Short-Rotation Willows as a Wastewater Treatment Plant: Biomass Production and the Fate of Macronutrients and Metals

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Abstract: Evapotranspirative willow systems (EWS) are zero-discharge wastewater treatment plants that produce woody biomass and have no discharge to surface or groundwater bodies. The influence of wastewater on the growth of three clones of *Salix alba* ('V 093', 'V 051' and 'V 160') and the distribution of macronutrients and metals in a pilot EWS receiving primary treated municipal wastewater was studied under a sub-Mediterranean climate. The influent wastewater, shoot number, stem height, and biomass production at coppicing were monitored in two consecutive two-year rotations. Soil properties and the concentrations of macronutrients and metals in soil and woody biomass were analyzed after the first rotation. *S. alba* clones in EWS produced significantly more woody biomass compared to controls. 'V 052' produced the highest biomass yield in both rotations (38–59 t DM ha^{-1}) and had the highest nitrogen and phosphorus uptake (48% and 45%) from wastewater. Nitrogen and phosphorus uptake into the harvestable woody biomass was significantly higher in all clones studied compared to other plant-based wastewater treatment plants, indicating the nutrient recovery potential of EWS. The indigenous white willow clone 'V 160' had the lowest biomass yield but absorbed more nutrients from wastewater compared to 'V 093'. Wastewater composition and load were consistent with the nutrient requirements of the willows; however, an increase in salinity was observed after only two years of operation, which could affect EWS efficiency and nutrient recovery in the long term.

Keywords: evapotranspirative willow system; resource recovery; sustainable wastewater treatment; short rotation coppice

1. Introduction

The coupling of domestic wastewater treatment with short-rotation willow coppice (SRWC) biomass production originated in Scandinavia in the 1980s, when agricultural willow plantations producing woody biomass for energy purposes were recognized as a potential treatment system for domestic wastewater [1,2]. Untreated domestic wastewater and nutrient-rich effluents from central wastewater treatment plants (WWTPs) with secondary treatment resulted in pollution of surface and groundwater, while on the other hand the profitability of SRWC was reduced due to the expensive mineral fertilizer and water requirements. So-called vegetation filter systems consisting of SRWC irrigated with domestic wastewater [3–6], industrial wastewater [4,7] or landfill leachate [8,9] have been explored and applied.

However, the irrigation of SRWC with wastewater cannot completely eliminate wastewater pollution, as some of the applied wastewater runs off or seeps into the environment; therefore, special attention must be paid to irrigation rates and timing [5,10,11]. Moreover, such applications are not suitable for areas with sensitive ground and surface waters. In response, zero-discharge evapotranspirative willow systems (EWS) were developed in Denmark in the 1990s [12,13], and their field of application shifted from agricultural biomass production to the wastewater treatment sector.

EWS are a special type of WWTP used to treat domestic wastewater from small settlements or individual households. The system consists of a 1.5-m deep watertight basin filled with soil and planted with willow clones (*Salix* sp.). The primary treated domestic wastewater is applied under pressure through an inlet pipe located 0.6 m below the ground surface. When properly designed, all influent wastewater and precipitation are evapotranspired on an annual basis, i.e., all influent wastewater is used for willow growth and evaporation [12,13].

EWS are now used in rural areas in all Scandinavian countries, the Baltic States, Poland, England [13], Ireland [14], and China [15] and there are pilot studies in Slovenia [16], France and Greece [4]. EWS provide efficient wastewater treatment and do not require large amounts of energy or skilled personnel for operation and maintenance. As such, they are a suitable technology for decentralized sustainable wastewater management. As EWS are zero-discharge systems that generate woody biomass from wastewater, they allow the direct recovery and reuse of resources and support the goals of the closed-loop concept. In addition, zero-discharge wastewater treatment systems also contribute significantly to the reduction of surface and groundwater pollution.

The potential of EWS for nutrient recovery and the effects of wastewater on willow growth and biomass production require further research attention. Most scientific work focuses on wastewater-irrigated SRWC; however, there is much less scientific research on EWS. In addition, research on EWS pays much attention to evapotranspiration rates and the hydraulic loadings of the system [17–19], but nutrient recovery and the fate of heavy metals are rarely addressed [12]. However, numerous authors have shown that the composition of the wastewater corresponds to the nutrient requirements of willows [12,20,21].

The efficiency in the uptake of pollutants from wastewater into woody biomass, and their accumulation in system media, are critical for planning, management, system performance evaluation and resource recovery. There is a need to estimate the mass balance of nutrients in EWS, i.e., their percentage of harvestable woody biomass and accumulation in the soil compared to their amount in the influent wastewater. The level of nutrients entering the system with domestic wastewater is similar to the level of nutrients in the willow biomass: the proportional requirement of willow for N, P and K (100:14:72) is similar to the proportion of these nutrients usually found in municipal wastewater (100:18:65) [20]. The exception is P, which was reported to be 30% higher in influent wastewater than in biomass; however, the P balance also depends on the use of P-containing detergents in the household(s) producing the wastewater [12]. Consequently, a significant increase in available P in the soil was reported [22], suggesting that soil may become saturated with P after a period of time. This may lead to a problem in SRWC vegetation filter treatment performance and reduces P recovery via woody biomass. Therefore, Lachapelle-T et al. [22] suggest that fertigation should be adjusted according to seasonal transpiration rates and plant nutrient requirements; however, in the case of EWS, wastewater is constantly applied according to the production in the household(s) and stored in the EWS as an elevated water level over the winter. Therefore, in the case of EWS, P accumulation in the system can be expected, resulting in a P-rich substrate that can be reused as fertilizer.

Fertigation with wastewater significantly increases willow yield compared to commercial rainfed- or potable water-irrigated SRWC [21,22]. The differences in yield increase depend on the characterization of the wastewater and the loading rate. Similarly, Curneen and Gill [17] reported the highest biomass and evapotranspiration for willow cultivars receiving septic tank effluent, compared to systems fed with secondary treated effluent and rainwater. The higher biomass production of willows fertigated with wastewater is reflected in larger stem diameter and plant height compared to non-fertigated plants [23]. However, when wastewater is applied to SRWC, not only do the nutrients increase biomass yield, but also the constant water availability [21]. In addition, water use by willows has been shown to be positively affected by N and P application [24]; however, permanent flooding had negative effects on most growth parameters in willows, except for the number of shoots per plant and root biomass [25].

Total woody biomass production is comparable in SRWC irrigated with wastewater and EWS, varying from 10 [2,12] to 22–26 [22] t dry matter (DM) ha^{-1} yr^{-1} in Denmark, Sweden and Canada, while under Mediterranean climatic conditions, aboveground biomass production can reach up to 64 t DM ha^{-1} in a two-year rotation [23], suggesting that climate may have a significant influence on system performance. In addition to climate, planting density, irrigation regimes, willow age and clone choice can also influence woody biomass production [5], while some authors find no differences between clones or irrigation regimes [26].

Plants used in EWS must have similar characteristics to those used in other phytotechnologies. They must be adapted to high nutrient and salinity levels that could increase in the system over time, and should have high transpiration rates, rapid growth and biomass production, high plantation densities, and coppicing ability. Willows and poplars have all these characteristics [27], supported also by their numerous and deep roots that provide a large root surface area [28]; however, willows show better tolerance to permanent flooding and anaerobic conditions than poplars [29,30], enabled by their important adaptations such as hypertrophied lenticels, aerenchyma, and adventitious roots [31]. Additionally, willows have higher water requirements than almost all agricultural crops [32], which is another important requirement for plants used in EWS.

Although willows are the first choice for this type of phytoremediation, different willow species and clones show different efficiencies in terms of growth, nitrogen and water use efficiency [33]. To date, most studies on EWS and SRWC for the treatment of municipal wastewater have been conducted in Europe and North America. In Denmark, the UK, Sweden, and Ireland, *Salix viminalis* has been mainly used, namely, the clones 'Jorr' (*S. viminalis*), 'Tora' (*S. viminalis* × *S. schwerinii* E. Wolf), and 'Bjørn' (*S. schwerinii* E. Wolf × *S. viminalis* L.) [11,12,18], while Rastas Amofah et al. [34], Sweden, used the frost-tolerant *S. viminalis* crossbreed 'Karin' ((*S. schwerinii* × *S. viminalis*) × *S. viminalis*) and 'Gudrun' (*S. burjatica* Nasarow × *S. dasyclados* Wimm). In Canada, research on an SRWC vegetation filter treating municipal wastewater was conducted on *S. miyabeana* 'SX67' [6,22]. *S. purpurea* [9,35], *S. amygdalina* [36] and other willow species were used to treat landfill leachate. *S. alba* var. 'Chermesina' was tested for phytoremediation in Poland [37], while *S. alba* clones used in this study were evaluated for their biomass production potential by Kajba and Andrić [38].

