An-Najah National University

Faculty of Graduate Studies

IMPACT OF PHARMACEUTICAL COMPOUND CARBAMAZEPINE IN WASTEWATER ON CUCUMBER QUALITY, QUANTITY AND GENES

By

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Dedication

I dedicated my dissertation work to my family and many friends. A special feeling of gratitude to most two people who meant to me so much to my loving parents for their engorgement and supporting me

A lot appreciate to my lovely brother and sister

Acknowledgment

First and foremost, I would like to accept praises and thanks from the Almighty God for allowing me to successfully complete this research project through His blessings.

My deep and sincere gratitude goes out to my research supervisor, Prof. Shehdeh Jodeh, and Raed Alkowni for giving me the opportunity to do this research and providing me with valuable advice throughout my research I am deeply indebted to my parents for their love, prayers, and sacrifices dedicated to my educating and preparing me for my future. I would like to say thanks to Dr. Subhi Samhan and Dr. Ziad Abdeen for their help and Abed-Alhameed for his help and giving advice about agriculture.

Many thanks to Middle East Desalination Research Center(MEDRC) for funding me.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

IMPACT OF PHARMACEUTICAL COMPOUND CARBAMAZEPINE IN WASTEWATER ON CUCUMBER QUALITY, QUANTITY AND GENES

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: Israa Maher Mohammad Qasem

Signature: Israa Qasem

Date: 7.2.2022

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ABSTRACT

The reuse of wastewater for irrigation in countries with water scarcity in agricultural sector was established but may introduce pollutants into environment as pharmaceuticals and personal care products. Many of these pharmaceutical compounds were detected and reported. Researchers discovered that there is potential of plant's uptake of these chemicals. Thus, this research aimed to study one of these pharmaceutical compound uptake and its effect on cucumber plants. Carbamazepine (CBZ) was one of the most pharmaceutical compound that found in treated wastewater and analyzed using High Performance Liquid Chromatography (HPLC). It is usually used to decrease nerve impulses that cause seizures and nerve pain, and use to treat epilepsy and neuropathic pain, bipolar affective disorder and acute mania. Carbamazepine was used as pollutants in this study on cucumber plant at different concentration ($1\mu g/L^{-1}$ and $25\mu g/L^{-1}$), tap water, treated waste water which was grown under greenhouse conditions from October to November 2021. Through the season as well as at the end of experiment, samples were collected from shoots and roots and stored at -20 for later analysis. LC-MS analysis revealed the absorbance of cucumber to CBZ in all collected samples but in different concentrations. Leaves were showed the part with highest concentration over all plant organs. The real time PCR analysis were applied to measure the gene expression level of two genes: Actin and Glutathione S-transferases (GSTs). Actin because it is conserved and essential proteins that involved in cell motility and ubiquitously expressed in all eukaryotic cells throughout the plant body and GST genes because they catalyze the detoxification of the herbicide atrazine and many herbicides and other toxic xenobiotic compounds in plants, as well as they are expressed as a response to environmental stress (temperature extremities, such as heat and cold, flood, salinity, drought, and metal toxicity) . For that, RNA extraction of samples were achieved by using silica method. Then RNAs quantity were quantified by using a spectrophotometer and quality of RNAs was verified by agarose gel

electrophoresis. The rt-PCR analysis demonstrated that after plant exposure to CBZ, Actin and GST gene were expressed at higher concentration in leaves and matched with our chemical analysis in LC-MC that CBZ concentrate more in leaves. From my research result I recommend that we must take precautions from Crops such as lettuce and other plant that we consumed the leaves part, whose edible parts are the leaves because leaves part is highly absorbable of pharmaceutical compound epically carbamazepine which may effect on human healthy.

Keywords: Sanitation, Agricultural Sector, Chemicals.

Chapter One

Introduction

1.1wastewater

If treated wastewater is introduced to soil pharmaceutical Compound(PC) may interact with soil in range of concentration (Kinney et al.,2006; Walters et al., 2010; Xia et al., 2010 Borgman and Chefetz, 2013). If soil continued irrigation with treated wastewater that led gathering of pharmaceutical compound in soil with higher level than irrigation with water. Experimental results showed that as 305–4060 and 274–1260 times erythromycin and carbamazepine accumulate in soil than their concentrations in the source irrigation water. The PC that are easy to dissolve in soil they have ability to migration and uptake by plant (Semple et al., 2004). Ability of PC to leaching may lead of these chemicals to migrate in to groundwater (Gibson et al., 2010; Bondarenko et al., 2012; Gottschall et al., 2012). In land that irrigated with treated wastewater Some PC have been dedicated like, Sulfamethoxazole, Trimethoprim, Carbamazepine, Meprobamate , and Primidone (Snyder et al., 2004; Avisar et al., 2009; Bondarenko et al., 2012). The major reason why PC present in environment is biodegradability (Ying et al.,2007; Xu et al., 2009; Yu et al., 2013). To evaluate the bioaccumulation of PCs in plants under hydroponic conditions many of experiment have been done and found the uptake mechanisms under simplified conditions (Redshaw et al., 2008;Bartha et al., 2010 Herklotz et al., 2010; Shenker et al.,2011; Calderón-Preciado et al., 2012; Tanoue et al., 2012; Wu et al., 2013 ; Dodgen et al., 2013, 2015). The Bioaccumulation Factors (BCF) of PCs in roots were found to vary widely, which were carried out at different concentrations. Some PCs such as fluoxetine, diazepam ,triclocarban and triclosan may be highly concentrated in roots, with BCF values up to 111-840 L/kg, while other PC such as diclofenac meprobamate, atorvastatin, acetaminophen and atorvastatin were less concentrated in roots, with less than 5 L/kg of BCF values generally. Diazepam, fluoxetine, dilantin, and carbamazepine are among the PCs with high values of BCF in leaves (Redshaw et al., 2008; Bartha et al., 2010; Herklotz et al., 2010; Shenker et al., 2011; Tanoue et al., 2012; Calderón-Preciado et al., 2012 ; Wu et al., 2013 ; Dodgen et al., 2013, 2015) .

Studies published in recent years suggest that wastewater have active pharmaceutical compounds (Ternes, 1998; Tixier et al., 2003; Clara et al., 2004; Miao et al., 2005;

Spongberg and Witter, 2008; Calisto and Esteves, 2009; Kummerer, 2009; Segura et al., 2009). The wastewater may contain a high level of nutrients, so it may reduce the need for other fertilizers. But in opposite site these pharmaceutical compound considering as warrying issue because its highly absorbable and resulting to accumulate in soil, sediment and tissue. Some study shows that drinking water contain concentration of pharmaceutical compound wich they are very low (1mg) but in different concentration found for irrigation are higher than 1mg (Tixier et al., 2003; Chefetz et al., 2008; Siemens et al., 2008). Exposure for long- term and low-level of PC can Couse human for health risk (Redshaw et al., 2008). This study addresses the possibility of PCs entering the food chain from irrigation plants using reclaimed wastewater. Increasing the population around the world continues with increasing urbanization the production of treated wastewater also on the rise, and that making the importance of disposal and recycling of these PC. Spain is predicted to have the highest wastewater reuse rates (> 1200 Mm3/year) (BIO by Deloitte, 2015) in the EU, with a total of 3222 Mm3/year in 2025. Agricultural sector with 70% of total water is the most one that use treated wastewater globally (Frenken and Gillet, 2012). In region with limited source of water they use recycled water as alternative option (Lorite et al., 2018).

PC compounds haven't been studied thoroughly in soil. CBZ degradation has been studied in some soils. (Li et al., 2013; Monteiro and Boxall, 2009; Yu et al., 2013 Salvia et al., 2014;). Knowing the fate of contaminants is important because, in addition to their presence, they can affect the health of the soil directly (Hammesfahr et al., 2008; Liu et al., 2009). In the years after the discovery of antibiotics just before World War II, pharmaceuticals have seen a dramatic increase in use (Henderson et al., 1999). 3000 active pharmaceuticals and more are use h human and veterinary medicine (Ternes, 2001; Petrovic. 2014.). Pharmaceuticals are partly resistant to degradation due to their physio-chemical properties, and consequently end up in the waste stream and eventually in waste treatment. (Heberer 2002; Długołecka, 2006; Chenxi et al., 2008). low removal efficiency in WWTPs of ≤ 10 % of Carbamazepine has been reported (Ternes TA. 1998; Paxéus N. 2003; Löffler et al., 2005) Radishes, ryegrass, lettuce, peppers, cucumbers and spinach peppers are among the plants that absorb and accumulate CBZ (Wu X et al., 2013 ; Carter et al., 2014)

1.2 Wastewater treatment

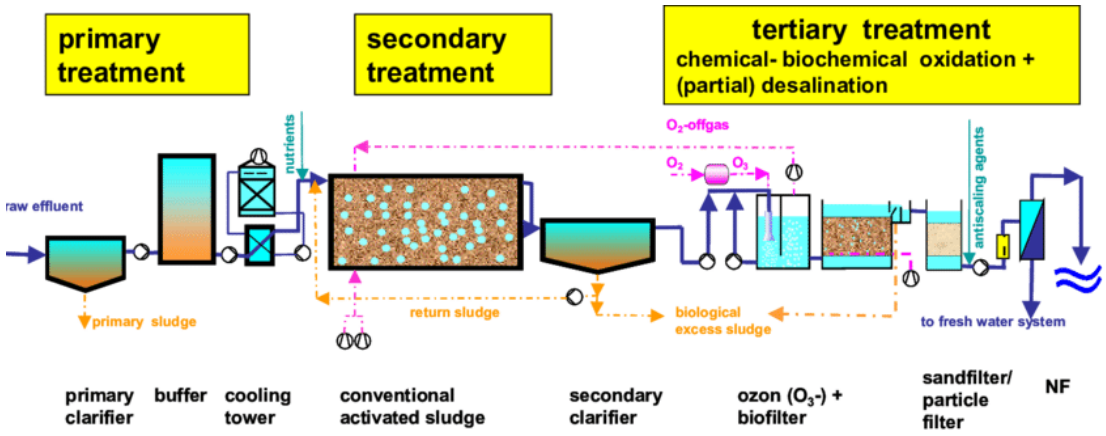
A problem would occur if we didn't treat billions of gallons of wastewater every day before releasing them into the environment. Wastewater treatment reduces pollutants to a level that is safe for the environment. If wastewater is not properly treated we will face many problems like excessive nutrients, such as Chlorine, Nitrogen, Phosphorus and Inorganic Chloramines compound, Bacteria, Viruses, Metals such as Mercury, Cadmium, Lead, Chromium and Arsenic, Other substances such as some personal care products and pharmaceutical all these pollutants are toxic and threat our health. Increasing population will limit fresh water resources in the future, which will result in an increasing use of reclaimed wastewater (Vörösmarty et al., 2000). To limit adverse effects on agricultural production and food, in the terrestrial environment it's crucial to anticipate the risks associated with pharmaceuticals. The counters that face problem in finding another resources for water they using wastewater treatment as alternative resource.

