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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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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

Highlights

- Primary effluent wastewater treatment using a fast-growing willow trees plantation

- Lysimeters show high nitrogen concentrations reduced to below regulatory limits
- 19 million litres of wastewater per hectare substantially increases biomass yield
- Wastewater tree stomatal size and pore index increased despite density decreases
- Phytofiltration can treat wastewater while producing sustainable biomass

1. Introduction

More than 3 trillion litres per year of municipal wastewater is discharged into surface water in Canada (CCME, 2006), 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 et al., 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 et al., 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 (Gnansounou and Dauriat, 2010; Hamelinck et al., 2005). Willow phytofiltration plantations could represent a solution for environmental wastewater treatment by combining high biomass yield, water demand and transpiration rates with contamination tolerance (Börjesson and Berndes, 2006; Brereton et al., 2016; Gonzalez et al., 2018; Grip et al., 1989; McCracken and Johnston, 2015) as well as improving local biodiversity and urban greening (Dzhambov and Dimitrova, 2015; Haughton et al., 2009; 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 (g_s) (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 g_s (Farquhar et al., 1980). The long-term modification of g_s is determined by developmental alterations to stomatal size and stomatal density, with smaller stomata and higher stomatal density traditionally associated with increased g_s (Drake et al., 2013). Franks and Beerling (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 et al., 2002).

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 and Ögren (2007) observed a reduction of between 24-57 % in stomatal conductance in four hybrid *Salix* species pot-grown over four months under moderate drought stress. Similarly, Pereina and Kozlowski (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 et al., 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 et al., 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. Materials and methods

2.1. 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 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 hours 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.

2.2. 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 was 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 (Brown, 1998). Chemical oxygen demand (COD) of water was measured according to APHA (2012) 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, 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. 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 (g_s) 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. 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}) (Escudero and Mediavilla, 2003; Rijkers et al., 2000).

A second set of leaf sample included three fully expanded leaves per tree (four trees per plot) destructively harvested for chlorophyll and carotenoids content determination and stored in the dark at -80°C. Extraction followed (Hiscox and Israelstam, 1980); 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 hours, 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* = (25.06 x 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 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 et al., 2005).

2.4. 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 hours. Biomass yields are estimated based on dry matter yields at the planting density of 16,000 trees ha^{-1}.

2.5. 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.

3. Results

3.1. Wastewater loads, soil pore water and soil characterisation

The wastewater loads applied during year two of the willow coppice growth cycle were 1,944 and 2,961 mm for WWD1 and WWD2 treatment doses, respectively; an equivalent of 19 and 30 ML ha^{-1} yr^{-1} (Figure 1). The chemical oxygen demand (COD) of the applied wastewater was an average of 290 mg COD L^{-1} (±29) (Table 1; Figure 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 - 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 1,245 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 1, Figure 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 - 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 wastewater 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 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; 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 5.6-6.0 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) 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). 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 20-38 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).

3.2. 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 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 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 26.5 C:N respectively, than for both wastewater treatments at 14.0 C: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 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 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 cm^2 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 1).

The nitrogen leaf content per unit area also increased significantly in wastewater irrigated trees, whereas no difference (ANOVA, $p > 0.05$) was identified between any treatments for the photosynthetic nitrogen use efficiency (PNUE), which ranged between 9.2-10.8 µmol CO_2 g^{-1} N s^{-1} (Figure 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 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 wastewater treatment was only identified as having significantly higher stomatal length than PW (9% increase). 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 x10^{-2} in WWD1 and 7.7 x10^{-2} in WWD2, when compared to both UI and PW treated trees, which averaged 6.3 x10^{-2} and 6.2 x10^{-2}, respectively (Figure 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 g_s ($r = 0.56$; $p < 0.001$), 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 ± 1.6 t 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 5).