Concentrations of heavy metals in plant tissues and media can be correlated with heavy metal concentrations in the environment; however, most heavy metals are stored in roots, and transport to aboveground tissues may be limited [39,40]. Furthermore, typical domestic wastewater contains low levels of heavy metals [41]; therefore, elevated concentrations are not expected in the woody biomass of EWS. On the other hand, heavy metals entering the EWS may accumulate in the roots and soil media over the long term, which may affect reuse options after the decommissioning of the facility. Therefore, in this study, we analyzed the concentrations of heavy metals in both woody biomass and soil media to assess the risks of reusing the system components.

The objective of this study was to investigate the potential of EWS to recover nutrients from primary treated municipal wastewater through the production of woody biomass and accumulation in the soil. In addition, the growth dynamics, biomass production and response of selected *S. alba* clones to wastewater irrigation in the sub-Mediterranean climate were investigated, to provide data for the proper design and operation of EWS as wastewater treatment plants in rural areas of the sub-Mediterranean region. In addition, *S. alba* was not tested in EWS before.

2. Materials and Methods

2.1. Pilot Evapotranspirative Willow System

The study was conducted on a 27 m^2 pilot EWS built next to a municipal WWTP in Ajdovščina, Slovenia (45°52′32″ N 13°54′20″ E). A detailed description and illustration of the pilot plant can be found in Istenič et al. [16]. Briefly, the pilot EWS consisted of

nine watertight treatment beds (each 3 m long, 1 m wide), filled with local soil (1.5 m deep) and planted with three clones of *S. alba* at a density of 1 tree per m^2. Each clone was tested in three parallel beds distributed in a Latin square to minimize environmental differences caused by positioning (north/south orientation, prevailing wind direction, etc.). In addition, control trees were planted around the EWS to avoid the edge effect and to be monitored as control plants.

Three *S. alba* clones from a selection of Croatian arborescent willows were tested, namely, two hybrids 'V 052' (*S. alba* L. var. *calva* G.F.W. Mey × *S. alba* L.) and 'V 093' (*S. alba* L. × *S. alba* var. *vitellina* (L.) Stokes) × *S. alba* L.) and one clone of the indigenous white willow 'V 160' (*S. alba* L.). The willows were provided as 1-year-old seedlings, planted and immediately cut back to 10 cm above ground level. The EWS was fed with primary treated municipal wastewater. The amount of water supplied was adjusted according to the water requirements of the willows—the water level in the treatment beds was maintained at approximately 1 m (0.5 m below the surface) during the monitoring period and was allowed to evapotranspire completely before the start of a new season. The pilot system was commissioned in March 2016 and monitored for four consecutive growing seasons until the end of the growing season in 2019. The monitoring period lasted 169, 115, 106 and 134 days, respectively, for the 4 consecutive years.

2.2. Wastewater and Soil Analyses

Grab samples of influent wastewater were taken weekly and analyzed for the typical parameters of municipal wastewater, namely, biochemical oxygen demand (BOD$_5$), chemical oxygen demand (COD), total phosphorus (TP), orthophosphate phosphorus (PO$_4$-P), total nitrogen (TN), ammonium nitrogen (NH$_4$-N), nitrite nitrogen (NO$_2$-N), nitrate nitrogen (NO$_3$-N), total suspended solids (TSS), settleable solids (SS), dissolved oxygen and oxygen saturation, temperature (T), pH and electrical conductivity (EC). Analyses followed the Standard methods for the examination of water and wastewater [42].

The mass loading of contaminants for each treatment bed in the EWS was calculated by multiplying the average contaminant concentrations in the wastewater by the total volume of water applied to each bed for each year, divided by the bed area. The mass loading is expressed in grams of added contaminants per m^2 of EWS.

A composite sample of the original fill soil was collected during the construction of the EWS. The grain size distribution was determined by sieving and sedimentation, according to SIST ISO 11277:2011, following USDA Textural Soil Classification [43] to define the soil texture class. After the first and second growing seasons, the soil was sampled according to the willow clones. Three samples were collected from each treatment bed using a soil probe and combined into one composite sample for each clone. Samples from the treatment beds and the sample from the original fill soil were analyzed for pH in 0.01 M CaCl$_2$ solution (SIST ISO 10390:2006), soil organic matter and organic carbon (SIST ISO 10694:1996), plant-available phosphorus (P$_2$O$_5$) and potassium (K$_2$O) (ÖNORM L 1087—modification with amonlactate extraction), total nitrogen (SIST ISO 13878:1999) and cation exchange capacity (CEC) (SIST ISO 13536:1996, modification by using KCl instead of BaCl$_2$). Ca, Mg, K and Na, as well as exchangeable acidity, the sum of base cations and base saturation were measured according to the Methods of Soil Analysis, ASA [44]. Heavy metal content was determined by extraction in aqua regia and analysis by ICP-MS.

2.3. Estimation of Willow Growth and Biomass Production

The number of shoots per stump was counted on each tree in the EWS and on control trees. Shoots were counted every month during the growing season; however, the number of shoots at the end of the growing season was used as the outcome. Stem height was measured every other week: the highest stem of each tree was measured with a wooden ruler. Mean and standard deviation were calculated for all control and test clones for each season.

The biomass production was measured at the 1st and 2nd harvest. All shoots from each test and control tree were harvested and weighed. Then, the shoots from each treatment bed were pooled and cut into woodchips. A sample of the woodchips was collected from each treatment bed for laboratory analysis of the moisture content. Based on the moisture content determined and the planting density (1 tree per m^2), the dry matter (DM) produced per hectare was calculated for each treatment bed. The mean and standard deviation of the beds with the same clone were calculated. Control trees were not grown in separate beds; therefore, all control trees of the same clone were combined into one sample.

The conversion of wastewater to biomass was calculated by dividing the DM produced in each treatment bed by the total amount of wastewater supplied to the bed during the 1st and 2nd rotation periods. The mean and standard deviation of all beds with the same clone were calculated.

2.4. Nutrient and Metal Content in the Woody Biomass

Samples of woodchips after the 1st rotation were further analyzed for carbon, nutrient and metal contents, namely, the total C content was analyzed according to SIST ISO 10694:1996, and total N content according to SIST ISO 13878:1999. P and the other 35 elements were analyzed by aqua regia digestion and ICP-MS.

The partitioning of P and N from wastewater (in g m^{-2}) between woody biomass and other compartments (accumulation in the root and leaf biomass, accumulation in the soil, and denitrification in the case of N) was studied by comparing the mass loads of TN and TP from wastewater, and the N and P content in the woody biomass produced (calculated by multiplying the N and P concentration in the dry matter by the total amount of biomass produced), while the N and P content in the other compartments together was calculated as the difference between the TN and TP, applied with the wastewater, and the content in the woody biomass. A similar procedure was also used by Lachapelle-T et al. [22].

2.5. Statistical Analyses

Microsoft Office Excel 2016 was used for statistical analysis of the data. The results are presented as mean and standard deviation of the mean. Significant differences were tested using one-way analyses of variance (ANOVA), with a significance level of 0.05 ($\alpha = 0.05$) between the mean values of shoot numbers in clones, test and control trees, and between the mean values of macronutrient and metal concentrations in control and test trees. When the results showed statistical significance, Student's *t*-test was used to further interpret the results and to show significant differences between the mean macronutrient and metal concentrations of different clones of the test trees.

3. Results

3.1. Wastewater Characteristics

The influent wastewater at the central WWTP in Ajdovščina is typical municipal wastewater with occasional elevated organic load (BOD_5 and COD), mainly originating from the food industry in the catchment area (Table 1). During the experiment, the influent wastewater also showed variable NH_4-N, NO_2-N and NO_3-N concentrations, which can be attributed to occasional nitrification in the primary clarifier from which the wastewater for the EWS was taken.

Table 1. Characteristics of municipal wastewater fed to the evapotranspirative willow system during four consecutive seasons.

