South Bohemia University in České Budějovice

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The effect of selected pharmaceuticals on growth, biomass and activity of soil microorganisms

Bachelor thesis

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Annotace

Kontaminace povrchových vod a půd léčivy je ekologický problém, který představuje do budoucnosti velké riziko nejen pro životní prostředí, ale i pro lidskou společnost. Ačkoliv byla přítomnost léčiv v půdě již prokázána a jejich chování bylo důkladně popsáno, stále chybí informace o vlivu léčiv na mikrobiální společenství, které tento biotop obývají. Tato práce se zaměřuje na změny ve složení a početnosti bakteriálních společenstev v zemědělských půdách kontaminovaných irbesartanem.

Annotation

Pharmaceutical pollution in surface water and soil is an ecological problem that can have serious consequences on the environment and even on human society in the future. Although the presence and behavior of pharmaceuticals in surface water and soil have been already described, there is still missing knowledge about the influence of these drugs on the microorganisms that inhabit the soil environment. The thesis was focused on changes in abundance and diversity of bacterial communities in agricultural soil under treatment with pharmaceutical irbesartan.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in a publicly accessible part of the IS STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Content

Abbreviations

Control – soil without irbesartan amendment

CARD-FISH - Catalysed Reported Deposition- Fluorescence in situ hybridization

DAPI - 4,6-Diamidine-2-phenylindole dihydrochloride

dw – dry weight

HI - high concentration irbesartan treatment

LI – low concentration irbesartan treatment

LDTD/APCI-MS/MS - laser diode thermal desorption with chemical ionization and tandem mass spectrometry

MI - moderate concentration irbesartan treatment

PhACs – pharmaceuticals

TCCs – Total cell counts

1. Abstract

The soil microbiota is a key component of terrestrial soil systems. They mediate important biochemical processes; they contribute to global cycling of nutrients (Richardson et al., 2011) and contribute to soil functionality and structure (C. Chenu, 1995). Recently, soil microbiota suffers for the increasing human impact in the terrestrial ecosystems. The soil nowadays suffers from increasing contamination of various types of pharmaceuticals (PhACs) and personal care products (Biel-Maeso et al., 2019). Presence of pharmaceuticals in soil can result in a decline in soil bacterial abundance and diversity (Boxall et al., 2012)

The most adverse effects have been observed in the case of human and veterinary antibiotics; however, even non-antibiotic PhACs can cause serious impact on soil microbiota, which in turn may cause alternations in soil functioning (Ding et al., 2010). In addition, the knowledge about microbial community responses, such as changes in microbial biomass, activity and diversity associated with the presence of non-antibiotic pharmaceuticals in the soil is missing.

Irbesartan is a very effective compound used for the treatment of high blood pressure. The biological activity of this compound in the human body lasts approximately 24 hours and thus the patients have to use the drug regularly. The human body has no appropriate enzymatic apparatus to degrade the irbesartan molecule a thus the body excretes it out without any chemical alternations (Chando et al., 1998). Frequent use of irbesartan causes its accumulation in wastewater, which is the main source of irbesartan pollution. Reclamation of the wastewater without sufficient treatment causes an accumulation of irbesartan in soil and ground waters. Despite the research on the presence and behavior of irbesartan in soil has been already investigated for example by (Kodešová et al., 2016) or (Klement et al., 2018), there is no information about interactions between irbesartan molecules and soil biota, including microbiota.

2. The aim of the work

Within this thesis, we have focused on the influence of irbesartan on the microbial community in arable soil (Chernozem Haplic) sampled in the Czech Republic. This work aimed to evaluate the response of soil microbial community to of irbesartan amendments in respect to their abundance and activity. We have studied the abundance of soil microorganisms by Enumeration of Total cell counts and Catalysed reported deposition- fluorescence in situ hybridization (CARD-FISH) technique. Moreover, we have studied the effect of irbesartan on the activity of soil microorganisms. For this purpose, we have used the L-[^{14}C]-leucine incorporation method.

