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קליטה וטרנסלוקציה של חומרים רפואיים על ידי צמחים: השפעת תוספת בוצה והשקיה בקולחים

**Uptake and translocation of pharmaceuticals by plants: Effects of biosolids application and irrigation with treated wastewater**

**אישור התוכנית:**

**תאריך:**

**חתימת הסטודנט:**

**חתימת המנחה:**

**חתימת ראש החוג:**

# **Introduction**

Pharmaceuticals and personal care products (PPCPs) are contaminants of emerging concern (CECs) that are commonly found in biosolids and effluents from wastewater treatment plants (WWTPs). In this study the focus will be on the pharmaceuticals (PCs) only. Land application of these biosolids and treated wastewater (TWW) use can transfer them into terrestrial and aquatic environments, giving rise to potential accumulation in plants. The increasing use of biosolids as manure and TWW for irrigation and the increasing concern for human health has led to numerous studies investigating the fate of PCs in the environment. Recent publications show uptake and accumulation of different PCs in soils, water sources and plants organs. Today only few articles are dealing with uptake focuses on field conditions under real environmental concentrations. Only these kinds of studies can indicate a real risk. In the present research we plan to expose different plants to biosolids and TWW under field-like conditions. Hopefully the generated results will assist to better understand uptake and actual exposure.

# **Literature review**

## Pharmaceutical compounds

Pharmaceutically active compounds are complex molecules with different functionalities, physicochemical and biological properties. Their molecular weight range typically between 300-1000 g mol-1. Each molecule has its own ionic nature, and under environmental conditions a molecule can be neutral, cationic, anionic or zwitterion according to their p*K*a and the pH of the surroundings. One way to classify PCs is according to their therapeutical purpose (e.g. antibiotics, anti-inflammatory, anti-epileptic, etc.). It is estimated that worldwide sales of pharmaceuticals in 2014 was higher than 900 billion U.S dollars. In 2010, the consumption of antibiotic drugs was more than 7×1010 standard units (defined as a single dose unit: i.e., pill, capsule, or ampoule) (Boeckel et al., 2014; Kummerer, 2004).

## Pharmaceutical compounds in the environment.

### Removal in wastewater treatment plants

Pharmaceuticals enter sewage effluents via urine, faeces or by improper disposal. The main sources of discharge are households and hospitals. Almost all sewage effluents are being treated in WWTPs and released to rivers or fields as irrigation water. As new compounds are continually being manufactured and released to the environment, a major concern regarding their fate has emerged. Pharmaceutical compounds are being partly removed in the WWTPs. Their fate depends on several factors such as physicochemical properties of pollutants, the type of treatment technology and climatic conditions (e.g., dilution of TWW, rainfall, temperature and level of sunlight) (Kasprzyk-Hordern et al., 2009). Miege et al. (2009) collected data from 117 scientific publications that checked the influents and effluents of WWTPs covering 184 PPCPs. Their study focused on 33 molecules represents 80% of the recorded data. According to their data, many of compounds are not completely removed in WWTPs. Carbamazepine and metoprolol show less than 20% removal, clofibric acid and diclofenac exhibited less than 60% removal. More recent articles still deal with this concern and show small removals (Karnjanapiboonwong et al., 2011; Golovko et al., 2014; Kwon and Rodriguez, 2014). These studies do not take into account the full removal of PPCP in WWTPs as only the dissolved concentrations are usually measured and reported while the sludge stays unmeasured. This probably does not change results dramatically for hydrophilic molecules, but it cannot be overlooked for more hydrophobic compounds.

