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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Uptake and translocation of sulfamethazine by alfalfa grown under hydroponic conditions

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ARTICLE INFO

Article history:
Received 11 March 2016
Revised 20 April 2016
Accepted 27 April 2016
Available online 2 July 2016

Keywords:
Alfalfa
Hydroponic culture
Antibiotics
Sulfamethazine
Concentrated Animal Feed Operations
Phytoremediation

ABSTRACT

Antibiotics are routinely used in intensive animal agriculture operations collectively known as Concentrated Animal Feed Operations (CAFO) which include dairy, poultry and swine farms. Wastewater generated by CAFOs often contains low levels of antibiotics and is typically managed in an anaerobic lagoon. The objective of this research is to investigate the uptake and fate of aqueous sulfamethazine (SMN) antibiotic by alfalfa (*Medicago sativa*) grass grown under hydroponic conditions. Uptake studies were conducted using hydroponically grown alfalfa in a commercially available nutrient solution supplemented with 10 mg/L of SMN antibiotic. Analysis of alfalfa sap, root zone, middle one-third, and top portion of the foliage showed varying uptake rate and translocation of SMN. The highest average amount of SMN (8.58 μg/kg) was detected in the root zone, followed by the top portion (1.89 μg/kg), middle one-third (1.30 μg/kg), and sap (0.38 μg/kg) samples, indicating a clear distribution of SMN within the sampled regions. The ultraviolet (UV) spectra of parent SMN and translocated SMN identified in different parts of the plant present the possibility of metabolization during the uptake process. Uptake of SMN using alfalfa grown under hydroponic conditions has potential as a promising remediation technology for removal of similar antibiotics from wastewater lagoons.

Introduction

Antibiotics used in intensive animal agricultural operations end up in a lagoon. These lagoons may be susceptible to flooding, potentially impacting surrounding watersheds. Antibiotics resist degradation under anaerobic conditions, but their removal prior to releasing treated water into the environment is important for microbial resistance management and to prevent environmental contamination. The major classes of antimicrobials including sulfonamides, macrolides, aminoglycosides, tetracyclines, and ionophores are routinely used in animal agriculture operations (Thiele-Bruhn, 2003). Various studies documented the use of pharmaceuticals in intensive animal agriculture practices and their subsequent occurrence in the environment (Daughton and Ternes, 1999; Kolpin et al., 2002; Campagnolo et al., 2002; Boxall et al., 2003, 2004). Once released into the environment, antibiotics are resistant to biodegradation and have a tendency to accumulate in the environmental matrices, and even at low concentration can adversely affect the aquatic and terrestrial environment (Homem and Santos, 2011). Restricting the usage of antimicrobials is one of the many ways to prevent the entry of antimicrobials in the environment. In intensive animal agriculture operations however, such options may not be economically feasible; especially in the

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http://dx.doi.org/10.1016/j.jes.2016.04.019
1001-0742/

United States where a large percentage (nearly one-third) of antimicrobials are being used at a sub-therapeutic level as a growth promoter (Levy, 2002). Although the occurrence of antimicrobials in the environment has been extensively studied, relatively few authors have investigated remediation protocols for their effective removal from the environment.

The studies that consider plant uptake of antimicrobials (Forni et al., 2002; Migliore et al., 2003; Boxall et al., 2006; Dolliver et al., 2007) do so from the viewpoint of soil media and focus on the remediation of land applied manure containing antibiotics. This approach may not be effective because of the heterogeneity of the soil and the complex interaction between manure, soil and antimicrobials. Antimicrobials tend to sorb on to the soil material and large amounts may remain in the sorbed phase, particularly in soils with substantial organic material and clay fractions (Kurwadkar et al., 2007). The sorbed antimicrobials may not be readily available for plant uptake and as such, the soil based phytoremediation approach may not be an effective management approach.

