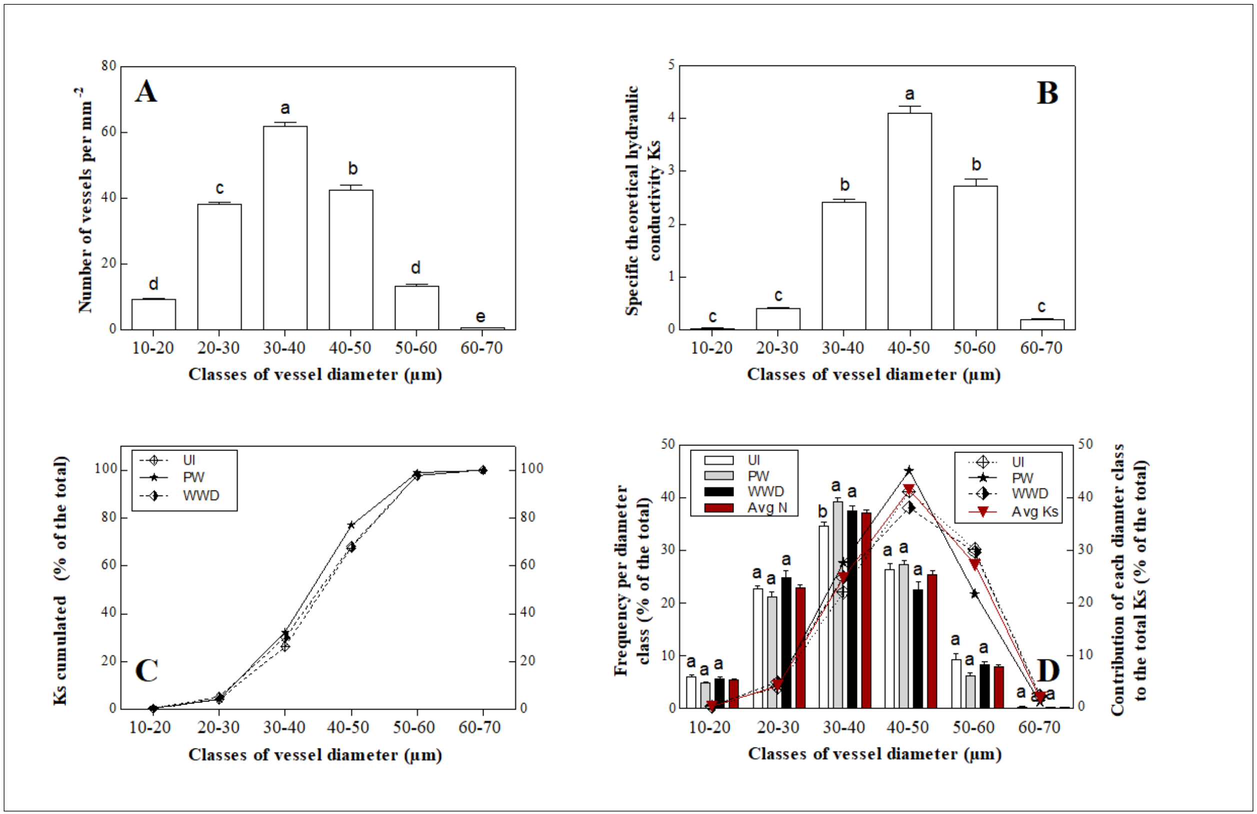

Figure 3. 2 : Average vessel density (N mm^{-2}) per vessel lumen diameter class (A), the theoretical sapwood area-specific hydraulic conductivity K_S (Kg m^{-1} pa^{-1} s^{-1}) per vessel lumen diameter class (B), the cumulated theoretical sapwood area-specific hydraulic conductivity K_S (Kg m^{-1} pa^{-1} s^{-1}) per vessel lumen diameter class (C), distributions of vessel diameters and their contribution to total hydraulic conductivity i.e. total K_S (D). For figure D, the histograms indicate vessel frequency per vessel diameter class and the lines indicate the contribution of each vessel lumen diameter class to total hydraulic conductivity K_S. Results represent the average values (mean ± standard error) for each irrigation treatment. Different letters in the same column group indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \leq 0.05$).

3.4.4 Wood composition analysis

The extractives content did vary significantly between the different irrigation treatments, with the content of WWD plants, 6.8 %, noticeably lower than the content of UI and PW biomass, i.e. 10.5 and 10.7 % (Table 3.5).

Total lignin content as well as its acid soluble and acid insoluble fractions, i.e. ''ASL'' and ''AIL'', were statistically similar within the different irrigation treatments, with respectively 27.7, 27.1 and 28.1 % for total lignin of WWD, UI and PW, 5.2, 5.1 and 5.3 % for acid-soluble lignin and 22.5, 22.1 and 22.8 % for acid insoluble lignin (Table 3.5). The cellulose content (glucan) differed significantly between UI, PW and WWD, with respectively 33.9, 36.2 and 39.5 % (Table 3.5). Hemicellulose content did not differ between treatments UI, PW and WWD and was respectively 16.1, 17.3 and 17.7 %. The content of the different hemicellulose sugar monomer components did not vary between treatments, although a statistically significant difference in arabinose content between the NW and the WWD biomass was detected. Respectively, for UI, PW and WWD, the content of different monomers was 11.9, 12.6 and 13.3 % for xylose, 1.3, 1.4 and 1.3 % for galactose, 1.2, 1.3 and 1 % for arabinose and 1.7, 2 and 2 % for mannose (Table 3.5).

Treatment	Glucan	Xylan	Galactan	Arabinan	Mannan	Hemicellulose
UI	33.9 ± 1.2 b	11.9 ± 0.5 a	1.3 ± 0.06 a	1.2 ± 0.1 ab	1.7 ± 0.1 a	16.1 ± 0.6 a
PW	36.2 ± 0.7 ab	12.6 ± 0.2 a	1.4 ± 0.03 a	1.3 ± 0.1 a	2.0 ± 0.1 a	17.3 ± 0.3 a
WWD	39.5 ± 1.1 a	13.3 ± 0.5 a	1.3 ± 0.05 a	1.0 ± 0.1 b	2.0 ± 0.1 a	17.7 ± 0.6 a
ANOVA *p* values	0.0237*	0.2900	0.2669	0.0482*	0.0789	0.2368
Treatment	Total Sugars	ASL	AIL	Total Lignins	Extractives	Mass closure
UI	50.0 ± 1.8 b	5.2 ± 0.05 a	22.5 ± 0.3 a	27.7 ± 0.3 a	10.5 ± 0.1 a	88.2 ± 1.7 a
PW	53.6 ± 1.0 ab	5.1 ± 0.07 a	22.1 ± 0.3 a	27.1 ± 0.3 a	10.7 ± 0.3 a	91.4 ± 1.0 a
WWD	57.2 ± 1.6 a	5.3 ± 0.06 a	22.8 ± 0.3 a	28.1 ± 0.3 a	6.8 ± 0.2 b	92.1 ± 1.5 a
ANOVA *p* values	0.0554	0.2268	0.2591	0.2207	0.0012*	0.2206

Table 3. 5 : Wood composition analysis of *Salix miyabeana* 'SX67' plants under different irrigation treatments. Results represent the average values (mean ± standard error) for each irrigation treatment. Different letters in the same column group indicate significant differences according to HSD-Tukey test for the irrigation treatments (p≤0.05).

3.5 Discussion

We investigated the effect of treatment of a willow plantation with primary wastewater on wood properties and composition, revealing complex cell wall and stem hydraulic architecture alterations only partially similar to the well documented effects induced by high nitrogen fertilization.

3.5.1 Biomass yield

Fertigation with primary wastewater effluent containing high concentrations of nitrogen significantly increased above ground biomass by 57 and 120 % compared to the control UI and the PW-irrigated plants for which waterlogging conditions may have impact plant growth as was observed in young *Salix nigra* plants (Pezeshki, Anderson and Shields, 1998), yet another flood tolerant willow species. In previous trials in the same plantation (Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015), irrigation with different loads of secondary treated wastewater effluent also led to an increase in biomass production over two years of growth. The maximum yield of 18.2 Mg ha^{-1} yr^{-1} then recorded, corresponds to 63.2 % less production compared to the present study (28.8 Mg ha^{-1} yr^{-1} over a season of growth (Jerbi *et al.*, 2020)). No such difference between the two trials was observed on control trees. Thus, depending on the nature (i.e. treated or untreated municipal or industrial), wastewater load and composition (nutrient content, EC, pH) the effect of fertigation on wood biomass may lead to drastic differences in terms of yield, xylem tissue structure and wood chemical composition (Pitre *et al.*, 2007a) and therefore broaden the opportunities for the bioproduct sector.

3.5.2 Wood density and mechanical parameters

Ranging from 0.32 to 0.39 g cm^{-3}, wood density was slightly lower in the present study than what was reported by Tharakan et *al.* (2003) for a group of 30 non-fertilized willow clones (0.36 to 0.48 g cm^{-3}). This was especially true for WWD plants as could be expected as a result of N fertilization (Pitre *et al.*, 2007a; Curran *et al.*, 2008; Hacke *et al.*, 2010).

Although we did not investigate fiber-cells, the low wood density of WWD plants compared to UI and PW suggests a likely alteration of fiber properties (Pitre *et al.*, 2007a; Watanabe *et al.*, 2008; Hacke *et al.*, 2010; Zanne *et al.*, 2010). Indeed, wood density is related to the morphology of the cells within the secondary xylem tissue i.e. the vessels, the ray parenchyma and the fibers (Poorter *et al.*, 2010) with the latter having the thickest cell walls and thus contributing the most. High N fertilization was reported to reduce wood density by both increasing fiber lumen and decreasing fiber cell wall thickness (Luo *et al.*, 2005; Pitre, Cooke and Mackay, 2007; Pitre *et al.*, 2010; Poorter *et al.*, 2010). Thus, besides its consequence on vessel to fiber area and density ratio, irrigation with primary wastewater may have induced such alteration of fiber cells that renders the stems more flexible to leaning under gravitation and wind circumstances (compare Figure 3.1A-B to Figure 3.1C) and consequently more susceptible to the formation of tension wood. Indeed, similarly to the morphological alteration described by Pitre *et al.* (2007a, 2010) on poplar following N addition, we also observed stronger cellulose staining in the inner part of cell wall fibres of stem section of primary wastewater irrigated trees (Figure 3.1M-O), hence likely suggesting an increase of tension wood proportion within WWD treated plants as well.

3.5.3 Stem hydraulic parameters

Contrary to what was first hypothesised, no difference were observed between the different irrigation treatments regarding the average vessel diameter even if it should be impacted by soil water status (Hacke *et al.*, 2010; Plavcová and Hacke, 2012) and/or increased because of nitrogen fertilization (Bucci *et al.*, 2006; Hacke *et al.*, 2006, 2010) and thus reflected by major changes of the theoretical specific conductance K_S in accordance to the Hagen–Poiseuille law (Hacke *et al.*, 2017).

The control UI plants did not receive water other from precipitation (about 1200 mm over the two-year trial) but showed similar hydraulic parameters (i.e. average vessel diameter and K_S) as those of PW which received high loads of potable water i.e. 3687 mm over the same period (Jerbi *et al.*, 2020). Because water scarcity causes xylem to exhibit narrower but more frequent vessels, this may suggest that the water was not the limiting factor for willow growth and development as both treatments UI and PW showed very similar physiological parameters (Jerbi *et al.*, 2020) as well as comparable hydraulic parameters (Table 3.2). In fact, the region where the experiment was set up is considered as a humid continental climate with generally high precipitation that may be more advantageous for a hydraulic architecture granting efficiency of water conductance (widest vessel lumen with less resistance) rather than to a ‘safe’ architecture to avoid cavitation and embolism caused by a drought that is unlikely to occur (Kotowska *et al.*, 2015).

Intriguingly, the hydraulic conductance K_S was significantly lower for WWD plants than PW and UI albeit various studies reported that high N fertilisation increased the production of vessels with larger lumen (Harvey and van den Driessche, 1999; Hacke *et al.*, 2010, 2017). Regardless fertigation regime, the majority of vessels were found in the diameter groups 20-30 µm, 30-40 µm and 40-50 µm which represented ~ 86 % of the total vessel (Table 3.3) as expected for shrub species with a higher proportion of narrow vessels than trees, i.e. ≤ 50 µm and practically no wide vessels i.e. > 200 µm (Wheeler, Baas and Rodgers, 2007; Anfodillo, Petit and Crivellaro, 2013). In fact, for most of the diameter classes, the density of vessels was lower for WWD fertilized plants than UI and PW, especially for the groups 30-40 µm and 40-50 µm. These results could be somewhat explained by the sampling strategy we used. In an effort to compare stems at equivalent developmental stages (wood that already transitioned to secondary growth before the irrigation began), samples were harvested at different heights for each treatment even though stem conducts widen basipetally and that vessel lumen diameter increases axially from the top canopy towards the roots (Larson and Isebrands, 1971; Anfodillo, Petit and Crivellaro, 2013; Hacke *et al.*, 2017).

3.5.4 Wood composition analysis

Difference between WWD plants from one hand and UI and PW from the other shows that fertigation with N-rich wastewater decreased the extractives content of WWD biomass. It is known that for a given species the composition of extractives is affected by growth conditions (Yang and Jaakkola, 2011). Fast-growing shrubs such as willows are generally associated with low bark to wood ratio. Because most of the extractives are generally located in the bark (Sassner, Galbe and Zacchi, 2006; Serapiglia *et al.*, 2009) such difference is likely to be associated with the higher growth rate of WWD plants and their lower bark content.

Several studies on willow cultivars have reported a compensatory relationship between cellulose and lignin synthesis (Guidi *et al.*, 2009; Serapiglia *et al.*, 2009, 2013a; Ray *et al.*, 2012). Although, WWD induced an increase of glucan content, lignin was similar between all treatments and rather coherent to what was reported for other willow cultivars (Serapiglia *et al.*, 2009, 2013b; Ray *et al.*, 2012). Furthermore, in a study comparing several willow genotypes grown at 45 degrees (forced to bend) in order to induce the formation of reaction wood, Brereton *et al.* (2012) also reported that glucan content did increase for plants with the most reaction wood fraction while lignin remain unchanged. Hence, the similarity between the different irrigation treatment for total lignin may suggest that irrigation with primary wastewater and/or with potable water did not affect much lignin synthesis and deposition during secondary cell wall formation (i.e. lignification phase).

Alike, cellulose content (Table 3.5 and Figure 3.1P) was similar to what was reported for the same cultivar as well as for other genotypes (Sassner *et al.*, 2006; Serapiglia *et al.*, 2008, 2009, 2013a; Ray *et al.*, 2012). The glucan content did differ between UI, PW and WWD with the higher content for plants that received wastewater. High nitrogen application was previously reported to impact the development of secondary xylem of various poplar genotypes mostly by altering fiber anatomy through the deposition of an additional layer with high cellulose content in the inner part of the fiber cell lumen i.e. the G-layer (Pitre *et al.*, 2007a-b, 2010). Although quantitatively assaying of the tension wood proportion is difficult (Brereton *et al.*, 2011) on the basis of stem section image analysis, the strong correlation (Figure 3.3, $r^2 = 0.59$, p = 0.0001) between glucan content (based on chemical gravimetric analysis) and the proportion of tension wood (based on numerical image analysis) suggests that the increase of cellulose content is likely due to the increase of the proportion of tension wood in the case of WWD treated plants.

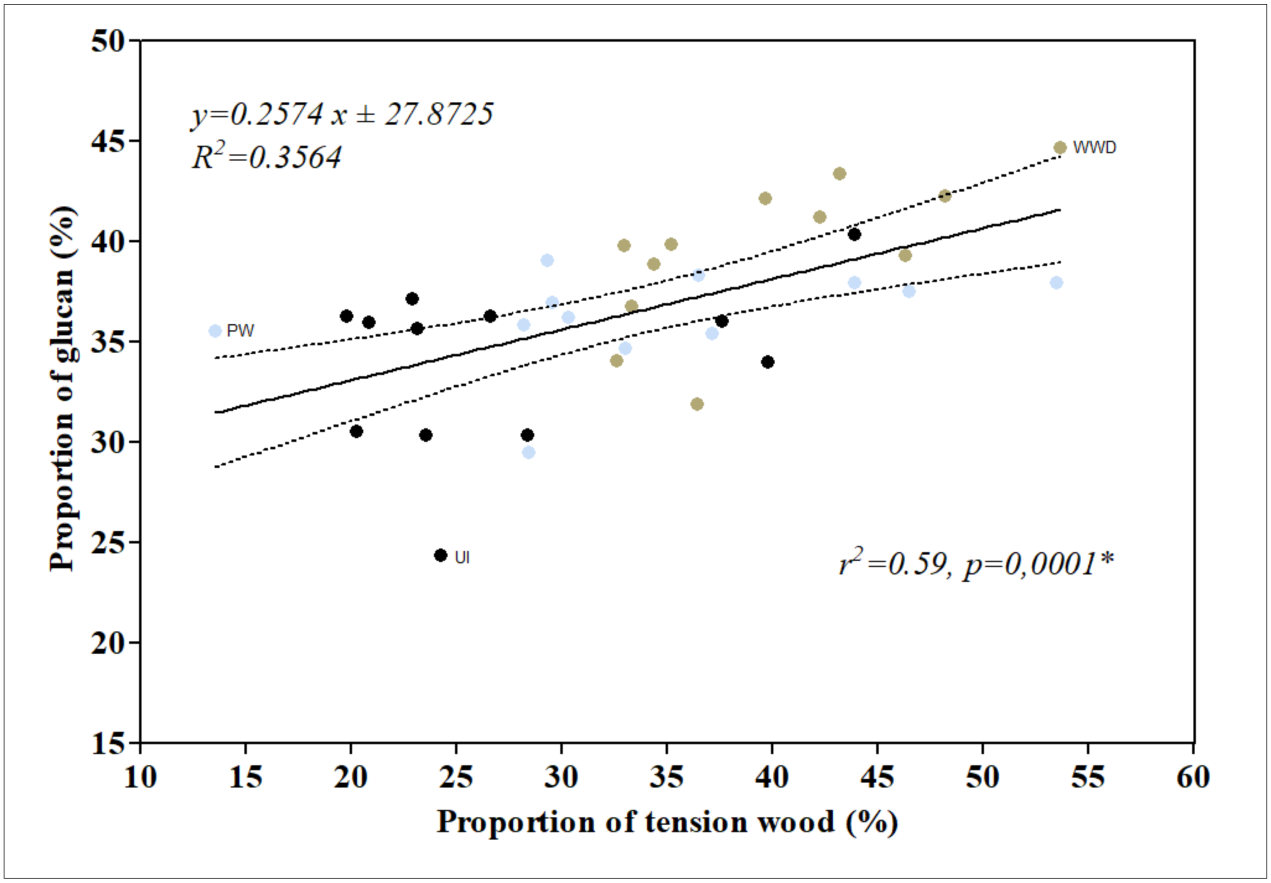

Figure 3. 3 : Linear regression between the tension wood proportion and the biomass glucan proportion of the cultivar *Salix miyabeana* 'SX67'. Black, blue and brown dots refers respectively to the data of UI, PW and WWD treatments.

3.6 Conclusions

Our findings reveal that the irrigation of willows with primary wastewater having high nitrogen load during two seasons of growth significantly altered wood chemical composition as well as cell wall structure. While fertigation increased the glucan content and the proportion of tension wood, it also resulted in the production of less dense wood with a significantly lower extractives fraction. This result may be of interest in the context of biofuel production and phytofiltration of municipal wastewater by SRWC. Consequently, it could provide the biofuel market with large amount of low cost-production raw material (i.e. biomass) with an increased content of glucan of higher energetic value for the conversion process (more biofuel produced per unit of biomass invested).

Credit authorship contribution statement

A. Jerbi: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **J. Laur**: Writing - review & editing. **K. Lajoie**: Investigation, Review & editing. **S. Barnabé**: Review & editing. **P.P. Gallant**: Software, Formal analysis, Review & editing. **M. Kalwahali Muissa**: Investigation, Review & editing. **M. Labrecque**: Writing - review & editing. **F.E. Pitre**: Writing - review & editing.