1.3 Stages of Wastewater Treatment

The wastewater treatment process has stages figure 1. first, wastewater is temporarily leaved to settle down where heavier solids such as rags, wood fragments, plastics, sand and grease are sink down to the bottom. Once settled, these materials are get rid of it. Then the water is pumped to next step of and chemicals are also added to remove phosphorus. biological degradation in this step is by aeration, by consuming the pollutants, microorganisms convert them into cell tissue, water, and nitrogen. Then filtration, by filtering through 10-micron polyester media. After disinfection, ultraviolet light is used to ensure that wastewater is virtually bacteriologically free. Disinfection is used after the filtration step to raise the quality of the water to optimum standards demand. It asked to remove of pathogens, to ensures that water is safe for drinking purposes.

Figure1

Stages of wastewater treatment



1.4 Actin gene

Actin are conserved and essential proteins that are important for cell motility and are all over expressed in all eukaryotic cells throughout the plant body. Necessary of cell cytoskeleton component. Plays a crucial role in cell division, determination of cell shape, organelle movement, cytoplasmic streaming, extension growth, fertilization, hormone transport and responses to external signals. Actin achieves these crucial functions by combine with many Actin-Binding Proteins (ABPs). Its expressed in mature pollen, organ primordia, young embryo sac, and pollen tubes. In vegetative organs few or no reproductive-gene expression is revealed, such as leaves, sepals, stem, root, and petals. Actins, in particular β -actin, play a crucial role in housekeeping functions essential for cell survival (Chang et al., 1998; Calvo et al., 2002). In higher plants, terrestrial plant Arabidopsis thaliana, in terrestrial plant the expression and structure of actin genes have been characterized, 2 actin pseudogenes, 8 functional actin genes have been reported (McDowel et al., 1996).

1.5 Glutathione S-Transferase

it discovered that atrazine and many herbicides and other toxic xenobiotic compounds in plants are catalyzed detoxification by GST (Lamoureux and Rusness, 1989; Timmerman, 1989; Dixon et al., 1998; Schröder et al., 2007). Molecular masses of around 50 kDa of GSTs proteins with ubiquitous and multifunctional enzymes encoded by number of gene families. Early investigation on some GST genes in GSTs in plant showed that they are

up-regulated by chemical, and microbial infections (Fodor et al., 1997; Sappl et al., 2004 & 2009). Other GST genes are inducible by biotic and abiotic stimuli (De Ridder et al., 2002; Wagner et al., 2002; Sappl et al., 2009; Dixon et al., 2011; Csiszár et al., 2014). In several plant species reported that GSTs play role in reducing oxidative damage (Cummins et al., 1999; Edwards et al., 2000; Kilili et al., 2004), oxidative stress tolerance are exhibited in transgenic plants that overproducing a GST gene (Roxas et al., 2000; Zhao and Zhang, 2006; Ji et al.,2010), furthermore they involve in controlling the programmed cell death (Kampranis et al.,2000). Particular groups of GSTs are induced in the early phase of bacterial, fungal and viral infections which numerous investigations proved that. Glutathione-S-transferases (GSTs) show four independent classes Alpha, Mu, Pi, Sigma which is dimeric enzymes (Buetler et al .1992, Neuefeind et al .1997) and Theta(Buetler et al .1992, Neuefeind et al .1995) . Classes Alpha, Mu and Pi are present only in animals and yeasts (Buetler et al .1992) in bacteria and plants these are not found (Pemble et al.1992). Heterogeneous Theta class (Buetler et al .1992; Meyer et al., 1991) is present in yeasts, humans , plants, bacteria, chickens, rats ,salmon and non-vertebrates such as flies and show absent in lower animals such as molluscs, Platyhelminthes and nematode (Taylor et al .1993). Zeta and Theta GSTs are found in both animals and plants, but the Phi and Tau classes are plant-specific.

1.6 Literature Review

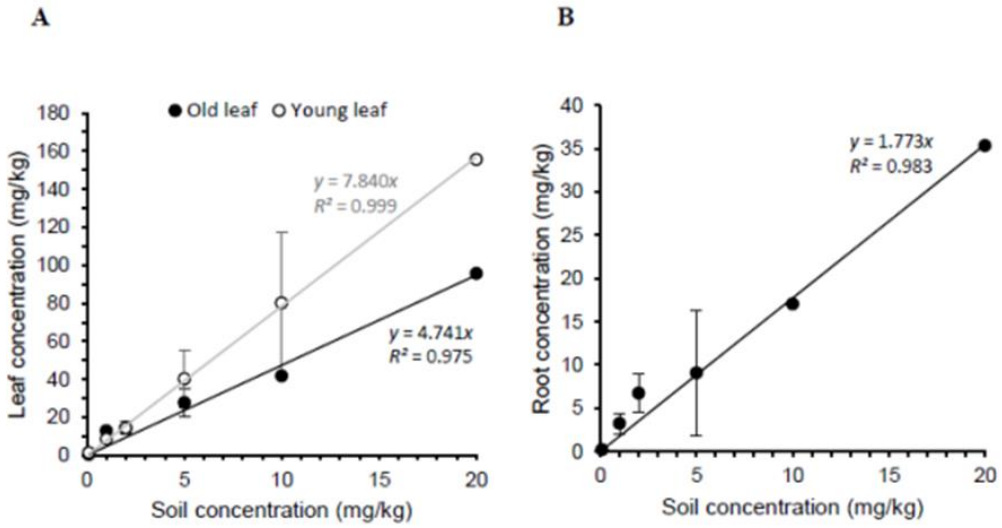
study demonstrates atenolol, carbamazepine, and triclosan uptake by lettuce, maze, and radish in a Mediterranean setting which irrigated with reclaimed water. And these PC have not been effectively removed from conventional wastewaters (Wang and Wang, 2016). The accumulation of PC in plant are widely reported in scientific literature (Boxall et al., 2006; Dettenmaier et al., 2009; Calderón-Preciado et al.,2011, 2013; Dodgen et al., 2015). This study provides data on the intake and translocation of atenolol, carbamazepine, which present in reclaimed water in an urban area in Spain. In lettuce soil The concentrations of ATN ranged from 0.6 to 2.1, TCS from 0.1 to 11.1 ng/g dry weight (d.w.), CBZ from 0.3 to 64.6. In maize soil the concentrations found for ATN ranged from 0.6 to 7.6, CBZ from0.3 to 185.8 and TCS from 0.3–11.6 ng/g d.w .In the radish soil the concentration of ATN ranged from of 0.3–14.0,CBZ from 0.2to 155.8 and TCS from 0.2–14.8 ng/g d.w. At the end of experiment, the concentrations of the three PC in the soils were in order: ATN < TCS <CBZ. And that match with study published by

(Carter et al. 2014; Hurtado et al). Which prove that CBZ was taken up to the greatest level in both the radish (52 μg/g) and ryegrass (33 μg/g). (Kinney et al.2006) The accumulation and translocation of the three PCs from soils to plants irrigated with row water were detected in the organs of plants containing CBZ among the four most common PCs detected in soils irrigated with row water. Radish plant produced the highest level of (ATN 10.5 ± 1.9 and TCS 6.0 ± 5.4 ng/g) in the root , while CBZ was in leaves (424.8±153.0ng/g). while maize plants contained the highest levels of ATN and CBZ in leaves (5.7 ± 0.9, 142.9 ± 43.2 and ng/g). The CBZ is Among three PC compound which accumulated in leaves with higher concentrations than root. And that agree with result reported by this research (Winker et al., 2010; Wu et al., 2010, 2012, 2013) reporting a different plant species grow in soils that contain high concentrations of CBZ. The reason why CBZ accumulate in leaves is the non-ionic properties of CBZ. In the direction of the transpiration stream, it can be moved from the xylem to the phloem with a tendency to accumulate in leaves.

Other study, bioaccumulation, toxicity and uptake of carbamazepine in soil plant systems. The soil in this study collected from Karoonda in South Australia (April, 2015). And work on Cucurbita pepo (Zucchini) plant. which shown ability to accumulate and translocate organic contaminant to shoot and root. This species use an indicator reclaimed wastewater and solids from water treatment. After plant were planted and grown the old leaves and young where separated based on size and at the end of experiment soil sample were taken to measure the concentration of carbamazepine Figure 2 .

Figure 2

Average uptake of CBZ into C.pepo leaves (A) and roots (B) after plants were grown from seed in CBZ-spiked soil for 14 weeks.