Removal of antibiotics using plant is a viable choice for antibiotics remediation if sufficient time is allowed for plant acclimatization and contaminant degradation (Salt et al., 1998; Singh and Jain, 2003; Aslund et al., 2007; Michelini et al., 2014). In uptake and translocation process, the plants can extract, detoxify, and/or sequester toxic antibiotics from Concentrated Animal Feed Operations (CAFO) wastewater, which may contain low levels of antibiotics. Lately this approach has emerged as a promising strategy for *in-situ* removal of numerous soil contaminants (Singh and Jain, 2003; Aslund et al., 2007; Kubiak et al., 2012; Favas et al., 2013). Hydroponic systems are a promising area for plant growth as they assure effective management of water, energy, and cost in confined spaces (Rius-Rui et al., 2014). A hydroponic culture constitutes a soil-less plant model system, which has been proven to allow uptake of the target compound with no interference from the soil matrix. Several authors have attempted hydroponic culture for remediating a variety of environmental pollutants (Kang et al., 2012; Rius-Rui et al., 2014; Das et al., 2014). Researchers have demonstrated the removal of pharmaceuticals found in biosolids and wastewater using hydroponically grown cabbage (*Brassica rapa* var. *pekinensis*) and a rapidly reproducing, specialized strain of the same species (Wisconsin Fast Plants) (Herklotz et al., 2010). The authors were able to detect all four of the tested pharmaceuticals in roots and leaves of the plants, as well as the stems of the Wisconsin Fast Plants. Two of the pharmaceuticals, carbamazepine and salbutamol studied by these authors, were also detected in the seed pods produced by the Wisconsin Fast Plants. The authors attributed the systemic distribution to symplastic uptake of pharmaceuticals as they cross the plant's epidermal tissue.

Given these and other results, the possibility of other fast growing plants sequestering a pharmaceutical from solution cannot be overlooked. For this research, alfalfa was chosen as a contaminant-tolerant, hydroponically-amenable plant that increases biomass reasonably quickly and requires irrigation or high rainfall to grow well (Flocco et al., 2002). Alfalfa is a perennial plant, which belongs to the Leguminosae (pea) family and bears abundant root systems, which has shown to remediate or tolerate various environmental pollutants (Bonfranceschi et al., 2009; Carrasco-Gil et al., 2013; Funes-Collado et al., 2013). Sulfamethazine (SMN) was selected as an antimicrobial for testing due to its widespread use in the beef, swine and poultry industries (Huang et al., 2011). Recently SMN has become a priority pollutant primarily because of its widespread detection in soil, surface water, drinking water and variety of agricultural produce (Dolliver et al., 2007; Hu et al., 2010; Huang et al., 2011; Awad et al., 2014). Furthermore, given the low molecular weight coupled with low sorption in soil media, SMN can be a prime candidate for plant uptake (Kurwadkar et al., 2007). The objective of this research is to demonstrate whether SMN can be taken up from a nutrient solution by hydroponically grown alfalfa in a greenhouse environment.

1. Materials and methods

1.1. Chemicals

SMN (CAS#1981-58-4; Assay ≥ 98%) was purchased from Sigma Aldrich, St. Louis, MO, USA. The nutrient solution for the hydroponic system was purchased from General Hydroponics, Sebastopol, CA, USA. The nutrient solution was primarily a performance pack and consisted of several formulations of nutrient solution. It was administered as recommended by General Hydroponics. HPLC grade acetonitrile, ammonium acetate, and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). A 1 g/L stock standard solution of SMN was prepared in nano-pure water (18.2 MΩ/cm at 25°C) and briefly stored in a refrigerator at 4°C prior to its use in the experiment. A sub-stock of 10 mg/L was used for studying the uptake of SMN by alfalfa.

1.2. Experimental design

Alfalfa was grown from seedlings using the commercially available hydroponic system. The fully automated system consists of an ECO 185 submersible pump equipped with an oil-free motor, filter, Eco-Air 4-air pump, and four high-output air stones (Fig. 1). The system circulates highly oxygenated bottom water which accelerates plant growth. Within a week, the seeds were sprouted and within two weeks, the alfalfa showed significant growth. The nutrients were fed according to the prescribed feeding schedule recommended by General Hydroponics. The nutrient losses were compensated by completely replenishing the entire nutrient solution every week. Once the plants were approximately one foot tall, a representative blank sample was procured from all corners of the hydroponic system.