3.7 Acknowledgments

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3.8 Supplementary material

Accuracy and reliability of the analysis method

Since vessels are not strictly cylindrical, the calculation of the specific hydraulic conductivity K_S based on the Hagen-Poiseuille law may overestimate the hydraulic conductivity (Tyree and Zimmermann, 2002; Quintana-Pulido *et al.*, 2018). However, the theoretical specific conductivity K_S calculated on the stem sections of the 2-year-old cultivar *S. miyabeana* 'SX67' (present study) i.e. 10.79, 9.99 and 8.85 Kg m^{-1} Mpa^{-1} s^{-1} respectively for UI, PW and WWD, was very similar to the K_S measured in vivo by experimental instrument XYL'EM apparatus (Bronkhorst, Montigny-les-Cormeilles, France) on similar age shoot of *Salix psammophila* i.e. 10.17±0.17 Kg m^{-1} Mpa^{-1} s^{-1} (Li *et al.*, 2016 a; b). Thus, calculation of the theoretical hydraulic conductance based on the vessels density and size (mostly the diameter) on full stem section may be a reliable approach to estimate the hydraulic capacity of specific genotype and predict their efficiency within different environmental constraints while reducing investment in term of time and resources.

ImageJ plug-in and analyses instructions

Vessels measurement

Count the numbers of vessels and calculate the lumen area of each individual vessel within the stem section. The recognizing of vessel lumen from fiber and ray cell lumen is based on area, roundness and circularity criterion. For each high-resolution scan, the multichannel images were imported into ImageJ using the plugin Bio-Formats and the "Bio-Formats Importer" command. The multichannel images were firstly converted to single images using the “RGB Color” command and then to grayscale with the “8-bit” command. The images were then converted to pure black and white the "Auto Local Threshold". The number of vessels were assessed with the "Analyze Particles..." method using ranges of sizes and circularities respectively 250-1000 pixel and 0.1-1(unitless). The false positives were then excluded using an experimentally determined criterion of a minimal value 0.65 for the sum of a particle given roundness and circularity.

Pith and Stem measurement

The area of the stem section and the pith were measured using lower-resolution images. The images were treated using the same set of commands previously described to obtain pure black and white images. The pith was selected using the “Analyze Particles” with a minimum size of 30000 pixels (minimum area to be considered as pith). The whole stem was measured using the “doWand” method (ImageJ command).

Tension wood measurement

The amount of tension wood was measured by counting the black pixels in the monochromes images of the wood stained with the chlorazol black. Subsequent eroding and dilating of the images eliminated the areas that were less pixel-dense (corresponding to the safranine staining) while maintaining a similar superficies in the denser areas i.e. whose were stained with the chlorazol B.

Cells lumen measurement

The images were treated as previously described to obtain pure black and white images. The images were then smoothed using the “smooth” command and the local maxima were counted using the "Find Maxima..." command. The amount of all the cells (i.e. lumens) present within the stem section was measured by counting the local maximums in the images. The number of fibers and ray cell in the image was obtained by subtracting the number of vessels previously calculated from the number of total cells. More details about cell-counting by ‘’Maxima’’ are described in (Grishagin, 2015).

Image command

Measure Vessels density and area

- run("Bio-Formats Importer", "open=[" + input + "] color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + serie);
- run("RGB Color");
- run("8-bit");
- run("Auto Local Threshold", "method=Bernsen radius=25 parameter_1=0 parameter_2=0 white");
- run("Analyze Particles...", "size=" + size + " circularity=" + circ + " exclude");

Measure the area of Pith, stem section and tension wood

- run("Bio-Formats Importer", "open=[" + input + "] color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + serie);
- run("RGB Color");
- run("8-bit");

Measure the Pith area:

- run("Auto Threshold", "method=Default white")
- run("Analyze Particles...", "size=30000-Infinity display add exclude");

Measure the stem section area (including pith):

- run("Auto Threshold", "method=Default white");
- doWand(getResult("X", nResults-1),getResult("Y", nResults-1), 10, "Legacy");
- run("Measure");

Measure the tension wood area:

- run("Auto Threshold", "method=MaxEntropy white");
- run("Options...", "iterations=5 count=4 edm=8-bit do=Erode");
- run("Options...", "iterations=5 count=4 edm=8-bit do=Dilate");

Measure cells (counting)

- run("Bio-Formats Importer", "open=[" + input + "] color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + serie);
- run("RGB Color");
- run("8-bit");
- run("Smooth");
- run("Find Maxima...", "noise=10 output=Count");

Supplementary tables and figures

	Precipitation		Irrigation	
Year	2016	2017	2016	2017
Treatment	mm	mm	mm	mm
UI	390	769	0	0
NW	390	769	1510	2177
WWD	390	769	1160	1944

Table S3. 1 : Precipitation, water and wastewater loads during the year’s growth trial (2016 and 2017).

Parameters	Symbol	Nutrient loads through wastewater irrigation Kg ha^{-1}		
		2016	2017	2016-2017
Chemical oxygen demand	COD	2650	5642	8292
Total nitrogen	TN	370	818	1188
Total Kjeldahl nitrogen	TKN	370	816	1186
Nitrates and nitrites	NOx-N	0.46	1.17	2
Total Phosphorus	TP	37	79	116
Orthophosphates	o-PO4	19	52	71
Calcium	Ca	1647	1904	3552
Magnesium	Mg	476	554	1030
Potassium	K	151	196	347
Sodium	Na	4304	2827	7131
Sulfate	SO4	394	1596	1990
Chlorure	Cl	7285	3697	10982

Table S3. 2 : Nutrient loads through primary wastewater irrigation during the year’s growth trial (2016 and 2017).

Variable	**Acronyms**	**Formula**	**Units**	**Definition**	**References**
Vessel lumen fraction	F	$\bar{A}N$	mm^2 mm^{-2}	Fraction of the xylem occupied by the vessels lumen. Provides reliable indication of stem mechanical strength and hydraulic conductivity	(Zanne *et al.*, 2010; Scholz *et al.*, 2013)
Non-vessel lumen fraction	NF	$NF = 1 - F$	mm^2 mm^{-2}	Proportion of the xylem which is not vessel lumen (not accounting for other tissue lumen such as fibers and ray parenchyma cells)	(Zanne *et al.*, 2010; Scholz *et al.*, 2013)
Vessel composition index	S	$S = \frac{\bar{A}}{N}$	mm^4	Measures the variation in the vessel composition within the transport space (vessel lumen fraction 'F') either as a change in average vessel area or in the vessels density	(Zanne *et al.*, 2010)
Vessel vulnerability index	VI	$VI = \frac{D}{N}$	µm mm^{-2}	Index of plant capabilities to withstand water stress or freezing	(Scholz *et al.*, 2013)
Mean hydraulic diameter	D_H	$D_H = \left(\frac{\sum D^4}{N}\right)^{\frac{1}{4}}$	µm	Corresponds to the lumen diameter of average Hagen-Poiseuille conductivity of given stem section i.e. the mean diameter that all vessels would have to correspond to the overall conductivity for the same numbers of vessels	(Choat *et al.*, 2011; Scholz *et al.*, 2013; Hacke *et al.*, 2017)

Table S3. 3 : Vessels parameters measured and calculated with acronyms, units and main definition.

Treatment	Density of vessels with diameter D, where a µm < D <= b µm						
	$10 \le D < 20$	$20 \le D < 30$	$30 \le D < 40$	$40 \le D < 50$	$50 \le D < 60$	$60 \le D < 70$	$10 \le D < 70$ All vessels
UI	10 ± 0.7 a	39 ± 1.7 a	60 ± 2.5 b	46 ± 2.5 a	16 ± 1.5 a	0.8 ± 0.2 a	172 ± 4 a
PW	9 ± 0.4 a	37 ± 1.5 a	68 ± 1.7 a	48 ± 1.4 a	11 ± 1.0 a	0.4 ± 0.1 a	173 ± 3 a
WWD	9 ± 0.6 a	38 ± 1.9 a	57 ± 2.3 b	35 ± 2.6 a	13 ± 1.1 a	0.6 ± 0.1 a	152 ± 4 b
ANOVA *p* values	0.1698	0.6008	0.0113*	0.0974	0.0807	0.3769	0.0023*

Table S3. 4 : Vessel density ($N\ mm^{-2}$) per vessel lumen diameter (D) class. Results represent the average values (mean ± standard error) for each irrigation treatment. Different letters in the same diameter class group indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \le 0.05$).

Treatment	The theoretical sapwood area-specific hydraulic conductivity Ks per vessel lumen diameter (D) range, where a µm < D <= b µm						
	$10 \le D < 20$	$20 \le D < 30$	$30 \le D < 40$	$40 \le D < 50$	$50 \le D < 60$	$60 \le D < 70$	$10 \le D < 70$ All vessels
UI	0.03 ± 0.002 a	0.42 ± 0.02 a	2.4 ± 0.1 b	4.4 ± 0.2 a	3.3 ± 0.3 a	0.3 ± 0.05 a	10.8 ± 0.2 a
PW	0.03 ± 0.001 a	0.40 ± 0.02 a	2.7 ± 0.1 a	4.5 ± 0.1 a	2.2 ± 0.2 a	0.1 ± 0.02 a	10.0 ± 0.3 b
WWD	0.03 ± 0.002 a	0.42 ± 0.02 a	2.2 ± 0.1 b	3.4 ± 0.2 a	2.6 ± 0.2 a	0.2 ± 0.04 a	8.8 ± 0.4 c
ANOVA *p* values	0.1761	0.6715	0.0034*	0.1155	0.1064	0.3787	<.0001*

Table S3. 5 : Theoretical sapwood area-specific hydraulic conductivity K_S ($Kg.\ m^{-1}.pa^{-1}\ s^{-1}$) per vessel lumen diameter (D) class. Results represent the average values (mean ± standard error) for each irrigation treatment. Different letters in the same diameter class group indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \le 0.05$).

Treatment	Average vessel perimeter (µm)	Vessel vulnerability index (VI)	Vessel Lumen fraction (F) ($mm^2\ mm^{-2}$)	Non lumen fraction (NF) ($mm^2\ mm^{-2}$)
UI	155 ± 2.7 a	0.21 ± 0.006 b	0.19 ± 0.003 a	0.81 ± 0.003 b
PW	152 ± 4.4 a	0.21 ± 0.004 b	0.19 ± 0.004 a	0.81 ± 0.004 b
WWD	153 ± 2.0 a	0.24 ± 0.006 a	0.16 ± 0.005 b	0.84 ± 0.005 a
ANOVA *p* values	0.8726	0.0068*	0.0013*	0.0013*

Treatment	Vessel size to number ratio (S) (mm^4)	Mean hydraulic	$\sum D4$ ($m^4\ mm^{-2}$)	Hydraulic conductivity K_h ($Kg\ m\ Mpa^{-1}\ s^{-1}$)
UI	6.5E-06 ± 2.3E-07 ab	40.0 ± 0.33 a	4.4E-10 ± 9.4E-12 a	6.2E-04 ± 2.3E-05 a
PW	6.3E-06 ± 1.5E-07 b	39.1 ± 0.26 b	4.1E-10 ± 1.3E-11 b	5.7E-04 ± 2.4E-05 a
WWD	7.1E-06 ± 2.0E-07 a	39.2 ± 0.30 b	3.6E-10 ± 1.5E-11 c	5.3E-04 ± 2.3E-05 a
ANOVA *p* values	0.0252*	0.0152*	<.0001*	0.1206

Table S3. 6 : Vessels parameters of *Salix miyabeana* 'SX67' plants under different irrigation treatments. The results represent the average values (mean ± standard error) for each irrigation treatment. For each variable, different letters indicate significant differences according to HSD-Tukey test for the irrigation treatments ($p \le 0.05$).

3.9 Synthèse du chapitre 3

Ici les résultats ont mis en lumière les propriétés anatomiques et chimiques du bois du cultivar *Salix miyabeana* 'SX67' en réponse à l'irrigation par des eaux usées et/ ou par de l'eau potable. Pour ce qui est de la composition de la biomasse ligneuse, les résultats ont montré que l'irrigation par des eaux usées ont provoqué une diminution de la fraction des extractibles ainsi qu'une augmentation de la fraction cellulose, sans toutefois que ceci soit accompagné d'une diminution de la lignine. Nous avons aussi constaté une différence de la densité des vaisseaux et de leur diamètre moyen en comparaison de ce qui a été observé sur des plantes témoins et/ou irriguées par l'eau potable. Toutefois, il ne peut être exclu que les différences notées soient en partie conséquentes de la méthode d'échantillonnage (région de la plante où les échantillons ont été pris). En effet, en prélevant des échantillons de même taille (section de tige de même diamètre) sur des arbres fertigués et des arbres témoins et/ou irrigués par de l'eau potable, qui précisement avaient une différence dans le diamétre des tiges à une même hauteur donnée (constaté visuellement). Ce faisant, il est possible que nous avons échantillé des tiges qui avaient une différence dans l'âge cambiale, faussant ainsi la réalité de la réponse morphologique observée des cellules du xylème aux différents traitements qui justement; ne va pas dans le sens où il y aurait une augmentation de la conductance hydraulique pour les plantes fertilisées. Toutefois, l'exactitude de l'approche expérimentale innovatrice utilisée dans ce chapitre, et qui consiste à mesurer la densité et la taille des vaisseaux à partir des coupes au complet et non pas que sur quelques régions d'intérêts (ROI) pour mesurer la densité et la taille des vaisseaux et calculer les capacités hydrauliques, a été validée en se basant sur des données de littérature sur des espèces similaires (e.g. *Salix psammophila*) avec le même âge cambial (2 ans).

Chapitre 4 | Effet de l’irrigation par les eaux usées sur l’anotomie et la composition du bois des saules et la répercussion sur le rendement de la saccharification enzymatique

Dans le chapitre précédent, nous avons vu que l’irrigation par des eaux usées avait provoqué une diminution de la fraction des extractibles ainsi qu’une augmentation de la fraction cellulose. Dans la mesure où la composition chimique d’une biomasse donnée définie sa récalcitrance à la déconstruction enzymatique et ainsi son potentiel pour la production de biocarburant, il est alors important de savoir dans quelle mesure une variabilité dans la composition chimique du bois de saules en lien avec son appartenance variétale et/ou en en réponse à des stimuli environnementaux affecte l’accessibilité à l’hydrolyse enzymatique et la capacité à libérer les sucres.

Wood composition analysis and saccharification yield of willow cultivars irrigated with wastewater

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4.1 Abstract

Conventional municipal wastewater treatment strategies can have both a high financial and environmental cost, often leading to discharge of undertreated wastewater into the ecosystem. In parallel, the cultivation of short rotation coppice willows for bioenergy can be limited due to their high-water demand, providing the potential for wastewater treatment using willow plantation, or 'phytofiltration', for both environmental and economic benefits. However, the effect of wastewater irrigation on biomass development and biofuel potential has not been investigated in different cultivars and different sites. Here, the effect of wastewater irrigation on basic wood density, vessel and fiber cell transverse frequencies and average areas, biomass composition and enzymatic saccharification were investigated in three-year old trees from thirteen willow cultivars and one poplar cultivar planted at either one of three sites in Canada or Northern Ireland. Analysis of variance identified significant anatomical and compositional variation between cultivars in all three sites, but that wastewater irrigation had little impact on biomass development. Vessel and fiber cell frequencies ranged between cultivars from between 129-178 and 4322-7058 per mm^2, with average transverse areas of 838-1278 and 120-190 μm^2, respectively. Glucan and lignin content varied from 42.3-50.7 % dry matter (DM) and 27.3-32.4 % DM, respectively, where glucose release varied from 50-160 mg g^{-1} of glucan or 30-90 mg g^{-1} of dry matter. In addition to glucan content, fiber cell frequency had a significant positive correlation with glucose release yields, whereas average fiber cell area had an inverse correlation with glucose release yields. Taken together these findings suggest both cell wall structure and tissue morphology could be important factors, in addition to cell wall compositional components, when considering cell wall polysaccharide recalcitrance to deconstruction within a lignocellulosic bioenergy process. A maintenance of biomass quality during irrigation indicates that the integration of dedicated bioenergy production with environmental wastewater treatment using willow phytofiltration is potentially feasible and a promising clean biotechnology.

Keywords: Willow, wastewater treatment, phytofiltration, bioenergy, biofuels, bioproducts

4.2 Introduction

Development of carbon-neutral biofuels as a renewable energy alternative has the potential to reduce fossil fuel dependency and contribute to reducing climate change. Lignocellulosic bioenergy remains an attractive route for achieving these environmental targets, such as the 30-per-cent reduction in emissions levels by 2030 set out by the Paris Agreement aimed at promoting a transition to a low carbon economy. Short rotation coppice willow has the potential to be a dedicated bioenergy crop which can also benefit the environment (Keoleian and Volk, 2005; Rowe, Street and Taylor, 2009; Djomo, Kasmioui and Ceulemans, 2011) , as well as improving local wellness and health (Dzhambov and Dimitrova, 2015; Van den Berg *et al.*, 2015). The selection of willow cultivars with biomass composition that is better suited to the conversion process is important to the feasibility of sustainable bioenergy (Stephenson *et al.*, 2010). The composition of major plant cell wall polymer groups can vary considerably in mature (2-3 year old) willow biomass between different varieties; for example, Serapiglia, Cameron, *et al* (2013) and Ray *et al* (2012) reported very similar cellulose content (extractives free) ranges from between 38.4-45.3 % and 38.7-45.5 % of dry matter (DM) in willow plantations containing 30 and 35 willow varieties cultivated in the US and UK, respectively. These compositional differences, alongside evidence of variation in cell wall structure, can contribute to substantial variation in recalcitrance to enzymatic deconstruction and the efficiency of the conversation process.

Some studies have explored how wood anatomy is associated with net biomass composition and recalcitrance to enzymatic deconstruction, and particularly secondary xylem cell frequencies and morphologies, in mature short rotation coppice willow trees. Zhou *et al* (2017) found fiber diameters decreased while vessel area and lignin increased in three year old secondary xylem of Salix psammophila compared to earlier wood. Brereton *et al* (2012) found associations of reduced recalcitrance to enzymatic saccharification in mature willow with increased reaction wood, characterised by gelatinous fiber rich tension wood, in plantations exposed to high levels of wind in the Orkney islands compared to the same cultivars grown in relatively more sheltered plantations, but did not measure vessel or fiber frequency or size.

While cell wall recalcitrance to deconstruction is an important roadblock to sustainable bioenergy, cultivation of biomass is always a substantial environmental and economic burden to lignocellulosic bioenergy production (Heller, Keoleian and Volk, 2003; Hamelinck, Hooijdonk and Faaij, 2005; Gnansounou and Dauriat, 2010). One approach to reducing both this environmental and economic burden of cultivation of willow as a dedicated bioenergy crop is through added-value cultivation via ‘phytofiltration’, the treatment of municipal or industrial wastewater using a willow plantation (Grip, Halldin and Lindroth, 1989; Börjesson and Berndes, 2006; Mccracken and Johnston, 2015). Effective treatment of wastewater using a willow phytofiltration plantation could have the potential to alleviate environmental impact of conventional wastewater treatment while also serving as an added-value cultivation practice for improved sustainability of willow bioenergy.

Wastewater treatment is broadly categorised as either primary, where suspended solids and some organic matter are removed, secondary, where organic matter is degraded using biological treatment, or tertiary where remaining solids, nutrient and emerging contaminants are removed by a range of polishing steps. In Canada, an estimated 100-270 billion liters of municipal wastewater are released without any treatment into the environment, around 1.5 trillion liters receives primary treatment before release, around 2.8 trillion liters receives secondary treatment before release, 1.4 trillion liters receives tertiary treatment before release annually (Statistics Canada, 2017). Recent research conducted by Sas *et al* (2021) established bioenergy potential was maintained in a Salix miyabeana 'SX67' field trial when irrigated with primary effluent wastewater, in addition to effective treatment of the wastewater and substantial biomass yield increases (Lachapelle-T., Labrecque and Comeau, 2019; Jerbi *et al.*, 2020).