Compared to ionic compounds, CBZ has a non-ionic behavior making it pass easily through root cells because it does not require energy to move through the root membranes (Malchi et al 2014.),(Carter et al . 2014). Studies indicate that Gaussian distribution is governed the uptake of organic compounds in plants with maximum translocation at log Kow1.5 to 2.0(Briggs e al. 1982.) In C. pepo the higher concentration of CBZ has been found in leaves (Carter et al. 2014.) (Carter et al. 2015.) In radish leaves the concentration of CBZ after 6 weeks was up to 52 mg/kg grown in soil with 1 mg/kg of CBZ (Carter et al, 2014.) The differences in concentration of CBZ between radish and C. pepo leaves depend on pharmaceutical compound physical and chemical properties but also the environmental growing conditions. The CBZ concentrations in the root of C. pepo were less than those found in the leaf. And that correspond with other study indicate that the root has lower concentrations of CBZ, w ere in the leaf is higher (Carter et al 2014; Wu X et al. 2014). An expirement on the uptake and translocation of ^{14}C-carbamazepine by plants and soil. there experiments were done in a greenhouse with soil treated with 3.86 mL of ^{14}C-CBZ stock solution into a 200.0 g of soil in 500 ml glass beaker then it mixed in fume hood until evaporating. ^{14}C-CBZ was initially leveled at 11,393dpm. After that, they planted celery, carrots, and pak choi in pots, and irrigated with water for 60 days for carrots and celery, and 40 days for pak choi. Then (carrot, celery and pak choi) was tested for up take ^{14}C from soil with initial concentration ^{14}C-CBZ at 167 ± 3.9 Bq g^{-1} (10001 ±

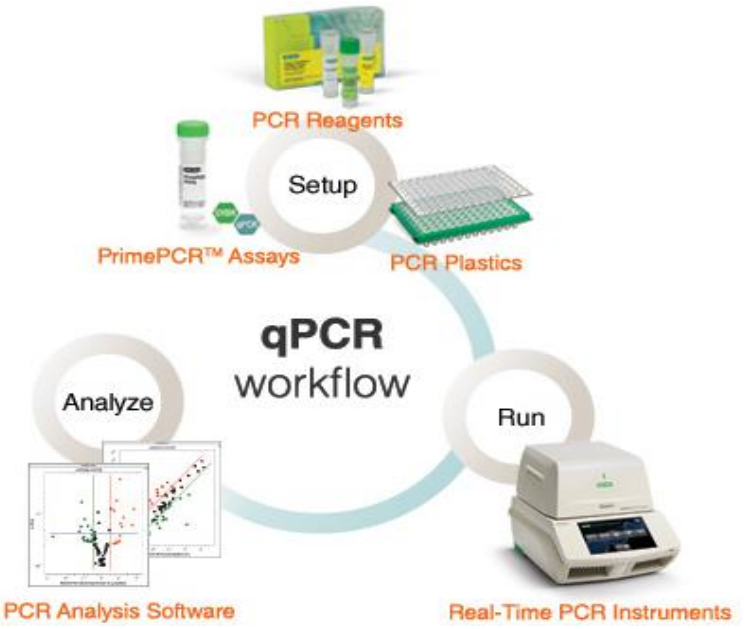
236 dpm g-1) soil (dry weight equivalent). And reflect that ^{14}C can be absorbed by three plant and transport to tissue (stems, root and leaves). Since the stem serves as a mass flow duct for transport of ^{14} C from roots to leaves, they detected that ^{14} C absorbed with a higher concentration in roots and transported to leaves, with a lower concentration in stems. (Carter et al., 2014). The accumulation of ^{14}C in the leaves may affect by the structure of plants. In celery ^{14} C was accumulate in the edge of the leaves. The same in carrot. but in pak choi it distributed homogeneously. That mean the different distribution in different tissue and vegetable because Features and characteristics of different physiological systems (e.g., phloem and lipid contents) (Wu et al., 2012). On day 10 the bioaccumulation factor in celery, carrot and pak choi were 7.6 ± 0.9, 3.6 ± 304 0.5 and 4.4 ± 3.5, respectively. On day 40, the BCF numbers increased to 25.5 ± 3.0, 305 23.6 ± 5.7, and 44.0 ± 5.6 for celery, carrot and pak choi respectively. In celery, the lowest BCF was found in the stem, between 7.02 ± 0.97 and 13.80 ± 0.77. Between 0 and 40 days, BCF values dramatically increased in leaves with a maximum BCF of 37.7 ± 3.6 at the end of the growing period. Studies demonstrate that higher accumulation of CBZ in leaves (Wu et al., 2013; Carter et al., 2014; Wu et al., 2014). For lettuce and pepper, CBZ accumulation was 7-10 times in leaves higher than in roots. In Plant tissues accumulation of CBZ in leaves are easy to cross the tissue and transported in the direction of transpiration, leading to a greater concentration (Goldstein et al., 2014; Malchi et al., 2014; Hurtado et al., 2016). The CBZ removal efficiency is low, and in some cases, even negative in some cases (Collado et al., 2014).

The Polymerase Chain Reaction (PCR), followed by reverse transcription (RT), represents the most powerful technique for increasing and detecting mRNA levels (Heid et al., 1996; Lockey, 1998). Real-time quantitative RT-PCR (qRT-PCR) is the method of choice for quantifying these low abundant expressed genes in any biological matrix. As compared to conventional RT-PCR, real-time qRT-PCR has many advantages, such as high sensitivity, high specificity, good reproducibility, and a wide dynamic range of quantification (Higuchi et al., 1993; Gibson et al., 1996; Orland et al., 1998; Freeman et al., 1999; Schmittgen et al., 2000; Bustin, 2000). The most sensitive and most reliable method is the qRT-PCR, especially when the RNA concentration is low, the structure is partly deteriorated, and the sample size is limited (Freeman et al., 1999; Steuerwald et al., 1999; Mackay et al., 2002).

There is enormous potential for real-time RT-PCR applications in transcriptome analysis, but it is important to understand its underlying principles of quantification. An optimal mRNA quantification process depends on standardized preanalytical steps (tissue sampling, storage, RNA extraction, and RNA quantity and quality control), optimal RT and PCR performance (in terms of specificity, sensitivity, reproducibility, and robustness), and precise post-PCT data processing (data acquisition, evaluation, calculation, and statistics) (Bustin, 2004; Pfaffl, 2004; Burkardt, 2000). In qRTPCR, there are two general types of quantification.

Figure 3

quantitative real-time RT-PCR technique.



The levels of expression of genes can be determined by either an 'absolute' quantification or by a relative or comparative real-time qRT-PCR (Pfaffl, 2004). Using a calibration curve, the PCR signal is linked to input copy number in the 'absolute' quantification approach (Bustin, 2000; Pfaffl and Hageleit, 2001; Fronhoffs et al., 2002). In addition to diluted PCR products, linearized plasmids, or spiked tissue samples, calibration curves can be generated using recombinant DNA or RNA.

In order to be reliable, such an absolute real-time RTPCR assay must be based on 'identical' amplification efficiencies both for the native mRNA and for the RNA or DNA used in the calibration curve (Souaze et al., 1996; Pfaffl, 2001). Because the

quantification is shown relative to the used calibration curve the ‘absolute’ quantification is misleading. It is necessary to correlate mRNA copy numbers with some biological parameters, like the mass of a tissue, DNA or RNA content, or the number of cells, or to compare to a commonly used reference gene copy number (such as ribosomal RNA or housekeeping genes (HKG)).

Real-time PCR can either be qualitative (the presence or absence of a sequence) or quantitative (copy number). In contrast, PCR is at best semi quantitative. Additionally, real-time qPCR data can be achevied without gel electrophoresis, resulting in reduced time and increased throughput. Finally, because real-time qPCR reactions are run and data are evaluated in a unified, closed-tube qPCR system, possibilities for contamination are low and the need for post amplification manipulation is eliminated in qPCR analysis.

Real-time qPCR technique have become the instrument of choice for the fast and sensitive determination and quantitation of nucleic acid in various biological samples, with many applications such as gene expression analysis, the detection of genetically modified organisms in food, and cancer phenotyping.

In research laboratories, qPCR technique are widely used for the quantitative measurement of gene copy number (gene dosage) in transformed cell lines or the presence of mutant genes. In combination with reverse-transcription (RT-PCR), qPCR technique can be used to precisely quantitate changes in gene expression, for example, an increase or decrease in expression in response to different environmental conditions or drug treatment, by measuring changes in cellular mRNA levels.

Relative quantification or comparative quantification measures the relative change in mRNA expression levels. Using SteadyState mRNA levels, it compares them across samples of a gene and presents them in relation to the levels of another gene. Relative quantification does not require a calibration curve or standards with known concentrations and the reference can be any transcript, as long as its sequence is known (Bustin, 2002).

In real-time RT-PCR experiments, relative quantities can be compared across multiple experiments, regardless of the units used to express them (Orlando *et al*., 1998; Vandesompele *et al*., 2002; Hellemans *et al*., 2006). It is the adequate tool to investigate

small physiological changes in gene expression levels. As reference genes, constant expressed genes can be amplified in the same tube in a multiplex assay (as endogenous controls) or can be amplified in a separate tube (as exogenous controls) (Wittwer *et al*., 2001; Livak, 1997, 2001; Morse *et al*.,2005). Multiple possibilities are obvious to compare a gene of interest (GOI) mRNA expression to one of the following parameters. A gene expression can be relative to: an endogenous control, e.g. a constant expressed reference gene, an exogenous control, e.g. an universal and/or artificial control RNA or DNA, an reference gene index, e.g. consisting of multiple averaged endogenous controls, and a target gene index, e.g. consisting of averaged GOIs analyzed in the study.

To determine the level of expression, the differences (Δ) between the threshold cycle (Ct) or crossing points (CP) are measured. As a result the mentioned methods might be called the ΔCP methods (Morse *et al*., 2005; Livak and Schmittgen, 2001). But the complexity of the relative quantification procedure can be increased. In a further step a second relative parameter can be added, e.g. comparing the GOI expression level relative to: a nontreated control, a time point zero and healthy individuals.