### Soils irrigated with treated wastewater

Pharmaceutical compounds (PCs) are introduced to arable land via intensive irrigation with TWW. In the field a PC may undergo several processes: sorption, chemo-degradation, photo-degradation, bio-degradation, mineralization, plant uptake, percolation or volatilization. Among these processes, sorption and degradation are considered the most important pathways for removal of PCs from agricultural soil (Qin et al., 2015). Sorption to soils will be determined by the physicochemical properties of the PC (e.g. p*K*a and *K*ow) and soil's characteristics (e.g. texture, clay and soil organic matter [SOM] type and content), which will determine the soil affinity to the specific PC (i.e., *K*d). While the percentage of SOM is not high in soils it plays an important role in the sorption process. Chefetz et al. (2008) examined the sorption of three PCs to different soil depths (0-5, 5-15 and 15-25 cm), they noted that the retardation factors increased as the fraction of SOM increased. For carbamazepine when the retardation factors for the 3 depths where normalized to organic carbon content (i.e., *K*oc) the trend tipped over. It has been speculated that the affinity of carbamazepine to the SOM is mainly governed by the nature of the SOM. The surface soil layer (0–5 cm), which has the highest SOM percent, seems to contain mostly partially decomposed and relatively polar organic materials, while the dipper depths contain more hydrophobic SOM due to relatively higher level of aromatic and alkyl moieties. Indeed Carbamazepine is believed to exhibit stronger sorption interaction with hydrophobic rather than polar SOM (Chefetz et al., 2008). Beside the SOM type and content, the physicochemical properties of the PC also contributed to the retardation factors values, comparing an anionic compound (i.e., naproxen; 1.8) to a neutral compound (i.e., carbamazepine; 5.8), was observed that the retardation factor for the neutral compound is higher due to electrostatic rejection between the anionic compound and the clays (Chefetz et al., 2008; Xu et al., 2009).

Degradation of pharmaceuticals and personal care products (PPCPs) was measured as well. Chen et al. (2013) followed the degradation, evaporation, leaching and storage of nine PPCPs in lysimeters for 10 years. Mass balance data reveled that more than 70% of the applied PCs were degraded in the sandy loam soil while less degradation was observed in the loamy sand soil. It was suggested that sorption to SOM or soil minerals reduced degradation as a result of reduced bioavailability. In both soils the majority of the PPCPs were detected in the upper 40 cm, and leaching below 90 cm was negligible. Yu et al. (2013) assessed the sorption and leaching of different PCs and noted that carbamazepine and gemfibrozil percolation was not negligible. When assessing degradation in aerobic and anaerobic states, it was found that more degradation occur in aerobic state and almost no degradation in anaerobic state. It led to the conclusion that PPCPs may degrade at high percentage in soil, but once it reaches the aquifers the compound will accumulate (Lin and Gan, 2011; Chen et al., 2013; Yu et al., 2013).

### Occurrence of pharmaceuticals in biosolids

Biosolids application in fields can act as a major sink for many PCs due to adsorption and absorption. On the other hand, biosolids application may be a potential route for PCs to be exposed to the environment. Removal of PCs in WWTPs can be substantial, whether the removal is due to solid partitioning or degradation is not fully understood. Partitioning into the solid phase depends mostly on the compound's hydrophobicity (i.e., *K*ow). The affinity coefficient (i.e., *K*d) of several PPCPs to the biosolids was positively correlated with their log *K*ow ranging from 1.26 to 5.48 (Dobbs et al., 1989; Xia et al., 2005). Field application of biosolids may contribute not only for the PC's concentration in the top layers of the amended soil, but also to the retardation factor. Column-leaching experiments in environmental concentrations revealed that application of biosolids generally increased the retardation factor for PCs with increased loading of biosolids. Whereas treated wastewater increased the mobility of weakly acidic in biosolids amended soils apparently due to higher pH in the soil solution caused by the TWW application (Borgman and Chefetz, 2013). Xia et al. (2010) noted that the concentrations of different compounds in the surface soil layer (0–15 cm) increased with increasing cumulative loadings of biosolids. However, this difference with cumulative loadings became less significant in soil samples below 30 cm depth. The concentrations of all four contaminants in soil decreased sharply within the top 30-cm depth and leveled off in deeper layers.

## Pharmaceuticals occurrence and fate in plants

### Uptake and translocation

Passive transport of water in the xylem is based on water potential gradient between the leaf and its surrounding atmosphere. This potential difference is the driving force for transpiration, which is affected by the climate conditions (e.g., temperature, humidity and wind). Water and nutrients may enter the root through three different routes: apoplastic, symplastic or trans-cellular. Apoplastic flow occurs through the intercellular space, while symplastic flow is intercellular through the plasmodesmata. Trans-cellular flow crosses the membranes of cells (Steudle, 2000; Gregory, 2008; Pallardy, 2010). Before entering the xylem, water and solutes need to cross the casparian strip, which act as a hydrophobic barrier (Enstone et al., 2003).