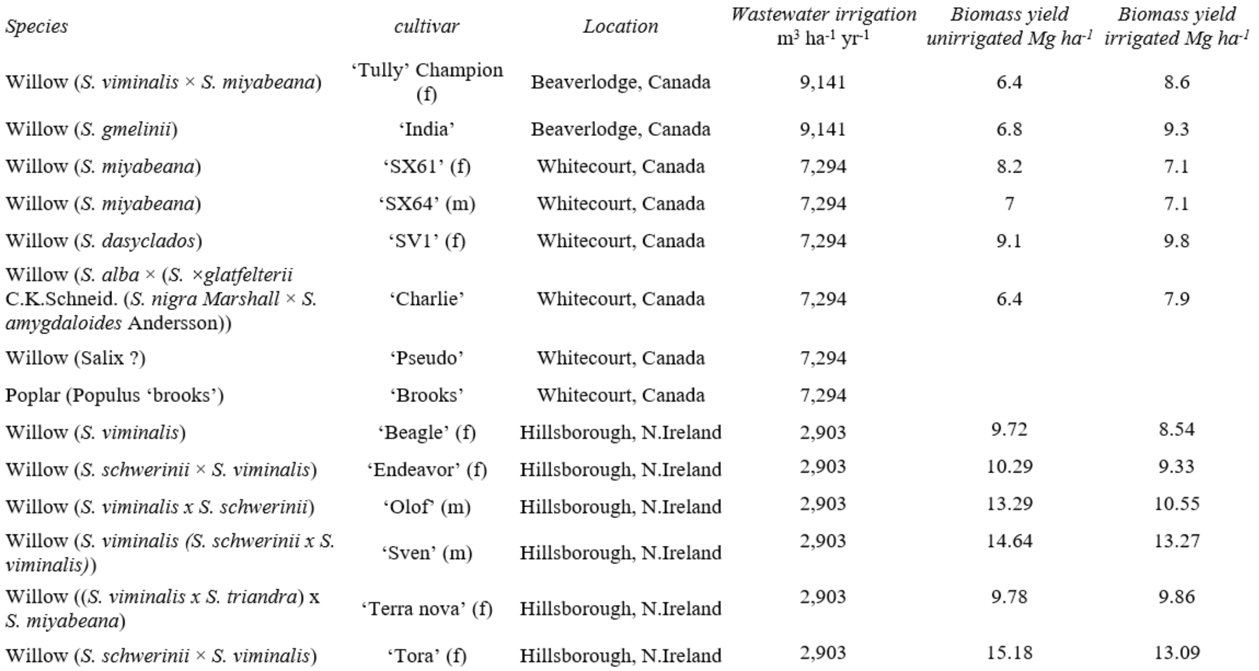
Although the potential for low-cost and environmental treatment of wastewater using willow plantations is promising, the impact of wastewater irrigation on willow wood development and potential for bioenergy has yet to be explored. The main goal of this research was to compare wood anatomy, biomass composition and recalcitrance to enzymatic deconstruction of several willow varieties cultivated in phytofiltration systems. The plant material used in the research comes from two experimental scale phytofiltration plantations in Canada and another one in Northern Ireland, i.e. with different cultivars, local conditions, plantation techniques and watering regimes. Setting up phytofiltration plantation comes with various technical hurdles and economical constraints that limit the number of species that can be assessed in a single study.

The present research is based on plant material collected from three distinct setups. Though the three experiments were conducted simultaneously in time (2012-2014), they were set independently with: (i) different species for each of the three sites (no replication); (ii) distinct geographical locations with contrasting temperature, seasonal precipitation and soil characteristics; (iii) distinctive loads and (iv) composition of effluents (wastewater with secondary treatment and primary dairy farm wastewater). Such variations between the three experiments limit the impact of any comparison between sites or the interpretation that can be made from the different parameters that were monitored but allows to look at a broad picture of the diversified willow genus.

4.3 Materials and methods

4.3.1 Study sites and plant material

The plantation sites were located in Beaverlodge and Whitecourt, Alberta, Canada, and Hillsborough, Northern Ireland. Environmental properties of each site: location, plantation design, temperature, precipitation, soil types are detailed in Nguyen, (2014) (Alberta sites) and Forbes *et al* (2017) (Hillsborough site). The properties of the wastewater are provided in the supplementary material. The genotypes cultivated at each site, wastewater irrigation loads yields and biomass yields are provided in Table 4.1.



Species	*cultivar*	*Location*	*Wastewater irrigation* $m^3\ ha^{-1}\ yr^{-1}$	*Biomass yield unirrigated Mg* ha^{-1}	*Biomass yield irrigated Mg* ha^{-1}
Willow (*S. viminalis* × *S. miyabeana*)	'Tully' Champion (f)	Beaverlodge, Canada	9,141	6.4	8.6
Willow (*S. gmelinii*)	'India'	Beaverlodge, Canada	9,141	6.8	9.3
Willow (*S. miyabeana*)	'SX61' (f)	Whitecourt, Canada	7,294	8.2	7.1
Willow (*S. miyabeana*)	'SX64' (m)	Whitecourt, Canada	7,294	7	7.1
Willow (*S. dasyclados*)	'SV1' (f)	Whitecourt, Canada	7,294	9.1	9.8
Willow (*S. alba* × (*S.* ×*glatfelterii* C.K.Schneid. (*S. nigra Marshall* × *S. amygdaloides* Andersson))	'Charlie'	Whitecourt, Canada	7,294	6.4	7.9
Willow (Salix ?)	'Pseudo'	Whitecourt, Canada	7,294		
Poplar (Populus 'brooks')	'Brooks'	Whitecourt, Canada	7,294		
Willow (*S. viminalis*)	'Beagle' (f)	Hillsborough, N.Ireland	2,903	9.72	8.54
Willow (*S. schwerinii* × *S. viminalis*)	'Endeavor' (f)	Hillsborough, N.Ireland	2,903	10.29	9.33
Willow (*S. viminalis x S. schwerinii*)	'Olof' (m)	Hillsborough, N.Ireland	2,903	13.29	10.55
Willow (*S. viminalis (S. schwerinii x S. viminalis)*)	'Sven' (m)	Hillsborough, N.Ireland	2,903	14.64	13.27
Willow ((*S. viminalis x S. triandra*) x *S. miyabeana*)	'Terra nova' (f)	Hillsborough, N.Ireland	2,903	9.78	9.86
Willow (*S. schwerinii* × *S. viminalis*)	'Tora' (f)	Hillsborough, N.Ireland	2,903	15.18	13.09

Table 4. 1 : Cultivar and plantation site. Biomass yield and wastewater irrigation rate are reported (further wastewater compositional details are available in supplementary material). Further site details are available in Nguyen (2014) (Alberta sites) and (Forbes *et al.*, 2017) Forbes et al (2017) (Hillsborough site). Cultivars names and pedigree are taken from Caslin, Finnan and McCracken (2012) and Kuzovkina (2015).

4.3.2 Plant sampling and biomass processing

The plants were harvested in the winter 2015 (following three years of growth i.e. 2012-2014) and the dry matter yields were calculated locally (in each site) from fresh plant weight and moisture content assessed using sub-samples taken from representative stems. The 112 harvested trees (14 cultivars x 2 treatments x 4 replicates) were air shipped immediately from the distinguish sites to our laboratory and allowed to air dry from June to December 2015. This large period of dry is essential to get moisture content below 10 %, since higher level may interfere with the sulfuric acid hydrolysis phase of the composition analysis protocol. Later, the whole trees were chipped to <7.5 cm using an Echo bear shredder model SC3306 and ground to between 180-850 µm using a Wiley mill.

4.3.3 Basic density, sectioning and histology

Two stem samples of 3-4 cm length were collected from the middle of a typical canopy stem of each of the 112 harvested trees and fixed in a FAA solution (3.7% formaldehyde, 5% acetic acid and 47.5% ethanol (Brereton *et al.*, 2015)). One stem sample was used for basic density measurements and the other for sectioning and histology. Basic density was measured by water displacement after vacuum infiltration with water, calculated as green volume by oven-dry weight at 105°C (Williamson and Wiemann, 2010). Sectioning was performed using a sledge microtome to 30-40 µm thickness. Sections were then stained with 1% aqueous Safranin O and 1% Chlorazol Black E in methoxyethanol (Brereton *et al.*, 2015). Sections were permanently mounted on glass slides with DPX medium (Brereton *et al.*, 2011, 2012).

4.3.4 Image acquisition and analysis

All 112 slides were scanned digitally using a linear whole slide scanner (Aperio ScanScope CS2, Leica, Germany) at 40x objective magnification. Raw image data were stored in Aperio SVS file format, a multi-layered compressed JPEG (further information on the image format can be found in Shawki *et al.*, 2020). The slide images varied in size from 0.2 to 0.4 Gb and were first viewed using Leica Aperio Imagescope digital slide viewer version 12 (Leica Biosystems, Aperio) to examine each entire slide for any potential problems that could interfere with analysis, e.g. air/dust spots, partial staining, missing xylem parts. The SVS image slides were then analyzed by a script developed in-house in Fiji image-processing software (www.fiji.sc, ImageJ, (Schindelin *et al.*, 2012). More details on the script and the image analysis methods are presented in the supplementary material. The Fiji script was run on a super-computing platform so that the SVS file could be opened as a big tiff file (approximately 20 Gigabytes) and the whole image analyzed in smaller fragments to examine the vessels and fibers density and area. The technique for distinguishing vessel lumen from the lumen of other xylem cells, i.e. fiber and parenchyma ray, was first tested experimentally by assessing a threshold of the area, the circularity and the roundness. Nevertheless, the lumen of non-vessels cells (fiber and parenchyma cells) was too low beyond the system threshold detection. The average fiber cell area was calculated by dividing the area outside vessel lumens (the non-vessel lumen fraction) by the number of non-vessels cells (fiber + ray parenchyma cells). Thus, the average fiber cell area mostly refer to the area occupied by the fiber lumen as well as the fiber cell wall, rather than the area of fiber lumen per-se. Average vessel area is generally reported separately for each of these ring types (Zanne *et al.*, 2010), however, we did not separate vessels from springwood and summer wood growth rings due to the technical difficulty of distinguishing between them among different growth rings.

4.3.5 Composition analysis

Prior to compositional analysis 5 g of ODW milled and sieved biomass was extracted with 95% ethanol according to the NREL protocol (Sluiter *et al.*, 2008), using a Dionex® Accelerated Solvent Extractor (ASE150) (100°C under 100 bar pressure and 3 x 5 min static cycles). The extracted biomass was then analysed for structural carbohydrates and lignin in accordance with the NREL protocol (Sluiter *et al.*, 2012). All sugars were quantified by high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) with a Bio-Rad Aminex HPX-87H column and refractive index detector. The HPLC data was anhydro corrected for the contribution of water to the molecular weight of sugars in monomer form compared to the polysaccharide form (sugars are termed glucan, xylan, arabinan, galactan and mannan when presented as a compositional proportion in polymeric form, however, this should not be mistaken with homogenous polysaccharides).

4.3.6 Saccharification analysis

The enzymatic hydrolysis was performed on 112 samples, 4 tree replicates of 14 cultivars and 2 treatments (control or wastewater irrigated) in accordance with the NREL protocol (Selig, Weiss and Ji, 2008) with minor modifications (Brereton *et al.*, 2011). Briefly, fresh biomass (20-80 mesh) corresponding to 100 mg of glucan DM was incubated with 5000 µL of 100 mM sodium citrate buffer (pH 4.8), 100 µg of 5% sodium azide (50 mg ml^{-1} MilliQ water), 100 µl of cellulase enzyme suite and demineralised water to bring the total reaction volume to 10 mL. The cellulase suite was Accellerase DUET® (DuPont Industrial Biosciences 2010), with specific activity of 2400-3000 endoglucanase CMC U/g (corresponding to 30FPU/g) oven-dry biomass, >400 pNPGU/g oven-dry biomass of β-glucosidase and >3600 ABX U/g oven-dry biomass of xylanase. For each of 112 samples, 3 technical replicates and 1 sample (no enzyme) control were incubated for 7 days at 50°C in a shaking rotary incubator (total of 448 reactions) along with enzyme blanks. Following incubation, samples were filtered (0.45 μm, nylon membrane, Whatman) and centrifuged prior to glucose concentration determination by HPLC using a Bio-Rad Aminex HPX-87H column and refractive index detector.

4.3.7 Statistics

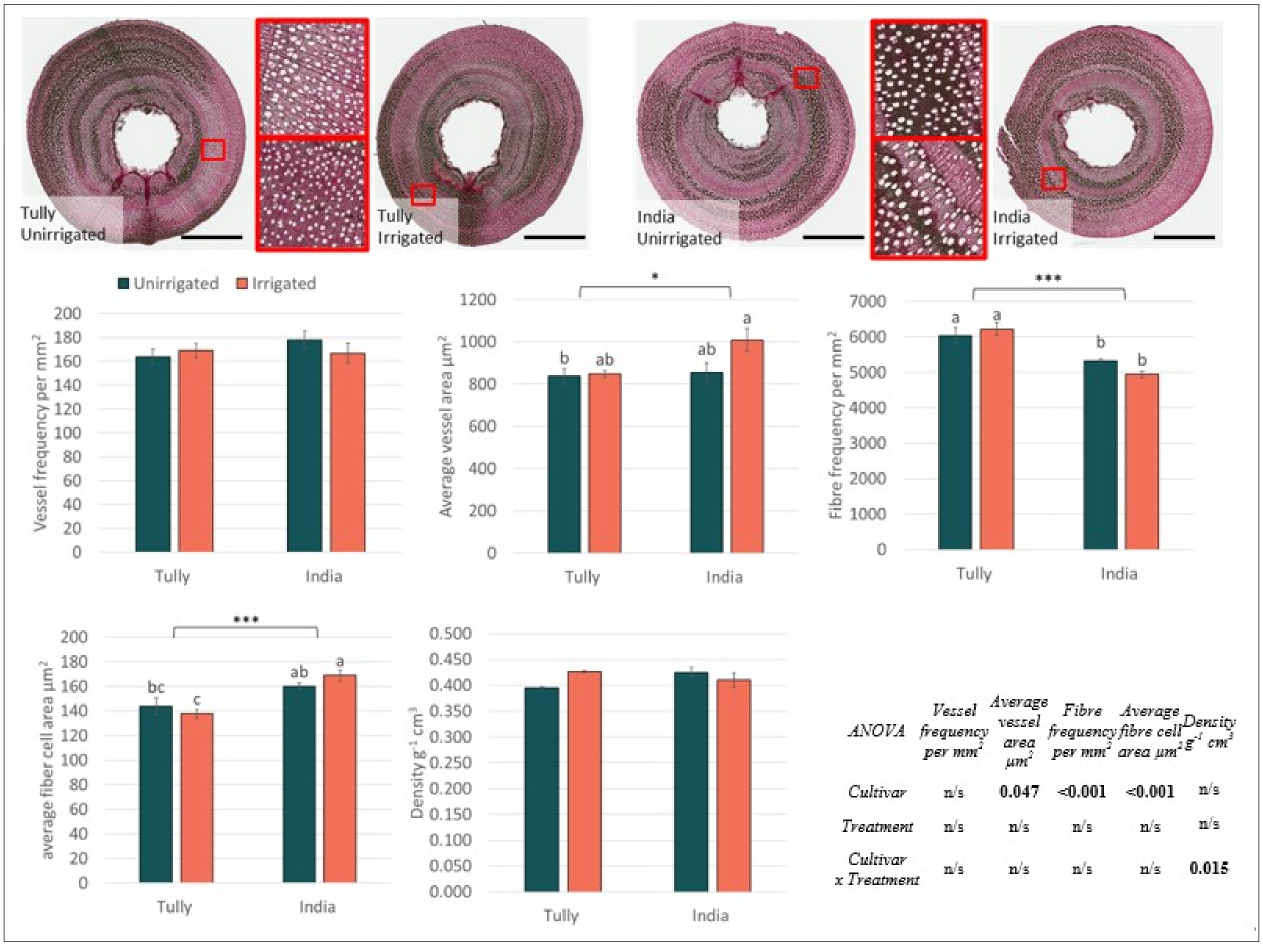
Significant difference using two-way ANOVA used $p < 0.05$. Pair-wise comparisons were conducted using Tukey's Honestly Significant Difference (HSD) post hoc test ($\alpha = 0.05$). Pearson correlations were performed using all 112 trees in order to have a general pattern portrait of associations within/ and between biochemical and anatomical variables. Statistical analyses was conducted using JMP version 11 (SAS Institute Inc, 2014).

4.4 Results

4.4.1 Beaverlodge site

4.4.1.1 Biomass density and wood anatomy

The willow plantation in Beaverlodge, Alberta, Canada, included the cultivars 'Tully' and 'India' (Table 4.1). Biomass yields were higher with wastewater irrigated trees, increasing from 6.4 to 8.6 Mg ha^{-1} for 'Tully' and from 6.8 to 9.3 Mg ha^{-1} for 'India'. Two-way analysis of variance in basic wood density and secondary xylem tissue parameters revealed significant differences ($p < 0.05$) between cultivars in average vessel area, fiber frequency and average fiber area, but no significant differences in none of the xylem parameters and the basic wood density due to wastewater irrigation, although a significant interaction between cultivar x irrigation treatment was detected in basic wood density (Figure 4.1). Post-hoc analysis using Tukey's test (HSD; $\alpha < 0.05$) indicated transverse fiber frequency was significantly higher in 'Tully' compared to 'India', at an average of 6130 (± 134) and 5128 (± 89) fibers per mm^2, respectively, while the average fiber area was significantly lower in trees of 'Tully' compared to 'India', at an average of 141 (± 4) and 164 (± 3) μm^2. Average vessel area was significantly higher in 'India' when wastewater irrigated, at 1010 (± 53) μm^2, than in 'Tully' controls, at 838 (± 36) μm^2. The vessel frequency and the basic wood density did not significantly vary between cultivars or treatment and ranged respectively from 164-178 vessels per mm^2 and 0.395-0.427 g cm^{-3} (Figure 4.1).



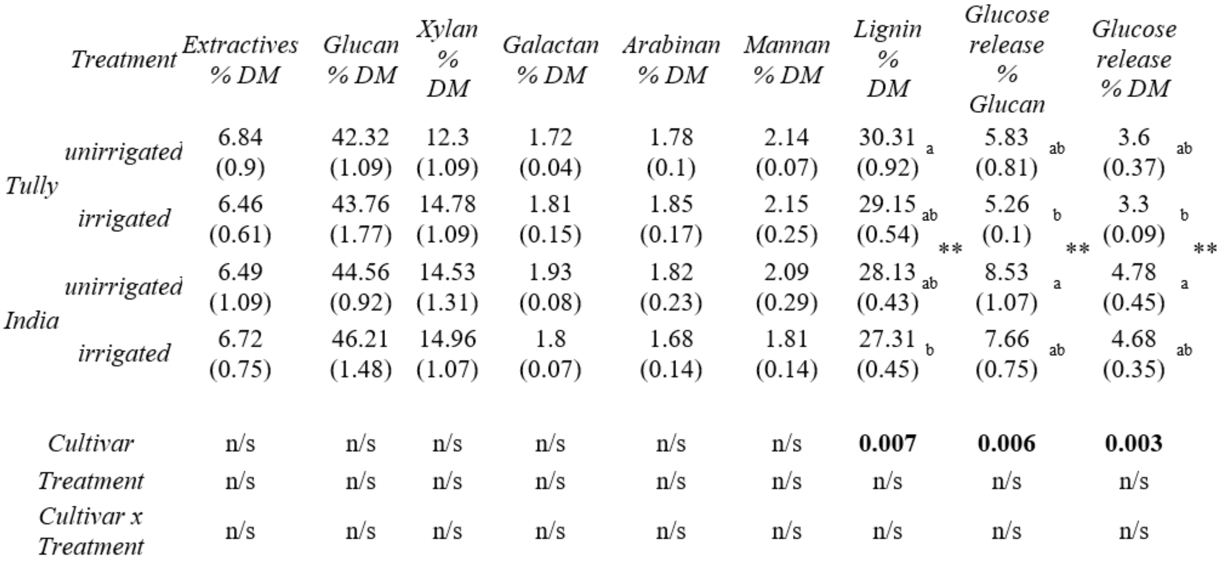
ANOVA	*Vessel frequency per mm^2*	*Average vessel area µm^2*	*Fibre frequency per mm^2*	*Average fibre cell area µm^2*	*Density g^{-1} cm^3*
Cultivar	n/s	**0.047**	**<0.001**	**<0.001**	n/s
Treatment	n/s	n/s	n/s	n/s	n/s
Cultivar x Treatment	n/s	n/s	n/s	n/s	**0.015**

Figure 4. 1 : Secondary xylem cell and measured tissue morphology parameters at the Beaverlodge site. Transverse sections of 30-40 µm taken from variable heights at a diameter of ~1 cm, scale bar = 2.5mm. Representative micrographs are each composed from >100 x40 high-res images (see example red box) allowing tissue analysis. Vessel frequency (mm^2), average vessel area (µm^2), fiber frequency (mm^2), average fiber area (µm^2) and basic wood density g cm^{-3} are shown using four replicate field-grown unirrigated and wastewater irrigated willow trees. T-tests are used to illustrate significant difference between cultivars using * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). Letters denote Tukey's HSD, α = 0.05. ANOVA values are shown with significance ($p < 0.05$) indicated in bold.