To achieve optimal relative expression results, appropriate normalization strategies are required to control for experimental error (Vandesompele *et al*., 2002; Pfaffl *et al*., 2004), and to ensure identical cycling performance during real-time PCR. The variations occur during the extraction and processing of the RNA, during PCR set-up and during the cycling process. All the relative comparisons should be made on a constant basis of extracted RNA, on analyzed mass of tissue, or an identical amount of selected cells (e.g. microdissection, biopsy, cell culture or blood cells) (Skern *et al*., 2005). It is important to normalize or equilibrate the relative expression data according to at least one of the following variables: sample size/mass or tissue volume, total amount of extracted RNA, total amount of genomic DNA reference ribosomal RNAs (e.g. 18S or 28S rRNA), reference messenger RNAs (mRNA), total amount of genomic DNA and artificial RNA or DNA molecules (= standard material).

There can be no overemphasis on the importance of choosing housekeeping or lineage specific genes. For a number of commonly used reference genes, processed pseudogenes have been shown to exist, e.g. for β-actin or GAPDH (Dirnhofer *et al*., 1995; Ercodani *et al*., 1988). It is possible for pseudogenes to generate specific amplification products independent of intact mRNA, and to result in specific amplification even in the absence

of intact mRNA. In order to determine the efficiency of RT and kinetic PCR, it is essential to develop universal, artificial, stable, internal standard materials that can be added before the preparation of RNA. (Bustin,2002). In a multiple pair-wise correlation analysis, more than one reference gene should be considered and a summary reference gene index should be obtained (Pfaffl *et al*., 2004). This represents a weighted expression of at least three reference genes and a more reliable basis of normalization in relative quantification can be postulated.

These aspects of qRT-PCR software tools for evaluating gene expression levels are increasingly appreciated .*geNorm* (Vandesompele *et al*., 2002) and *BestKeeper* (Pfaffl *et al*., 2004) allows for an accurate normalization of real-time qRT-PCR data by geometric averaging of multiple internal control genes. With the geNorm Visual Basic applet for Microsoft Excel®, the applet calculates a gene expression normalization factor for each tissue sample based on the geometric mean of ten tested genes in the cDNA sample panel geNorm's normalization strategy is crucial for accurate kinetic RT-PCR expression profiling, which can be used to study the biological significance of small expression differences (Vandesompele *et al*., 2002). These normalizing strategies are summarized and described in detail elsewhere (Huggett *et al*., 2005; LightCycler® Relative Quantification Software, 2001).

GOI expression relative to another gene, usually to an adequate reference gene, can be calculated on the basis of 'delta Cp' (ΔCp,24) or 'delta delta Ct' (ΔΔCt) values (Livak and Schmittgen, 2001). A variety of mathematical models are available today to calculate the relative expression ratio (R), based on a comparison of distinct cycle differences.

The CP value can be determined by various algorithms, e.g. CP at a constant level of fluorescence or CP acquisition according to the established mathematic algorithm.

Three general procedures of calculation of the relative quantification ratio are established:

1- The so-called 'delta Ct 'or 'delta-delta Ct' method.

$R = 2[CP\ sample - CP\ control]$ (eq. 1)

$R = 2\Delta CP$ (eq. 2)

$R = 2–[\Delta CP\ sample – \Delta CP\ control]$ (eq. 3)

$R = 2–\Delta\Delta CP$ (eq. 4)

The method which is used in our research.

2- The efficiency corrected calculation models, based on ONE sample.

3- An efficiency corrected calculation models, based on MULTIPLE sample and on MULTIPLE reference genes, so-called REF index, consisting at least of three reference genes.

One or more nonregulated reference genes (REF) are used to normalize the target gene expression, e.g., derived from classical and frequently described reference genes (Bustin, 2000; Vandesompele *et al*., 2002; Pfaffl *et al*., 2005). In this approach, the major problem is that the most common reference-gene transcripts from stable housekeeping genes may be influenced by the applied treatment. Expression of mRNA can be regulated, and levels of expression vary significantly during treatment, between tissues and/or individuals (Pfaffl, 2004; Schmittgen and Zakrajsek, 2000).

However, qRT-PCR is influenced by numerous variables and appears as a multifactorial reaction. In order for relative quantification to generate useful and biologically relevant results, it must be highly validated. Reference genes as external standards have the main disadvantage that they don't include internal controls for RT and PCR inhibitors. All quantitative PCR methods assume that the target and the sample amplify with similar efficiency (Wittwer *et al*., 2001; Livak and Schmittgen, 2001). In external references, there is a risk that some samples may contain substances that greatly affect the PCR amplification efficiency. As discussed earlier (Pfaffl, 2004), sporadic RT and PCR inhibitors or enhancers can occur.

During real-time fluorescence analysis, each sample generates its own amplification history. As we know from laboratory practice, biological replicates, even technical replicates, result in significantly different fluorescence curves as a result of sample-to-sample variations. Inhibitors and enhancers that affect RT and PCR efficiencies, along with variations in the RNA pattern, cause changes in PCR efficiencies.

Thus the shapes of fluorescence amplification curves differ in the background level (noisy,constant or increasing), the take-off point (early or late), the steepness (good or bad efficiency), the change-over to the plateau phase (quick or steady), and in the appearance of the PCR plateau (constant, in or decreasing trend) (Tichopad *et al*., 2003; Tichopad *et al*., 2004). PCR amplification efficiency is the most important factor affecting amplification kinetics, and is strongly influenced by the PCR reaction components. Therefore, CP determination of the threshold level and in consequence the accuracy of the quantification results are influenced by the amplification efficiency. In real-time gene quantification, efficiency evaluation is an essential marker and correction is necessary (Rasmussen, 2001; Liu and Saint, 2002a; Liu and Saint, 2002b; Tichopad *et al*., 2003; Meijerink *et al*., 2001).

Up to now only one software package can automatically determine the realtime PCR efficiency sample-by-sample. In the Rotor-Gene™ 3000 software package (Corbett Research), it is called the comparative quantification. Based on the fluorescence increase during the exponential phase of PCR, the amplification rate is calculated . Further algorithms and methods are described in recent publications to estimate the real-time PCR efficiency. These can be grouped in direct and indirect methods. Methods that are direct are based either on dilutions or on measurements of the relative fluorescence during the exponential phase . Indirect methods, on the other hand, are based on fitting mathematical models, such as sigmoidal, logistic, or exponential curves, to determine efficiency.

The amplification rate is calculated on the basis of a linear regression slope of a dilution row. Efficiency (*E*) can be determined (Higuchi *et al*., 1993; Rasmussen, 2001). But the real-time PCR efficiency should be evaluated sample-by-sample, which is quite laborious and costly, wastes template, and takes time if the dilution method is used. As an alternative, a pool of RNAs from all samples can be used to accumulate all possible 'positive and negative impacts' on kinetic PCR .

In the calibration curve as well as in native sample RNA, linear relationship between CP and logarithm of starting copy number should exist for up to five orders of magnitude (Muller *et al*., 2002). The advantage of the dilution method is that it is highly reproducible and constant within one transcript and tissue.

The disadvantage of this approach is the high efficiencies, often higher than two (E > 2.0), which is practically impossible on the basis of the PCR amplification theory. This indicates that this efficiency estimation is more or less not the best one and it will overestimate the 'real' amplification efficiency.

Efficiency calculation from the fluorescence increases in the exponential phase of fluorescence history plot (in log. scale) (*Figure 3.4*). Fitting can be done by eye, or more reliably by software applications like LinRegPCR (Ramakers *et al*., 2003) or DART-PCR (Peirson *et al*., 2003). During the analysis, the investigator must decide which fluorescence data should be included and which should be omitted. Based on at least four data points, a linear regression plot is drawn, where the slope of the regression line indicates the PCR efficiency . Therefore this method is more or less arbitrary and dependent on the chosen data points.

The advantage of both direct methods is the independency of the background fluorescence. The indirect curve fit, like sigmoidal, logistic, and exponential ones, will be interfered with by a rising trend in the background fluorescence, as we know from several application . For probe-based detection, background levels tend to be high and fluctuating, while for SYBR® Green I applications, background levels are low and constant.

The CP value is the central value in real-time PCR applications. Everything is related to this single point. But little effort has been put into standardizing and optimizing the determination of this parameter, which is so important to quantification . Most software measures the CP at a constant fluorescence intensity using the so-called "threshold cycle method" or "fit point method." There are, however, other options to explore.

Pre-adjusted fluorescence data and preadjusted CP are displayed on most real-time platforms. Various approaches are used to obtain the CP value after an automatic background adjustment, e.g., at a constant level of fluorescence. At the threshold fluorescence, these constant threshold approaches presume that all samples have the same DNA concentration. However, determining the degree of background fluorescence might be difficult. Drift-ups and drift-downs over the course of the experiment frequently create considerable background fluorescence changes in real-time PCR assays. Averaging over a drifting backdrop will boost the threshold level by overestimating variation (Livak,

1997, 2001; Rasmussen, 2001; Wilhelm *et al*., 2003). Fitting the intersecting line at 10 standard deviations above baseline fluorescence level yields the threshold level. The threshold level is determined by fitting the intersecting line at 10 standard deviations above the baseline fluorescence level. (Livak, 1997, 2001). The fit point approach requires the user to remove uninformative background points, enter the number of log-linear points to exclude plateau values, and then fit a log line to the linear component of the amplification curves. These log lines are projected back to a common threshold line, and the CP value is found at the junction of the two lines. The robustness of this approach is its main advantage. The flaw is that it is difficult to automate, necessitating a lot of user interaction, which is more or less arbitrary. (Rasmussen, 2001, LightCycler® Software, 2001). The main issue is comparing a large number of biological samples. Within one run or between runs, the researcher will have difficulty defining a steady background for all samples. The development of a new and user-friendly CP acquisition approach was prompted by these sample-to-sample changes in variance and absolute fluorescence readings. There are numerous mathematical models to determine the amplification rate, including a logistic or sigmoidal model, as stated in the previous section. The optimal CP can also be determined using these mathematically fit models. They are more or less independent of background levels, or they are estimated using background fluorescence, and they are used in the CP determination model (Tichopad *et al*., 2004; Wilhelm *et al*., 2003).

