THE EFFECTS OF SECONDARY TREATMENT ON NODULATION OF ALFALFA (*MEDICAGO SATIVA* L.) AND NUTRIENTS SUPPLY IN DOMESTIC WASTEWATER

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Abstract. The use of domestic wastewater in irrigation is becoming widespread. The aim of this study was to determine the effect of Secondary Treated wastewater (STW) irrigation on alfalfa nodulation, agronomic parameters, exchangeable cations and heavy metals in soil and plant. Three levels of STW diluted with distilled water and distilled water were applied for 6 months with three replications in randomized plots in laboratory. Negative binomial regression was applied to model the overdispersion. Diluted STW reduced the total and active nodule number by at least 79.5% and 91.2%, respectively. Diluted STW decreased total biomass of alfalfa compared to distilled water. STW increased the EC and Mg ratios in the soil and the Na ratio in the soil, root and shoot. However, it did not affect the K and Ca ratios in the soil, root and shoot. Co, Ni, As and Pb uptake were inhibited in roots, however elements such as Fe, Cu, Mo, Mn, Al, Cr and Cd accumulated in high concentration in the root. Increasing Zn concentration in shoot may be attributed physiological selectiveness of the alfalfa. Consequently, the use of STW cannot be recommended in alfalfa irrigation as it could reduce the biomass and prevent nodulation.
Keywords: *houshold treated wastewater, alfalfa, nitrogen fixation, negative binomial regression, elements, yield*

Introduction

Water is the source of life. As a result of development, water consumption at the beginning of the 21st century has been increased by more than 6 times, as compared to the beginning of the 20th century; the share of irrigation for agricultural purposes was 70% (FAO, 2013). In our country, where 86.1% of its total area is dominated by arid and semi-arid (Cebeci et al., 2019), this growth rate will inevitably increase with global warming.

Local governments and policy makers are making a concerted effort to use recycled water for irrigation, instead of discharging it into natural water bodies (Rahman et al., 2016). Although the use of treated wastewater in agriculture is a valid alternative, it is considered a necessity in some cases (Niemczynowicz, 1999). The world's annual consumption of N, P and K (N + P_2O_5 + K_2O) in agriculture was 186.67 million tons in 2015, and it was expected to reach 201.66 million tons by the end of 2020 (FAO, 2017). The use of treatment water in agriculture will contribute to the clean water ecosystem by reducing the discharge (Toze, 2006), an alternative to the use of chemical fertilizers in terms of macronutrients, with economic and ecological importance. Although the effect of treated wastewater on plant's growth depends on the chemical properties of the water used and the plant species (Shahrivar et al., 2019). The treatment method is also an

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important factor determining the wastewater quality (Singh et al., 2012). Theoretically, the treatment water can be used in irrigation to meet the increasing water demand in agriculture to benefit from the nutrients it contains, and to prevent environmental problems that may arise in the case of mass discharge. However, the reaction of plants belonging to different families or even species to various contents of treatment water, especially salt, is different (Katerji et al., 2003).

The fixation of atmospheric nitrogen in a year is 175 million tons (Haktanır and Arcak, 1997), it is estimated that it will constitute 86.6% of all nitrogen consumed in 2020. Legumes are the plants that can best utilize atmospheric nitrogen. N fixation of perennial forage legumes is of great importance. The most produced forage crop in Turkey is alfalfa (662,889 ha) (TUIK, 2021), the annual symbiotic nitrogen fixation is 125-335 kg ha^{-1} (Haktanır and Arcak, 1997). This is more than the world's average nitrogen consumption per unit area (TAGEM, 2018). In order to obtain a high yield from alfalfa, which has a high cation exchange capacity, many cationic macro and micro nutrients, especially K, Ca and Mg, are needed (Ottman, 2010; Al-Amoodi, 2011). Alfalfa is the main source of roughage, which is mowed 8-9 times a year in irrigated conditions, depending on the vegetation period, and an effective crop rotation plant in terms of great importance in the sustainable agriculture and organic production (Radović et al., 2009). One of the most important reasons for alfalfa rotation in the sustainable field agriculture is to reduce the use of chemical nitrogen by making use of symbiotic nitrogen fixation.

It has been reported that treated wastewater can be used for germination and plant growth after dilution (Dash, 2012). Untreated municipal wastewater provides higher germination and seedling performance when compared to the treated wastewater (Gassama et al., 2015). Salinity (EC), pH, sodium absorption rate and some metals, which are accepted as the physicochemical characteristics of the treated wastewater, are important parameters in terms of agricultural applications. The treated wastewater increases the salt and sodium concentration of the soil compared to other cations (Becerra-Castro et al., 2015), causing hyperosmotic pressure, oxidative stress and ion toxicity that can limit plant's growth and productivity (Ngara et al., 2012; Levy and Tai, 2013). Sodicification impairs the aggregate and structure stability of the soil, making it compacted and decreasing its permeability and conductivity, thus negatively affecting soil microorganisms (Becerra-Castro et al., 2015). The increase of soil salinity causes a decrease in the number of bacteria and fungi (Pankhurst et al., 2001), microbial diversity and biomass (Ke et al., 2013; Chen et al., 2017). Salinity and sodicity do not only affect the chemical and physical properties of the soils, but also greatly damage their microbial and biochemical properties (Rietz and Haynes, 2003), also causing the delay of mineralization and nitrification (Azam and Ifzal, 2006). In most irrigation studies with wastewater, the effects of wastewater on soil microorganisms activity have been neglected. Therefore, there is a great knowledge gap regarding soil microorganism, health and efficiency of wastewater (Becerra-Castro et al., 2015).