4.4.1.2 Biomass composition and enzymatic saccharification

The assessment of biomass composition and enzymatic saccharification revealed significant variation (ANOVA, $p < 0.05$) between cultivars in total lignin content and glucose release yields but no significant variation between cultivars in glucan content or any hemicellulose monomer content. No significant variation was detected in any of the parameters due to wastewater irrigation (Table 4.2). The extractives content of trees from both Beaverlodge cultivars were similar and ranged from 6.4-6.8 % dry matter (DM), as was glucan content which ranged from 42.3%-46.2 %

DM and hemicellulose content which ranged 17.9-20.6 % DM. The hemicellulose constituent monomers ranged from 12.3-15.0 xylose % DM, 1.7-1.9 galactose % DM, 1.7-1.9 arabinose % DM and 1.8-2.2 mannose % DM. Lignin content was significantly higher in 'Tully' compared to 'India', and unirrigated 'Tully' had the highest lignin content of 30.3 (± 0.9) % DM while wastewater irrigated 'India' had the lowest lignin content of 27.3 (± 0.5) % DM. Glucose release from enzymatic saccharification yields can be expressed as a proportion of the initial glucan present in the biomass, which is indicative of glucose accessibly independent of glucan content, or as a proportion of total DM, which includes variation in glucan content as substrate. Enzymatic saccharification revealed significantly higher glucose release yields in 'India' compared to 'Tully', with the highest yields of 85 (± 11) mg g^{-1} glucan and 48 (± 5) mg g^{-1} DM for control trees of 'India' and the lowest yields of 53 (± 10) mg g^{-1} glucan and 33 (± 9) mg g^{-1} DM for wastewater irrigated trees of 'Tully' (Table 4.2).



	Treatment	*Extractives % DM*	*Glucan % DM*	*Xylan % DM*	*Galactan % DM*	*Arabinan % DM*	*Mannan % DM*	*Lignin % DM*	*Glucose release % Glucan*	*Glucose release % DM*
Tully	*unirrigated*	6.84 (0.9)	42.32 (1.09)	12.3 (1.09)	1.72 (0.04)	1.78 (0.1)	2.14 (0.07)	30.31 ^{a} (0.92)	5.83 ^{ab} (0.81)	3.6 ^{ab} (0.37)
	irrigated	6.46 (0.61)	43.76 (1.77)	14.78 (1.09)	1.81 (0.15)	1.85 (0.17)	2.15 (0.25)	29.15 ^{ab} (0.54) **	5.26 ^{b} (0.1) **	3.3 ^{b} (0.09) **
India	*unirrigated*	6.49 (1.09)	44.56 (0.92)	14.53 (1.31)	1.93 (0.08)	1.82 (0.23)	2.09 (0.29)	28.13 ^{ab} (0.43)	8.53 ^{a} (1.07)	4.78 ^{a} (0.45)
	irrigated	6.72 (0.75)	46.21 (1.48)	14.96 (1.07)	1.8 (0.07)	1.68 (0.14)	1.81 (0.14)	27.31 ^{b} (0.45)	7.66 ^{ab} (0.75)	4.68 ^{ab} (0.35)
Cultivar		n/s	n/s	n/s	n/s	n/s	n/s	**0.007**	**0.006**	**0.003**
Treatment		n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Cultivar x Treatment		n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s

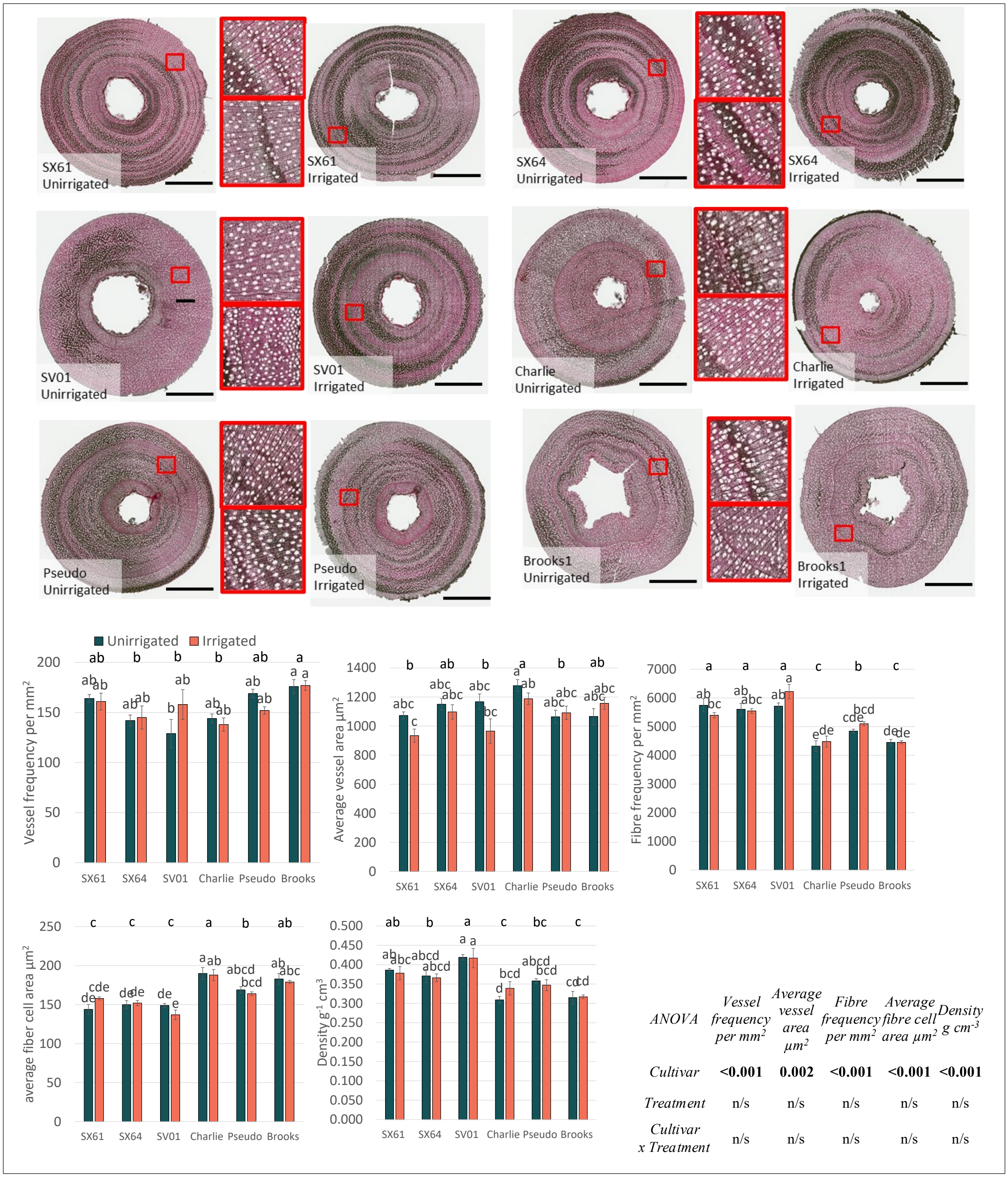
Table 4. 2 : Biomass composition and enzymatic saccharification at the Beaverlodge site. Letters denote Tukey's HSD, α = 0.05. ANOVA values are shown with significance ($p < 0.05$) indicated in bold.

4.4.2 Whitecourt site

4.4.2.1 Biomass density and wood anatomy

The plantation in Whitecourt, Alberta, Canada, included the willow cultivars 'SX61', 'SX64', 'SV1', 'Charlie' and 'Pseudo' as well as the poplar cultivar 'Brooks'. Biomass yields varied from between 7 and 9.8 Mg ha^{-1} (Table 4.1), although biomass yields for the cultivars 'Charlie' and 'Pseudo' were not reported due to relatively poor performance. Two-way analysis of variance of secondary xylem tissue parameters revealed significant differences ($p < 0.05$) between cultivars in all measured parameters: vessel frequency, average vessel area, fiber frequency, average fiber area and basic wood density (Figure 4.2), but no significant treatment or cultivar x treatment interaction effects.

Post-hoc analysis using Tukey's test (HSD; $\alpha < 0.05$) revealed that the highest vessel frequency was observed in the poplar cultivar 'Brooks', regardless of treatment, while the lowest was the unirrigated willow cultivar 'SV1', at 177 (± 4) and 129 (± 14) vessels per mm^2 respectively (Figure 4.2). Average transverse vessel area was 1233 (± 31) μm^2 in the cultivar 'Charlie', significantly higher than 1003 (± 36) μm^2 in 'SX61', 1066 (± 60) μm^2 in 'SV1' and 1078 (± 30) μm^2 in 'Pseudo'. Fiber frequency was significantly higher in all three cultivars 'SX61', 'SX64' and 'SV1', ranging from 5571-5970 fibers per mm^2, than the cultivars 'Charlie', 'Pseudo' and 'Brooks' which ranged from 4402-4973 fibers per mm^2. An inverse pattern was observed with the average transverse fiber areas of cultivars 'SX61', 'SX64' and 'SV1', ranging from 143-151 μm^2, significantly lower than those of 'Charlie', 'Pseudo' and 'Brooks', ranging from 166-189 μm^2, respectively. The lowest basic wood density was 0.309 (± 0.009) g cm^{-3} and 0.315 g cm^{-3} for unirrigated 'Charlie' and 'Brooks', respectively, while the highest density was 0.417 (± 0.025) and 0.419 (± 0.007) g cm^{-3} for irrigated and unirrigated 'SV1', respectively (Figure 4.2).



ANOVA	*Vessel frequency per mm²*	*Average vessel area µm²*	*Fibre frequency per mm²*	*Average fibre cell area µm²*	*Density g cm⁻³*
Cultivar	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**
Treatment	n/s	n/s	n/s	n/s	n/s
Cultivar x Treatment	n/s	n/s	n/s	n/s	n/s

Figure 4. 2 : Secondary xylem cell and tissue morphology at the Whitecourt site. Transverse sections of 30-40 µm taken from variable heights at a diameter of ~1 cm, scale bar = 2.5mm. Representative micrographs are each composed from >100 x40 high-res images (see example red box) allowing tissue analysis. Vessel frequency (mm^2), average vessel area (µm^2), fiber frequency (mm^2), average fiber area (µm^2) and basic wood density g cm^{-3} are shown using four replicate field-grown unirrigated and wastewater irrigated willow trees for each cultivar. Letters denote Tukey's HSD, α = 0.05. ANOVA values are shown with significance ($p < 0.05$) indicated in bold.

4.4.2.2 Biomass composition and enzymatic saccharification

Analysis of variance of biomass composition and enzymatic saccharification parameters identified significant variation between cultivars at the Whitecourt site for all parameters with the exception of xylan content. Significant variation was detected in extractives content and enzymatic saccharification yields due to wastewater treatment, and a significant cultivar x wastewater interaction effect was observed for extractives content, arabinose content and enzymatic saccharification yields (Table 4.3). Tukey’s post hoc analysis indicated extractives content was significantly higher in trees of the cultivars ‘SX61’, ‘SX64’, ‘Pseudo’ and ‘Brooks’, ranging between 4.1-5.2 % DM, than trees of the lower ‘SV1’ and ‘Charlie’, which had extractives ranging from 3.2-3.9 % DM. Glucan and lignin content were similar between all cultivars, ranging between 28.7-29.7 lignin % DM and 46.4-48.9 glucan % DM, with the exception of ‘Charlie’, which had significantly higher lignin content of 32.4 (± 0.2) and 31.6 (± 0.5) % DM and a significantly lower glucan content of 42.5 (±0.06) and 45.6 (± 0.8) % DM than all cultivars for unirrigated and wastewater irrigated trees, respectively. Total xylose content did not vary significantly between cultivars, ranging from 14.2-16.6 % DM, but significant differences were identified between cultivars in galactan, arabinan and mannan contents. ‘Brooks’ had the lower galactan content of 1.2 (±0.1) % DM in unirrigated trees while ‘SV1’ and ‘Charlie’ had significantly higher galactan, which ranged from 1.6-1.7 % DM. ‘SX61’, ‘SX64’ and ‘SV1’ had significantly higher arabinose content of 1.3-1.7 % DM compared to ‘Pseudo’ and ‘Brooks’, which ranged from 1.0-1.2 arabinan % DM. ‘Brooks’ had the highest mannan content of 2.7 (± 0.2) and 2.9 (± 0.2) % DM, while ‘Pseudo’ had the lowest mannan content of 1.4 (± 0.1) and 1.3 (± 0.2) % DM for unirrigated and wastewater irrigated trees respectively (Table 4.3).

	Treatment	Extractives % DM	Glucan % DM	Xylan % DM	Galactan % DM	Arabinan % DM	Mannan % DM	Lignin % DM	Glucose release % Glucan	Glucose release % DM
SX61		a	a		ab	a	b	b	c	c
	unirrigated	5.03 (0.18) ab	48.7 (0.44) a	14.54 (1.08)	1.58 (0.1) ab	1.42 (0.07) abc	2.22 (0.12) ab	28.99 (0.23) c	7.34 (0.36) bc	4.45 (0.14) cd
	irrigated	4.23 (0.13) abcd	47.42 (0.88) a	14.34 (0.32)	1.57 (0.05) ab	1.43 (0.1) abc	1.85 (0.09) bc	29.38 (0.55) c	7.51 (0.29) bc	4.35 (0.20) cd
SX64		a	a		ab	a	bc	b	bc	bc
	unirrigated	4.60 (0.45) ab	47.81 (1.39) a	14.23 (0.95)	1.58 (0.06) ab	1.69 (0.16) a	1.94 (0.06) bc	29.48 (0.08) c	7.70 (0.39) bc	4.61 (0.26) c
	irrigated	4.05 (0.37) bcd	46.98 (0.49) a	16.13 (1.83)	1.62 (0.11) ab	1.21 (0.15) bc	1.5 (0.21) bc	29.92 (0.44) bc	9.76 (0.6) ab	5.69 (0.29) abc
SV1		b	a		a	a	bc	b	a	a
	unirrigated	3.19 (0.39) d	47.57 (1.05) a	16.66 (0.57)	1.74 (0.03) a	1.3 (0.08) abc	1.88 (0.04) bc	28.97 (0.63) c	11.1 (0.35) a	6.23 (0.25) ab
	irrigated	3.89 (0.17) bcd	48.1 (1.01) a	15.24 (1.2)	1.71 (0.05) ab	1.46 (0.09) ab	1.76 (0.23) bc	28.67 (0.34) c	11.06 (0.33) a	6.43 (0.08) a
Charlie		b	b		a	ab	c	a	b	bc
	unirrigated	3.34 (0.16) d	42.54 (0.63) b	14.28 (0.87)	1.64 (0.08) ab	1.22 (0.05) abc	1.42 (0.07) c	32.36 (0.21) a	9.59 (0.68) ab	4.94 (0.32) bc
	irrigated	3.38 (0.06) cd	45.58 (0.79) ab	16.01 (1.65)	1.77 (0.15) a	1.15 (0.09) bc	1.62 (0.18) bc	31.59 (0.45) ab	8.66 (0.85) ab	4.94 (0.48) bc
Pseudo		a	a		ab	b	c	b	bc	b
	unirrigated	5.22 (0.07) a	46.82 (1.14) a	15.03 (0.3)	1.66 (0.07) ab	1.22 (0.09) abc	1.44 (0.03) c	29.74 (0.61) bc	7.33 (0.68) bc	4.39 (0.39) cd
	irrigated	4.10 (0.13) abcd	48.87 (0.63) a	16.25 (1.14)	1.39 (0.17) ab	0.96 (0.09) c	1.32 (0.21) c	28.95 (0.4) c	10.34 (0.51) a	6.19 (0.25) ab
Brooks		a	a		b	b	a	b	d	d
	unirrigated	4.51 (0.08) abc	46.38 (0.31) ab	15.11 (1.13)	1.24 (0.14) b	1.04 (0.01) bc	2.73 (0.2) a	29.36 (0.1) c	5.37 (0.35) c	3.1 (0.19) d
	irrigated	4.27 (0.16) abcd	46.95 (0.46) a	15.99 (0.54)	1.53 (0.1) ab	1.07 (0.08) bc	2.92 (0.19) a	28.72 (0.25) c	5.55 (0.46) c	3.21 (0.26) d
Cultivar		**<0.001**	**<0.001**	n/s	**0.021**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Treatment		**0.020**	n/s	n/s	n/s	n/s	n/s	n/s	**0.017**	**0.003**
Cultivar x Treatment		**0.006**	n/s	n/s	n/s	**0.026**	n/s	n/s	**0.005**	**0.008**

Table 4. 3 : Biomass composition and enzymatic saccharification at the Whitecourt site. Letters denote Tukey's HSD, α = 0.05. ANOVA values are shown with significance ($p < 0.05$) indicated in bold.