4. Discussion

4.1. 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 et al., 1991; Levy et al., 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 1; Figure 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. 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) established by the European Council (Miguel et al., 2014; Union, 1991). 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 et al., 1991; Levy et al., 2011), similar to total nitrogen reduction levels of 30-60 % in conventional treatment systems (Levy et al., 2011; Nourmohammadi et al., 2013; Tazkiaturrizki et al., 2018).

The nitrogen concentrations of the wastewater applied here were similar to those commonly reported for untreated sewage/municipal wastewater (Asano, 1994; FAO, 1992; Levy et al., 2011; Sedlak, 2018). Although positive yield increases have been observed when fertilizing willow at rates as high as 590 kg N ha^{-1} (Cavanagh et al., 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 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 less than 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 3,500% and 8,800% 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; MDDELCC, 2019) and total nitrogen concentration in the soil pore water was reduced to 5-13 % of that in wastewater (Figure 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 et al., 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; MDDELCC, 2015) and soil analysis revealed no significant differences in phosphorus between different treatments (Table 1, Figure 2). Taken together, this suggested complete removal of the high phosphorus 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; 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 et al., 1991) and 90 mg Cl L^{-1} (Levy et al., 2011). Although chloride can be growth-limiting, these high applications (cumulative wastewater 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 (Belaid et al., 2010; Halliwell et al., 2001). 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.

4.2. 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 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 (Bacon, 2009; Hacke et al., 2010; Merilo et al., 2006), 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 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 (Benomar et al., 2012; Escudero and Mediavilla, 2003). 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) (Goodman et al., 2014; Guo et al., 2011). 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 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 8-26 %. 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 (Bacon, 2009; Chaves, 2002; Mooney et al., 1997)**.** 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 1). This demonstrates that water availability did not drive the variation in stomatal conductance observed between the treatments and aligns with findings by Bowman and Conant (1994) that stomatal conductance positively associates with nitrogen fertilisation in willow.

Natural variation in stomatal conductance is primarily determined by the density (Franks and Beerling, 2009; Schlüter et al., 2003) and/or the size of stomata (Bucher et al., 2016; Lawson and Blatt, 2014). 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 (Figure 4, Figure 5, Supplementary file 1). High correlation of the stomatal conductance and pore length has previously been observed in *Salix* (Aasamaa et al., 2002). 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 (Butler et al., 2013; Galmés et al., 2007), 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 et al., 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.

4.3. 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 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.

5. 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.

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.

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Supplementary information

Supplementary file 1 - Field site precipitation, soil properties prior to treatment, additional wastewater, soil and soil pore water composition, Pearson correlations.

CRediT author statement

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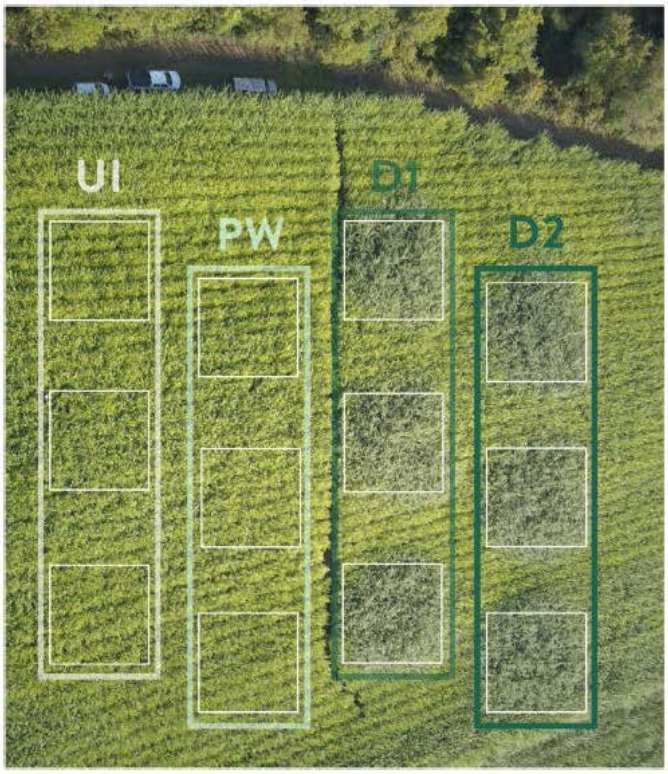
Tables

Table 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.