Uptake of a PC depends on its release from the soil solid phase to soil solution, and from the soil solution to the cell membrane and cytoplasm. This process is complex and also depends on the environmental conditions (e.g., temperature, humidity, wind, water content is the soil and more). Apart from the soil's properties, the compound's physicochemical properties or the environmental ones, the plant's properties influence the uptake as well (e.g., lipid content of the root membrane, metabolism systems, growth rates and foliage mass) (Wu et al., 2013, 2014).

Briggs et al. (1982) assessed the uptake and translocation of nonionic organic compounds. They noted that uptake of nonionic compounds increased with increasing lipophilicity within the range of log *K*ow 1.5-5. They conclude that the mechanisms controlling uptake of nonionic compounds are partitioning for hydrophobic compounds and uptake into the aqueous phase in roots, between and within the root cells for more polar compounds. Translocation was also *K*ow depended and was maximized with log *K*ow ~2, more hydrophobic compounds did not reach the hydrophilic xylem in high concentrations. Compounds that are less hydrophobic (log *K*ow < 2) are hindered while crossing the hydrophobic cell membrane. Wu et al. (2013) measured the bio-concentration factor (concentration in organ / concentration in matrix) for the roots for neutral PPCPs versus the log *D*ow ; *f*n is the neutral fraction of a compound)in cucumber, spinach, lettuce and pepper. They found a linear correlation between the two parameters. When calculating the translocation factor versus log *D*ow a generally negative correlation was found indicating that compounds with strong hydrophobicity (i.e., large *D*ow) tended to remain in the roots with limited in-plant redistribution.

Uptake of ionic compounds is more complex due to their capability to be charged according to their p*K*a and the surrounding pH. The activity ratio *K*D between neutral and dissociated state is describe by the Henderson-Hasselbalch equation: where i is 1 for acid and -1 for basis. The fraction of the neutral species *f*n at a given pH is. The neutral form of the weak acid or basis can be sorbed to soil, dissolved in the soil solution, in the cell (e.g., cytoplasm or vacuole) or sorbed to the cell membrane. The dissociated fraction may exist in the same state, except for hydrophilic sorption which is negligible. When the pH and the p*K*a are equal, the acid is 50% dissociated, and the fraction of the neutral form is equal to the ionic form. Soils may range from an acidic pH of ~5-6 to basic pH of ~7-8.5 according to the climate and source of the soil. Plant cells are separated from their surrounding by membrane. Out of the cell (i.e., the rhizosphere) pH can range within 5-6.5, inside the cell (i.e., the cytoplasm) pH is ~7, and in the vacuole pH is ~5.5. A neutral compound in the cell cytosol might be positively charged in the vacuole, resulting in its trapping within the vacuole hindering its translocation (i.e., ion trapping). Positively charged molecules may be sorbed to the cell wall (Trapp, 2000, 2004). These different environmental condition and plant compartments influence the transport and accumulation of weak acids or bases.

Recent studies have evaluated uptake of PPCPs under different conditions such as hydroponics (Herklotz et al., 2010; Shenker et al., 2011; Dodgen et al., 2013; Wu et al., 2013), greenhouse (Wu et al., 2010; Shenker et al., 2011; Goldstein et al., 2014), and field conditions (Wu et al., 2014; Malchi et al., 2014). Yet a small portion of these studies assessed uptake under environmental concentrations ~1 µg L-1. All the studies have reported an uptake and translocation of several PPCPs. Wu et al. (2014) assessed the uptake and translocation of 19 PPCPs in 8 vegetables under field conditions. The accumulation of PPCPs in plants generally decreased in the order of root > leaf > stem > fruit. The different PPCPs accumulated preferentially in different plant organs according to their physicochemical properties. For example, triclosan and triclocarban, concentrated mainly in in the roots, suggesting that these two hydrophobic chemicals (log *K*ow 4.7−4.9) were more likely to accumulate in roots and not translocate easily to the shoot. Malchi et al. (2014) reported a different pattern, neutral PCs (carbamazepine, caffeine, lamotrigine) were found in higher concentrations in leaves than in the roots. Probably due to different plant type and growing conditions between the two studies. Wu et al. (2010) also examined uptake of 5 PPCPs under two treatments simulating biosolids application and TWW irrigation. They noted that biosolids application resulted in higher plant concentrations, likely due to higher loading in the biosolids. However, compounds introduced to the soil by irrigation in contrast to biosolids appeared to be more available for uptake.