Domestic treated wastewater can be either stimulating or antagonistic to resident soil microorganisms. In particular, there has not been enough study on how Rhizobium sp bacteria is affected from wastewater use. Nodulation and nitrogen fixation that occur with Legume-Rhizobium compatibility are sensitive to water quality. Therefore, poor water quality is considered as one of the main causes of unsuccessful symbiosis, as well as reducing yield by preventing the legume plant's growth (Desouky Shetta, 2016). Salinity, which reduces water quality, not only affects nitrogen uptake, but also reduces the biological nitrogen contribution of the soil (Katerji et al., 2003). So, the effects of plant nutrients and metals concentration, Na ratio and salinity on nodulation and nodule activity in wastewater are not well known.

Therefore, in this study, it was aimed to investigate the effects of Secondary Treated Domestic Wastewater (STW) on alfalfa nodulation and activity, yield characteristics, exchangeable cations and heavy metal contents in the plant and soil, and some physicochemical properties of the soil.

Materials

The research was carried out in Van Yuzuncu Yil University, Faculty of Agriculture, Department of Field Crops. The Bilensoy 80 variety of alfalfa (*Medicago sativa* L.) moderately resistant to salt (2-5 dS/m) was used as the plant material in the experiment. The soil was taken from a 0-30 cm depth in the field where alfalfa had been produced before. The soil was thoroughly mixed before being placed in the pots and the samples were taken for chemical and physical analysis.

The wastewater used in the experiment was taken from the Ministry of Environment and Urbanization, Housing Development Administration (TOKI) residences. This process is biological treatment, in which nitrogen, phosphorus and organic substances are removed (Jin et al., 2014), it consists of the parts shown in *Figure 1*. The discharge water obtained from this process, which is defined as Secondary Treated Wastewater is used for irrigation of dairy pasture, horticultural crops and playgrounds (Bootha et al., 2003). High amount of secondary treated wastewater effluent is commonly discharged into rivers and streams, while some of it is transferred to advanced treatment process to obtain higher quality wastewater (Wang et al., 2018). Advanced treatment is optionally applied in this treatment. For this study, effluent without advanced treatment was used. The discharge point of treatment is 34^o 59′ 14.30″ E, 42^o 53′ 265.20″.

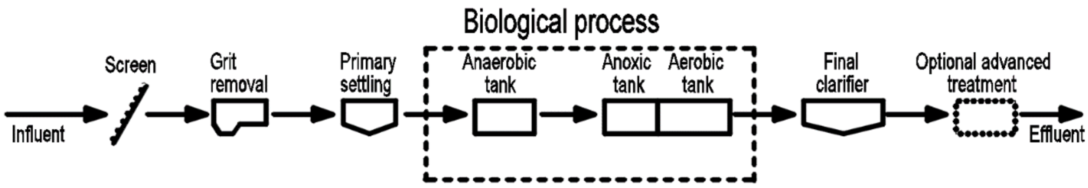

Figure 1. *Flow of chart secondary treatment process wastewater treatment of Edremit, Van*

Methods

Experimental Plan

The experiment was conducted in 3 replications according to a randomized plots trial plan in 2017; this was done in a climate room with 60-70% humidity and a 16-hour light/8-hour dark period; the room was enlightened by a cool fluorescent lamp (100 µmol m^2s^{-1}) under 24/21 ºC day/night lighting conditions for 6 months. Before the treatment wastewater was applied, 102 mg N and 272 mg P_2O_5 were applied to all pots with a volume of 22.5 cm X 7.5 cm X 10 cm, at a rate of 30 kg ha^{-1} N and 8 kg ha^{-1} P_2O_5 (Türk et al., 2018). 100% distilled water was applied to the control pots; Secondary Treated wastewater diluted with 25%, 50% and 75% distilled water was applied to the other pots. According to Obi (1974) method’s, as given follow, each pot with a field

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capacity of 26.5% was regularly irrigated with 400 ml of distilled water and a mixture of STW was diluted with distilled water at 8-day intervals. In each pots 24 seeds were planted and the first irrigation was started on 17.12.2017. During the trial, 23 irrigations were carried out and new wastewater was taken from the discharge point of the treatment plant for each irrigation. Emergence counts were made 5 days after sowing, at 3-day intervals, for a month. Agronomic evaluations were made according to the total number of plants in the pot.

Calculation of Field Capacity

The cylindrical vessel filled with soil was placed in a basin with distilled water up to 5 cm and waited 24 hours. After 24 hours, the cylindrical vessel was taken from the basin and waited until the drainage stopped. At this stage the soil is at field capacity. After the drainage has stopped, the soil in the cylindrical container was weighed after drying in an oven at a temperature of 105°C for 24 hours. The field capacity or saturation percentage was calculated (on gravimetric basis %) as follow (Obi, 1974):

$$SP\ (FC) = \frac{K-J}{J-C} = \frac{4329{,}92-3581{,}55}{3581{,}55-762{,}01} = 0.2654 = 0.2654 * 100 = \%\ 26.5 \qquad \text{(Eq.1)}$$

where,
SP (FC)= Saturation percentage
K= Weight of container + wet sample
J = Weight of container + dry sample
C = Weight of container.