	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**

Figures



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 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 x 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.

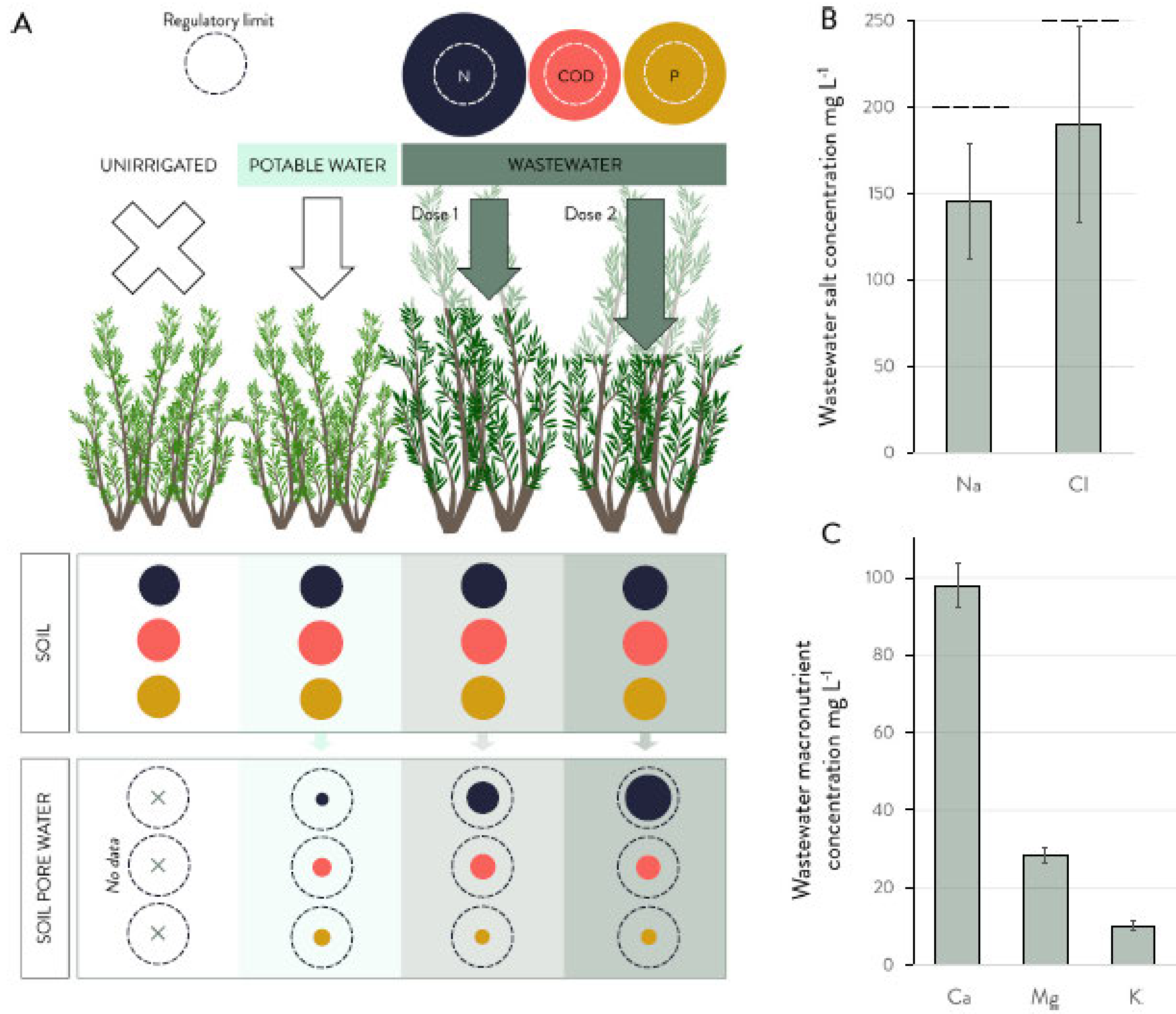


Figure 2 Phytofiltration reduction of wastewater contaminants below regulatory limits