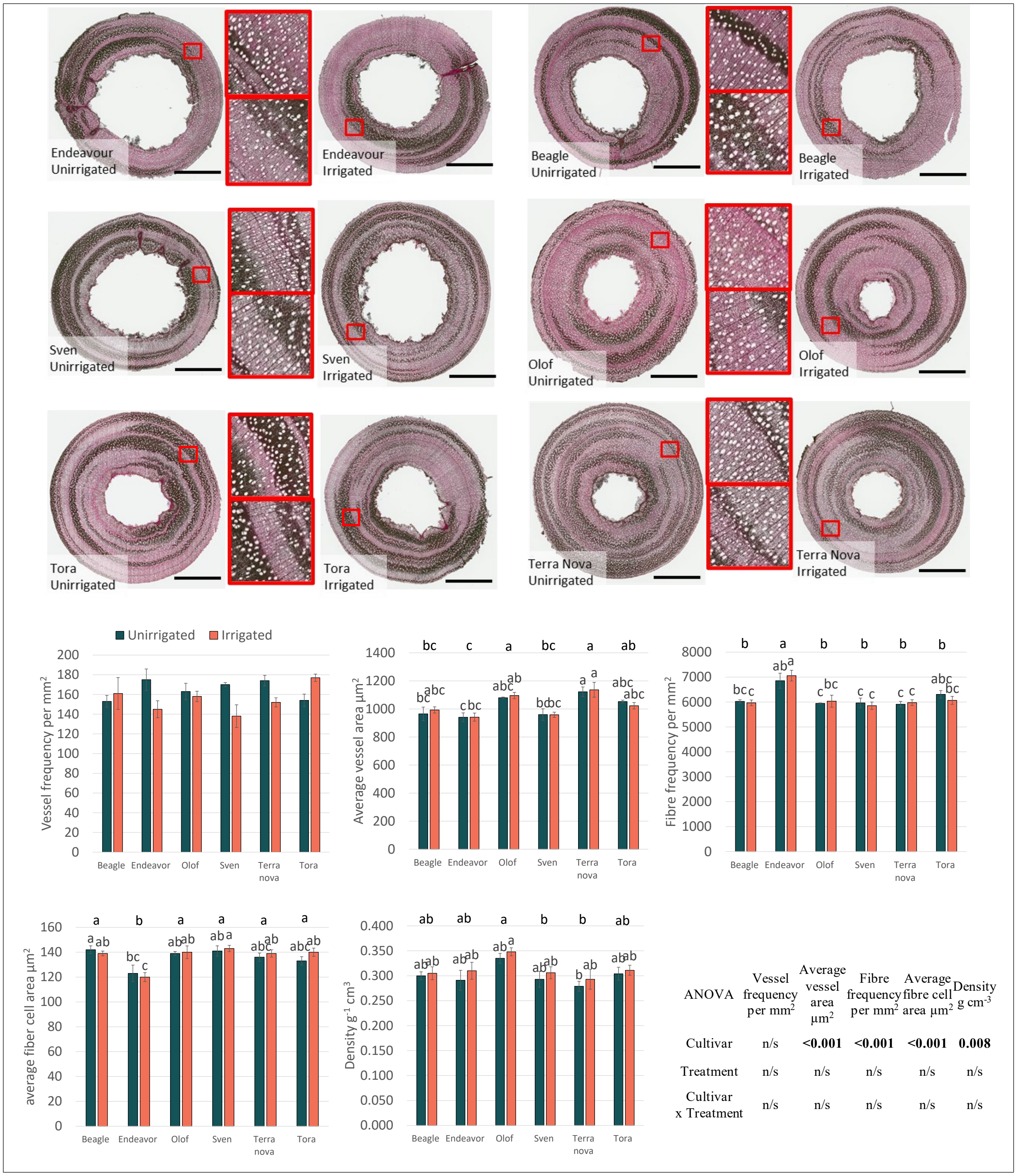
Glucose release varied substantially and significantly between cultivars, with 'SV1' the highest yielding cultivar at 111 mg g^{-1} glucan for both unirrigated and irrigated trees, while 'Brooks' had the lowest yields of 54 (4) and 56 (5) mg g^{-1} glucan for unirrigated and irrigated trees, respectively (Table 3). All cultivars had similar or slightly higher glucose release yields when irrigated with wastewater with the exceptions of the cultivars 'SX64' and 'Pseudo', which respectively increased substantially from 77 (± 4) to 98 (±6) mg g^{-1} glucan and from 73 (± 7) to 103 (±5) mg g^{-1} glucan (Table 4.3).

4.4.3 Hillsborough site

4.4.3.1 Biomass density and wood anatomy

The willow plantation in Hillsborough, Northern Ireland, included the cultivars 'Beagle', 'Endeavour', 'Olof', 'Sven', 'Terra Nova' and 'Tora'. The biomass yields ranged from 9.7 to 15.2

Mg ha^{-1} yr^{-1} respectively for ‘Beagle’ and ‘Tora’ (Table 4.1) and there was a general trend of reduced yield due to wastewater irrigation which was highest at 20% in the cultivar ‘Olof’. Two-way analysis of variance revealed significant differences ($p < 0.05$) between cultivars in the measured parameters of transverse average vessel area, fiber frequency, average fiber area and basic wood density, but not vessel frequency, whereas no significant effect was detected due to wastewater irrigation or interaction between cultivar x irrigation treatment (Figure 4.3). Tukey’s post-hoc analysis test revealed average transverse vessel area was highest in the cultivars ‘Olof’ and ‘Terra Nova’, ranging from 1137-1080 µm^2, and was lowest in ‘Endeavor’ which was 941 (± 31) and 942 (± 28) µm^2, in unirrigated and wastewater irrigated trees, respectively. Fiber frequency was significantly higher in ‘Endeavour’, at 6854 (± 302) fibers per mm^2 in unirrigated trees and 7058 (± 215) fibers per mm^2 in wastewater irrigated trees, than the five other cultivars ‘Beagle’, ‘Olof’, ‘Sven’, ‘Terra Nova’ and ‘Tora’, which ranged from 5851-6305 fibers per mm^2. Conversely, the average transverse fiber area of ‘Endeavor’ was 123 (± 6) µm^2 in unirrigated trees and 120 (± 4) µm^2 in irrigated trees, which was significantly lower than all five cultivars which ranges from 133-143 µm^2. Significant differences were detected in basic wood density with the wastewater irrigated trees from ‘Olof’ having the highest density of 0.348 (± 0.008) g cm^{-3} and unirrigated trees from control trees from ‘Terra Nova’ having the lowest density of 0.279 (± 0.010) g cm^{-3} (Figure 4.3).



ANOVA	Vessel frequency per mm^2	Average vessel area µm^2	Fibre frequency per mm^2	Average fibre cell area µm^2	Density g cm^{-3}
Cultivar	n/s	**<0.001**	**<0.001**	**<0.001**	**0.008**
Treatment	n/s	n/s	n/s	n/s	n/s
Cultivar x Treatment	n/s	n/s	n/s	n/s	n/s

Figure 4. 3 :Secondary xylem cell and tissue morphology at the Hillsborough site. Transverse sections of 30-40 µm taken from variable heights at a diameter of ~1 cm, scale bar = 2.5mm. Representative micrographs are each composed from >100 x40 high-res images (see example red box) allowing tissue analysis. Vessel frequency (mm^2), average vessel area (µm^2), fiber frequency (mm^2), average fiber area (µm^2) and basic wood density g cm^{-3} are shown using four replicate field-grown unirrigated and wastewater irrigated willow trees for each cultivar. Letters denote Tukey's HSD, α = 0.05. ANOVA values are shown with significance ($p < 0.05$) indicated in bold.

4.4.3.2 Biomass composition and enzymatic saccharification

Analysis of variance in biomass composition and enzymatic saccharification parameters identified significant variation between cultivars in xylan and mannan content but not in glucan, arabinan, galactan, lignin content, or glucose release yields. A significant wastewater treatment effect was identified in xylan content as well as glucose release yields, and no cultivar x wastewater treatment interaction was detected (Table 4.4). Extractives, glucan, galactan and arabinan contents ranged from between 2.8-4.2 % DM, 46.5-50.7 % DM, 1.4-1.8 % DM and 1.0-1.3 % DM, respectively. The lowest xylan content was detected in unirrigated trees from the cultivar 'Sven', at 12.4 (± 0.6) xylan % DM, while the highest was detected in wastewater irrigated trees from the cultivar 'Tora', at 17.6 (± 0.6) xylan % DM. Mannose content was highest in the cultivar 'Beagle', at 2.8 (± 0.2) % DM in unirrigated and 2.5 (± 0.2) % DM in wastewater irrigated trees, while the lowest mannan content was observed in cultivars of 'Endeavour' and 'Terra Nova', which ranged from 1.6 – 2.0 % DM (Table 4.4). Lignin was relatively stable between cultivars with the only significant difference detected between wastewater irrigated trees from the cultivars 'Endeavour' and 'Tora' with 30.5 (± 0.7) and 27.8 (± 0.6) lignin % DM, respectively. Similarly, glucose release yields were relatively similar with significant differences only detected between wastewater irrigated trees from 'Endeavour' with 100 (± 10) mg g^{-1} glucan and 57 (± 7) mg g^{-1} DM, and unirrigated trees from 'Sven' with 158 (± 14) mg g^{-1} glucan and 90 (± 7) mg g^{-1} DM (Table 4.4).

	Treatment	*Extractives % DM*	*Glucan % DM*	*Xylan % DM*		*Galactan % DM*	*Arabinan % DM*	*Mannan % DM*		*Lignin % DM*	*Glucose release % Glucan*	*Glucose release % DM*
Beagle	*unirrigated*	3.44 (0.41)	50.74 (0.81)	16.24 (1.02) ab	a	1.48 (0.24)	1.07 (0.09)	2.79 (0.15) a	a	28.19 (0.7) ab	12.13 (0.45) ab	7.31 (0.18) ab
	Irrigated	3.24 (0.31)	48.8 (0.47)	16.17 (0.8) ab		1.38 (0.06)	1.12 (0.05)	2.54 (0.19) ab		28.67 (0.29) ab	11.54 (0.99) ab	6.70 (0.59) ab
Endeavor	*unirrigated*	3.59 (0.7)	48.29 (0.45)	14.51 (0.57) ab	ab	1.63 (0.1)	1.11 (0.10)	1.62 (0.06) d	c	29.41 (0.34) ab	12.71 (1.62) ab	7.60 (1.00) ab
	Irrigated	3.49 (0.49)	46.48 (1.45)	15.42 (1.18) ab		1.79 (0.07)	1.06 (0.07)	1.98 (0.18) bcd		30.53 (0.68) a	10.04 (1.02) b	5.73 (0.69) b
Olof	*unirrigated*	2.77 (0.06)	49.27 (0.43)	13.31 (1.4) ab	ab	1.57 (0.08)	1.03 (0.07)	2.11 (0.14) bcd	b	29.09 (0.4) ab	15 (0.39) ab	8.59 (0.15) ab
	Irrigated	4.02 (0.76)	49.38 (2.20)	16.16 (1.21) ab		1.78 (0.03)	1.14 (0.10)	2.35 (0.15) abc		29.24 (0.77) ab	11.62 (1.07) ab	7.00 (0.83) ab
Sven	*unirrigated*	4.05 (0.25)	48.87 (1.03)	12.38 (0.59) b	b	1.58 (0.03)	1.17 (0.06)	2.59 (0.1) ab	ab	29.16 (0.43) ab	15.77 (1.35) a	8.96 (0.71) a
	Irrigated	4.18 (0.24)	47.26 (0.6)	13.59 (0.8) ab		1.6 (0.04)	1.25 (0.10)	2.24 (0.13) abcd		28.81 (0.18) ab	13.22 (1.42) ab	7.49 (0.75) ab
Terra nova	*unirrigated*	3.24 (0.38)	49.18 (1.18)	12.91 (1.27) ab	ab	1.65 (0.03)	1.20 (0.07)	1.83 (0.13) cd	c	29.11 (0.83) ab	13.81 (1.02) ab	7.89 (0.45) ab
	Irrigated	3.74 (0.35)	48.95 (0.28)	15.27 (0.64) ab		1.56 (0.09)	1.30 (0.09)	1.79 (0.06) cd		28.77 (0.6) ab	12.05 (0.74) ab	6.99 (0.35) ab
Tora	*unirrigated*	3.17 (0.27)	48.17 (0.58)	13.37 (0.8) ab	ab	1.46 (0.02)	1.10 (0.05)	2.6 (0.08) ab	ab	29.54 (0.06) ab	11.21 (1.16) ab	6.36 (0.60) ab
	Irrigated	3.03 (0.17)	49.98 (0.96)	17.57 (0.58) a		1.49 (0.17)	0.98 (0.06)	2.47 (0.05) ab		27.79 (0.62) b	12.39 (1.68) ab	7.22 (0.83) ab
Cultivar		n/s	n/s	**0.032**		n/s	n/s	**<0.001**		n/s	n/s	n/s
Treatment		n/s	n/s	**0.001**		n/s	n/s	n/s		n/s	**0.019**	**0.017**
Cultivar x Treatment		n/s	n/s	n/s		n/s	n/s	n/s		n/s	n/s	n/s

Table 4. 4 : Biomass composition and enzymatic saccharification at the Hillsborough site. Letters denote Tukey's HSD, α = 0.05. ANOVA values are shown with significance ($p < 0.05$) indicated in bold.

4.4.4 Trait correlation

Pearson correlation was used to identify significant ($p < 0.05$) associations between transverse tissue and cell morphology, cell wall composition and cell wall accessibility to hydrolysing enzymes across all 112 trees (Table 4.5). The Vessel frequency had a significant inverse correlation of r = -0.42 with average vessel area and the latter had an inverse correlation of-r = -0.27 with basic wood density. Fiber frequency had a very strong inverse correlation coefficient of r = -0.97 with average fiber cell area, and of r = -0.20 with lignin but positive correlations of r = +0.33 glucan content, r = +0.39 glucose release % glucan and r = +0.44 glucose release % DM. Average fiber cell area showed the converse pattern with a positive correlation of r = +0.26 with lignin content but an inverse correlation of r = -0.40 with glucan content, r = -0.41 glucose release % glucan and r = -0.46 glucose release % DM. Basic wood density had a positive correlation of r = +0.49 with extractives and r = +0.31 with galactan content as well as a high correlation of r = +0.61 with arabinan content, but had an inverse correlation of r = -0.31 with glucan content, r = -0.41 glucose release % glucan and r = -0.40 glucose release % DM. Glucan content had an inverse correlation of r = -0.33 with galactan, r = -0.50 with arabinan and r = -0.45 with lignin but had a positive correlation of r = +0.25 with mannan and r = +0.44 glucose release % glucan and r = +0.52 glucose release % DM. Xylan content had a negative correlation of r = -0.28 with arabinan content while galactan had a positive correlation of r = +0.49 with arabinan content. Xylan, galactan and mannan content had no significant correlation with glucose release, whereas arabinan content had a significant inverse correlation of r = -0.37 with both glucose release yields. Lignin had an inverse correlation of r = -0.28 with glucose release expressed as a proportion of DM but no significant correlation with glucose release as a proportion of glucan (Table 4.5).

Pearson Correlation r	*Vessel frequency per mm^2*	*Average vessel area µm^2*	*Fibre frequency per mm^2*	*average fibre cell area µm^2*	*Density g cm^{-3}*	*Extractives % DM*	*Glucan % DM*	*Xylan % DM*	*Galactan % DM*	*Arabinan % DM*	*Mannan % DM*	*Lignin % DM*	*Glucose release % Glucan*	*Glucose release % DM*
Vessel frequency per mm^2	1													
Average vessel area µm^2	**-0.442*****	1												
Fibre frequency per mm^2	0.043	**-0.393*****	1											
average fibre cell area µm^2	-0.139	**0.334*****	**-0.973*****	1										
Density g cm^{-3}	-0.072	**-0.271****	-0.043	0.079	1									
Extractives % DM	0.180	**-0.373*****	-0.154	0.167	**0.491*****	1								
Glucan % DM	-0.024	0.137	**0.326*****	**-0.395*****	**-0.309*****	**-0.518*****	1							
Xylan % DM	**-0.285****	0.141	-0.118	0.142	-0.013	-0.164	0.133	1						
Galactan % DM	-0.058	-0.134	0.093	-0.045	**0.311*****	**0.319*****	**-0.333*****	0.111	1					
Arabinan % DM	0.100	**-0.368*****	0.008	0.011	**0.608*****	**0.667*****	**-0.500*****	**-0.282****	**0.486*****	1				
Mannan % DM	0.175	-0.155	0.124	-0.143	-0.178	-0.064	**0.247****	-0.124	**-0.194***	-0.153	1			
Lignin % DM	-0.163	**0.217***	**-0.197***	**0.261****	-0.149	**-0.245****	**-0.447*****	-0.123	0.169	-0.034	**-0.284***	1		
Glucose release % Glucan	-0.083	0.026	**0.388*****	**-0.408*****	**-0.413*****	**-0.488*****	**0.435*****	-0.003	-0.052	**-0.373*****	0.001	-0.156	1	
Glucose release % DM	-0.082	-0.001	**0.435*****	**-0.457*****	**-0.398*****	**-0.455*****	**0.520*****	0.006	-0.059	**-0.374*****	0.020	**-0.237****	**0.988*****	1

Table 4. 5 : Trait correlations. Pearson Correlation coefficients for measured parameters using all 112 trees. Significant correlations are highlighted in bold and stars indicate: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Discussion

4.4.5 Biomass density and wood anatomy

The two willow cultivars grown at the Beaverlodge plantation in Alberta, Canada, differed in secondary xylem tissue structure, with 'Tully' having a higher number of small fiber cells than 'India', while vessel size and frequency was similar between the two (Figure 4.1). Slower growth of smaller fiber cells with thicker secondary cell walls is considered to lead to increased wood density (Martínez-Cabrera *et al.*, 2009; Cobas *et al.*, 2013; Berthod *et al.*, 2015; Brereton *et al.*, 2015). However, the differences in tissue structure here indicate little difference in basic wood density between the two cultivars despite a 20% increase in fiber cell frequency and 15% reduction in fiber cell size in 'Tully' compared to 'India'. Vessel cell frequency was similar between the cultivars, suggesting fibers and vessels can vary independently between cultivars. This illustrates the cellular specialisation (Sperry, Hacke and Pittermann, 2006), or division of labour, in woody angiosperms of vessels for hydraulic architecture (being the major water conducting cells) and fibers for major structural strength, when compared to tracheids of most gymnosperm trees (which serve both roles).

The five willow and one poplar cultivars grown in the Whitecourt plantation in Alberta, Canada, also had significant differences between cultivars in the measured tissue architecture parameters and no variation due to wastewater irrigation. The cultivars 'SV1', 'SX61' and 'SX64' had a generally higher wood density which was accompanied by a higher number of fiber cells and smaller average fiber cell transverse area relative to the lower wood density willow cultivars of 'Charlie' and 'Pseudo' as well as the poplar cultivar 'Brooks' (Figure 4.2). These findings suggest two distinct cultivar strategies based on the ratio between fiber size and frequency. Studies investigating variation in vessel development under water stress conditions find a common phenotype of reduced vessel area but increased vessel frequency, considered a developmental response which improves resistance to cavitation (Jacobsen *et al.*, 2005; Junghans *et al.*, 2006; Fichot *et al.*, 2009). Doffo *et al* (2017) found that both drought and flooding reduced average transverse vessel cell area in Salix alba and Salix matsudana x S. alba, but that only drought stress also reduced vessel frequency, with flooding potentially eliciting an anatomical phenotype to limit water transport or when water transport becomes less of a developmental priority compared to the necessary cavitation resistance required under drought conditions. The average vessel areas were relatively stable likely suggesting that control plants did not struggle with neither water shortage, nor flooding stress, although there were significant reductions in vessel area in wastewater irrigated ‘SX61’ and ‘SV1’ trees compared to unirrigated trees from the cultivar ‘Charlie’.

The six willow cultivars grown at the Hillsborough plantation, Northern Ireland, varied in all xylem tissue architecture parameters with the exception of vessel cell frequency (Figure 4.3). Trees from the cultivars ‘Terra Nova’ and ‘Olof’ had the largest average transverse vessel cell area, while ‘Endeavour’ had a higher fiber frequency (~16 %) and lower average fiber cell area (~13%) compared to the other cultivars. However, unlike the Whitecourt plantation, the higher frequency of smaller fiber cells in trees of the ‘Endeavour’ did not translate to increase wood density, with trees from ‘Olof’ having the highest density. Wastewater irrigation again had no significant effect on the measured xylem tissue parameters, suggesting these willow cultivars are resilient to this high-water load irrigation treatment in terms of plant development.

Taken in isolation, the Whitecourt plantation indicates that variation in transverse fiber cell area and fiber frequency could be major determinants of biomass density, but findings from the Beaverlodge and Hillsborough plantations suggests these factors are insufficient to explain difference in basic density. For example, ray parenchyma make up a very small proportion of transverse area and therefore were not quantified here but have been observed to correlate with basic wood density in sycamore (Taylor, 1975). Fast-growing tree species commonly have lower wood density (Muller-Landau, 2004; Cobas *et al.*, 2013; Berthod *et al.*, 2015; Brereton *et al.*, 2015). This is reflected when comparing the Beaverlodge and Hillsborough plantations (Figure 4.1 and 4.3), as Beaverlodge cultivars had the highest wood density and the lowest biomass yield (Figure 4.1 and Table 4.1), while the Hillsborough cultivars had the lowest wood density and the highest biomass yield (Figure 4.3 and Table 4.1). Similarly, although total vessel area and total fiber area are mutually dependent, in making up >99% of the transverse area of secondary xylem (the small proportion of ray parenchyma was not measured here), variation in the fibers cell frequency was not the inverse of vessel cell frequency between or within the sites. This is surprising given the intuitive relationship of the ratio between large lumen, thin-walled vessels and thick secondary cell walled fibers. The significant differences in these xylem tissue parameters between cultivars (Figure 4.1-4.3) and sites (compare Figure 4.1, 4.2 and 4.3), in particular those of fiber size, have the potential to underly variation in second-generation lignocellulosic bioenergy processes which depend on fiber secondary cell wall recalcitrance to enzymatic deconstruction (Wildhagen *et al.*, 2018). The lack of observable xylem tissue changes due to wastewater irrigation, however, could be further evidence to that presented by Sas *et al* (2021) that phytofiltration of wastewater could be possible without detriment to downstream biomass properties.