As supplementary information, the thesis includes data on the concentrations of irbesartan in soil in different times of incubation to elucidate whether the irbesartan undergoes degradation in soil. This analysis was done with help of analytical methods - liquid chromatography (LC) and mass spectrometry (MS) laboratory of Faculty of Fisheries and Protection of Waters, the University of South Bohemia in České Budějovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses in Vodňany.

The data was evaluated to verify the following hypotheses that we stated to elucidate the effect of irbesartan amendments on the soil microorganisms.

Hypotheses:

1. Irbesartan amendments would negatively influence microbial biomass and activity in Chernozem Haplic

2. The response of soil microbial community in terms of abundance and activity would be affected by irbesartan dose

3. Soil microbial community would be negatively affected by irbesartan in a short time and would recover in long term

3. Theoretical background

3.1 Soil functions on Earth

Soil is an important element for sustainability of the most of the ecosystems on the Earth due to the functions that it permits to the environment such as regulation, buffering, and mediation of chemical processes and habitat function. Efficacy of these functions is usually in tight connection with soil texture and thus differs among soil types (Redemeier, 2006).

A major part of bulk soil is occupied by soil particles that form a solid phase of the soil. A classification, which is based on the size of particles, splits soil particles to coarse fragments (< 2 mm) and fine earth fragments (> 2 mm), which can be further segregated to sand (200 – 63 μm), slit (63 – 2 μm) and clay (< 2 μm). A proportion of these particles in the solid phase indicates the texture of the soil, which has a direct influence on other soil properties among those belongs also the water holding capacity (Matthews, 2014).

The ability of soil to hold water is crucial for the growth and nutrition of plants, chemical reactions in soil, and the life in the soil in general. The water holding capacity decreases with the increasing size of the soil particles. The amount of water that can be captured in the soil is also affected by soil organic material that can absorb water molecules (Minasny et al., 2018). In soil the water forms a soil solution, in which all water-soluble substances including nutrients and gasses are dissolved. The soil solution provides the nutrients and gasses to organisms, stabilize the chemical environment in soil and serves as a medium form bio/geochemical transformation of nutrients in soil (De Neve et al., 2003).

Soil serves as an ideal medium for significant environmental processes such as decomposition of organic material and subsequent formation of mineral nutrients. Soil microorganisms represent the main motive forces that facilitate these processes (Schulz et al., 2013). The soil microorganisms and their particular influence on soil formation and quality will be discussed in more detail in the following chapter. Along with the naturally present complex organic compounds, different xenobiotics can be degraded or even mineralized in soils by action of microorganisms (Barra Caracciolo et al., 2015).

Besides soils are bases for various chemical cycles and terrestrial life, they represent the largest terrestrial carbon (C) storage on the planet. A rough estimation of the C mass stored in soil is about 1500 Pg, which is twice higher than the C mass in the atmosphere as CO_2 (816 Pg of C). As a consequence, the soil is considered to be a powerful regulator of carbon dioxide concentration in the atmosphere and thus can mitigate the greenhouse effect (Scharlemann et al., 2014).

In this thesis, our attention will be focused on Chernozem Haplic, which constitute significant type of soil in Europe according to Soil Atlas of Europe (Virto et al., 2015). Due to its high fertility, Chernozems are highly valuable soils in agriculture for the cultivation of various crops. Generally, Chernozem are soils characterized by deep organic layer, rich in organic carbon, ranging between 2 % and 4.5% (vol.) of soil, and nitrogen in concentration 43 mg kg^{-1}, with neutral soil reaction (pH ≈ 7) (Vysloužilova et al., 2016). When we would talk about soil texture, in the case of Chernozem the slit forms roughly 60% (vol.) of the soil content and predominates over the other particles. Besides, Chernozem are in general rich in clay content 25 - 29% vol.

3.2 Role and structure of soil microbiota

Due to their very flexible metabolism, microorganisms can accommodate all soil types on the Earth. In the terrestrial ecosystems, microorganisms represent a fundamental component of edaphon and are responsible for many soil functions.