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; MDDELCC, 2019), limits for COD set by the European commission are 125 mg COD L^{-1} (Miguel et al., 2014; Union, 1991), and the limit for phosphorus release into surface waters in Quebec is 1 mg P L^{-1} (CCME, 2002; MDDELCC, 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; MDDELCC, 2019) but have not yet been established for calcium, magnesium or potassium discharge. See Supplementary file 1 for more extensive composition data.

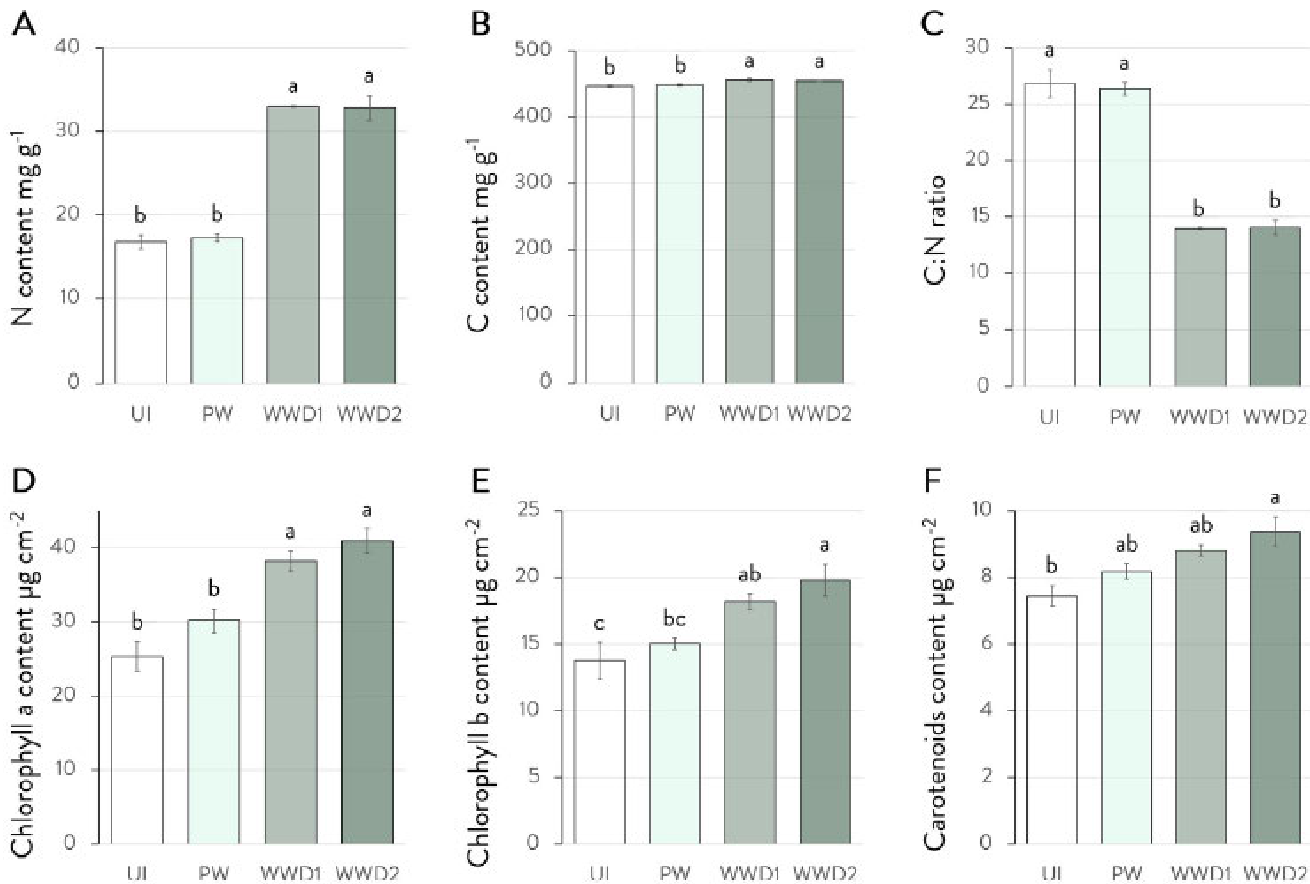


Figure 3 Leaf composition varies in response to wastewater treatment

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 ($\alpha = 0.05$).