Physical and Chemical Analysis of Plant and Soil

Soil samples were air-dried and passed through a 2 mm sieve. Among the physicochemical properties, soil reaction (Mclean, 1983), electrical conductivity (Richards, 1954), texture (Bouyoucus, 1951) and pH (1:2 soil-water) (Gavlak et al., 1994) were determined according to the mentioned methods. Total macro and micro elements and metals in the soil and plants were extracted using nitric acid (HNO_3) % 67-70 and perchloric acid ($HCLO_4$) % 70 HNO_3/ 4$HCLO_4$ of the mixture (Khan and Frankland, 1983). Aluminum (Al) and molybdenum (Mo) concentrations in the filters were converted to mg/kg after being determined by ICP-MS (Seri No: IC3D2a25110) due to their low concentrations. Phosphorus (P), Potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), cobalt (Co), nickel (Ni), selenium (Se), arsenic (As), lead (Pb), cadmium (Cd) and chromium (Cr) were determined by ICP-OES (ICAP6300 DUO).

Treated Wastewater Water Analyses

New treated wastewater samples taken for each irrigation were collected regularly during the trial and kept at -18 0C for physicochemical analysis. The pH of the treatment water (Mclean, 1983) and electrical conductivity (Richards, 1954), which are important for irrigation, were determined. The treated wastewater samples taken for each irrigation were mixed at the end of the experiment. The element contents of wastewater were determined in ICP-MS and ICP-OES. Aluminum (Al) and molybdenum (Mo) concentrations in the solution were converted to µg/kg after being determined in ICP-MS (Seri No: IC3D2a25110) due to their low concentration. Nitrate (NO_3) in solution,

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Calcium (Ca), Magnesium (Mg), Sodium (Na), Iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), nickel (Ni), cobalt (Co), selenium (Se), arsenic (As), lead (Pb), cadmium (Cd) and chromium (Cr) concentrations in solution were determined by ICP-OES (ICAP6300 DUO).

The nitrate concentration (22.63 mg/L) in the secondary treated wastewater used in this study is 1/10 of 5mM nitrate. Ohyama et al. (2011) report that 5 Mm nitrate is sufficient to prevent nodule formation, and this concentration has an effect equivalent to 230 mg/L NO_3. However, even if it is applied by diluting every eight days, it can reach a level that prevents nodule formation by increasing the nitrate concentration in the soil. Other parameters examined in Secondary treated wastewater were determined to be lower than the limit value.

Agronomic Data

The first mowing was done 29 days after sowing. Cuttings (10% flowering period) were done at 20 day intervals and plant height was measured before each cutting. Plant dry weight was determined by keeping fresh samples at 65 °C to reach a constant weight. Fresh and dry weights of the root (after removing nodules) and shoot samples, consisting of a total of 9 mowing, were calculated as g/pot (Zhao et al., 2020).

Nodule Counting

After the pots were left completely submerged for 10-12 hours, the roots were carefully separated from the soil and rinsed with distilled water; the nodules were counted rapidly (so that the inner color of the nodule did not change) per pot (Zhao et al., 2020). Nodules with pink inner color were accepted as “active nodules”, but those with white, yellow, grayish or brown colors were considered as “inactive nodules” (Uyanık et al., 2011; Zhao et al., 2020).

Statistical Analysis

Data was applied to the General linear model and the difference between the means which were significant as a result of the analysis of variance was determined according to the Duncan multiple comparison test. Firstly, Poisson regression was applied to model the factors affecting the nodule numbers. However, since the Poisson variance was much larger than the Poisson mean (overdispersion) as a result of the Poisson regression, negative binomial regression was applied (Yeşilova et al., 2010; Yeşilova and Denizhan, 2016). In addition, the Kruskal Wallis test was applied to determine whether there was a difference between the applications in terms of total nodules and active nodules. Pearson correlation was also performed to determine the effect (relationship) of chemical and physicochemical factors affecting the total and active nodule in the soil and root. Only the factors important with nodulation are shown in the *Table 1*.

Results and Discussion

Nodule Number and Activity

Secondary Treated wastewater application rates affected the dispersion of nodules quite differently and no nodule formation (zero “0” value) was detected in the repetitions of many applications. The Kruskal Wallis test was used to determine whether there was a difference between the treatments in terms of total nodules and active nodules (*Table 2*).

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As a result of the Kruskal Wallis test, the difference between applications in terms of the total nodule and active nodule was statistically significant ($p<0.05$). According to the Mann-Whitney test applied to determine the differences between STW applications, only the difference between the control and the other irrigation level was statistically significant. However, the difference between the three STW levels was insignificant.