4.4.6 Biomass composition and enzymatic saccharification

When investigating biomass composition and enzymatic saccharification yields, lignin content and enzymatic saccharification varied significantly between the cultivars 'Tully' and 'India' at the Beaverlodge plantation, while glucan and hemicellulose monomers did not vary significantly. While around 7 % higher lignin content in tree of the cultivar 'Tully' may be relatively small in absolute terms, lignin content has been shown to not vary substantially in previous willow trials (Serapiglia *et al.*, 2009; Brereton *et al.*, 2012; Ray *et al.*, 2012; Serapiglia, Cameron, *et al.*, 2013; Serapiglia, Humiston, *et al.*, 2013). Enzymatic saccharification revealed 30% lower glucan accessibility in 'Tully', consistent with the well-characterised role of lignin in underlying cell wall recalcitrance to deconstruction but somewhat surprising as previous work in willow has suggested natural variation in glucose release yields between cultivars (as opposed to a strong relationship within cultivar) does not correlate with lignin content (Studer *et al.*, 2011; Ray *et al.*, 2012; Serapiglia, Humiston, *et al.*, 2013).

At the Whitecourt plantation, the cultivar 'Charlie', which had both low biomass yield and wood density (Table 4.1 and Figure 4.2) , had significantly higher lignin content (~10%) and significantly lower glucan content (~11%) than the five other cultivars, which were all relatively similar at ~29 % and ~47 % of DM, respectively. Both the lignin and glucan content were higher than previous studies in the UK (Ray *et al.*, 2012) and US (Serapiglia *et al.*, 2009; Serapiglia, Cameron, *et al.*, 2013; Serapiglia, Humiston, *et al.*, 2013) which averaged 22-26% lignin content and 38-45% glucan content but higher hemicellulose proportions of 21-33 %. Significant variation in galactose, arabinose and mannose is suggestive of structural cell wall differences and has been associated with variation in enzymatic saccharification (Escamez *et al.*, 2017). The cultivar 'SV1' had the highest glucose release yields within the Whitecourt plantation. 'SV1' has been previously reported (Serapiglia, Cameron, *et al.*, 2013) as having lower cell wall recalcitrance when compared to other cultivars. As the glucan content of 'SV1' was similar here to other cultivars, the higher sugar release yields compared to 'Pseudo' or 'SX61' are unlikely to be determined by differences in substrate (glucan) content alone, similar to previous findings in willow (Ray *et al.*, 2012). Interestingly, extractives varied due to wastewater irrigation as well as between cultivars, decreasing by 5.3-21.5% in treated trees for most of the cultivars Biomass extractives comprise soluble sugars, protein and lipids as well as potentially valuable phytochemicals such as phenolics in willow. A net decrease in extractive concentrations of wastewater treated willow was also observed in research by Sas *et al* (2021) and reflected the alterations in specific secondary metabolites likely employed by the tree to tolerate the stress induced by wastewater treatment. These changes could represent a potential opportunity for targeted alteration of bioproducts, a current research target for added-value of dedicated bioenergy and sustainable biomass production systems (Devappa, Rakshit and Dekker, 2015; Brereton *et al.*, 2017; Sas *et al.*, 2021). Similarly, not only were there significant differences in glucose release yields between cultivars, with the 'SV1' outperforming other cultivars, but wastewater irrigation also increased glucose release yields for most of the cultivar and the cultivars 'SX64' and 'Pseudo' increasing respectively over 26% and 40%, raising the possibility that wastewater treatment could help improve the bioenergy potential of willow.

At the Hillsborough plantation, significant variation was observed between the six willow cultivars in the major hemicellulose backbone monomers xylose and mannose but not in glucan content or lignin content, and the variation in composition was not reflected in significant differences in sugar release yields between cultivars. Wastewater irrigation did induce broad changes in cell wall development as xylose content increased by 6.2% to 31.4% across all cultivars (excepting 'Beagle'). Extractives content was not altered by wastewater irrigation as in the Whitecourt plantation and sugar release yields had the converse response, reducing by around 12% in treated trees. Interestingly, the glucose release from enzymatic saccharification decreased for most of the cultivars of Hillsborough. Such result may be related to one the effluent characteristics; electrical conductivity being very higher i.e. 4.6 dS m^{-1} (Table S4.1) when compared to the effluents used in both Alberta sites. This increase in cell wall recalcitrance for Hillsborough cultivars, contrary to the reduction of cell wall recalcitrance due to wastewater irrigation in the Whitecourt plantation, illustrates how site-specific factors such as environmental conditions and wastewater composition could alter yields within an integrated wastewater treatment and sustainable bioenergy system, highlighting a major drawback when comparing natural phytotechnology approaches to conventional wastewater treatment approaches.

The range of glucose release from enzymatic saccharification across all three sites and the fourteen cultivars (Table 4.2, 4.3 and 4.4), 6-16 % of available glucan, was similar to yields reported by Serapiglia, Humiston, *et al* (2013) and Ray *et al* (2012) for 30 and 35 willow varieties cultivated in the US and UK, respectively. Xylan and lignin were relatively stable between the three sites, but glucan content varied by 10%, the highest being measured in trees cultivated at Hillsborough (compare Table 4.2, 4.3 and 4.4). Glucose release varied substantially, with the Hillsborough site yielding 85% more glucose when expressed (normalised) as a proportion of glucan and 79% more glucose when expressed as a proportion of DM compared to trees cultivated at Beaverlodge (compare Table 4.2, 4.3 and 4.4). While willows with reduced cell wall recalcitrance could improve bioenergy potential (Brereton *et al.*, 2012; Ray *et al.*, 2012), interactions with different pretreatment technologies and any changes in fermentation inhibitor production need to be explored before any benefits from wastewater irrigation could be realised.

4.4.7 Trait correlation

Average vessel area was inversely correlated with vessel frequency, it also has a weakly inverse correlation with basic wood density. Within the fourteen cultivars, the proportion of vessels per wood tissue ranged from 14 to 20 %, which likely explaining the moderate correlation between the two vessel traits. The wood of broadleaved species is made up of three tissue types; fibers, parenchyma and vessel, with the latter making up only a small proportion (relatively to the fibers) of the wood i.e. 3–23% (Poorter et al., 2010). Therefore, it may be expected that the trade-off between the two vessel trait i.e. vessel area and the vessel frequency will be less noticeable than in the wood of coniferous species as it’s composed of single tissue type i.e. tracheids (Preston, Cornwell and DeNoyer, 2006; Sperry, Hacke and Pittermann, 2006; Poorter et al., 2010; Zanne et al., 2010). Mainly, the vessel area and the vessel density conjointly contribute to the wood basic density by affecting the amount of the lumen space in the wood. Therefore, we might expect that both parameters will vary inversely with the wood basic density. However, the wood density in the present study did not correlate with the vessel frequency being only and weakly correlated with average vessel area. Such results, was previously reported within many species, with Vessel fraction variously showing either negative or no correlation with wood density (Martínez-Cabrera et al., 2009; Poorter et al., 2010; Ziemińska et al., 2013).

Fiber frequency was very strongly inversely correlated with average fiber cell area and both did not correlate with wood basic density. The fibers beside vessel and parenchyma are the three main cell types of the wood tissue, with different structural characteristics and relative proportions of those cell types within the xylem thereby affecting the wood basic density (Martínez-Cabrera *et al.*, 2009; Ziemińska *et al.*, 2013). While the vessel lumens having essentially zero density, most of the wood basic density is driven by the fiber traits and to lesser extent to vessel walls and parenchyma (Preston, Cornwell and DeNoyer, 2006; Martínez-Cabrera *et al.*, 2009; Ziemińska *et al.*, 2013). However, both of the fiber traits i.e. fiber frequency and average fiber cell area were not correlated with basic wood density, which suggest that the density here may have been driven by others fiber traits such fiber wall fraction, fiber wall to lumen ratios and total wall areas (Martínez-Cabrera *et al.*, 2009; Ziemińska *et al.*, 2013).

The fiber frequency had positive correlations with glucan content and the proportion of glucose released as well as having negative correlation with lignin content. As an increase of fiber frequency within the wood tissue means more fiber cell per unit area (i.e. more cell wall material) and consequently an increase of total wall area, this will result in increase of glucan content per unit of biomass. Also the increases of fiber frequency have shown to be associated with a decreases of fiber lumen diameter and an increases in fiber cell wall thickness (McDonald, Williamson and Wiemann, 1995; Hori *et al.*, 2020; Janssen *et al.*, 2020), with the latter parameters being frequently associated with wood recalcitrance (thicker fiber cell wall resulting higher glucose yield potential) (Escamez *et al.*, 2017)). Thus, the increase of the glucan content and the proportion of glucose released might likely be a result of an increase of the fraction of cell wall material (increase of fiber per unit area and inevitably a decrease of fiber lumen ratios) as well as the increase of the thickness of the cell wall material in-see.

The basic wood density was highly positively correlated with the extractives content. This is in line with previous statements (Pitre, Cooke and Mackay, 2007) as in opposition to fast growing cultivars, the plants with low growth rate have denser wood (Table 4.1, Figure 4.1 and 4.3) with low wood to bark ratios and therefore high extractives content.

The basic wood density showed inversely correlated with glucan content and glucose release but was not correlated with lignin content. This association between density and glucose release from enzymatic saccharification is contrary to the findings of Serapiglia, Humiston, *et al* (2013) where no correlation was identified, but similar to findings by Wang *et al* (2018), Murphy *et al* (2021) and Djioleu *et al* (2012) who identified relationships between lower density and higher glucose release in woody angiosperms.

Lignin content had high negative correlation with glucan content, thus, highlighting the compensatory relationship between synthesis of those both wood cell wall major components (Ray *et al.*, 2012; Serapiglia, Humiston, *et al.*, 2013). Lignin content had a weak inverse correlation with glucose release expressed as a proportion of DM but not when glucose release was expressed as a proportion of glucan, suggesting that lignin can drive cultivar differences in glucose release based on composition, or substrate availability, but not in terms of cell wall recalcitrance in willow. This aligns with the findings of Ray *et al* (2012) and Serapiglia, Humiston, *et al* (2013) who also observed that variation between willow varieties in glucose release was independent of lignin content (whereas within variety variation is commonly found to be associated with lignin content) but may be related to interactions (linkages) between the majors wood polymers (cellulose, hemicellulose and lignin).

The high correlation between the galactose and arabinose content may suggest variation of a prevalent arabinogalactan. Recalcitrance of woody biomass is traditionally considered to be affected by factors including the cellulose (crystallinity and degree of polymerization) and lignin (content, S:G ratio). Escamez *et al* (2017) have previously associated increased arabinose content with cell wall recalcitrance and highlighted that the less abundant cell wall matrix polysaccharides such as arabinose might be important targets for improved lignocellulosic yields in poplar. The correlation between cell wall recalcitrance and both galactose and arabinose support this focus on factors beyond cellulose and lignin into xylem tissue anatomical factors, such as fiber cell frequency.

4.5 Conclusions

Compositional analysis of willow biomass across the three field trials suggests, unsurprisingly, that increased glucan content is related to improved glucose accessibility and final glucose release yields. However, lignin content was not associated with variation in glucose accessibility but did have a weak inverse correlation to final glucose release yields. Significant associations were also identified between secondary xylem wood anatomy and cell wall glucose accessibility, with a greater number of smaller fiber cells leading to improved glucose release yields. While the biomass compositional and enzymatic saccharification results suggest there is not an improvement in the feedstock for a potential bioenergy process due to wastewater irrigation, there was also no observed corresponding penalty or negative impact of treatment to biomass quality at the three field trials. Cultivation of biomass is often seen as the most substantial hurdle for economically feasible bioenergy production. By aligning feedstock production strategy with environmental wastewater treatment, these findings suggest willow cultivation could serve as a positive value-stream, in terms of both financial and environmental benefits.

Credit authorship contribution statement

A. Jerbi: Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **J. Laur**: Writing - review & editing. **M. Kalwahali Muissa**: Investigation, Review & editing. **P.P. Gallant**: Formal analysis, Review & editing. **R. Krygier:** Review & editing. **C. Johnston:** Review & editing. **M. Blank:** Review & editing. **Barnabé**: Review & editing. **M. Sarrazin**: Formal analysis, Review & editing. **M. Labrecque**: Writing - review & editing. **N.J.B. Brereton**: Conceptualization, Methodology, Writing - original draft, Writing - Review & editing. **F.E. Pitre**: Conceptualization, Methodology, Writing - Review & editing.

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4.7 Supplementary material

ImageJ plug-in and analyses instructions

Vessels measurement

Count the numbers of vessels and calculate the lumen area of each individual vessel within the stem section. The recognizing of vessel lumen from fiber and ray cell lumen is based on area, roundness and circularity criterion. For each high-resolution scan, the multichannel images were imported into ImageJ using the plugin Bio-Formats and the "Bio-Formats Importer" command.

The multichannel images were firstly converted to single images using the “RGB Color” command and then to grayscale with the “8-bit” command. The images were then converted to pure black and white the "Auto Local Threshold". The number of vessels were assessed with the "Analyze Particles..." method using ranges of sizes and circularities respectively 250-1000 pixel and 0.1-1(unitless). The false positives were then excluded using an experimentally determined criterion of a minimal value 0.65 for the sum of a particle given roundness and circularity.

Cells lumen measurement

The images were treated as previously described to obtain pure black and white images. The images were then smoothed using the “smooth” command and the local maxima were counted using the "Find Maxima..." command. The amount of all the cells (i.e. lumens) present within the stem section was measured by counting the local maximums in the images. The number of fibers and ray cell in the image was obtained by subtracting the number of vessels previously calculated from the number of total cells. More details about cell-counting by ‘’Maxima’’ are described in (Grishagin, 2015).

Image command

Measure Vessels density and area

- run("Bio-Formats Importer", "open=[" + input + "] color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + serie);
- run("RGB Color");
- run("8-bit");
- run("Auto Local Threshold", "method=Bernsen radius=25 parameter_1=0 parameter_2=0 white");
- run("Analyze Particles...", "size=" + size + " circularity=" + circ + " exclude");

Measure the area of Pith, stem section and tension wood

- run("Bio-Formats Importer", "open=[" + input + "] color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + serie);
- run("RGB Color");
- run("8-bit");

Measure the Pith area:

- run("Auto Threshold", "method=Default white")

- `run("Analyze Particles...", "size=30000-Infinity display add exclude");`

Measure the stem section area (including pith):

- `run("Auto Threshold", "method=Default white");`
- `doWand(getResult("X", nResults-1),getResult("Y", nResults-1), 10, "Legacy");`
- `run("Measure");`

Measure the tension wood area:

- `run("Auto Threshold", "method=MaxEntropy white");`
- `run("Options...", "iterations=5 count=4 edm=8-bit do=Erode");`
- `run("Options...", "iterations=5 count=4 edm=8-bit do=Dilate");`

Measure cells (counting)

- `run("Bio-Formats Importer", "open=[" + input + "] color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_" + serie);`
- `run("RGB Color");`
- `run("8-bit");`
- `run("Smooth");`
- `run("Find Maxima...", "noise=10 output=Count");`

		Beaverlodge	Whitecourt	Hillsborough
pH		7.70	7.40	6.05
Elect. Cond.	(dS m^{-1})	1.45	0.79	4.61
NO_3-N	(mg L^{-1})	2.53	23.36	-
NH_4-N	(mg L^{-1})	11.34	0.28	-
N	(mg L^{-1})	-	-	183.80
P	(mg L^{-1})	4.04	1.58	56.15
K	(mg L^{-1})	-	-	568.20
Na	(mg L^{-1})	114.28	83.78	80.40
Mg	(mg L^{-1})	44.72	16.16	44.88
Ca	(mg L^{-1})	111.22	67.93	129.51
BOD	(mg L^{-1})	13.65	7.14	2752.50

Table S4. 1 : Wastewater compositional within the three sites.

4.8 Synthèse du chapitre 4

Les résultats que nous venons de présenter sont ceux de trois expériences distinctes géographiquement, mais comparables dans la durée et la période de traitement (trois années de croissance c.-à-d. 2012-2014). L'effluent appliqué pour chacune de ces expériences est différent. Pour les sites en Alberta (Beaverlodge et Whitecourt), il s'agissait d'eaux usées avec traitement secondaire alors que ce sont des effluents de laiterie non traités qui étaient en cause pour le site d'Irlande du Nord (Hillsborough). Aussi, les génotypes de saules et/ou de peuplier cultivés dans chacun des trois sites ne sont pas répliqués ce qui limite la portée des analyses et des interprétations.

Bien que nous n'ayons pas fait des statistiques pour la biomasse aérienne faute d'avoir les données brutes, nous avons constaté que pour les plantes de Beaverlodge et de Whitecourt qui ont été irriguées par des eaux usées avec traitement secondaire, il y a eu une augmentation de biomasse aérienne (plus manifeste pour les cultivars de Beaverlodge que ceux de Whitecourt), alors que pour les plantes irriguées par des effluents de laiterie à Hillsborough, la productivité était moindre que celles des plantes témoins. Nous pensons que cette diminution peut être reliée à la composition de l'effluent et plus précisément à son électroconductivité qui était considérablement élevée.

Pour tous les sites, les résultats n'ont pas montré de différence significative entre les plantes témoins et celles irriguées pour aucune des variables anatomiques ni pour la densité du bois. Ces résultats peuvent suggérer que ces cultivars ont été résilients à l'effet d'application des diverses doses d'effluents sans induire de modifications dans la structure de leur bois (xylème). Pour chacun des trois sites, nous avons observé des variations pour la plupart des paramètres anatomiques du xylème entre les différents cultivars, notamment dans la fréquence et le diamètre des fibres. Si ces deux paramètres ont été les déterminants majeurs de la variabilité de la densité du bois chez les cultivars de Whitecourt, ce n'est pas le cas pour les cultivars des deux autres sites, ce qui suggère que la variation de la densité du bois chez les saules peut se faire selon différentes stratégies en impliquant possiblement d'autres traits anatomiques tels que l'épaisseur des parois cellulaires des vaisseaux et des fibres et le rapport paroi-cellulaire/lumen des fibres.

D'une façon générale, la réponse des plantes à la fertilisation par des effluents organiques a varié d'un site à un autre. Alors que pour Beaverlodge il n'y a pas eu d'effets de la fertigation sur la composition du bois ainsi que sur sa récalcitrance à la saccharification, il a été observé une diminution du taux d'extractibles et une augmentation des rendements en sucre pour certains cultivars de Whitecourt et une augmentation du contenu en xylose et une diminution des sucres libérées pour la plupart des génotypes de Hillsborough. Ces résultats sont partiellement en accord avec une des hypothèses (fondée sur le constat de précédentes recherches) puisque la fertilisation azotée n'a pas induit la formation d'un bois plus riche en cellulose, faible en lignine et donc moins récalcitrant à la saccharification enzymatique. La non-variation de la récalcitrance pour les cultivars de Beaverlodge et sa diminution pour les cultivars de Hillsborough suite à la fertigation peuvent suggérer que la composition des effluents utilisée à Beaverlodge et à Hillsborough a été respectivement plus faible en azote et ayant une forte électroconductivité ce qui a possiblement affecté la formation du bois (xylogénèse).

Pour chacun des sites, il y a eu une variation dans la composition du bois ainsi que dans les rendements de la saccharification des différents cultivars. À Beaverlodge, la teneur en lignine et les rendements de la saccharification enzymatique ont varié inversement et significativement entre les deux cultivars du site ce qui est en accord avec le rôle bien caractérisé de la lignine dans la récalcitrance de la paroi cellulaire à la saccharification enzymatique. À Whitecourt, toutes les mesures de la composition (excepté le contenu en xylane) ainsi que les rendements en sucres ont varié significativement ce qui laisse penser que des différences structurelles dans la paroi cellulaire observées entre les différents cultivars auraient pu être associées à une variation dans la saccharification enzymatique du bois de ces cultivars. À Hillsborough, la teneur en xylane et en mannane (tous deux considérés comme les majeurs monomères formant l'hémicellulose) ont été les seules composantes à varier significativement entre les différents cultivars. Toutefois, cette variation ne s'est pas traduite par une différence entre les cultivars pour le taux de glucose libéré (rendement en glucose).