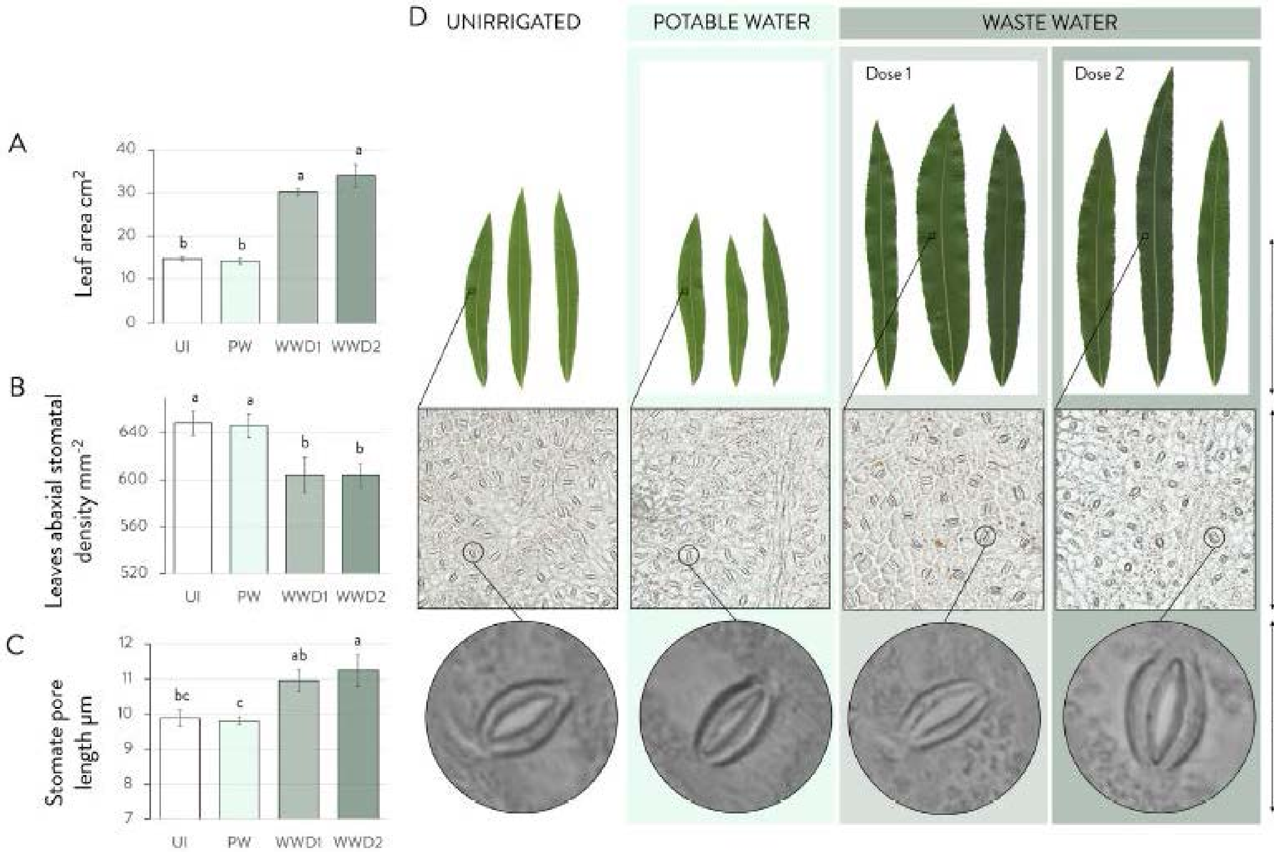


Figure 4 Leaf area, stomatal density and stomatal size vary due to wastewater treatment

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 (α = 0.05).

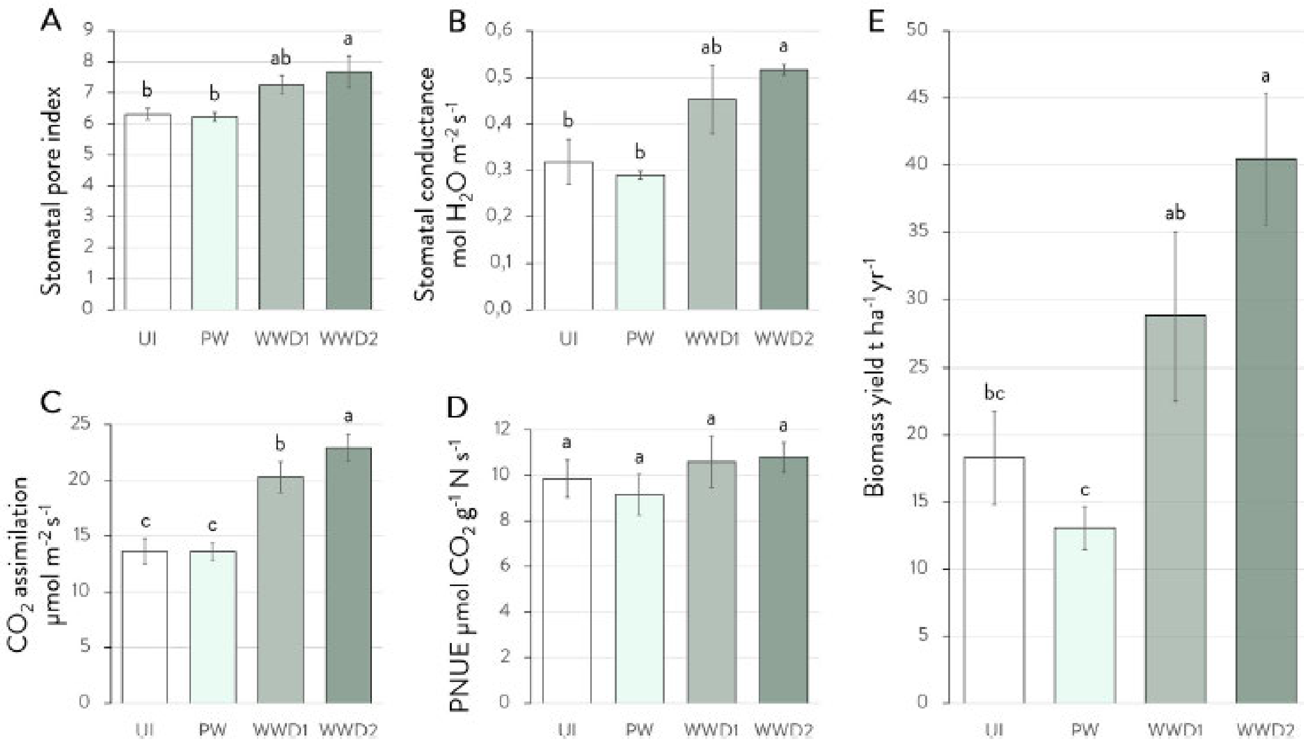


Figure 5 Wastewater treatment increases stomatal pore index, photosynthesis and biomass yield

A) stomatal pore index, B) stomata conductance (gs) C) net CO2 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).