In LightCycler® (Roche Applied Science) and Rotor-Gene™ (Corbett Research) software packages these approaches are already implemented. The CP is automatically recognized and measured at maximal fluorescence acceleration. (Ramussen, 2001; LightCycler® Software, 2000). Although the specific mathematical algorithm used is yet unknown, it is quite similar to a logistic fit. The 'take of point' in the Rotor-Gene family is also estimated using comparative quantification and a sigmoidal model. With good agreement, both the sigmoidal and polynomial curve models function well. ($P<0.001$; $r>0.99$) (Tichopad *et al*., 2004; Liu and Saint, 2002a; Liu and Saint, 2002b; Rutledge, 2004). The sigmoidal exponential function was more precise, and it could improve the CP measurement's exactness and precision, as well as the amplification efficiency rate (Wilhelm *et al*., 2003).

The creation of precise and reliable gene expression analysis and quantification software is a key task. Future advances are aiming for a "one-size-fits-all" detection and application software, which appears to be the best solution. Real-time qRT-PCR is the method of choice for expression profiling in biological research and clinical diagnostics. On one hand, cyclers and chemistry advanced much more quickly than detection and analysis software. However, a precise and simple mathematical and statistical analysis of the raw data is required. (cycle threshold/crossingpoint values or molecules quantified) The administration of expanding data collections, as well as the analysis of gene expression, have become critical challenges. 96-well applications are currently the industry standard in research laboratories, but high-throughput 384-well applications will create massive amounts of data in the near future. The data need to be grouped (Hellemans *et al*., 2006) and standardized by intelligent algorithms. To detect samples with differing efficiencies, real-time qPCR data should be examined using an automated statistical method, such as Kinetic Outlier Detection (KOD). (Bar *etal*., 2003). Statistical data analysis or CP values are often accomplished using basic parametric tests like analysis of variance or t-tests. Parametric tests rely on unproven assumptions, such as the normality of distributions (Sheskin, 2000). Normal distributions may not be predicted in absolute or relative quantification analysis, where the quantities of interest are obtained from ratios and variances can be substantial, and it is unclear how a parametric test should be designed (Pfaffl *et al*., 2002; Sheskin, 2000). Currently, the relative quantification data analysis and software programs listed below are accessible.

The first commercially available software was the LightCycler® Relative Quantification Software (2001). It can be used to calculate and compare relative quantification results of triplicates of a target versus a calibrator gene. Target genes are corrected via a reference-gene expression and calculates on the basis of the median of the performed triplets. Real-time PCR efficiency correction is possible within the software and is calculated from the calibration curve slope, according to the established eq. 9, ranging from E = 1.0 (minimum value) to E = 2.0 (theoretical maximum and efficiency optimum). A given correction factor and a multiplication factor, which are provided in the product specific applications by Roche Molecular Biochemicals (LightCycler® Relative Quantification Software, 2001), have to be incorporated in eq. 6. Importantly, no statistical comparison of the results by a statistical test is possible.

Recently, Q-Gene, a software tool capable of performing statistical tests on real-time data, was developed (Muller *et al*., 2002). Q-Gene facilitates the planning, execution, and analysis of quantitative real-time PCR operations. Graphical representation of the expression outcomes was used. It is feasible to adjust efficiency using the dilution method. Q-Gene can handle high-throughput quantitative real-time PCR studies (96-well and 384-well format) and greatly simplifies and rationalizes experimental set-up, data analysis, and data administration while assuring maximum reproducibility. The Q-Gene Statistics Add-In is a collection of VBA programs for performing commonly used parametric and non-parametric statistical tests quickly and with menu guidance. A paired or unpaired Student's test, a Mann-Whitney U-test, or a Wilcoxon signed-rank test can be used to determine the level of significance between any two groups' expression values. Furthermore, between two matched sets of expression values, Pearson's correlation analysis can be used. Furthermore, all statistical algorithms compute the mean values of both investigated groups as well as the % difference between them. (Muller *et al*., 2002).

Software that is comparable Colleagues recently developed qBASE to provide solutions for comparing additional real-time setups (Hellemans *et al*., 2006). QBASE is an Excel®-based application for managing and analyzing real-time quantitative PCR data. By hierarchically arranging data into projects, experiments, and runs, the qBASE browser allows data storage and annotation while keeping track of all real-time PCR runs. It works with many of the current PCR equipment' export files and gives you quick access to all of your data, both raw and processed. The qBASE analyzer includes a simple plate editor, does quality check, translates CP data into normalized and rescaled numbers with proper error propagation, and shows results in tabular and graph format. One of the program's main advantages is that it has no restrictions on the amount of samples, genes, or replicates that can be used, and that data from many runs can be pooled and analyzed simultaneously (Hellemans *et al*., 2006). On the basis of the geNorm normalization technique, the ability to use up to five reference genes allows for reliable and robust normalization of gene expression levels. (Vandescompele *et al*., 2002). qBASE 78 Real-time PCR facilitates data sharing between users and exports tabulated data for statistical analysis using specialised software.

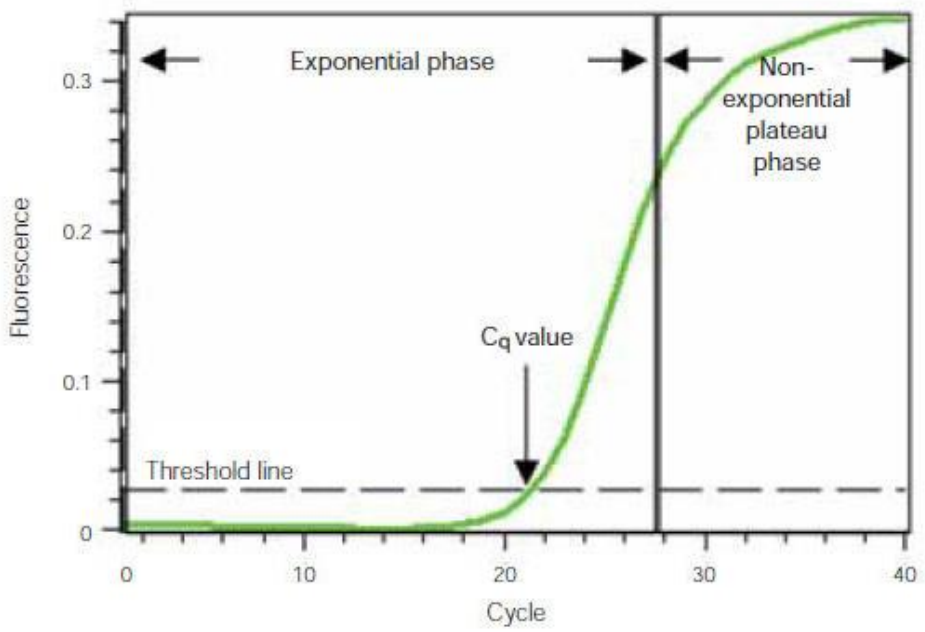
To know how real-time PCR works, a qPCR analysis using a typical amplification plot (Figure 4). In this plot is illustrate, the number of PCR cycles is shown on the x-axis, and

in y-axis the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube is shown.

The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase. During the exponential phase, in each cycle the amount of PCR product approximately doubles. As the reaction proceeds, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows down and enters the plateau phase (cycles 28–40).

Figure 4

Amplification plot. Baseline-subtracted fluorescence versus number of PCR cycles.



Initially, fluorescence remains at low levels, and not detectable at (cycles 1–18, Figure 4) even though product concentrate exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescence signal. The cycle number is called the quantification cycle, or C_q. the C_q value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template in the reaction based on the known exponential function describing the reaction progress.

The C_q of a reaction is determined mainly by the amount of template present at the beginning of the amplification reaction, relatively little amplification cycles will be need to accumulate enough amount product to give a fluorescence signal above background. If

a large amount of template is present at the start of the reaction That mean, the reaction will have a low, or early, C_q. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescence signal to rise above background. Thus, the reaction will have a high, or late, C_q. This relationship forms the basis for the quantitative aspect of real-time PCR.

For RNA isolation and the quantification of gene expression, sample material should be as homogeneous as possible. If the samples are heterogeneous, pinpointing the expression pattern of target gene may be difficult. When tissue sample consists of many different cell types, use one of the many methods that are available for separating and isolating specific cell types, for example, tissue dissection, needle biopsies, and laser capture microdissection. The collected cells can then be used to obtain the RNA samples.

Most real-time RT-qPCR applications can be performed with either total RNA or poly(A+) RNA. Working with RNA requires eliminating RNases from solutions, supplies, and lab ware. Ready-to-use RNase-free solutions can be purchased, or solutions must be treated with diethyl pyrocarbonate (DEPC) and then autoclaved. A DEPC treatment or a 3-hour bake at 250°C can also inactivate RNases on lab ware.

DNase treatment is needed when Prepared RNA samples to prevent potential amplification of any contaminating genomic DNA, which could lead to overestimation of the copy number of an mRNA. When starting material is start to eliminate DNase treatment might be unwise because of the possibility of losing RNA during additional manipulations. The amplification of potentially contaminating genomic DNA can be precluded by designing transcript-specific primers, for example, primers that span or amplify across splice junctions.