Microbiota are a key drivers of decomposition of organic matter and nutrient cycling, which has a direct link to plant biomass production. The decomposition of organic matter and the formation of humus substances are fundamental processes for maintaining soil quality and fertility (De Neve et al., 2003). The organic molecules in dead bodies are usually utilized as a source of energy for a broad range of soil heterotrophic microorganisms, resulting in the production of mineralized products (CO_2, PO_4^{3-}, NO^{2-}, etc.), which can be utilized by plants and other organisms as nutrients (Sculte and Kelling, 1994). Apart from the higher plants, a certain amount of nutrients is consumed by soil microbiota themselves. This immobilization of nutrients is an important process that keeps nutrients in soil over long periods through the natural turnover of microbial biomass. As consequence nutrients are released back in small doses for a long time, which is a beneficial process for plant nutrition and prevent the vanishing of nutrient from soil (Edwards, 1998).

Even though the organic molecules serve as a source of energy, their oxidation does not always end up with the formation of mineralized products. Since the plant and animal bodies comprise very complex organic molecules (lignin, cellulose, proteins, etc.), their complete decomposition to mineral inorganic substances is not always accomplished. This decomposition sometimes accompanied with fermentation gives rise to humus substances in soil (Kononova, 1966). Humus substances, usually composed of complex long-chain molecules, have a significant role in soil productivity as well as for enhancement of soil water holding capacity (Eshwar et al., 2017).

Soil microbes contribute to water retention and affect soil structure by producing an extracellular matrix of polysaccharides. The extracellular matrix is in general formed by several types of carbohydrates that are polymerized to the chains (Friedman et al., 2005). Besides protection against environmental threats, extracellular matrix contributes to the formation of aggregates of greater size a thus change the structure of the soil. In particular, the extracellular matrix absorbs on the surface of clay particles and form the so-called organomineral network, which prevents its vanishing from the soil and has a strong influence on the water holding capacity of the soil ((Chenu, 1993).

Soil microbiota can be considered as a group of soil organisms consisted of *Bacteria*, *Archaea*, and microscopic fungi. One gram of arable soil contains approximately $10^8 – 10^9$ individual bacteria cells, $10^7 – 10^8$ actinomycetes, and $10^5 – 10^6$ fungal individuals. In general, microbiota represents 80% of the living part of soil organic matter and 5.6% of organic carbon in the soil (Torsvik et al., 2006).

Bacteria represent one of three domains of life on Earth. They belong to prokaryotic microorganisms and are most widespread among all organisms around the world. Bacteria carry out different life strategies such as heterotrophy, phototrophy or chemolithotrophy (Radhey, 2000). Six bacterial phyla are significant in soil habitat: *Proteobacteria, Acidobacteria, Actinobacteria*, *Firmicutes* and *Bacteriodetes* (Fierer et al., 2007).

Proteobacteria contribute intensively to nutrient cycling in soil, due to various symbiotic interactions with plant roots (nitrogen-fixing *β-Proteobacteria* on legumes), but the phyla includes also many saprophytes facilitating wide spectra of biochemical reactions in soil (Van Elsas et al., 2006). The degradation of various organic molecules including complex compounds and xenobiotics is also permitted by soil *Proteobacteria*,

mainly due to *γ-Proteobacteria*, which stand behind decomposition of various analgesics such as naproxen or ibuprofen (Barra Caracciolo et al., 2015).

Another important phylum for soil ecosystem is *Acidobacteria*. This phylum represents about 50% of the total bacterial soil community, especially in soil with lower pH. These mostly aerobic chemoorganotrophes can metabolize broad spectra of carbohydrates including polysaccharides and belong to important denitrifiers in the nitrogen cycle. Besides polysaccharide decomposition, these microorganisms can also facilitate degradation of more complex organic compounds including xenobiotics (Kuramae et al., 2019).

The *Actinobacteria* are high abundant bacterial phyla in agricultural and forest soils. These bacteria are usually indigenous organisms, which contribute to many biochemical processes in soil due to their versatile metabolism. These bacteria are important for the soil ecosystems due to their ability to decompose recalcitrant and complex organic molecules or as producers of many natural antibiotics and geosmin. (Yadav et al., 2018). Some members of *Actinobacteria* phylum become known for their ability to decompose xenobiotics and pharmaceuticals in soil. As an example, the biodegradation of Sulfamethizole or Carbamazepine by *Rhodococcus rhodochrous* might be given (Gauthier et al., 2008).