	BOD_5	COD	TP	PO_4-P	TN	NH_4-N	NO_3-N	NO_2-N	TSS	SS	O_2	O_2	T	pH	EC
Unit					**mg/L**					**mL/L**	**mg/L**	**%**	**°C**		**µS/cm**
Average	452	739	5.75	2.84	50.6	23.3	0.298	0.092	207	7.4	2.87	33.0	23.9	6.68	441
SD	182	294	2.45	2.13	14.4	10.5	0.792	0.307	161	10.3	2.47	27.0	4.8	0.58	482
Nr	40	40	40	40	36	36	36	36	20	14	19	19	22	39	39

The three clones of *S. alba* received different amounts of wastewater depending on water use by evapotranspiration; consequently, mass loading rates varied among rotation periods and clones, and according to the influent composition (Table 2). Mass loading rates for all parameters were lowest in the 1st year of the 1st rotation, because water use by the willows was lowest (willows at age 1/2; 1-year-old stem with 2-year-old root system). In the 2nd year, the root systems and trees were more developed, resulting in higher water use. Consequently, more wastewater was supplied, and mass loading rates increased. Mass loading rates also increased slightly after the first coppicing (aged 1/4) and remained in the same range in the 2nd year of the 2nd rotation (aged 2/5). In the 2nd rotation, 'V 160' received lower mass loading rates due to lower water demand.

Table 2. Annual mass loading rates of evapotranspirative willow system beds in g m^{-2}, according to the three willow clones tested ('V 093', V 052' and 'V 160') for four consecutive years. Mean and standard deviation are given (N = 3).

	BOD_5	COD	TP	PO_4-P	TN	NH_4-N	NO_3-N	NO_2-N
				1st rotation, (aged 1/2)				
'V 093'	181 ± 9	278 ± 14	2.84 ± 0.15	1.50 ± 0.08	17.2 ± 0.9	11.1 + 0.1	0.023 ± 0.001	0.015 + 0.001
'V 052'	183 ± 64	281 ± 98	2.87 ± 1.0	1.51 ± 0.53	17.4 ± 6.1	11.2 ± 3.9	0.023 ± 0.008	0.015 ± 0.005
'V 160'	170 ± 21	262 ± 32	2.67 ± 0.32	1.41 ± 0.17	16.2 ± 2.0	10.4 ± 1.3	0.022 ± 0.003	0.014 ± 0.002
				1st rotation, (aged 2/3)				
'V 093'	392 ± 61	600 ± 93	8.62 ± 1.33	6.32 ± 0.98	53.6 ± 8.3	34.7 ± 5.4	0.278 ± 0.043	0.072 ± 0.111
'V 052'	323 ± 114	496 ± 174	7.12 ± 2.51	5.21 ± 1.84	44.3 ± 15.6	28.6 ± 10.1	0.230 ± 0.081	0.060 ± 0.021
'V 160'	326 ± 55	499 ± 84	7.17 ± 1.21	5.25 ± 0.89	44.6 ± 7.5	28.8 ± 4.9	0.231 ± 0.039	0.060 ± 0.010
				2nd rotation, (aged 1/4)				
'V 093'	485 ± 7	793 ± 11	6.17 ± 0.08	3.05 ± 0.04	54.3 ± 0.7	25.0 ± 0.3	0.319 ± 0.004	0.099 ± 0.001
'V 052'	469 ± 54	766 ± 88	5.97 ± 0.69	2.95 ± 0.34	52.4 ± 6.1	24.2 ± 2.8	0.309 ± 0.036	0.095 ± 0.011
'V 160'	406 ± 103	665 ± 169	5.17 ± 1.31	2.56 ± 0.65	45.5 ± 11.6	21.0 ± 5.3	0.268 ± 0.068	0.083 ± 0.021
				2nd rotation, (aged 2/5)				
'V 093'	592 ± 31	1019 ± 54	4.89 ± 0.26	1.30 ± 0.07	68.6 ± 3.6	18.8 ± 1.0	0.599 ± 0.032	0.182 ± 0.010
'V 052'	619 ± 130	1064 ± 224	5.11 ± 1.08	1.35 ± 0.29	71.6 ± 15.1	19.7 ± 4.1	0.626 ± 0.132	0.190 ± 0.040
'V 160'	471 ± 148	810 ± 254	3.89 ± 1.22	1.03 ± 0.32	54.5 ± 17.1	15.0 ± 4.7	0.476 ± 0.149	0.145 ± 0.045

3.2. Willow Growth and Biomass Production

As expected, the number of shoots per stump increased significantly in the 2nd rotation period, confirming that coppicing stimulates the formation of multiple shoots (Figure 1). In the 1st rotation period, the test willows of 'V 052' and 'V 160' had significantly more shoots compared to the control trees, while in the case of 'V 093' the situation was reversed, and the control trees produced more shoots. Moreover, the test trees of 'V 093' had significantly fewer shoots (5.8 ± 0.3) compared to 'V 052' and 'V 160' (7.6 ± 0.7 and 7.5 ± 0.7, respectively). In the 2nd rotation, the differences between the clones became more evident: 'V 052' produced 27 ± 7 shoots per stump, which was significantly more than 'V 093' and 'V 160', which produced 17 ± 5 and 17 ± 6 shoots per stump, respectively. All the clones developed significantly more shoots in the EWS compared to control trees. However, in the 2nd year of the 2nd rotation, the number of shoots reduced in all test clones. The greatest reduction was observed in clones 'V 160' and 'V 052' (27% and 25%, respectively), while the reduction in 'V 093' was only 12%. This reduction was not observed

in control trees, which already had a lower number of shoots. In 2019, only one control tree of 'V 160' survived.
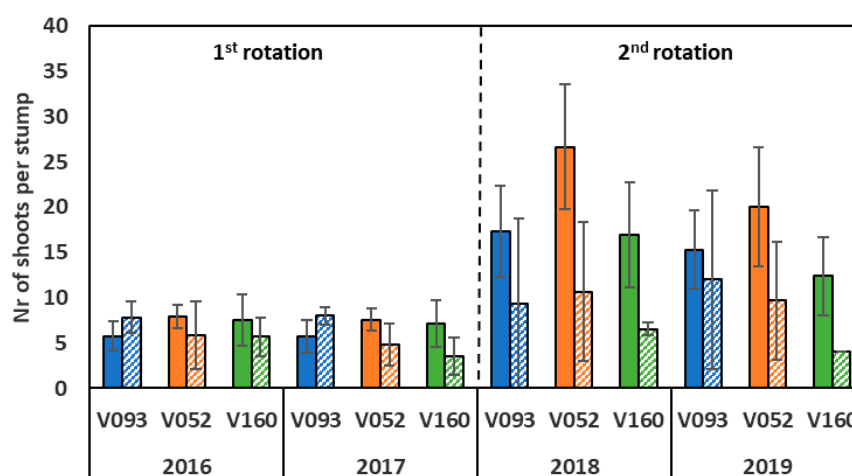

Figure 1. Mean number of shoots per stump at the end of each growing season for three clones of *S. alba* ('V 093', 'V 052' and 'V 160') in the evapotranspirative willow system (full columns) and controls (dashed columns). Mean and standard deviation are given (*N* = 9 for test trees and 1–6 for control trees).

In the 1st year of the 1st rotation, the test willows reached about 2.5 m and were 20–40 cm taller than the control trees (Figure 2). In the 2nd growing season, the willows in the EWS grew another 2 m, with 'V 052' being taller (479 ± 50 cm) than 'V 093' (463 ± 37 cm) and 'V 160' (431 ± 65 cm). The difference from the control trees increased: test trees for 'V 052', 'V 093' and 'V 160' were 1.2, 1.6 and 2.1 m taller than the control trees, respectively. After the first coppicing, the willows grew back successfully and rapidly, reaching 3.4 ('V 160') and 3.8 m ('V 093' and 'V 052'). In the 2nd growing season of the 2nd rotation, the difference between the clones increased and showed the same trend as in the 2nd growing season of the 1st rotation, with 'V 052' being the tallest (501 ± 52 cm), followed by 'V 093' (498 ± 18 cm) and 'V 160' (423 ± 27 cm), which was again the shortest.