Accurate nucleic acid quantification is important for gene expression analysis, especially when total RNA amounts are used to normalize target gene expression. RNA concentration and purity are commonly determined by measuring the ratio of UV absorbance at 260 nm and 280 nm.

quantification of gene expression by RT-qPCR with two methods: two-step RT-qPCR and one-step RT-qPCR. In both cases, RNA is reverse transcribed into cDNA, and the cDNA is then used as the template for qPCR amplification. *One-step* and *two-step* refer

to whether the RT and real-time PCR amplification are performed in the same or separate tubes. In the two-step method, RNA is first transcribed into cDNA in a reaction using reverse transcriptase. An aliquot of the resulting cDNA is then used as a template for multiple qPCR reactions. In the one-step method, RT and qPCR are performed in the same tube.

1.7 Objective

The main scope of this research:

1. Detect the impact of a selected pharmaceutical compound(CBZ) in wastewater on quantitative and qualitative characteristic on cucumber
2. Determine if a selected pharmaceutical compound(CBZ) had been absorbed by cucumber fruit .
3. Determine the effect of a selected pharmaceutical compound on some defined gene(s) in cucumber.
4. Identify the part of cucumber plant that is highly absorbable for the selected pharmaceutical compound(CBZ).

Chapter Two

Material and Methods

2.1 Field Survey and Sample Collection

The sample were collected from West Nablus wastewater treatment plant. Since the beginning of the nineties, efforts have been made to establish a western plant located in the eastern Deir Sharaf lands. The municipality of Nablus obtained the necessary funding as a grant in 1998 from the Federal Government of Germany. The quantities of treated water currently up to 10,000 cubic meters per day, bearing in mind that its capacity reached 14,000 cubic meters per day.

Figuer 5

West Nablus wastewater treatment plant

The purification plants aim to:

1-Preventing the spread of unpleasant odors and diseases associated with open sewage water flowing along Wadi Al-Zumer in the west and Wadi Al-Sajur in the east.

2- protect water source surface and springs from polluotion.

3-Using treated wastewater in agriculture and increasing the amount of available drinking water.

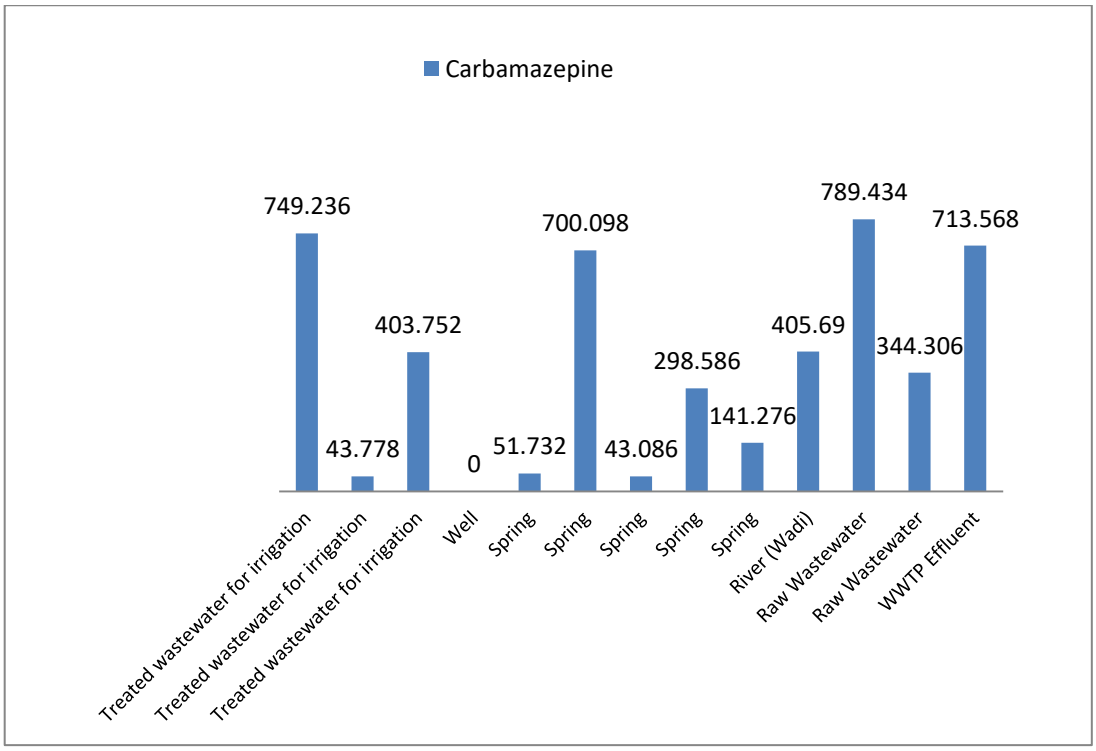
4-To prevent farmer from using untreated wastewater in agriculture in order to preserve public health.

2.2. Sample analysis

After sample were collected it analyzed by HPLC (High-performance liquid chromatography), which is high-pressure liquid chromatography, it's a technique used to separate, quantify and identify each component in a mixture. The most commonly used instrument in plant tissue is Liquid Chromatography (LC) along with Mass Spectrometry (MS) to analyze multiple PPCPs extracts.

Figure 6

carbamazepine concentration (Courtesy provided by Dr. Subhi Samhan/ PWA; personal communication)



It turns out after analysis that the highest pharmaceutical concentration is carbamazepine.

2.3. Carbamazepine

Which is a drug sold under the trade name Tegretol table 1. It was discovered in 1953 by Swiss chemist Walter Schindler. And marketed in 1962 Moshé S (2009). Its available as a generic medication Figure4 . In wastewater treatment plants, it showed only limited removal efficiency (Ternes, 1998; Zhang et al., 2008a). CBZ has been descovred in groundwater, drinking water, wastewater (Kummerer, 2001; Tixier et al., 2003; Chefetz et al., 2008). Several studies demonstrated that plant can take up PCs when it introduces by irrigation water (Kumar et al., 2005; Boxall et al., 2006; Dolliver et al., 2007; Redshaw et al., 2008; Farkas et al., 2009; Herklotz et al., 2010)

Figure 7

carbamazepine tablet as sold in local pharmacies

2.4 Medical uses

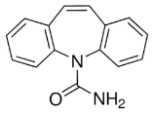
Used to decreasing nerve impulses that cause seizures and nerve pain, and use to treat epilepsy and neuropathic pain, bipolar affective disorder and acute mania.

2.5Adverse effects

1. Increased risks of hyponatremia Gandelman MS (March 1994).
2. if the person stops taking the drug there is possible Risk of seizures.
3. Blurred vision or double vision.
4. Continuous back-and-forth eye movements.
5. Increased risks of suicide.

Table 1

Selected chemical and physical properties of carbamazepine

Chemical formula	**$C_{15}H_{13}ClN_2O$**
structure	
molecular weight (g mol^{-1})	236.27
aqueous solubility (mg l^{-1})	125.0±2
log k_{ow}	2.45

2.6. Greenhouse experiments

In loess (sandy)soil plant were grown in greenhouse. In Table 2 the major soil properties are presented with natural full sunlight from October to December, with four line of cucumber plant each line composed of 20 sample of cucumber and each one irrigated with different source of water which is fresh water , treated wastewater which was obtained from (west Nablus water purification plant) CBZ concentration in this water were detected an of about (17-25Mg), and irrigated with two different concentration of water spiked with CBZ (25Mg-1Mg).it's important to notice that all sample were grown under same condition(tempteture and humidity)and Standard agronomic practices for cucumber plant in the felid were performed (pest and weed management)according to local management practice. Fruit were harvested depending on the size that is approved and a length of about 12 cm, they washed and kept under -20 ^{O}C at the end of experiment until extraction, the all aboveground plant was separated, cut, rinsed with deionized water, left to drain, frozen, and kept at -20 C until analysis, when plants were about 3 months old.

Figure 8

the greenhouse before planting in Deir Balut.

Soil properties are very important for agricultural production and sustainable soil use. The amount and rate of water, oxygen, and nutrients absorbed by plants largely depend on the root's ability to absorb soil solution and the ability of the roots to hold onto the soil's molecules

Table 2

Major properties of soil

soil sample	Clay(%)	Silt(%)	Sandy(%)	Organic matter content(%)	$CaCo_3$	pH
Tap water	62	8	12	2.7	14	6.88
25 µg/L	63	6	13	2.6	16	7.23
1 µg/L	64	6	14	2.6	16	7.11
Soil before plant	67	7	15	2.5	17	6.82

2.7 Extraction and LC–MS analysis

2.7.1 soil preparation for analysis

2 g soil is added on 5 mL of methanol then vortex for 30 seconds and then centrifuging for 30 min. then the supernatant was collected and adding 5 mL methanol and then 5 mL acetone then vortex for 30 Sec and then centrifuging for 30 min. Before analysis, the supernatant was evaporated with N gas and reconstituted in 1 mL methanol. To ensure that the concentrations were within the calibration range, soil samples were diluted by ≥2 mg/kg.

2.7.2 plant preparation for analysis

2g of plant part was added on 5 mL of methanol then vertex for 30 seconds and then centrifuging for 30 min. Following collection of the supernatant, 5 mL of methanol and 5 mL of acetone were added, followed by vortexing for 30 seconds, centrifuging for 30 minutes, and then dilution with Milli-Q water to a maximum solvent concentration of 10%. After the samples were loaded, they were eluted by adding 6 ml methanol and 3 mL methylene chloride Prior to analysis, the eluted sample was evaporated by N gas and reconstituted in 1 mL methanol. The concentrations were again diluted as necessary to ensure they were within the calibration range.

2.7.3 LC-MS analysis

We used a Waters Atlantis T3 C18 100 x 2.1 mm for chromatographic separation (3 µm particle size) With a guard cartridge attached to the column (Waters, Ireland), and operating at a 250 l/min mobile phase flow rate. The mobile phase was composed of two eluents, namely (A) 10 mM ammonium acetate in 0.1 % formic acid and (B) methanol using a binary gradient, programmer over 15 min. 4 min for relative flow rate of (A) was 95 % for 2 min, 2 % and held for 6 min before returning to 95 % by 10.10 min. with atmospheric pressure electrospray ionization (ESI) in positive mode the Mass spectroscopy was undertaken using.