Table 1. *Pearson's Correlation Coefficients (r) between both total-active nodule and soil and root elements, and heavy metals concentration in alfalfa**

	Soil			
	Mg	Mo	OM	EC
Total Nodule	-0.611*	0.614*	0.589*	-0.618*
Active Nodule	-0.612*	0.600*	0.596*	-0.670*
	Root			
	Na	Mo	Al	As
Total Nodule	-0.705*	0.693*	0.842**	0.859**
Active Nodule	-0.684*	0.737*	0.880**	0.885**

*Correlation is significant at the P<0,05 level, **Correlation is significant at the 0,01 level

Table 2. *Kruskal Wallis test results (number nodule per plot) **

	Treatments	**N**	**Mean**	**Std. Deviation**	**Mean Rank**
Total Nodule	Control	3	75.33	29.738	11.00[a]
	25% STW	3	2.23	4.041	4.00[b]
	50% STW	3	6.67	7.638	6.00[b]
	75% STW	3	5.00	7.810	5.00[b]
	Total	12			
Active Nodule	Control	3	78.00	28.478	11.00[a]
	25% STW	3	6.33	6.807	4.00[b]
	50% STW	3	16.00	13.389	5.83[b]
	75% STW	3	13.67	16.503	5.17[b]
	Total	12			

* Means with different letters in the same column are significantly different (P <0,05) according to Duncan multiple comparison test

Total Nodule

The count data show the Poisson distribution. Since total and active nodules were obtained based on counts, Poisson regression was applied to model the influential factors. In the Poisson distribution, the mean and variance are equal. If variance is greater than mean, it is called overdispersion; if it is small, it is called underdispersion. In this study, the mean and variance of the active nodule were obtained to be 22.33 and 1209.82, respectively; also, the mean and variance of the total nodule were 28.50 and 1145.36, respectively. As can be seen, the variances in both active and total nodules were considerably higher than the means. As a result, the overdispersion parameters for active and total nodules were obtained to be 10.73 and 9,992, respectively. Poisson regression cannot be applied if the overdispersion parameter is greater than 1 (Yeşilova et al., 2010). Instead, negative binomial regression modeling overdispersion is applied. In this study,

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as a result of negative binomial regression, the overdispersion parameter for active and total nodules was 1.073 and 1.007, respectively. Thus, parameters were obtained after overdispersion was controlled. As a result of negative binomial regression, in which the control group was taken as the reference category, the effects of STW at different rates on the total nodule were found to be statistically significant ($p<0.01$). In nonlinear regression parameters coefficients need to be linearized for interpretation. Therefore, the parameter coefficients are linearized by taking exponential function. For example; total number nodules were decreased by 91.9% ($p<0.01$) with 25% (the value of 91.9% in the 25% STW group was obtained with the 1-exp (-2.511) equation), 79.5% ($p<0.05$) with 50% (the value of 79.5% in the 50% STW group was obtained with the 1-exp (-1.584) equation), and 82.5% with 75% application of STW ($p<0.01$) (the value of 82.5% in the 75% STW group was obtained with the 1-exp (-1.742) equation) compared to the control (*Table 3*).

Table 3. *Maximum Likelihood Parameter Estimates for the total nodule*

Analysis of the Maximum Likelihood Parameter Estimates						
Parameters	**Estimate**	**Standard Error**	**Wald 95% Confidence interval**		**Wald Chi-Square**	**p-value**
İntercept	4.357	0.468	3.440	5.273	86.84	0.0001
Control reference category						
25 % STW	-2.511	0.697	-3.877	-1.145	12.99	0.0003
50 % STW	-1.584	0.674	-2.904	-0.264	5.53	0.0187
75 % STW	-1.742	0.676	-3.067	-0.416	6.63	0.0100

Active Nodule

As with the total nodule, as a result of negative binomial regression in which the control group was taken as the reference category, the effects of STW rates on the active nodule were found to be statistically significant ($p<0.01$). Accordingly, the number of active nodules was decreased to 96.9% ($p<0.01$) with 25%, 91.2% ($p<0.05$) with 50%, and 93.4% with 75% ($p<0.01$) application of STW (*Table 4*).

Table 4. *Maximum Likelihood Parameter Estimates for the active nodule*

Analysis of Maximum Likelihood Parameter Estimates						
Parameters	**Estimate**	**Standard Error**	**Wald 95% Confidence interval**		**Wald Chi-Square**	**p-value**
İntercept	4.322	0.696	2.958	5.685	38.60	0.0001
Control reference category						
25 % STW	-3.475	1.052	-5.536	-1.413	10.91	0.0010
50 % STW	-2.425	1.007	-4.398	-0.452	5.80	0.0160
75 % STW	-2.713	1.015	-4.702	-0.723	7.14	0.0075

STW ratios had greater adverse effects on the active nodule (*Table 2* and *Table 3*). It has been reported that STW causes the loss of the infectivity of bacteria rather than bacteria themselves (Sayed, 2003). It is estimated that the nitrate content of STW is 10 times higher than the maximum allowable nitrate amount (*Table 5*) affecting nodulation

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and nodule activity. In particular, nitrate nitrogen inhibits both nodulation and active nodule (Luciński et al., 2002). Excess nitrogen fertilizers can reduce the carbon/nitrogen ratio of legumes, causing the regression of nitrogen fixation in long-term applications (Graham, 1981). Furthermore, rhizobium sp bacteria in the nodule become parasitic, so they start to benefit from the nitrogen of the plant and degenerate the nodule (Hansen, 1994). The results of these investigations support the decrease in the number of active nodules due to STW application in this study. The high decrease in the number of active nodules could be due to the increased N with frequent irrigation, rather than the N ratio of STW. In addition, it has been reported that the excess nitrogen can prevent nodulation indirectly by preventing the formation of infection filaments (Rawsthorne et al., 1985; Sezgin, 2018).