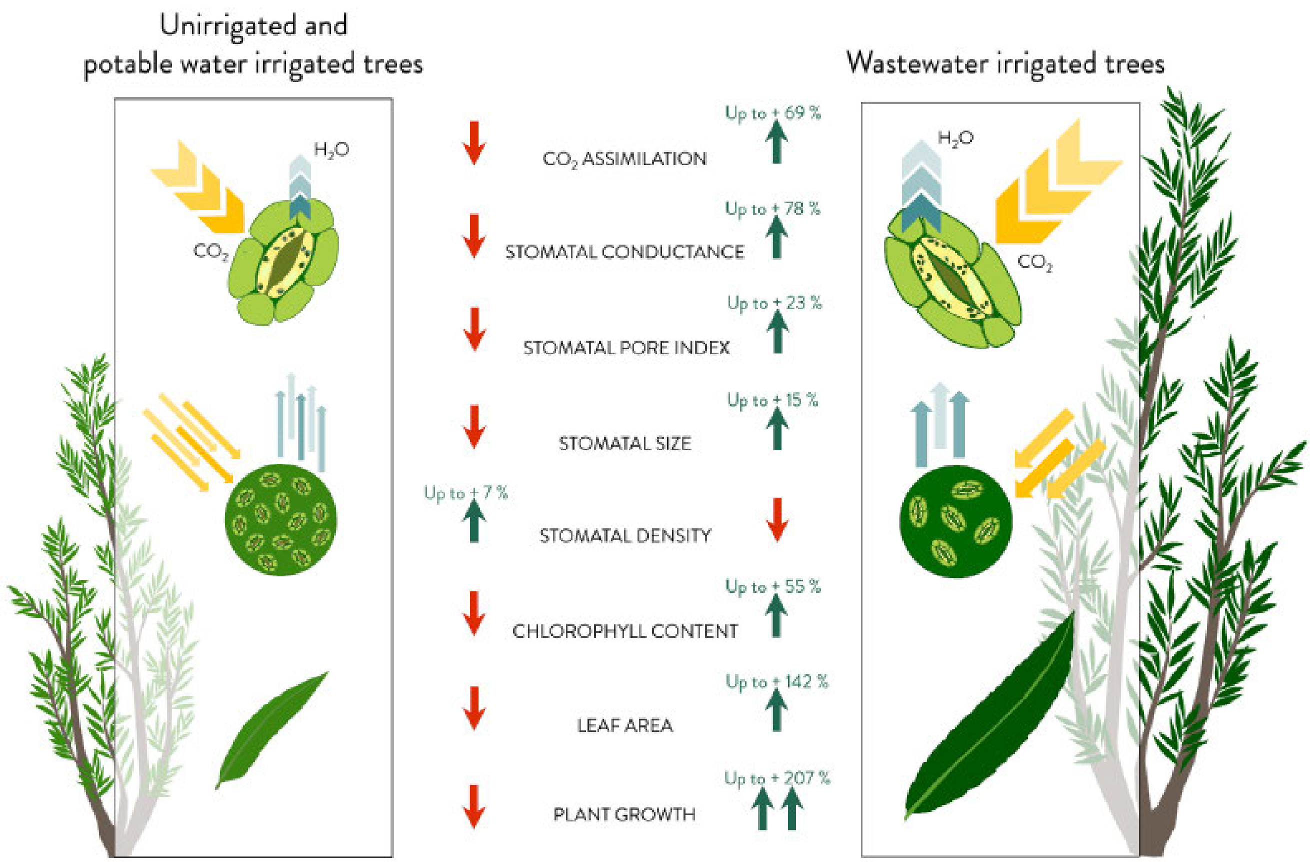


Figure 6 Willow wastewater phenotype

Summary schematic of tree modifications following primary effluent wastewater irrigation.