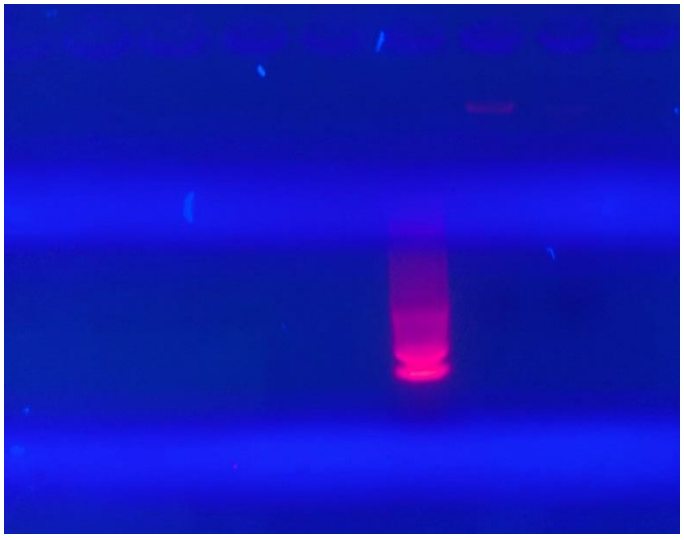
2.8. Quantitative real time PCR

2.8.1 RNA extraction by silica

After cucumber fruit, root and leaves was frozen we grind 300 mg of plant tissue in small plastic bags with grinding buffer 3ml/300mg with 0.5% of sodium metabisulite (as antioxidant),using a pestle to crush then we collect 500ml of the aqueous phase in to another tube and added 150Ml of 10%Na-sarkosyl then vortex and incubate at 70c for 10 min with intermittent shaking then put them on ice for 5 min before centrifuge at 13000 rpm for 3 min then transfer 500 ml of the supernatant to new tube then add to them 250 ml ethanol 500 ml NaI 6M and 35 ml re-suspend silica then incubate at room temperature for 10 min with intermittent shaking then centrifuge 6000rpm for 1 min then collect the pellet and wash it by adding 500ml of cold washing buffer then centrifuge6000rpm for 1 min then repeat the washing steps then let the pellet dry for a few minutes at room temperature by putting the tube upside down on tissue paper then re- suspend the pellet in 150 ml H2O then incubate at 70c for 4min then centrifuge 13000 rpm for 3 min then collect the supernatant to a new tube and store at -20c.then run RNA on gell electrophoresis to ensure that there is RNA exist in our sample .

Figure 9

RNA band for leaf sample



2.8.2. Quantitative real time PCR

After Preparing two Eppendorf tube one for Actin gene and other GST gene and put in each one of them 10 ml of master mix then 5 ml of distal water then 1 ml of forward and reverse primer for both Actin and GST genes. Forward for Actin 5'-qaqaqqGGG ATG GAG AAG TTT GGT GGT GG-3' Reverse for Actin 5'-CTT CGA CCA AGG GAT GGT GTA GC-3'. Forward for GST 5'-ACT CGT TTT TGG GCT TT-3' Reverse for GST 5'-CGA TTC AAC TCC CTC TGC TT-3'. By hylabs company. After prepare the primers for Actin by adding 100ml of forward primer and 100ml of reverse primer and 800ml of distal water. then prepare the primers for GST 100ml of forward primer and 100ml of reverse primer and 800ml of distal water. Then finally adding 1ml of enzyme. In real time PCR Eppendorf tube put 17ml of previous component and 3ml of RNA sample. Then but the samples in q real time PCR machine and wait for result. A spectrophotometer was used to measure RNA concentrations.

Chapter Three

Result and discussion

3.1 Green house experiment: effect of water quality

This experiment utilized reclaimed wastewater used mainly for cotton and corn irrigation by farmers. in our experiment carbamazepine was detected in cucumber fruit, leaves, root and stem but in different concentration Table 3.

Table 3

Carbamazepine(CBZ)concentration (average ± standred deviation) detected in cucumber plant grown in sandy soil.

Plant part	Tap water	spiked sample with CBZ 25 mg
Fruit	1.16±0.13 µg/L	3.52±0.62 µg/L
Leaves	2.36±0.87 µg/L	5.53±1.06 µg/L
Root	1.23±0.03 µg/L	3.12±0.08 µg/L
Stem	1.86±0.06 µg/L	4.32±0.75 µg/L
soil	1.32±0.09 µg/L	2.65±0.85 µg/L

The concentration observed in plant irrigated with fresh water with 25mg CBZ Figure 9 with highest concentration that has been detected in leaves with concentration (5.5±1.06) followed by stem with concentration (4.32 ± 0.75), root (3.12±0.08) and fruit (3.52±0.62). the same result observed in plant irrigated with fresh water Figre11 the highest concentration of CBZ was detected in leaves comparison with fruit (1.16±0.13), root (1.23±0.03) and stem (1.86±0.06). CBZ concentration in soil that irrigated with concentration 25 mg at the beginning of experiment was (0.71±0.03mg). but at the end of experiment the CBZ concentration was (2.65±.83) that mean the higher concentration that observed at the end of experiment are due to CBZ accumulation and adsorption in soil. In water with 25mg CBZ the concentration was focus in leaves >stem >fruit >root. But in fresh water leaves> stem >root >fruit. It notices that carbamazepine that have been introduce to cucumber in our experiment and accumulate in leaves more than other part match with other study.

1- Uptake of carbamazepine by cucumber plants A case study related to irrigation with reclaimed wastewater. 2- Uptake of atenolol, carbamazepine and triclosan by crops irrigated with reclaimed water in a `Mediterranean scenario.3- Bioaccumulation, uptake and toxicity of carbamazepine in soil plant systems. The reason why carbamazepine is the most one that uptake by plant. That CBZ has non-ionic properties that might allow it to move from the xylem to the phloem in the direction of transpiration, causing it to accumulate in the leaves. It's important to note that CBZ in Both WWTPs and in soil, it has a half-life of over 365 days (Walters e. 2010). Because of the long half-life, there is concern about bioaccumulation in terrestrial systems.

Figure 10.A

cucumber sample that have been irrigated with spiked water 1µg/L of carbamazepine concentration

Figure 10.B

cucumber sample that have been irrigated with spiked water with 25 µg/L of carbamazepine concentration

Figure 10.C

Cucumber sample that have been irrigated with treated wastewater with 789.43 Mg/L

Figure 10.D

Cucumber sample that have been irrigated with fresh water from Deir Balut.

3.2 quantitative real time PCR for Actin and GST genes

To detect if cucumber affected by CBZ the expression level of Actin and GST genes were analyzed Figure12,13. First we extract RNA by silica for fruit, root, stem and leaves. A spectrophotometer was used to measure the concentration of RNA. And quality of RNA was assessed by agarose gel electrophoresis. But the amount of RNA that have been extracted for fruit, root and stem was low comparison with leaves was high. The numbers that have been obtain from spectrophotometer for root, stem and fruit was low. The same result in agarose gel electrophoresis, there was no band for fruit, root and stem comparison with leaves the band has been notice. After several trails and with different process in RNA extraction by using new silica beads or different Wight of sample or preparing new solution need for extraction. So the leaves are the only candidate for analysis by qRT- PCR. expression levels were calculated by the ΔΔCt method figuer14. however, as mentioned in many scientific article there is difficulty in RNA extraction (Deepa et al. 2012). There is study on rice roots RNA extraction, as plant growth progressed the total amount of RNA decreased, and extract RNA from roots using a commercial RNA isolation kit was impossible to an enough amount (Matsunami et al., 2018). Because low amount of active cells in the root. Another reason for the lack of extraction of RNA is lignin accumulation (Yamaguchi et al., 2010). In addition, osmotic stress may lead to the accumulation of soluble sugars (Darko et al., 2019). Adult root has lysigenous aerenchyma which is (programmed cell

death) (Kawai et al. 2008). Polysaccharides and polyphenolic they affecting the quality and quantity of RNA isolated (Asif et al. 1987). During ripening or in response to external stimuli, they accumulate. Precipitate and contaminate the RNA during the extraction. according to cucumber fruit there was little or no RNA because the fruit is full of water. It's important to notice that Actin gene is reference gene which must work in all sample despite over expression or down regulation. On the basis of which its determined RNA concentration and established until determine the difference in expression. See the Graphics in the Appendix A,1,2,3.

Chapter Four

Conclusions

It’s important to notice that CBZ is absorbed by all part of cucumber but in different concentration but in higher concentration in leaves. It found that CBZ did not effect on quality and quantity of the cucumber plant, as the number of cucumber leaves, flower and fruits were compered between each all sample also the length of the plant and roots were measured and compared and show that there is no obvious difference. Leaves have a significantly higher uptake than fruits, roots, and stems that obtained in this study may reveal that Crops such as lettuce require greater attention or other plant that we consumed the leaves part, whose edible parts are the leaves. And plant absorption of these pharmaceutical compound depends on their physical and chemical properties. how much CBZ is to be consumed through cucumbers contaminated with CBZ based on our data (Table 3). Exposure to PCs does not appear to be hazardous to human health in water, according to studies published recently (Bruce et al., 2010). Therefore, irrigating crops with reclaimed wastewater should be investigated especially.in our study didn’t focus only carbamazepine absorption in plant it also focusses on plant gene. The actin gene encodes a protein that contributes to cell motility and GST gene which catalyzed the detoxification of the herbicide .it detected by measure their expression level in cucumber by quantitative real time PCR and the result was 1.9 for spiked water with 25Mg of CBZ, 1 for tap water, 2.2 for treated wastewater. the result was between 1.9> 1 >2.2. In our experiment the accumulation of CBZ in leaves match with expression level for Actin and GST genes which reveal that CBZ accumulate in high concentration in leaves.