Firmicutes. A characteristic feature of this group is the zymogenic behavior. The members of this group metabolize readily available organic substrates in the soil, which presence is a limiting factor for their growth. Thus, these bacteria are frequently abundant in the rhizosphere where they settle on plant residues or interact with mycorrhizal fungi. Some members of genera *Bacillus* and *Paenibacillus* have even exceeded an association with fungi or plants in an endosymbiotic relationship (Van Elsas et al., 2006).

Bacteroidetes includes bacteria with diverse life strategies; however, the common trait for the whole phylum is heterotrophic metabolism. Bacteroidetes comprise both anaerobes and aerobes that contribute to the decomposition of organic matter in the soil (Fierer et al., 2007).

Archaea are microorganisms known for their very diverse metabolism and life strategies as well as for their ability to accommodate various habitats. They play a key role in carbon and nitrogen cycles in the locations with harsh conditions, where the bacteria

would not be able to survive, however, they belong to abundant prokaryotes even in common terrestrial ecosystems (Killham et al., 2015). Despite they are involved in biochemical processes in soil, there was no biodegradation or even interaction between *Archaea* and pharmaceuticals observed and thus they will not be discussed further.

Along with prokaryotes, microscopic fungi are significant members of soil microbial communities. In comparison to metabolically diverse *Bacteria* and *Archaea*, in the fungi group, there are only heterotrophic organisms with a disability of active movement. Similarly to *Bacteria* and *Archaea* fungi includes many symbionts of higher plants, however, there are also important human, animal, and plant pathogens (Giri et al., 2005). Their main role in the soil ecosystem is the decomposition of various organic substances including complex molecules (nature polymers, heterocycles, xenobiotics. etc.) (Finlay, 2006). However, we did not focus on fungi in this study, since prokaryotes are dominating microbial communities in agricultural soils and thus they will drive likely the most important processes in there.

3.3 Pharmaceutical pollution in soil

Environmental pollution has a detrimental effect on soil quality and health. Nowadays, the soil suffers for the contamination with so-called emerging pollutants, comprising of pharmaceuticals (PhACs), personal care products, antimicrobial products, food additives, and veterinary products, where pharmaceuticals represent one of the prominent members in this group (Biel-Maeso et al., 2019).

The annual consumption of PhACs in Europe ranges from thousands to millions of kilograms per year. The average consumption varies significantly between countries as well as with the sort of disease the drug is used for. The majority of consumed PhACs represent analgesics and anti-inflammatory drugs. The antibiotics are the second most commonly used group of pharmaceuticals, followed by antiepileptic drugs. The annual consumption is concluded in Figure 1 based on data provided by (Monteiro et al., 2010).