En comparant les différents cultivars (sans tenir compte des traitements), les résultats ont montré des différences dans les mesures de composition surtout la teneur en cellulose (glucose) entre les cultivars d'un même site, ainsi que ceux provenant de différents sites (même si cette dernière affirmation repose davantage sur des constats visuels et non quantitatifs). Toutefois, les différences entre les cultivars issus de différents sites étaient plus prononcées que celle entre les génotypes d'un même site avec Beaverlodge et Hillsborough ayant respectivement les plus petits et les plus grandes teneurs en cellulose.

Nous avons aussi trouvé de grandes différences de l'indice la récalcitrance du bois mesurée en tant que quantité de glucose libéré par fraction de glucane entre les cultivars provenant d'un même site ainsi qu'entre ceux provenant de sites distincts. D'une manière semblable à celle de la teneur en cellulose; les différences dans le rendement en glucose étaient plus prononcées pour les cultivars issus de différents sites que ceux provenant d'un même site, Hillsborough étant le site où les plantes avaient les plus grands rendements en glucose (les moins récalcitrants).

Les résultats conjoints de la teneur en cellulose et de la saccharification enzymatique ont permis de faire un constat assez intéressant. En effet, dans chacun des trois sites nous avons trouvé que certains cultivars ont montré un plus grand rendement en sucre que d'autres alors que ces derniers avaient une teneur en cellulose plus élevée. Ce résultat va à l'encontre du principe qu'un bois avec une plus grande fraction de cellulose serait moins récalcitrant à l'hydrolyse enzymatique et libérera davantage de sucres. Toutefois, il vient supporter les résultats d'autres recherches stipulant que la récalcitrance d'une biomasse donnée n'est pas exclusivement liée à son contenu en cellulose, mais que d'autres facteurs tels que le contenu et la nature de la lignine peuvent interférer sur l'hydrolyse du bois par les enzymes.

Les mesures de corrélation ont permis d'identifer des associations significatives entre des variables anatomiques du xylème secondaire et les mesures d'accessibilité au glucose (recalictrance) de la paroi cellulaire. Un plus grand nombre de petites fibres conduisant à de meilleurs rendements en glucose suite à la saccharification que des fibres avec un grand diamètre mais qui sont moins fréquentes.

Chapitre 5 | Synthèse générale

5.1 Rappel de la problématique

Au Canada, il y a plus de 5.9 trillions de litres d'eaux usées municipales qui sont déversés chaque année dans les eaux de surface (Statistics Canada, 2017) dont plus de 150 milliards de litres sont non traités ou non suffisamment traités (ECCC, 2017). Le traitement préalable de ces eaux dans les stations d'assainissement représente une part conséquente des budgets des municipalités (Jerbi *et al.*, 2020) et permet généralement de réduire les fractions solide, organique, azotée et phosphorée des effluents avant leur déversement (Levy, Fine and Bar-tal, 2011). Toutefois, ces traitements n'étant pas totalement efficaces, des grandes quantités de contaminants (essentiellement azotés) se retrouvent alors dans les cours d'eaux ce qui cause des problèmes majeurs tant pour l'environnement, avec la prolifération des organismes autotrophes tel que les algues et les cyanobactéries, que pour la santé humaine avec la méthémoglobinémie et d'autres maladies de la thyroïde (Levy, Fine and Bar-tal, 2011; Guidi Nissim *et al.*, 2015; Pettygrove and Takashi Asano, 2018; Ngatia and Taylor, 2019) .

L'utilisation de plantation d'espèces ligneuses à croissance rapide en tant que filtre végétal comme alternative aux traitements traditionnels permet de valoriser les effluents municipaux et de réduire les risques de contamination environnementale, tout en améliorant les rendements de la production végétale des cultures en place. Plusieurs études (Dimitriou and Aronsson, 2011; Holm and Heinsoo, 2013; Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015, 2020) ont témoigné de l'efficacité des saules pour filtrer de grandes quantités d'effluents organiques et produire davantage de biomasse lignocellulosique. Plusieurs études ont aussi rapporté des altérations dans la composition chimique et la structure du bois de certaines espèces de peupliers et de saules lorsque ceux-ci étaient amendées par de l'azote durant leur phase de croissance (Luo *et al.*, 2005; Pitre, Cooke and Mackay, 2007; Pitre *et al.*, 2010).

En considérant le contexte de la transition écologique, il est important que des recherches soient faites pour étudier l'effet de l'irrigation par des effluents municipaux sur la composition chimique d'un bois des végétaux utilisés afin de déterminer les répercussions de ces altérations sur le rendement en biocarburant.

5.2 Rappel des objectifs

Les objectifs généraux de mon projet de doctorat étaient (1) d'évaluer l'effet de l'irrigation par des eaux usées chargées en azote sur la croissance et le développement des plants de saule dans un contexte de phytofiltration; (2) à la suite de la récolte de la biomasse, de déterminer s'il y a eu une altération au niveau de la structure physique et/ou de la composition chimique du bois suite à cette fertigation et (3) son implication dans un contexte de production de biocarburant. Pour ce faire, trois volets d'études ont été menés regroupant les résultats de quatre expériences.

5.3 Retour sur les hypothèses et faits saillants (résumé des principaux résultats)

5.3.1 Chapitre 2| Effet de l'irrigation par des eaux usées sur les paramètres physiologiques et le développement d'une culture de saules *Salix miyabeana* 'SX67'

Le premier objectif de ma thèse était de comprendre les effets de l'application d'effluents municipaux avec traitement primaire donc, fortement chargés en azote, sur la physiologie et la morphologie des parties aériennes des saules, essentiellement les feuilles, ainsi que sur la productivité de ces cultures. Les hypothèses associées à cet objectif étaient que la fertigation par des effluents chargés en azote allait induire des altérations morphologique, chimique et physiologique des feuilles avec augmentation de leur surface, de la concentration en azote et de leur capacité d'échanges gazeux, ce qui induira une augmentation de la croissance et du développement des plants.

La première expérience consistait à l'irrigation d'une culture de saules *Salix miyabeana* 'SX67' par différentes doses d'effluents municipaux avec traitement primaire et ayant donc gardé une grande partie de la charge organique et azotée initiale (c.-à-d. celle des eaux usées brutes). Les résultats ont montré que l'azote a induit une augmentation de la surface foliaire, de l'azote foliaire et de la concentration des pigments chlorophylliens permettant de maximiser la surface photosynthétique ainsi que le rendement quantique par unité de surface foliaire. Nous avons également constaté que l'irrigation par les eaux usées avait induit une altération majeure de la distribution et la taille des stomates avec notamment une diminution de 6,5-6,8 % de leur nombre par unité de surface et une augmentation de 11,1-15,3 % de la longueur des pores de ces stomates (c.-à-d. ostiole). Cette modification s'est traduite par une augmentation de l'index du pore stomatique, c.-à-d. de la surface cumulative des pores de stomates pour une surface donnée de feuille, engendrant inévitablement une augmentation de la conductance stomatique et de la photosynthèse et conséquemment une augmentation de la croissance et du développement des plantes.

Nos résultats ont aussi montré l'efficacité du système filtre à base d'une plantation de saules *Salix miyabeana* 'SX67' pour décontaminer de grandes quantités d'eaux usées, potentiellement jusqu'à 30 millions de litres par année. Le système est également efficace pour capter de grandes quantités de nutriments surtout l'azote jusqu'à 817 kg N ha^{-1} au niveau des parties végétatives et dans le sol. Enfin, bien que la fertigation ait occasionné une augmentation du niveau de la demande chimique en oxygène ainsi que du nitrate dans les solutions de sol (eaux de percolation) pour la plus grande dose d'eaux usées, ces valeurs sont toutefois restées sous les standards recommandés par les instances environnementales (Union, 1991; CCME, 2002; Miguel *et al.*, 2014; MELCC, 2019).

5.3.2 Chapitre 3| Effet de l'irrigation par les eaux usées sur le développement du xylème secondaire, la densité du bois et la composition du bois de saules *Salix miyabeana* 'SX67'

Le deuxième objectif de ma thèse était d'identifier d'éventuelles altérations dans la composition chimique et de la structure anatomique du bois des saules suite à une irrigation par des effluents municipaux fortement concentrés en azote. Les hypothèses associées à cet objectif sont que la fertigation par des eaux usées avec traitement primaire de façon similaire a une hyper-fertilisation (Luo *et al.*, 2005; Pitre, Cooke and Mackay, 2007; Pitre *et al.*, 2010), va favoriser la formation d'une structure anatomique contribuant davantage à une efficacité hydraulique du bois qu'à une rigidité mécanique; stimuler davantage la formation du bois de tension et altérer la composition chimique du bois. La deuxième étude de mon projet de doctorat fait suite à la première expérience et consiste donc à analyser les propriétés physiques et chimiques du bois de saule *Salix miyabeana* 'SX67' après deux années de croissance.

Les analyses histologiques du bois de saule ont montré que les plants irrigués par les eaux usées (en comparaison au bois des plantes non irriguées et/ou irriguées par l'eau potable) avaient moins de vaisseaux par unité de surface de xylème secondaire ainsi qu'une plus faible proportion de gros vaisseaux, ce qui a inévitablement conduit à une plus faible conductance hydraulique théorique des tiges des saules fertilisés. Ces résultats vont à l'encontre de notre hypothèse stipulant qu'il y aurait une augmentation de la conductance hydraulique pour les plantes fertilisées afin de supporter une plus grande demande évaporative occasionnée par l'accroissement de la surface foliaire ainsi qu'une augmentation de la transpiration pourtant constatée lors de la première expérience. Ce constat surprenant pourrait découler de l'effet concomitant imposé en champ de l'inondation et de la fertilisation. Il pourrait aussi être le résultat de notre stratégie d'échantillonnage des tiges pour les analyses histologiques. En effet, dans un souci de comparer des tiges à des stades de développement équivalents, alors que le bois avait amorcé sa croissance secondaire, nous avons chosi de prélever des sections à des niveaux plus élevés pour les plantes fertiguées que pour les plantes non irriguées et irriguées par l'eau potable. Toutefois, nous n'avons pas considéré le fait que pour les espèces ligneuses dicotylédones, l'architecture hydraulique diffère entre les différentes parties de la plante, avec des vaisseaux qui s'élargissent davantage vers la base des tiges (Anfodillo, Petit and Crivellaro, 2013; Kotowska *et al.*, 2015; Hacke *et al.*, 2017; Olson *et al.*, 2020). Il est donc possible que nos interprétations aient été influencées par cette particularité anatomique.

Les analyses histologiques ont aussi montré que pour les plantes irriguées par des eaux usées il y avait une altération de la paroi cellulaire des fibres avec la formation d'une extra-couche du côté interne de celle-ci. La coloration par le Chlorazol indiquant une forte teneur en cellulose. Cette structure typique reconnue comme celle du bois tension a été rapportée dans certaines études chez des espèces de peupliers lorsque ces dernièress ont été fertilisées par l'azote durant leur phase de croissance. Les analyses gravimétriques ont montré que la teneur en cellulose a augmenté de 16 % pour les plantes fertiguées en comparaison des plantes témoins, sans toutefois que ceci soit accompagné d'une diminution de la lignine. Ceci peut suggérer que l'augmentation de la fraction cellulose soit due, tout au moins en partie, à une augmentation de la proportion du bois de tension Les analyses gravimétriques de la biomasse ont aussi montré une diminution de 35,3-36,4 % de la teneur en extractibles du bois des saules irrigués par les eaux usées. Une augmentation du taux de croissance, comme celle observée chez les plantes qui ont été fertilisées par les eaux usées riches en azote, est généralement associée à une forte production du bois aux dépens de la partie écorce. Étant donné que la majeure partie des extractibles est située dans les écorces, une diminution de celles-ci dans la biomasse lignocellulosique résulte indéniablement en une diminution de ces extractibles.

5.3.3 Chapitre 4| Effet de l'irrigation par les eaux usées sur la composition du bois des saules et le rendement de la saccharification enzymatique

Le troisième objectif de ma thèse était d'investiguer l'effet de l'irrigation par des effluents organiques sur la structure anatomique et la composition chimique du bois de différents génotypes de saules (ainsi que d'un cultivar de peuplier) et d'identifier l'effet d'éventuelles altérations de la structure et/ou de la composition sur le rendement en glucose. Les hypothèses associées à cet objectif, sont que l'irrigation par des effluents va affecter la structure anatomique et la composition chimique des plantes de façon variable selon les génotypes . De même, les rendements en sucres, suite à la saccharification de la biomasse seront différents selon le génotype avec davantage de glucose libéré pour les plantes irriguées par les effluents.

Cette partie de mon projet de doctorat reposait sur les résultats de trois expériences conduites dans des régions distinctes géographiquement (deux en Alberta et une en Irlande du Nord) mais conjointes dans la durée et la période de traitement (trois années de croissance). Pour chacune de ces expériences, les génotypes utilisés ainsi que le type et la composition de l'effluent appliqué étaient différents, ce qui limite nos interprétations suivant les résultats obtenus quant aux différences dans la structure anatomique, la composition chimique et/ou le rendement en sucre.

Nous n'avons pas fait d'analyses statistiques pour les rendements en biomasse faute d'avoir les données brutes, toutefois, nous avons fait certains constats suite à la fertigation des plantes. Ainsi, la plupart des cultivars des plantes de Beaverlodge et de Whitecourt qui ont été irriguées par des eaux usées avec traitement secondaire présentaient de meilleurs rendements en biomasse. L'augmentation ayant été plus manifeste pour les cultivars de Beaverlodge que ceux de Whitecourt.

Pour ce qui des plantes de Hillsborough (Irlande du Nord), nous avons constaté des rendements moins élevés pour les plantes fertiguées (par des effluents de laiterie non traités) que ceux des témoins. La même tendance a d'ailleurs été observée dans une étude précédente (Forbes *et al.*, 2017) sur les mêmes plantes et avec le même type d'effluents. Ceci peut être relié à la composition des effluents et plus précisément à leur forte électroconductivité qui était de 4,6 dS m^{-1} (Table S4.1) dans la présente étude et de 3,1- 4,8 dS m^{-1} dans l'étude de Forbes *et al* (2017) (valeur moyenne faite sur quatre années de 2008-2011) ce qui a possiblement perturbé la formation du bois (xylogénèse) ainsi que la croissance des plantes.

Structure anatomique et densité du bois

Bien que les deux cultivars de Beaverlodge ait montré une tendance inverse pour les traits des fibres investigués, avec un plus grand nombre de fibres de petit diamètre pour 'Tully' et inversement moins de fibres par unité de surface, mais avec un plus grand diamètre pour 'India', nous n'avons pas détecté de différence dans la densité du bois entre ces deux cultivars. Ceci demeure intriguant étant donné qu'en majeure partie, la densité du bois est déterminée par les caractéristiques des fibres (et dans une moindre mesure par les parois vasculaires et/ou des cellules du parenchyme).

Les génotypes de Whitecourt (cinq cultivars de saule et un peuplier) ont montré des différences significatives pour l'ensemble des paramètres anatomiques ainsi que pour la densité du bois. Tout comme pour les cultivars de Beaverlodge, ceux de Whitecourt ont aussi montré une tendance inverse pour les traits des fibres, avec un plus grand nombre de fibres de petit diamètre pour les cultivars 'SV1', 'SX61' et 'SX64' et inversement moins de fibres par unité de surface, mais avec un plus grand diamètre pour 'Charlie', 'Pseudo' et 'Brooks'. Toutefois, cette variabilité dans la fréquence des fibres et de leur diamètre pour les cultivars de Whitecourt (contrairement à celle de Beaverlodge) explique bien la différence dans la densité du bois, avec un plus grand nombre de fibres de petit diamètre contribuant davantage à augmenter la densité du bois que des fibres avec un plus grand diamètre, mais qui sont moins nombreuses.

Les six cultivars étudiés à Hillsborough ont montré des variations pour tous les paramètres anatomiques à l'exception de la fréquence des vaisseaux. Les cultivars 'Terra Nova' et 'Olof' étant ceux avec le plus grand diamètre des vaisseaux, alors que le cultivar 'Endeavour' était celui qui avait la plus grande fréquence de fibres (~ 16%) et le plus petit diamètre moyen (~ 13%) par rapport aux autres cultivars. Toutefois, cette fréquence plus élevée de fibres de petit diamètre observée chez 'Endeavour', ne s'est pas traduite par une augmentation de la densité du bois comme c'était le cas pour les cultivars de Whitecourt, puisque le cultivar 'Olof' était celui avec la plus grande densité du bois.

Dans l'ensemble, les résultats des trois sites suggèrent que la variation dans la fréquence et le diamètre des fibres seraient les éléments déterminants principaux pour expliquer les variations de la densité du bois pour les cultivars de Whitecourt. Cependant, cette implication aurait été limitée ou inexistante pour les cultivars des deux autres sites. De tels résultats, suggèrent que la variation de la densité du bois peut se faire selon des processus différentes et celle-ci n'est pas uniquement due à la variation dans la fréquence et le diamètre des fibre. Des facteurs tels i) l'épaisseur de la paroi des vaisseaux et des fibres, ii) la fraction de la paroi cellulaire des fibres dans le xylème, iii) le rapport paroi cellulaire/lumen des fibres et la surface totale des parois cellulaires et iv) la densité des cellules parenchymateuses radiales, pourraient aussi expliquer cette variation.

Pour tous les sites, les résultats n'ont pas montré de différence significative entre les plantes témoins et celles irriguées pour aucune des variables anatomiques ni pour la densité du bois. Ce résultat semble suggérer que ces cultivars ont été résilients à l'application des diverses doses d'effluents.

Composition de la biomasse et saccharification enzymatique

À Beaverlodge, alors que le contenu en sucres cellulosique et hémicellulosique des cultivars 'Tully' et 'India' était comparable, la teneur en lignine et les rendements de la saccharification enzymatique de ceux-ci variaient d'une façon significative. Le contenu en lignine est reporté être relativement stable chez les saules. Toutefois, nous avons détecté une augmentation de 7% pour 'Tully' par rapport à 'India' et qui s'est accompagnée d'une diminution des rendements en sucres de près de 30 %. Ces résultats sont en accord avec le rôle bien caractérisé de la lignine dans la récalcitrance de la paroi cellulaire à la déconstruction enzymatique. Les analyses de la composition et de la saccharification enzymatique des cultivars de Beaverlodge n'ont pas permis de détecter d'effet de la fertigation.

Pour les cultivars de Whitecourt, les analyses ont montré des différences significatives pour toutes les mesures de la composition (excepté le contenu en xylane) ainsi que pour les rendements en sucres. Ces résultats peuvent suggérer que des différences structurelles dans la paroi cellulaire auraient pu être associées à une variation de la saccharification enzymatique. Parmi les cultivars de Whitecourt, 'SV1' était celui avec les rendements les plus élevés, ce qui est en accord à des recherches faites sur de nombreux génotypes de saules (Serapiglia, Humiston, *et al.*, 2013) et qui reportaient 'SV1'comme étant le cultivar avec la moindre récalcitrance de la paroi cellulaire à la déconstruction enzymatique. Fait intéressant, pour un bon nombre de cultivars, le taux d'extractibles a baissé suite à la fertigation, avec une diminution oscillant entre 5,3% et 21,5% respectivement pour les cultivars 'Brooks' et 'Pseudo'. Des résultats similaires ont été observés chez un autre cultivar de saules (*Salix miyabeana* 'SX67') lorsque irrigué par des eaux usées (Sas *et al.*, 2021), ce qui peut suggérer qu'il y a eu une éventuelle altération de certains métabolites secondaires, probablement en réponse à l'irrigation avec des eaux usées. Dans le contexte de la production bioénergétique, de tels changements peuvent être d'intérêt en apportant une valeur ajoutée au produit final par des altérations ciblées de certains bioproduits, contribuant ainsi à la pérennité des systèmes de production. Autre fait saillant, pour certains cultivars de Whitecourt dont les plantes ont reçu des eaux usées, il y a eu une augmentation du rendement en glucose. Celle-ci était faible pour 'SX61' et 'Brooks' avec respectivement 2,3 et 3,5%, mais plus grande pour 'SX64' et 'Pseudo' avec respectivement 26,8% et 41% d'augmentation. Ce résultat laisse présumer que les eaux usées pourraient améliorer le potentiel bioénergétique de certains saules en produisant une biomasse moins récalcitrante à la saccahrification enzymatique permettant de produire davantage d'éthanol par unité de biomasse.