Table 5. *Some properties and limit values of soil and STW used in the experiment**

Measurement	Soil	STW	Limit value*
Texture	Loamy	-	-
Sand (%)	29.20	-	-
Clay (%)	42.80	-	-
Silt (%)	28.0	-	-
pH	8.45	7.81	6-9
EC (dS/m)	0.285	0.654	0.250-3
Organic matter (%)	1.27	-	-
Nitrate (mg/kg, mg/L, mg/L)	18.83	22.63	5-20
K(%)	0.036	0.003	<0.005
Ca (%)	0.627	0.013	75-200
Mg (%)	0.147	0.006	50-150
Na (%)	0.045	0.013	0.025
Fe (mg/kg)	102.2	0.004	0.3-1
Cu (mg/kg)	0.113	4.41	0.2-5
Zn (mg/kg)	0.213	8.84	2-10
Mn (mg/kg)	2.471	9.02	0.2-10
B (mg/kg)	0.665	-	0.5-2
Al (µg/kg)	0.309	0.06	5-20
Mo (µg/kg)	nd	nd	0.01-0.05
Se (mg/kg)	nd	nd	0.02-0.2
Ni (mg/kg)	0.448	3.93	0.2-20
Co (mg/kg)	0.071	0.283	0.05-5
As (mg/kg)	0.116	0.891	0.1-2
Pb (mg/kg)	0.026	0.159	5-10
Cd (mg/kg)	0.0006	0.025	0.01-0.05
Cr (mg/kg)	0.377	1.070	0.1-1

* Regulation Amending the Regulation on Surface Water Quality Management, April 15, 2015 and Official Gazette No. 29327 of Turkish Republic, converted from the European Union legislations

STW applications increased the Mg and EC ratios, while decreasing the organic matter ratio and Mo concentration in the soil. Thus, their relationship with the total and active nodule was found to be significant ($P<0.05$). Especially, the significant increase in Mg and EC ratios (*Table 6* and *Table 7*) and the significant decrease in nodulation (*Table 2*) caused the correlation coefficient to be negative. When the total and active nodule was correlated with all elements in the root, the relationship with Na was only negative and significant ($P<0.05$), while the relationship with Mo ($P<0.05$), Al and As ($P<0.01$) was positive and significant (*Table 1*).

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Table 6. *Effect of STW* dilution at different ratios on the concentrations of exchangeable cations in the soil, root and shoot (%)***

	Treatments	K	Ca	Mg	Na
Soil	Control	0.033	0.480	0.187[b]	0.300[b]
	25% STW	0.033	0.470	0.217[b]	0.333[ab]
	50% STW	0.027	0.420	0.200[b]	0.300[b]
	75% STW	0.033	0.483	0.253[a]	0.433[a]
Root	Control	1.720	0.900	0.557[a]	0.270[b]
	25% STW	1.647	0.930	0.540[ab]	0.347[ab]
	50% STW	1.826	1.133	0.650[a]	0.373[a]
	75% STW	1.600	0.997	0.370[b]	0.370[a]
Shoot	Control	3.727	2.450[ab]	0.743[b]	0.183[c]
	25% STW	4.113	2.867[a]	0.733[b]	0.227[bc]
	50% STW	3.953	2.407[ab]	0.930[a]	0.320[a]
	75% STW	3.925	2.220[b]	0.690[b]	0.257[b]

*STW: Secondary Treated Wastewater, **Means with different letters in the same column are significantly different (P <0,05) according to Duncan multiple comparison test

Table 7. *Effect of STW dilution at different ratios on some physicochemical properties of soil and agronomic properties of alfalfa**

Treatments	OM (%)	pH	EC (dS/m)	EN (no/pot)	FN (no/pot)	PL (cm)	DW (per/pot)	DWR (per/pot)	TN** (no/pot)	AN** (no/pot)
Control	1.59	8.42	0.20[c]	15.33	8.33	12.40	1.96	0.76	78.0	75.3
25% STW	1.27	8.44	0.26[b]	10.67	10.00	11.78	1.44	0.59	6.3	2.3
50% STW	1.14	8.43	0.34[a]	12.00	11.67	10.00	1.24	0.62	16.0	6.8
75% STW	1.07	8.54	0.36[a]	16.00	13.00	9.76	1.84	0.80	13.0	5.0

*Means with different letters in the same column are significantly different (P <0,05) according to Duncan multiple comparison test. Organic Matter (OM), Electrical Conductivity (EC), Emerging Number (EM), Final Count (FC), Plant Length (PL), Dry Weight Shoot (DWS), Dry Weight Root (DWR), Total Nodule (TN) and Active Nodule (AN), **Since total nodule and active nodule data showed Poisson dispersion, Kruskal Wallis test was applied (*Table 2*) It is given here for information purposes