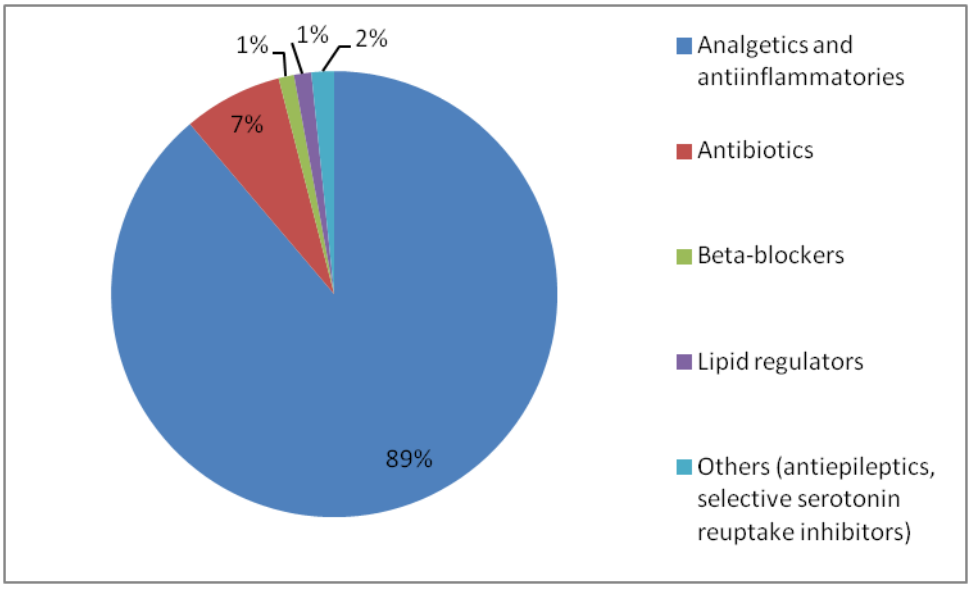

Figure 1.: An annual consumption of PhACs in three countries of western Europe (United Kingdom, France and Spain) per year 2010. Data are expressed in mass ratio concerning the total PhACs annual consumption (9.51x10^{6} kg yr^{-1})(Monteiro et al., 2010)

The main source of PhACs pollution comes from insufficiently treated wastewater. This is called point pollution. It usually includes wastewater coming from industry, hospitals, and households. This wastewater is concentrated on wastewater treatment plants (WWTP, hotspots), where it is treated and cleaned, and then released into the environment (Geissen et al., 2015). According to data obtained in 2015, a major part of the citizens of the Czech Republic (82.4%), are connected with the sewer system, and 97% of wastewater caught in is treated. Quality of water treatment correlates directly with population density in a certain locations. The higher the number, the better treatment is provided (Wanner, 2015). WWTPs in the Czech Republic utilize as most countries in central Europe, modern mechanical-biological treatment systems, which sufficiently eliminate most of the waste such as detergents, dyes, nutrients, or petrol (Wanner, 2015). The presence of PhACs in wastewater became known just several years ago. The main contribution to this finding brought analytical methods developed at the end of the 20th century that enabled scientists to detect these compounds in environmentally relevant concentrations (Erıc et al., 2017). Despite there have already been developed methods, which can eliminate some of PhACs from wastewater, their use is not common in wastewater plants, since there is no regulations, which would

force the plants to use these technologies in the process of wastewater treatment (Stein et al., 2018).

Additionally, the practice in conventional animal husbandry also plays a significant role in the dissemination of emerging pollutants into soil and water (diffuse pollution). PhACs, especially antibiotics, are used for treatment and prevention against animal diseases due to their high efficiency. Due to this practice the antibiotics were found abundant in manure that is utilized as an organic fertilizer (Kyselková et al., 2015), which cause that the concentrations of such PhACs have been detected also in the fields and meadows far distanced from hotspots sources of pollution (Grenni et al., 2018).

A release of wastewater, which was not treated for the emerging pollutants, causes the accumulation of PhACs in surface water. Average concentrations of PhACs in effluent water, released from WWTP, were determined to be in the range of 3-41 $\mu g\ l^{-1}$ (Biel-Maeso et al., 2018). The presence of PhACs in the effluent of WWTPs has a high potential risk for the environment. Especially in regions with small water reservoirs, where the wastewater is reclaimed for irrigation of crops, the correlation between PhACs amendments in soil and irrigation was revealed. The concentration of PhACs in arable soil was detected in range 2-15 ng g^{-1} dw soil. The most abundant groups detected were analgesics and anti-inflammatory compounds, blood lipid regulators, and antibiotics (average concentrations 4.02 ng g^{-1}, 4.02 ng g^{-1}, and 0.97 ng g^{-1} dw soil respectively) (Biel-Maeso et al., 2018).