### Plant Metabolism

Plant metabolizes toxicants through a multiple process that converts the parent compound to a more polar molecule. Phase I metabolism converts biologically active toxicants into less active compounds via hydrolysis or oxidation, but occasionally into more toxic metabolites through bioactivation. The main enzymes in phase I is cytochrome P450 family (i.e., CYP450) which unmask or introduce polar groups on the drug to make it more soluble in water. Phase II metabolism includes conjugation reactions that link the products of phase I with plant constitutes such as sugars, amino acids glutathione or other small molecules. The main enzyme family in phase II reaction is transferase family that attaches small endogenous polar molecules of the drug (Hock and Elstner, 2004).

Phase I and phase II metabolism of carbamazepine was estimated in sweet potato and carrot by Malchi et al. (2014). While the parent compound was dominant in the soil and root of the vegetables (~90%), the portion of the metabolites: 10, 11-epoxycarbamazepine and 10, 11-dihydroxycarbamazepine in the leaves were dominant (~70%), suggesting significant metabolism of carbamazepine occurs in leaves. Comparison between carbamazepine metabolites in cucumber and tomato leaves and fruit, revealed that fraction of metabolites in leaves were higher than in fruits, suggesting again that metabolism occurs mainly in the leaves (Goldstein et al., 2014). Only evaluation and estimation of the parent compound and its metabolites will reveal the whole picture of uptake and translocation of PCs.

# **Hypothesis**

Biosolids application will have an impact on the PCs concentration in the soil solution. Hydrophobic PCs will be absorbed to the biosolids, and as a result their bioavailability will be lower. Biosolids addition can affect the bioavailability of hydrophilic compounds as well since there are many polar groups in the SOM. These affinities to SOM will be affected by the soil pH. Generally, in acidic soils the polar groups tend to be neutral while in basic soils the polar groups tend to be more negatively charged. Since irrigation with TWW introduces PCs to the soil/plant on a daily basis while biosolids in soils can act as a sink for PCs, we expect that uptake and accumulation of PCs from TWW irrigation treatments will be higher than treatments with biosolids application.

# **Objectives**

The main objective of the research is to examine uptake and translocation of several PCs in plants grown in soils amended with biosolids and/or irrigated with TWW. We also aim to understand better the plant role in uptake of PCs, planting different types of plants; leafy vegetables versus fruit vegetables and to evaluate the uptake in rain-fed plants grown in lysimeters exposed to biosolids and TWW. Examine metabolism of chosen PCs in different plant parts.

# **Materials and Methods**

## Research plan

Three types of plants will be grown in the summers of 2014 and 2015. Plants will be exposed to biosolids and treated wastewater (TWW) containing different pharmaceuticals in a range of concentrations. Treated wastewater and biosolids are provided by a conventional activated-sludge wastewater-treatment facility in the city of Kiryat Gat, Israel. The case of rain-fed cultivation will be checked as well. Wheat will be grown in lysimeters exposed to TWW and biosolids application. All plants will be analyzed to evaluate and quantify PCs concentrations.