Among the physicochemical factors in the soil, only Mg, Mo, organic matter and EC were significantly correlated with the total number of nodules and active nodules (P<0.05). The relationships between Mg and EC, which are the most important factors of soil salinity, and active nodule were found to be negative and significant (P<0.05) (*Table 1*). The relationship between the total and active nodule, and organic matter and Mo was positive and significant (P<0.05). The control plot produced more inoculation (Allito et al., 2020), and more decomposed material due to its well-developed root system. The low number of active nodules could be attributed to the fact that salinity affects the N fixation process, indeed, it has been reported that the fixation more sensitive than Rhizobium and plants (Zurayk, 1998). In another study reported that Mg, Na and EC, which are the sources of salinity, were influence nodulation directly or indirectly by affecting the rhizobium bacteria (HanumanthaRao et al., 2016). Salinity causes the aging of the nodule from the beginning of nodule formation to the fixation process

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(Balasubramanian and Sinha, 1976), especially by reducing leghemoglobin during the fixation process (HanumanthaRao et al., 2016).

The negative and significant relationship of the Na ratio in the root with the total and active nodule number is consistent with the studies whose results were reviewed above. This finding is also one of the reasons why the number of active nodules is lower than that of the total nodules. It is stated that Rhizobium spp population decreases with the increase of EC; most agricultural legumes cannot survive at 6.7 $mScm^{-1}$ salt concentration (Kaur et al., 2015). The relationship between soil and root Mo and root Al concentrations with active and total nodules were positive and significant ($P<0.05$, $P<0.01$, respectively) (*Table 1*). The findings that sufficient Mo concentration increases N fixation (Silva et al., 2017; Ma et al., 2019) support the positive and significant relationship of Mo concentration with active and total nodule number in this study (*Table 1*). However, this is due to control, not to STW (*Table 8*). The positive and very significant ($P<0.01$) relationship of Al in the alfalfa root in the control plot with the total and active nodule were estimated to be due to the cumulative property of the alfalfa root (*Table 1*, *Table 8*) not associated with nodulation. Findings that Al toxicity mostly occurs in soils with low pH (Jaiswal et al., 2018) support the conclusion of our study that the alkalinity of the trial soil caused the correlation between soil Al concentration and nodulation to be insignificant (Since it does not comply with the text, only the elements and physicochemical properties that are significant relation to nodulation are given in the *Table 1*).

Soil, Root and Shoot Exchangeable Cations

The effect of STW on the soil exchangeable cations ratio was insignificant, except for Mg and Na. The ratios of K, Ca, Mg and Na in the soil, root and shoot were determined as shoot > root > soil, respectively. While the Mg and Na ratios were increased significantly ($P<0.05$) in the soil, there was only a significant increase of the Na ratio in the root ($P<0.05$). The increases and decreases of Mg in the root and Ca and Mg in the shoot were significant, though unstable. There was a gradual and numerical increase in the K ratio of the shoot. Application of STW at the rate of 75% decreased the rate of exchangeable cations in the root and shoot, as compared to its application at the rate of 50%. It has been stated that treated municipal wastewater contains less nutrients in comparison to the untreated one (Gassama et al., 2015), and exchangeable cations concentration being similar to that of irrigation water (Elfanssi et al., 2018). These findings support the results from this study.

STW did not significantly affect the ratios of exchangeable cations in the soil, root and shoot, except for Na and Mg. This could be attributed to the pot soil, which was an alkaline, and had been previously planted with alfalfa. In some previous studies (Schipper et al., 1996; Galavi et al., 2010; Alnaimy et al., 2021), it has been reported that treated water increases the Mg concentration in the soil. It has been reported that the treated wastewater increases the salt and Na content of the soil; the intake of the required elements is insufficient due to the accumulation of Na (Morugán-Coronado et al., 2011; Castro et al., 2013). The ratio of K in the shoot of alfalfa (3.930% on average) was approximately 113 times higher than that in the soil (0.032% on average) (*Table 6*). The findings that herbaceous legumes could accumulate K up to 5% under saline soil conditions (Arienzo et al., 2009) supporting the results of this study.

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Table 8. *Effect of STW dilution at different ratios on the concentrations of exchangeable cations in soil, root and shoot (mg/kg)*[*]