Accumulation of PhACs in soil constitutes a potential risk for the terrestrial ecosystems as well as for the human society. The exposure of the PhACs to plant tissues (roots, leaves) cause their uptake and subsequent accumulation. The presence of PhACs has detected in many ordinary crops and vegetables in concentrations ranging between 10-40 $\mu g\ g^{-1}$ dry weight (Kodešová et al., 2019). These concentrations were assessed for any potential toxic effect on the plants and herbivores that feed on those including human entities. Since the level of PhACs accumulated in plants were detected to be below the phytotoxic levels or therapeutic dose, the exposure of PhACs in plant tissues was assessed of low toxicity, however, the exposure has not been tested comprehensively and there are still many issues not clear (Bartrons et al., 2017). Despite the exposition PhACs in environment in low concentration might not endanger the health of humans or animals and plants employed in agriculture, a different assumption was taken in the case of their micro-symbionts. These are important and in some cases

even crucial components of metabolism of many organisms. The communities of symbiotic microbiota, as well as those living free in the soil, can be significantly affected due to the long-term contact with PhACs and other micropollutants (Boxall et al., 2012).

The human and veterinary antibiotics represent a group of PhACs with the strongest adverse effect on soil microbial community. The antibiotics cause a disturbance in ecosystem stability and nutrient cycling. Since their effects are selective for different groups of soil microbiota, they disturb the balance in the diversity of soil microbiota communities (Ding et al., 2010). Besides their adverse influence on soil microbiota, the interaction between bacteria and antibiotics can become a serious problem for human society as well. The contact between antibiotics and soil microbiota causes a selective propagation of bacteria with genes that allows resist the effects of antibiotics. Due to the horizontal gene transfer, these genes can be transferred among the bacteria without species boundaries including human and animal pathogens (Sanderson et al., 2016).

Although antibiotics are a big threat for microbiota in soil, there are other types of PhACs accumulated in the soil, mainly broadly used analgesics and anti-inflammatory compounds. In addition, many other recalcitrant types of drugs (blood lipid regulators, antidepressants, etc.) have been detected in the soil environment (Patrolecco et al., 2013). The behavior of these compounds against the soil microbiota varies significantly within the individual groups. The smallest effect on soil microbiota was found in the case of the most abundant group of PhACs (analgesics and anti-inflammatory). Most of these compounds become toxic only at concentrations levels above the environmental values and are biodegradable under aerobic conditions in soil (Barra Caracciolo et al., 2015).

3.4 Irbesartan

Irbesartan represents one of the most efficient functional components in drug products utilized for hypertension treatment and kidney protection. From a clinical point of view, irbesartan binds in the human body to angiotensin II receptors and reduce the production of cortical hormones aldosterone and renin (Chando et al., 1998). The biological activity of irbesartan is approximately 24 hours so people suffering from hypertension are obliged to intake this drug daily. Casual dosage for irbesartan pills fluctuates among individual patient usually in the range from 50 to 300 mg per dose.

The human body can't metabolize the whole molecule of irbesartan, but forms metabolites (monohydroxy-, dihydroxy, keto- or carboxyl- modified metabolites) that have been transformed by oxidation from the original molecules (Chando et al., 1998).

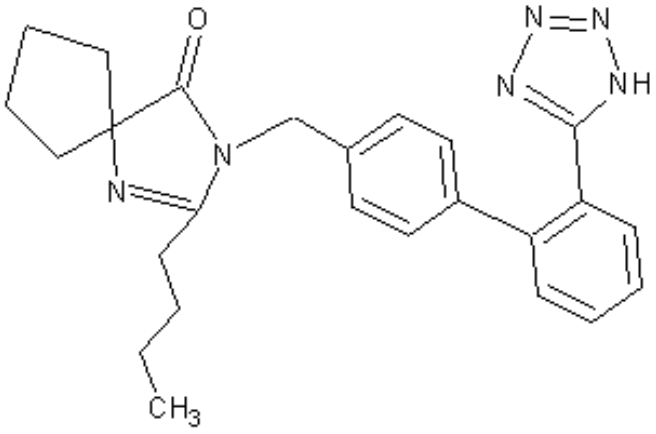

Fig.2: Molecule of irbesartan

Discharge of irbesartan and its metabolites in the surface water cause accumulation of these compounds in water and subsequently in soil. Irbesartan, as the other pharmaceuticals, has a potential risk for organisms accommodating water and soil habitat and even for human health, since its presence has been detected also in the drinking water (Bottoni et al., 2010). The behavior of the irbesartan molecule in the soil is variable with respect to the pH of soil solution and soil texture. According to its pKa values (pKa1 = 4.12; pKa2 = 7.4), irbesartan molecules in the soil can be found in three different ionic forms; cationic, neutral and anionic. The occurrence of irbesartan forms considering soil solution pH as a variable was described by (Klement et al., 2018) with liquid chromatography-tandem mass spectrometry (LC-MS/MS) and can be seen in Figure 3.