## Crops, soils and growing conditions.

Plants will be grown in lysimeters (1 m height × 0.5 m2 surface area) containing soils (classified as Loessial Arid Brown Calcisol soils) from three locations in the northwest Negev region of Israel (Sa’ad, Nir Oz and Ein Hashlosha). Plants will be irrigated with tertiary treated wastewater (TWW) or fresh water (FW). Biosolids (60 m3 hectare-1) will be added to each of the soils irrigated with FW. Two more treatments For Ein Hashlosha soil will be added: irrigation with TWW and biosolids application (60 m3 hectare-1), and higher dose of biosolids application (180 m3 hectare-1) with FW irrigation. The experiment will be performed in triplicates for each soil, water and biosolids combination. To ensure exposure in the tomato experiment, PCs at environmentally relevant concentrations will be added to the treated wastewater throughout the growing period. Concentration of the PCs in the irrigation water will be measured throughout the growing season. At harvest, plant materials and soil samples (0−60 cm) will be collected from each lysimeter.

Table 1. Selected physicochemical properties of the different PCs investigated.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Chemical | Formula | MW | Aqueous solubility (mg L-1) | Log *K*ow\* | p*K*a | Log D | Charge | Uses |
| (g mol-1) | pH 6.5 | pH 7 |
| Bezafibrate | C19H20ClNO4 | 361.83 | 0.355 | 4.25 | 3.73 | 1.13 | -1 | Anti- lipedemic |
| Clofibric acid | C10H11ClO3 | 214.65 | 583 | 2.57 | 3.2 | 0.14 | -1 |
| Gemfibrozil | C15H22O3 | 250.34 | 10.9 | 4.77 | 4.48 | 2.23 | -1 |
| Diclofenac | C14H11Cl2NO2 | 296.16 | 2.37 | 4.51 | 4.15 | 2.43 | -1 | Anti-inflammatory agent |
| Ibuprofen | C13H19O2 | 206.29 | 21 | 3.97 | 4.91 | 1.44 | -1 |
| Ketoprofen | C16H14O3 | 254.29 | 51 | 3.12 | 4.45 | 0.51 | -1 |
| Naproxen | C14H14O3 | 230.27 | 15.9 | 3.18 | 4.15 | 1.12 | -1 |
| Carbamazepine | C15H12N2O | 236.27 | 112 | 2.45 | ---/0 | 2.23 | 0 | Anti-convulsant |
| Lamotrigine | C9H7Cl2N5 | 256.1 | 488 | 2.57 | 5.3 | 2.08 | 0 |
| Caffeine | C8H10N4O2 | 194.19 | 2.06×104 | -0.07 | 10.4 | 0.11 | 0 | stimulant |
| Metoprolol | C15H25­NO3 | 267.36 | 4780 | 1.88 | 9.68 | -0.7 | 1 | β blocker |
| Sildenafil | C22H30N6O4S | 474.59 | 3.2×103 | 2.75 | 6.4; 7.4 | 0.35 | 0 | vasoactive agent |
| Sulfapyridine | C11H11N3O2S | 249.29 | 268 | 0.35 | 2.45; 5.6 | 0.15 | -1 | Antibacterial agent |
| Sulfamethoxazole | C10H11N3O3S | 253.28 | 910 | 0.89 | 2.3 | 0.58 | 0 |
|  |  |  |  |  |  |  |  |  |

## Sample preparation, extraction, and analysis.

Freeze-dried plant material will be ground to a fine powder (magic bullet 17, Homeland Housewares, USA) and extracted using an accelerated solvent extractor (ASE350, Dionex, Sunnyvale, CA). Ground plant material (1 g) will be placed in 10 mL extraction cells between two layers of florisil (2 g) (Mg2O4Si, Alfa Aesar, Ward Hill, MA). Glass-fiber filters (27 mm) will be placed at the bottom of the cells. The packed cells will be extracted in two static cycles (5 min) with 100% methanol at 80 °C under a constant pressure of 10.34 MPa. Freeze-dried soil samples (5 g) will be similarly prepared and extracted with three static cycles (15 min) with acetonitrile/water (70:30, v/v) at 100 °C under a constant pressure of 10.34 MPa. All extracts will be evaporated to dryness and redissolved in 980 μL acetonitrile/water/acetic acid (30;70;0.1), thereafter spiked with 20 μL of a mixture of stable isotope labeled internal standards in acetonitrile, sonicated (37 kHz, 15 min), centrifuged at 17,000 g for 20 min, and filtered (0.22 μm PTFE). The final solutions will be analyzed by an Agilent 1200 Rapid Resolution LC system (Agilent Technologies Inc., Santa Clara, CA) equipped with a Gemini C-18 column (150 × 2 mm, 3-μm particle size; Phenomenex, Torrance, CA, USA), coupled to an Agilent 6410 triple quadruple mass spectrometer with an ESI ion source (Agilent). A binary gradient of 1.5% acetic acid in deionized water and 0.05% acetic acid in acetonitrile will be used for separation of the PCs (Goldstein et al., 2014; Malchi et al., 2014).