Prior to changing the nutrient solution with an antibiotic solution, the entire system was thoroughly cleaned. The plants, including roots, were thoroughly rinsed and re-grown in 15 gal of a nutrient solution containing 10 mg/L of SMN. The same nutrient feeding schedule was followed as during the growth phase. Water loss due to transpiration was compensated with nutrient solution containing the antibiotic dose. The dose of 10 mg/L of SMN was based on a screening-level phytotoxicity study which demonstrated that alfalfa exposed to concentration greater than 10 mg/L over 5 days experienced adversely affected germination, total length, root length and shoot length

Fig. 1 – Hydroponic system with fully grown alfalfa plants with its root zone and the nutrient solution. Schematic shows the Model 600 Pressure Chamber Instrument with the sap extraction in progress.

(effective concentration resulting in 25% reduction in species germination (EC_{25}) > 10 mg/L) (Hills et al., 2011). Sampling of the roots, sap, top portion and middle of the plant was conducted after 72 hr of contact between the plants and the nutrient solution. It should be noted that SMN is an ionic compound, characterized by two acid dissociation constants, first dissociation is characterized by the protonation of $–NH_3^+$ group at lower pH values (pH 2–3) whereas at higher pH values (pH 5–11) result in the deprotonation of $–SO_2NH^-$ group. This ionic behavior of SMN indicates that it will exist partially as an anion in the environment and as such loss due to volatilization is less likely (Sakurai and Ishimitsu, 1980; HSDB Hazardous Substances Data Bank, 2014; Tolls, 2001).

1.3. *Sample collection and extraction procedure*

For the extraction of SMN, plants were selected from the corners as well as from the center of the hydroponic growth chamber to obtain the representative plant sample. Fresh plant samples were equally divided into three parts — roots, middle one-third, and top portion and SMN was extracted, using a similar extraction process as proposed by Dolliver et al. (2007). Sap was extracted from the entire plant and diluted prior to analysis. To extract plant sap, freshly collected plant stems were inserted into a pressure chamber (Model 600, PMS Instrument Company, Albany, OR, USA). At around 15 bar pressure level, the sap started dripping from the stem (Fig. 1). The extracted sap samples were immediately analyzed using High Performance Liquid Chromatography (HPLC) system.

For chemical extraction, the plant was divided into three parts: the lower root zone, middle part, and upper leafy part of the plant. To begin the extraction process, a 10 g plant sample from root zone, middle third part, and the upper third part was individually fortified with 10 mL of methanol, 500 μL of HCl acid, and 50 mL of nano-pure water. The plant, along with the extraction chemicals were pulverized for 3 min using a commercially available extractor fitted with a 600 watt motor. The pulverized plant pulp was centrifuged at 3000 r/min for 15 min. The centrifuged plant pulp was immediately filtered using a 0.2 μm syringe filter (Acrodisc® syringe filters with HT Tuffryn® and Versapor®) (Pall Life Sciences, Ann Arbor, MI, USA). The extracted samples were immediately analyzed using the HPLC system.

1.4. *Analytical methods*

The qualitative and quantitative analysis of SMN from the plant extract was achieved with the Waters HPLC system (Waters Corp. Milford, MA, USA), equipped with an e2695 Separations Module and 2998 Photodiode Array (PDA) detector. The separation profile was analyzed using the Waters Empower 3 Chromatography data software. The separation of SMN was carried out isocratically with a mobile phase A consisting of 90% by volume of 20 mmol/L ammonium acetate solution adjusted to pH 5.7 with glacial acetic acid and 10% acetonitrile. Mobile phase B contained 20% of mobile phase A and 80% acetonitrile. Waters e2695 module has a built-in degassing ability and with fluidics module that effortlessly