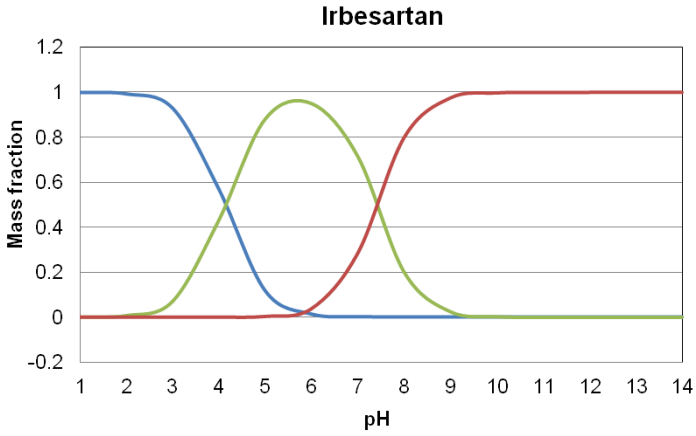

Figure 3.: Mass fractions of irbesartan in cationic (blue), neutral (green) and anionic (read) form detected by LC-MS/MS under different pH values (Klement et al., 2018)

The form of irbesartan molecules has a crucial effect on its mobility in soil and through the soil into the surface and subsurface water. The mobility of PhACs in soil generally depends on their affinity to the components of soil bulk and its physicochemical properties (Klement et al., 2018). As it was already mentioned, a pH strongly influences compound's behavior, however, this effect is always in combination with other soil properties such as organic carbon content, cation exchange capacity, basic sorption complex, the soil texture, which also play a significant role in the adsorption of PhACs on soil particles (Kodešová et al., 2016). In that study, the sorption of the irbesartan molecule was relatively small in comparison to another two molecules (fexofenadine and citalopram) in Haplic Chernozem soils. The proportion between irbesartan remaining in soil solution and the total irbesartan introduced to the soil bulk ranged between 17.6 – 61.9%. The sorption of irbesartan was found to have a positive correlation only with exchange acidity, soil hydrolytic acidity, and sand content. Thus, in general, it can be concluded that irbesartan molecules undergo the best sorption in their cationic form (Klement et al., 2018). It can be concluded, that a significant part of irbesartan, especially in soils with neutral pH reaction, is dispersed in the soil solution and thus readily available for uptake by plants or soil microorganisms.

Despite the well-documented presence of irbesartan in the environment, the effects of this compound on the water and soil organisms, with the focus on soil microbial community responses haven't been studied so far.

4. Materials and methods

4.1 Soil sampling and processing

The preparation of the soil samples was the first step in the work approach. In our experiment, we have used the Haplic Chernozem situated in Czech University of Life Sciences campus (Suchdol, Prague, Czech Republic, 50°7′48.01″N, 14°22′24.88″E). The soil was selected according to previously obtained data (Kodešová et al., 2016) mainly due to its pH reaction (pH= 7.97) which determines the form of irbesartan and content of organic matter (2.09%) in the soil that is in direct correlation with ability of soil to absorb the irbesartan (Klement et al., 2018). The soil sample was taken from the soil surface horizon (0-25cm) in November 2017 and February 2018, then air-dried, sieved through a 2-mm sieve, and stored at 4 C° until use (no longer ten few days). We would like to thank Aleš Klement (Czech University of Life Sciences, Prague) who has sampled the soil for this study.