The clones of *S. alba* in EWS produced on average between 34–38 and 33–59 t DM ha^{-1} for the 1st and 2nd rotation, respectively (Figure 3). Biomass production was much higher compared to the controls, reaching a maximum of 14 t DM ha^{-1}. The standard deviations of the mean biomass production between the treatment beds of the same clone were high because the beds in the pilot EWS had different positions to sun and wind, resulting in different growing conditions. In the 2nd rotation, biomass production of 'V 093' and 'V 052' increased by 21% and 55%, respectively, compared to the 1st rotation, while it remained in the same range for 'V 160'. Additionally, the increase in conversion of wastewater to woody biomass was even more obvious: the conversion increased significantly in the 2nd rotation, when the clones produced 5.8 ± 1.1, 6.8 ± 0.5 and 8.5 ± 0.6 kg DM per m^3 of wastewater, for 'V 160', 'V 093' and 'V 052', respectively. Moreover, 'V 052' showed significantly higher conversion compared to the other two clones in the 2nd rotation (Figure 4).
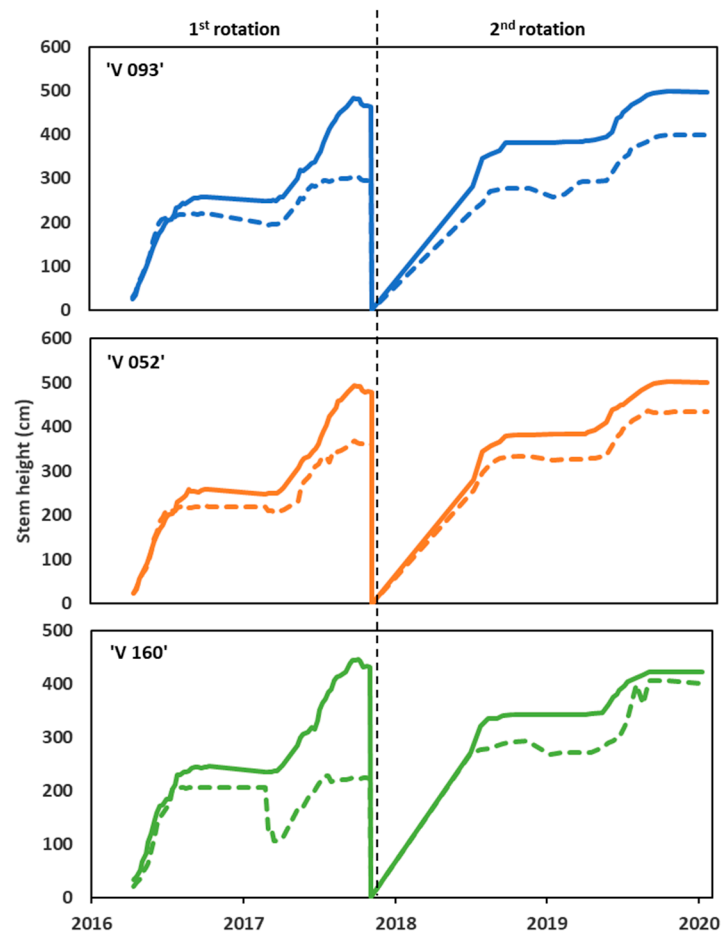
Figure 2. Mean **st**em height in two consecutive two-year rotations for three clones of *S. alba* ('V 093', 'V 052' and 'V 160') in evapotranspirative willow system (solid lines) and controls (dashed lines) (*N* = 9 for test trees and 1–6 for control trees).
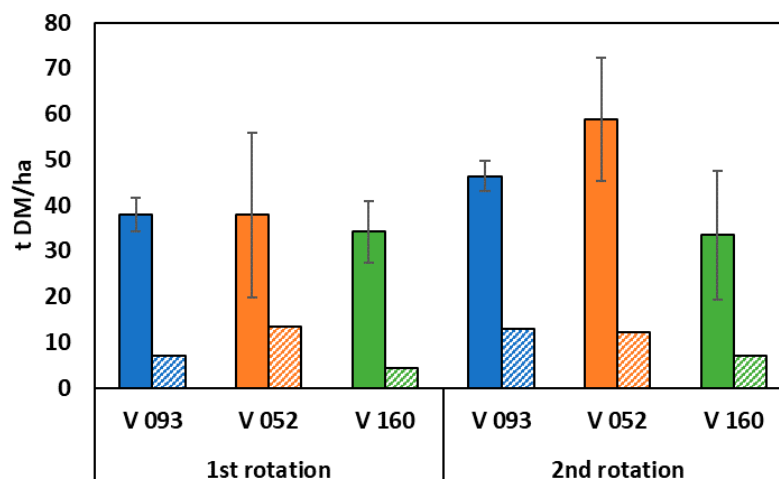
Figure 3. Mean biomass production in the 1st and 2nd rotation for three clones of *S. alba* ('V 093', 'V 052' and 'V 160') in evapotranspirative willow system (solid-filled columns) and controls (pattern-filled columns) (*N* = 3 for evapotranspirative willow system and 1 for control).
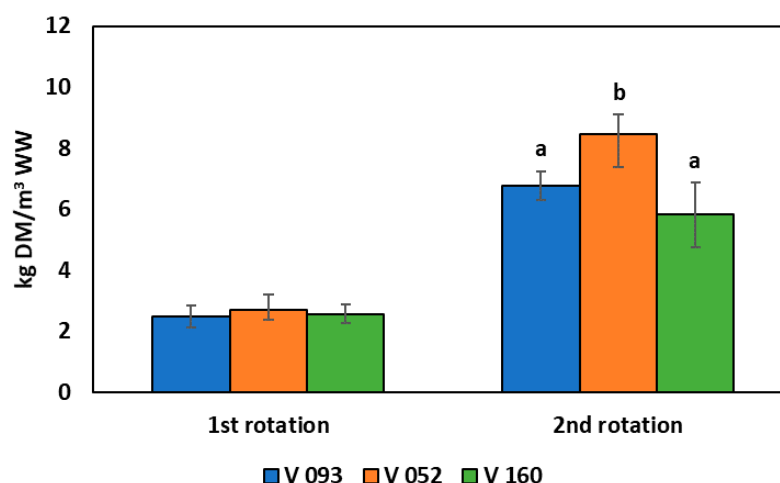
Figure 4. Mean biomass dry matter (DM) produced per m³ of added wastewater for three clones of *S. alba* ('V 093', 'V 052', 'V 160') in evapotranspirative willow system for the 1st and 2nd rotation. Letters a, b, show statistically significant differences between clones in the 2nd rotation (*N* = 3).

3.3. Fate of Macronutrients

Macronutrients (C, N, P, K, Ca, Mg, and S) are major components of plant biomass. For willows in the EWS, they are derived from the atmosphere (C), soil, and wastewater (N, P, K, Ca, Mg, and S). The EWS was filled with locally available clay soil (Table 3). The properties of a soil composite sample before the addition of wastewater indicate a relatively fertile soil, with high P and organic matter content (Table 4). Irrigation with wastewater for two consecutive seasons of the 1st rotation resulted in an increase in some soil parameters. There was no increase in soil organic matter and organic carbon, indicating the efficient decomposition of organic matter from wastewater. Total N increased after the 1st growing season, indicating excessive N input to the young willows; however, in the 2nd growing season, despite the much greater N load from wastewater, there was no further N accumulation, probably due to the intensive growth of willows and possible denitrification. Due to N uptake, the C/N ratio also increased in the 2nd growing season. Similarly, the P supplied by the wastewater also seemed to meet the P demand of the willows, since there was neither an accumulation of P_2O_5 nor a decrease in the soil. On the other hand, K_2O content, which was already relatively low in the original soil, increased with the addition of wastewater, suggesting that the K requirement of the willows was met and excess K_2O accumulated in the soil, or that K uptake was displaced by other minerals. This is consistent with the results of cation analysis, namely, that soil concentrations of Ca and K cations increased in the 2nd year; however, changes in Mg concentrations showed no such trend. In contrast to the other cations, Na concentrations increased over the years, indicating salt accumulation in the system.

Table 3. Texture of the soil used to fill up the evapotranspirative willow system.

Parameter	Percentage/Classification
Sand	26.1
Silt—coarse	18.1
Silt—fine	30.4
Silt—total	48.5
Clay	25.4
Classification *	clay

* according to USDA textural soil classification.

Table 4. Soil properties in the evapotranspirative willow system before wastewater addition (Start), after the 1st and 2nd year of the 1st rotation. Mean and standard deviation are given for the first and second year ($N = 3$).

|	Unit	Start *	1st Year	2nd Year
pH in CaCl$_2$		7.1	7.1 ± 0.06	7.3 ± 0.06
P$_2$O$_5$	mg/100 g	144	162 ± 46.6	177 ± 3.13
K$_2$O	mg/100 g	13.0	13.7 ± 0.66	14.4 ± 0.32
Organic matter	%	7.0	7.5 ± 0.3	7.2 ± 0.5
Organic carbon	%	4.1	4.3 ± 0.15	4.2 ± 0.3
TN	%	0.43	0.48 ± 0.01	0.40 ± 0.04
C/N ratio		9.5	9.0 ± 0.32	10.5 ± 0.40
Ca	mmol/100 g	35.2	30.0 ± 0.68	36.8 ± 1.57
Mg	mmol/100 g	2.74	3.11 ± 0.12	2.87 ± 0.09
K	mmol/100 g	0.30	0.20 ± 0.02	0.30 ± 0.02
Na	mmol/100 g	0.05	0.17 ± 0.02	0.43 ± 0.10
Exchangable acidity	mmol/100 g	3.55	NA	4.80 ± 0.22
Sum of base cations	mmol/100 g	38.3	33.4 ± 0.78	40.4 ± 1.74
CEC	mmol/100 g	41.8	NA	45.2 ± 1.54
Base saturation	%	91.6	NA	89.4 ± 0.84

* before application of wastewater. .