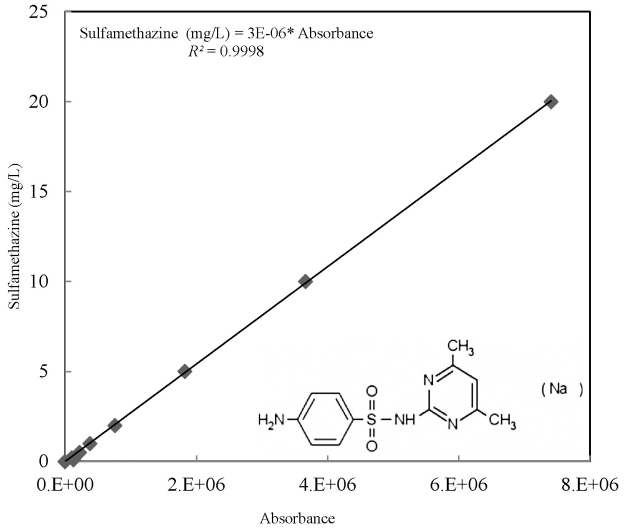

Fig. 2 – Standard curve used in the quantification of sulfamethazine.

facilitate isocratic separation with 80% of mobile phase A and 20% of mobile phase B. Chromatographic separations were achieved using a reverse phase Phenomenex Kinetex 2.6 μm C18 (50 × 2.1 mm internal diameter (ID)) column. An injection volume of 50 μL and flow rate of 0.7 mL/min with total run-time of 10 min was used. Under these chromatographic conditions, the retention time for SMN was observed to be 3.3 min at 266.3 nm maximum absorbance. All samples (sap, root, middle one-third, and top portion) were further diluted in 1:4 ratios with nano-pure water prior to their chromatographic separation. A standard curve for SMN and its absorbance were plotted for various concentrations (5, 2, 1, 0.5, 0.2, and 0.1 mg/L) (Fig. 2).

2. Results and discussion

2.1. Uptake in the root zone

Experimental results show that the roots are the prime pathways for SMN uptake from a hydroponic solution. Since the roots were directly in contact with the nutrient solution and SMN, the root samples were thoroughly rinsed prior to initiating the extraction procedure. Despite this precaution, the roots consistently showed higher concentrations compared to the other parts of the plant. The observed average aqueous phase concentration in the root zone was found to be 8.58 μg/kg (μg of SMN uptake per kilogram mass of root) (Table 1 and Fig. 2). The highest recorded concentration in the root zone was 13.11 μg/kg, while the lowest observed concentration was 4.36 μg/kg. The variation in the concentration of SMN could be attributed to a variation in transpiration rate, which is influenced by the leaf morphology, stomatal mechanism and growth stage of individual plant.

Spectra of SMN in the root sample are similar to the spectra of the parent SMN compound (Fig. 3). This similarity in spectra demonstrates that the extraction process did not result in reaction with SMN. This is particularly important because SMN lacks functional groups that hydrolyze under environmental conditions (Lyman et al., 1990). The retention of a spectral signature as well as low possibility of hydrolysis indicates that the extraction process was effective and the detected concentration of SMN accurately represents the concentration present in the root zone. The uptake of contaminants *via* plant roots can be either through diffusion or through chemical potential gradient, depending on the contaminant and plant species (Carvalho et al., 2014). Uptake of SMN in *Phragmites australis* grown under hydroponic condition suggested that root being in direct contact with antibiotic solution, large accumulation of SMN in root system could be *via* biological uptake and physical–chemical absorption (Liu et al., 2013).