Les analyses de la composition et de la saccharification enzymatique des six cultivars de Hillsborough n'ont pas permis identifier de différence dans la teneur en extractibles, en lignine, en glucane, en arabinane, en galactane, mais plutôt des variations dans la teneur en xylane et en mannane (ces deux derniers étant les principaux monomères de l'hémicellulose). Toutefois, cette variation de composition ne s'est pas traduite en une différence dans le taux de glucose libéré (rendement en glucose) entre les cultivars. Pour presque tous les cultivars (à l'exception de 'Beagle'), l'irrigation par les eaux usées a induit une augmentation de la teneur en xylose entre 6,2% et 31,4%. Toutefois, il n'y a pas eu de différence pour la teneur en extractibles suite à la fertigation comme celle observée chez les cultivars de Whitecourt. Aussi, une diminution de 6,3 à 22,0% a été observé pour les rendements en glucose pour tous les cultivars de Hillsborough, à l'exception de 'Tora', ce qui est en opposition à l'augmentation du rendement en glucose observée à Whitecourt pour bon nombre de cultivars. Ces résultats, peuvent suggérer que des facteurs spécifiques aux differents sites tels que les conditions environnementales et la composition des eaux usées pourraient avoir influencé la récalcitrance de la biomasse.

D'une façon générale, la réponse des plantes à la fertilisation par des effluents organiques a varié d'un site à un autre. Alors que pour Beaverlodge il n'y a pas eu d'effets de la fertigation sur la composition du bois ainsi que sur sa récalcitrance à la saccharification, il a été observé d'une part une diminution du taux d'extractibles et une augmentation des rendements en sucre pour certains cultivars de Whitecourt et, d'autre part, une augmentation du contenu en xylose et une diminution des sucres libérées pour la plupart des génotypes de Hillsborough. Ces résultats sont partiellement en accord avec certaines des hypothèses postulées qui stipulent qu'il y aurait une variation de la composition du bois suite à la fertigation avec une augmentation des rendements en sucres (essentiellement le glucose) pour les plantes fertiguées ayant bénéficié d'un apport supplementaire en nutriments, notamment en azote. Il est à préciser que cette hypothèse a été fondée sur le constat de certaines recherches faites sur des espèces de peuplier (Pitre *et al.*, 2007, 2010; Pitre, Cooke and Mackay, 2007) stipulant qu'une forte fertilisation azotée favoriserait la formation d'un bois plus riche en cellulose et ayant moins de lignine et donc moins récalcitrant à l'hydrolyse enzymatique (Brereton *et al.*, 2012; Ray *et al.*, 2012; Serapiglia, Humiston, *et al.*, 2013). Ainsi, la faible et/ou l'absence de variation dans la composition pour certains cultivars tels que 'Tully' et 'India' de Beaverlodge, peut suggérer que l'apport en azote n'était pas suffisant pour induire des changements dans la composition chimique du bois propre à chacun des cultivars.

En comparant les différents cultivars indépendamment des traitements, nous avons constaté des différences dans la composition du bois et plus spécifiquement pour le taux de glucane. Bien que ce taux ait varié légèrement et pas toujours significativement entre les cultivars d'un même site, nous avons constaté des écarts entre les cultivars des différents sites, avec les plus petits et les plus grands taux respectivement pour Beaverlodge et Hillsborough.

De même, nous avons aussi observé des différences dans la récalcitrance du bois mesurée en termes de quantité de glucose libéré par fraction de glucane dans la biomasse (mg glucose libéré/g de glucane) des différents cultivars, qu'ils soient d'un même site et/ou provenant de sites différents. La récalcitrance s'est révélée clairement différente entre les cultivars d'un même site surtout pour ceux de Beaverlodge et de Whitecourt avec écart respectif de 46% et 103 % entre leur cultivar les moins et les plus récalcitrants. Bien que pour Hillsborough, l'écart de récalcitrance était moindre, (27 %), néanmoins, les rendements des cultivars de ce dernier site étaient les plus élevées que ceux des sites d'Alberta (Beaverlodge et Whitecourt). Même que le plus récalcitrant cultivar de Hillsborough, c.-à-d. 'Endeavour' avait un rendement supérieur 11,4 mg g^{-1} glucane, à celui de 'SV01'; le moins récalcitrant de tous les cultivars de l'Alberta avec 11,1 mg g^{-1} glucane.

Le degré d'accessibilité des enzymes aux sucres formant les polymères glucidiques du bois (essentiellement les molécules de glucose à partir de la cellulose) est fortement relié à la composition chimique de ce dernier (Serapiglia, Humiston, *et al.*, 2013). Ainsi, le bois avec une plus grande fraction de cellulose et moins de lignine est réputé être moins récalcitrant à l'hydrolyse et libère davantage de sucres (Brereton *et al.*, 2012; Ray *et al.*, 2012; Serapiglia, Humiston, *et al.*, 2013). Dans ces conditions, nous pouvons présumer que nos cultivars, avec une plus grande fraction de cellulose, auront une plus faible récalcitrance c.-à-d. un rendement plus élevé en glucose libéré par g de glucane dans la biomasse. Ceci n'était toutefois pas le cas et pour bien des cultivars à différents sites. À Beaverlodge, par exemple, bien que le taux de glucane des deux cultivars 'Tully' et 'India' ait été assez similaire avec respectivement 43% et 45,4 %, nous avons observé une variation dans leur récalcitrance très distincte avec 5,5% et 8,1 % (soit 46% de différence). À Whitecourt, bien que le taux de glucane de 'SV01' (47,8%) était respectivement similaire ou inférieur à celui de 'Pseudo' et 'SX61' (47.8% et 48.1%), le rendement en sucres de 'SV01' 11,1 % était supérieur en comparaison de celui de ces deux cultivars 8,8% et 7,4 %. Aussi à Whitecourt, alors que 'Charlie' était le cultivar avec le taux de cellulose le moins élevé du groupe, son taux de glucose libéré de 9,1% était plus élevé que celui de ces derniers cultivars avec des valeurs respectives de 7,4%, 8,7%, 8,8% et 5,5 %. Enfin à Hillsborough, 'Sven', le cultivar avec le plus haut rendement de glucose 14,5 % était parmi les cultivars avec le plus faible taux de cellulose (48,1%). À la lumière de ces résultats, il apparait vraisemblable que la récalcitrance d'une biomasse donnée n'est pas exclusivement liée à son contenu en cellulose (contenu en glucane), mais que d'autres facteurs (tel que le contenu en lignine et la nature des groupes phénoliques de la lignine ainsi que les ratios des unités de syringyle (S) et de guaïacyle (G) (Serapiglia, Humiston, *et al.*, 2013) peuvent interférer sur l'hydrolyse de la macrostructure du bois par les enzymes saccharifiantes.

Même si nous n'avons pas prétraité la biomasse (ce qui peut augmenter jusqu'à cinq fois le taux de glucose libéré suite à la saccharification enzymatique) (Serapiglia, Humiston, *et al.*, 2013), les valeurs obtenues de rendement de glucose libéré par fraction de glucane (mg g^{-1} glucane) nous donnent une idée de la récalcitrance d'une biomasse qui aurait été prétraitée (Ray *et al.*, 2012).

Bien que nous ne présentions pas les rendements théoriques en éthanol (EtOH), il est raisonable de penser que les cultivars de Hillsborough qui sont les moins récalcitrants et qui libèrent davantage de sucres, aient un meilleur rendement de bioéthanol par unité de biomasse sèche (L EtOH Mg^{-1} biomasse). Étant donné que les cultivars de Hillsborough ont aussi été les plus productifs, il est à prévoir qu'ils auront également les meilleurs rendements éthanoïques théoriques par surface cultivée (L EtOH/ hectare). On peut ainsi présumer que les cultivars de Hillsborough seront les plus appropriés pour la production de bioéthanol du fait de leur forte productivité de biomasse dont la composition chimique est plus propice à la saccharification enzymatique. Cependant, des essais devront être faits pour tester la résilience de ces génotypes dans un contexte climatique et environnemental Nord-Américain.

Les résultats ont montré que le contenu en glucane était fortement et inversement corrélé à celui en lignine, mettant ainsi en évidence la relation compensatoire qui existe entre la synthèse de ces deux composantes majeures de la paroi cellulaire. Bien que le contenu en glucane se soit révélé fortement et positivement corrélé aux rendements en glucose (exprimés sous deux formes soit par mg glucose g^{-1} glucane ou par mg glucose g^{-1} MS de plante), le contenu en lignine n'est pas apparu corrélé avec le rendement en glucose exprimé en terme de glucane, mais avait une faible corrélation avec le rendement en glucose exprimé en terme de biomasse sèche. Ce résultat suggère que la différence dans la récalcitrance du bois entre différents cultivars n'est pas essentiellement due à l'écart dans le contenu en lignine entre ces cultivars, mais pourrait possiblement être reliée à d'autres facteurs tels que la composition chimique des différents polymères formant le bois ainsi que des interactions qui existent entre eux.

Les résultats ont aussi révélé des associations significatives entre des variables anatomiques et biochimiques notamment une corrélation positive entre la fréquence des fibres et la proportion de glucose libérée suite à la saccharification. Du bois avec des fibres de petit diamètre, mais qui sont en plus grand nombre par unité de surface conduirait à un meilleur rendement en glucose que du bois contenant des fibres de plus grand diamètre, mais qui sont moins fréquentes.

5.4 Synthèse et discussion des résultats

Le concept de la phytofiltration des eaux usées par des cultures de plantes à croissance rapide tel que les saules et les peupliers a vu le jour en Suède il y a plus de quarante ans et, depuis, bon nombre d'études ont montré l'efficacité de tels systèmes à retirer la majorité de la fraction organique et azotée des effluents et à améliorer la croissance et la productivité des plantes en culture (Perttu and Kowalik, 1997). Toutefois, l'approche expérimentale de ces recherches était généralement limitée à l'étude de l'impact environnemental de la fertigation (essentiellement au niveau du sol et/ou des eaux de surface et souterraines) ainsi qu'à ses répercussions sur les propriétés morphologiques des des plantes fertiguées (Dimitriou and Aronsson, 2011; Holm and

Heinsoo, 2013; Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015). L'originalité de mon projet de doctorat réside dans sa structure qui en plus du volet environnemental consistant en la gestion des eaux municipales par des cultures filtrantes de saules et la production de biomasse, intègre aussi un volet valorisation de la biomasse produite en tant que matière première pour la production des biocarburants. En effet, outre la production de grandes quantités de biomasse, nos résultats ont montré que l'irrigation des saules par des effluents municipaux riches en azote favorisait la formation d'un bois avec des qualités particulièrement appropriées pour l'industrie des biocarburants. Un bois avec une plus grande fraction de glucan (chapitre 3) et une récalcitrance moins grande (chapitre 4), ce qui permettra d'avoir un meilleur rendement énergétique par unité de biomasse produite.

Une autre originalité apportée dans cadre de la présente thèse est l'utilisation de méthodes d'analyses histologiques novatrices que nous avons développées et qui consistent en l'analyse numérique intégrale de coupes histologiques et non pas de régions d'intérêts (ROI) choisies aléatoirement ce qui renforce davantage la précision des résultats.

5.5 Principales limites, possible source d'erreur et questionnements soulevés

Les résultats et les conclusions apportés par la présente thèse constituent un apport important aussi bien dans le domaine de la phytoremédiation (plus précisément la phytofiltration) que dans le secteur de production bioénergétique. Toutefois, quelques limites dans les études effectuées doivent être mentionnées.

Dans le volet portant sur l'anatomie du xylème une limite claire de l'étude est la méthodologie utilisée pour les analyses histologiques. Le fait d'avoir comparé des échantillons provenant de différentes parties des tiges des plantes a certainement rendu l'interprétation des résultats plus difficile. En effet, au moment de l'échantillonnage des tiges à la mi-saison de la seconde année de croissance, les plantes soumises à différents traitements montraient déjà de bonnes différences quant au diamètre de leurs tiges. Ceci rendait difficile une comparaison histologique des échantillons prélevés à la même hauteur. Dans un effort d'avoir des sections de tige de taille avec des diamètres similaires pour les plantes non irriguées et irriguées par l'eau potable et les plantes irriguées par les eaux usées, nous avons échantillonné à un niveau plus élevé pour les plantes fertiguées afin de comparer des stades de développement équivalent. Cependant, l'architecture hydraulique varie selon le long des tiges avec notamment des vaisseaux plus larges vers la base des plantes. Ainsi, lorsque on compare les paramètres hydrauliques de différents arbres, il est important de prendre en considération ce gradient vertical de la conductivité. Une possible approche consisterait à subdiviser une tige en différents zone selon leur hauteur et à comparer la conductance hydraulique des échantillons appartenant au même niveau.

Dans la troisième étude, celle regroupant les résultats des trois expériences de Beaverlodge, Whitecourt et Hillsborough, nous avons eu plusieurs limites pour cette étude. D'emblée, les trois expériences bien qu'ayant été conduites pendant une même période et durée (trois années de croissance c.-à-d. 2012, 2013 et 2014), sont très distinctes géographiquement avec des conditions pédoclimatiques assez contrastées. De plus, pour chacune de ces expériences les génotypes de saules ainsi que le type de l'effluent appliqué sont différents (eaux usées avec traitement secondaire et effluents de laiterie non traités). Cette situation a limité nos interprétations quant aux différences dans la composition et les rendements en sucres qui ont été observées.

Une autre limite à l'étude est possiblement la méthodologie utilisée dans le protocole de l'extraction de la biomasse ligneuse préalablement aux mesures des contenus en sucres et en lignine. En effet, la teneur des extractibles mentionnée dans notre étude pour la plupart des génotypes était inférieure à celles rapportées dans d'autres études pour les mêmes cultivars, ce qui suggère une extraction partielle et non complète des échantillons pour nos échantillons. Alors que le protocole du NREL (National Renewable Energy Laboratory) (Sluiter *et al.*, 2008) recommande une extraction de la biomasse lignocellulosique en étapes avec l'eau en premier et un solvant organique après (habituellement l'éthanol), nous n'avons fait qu'une une seule extraction avec de l'éthanol 95% (95% EtOH et 5% H_2O) et ce, en raison de limitations logistiques et technique à l'époque de ces analyses. Aussi, nous pensons que les conditions de conservation et de stockage de la biomasse avant les extractions ont aussi influencé le taux d'extractibles que nous avons obtenu. En effet, immédiatement après le recépage d'un arbre, sa teneur en extractibles commence à diminuer avec une exposition prolongée à l'air (longue durée de stockage) pouvant affecter les doubles liaisons des extractibles et générant des radicaux libres fortement oxydants (dégradation moléculaire des extractibles) (Yang and Jaakkola, 2011). Étant donné le long délai occasionné par les diverses manipulation, récolte des arbres (avril 2015), leur broyage (décembre 2015) et leur extraction (avril-mai 2016), la teneur en extractibles a pu être affectée surtout du fait du stockage sous forme de copeaux pour plus de quatre mois (particules plus enclines à être oxydées/dégradées).

Autre que la sous-estimation du taux d'extractibles, nous pensons que l'extraction partielle de la biomasse a possiblement interférée avec les taux de la cellulose (glucose) et de la lignine. En effet les extractibles, si ils ne sont pas totalement retirés de la biomasse antérieurement à l'hydrolyse acide (lors du protocole de détermination des sucres et de la lignine (Sluiter *et al.*, 2012)), ils peuvent être dégradés et précipités avec la lignine insoluble, ce qui peut résulter en une surestimation de la lignine (Serapiglia *et al.*, 2009; Ray *et al.*, 2012).

5.6 Apport des résultats de recherche et possible retombées

Une des raisons majeures de la présente étude était d'investiguer la capacité d'un système filtre végétale à base à d'une culture de saules (*Salix miyabeana* 'SX67') à traiter de grands volumes d'eaux usées avec traitement primaire et qui donc, étaient fortement chargés en matière organique et en azote.

En nous basant sur nos précédentes études (Guidi Nissim *et al.*, 2015; Jerbi *et al.*, 2015) alors que l'application de 15 600 m^3 ha^{-1} d'eaux usées avec traitement secondaire (au terme de deux années de croissance) sur la même culture de saules n'avait pas occasionné de lessivage de l'azote et a permis d'augmenter la productivité de 83%, nous avons essayé de tester la limite supérieure d'un tel système en terme de quantité d'effluents pouvant être traitée sans que cela n'occasionne des répercussions négatives pour l'environnement. L'irrigation de 30 000 m^3 ha^{-1} d'eaux usées avec traitement primaire au cours d'une seule saison de croissance, a fait augmenter de 8 800% la concentration du nitrate dans les eaux percolées (c.-à-d. dans les lysimètres) en comparaison avec l'irrigation par l'eau potable. Toutefois, cette concentration est restée sous la limite d'acceptation des normes d'eau potable du Québec soit 10 mg N L^{-1}(CCME, 2002; MELCC, 2019). Ce volume de 30 000 m^3 ha^{-1} an^{-1} pourrait être considéré comme seuil d'application pour la mise en place un système filtre à base de saules. Néanmoins, les capacités physiologiques du cultivar de saule qui sera utilisé dans le système filtre et la nature des effluents qui seront appliqués nécessitent considération.

Les résultats de ma troisième étude (chapitre 4) ainsi que celle de la deuxième étude (chapitre 3) peuvent être d'un grand d'intérêt pour les industries des biocarburants et des bioproduits. En effet, les différences dans la composition et dans l'accessibilité aux sucres dans le bois des différents cultivars de saules soumis aux différents traitements de fertilisation peuvent influencer le choix de culture ou de la fertilisation en fonction des besoins de l'industrie. Par exemple, des cultivars avec une bonne production aérienne et une structure cellulaire plus accessible à la dégradation enzymatique peuvent été utilisés pour la production de bioéthanol cellulosique, alors que des cultivars avec des taux plus élevés en composés phénoliques et en lignine pourraient servir respectivement pour la chimie verte et pour la combustion.

5.7 Perspectives

Les concepts de la phytoremédiation des sols et des eaux se sont beaucoup développés au courant des dernières années notamment grâce à l'amélioration des techniques de culture des plantes filtrantes ainsi qu'à la sélection de génotypes plus tolérants aux stress et plus performantes en termes de dépollution. Toutefois, la phytofiltration demeure un des concepts les plus prometteurs de la phytoremédiation et le plus enclin à être appliqué rapidement sur le terrain compte tenu de son court délai d'action intimement lié à la croissance des plantes et à l'accessibilité des contaminants en cause (essentiellement de l'azote et du phosphore). À l'opposé, la dépollution des sols contaminés par des métaux lourds et/ou des polluants organiques qui peut s'étendre sur plusieurs décennies.

Dans le contexte climatique actuel, les lois internationales imposent aux pays et gouvernements de réduire leurs émissions des gaz à effet de serre et de favoriser une transition vers l'utilisation d'énergies renouvelables. Les résultats apportés par nos recherches cadrent très bien avec cette perspective. L'irrigation avec des effluents organiques permettrait d'augmenter le taux de carbone atmosphérique assimilé par les plantes (c.-à-d. la photosynthèse) tout en produisant une matière première renouvelable pour le marché des biocarburants.

Outre les avantages économiques et environnementaux qui résultent de cette pratique, la production de biocarburants à partir de la biomasse issue de cultures filtrantes a aussi des avantages sociétaux en favorisant la création d'emplois locaux ainsi que la mise en place de pôles industriels particulièrement dans les zones rurales.

Chapitre 6 | Bibliographie

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