In the woody biomass, the amount of C did not differ significantly between the control and test trees (Table 5); however, 'V 093' showed a trend of storing more C compared to the other two clones in both control and test trees. There was no statistically significant difference in N and P contents between the test and control trees. When comparing the test clones, 'V 052' appeared to accumulate more N and P compared to the other two clones; however, the difference was not always statistically significant. The test trees showed significantly lower K and Ca contents and significantly higher S concentrations compared to the control trees. There was no significant difference in Mg content between the test and control trees; however, 'V 160' accumulated significantly more Mg in the test trees compared to the other two clones.

Table 5. Macronutrient concentrations (in g kg^{-1} DM) in the woody biomass of three clones of *S. alba* ('V 093', 'V 052' and 'V 160') in the evapotranspirative willow system and controls after the 1st rotation. Mean and standard deviation are given ($N = 2$ for control and 3 for test trees). *P*-value indicates statistical significance between control and test trees ($N = 6$ for control and 9 for test trees), and superscripts a and b indicate statistically significant differences between clones of the test trees.

	Control Trees			Test Trees			
	'V 093'	'V 052'	'V 160'	'V 093'	'V 052'	'V 160'	*P*
C	477 ± 1.8	473 ± 0.28	475 ± 0.0	477 ± 0.40 [a]	474 ± 0.31 [b]	474 ± 1.8 [ab]	0.845
N	7.6 ± 0.07	10 ± 0.2	7.6 ± 0.21	6.5 ± 1.0	7.9 ± 0.76	7.1 ± 0.73	0.081
P	0.99 ± 0.16	1.1 ± 0.40	0.91 ± 0.07	0.92 ± 0.23 [ab]	1.2 ± 0.04 [a]	1.0 ± 0.08 [b]	0.775
K	2.4 ± 0.07	2.9 ± 0.28	2.2 ± 0.14	1.8 ± 0.25	2.2 ± 0.21	2.0 ± 0.25	0.029
Ca	8.1 ± 2.8	8.2 ± 2.1	9.1 ± 0.07	5.0 ± 0.25	5.7 ± 0.78	5.8 ± 1.2	0.005
Mg	0.93 ± 0.18	1.1 ± 0.1	0.84 ± 0.16	0.75 ± 0.06 [b]	0.75 ± 0.04 [b]	0.87 ± 0.04 [a]	0.060
S	1.6 ± 0.42	1.7 ± 0.07	1.1 ± 0.50	1.9 ± 0.27	1.8 ± 0.23	1.9 ± 0.15	0.044

The results on the distribution of nutrients from the wastewater into the woody biomass of *S. alba* and the other compartments of the EWS show that 52–65% and 55–69% of N and P, respectively, were degraded and accumulated in the soil, root system and leaves, while 35–48% and 31–45% were stored in the woody biomass (Figure 5). 'V 052' showed the highest accumulation of nutrients (48% and 45% for N and P, respectively) and 'V 093'

the lowest (35% and 31% for N and P, respectively). The distribution of K could not be presented, as it was not measured in the influent wastewater.
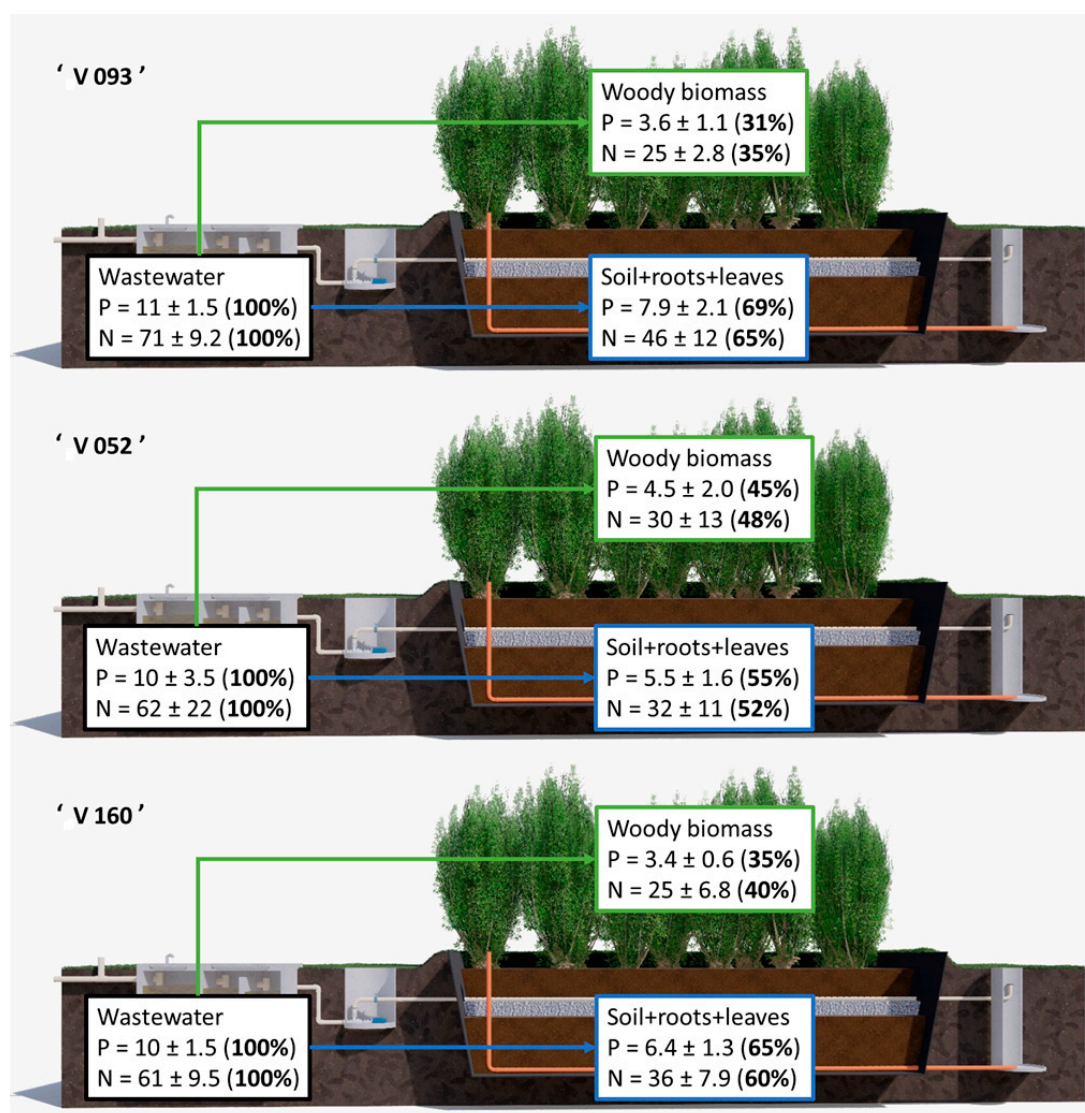
Figure 5. Distribution of total nitrogen (N) and phosphorus (P) from wastewater in g m^{-2} between woody biomass of *S. alba* and other compartments (accumulation in soil, roots and leaves and denitrification in the case of N) in the evapotranspirative willow system after the 1st rotation for three investigated clones ('V 093', 'V 052' and 'V 160').

3.4. Fate of Metals

The concentrations of metals in the woody biomass after the 1st rotation showed some significant differences between the control and test trees and between the clones of the test trees (Table 6). The concentrations were compared with the heavy metal concentrations in the soil (Table 7). After two growing seasons of irrigation with municipal wastewater, most of the heavy metals measured showed a slight increase in soil concentrations. There was no increase for Cd and As, while Cr and Pb were increased only in the soil of 'V 160'. The heavy metals studied are below the critical levels given in the decree on limit values, alert thresholds and critical levels of dangerous substances into the soil (OG RS, 68/96, 41/04).