2.2. Uptake in middle one-third zone

Detection of SMN in the middle one-third zone varied from 0.85–1.56 μg/kg (Table 1 and Fig. 2). This variation is due to the varying retention of SMN in the middle one-third part of the plant. The SMN concentration in the middle one-third zone was also lower than that both the root zone and the top portion. The middle part is a transitory zone between the active root zone and the shoots that transpire water as such retention of SMN in the middle zone is less likely. Similar observation was also reported by Liu et al. (2013) in their uptake study of SMN by *P. australis* grown under hydroponic condition. The authors reported that SMN accumulated *via* passive absorption with distribution following the sequence root > leaf > stem. Furthermore, since the middle part also exhibits variation in the amount of plant leaves and stem

Table 1 – Distribution of sulfamethazine in different parts of the plants. The concentration is expressed in terms of the amount of sulfamethazine taken up on mass basis (μg/kg).

Samples	Concentration of SMN in different parts of plants (μg/kg)			
	Root sample	Middle-1/3rd	Top-1/3rd	Sap sample
Sample-A	10.45	0.85	1.64	0.05
Sample-B	13.11	1.36	1.37	0.79
Sample-C	6.41	1.56	2.68	0.65
Sample-D	4.36	1.45	1.87	0.01
Mean	8.58	1.30	1.89	0.38
Standard deviation	3.94	0.31	0.57	0.40
Concentration at $\alpha = 0.05$	8.58 ± 6.26	1.30 ± 0.50	1.89 ± 0.90	0.38 ± 0.64

SMN: sulfamethazine.