# **Preliminary results**

Tomato plants were exposed to 14 PCs through the irrigation with TWW and biosolids application. The treatments in the experiment were: 1. irrigation - fresh water (FW) and TWW. 2. Soils – Sa'ad (H), Ein Hashlosha (M), and Nir Oz (L). 3. Biosolids application- 60 m3 hectare -1 (BS), 180 m3 hectare -1 (BSS). Uptake was observed mainly for the nonionic compounds that can cross the cell membrane easily. In the tomato leaves three compounds were found: carbamazepine, lamotrigine and caffeine, 35.45 ± 2.25; 9.91 ± 2.62; 14.67 ± 10.33 ng g-1, respectively. The amount of PCs that were taken up with biosolids application was smaller: carbamazepine, 5.92 ± 1.78 ng g-1 and caffeine, 5.7 ± 1.15 ng g-1 (Figure 1). In the fruit only carbamazepine was found: 1.29 ± 0.25 ng g-1 with TWW irrigation and 0.21 ± 0.03 ng g-1 with biosolids application (Figure 2). Since the analyzed PCs are not volatile under the experimental conditions and there was no direct contact between the TWW or the biosolids and the plants upper part we can assume that the seen PCs were taken up and translocated by the plant. The other compounds (mostly acidic PCs) were not taken up probably due to percolation, degradation or sorption to the soil according to their physicochemical properties and for that reason were not taken up by the plant.

PCs concentration was higher under TWW irrigation than biosolids application (figure 1 and 2). Probably due to lower loading of the biosolids compared with TWW irrigation that added PCs to the soil solution on a daily basis. The number of PCs in the fruit and their concentrations were lower than in the leaves.

Carbamazepine metabolites were observed in the fruit and leaves of the tomato. In the plants leaves irrigated with TWW, two main metabolites were detected: 10, 11-epoxide-carbamazepine (ep-CBZ) and 10, 11-dihydro-10, 11-dihydroxy-carbamazepine (diOH-CBZ), 86.99 ± 8.38 and 13.22 ± 1.24 ng g-1 respectively. The molar fraction of the two compounds together were approximately 0.74 while carbamazepine was ~0.24. In the fruit irrigated with TWW only diOH-CBZ was found: 2.3 ± 0.6 ng g-1. The metabolite molar fraction was approximately 0.64. It is observable that the metabolites contribute a lot to the amount of PCs in the plant and cannot be neglected. With biosolids addition uptake was smaller, thus assessing the metabolism was more problematic.

Figure 1. Concentration of 3 pharmaceuticals: carbamazepine, lamotrigine and caffeine, and 2 carbamazepine metabolites: 10, 11-epoxide-carbamazepine (ep-CBZ) and 10, 11-dihydro-10, 11-dihydroxy-carbamazepine (trans diOH-CBZ) in the tomato leaves under different variables combinations- water irrigation: treated wastewater (T) and fresh water (F); soils: Sa’ad (H), Ein Hashlosha (M) and Nir Oz (L); and biosolids application: 60 m3 hectare-1 (BS) and 180 m3 hectare-1 (BSS). The bars are standard deviations for 3 repeats.

Figure 2. Concentration of carbamazepine and its metabolite: 10, 11-dihydro-10, 11-dihydroxy-carbamazepine (trans diOH-CBZ) in the tomato fruit under different variables combinations- water irrigation: treated wastewater (T) and fresh water (F); soils: Sa’ad (H), Ein Hashlosha (M) and Nir Oz (L); and biosolids application: 60 m3 hectare-1 (BS) and 180 m3 hectare-1 (BSS). The bars are standard deviations for 3 repeats.

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