The control trees accumulated significantly more Fe, Mo, Sr, Ba, Ti and B compared to the test trees, although the test trees had higher available Fe and Mo concentrations in the soil (Sr, Ba, Ti and B were not measured in the soil). On the other hand, Na and Ag occurred at significantly higher concentrations in the test trees, which may be related

to their presence in wastewater. The difference in heavy metal accumulation between clones was significant only for Cu and Mn: 'V 052' accumulated significantly higher concentrations of Cu and 'V 160' accumulated significantly higher concentrations of Mn, but the concentrations are much lower compared to soil, so there is no obvious transport of metals from soil to aboveground tissues.

Table 6. Metal concentrations in mg kg^{-1} DM in woody biomass for three clones of *S. alba* ('V 093', 'V 052' and 'V 160') in the evapotranspirative willow system and controls after the 1st rotation. Mean and standard deviation are given (*N* = 2 for control and 3 for test trees). *P*-value indicates statistical significance between control and test trees (*N* = 6 for control and 9 for test trees) and superscripts a, b, c, indicate statistically significant differences between clones of the test trees.

| | Control Trees | | | Test Trees | | | |
	'V 093'	**'V 052'**	**'V 160'**	**'V 093'**	**'V 052'**	**'V 160'**	**P**
Fe	105 ± 7	95 ± 7	75 ± 7	57 ± 6	63 ± 6	67 ± 6	0.003
Al	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
Na	20 ± 0	15 ± 7	25 ± 7	33 ± 6	33 ± 15	47 ± 12	0.003
Mo	0.04 ± 0.03	0.06 ± 0.00	0.05 ± 0.01	0.01 ± 0.01	0.02 ± 0.00	0.03 ± 0.02	0.008
Cu	7.8 ± 1.7	11.1 ± 1.0	8.8 ± 2.2	6.2 ± 0.6 [c]	10.0 ± 0.5 [a]	7.9 ± 0.4 [b]	0.271
Pb	1.22 ± 1.11	0.19 ± 0.04	0.16 ± 0.00	0.12 ± 0.01	1.55 ± 2.48	0.14 ± 0.03	0.885
Zn	46 ± 5	58 ± 24	40 ± 7	42 ± 2	42 ± 4	45 ± 5	0.436
Ni	0.70 ± 0.28	0.45 ± 0.07	0.30 ± 0.14	0.23 ± 0.12	0.30 ± 0.17	0.53 ± 0.40	0.343
Co	0.10 ± 0.01	0.11 ± 0.01	0.06 ± 0.00	0.35 ± 0.33	0.06 ± 0.01	0.08 ± 0.02	0.322
Mn	9.0 ± 2.8	10.0 ± 4.2	9.0 ± 1.4	9.3 ± 1.2 [b]	8.7 ± 0.6 [b]	15.3 ± 2.5 [a]	0.265
As	0.25 ± 0.07	0.40 ± 0.00	<LOD	0.29 ± 0.36	0.20 ± 0.10	0.11 ± 0.07	0.678
U	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.254
Th	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000
Sr	10.8 ± 4.6	10.9 ± 0.2	10.8 ± 1.8	6.6 ± 0.8	7.0 ± 1.0	7.6 ± 0.7	0.007
Cd	0.88 ± 0.56	0.72 ± 0.01	0.57 ± 0.04	0.43 ± 0.07	1.00 ± 0.96	0.49 ± 0.01	0.713
Sb	0.03 ± 0.01	0.02 ± 0.01	<LOD	<LOD	0.02 ± 0.00	<LOD	0.139
Bi	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.254
V	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
La	0.02 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	0.363
Cr	2.50 ± 0.85	2.05 ± 0.07	1.95 ± 0.21	2.17 ± 0.25	2.20 ± 0.20	2.50 ± 0.61	0.608
Ba	4.65 ± 2.05	4.85 ± 0.78	3.65 ± 0.07	2.20 ± 0.00	3.70 ± 3.03	2.40 ± 0.61	0.046
Ti	13.5 ± 0.7	27.0 ± 2.8	24.5 ± 9.2	4.0 ± 2.6	11.7 ± 7.8	18.7 ± 9.6	0.037
B	15.5 ± 4.9	14.5 ± 0.7	13.0 ± 0.0	9.3 ± 0.6	9.7 ± 1.5	9.7 ± 1.5	0.004
W	<LOD	0.20 ± 0.00	0.14 ± 0.09	2.29 ± 2.27	0.15 ± 0.13	0.15 ± 0.13	0.201
Sc	0.30 ± 0.14	0.25 ± 0.07	0.20 ± 0.00	0.23 ± 0.06	0.30 ± 0.00	0.27 ± 0.06	0.674
Tl	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.254
Se	0.30 ± 0.14	0.20 ± 0.00	0.20 ± 0.14	0.20 ± 0.00	0.23 ± 0.06	0.17 ± 0.06	0.488
Te	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.254
Ga	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000
Ag	3.21 ± 2.54	3.50 ± 0.71	3.00 ± 0.00	4.67 ± 1.53	6.67 ± 1.15	9.67 ± 1.53	0.002
Au	<LOD	<LOD	<LOD	<LOD	0.11 ± 0.17	<LOD	0.347
Hg	4.00 ± 1.41	2.50 ± 0.71	3.00 ± 1.41	1.90 ± 1.15	2.33 ± 1.53	3.33 ± 0.58	0.321

Table 7. Heavy metal concentrations in mg kg^{-1} of soil in the evapotranspirative willow system before wastewater addition (Start) and after the 1st rotation for the three clones of *S. alba* ('V 093', 'V 052' and 'V 160').

	Start	**After 1st Rotation**		
		'V 093'	**'V 052'**	**'V 160'**
Fe	28,700	29,200	29,300	31,000
Cd	0.7	0.6	0.7	0.6
Cu	66.2	67.2	66.5	72.8
Ni	80.9	78.1	81.3	86.6
Pb	36.4	34.1	35.5	42.2
Zn	153	161	172	166
Cr	63	60	59	67
Hg	0.56	0.79	0.76	0.65
Co	16.1	16.3	16.5	18.3
Mo	1.2	1.3	1.3	1.5
As	8.7	8	8.2	8.1
Mn	1012	1146	1110	1069

4. Discussion

4.1. Wastewater

Mass loading rates to the EWS were dependent on the composition of the influent wastewater, willow water use, and root system development. The most significant increase in mass loading rate was from the 1st to the 2nd year of the 1st rotation, when the most vigorous root development is assumed. Since the loading rate increased only slightly or remained in the same range in the following years, it is assumed that the root system developed almost to full capacity in the 2nd growing season. This is also in agreement with Rytter [45], who reported the highest increase in total belowground production (coarse and fine roots) in the 2nd growing season after planting.

The organic and nutrient loading rates in this study, i.e., 1.6–7.9 g COD, 0.10–0.53 g TN, and 0.02–0.08 g TP, applied per m^2 per day during the growing season, were in the same range as those reported by Amiot et al. [46], who applied 3.4–5.1 g COD, 0.48–0.72 g TN, and 0.05–0.07 g TP per m^2 per day to an SRWC vegetation filter, and reported efficient removal of pollutants from domestic wastewater during the growing season.

4.2. Willow Growth and Biomass Production

Cutting back is known to stimulate the formation of multiple shoots; however, different clones, planting densities, irrigation regimes, available nutrients, and plant age may result in significantly different numbers and heights of shoots [5,47]. The *S. alba* clones in this study developed 12–20 shoots per stump at the end of the 2nd rotation, while Holm and Heinsoo [5] reported 1.5–7.1 shoots per stump for clones of *S. viminalis*, *S. burjatica*, *S. dasyclados*, and *S. schwerinii* irrigated with municipal wastewater in Estonia. In addition, the willows in the EWS had higher and significantly more shoots compared to the controls, confirming the positive effects of adequate water and nutrient supply from wastewater. Although the controls were planted at the edges of the system and had more space available, they still developed fewer shoots compared to the densely vegetated willows in the EWS. The reduction in shoot number in the 2nd year of the 2nd rotation might indicate that in some clones a certain number of shoots becomes dominant and overtakes the growth while weaker shoots die off. In our study, this was particularly observed for clones 'V 160' and 'V 052'.