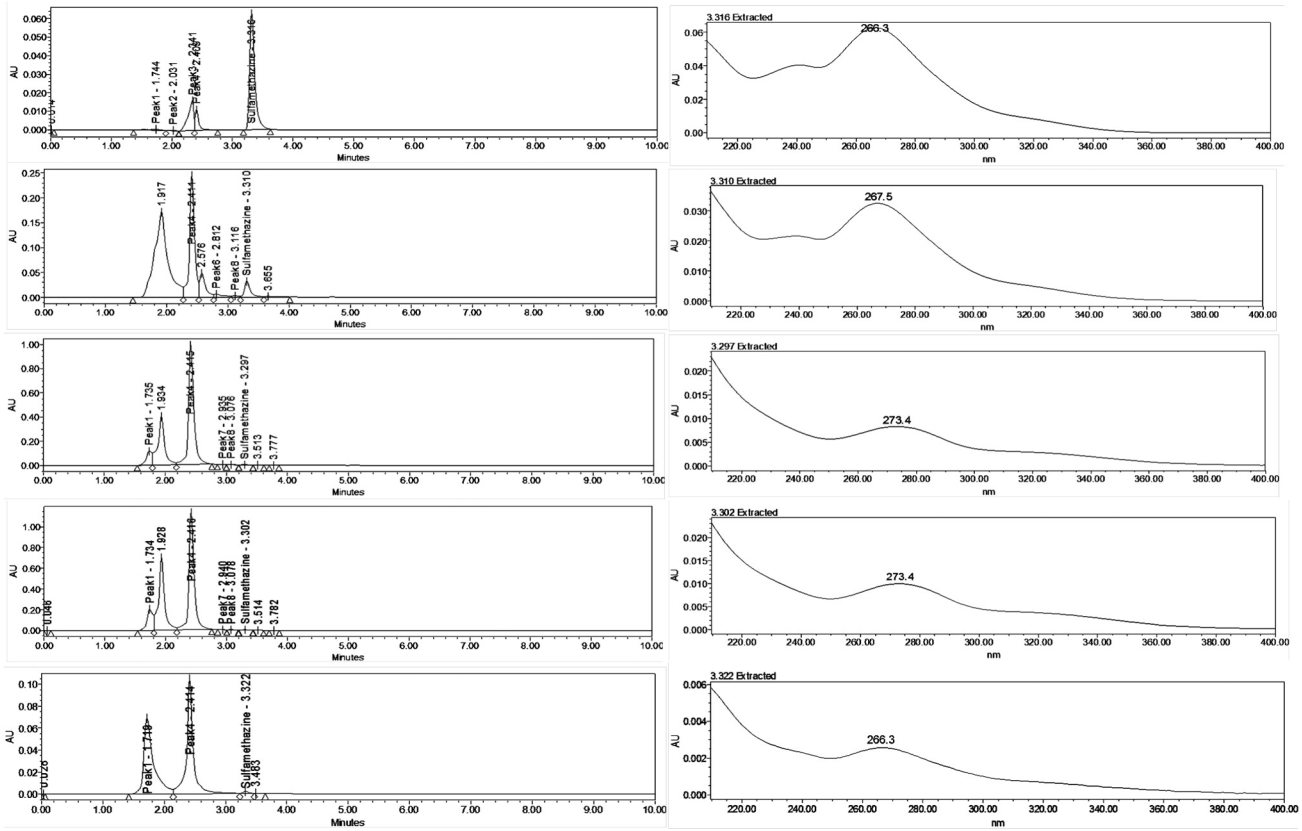

Fig. 3 – Schematic representation of spectral variation of sulfamethazine in different parts of the plant. Presented here is a distinct comparison between the chromatogram and spectra of sulfamethazine standard (top) and sulfamethazine in root (second), middle one-third (third), top portion (fourth), and sap (last) samples. All samples were scanned for their absorbance spectra at 180–400 nm.

thickness, a variation in middle zone concentration was to be expected. Additionally, the lower concentration of SMN in the middle one-third part of the plant compared to the upper one-third indicates that the retention of SMN may be dependent on moisture content. This is evidenced by the fact that the top portion has higher moisture content (80.34%) compared to the root zone (73.36%). Similar distribution was also reported by Liu et al. (2013). The authors attributed higher concentration of SMN in the leaf compared to stem due to the high transpiration stream that translocated SMN in plant leaves and stem simply acted as a conduit for conduction of SMN resulting in relatively lower accumulation of antibiotic in the stem.

2.3. Uptake in the top portion

The middle zone is proximal to the active root zone yet we detected higher concentration of SMN in the top portion part than the middle one-third part. This is due to the higher transpiration rate through leaves of the plant. Furthermore, transpiration could also lead to the concentration of SMN in the top portion of the plant. The average concentration in the upper zone was observed to be 1.89 μg/kg with the concentration ranging from 1.37–2.68 μg/kg (Table 1 and Fig. 2).

In plants, cell walls are often considered an important sink for environmental pollutants as a possible defense mechanism. It is important to note that SMN is an ionizing compound whose behavior is highly dependent upon the pH. Once SMN is added to the solution it speciates to cationic, neutral, anionic and zwitterionic (≤2%) form depending on the pH of the solution. During the study, the pH of the hydroponic solution was found to be in the range of 6.0 to 6.5. This is precisely the pH range, in which a large percentage (nearly 91% to 97%) of SMN is in neutral form. Predominance of neutral species during the experimental conditions has facilitated the translocation of SMN *via* the xylem vessel. Ionizable organic compounds in predominantly neutral form facilitate root uptake, while at the same time reducing bioaccumulation in plants (Trapp, 2009). Consequently finding a higher concentration of SMN in the top portion of the plant compared to the concentration in the middle one-third part could be attributed to transport *via* xylem vessel. This also indicates that SMN did not sorb strongly to the plant tissues once it entered the transpiration stream and instead traveled all the way to the top portion of the plant where transpiration is expected to be most prevalent.

2.4. Concentration in the sap samples

The lowest concentration of SMN was observed in the sap recovered from the whole plant. The average detected sap concentration of SMN was found to be 0.38 μg/kg with the overall concentration varying from 0.05–0.79 μg/kg (Table 1

and Fig. 2). Low concentrations in the sap samples suggest a possibility that SMN could be tightly held in other parts of the plant such as the xylem or vacuoles and therefore could not be fully extracted *via* the sap.