	Treatments	Fe	Cu	Al	Zn	Mo	Co	Se	Ni	Mn	Cr	As	Pb	Cd
Soil	Control	4.957	1.380	0.005	0.330^{ab}	0.003^{a}	14.35^{b}	nd	109.5	1.927	75.00^{a}	33.19	4.333	0.337
	25% STW	4.980	1.267	0.004	0.303^{b}	0.001^{c}	14.49^{b}	nd	111.1	1.830	63.90^{ab}	33.61	4.363	0.347
	50% STW	4.517	1.270	0.004	0.260^{b}	0.002^{ab}	14.40^{b}	nd	110.8	1.797	48.25^{b}	27.14	4.287	0.287
	75% STW	4.447	1.247	0.005	0.420^{a}	0.002^{b}	15.12^{a}	nd	115.3	1.810	80.85^{a}	35.03	4.450	0.370
Root	Control	2530.5	28.05	9.381^{a}	31.830	0.032^{a}	1.947	nd	36.37^{b}	57.05^{c}	16.88	14.11^{a}	1.330^{b}	0.450^{a}
	25% STW	2729.0	27.22	0.834^{b}	29.653	0.021^{b}	1.883	nd	22.61^{c}	70.65^{b}	13.89	8.16^{b}	1.180^{c}	0.320^{b}
	50% STW	3338.8	29.82	0.919^{b}	31.153	0.019^{b}	2.240	nd	50.96^{a}	77.31^{a}	18.48	9.64^{ab}	1.270^{b}	0.443^{a}
	75% STW	3296.6	29.86	0.913^{b}	28.977	0.017^{b}	2.227	nd	22.44^{c}	71.81^{b}	17.39	8.11^{b}	1.430^{a}	0.370^{ab}
Shoot	Control	187.3^{a}	16.47	1.068^{a}	45.943	0.010^{b}	0.267	0.067	5.950^{b}	37.56^{a}	1.757^{ab}	1.763^{a}	0.267^{c}	0.067^{b}
	25% STW	158.9^{ab}	16.20	0.742^{b}	44.790	0.007^{b}	0.233	0.037	3.233^{c}	36.31^{ab}	1.163^{b}	1.120^{b}	0.243^{c}	0.050^{b}
	50% STW	134.0^{b}	16.38	0.636^{b}	42.230	0.008^{b}	0.240	0.073	7.100^{a}	34.83^{ab}	2.307^{a}	1.057^{b}	0.627^{a}	0.120^{a}
	75% STW	129.4^{b}	14.73	0.615^{b}	42.413	0.017^{a}	0.216	nd	3.517^{c}	34.27^{b}	1.883^{ab}	1.027^{b}	0.427^{b}	0.070^{b}

[*]Means with different letters in the same column are significantly different (P <0,05) according to Duncan multiple comparison test, nd: non detected

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Soil, Root and Shoot Trace Elements and Heavy Metals

STW applications did not affect the concentration of micronutrients and heavy metals in the soil, except for a few elements; only was the Co concentration significantly increased, as compared to the control. While 75% ratios of STW did not affect the concentrations of Zn and Cr in the soil, they were decreased significantly with 25% and 50% applications compared to the control. Among the investigated elements in the soil, only the Mo concentration was raised gradually and significantly with increasing STW applications, but it was less than that of the control at all application levels. Fe, Zn, Cu and Mn concentrations were lower in treated municipal wastewater than in the untreated one (Gassama et al., 2015), irrigation with tap water and STW did not cause soil heavy metals accumulation, and there was no significant difference between them (Elfanssi et al., 2018). The findings of the researchers mentioned above are consistent with the results of our study.

The concentrations of Co, Ni, Cr, As and Pb in the root were much lower than those in the soil; the concentration of Co in the root was 14 times less than that in the soil. In contrast, the accumulation of Fe, Zn and Cu in the roots was much higher than in the soil and the effect of application on these elements was insignificant. However, it was found that Al accumulation with distilled water and Mn accumulation were significantly increased by applying STW treatments (*Table 8*). It is estimated that the excess of Fe, Zn, Cu, Mn and Al in the roots is due to the cumulative properties of alfalfa and frequent irrigation. Ike et al. (2007)'s findings that frequent irrigation (every eight days) increases the bioavailability of cationic metals confirm our results.

STW decreased only the Mo concentration gradually and significantly in the root. The increase in Fe, Cu, Co and Cr concentrations, and the decrease in Zn and Ni concentrations in the root, caused by STW application, were insignificant. STW caused the concentrations of Al, Mo, As and Cd to decrease, while those of Mn and Pb were increased significantly. The most distinctive and significant increase was determined for the 50% STW application in the Mn content of the root, while the highest Al, Mo, As and Cd contents were recorded in the control ($P<0.05$) (*Table 8*). Alfalfa root accumulated Fe, Al, Cu, Mo, Ni, Mn, Cr, As, Pb and Cd, limiting their transport to the shoot. The results obtained from this study are similar previous study (Agnello, 2014) that alfalfa roots preferentially accumulate heavy metals in the root and reduce their transport to the shoot. The only metal and micronutrient transported to the shoot without accumulation by the alfalfa root was Zn (*Table 8*).

Because alfalfa was consumed together, the shoot and leaves were analyzed together. Except for Zn and Se, the concentration of metals in the shoot was less than that in the root. The zinc concentration in alfalfa leaves and it is transfer factor from soil to above-ground organs (0.77) were found to be higher than other metals (Chaoua et al., 2019). Tap water increased the Zn concentration in the root and shoot, as compared to the treated water (Hussain et al., 2019), so that the concentrations of metals accumulated in the roots and shoots were not related to the treated wastewater application rates. As a matter of fact, that the metals except for Fe, Al, Mo and As were not affected by the application rates of STW. In studies on heavy metal uptake and transport in the organs of alfalfa (WHO/FAO, 2007; Mbarki et al., 2018; Ghaderpour et al., 2018; Rezaeian et al., 2020), it was reported that the Zn, Cu, Pb, Cr, As and Cd concentrations in shoot were low. This can be attributed to the accumulation of many heavy metals by alfalfa root as mentioned above and the low metal concentration of STW.