The annual biomass production (17–19 and 17–30 t DM ha^{-1} y^{-1}, for willows aged 2/3 and 2/5, respectively) was in the same range or higher than that reported by Kajba and Andrić [38] (10–19 and 19–24 t DM ha^{-1} y^{-1}, for willows aged 2/3 and 2/5, respectively), who tested biomass production of several clones, including those tested in this study, under controlled conditions in a nursery in Croatia. Similar biomass production was also reported by Lachapelle-T. et al. [22], namely, 22–26 t DM ha^{-1} y^{-1} for *S. miyabeana* aged

1/8, irrigated with wastewater under Canadian humid continental climatic conditions. However, both studies were conducted at almost the same geographical latitude (45°51′29″ N and 45°52′32″ N for Canadian and this study, respectively) that confirms the concordance between the studies, and shows that the latitude and the corresponding total amount of incoming solar energy might have more impact on biomass production than climate.

Willows in short-rotation plantations usually achieve the highest annual increment three to four years after planting, and it has been reported that an increase in the 2nd rotation is 18–62% compared to the 1st rotation [48]. Similarly, in our study, biomass production in 'V 093' and 'V 052' increased by 21% and 55% respectively, while there was no increase in 'V 160'. In subsequent rotations, the well-established root system supports the growth of aboveground shoots by providing the stored fixed carbon [27], thus maintaining the high annual increment.

The well-established root system together with the increased number of shoots also seems to increase the conversion of wastewater to biomass, as significantly more DM per m^3 of wastewater was produced in the 2nd rotation in all three clones, including 'V 160', which had the same total biomass production (34 and 33 t DM ha^{-1}), but produced 2.6 and 5.8 kg DM per m^3 of wastewater in the 1st and 2nd rotation, respectively.

The stimulatory effect of wastewater on willow growth can be demonstrated by comparing the biomass production of willows irrigated with wastewater and control willows irrigated with fresh water. In this study, control willows received only rainwater (about 1330 mm per year) and produced 5.5 t DM ha^{-1} y^{-1}, while biomass yield increased by 318% in EWS, which is much higher compared to other studies: Börjesson and Berndes [21] reported a 30–100% increase in wastewater-fertigated willow plantations compared to rainfed systems in Sweden, and Lachapelle-T. et al. [22] reported an 83–117% increase compared to *S. miyabeana* irrigated with potable water under Canadian climatic conditions. Similarly, Fabio and Smart [49] reported a 61% yield increase in willows irrigated with municipal waste, sludge, or wastewater, which was even higher than the yield increase from synthetic fertilizers (48%). This confirms that water is also a limiting factor for willow growth and that wastewater has a dual stimulating effect—it provides nutrients and increases water availability. The significantly higher yield increase in our study demonstrates the increased importance of water availability in the sub-Mediterranean climate. Despite the high annual precipitation (1330 mm), uneven distribution of rainfall, high average annual temperatures (13.5 °C), high solar radiation and strong winds caused high evapotranspiration (reference ET0 975 mm) and lower water availability for control willows, resulting in lower biomass production.

4.3. Fate of Macronutrients

The original soil in EWS was already nutrient-rich before the addition of wastewater. The P_2O_5 content of 144 mg in 100 g was far above the target values of fertile soil in agricultural production (13–25 mg in 100 g), and the organic content of 7% was higher than the normal organic content (3%) in the surrounding agricultural lands. To our knowledge, there are no upper limits for available P content in the soil. On the other hand, the values of K_2O were lower (13 mg in 100 g) compared to the target values in agriculture in Slovenia (20–30 mg in 100 g) [50]. The cation exchange capacity (CEC) was relatively high, as expected, due to the high content of clay and organic matter. Irrigation with wastewater caused changes in some soil chemical properties, as is well documented for agricultural wastewater reuse [51].

Many authors reported that wastewater meets the nutrient requirements of willows and that P may be present in excessive concentrations [12,20,22]. Thus, N, P and K are expected to be utilized by willows, while excess amounts are immobilized in the soil and N can be denitrified and released to the atmosphere in the form of N_2. Production of NO_X and N_2O in the denitrification process is not expected, due to high concentrations of dissolved organic carbon applied by wastewater and anoxic conditions in the system.

4.3.1. Carbon and Organic Matter

The primary treated wastewater applied to the EWS contained mainly organic matter and fewer inorganic nutrients. Despite significant amounts of organic matter applied to the EWS (2620–10,640 kg COD ha^{-1}), there was no significant increase in soil organic matter or organic carbon content, as is consistent with Lachapelle-T et al. [22], indicating the efficient decomposition of organic matter in the EWS. The C stored in the willow biomass was derived from the atmosphere. In this study, the C content in dry biomass was equal among clones and between test and control trees, varying between 473 and 477 mg C kg^{-1} DM, which is lower compared to the 502 mg C kg^{-1} DM reported by Stolarski et al. [52] in *S. alba* and the 491–518 mg C kg^{-1} DM reported by Matthews and Lamlom and Savidge [53,54] for different willow species. This variability is consistent with the findings of Stolarski et al. [52] that C content in willows differs according to location and genotype.

4.3.2. Nitrogen

The fertilization of commercial SRWC is usually based on N requirements [49]. Additionally, N uptake by willows is an important design parameter for SRWC vegetation filters [22]. The influence of N uptake by willows was also observed in this study, as the intensive growth of willows in the 2nd season reduced the percentage of total N accumulated in the soil during the 1st season. Total N applied via wastewater during the 2nd growing season in this study was 440–540 kg N ha^{-1}, which is in the same range as that reported by Lachapelle-T et al. [22] (370–580 kg N ha^{-1}). The uptake into woody biomass corresponds to 35–48% of the total N applied via wastewater, and the rest of the applied N (52–65%) was stored in roots and leaves or denitrified. The distribution is similar to that of Lachapelle-T et al. [22], i.e., 18–59% was stored in woody biomass, and 35–70% was accumulated in soil, roots, and leaves or denitrified. In both studies, the amount of N applied was much higher compared to the recommended N fertilization in commercial SRWC, which ranges from 40–180 kg N ha^{-1} [32,49]; however, N uptake was in the same range: 250–300, 88–260 and up to 311 kg N ha^{-1} for this study (*S. alba* aged 2/3), wastewater-irrigated SRWC (*S. viminalis* aged 3/3 and *S. miyabeana* aged 1/8) [17,22], and commercial SRWC (different species and ages) [49], respectively. Additionally, in this study, N concentrations in the woody biomass of control (rainfed) and test (wastewater-irrigated) trees of different clones did not differ significantly. This suggests a universal N uptake capacity of willows regardless of climate, N application and/or willow species/clones, and that excess applied N is subject to denitrification, accumulation in the soil, or leaching in the case of SRWC.

4.3.3. Phosphorus

Despite high P$_2$O$_5$ concentrations in the initial soil, P$_2$O$_5$ concentrations did not increase during the first two growing seasons in this study. The total P applied via wastewater in the 2nd growing season was higher in this study (71–86 kg P ha^{-1}) compared to that in Lachapelle-T et al. [22] (37–58 kg P ha^{-1}), while the percentage of total applied P incorporated into the woody biomass was similar (31–45% and 18–59% for this and the Canadian study, respectively) resulting in different P concentrations in the woody biomass: 34–45 and 9–26 kg P ha^{-1} for this study on *S. alba* (aged 2/3) and Lachapelle-T et al. [22] on *S. miyabeana* (age 1/8), respectively. Again, a different P uptake was reported by Curneen and Gill [17], namely, 28–35 kg P ha^{-1} for *S. viminalis* (aged 3/3). Additionally, in our study, there was no significant difference in P accumulation between the control (rainfed) and test (wastewater-irrigated) trees, while 'V 052' accumulated more P compared to the other two clones in EWS. This indicates differences in P uptake between different willow species/clones. The rest of the applied P was stored in leaves and roots and immobilized in the soil in a form unavailable to plants. The recommended P application is 24 kg P ha^{-1} [32], which is much lower compared to our and similar studies, suggesting that EWS might accumulate P in the soil during long-term operation. When the soil is saturated, an increase in P that is available in the soil is expected, as also observed by Lachapelle-T et al. [22].

4.3.4. Potassium

Potassium is not a basic wastewater parameter and is therefore rarely monitored in municipal wastewater. Its concentrations in wastewater range from 10–30 mg/L [55], which in the case of our wastewater load would result in 100–353 kg K ha^{-1} applied in the 2nd growing season, which is in the same range or higher than the recommendation for commercial SRWC, i.e., 120–155 kg K ha^{-1} [32]. In this study, K$_2$O levels were slightly elevated in the soil of the EWS, suggesting that the applied wastewater may have met the K requirement of the willows and excess K was accumulated in the soil. Accumulation of K in the woody biomass was 70–87 kg K ha^{-1}. According to K concentrations in wastewater as reported in the literature [55], this would result in 20–80% uptake of applied K into woody biomass, which is too wide a range to draw any conclusions regarding the distribution of applied K in the system. However, the accumulation of K in the woody biomass was much higher than the 21–29 kg K ha^{-1} reported by Curneen and Gill [17], and lower than the 85–123 kg K ha^{-1} reported by Gregersen and Brix [12], suggesting possible differences between willow species and clones and/or a response to the elevated environmental K concentrations (loads), as also observed for P but not for N. Similarly, Adegbidi et al. [56] also reported a significant increase in K and P, but also N in the case of fertilizer application. Although the applied wastewater appeared to have sufficiently high K concentrations for the needs of the willows and excess K was accumulated in the soil, the control willows in this study had significantly higher K content in the woody biomass, questioning the availability of K in the EWS.