2.5. *Translocation of SMN in alfalfa*

From the above analysis it is evident that uptake of SMN by alfalfa is possible under hydroponic conditions. Upon exposure, hydroponically grown alfalfa was able to systemically uptake and translocate SMN. The uptake rate when compared across different parts of alfalfa, it is clear that there is significant difference ($p < 0.05$) between concentration observed in middle part and root zone, between root zone and sap sample and top portion of the plant. This variation in uptake and translocation could be attributed to chemical characteristics of SMN, particularly ionization behavior, hydrophobicity, and the physiological characteristics of alfalfa. Various researchers have established the uptake and translocation of organic pollutants based on the hydrophobicity of the pollutants (Briggs et al., 1982; Burken and Schnoor, 1998). Based on this model, SMN with $\log K_{OW} < 1$ and predominantly neutral charge observed under experimental conditions, it is possible that SMN would be easily translocated *via* the xylem vessel (Mathews and Reinhold, 2013). Studies conducted by some researchers demonstrated that translocation of SMN from root to foliage is primarily due to its hydrophilic nature and low logarithmic octanol water partition coefficient ($\log K_{OW} = 0.27$) (Kumar et al., 2005; Dolliver et al., 2007). In general, organic compounds with $\log K_{OW} < 1.8$ may not partitioned through lipid membranes in epidermal root cells whereas compounds with $\log K_{OW} > 1.8$ will not enter the xylem and will not be translocated (Mathews and Reinhold, 2013).

Uptake rate may also vary among the antibiotics belonging to the same class even for the same plant species. For example, uptake of other sulfonamides such as sulfadiazine, and sulfamethoxazole from wastewater using three varieties of Italian ryegrass — Dryan, Tachimasari and Waseyutaka shows that sulfonamides can be directly absorbed by ryegrass through its roots; however the uptake rate varies with the type of antibiotics (Xian et al., 2010). Plant's physiological status such as the presence of moisture content and potential phytotoxicity could directly affect the plant's accumulation/uptake strategy. In summary, uptake and translocation of SMN is dependent on plants physiological status during the uptake as well as the chemical characteristics of a target compound.

3. Conclusion

The alternative methods for remediation of pharmaceuticals from environmental matrices are currently being evaluated by various researchers. Particularly remediation of pharmaceuticals using hydroponically grown plants has gained momentum due to potential for uptake, metabolism and degradation of these micro-pollutants. For example, hydroponically grown vetiver grass (*Chrysopogon zizanioides* L.) has shown promising results with regard to uptake, translocation, and transformation of tetracyclines (Datta et al., 2013). Similarly uptake of aspirin and tetracycline using *Brassica juncea* has shown great potential with average remediation rate of aspirin and tetracycline was approximately 90% and 71%, respectively (Gahlawat and Gauba, 2015). In the present study, the variation in concentration in different parts (from 0.38 μg/kg in sap samples to 8.58 μg/kg in the root zone) of the plant indicates different retention rates of SMN. The presence of a higher concentration of SMN in the root zone was expected because of the direct contact between the roots and SMN-mixed nutrient solution. The concentration of SMN was greater in the roots than the concentration in shoots. This observation is consistent with the findings reported by several researchers. The middle one-third part of the plant showed significantly lower concentration than the upper one-third, indicating that the retention of SMN is mostly confined to the leafy top portion than in the middle stem part. Sap had the lowest concentration of SMN, suggesting that plants may have the ability to retain or metabolize SMN. Further research is warranted to better understand the uptake and translocation of SMN by alfalfa grown under environmental waters (CAFO lagoon and wastewater) to understand the matrix interference in the uptake of SMN. Hydroponic based remediation of pharmaceutical is very relevant because of the very low levels of pharmaceuticals found in wastewater lagoons, more so this remediation process can be implemented *in-situ*, requires less operation and maintenance and does not involve any additional chemicals.

Acknowledgments

Lead author would like to acknowledge the Office of Faculty Research for partially supporting this research study. Our sincere thanks go to Mr. Aaron Abrams for donating the Hydroponic System. The assistance from work–study students is also greatly appreciated. The authors would like to thank an anonymous reviewer for providing quality reviews for the manuscript.

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