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Some Physicochemical Properties of Soil

STW had a negative effect on the physicochemical properties of the soil (*Table 7*). Soil salinity (EC) was increased significantly (P<0.05), but pH was raised numerically. With 75% application of STW, the 0.12 increase in H^+ concentration relative to the control means a one-fold decrease in the H^+ concentration of the soil. A ten-fold increase or decrease in the H^+ ion concentration causes an increase or decrease in pH by one unit (Svobodova, 1993). Therefore, although the increase in pH is statistically insignificant, it is important in terms of plant production.

STW gradually reduced soil organic matter up to 32% in the highest (75%) application rate compared to the control. The Na concentration of STW used in this study was quite higher than the limit value estimated to cause a decrease in organic matter. Treated wastewater negatively affected soil organic carbon due to salinity (Ganjegunte et al., 2018) and reduced the biomass input and decomposition (Setia et al., 2013). In addition, wastewater treatment sludge water contains easily soluble organic matter (Wang et al., 2015) and provides a rapid increase in the microorganism population and causes the decaying root particles to become more mineralized, which is defined as the "priming effect" (Blagodatskaya and Kuzyakov, 2008), resulting in a decrease in soil organic matter (Wang et al., 2015; Al-Tamimi et al., 2016). The researchers' findings support the gradual decrease in organic matter in this study due to wastewater ratio.

With increasing STW application rates, soil EC also increased significantly (*Table 7*). The amount of Na in both soil and STW was thought to cause an increase in EC. It has been determined in many studies that frequent and long-term STW applications can increase the soil EC due to its high Na content (Morugán-Coronado et al., 2011; Elfanssi et al., 2018; Shahrivar et al., 2019, 2020; Ganjegunte et al., 2019).

Some Agronomic Characteristics of Alfalfa

At the 25% (10.67 units) and 50% (12.0 units) application rates of STW, the number of emergences was less than the control (15.3 units). However, the 75% application rate was similar to the control. Despite homogeneous conditions, the high difference between replications caused the difference between treatments was insignificant (*Table 7*). Distilled water increased the plant height, hay yield and root dry weight of alfalfa numerically, compared to STW. This could be attributed to the application of DAP (Diammonium Phosphate) as a basic fertilizer to all pots. As a matter of fact, the shoot dry weight of 8 plants in the control was 1.960 g/pot, while the dry weight of 13 plants with the highest STW application rate (75%) was 1.843 g/pot. The average yield per pot, as determined by the number of plants, was 60% higher than the highest STW application rate (*Table 7*). This could be attributed to the fact that basic fertilization (DAP) increased the yield in the control. Hay yield and plant height were higher with distilled water, contrary to some previous study (Elfanssi et al., 2018). Since Elfanssi et al. (2018), supported the findings of alfalfa according to the results of the "barley", which is a Poacea, could be criticized both ecologically and physiologically. The fact that treated and untreated water significantly reduces root and shoot parameters of alfalfa seedlings supports our results. Even if alfalfa is resistant to moderate salinity (Bernstein, 1961; Orloff, 2007), STW decreased the alfalfa yield by increasing Mg, Na and EC in the soil. It is considered that STW, which is frequently applied, decreases the uptake of K and Ca cations by increasing the Na concentration of the soil (Hasegawa et al., 2000; Viegas et al., 2001), thus causing a decrease in the total biomass of alfalfa.

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Conclusion

STW application rates affected the dispersion of nodules quite differently; a zero “0” value (no nodule formation) was detected in the repetitions of many applications. Firstly, Poisson regression was applied to model the factors affecting the nodule numbers. However, since the Possion variance was much larger than the Poisson mean (over dispersion) as a result of the Poisson regression, negative binomial regression was applied. In addition, the Kruskal Wallis test was applied to determine whether there was a difference between the applications in terms of the total nodules and active nodules.

The STW significantly decreased total number of nodules, as well as the number of active nodules. Although STW application rates increased the number of plants per pot, it numerically decreased the total biomass fresh and dry weight, as compared to distilled water. STW applications did not affect the availability of K and Ca in alkaline soil, but it increased the availability of Mg and Na compared to distilled water. Increasing STW ratios caused a significant increase of both Na and Mg with EC, resulting in a decrease of nodulation and total biomass. The Na ratio was relatively higher in the root, as compared to the soil and shoot with STW irrigation. The concentrations of Co, Ni, Cr, As and Pb in the soil were higher than those in the root above grand biomass. Alfalfa took up Fe, Cu, Mo, Mn, Al and Cd elements, but accumulated them in high concentrations in the root. As an exception, the micronutrient Zn which is also a heavy metal, was increasingly transported from the soil to the root and the aboveground biomass. Although the decrease in the soil organic matter and the increase in pH are statistically insignificant, depending on the application rates of STW, however their gradual changes should be considered in terms of soil fertility.

Although irrigation with STW did not affect the survival of alfalfa but it reduced the total biomass, which is the main cultivation purpose. It also inhibited biological N fixation by reducing nodulation. Even if diluted with distilled water, irrigation of alfalfa's by STW mean end of rotational manifestation with alfalfa. Therefore, secondary treated domestic wastewater is not recommended to be used in alfalfa irrigation to meet nutritional needs or to eliminate water scarcity.

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