4.3.5. Sulfur

The S content in control and test trees (1.1 to 1.9 mg S kg^{-1} DM) was much higher than the 0.57 mg S kg^{-1} DM determined by Stolarski [57] in shoots of *S. alba*. The difference may be due to the different S uptake of different clones of *S. alba* and the location [52], suggesting that the studied clones 'V 093', 'V 052' and 'V 160', when grown in a sub-Mediterranean climate, may emit more SO$_2$ during combustion when used as an energy crop. On the other hand, they allow a higher uptake of S from wastewater and have better S recycling potential.

4.3.6. Calcium and Magnesium

Different willow species can accumulate different concentrations of Ca and Mg [56,58]. In this study, the test trees of 'V 160' accumulated more Mg compared to the other two clones. Since fertilization has been shown not to increase Ca and Mg uptake in willows [56], the excess of these elements supplied via wastewater can be expected to accumulate in EWS in the long term. In our study, there was a slight increase in soil Ca concentration and no apparent changes in Mg concentration, which may indicate an adequate amount of available Ca and Mg for willow growth. An increase in soil Ca was also observed by Lachapelle-T et al. [22] when SRWC was irrigated with higher wastewater loads. Despite the slight increase in soil Ca in the EWS, the test trees had significantly lower Ca concentrations compared to the control trees. This is consistent with significantly lower K concentrations in the test trees and a significant increase in Na concentration in the soil, indicating increasing salinity in a zero-discharge EWS. Salinity can lead to nutrient deficiencies or disproportions, due to the competition of Na$^+$ with K$^+$ and Ca^{2+} in the soil–root system [59]. Increasing salinity in EWS was recognized as a potential problem already when systems were designed [12]; however, the systems in Denmark have been operated for more than 20 years with no apparent detrimental effects of salinity on willow growth or evapotranspiration [13]. According to Gregersen and Brix [12], the electrical conductivity in EWS beds did not increase during the first two years of operation and did not show an increase in salinity; however, the results of the current study on soil Na concentration and Ca and K uptake indicate that an increase in salinity and nutrient imbalance can already be observed during the first years of operation. For an efficient EWS operation, suitable willow species and clones tolerant to increasing salinity need to be selected, and

more attention should be paid to the response of willows (evapotranspiration, nutrient and metal uptake) to the combination of long-term flooding and salinity, as the combination of these two plant stressors is known to be stronger than either stress alone [31].

4.3.7. Removal of Nutrients by Harvesting

Biomass harvesting removes some nutrients from the system, representing nutrient recovery potential. In the current study, one-third to one-half of the applied N and P was accumulated in the harvestable woody biomass and the remainder was stored in the soil, denitrified, or taken up by roots and leaves, as is consistent with Lachapelle-T et al. [22]. This is a significant amount compared to constructed wetlands as the most common plant-based wastewater treatment systems. For example, harvesting *Phragmites australis* in a constructed wetland can only remove about 4% and 5% of applied N and P, respectively [60]. In SRWC systems, harvesting is usually done after the leaves have fallen, so the nutrients stored in the leaves remain on-site and are recycled through decomposition in the topsoil. This internal nutrient cycling reduces the need for fertilizers. However, to recover more nutrients from the wastewater in EWS, the biomass could be harvested before leaf abscission. In addition, the harvesting cycle can significantly affect nutrient removal and nutrient use efficiency, which also needs to be considered in the operation and management of EWS. In fact, Adegbidi et al. [56] found that the annual harvest cycle had the lowest nutrient efficiency and the highest nutrient removal, and suggested this type of cycle for nutrient phytoremediation in vegetation strips. Similarly, an annual harvest cycle should be considered for higher nutrient recovery in EWS. Willow woodchips produced in this way can be used as a soil amendment in agriculture [61] to return nutrients to the food chain.

Despite the high potential for nutrient removal through harvesting, EWS soil may become saturated with nutrients after a certain period of operation. The nutrient-rich media can be used as a fertilizer or soil amendment [12], which can also return nutrients to the food chain; however, the presence of heavy metals and persistent organic pollutants must be investigated prior to application. By reusing the soil and utilizing the willow biomass, the material cycle of EWS can be closed.

4.4. Fate of Metals

Concentrations of heavy metals in the woody biomass and soil media can affect the potential for their reuse. Typical concentrations of heavy metals in municipal wastewater are expectedly low, ranging from a few to a few hundred $\mu g \ L^{-1}$ [41]; however, heavy metals showed a slight increase in soil concentrations, due to high wastewater loads and zero-discharge operation of EWS, which was also observed by Gregersen and Brix [12]. Studies of SRWC irrigated with wastewater or landfill leachate generally do not report elevated heavy metal concentrations in soil, due to leaching to surrounding water bodies or to the subsurface [8,35]; however, in the case of EWS, all influent heavy metals are retained in the system and are available for uptake by willows or accumulate in the soil. As salinity increases over the long term, the availability and uptake of heavy metals may change and the accumulation of heavy metals in the soil between years may not be linear.

The response of willows to increased salinity from irrigation with wastewater and landfill leachate has been reported in many studies [62–64]; however, the effect of salinity on heavy metal uptake by willows has not been directly investigated. It is known from plant nutrition research that salinity can affect plant micronutrient concentrations differently, depending on the plant species and salinity level [59]. In this study, test trees under higher salinity conditions accumulated less Fe, Mo, Sr, Ba, Ti, and B compared to control trees, even when these elements were present in the soil at higher concentrations (e.g., Fe and Mo), indicating a possible competition of these cations with salt ions, particularly Na.

In addition to increasing salinity, the dynamics of heavy metals in EWS are also affected by waterlogging—during the winter period (low evapotranspiration), influent wastewater accumulates in the system, leading to soil saturation and anaerobic conditions that can convert metals such as Fe and Mn to reduced and more soluble forms [31].

The uptake of heavy metals by willows depends on both heavy metal concentrations in the soil and willow species and clones [38,65]. In agreement with this, our study found some significant differences in heavy metal accumulation among clones. In addition, studies on phytoremediation of heavy metal contaminated soils generally report higher heavy metal concentrations in the woody biomass of willows in response to higher concentrations in the adjacent soil. Reports of heavy metal accumulation and uptake in EWS are rare, and report low concentrations of the heavy metals studied in plant tissues [12].

5. Conclusions

Evaluation of nutrient recovery from primary treated wastewater by a zero-discharge willow system under a sub-Mediterranean climate was conducted on a pilot EWS, using three *Salix alba* clones ('V 093', 'V 052' and 'V160'). The growth dynamics and biomass production of selected clones were investigated. The study showed that *S. alba* clones were suitable for use in EWS and produced significantly more biomass when irrigated with wastewater. 'V 052' was the highest, produced the highest number of shoots and had the highest biomass yield (38–59 t DM ha^{-1}) in both rotations. In addition, 'V 052' had the highest N and P uptake (48 and 45%) from wastewater, and the highest conversion of wastewater to biomass (8.5 kg DM per m^3 of wastewater). The indigenous white willow clone 'V 160' was the shortest and had the lowest biomass yield and wastewater to biomass conversion. Nevertheless, 'V 160' took up more nutrients from wastewater compared to 'V 093'. The uptake of N and P from wastewater into harvestable wood biomass was significant compared to other plant-based wastewater treatment systems, indicating a good nutrient recovery potential of EWS. Wastewater composition and loading were consistent with willow nutrient requirements; however, the uptake of macronutrients and metals may be hindered or altered by increasing salinity caused by EWS zero-discharge operation. Increased salinity has been noted as increased Na concentration in the soil and woody biomass, and decreased Ca and K uptake after only two years of operation; however, the plants in Denmark have been in full operation for 20 years, and to our knowledge there have been no reports of decreased willow growth, evapotranspiration, or other deleterious effects that may be caused by increased salinity, indicating the potential adaptability of *Salix* spp. that should be further investigated.

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