List of Abbreviation

Abbreviation	Meaning
ABP	Actin Binding Protein
CBZ	Carbamazepine
GST	Glutathione S transferase
HPLC	High Performance Liquid Chromatography
ISR	Induce Systemic Resistance
PC	Pharmaceutical Compound
qRt-PCR	Quantitative Real Time Polymerase Chain Reaction
PWA	Palestinian Water Authority
MEDRC	Middle East Desalination Research Center
mg\L	Milligram per litter
ΔΔCt	difference of expression between two genes whereas Cycle threshold is specific to the expression of one gene
BCF	Bio accumulation Factor
WWTPs	Wastewater treatment plants
ATN	Atenolol
TCS	Triclosan
d.w	dry weight
mg/Kg	Milligram per Kilogram
^{14}C-CBZ	Radioautographic Carbamazepine
Bq g^{-1}	Becquerel per gram
Dpm	disintegrations per minute
PPCP	Pharmaceutical and Personal Care Products
ng	Nanogram
µg/L	Microgram per litter

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Appendices

Appendix A

Figure A.1

Expression level for Actin gene

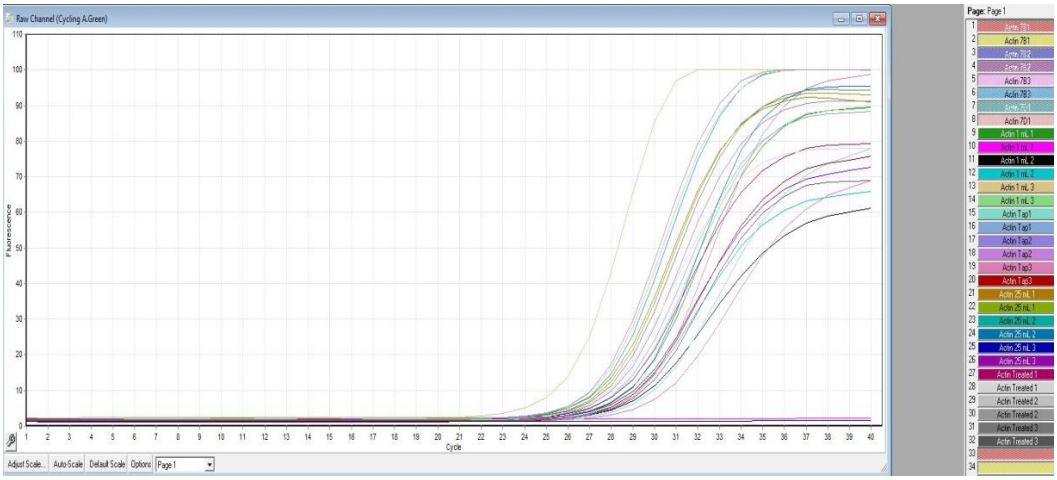

Figure A.2

Expression level for GST gene

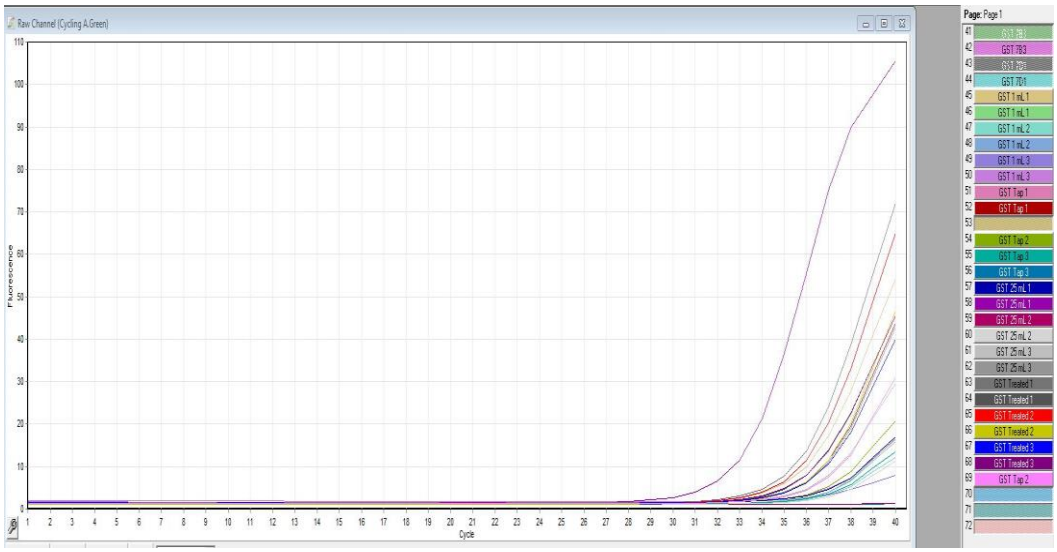

Figure A.3

ΔΔCt method used to calculate the gene expression value for q real time PCR.

	Ct1	Ct2	Avg.Ct		Ct1	Ct2	Avg.Ct	ΔCt	ΔΔCt	2^-ΔΔCt		
Actin 1 mL	23.65	23.06	23.355	GST 1 mL	32.32	31.76	32.04	8.685	0.12	0.920188	avgofCBZ	0.589502
Actin 1 mL	20.38	23.04	21.71	GST 1 mL	32.4	32.05	32.225	10.515	1.95	0.258816	avgof 25 Ml	1.925189
Actin 25 mL	21.88	21.87	21.875	GST 25 mL	31.9	27.09	29.495	7.62	-0.945	1.925189	avg of tap 2	1
Actin Tap	22.53	23.64	23.085	GST TAP	31.61	31.69	31.65	8.565	0	1	avg of treated	2.235916
Actin Treated	22.87	22.06	22.465	GST Treated	29.4	30.24	29.82	7.355	-1.21	2.313376		
Actin Treated	22.41	23.63	23.02	GST Treated	30.66	30.29	30.475	7.455	-1.11	2.158456		
					Avg.ΔCt of control		8.565					

The delta delta Ct as well, several samples are positive, others have negative signs. It's normal to have negative delta-delta values, because if we power them with 2 we will get below 1 for negative values and above 1 for positive. That means downregulation (below 1) or upregulation (above 1) of our samples compared to control.

جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

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إشراف

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أ.د. شحدة جودة

قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجه الماجستير في العلوم الحياتية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

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الملخص

تم إعادة استخدام مياه الصرف الصحي للري في البلدان التي تعاني من ندرة المياه في القطاع الزراعي، ولكنها قد تؤدي إلى إدخال ملوثات في البيئة مثل الأدوية ومنتجات العناية الشخصية. تم الكشف عن العديد من هذه المركبات الصيدلانية والإبلاغ عنها. اكتشف الباحثون أن هناك إمكانية لامتصاص النبات لهذه المواد الكيميائية. لذلك كان الهدف من هذا البحث دراسة امتصاص أحد هذه المركبات الصيدلانية وتأثيره على نباتات الخيار. كان الكاربامازيبين أكثر المركبات الصيدلانية الموجودة في مياه الصرف الصحي المعالجة بواسطة الكروماتوغرافيا السائلة عالية الأداء. الذي يستخدم عادة لتقليل النبضات العصبية التي تسبب النوبات وآلام الأعصاب، ويستخدم لعلاج الصرع وآلام الأعصاب والاضطراب العاطفي ثنائي القطب والهوس الحاد. تم استخدام الكاربامازيبين كملوث في هذه الدراسة على نبات الخيار بتركيزين مختلفين (1 ميكروغرام / لتر و25 ميكروغرام / لتر)، مياه الصنبور، مياه الصرف الصحي المعالجة والتي تمت زراعتها في بيت بلاستيكي من أكتوبر إلى نوفمبر 2021. خلال الموسم وكذلك في نهاية التجربة، تم جمع العينات من النبتات والجذور وتخزينها لتحليلها لاحقًا. كشف تحليل الكروماتوغرافيا السائلة عالية الأداء عن امتصاص الخيار للكاربامازبين في جميع العينات التي تم جمعها ولكن بتركيزات مختلفة. أظهرت الأوراق الجزء الأعلى تركيزًا في جميع أعضاء النبات. تم تطبيق تحليل ريال تايم بي سي ار لقياس مستوى التعبير الجيني لجينين: جين الاكتين وجين جي اس تي. الأكتين لأنه محفوظ وبروتينات أساسية تشارك في حركة الخلية ويتم التعبير عنها في كل مكان في جميع الخلايا حقيقية النواة في جميع

أنحاء الجسم النباتي وجينات جي اس تي لأنها تحفز إزالة السموم من مبيدات الأعشاب الأترازين ومبيدات الأعشاب المختلفة والمركبات الغريبة الحيوية السامة الأخرى في النباتات، وكذلك يتم التعبير عنها كاستجابة للإجهاد البيئي (درجات الحرارة القصوى، مثل الحرارة والبرودة والفيضانات والملوحة والجفاف وسمية المعادن) . لذلك تم استخلاص عينات من الحمض النووي الريبي باستخدام طريقة السيليكا. ثم تم قياس كمية الحمض النووي الريبي باستخدام مقياس الطيف الضوئي؛ وتم التحقق من جودة الحمض النووي الريبي بواسطة الاغاروز الكهربائي للهلام. أظهر تحليل أنه بعد تعرض النبات للكاربامازبين، تم التعبير عن الجين بتركيز أعلى في الأوراق ومطابقًا لتحليلنا الكيميائي في الذي يركز في الأوراق من نتيجة بحثي، أوصي بضرورة اتخاذ الاحتياطات من المحاصيل مثل الخس والنباتات الأخرى التي استهلكناها جزء الأوراق، والتي تكون الأجزاء الصالحة للأكل هي الأوراق لأن جزء الأوراق قابل للامتصاص بدرجة عالية من المركب الصيدلاني كاربامازيبين الذي قد يؤثر على صحة الإنسان.

الكلمات المفتاحية: الصرف الصحي، القطاع الزراعي، المواد الكيميائية.