4.2 Experimental setup and soil sampling

The soil samples collected by Aleš Klement (Czech University of Life Sciences, Prague) were homogenized and air-dried. In the next step, 50g of the homogenized soil was transferred into PE bottles, wetted with 6 ml of autoclaved tap water, and put into an incubator for three days to pre-incubate the soil samples. After the pre-incubation period, the soil samples were amended with 6 ml of autoclaved tap water without the irbesartan (control), with a low dose of irbesartan (LI, 1.23 μg g^{-1} dw soil), a moderate dose of irbesartan (MI, 12.3 μg g^{-1} dw soil) and a high dose of irbesartan (HI, 123 μg g^{-1} dw soil). The amount of water added was calculated according to the field water holding capacity described in the previous study (Klement et al., 2018). We have performed 2 experiments; first in December 2017 and the second one in March 2018. The first one was performed as a pilot and served for the optimization of the second experimental setup. In this thesis, we will focus on the results of the second experimental trial (March 2018). The data from the pilot experiment were used for assessing optimal dilution factor for enumerating the Total cell counts in soils procedure (chapter 4.5.1) that helped us to prepare an optimal protocol for soil extract preparation and as well to adopt all methodology. The results from analyses of manipulated soils from the first experimental trial (December 2017) were not reliable due to side effects caused by

ethanol presence in spiking solutions and consequently its residues in soil extracts (more in chapter 4.3.).

All soil samples were carried out in three replicates and incubated at 20°C in the dark. The soils were sampled destructively for further analysis within three weeks. The incubation times were 1-4 hours, 1day, 3 days, 7 days, 14 days and 21 days according to the observed half- time (DT50) for irbesartan in studied soil in the previous experiment, which was 29 days (Klement et al., 2018). The samples were divided into several aliquots and stored accordingly, each serves for different analysis according to Table 1.

Table1.: Destructive sampling of Chernozem Haplic soil after incubation with irbesartan in the dark.

Analyses	Weight of the sample (g)	Storage conditions
calculation of dry soil weight	5	No storage, analysed immediately
analytical chemistry (LDTD/APCI-MS/MS)	5	Frozen at -20 C
soil-water extracts (enumeration of total cell counts, 14-C leucine incorporation, CARD-FISH technique)	5	No storage, analysed immediately

4.3 Preparation of stock and spiking solutions

To ensure homogenous distribution of irbesartan to soil samples we prepared the stock and spiking solutions. Upon ambient conditions, irbesartan is a white powder. Due to its rather non-polar properties and low water solubility, we were supposed to use the non-polar solvent. At first, we prepared a stock solution by dissolving irbesartan in ethanol as it was described previously (Gao et al., 2008), but we have found it not suitable for our purpose. The reason is that ethanol has interfered with measurements of microbial biomass and likely enriched ethanol-feeding microorganisms at day 7 in all treatments, which was not the purpose of the study. Thus, we dissolved the irbesartan in DMSO as a final choice, because DMSO was found to be a very good solvent for irbesartan due to

its good miscibility with water and ability to dissolve irbesartan molecules. Although the DMSO is toxic for bacterial cells, the response of bacterial cells on DMSO concentration was found significant from concentrations above 2% vol. (Wadhwani et al., 2012), which is beyond the concentrations we used in this work.

Stock solution 1 mg ml^{-1} was used to prepare spiking solutions as follows. 500 ml of irbesartan water-DMSO solution of concentration 1 mg ml^{-1} was prepared from 500 mg of irbesartan (≥ 98%), purchased from CHEMOS GmbH (Regenstauf, Germany), which was dissolved in 10 ml of DMSO (Sigma Aldrich, 99.9 %) and subsequently diluted up to 500 ml with distilled water. The concentration of DMSO in the stock solution was determined to be 2.5 % (vol.). The sparking solutions of concentrations 8 µg ml^{-1},80 µg ml^{-1} and 800 µg ml^{-1} were prepared by dilution of 4 ml, 40 ml, and 400 ml of stock solution respectively in 500 ml vials.

4.4 Preparation of soil extracts

To obtain soil extract with an optimal ratio between the number of microbial cells and interfering soil particles, it was necessary to estimate the proportion of centrifugation velocity and soil dilution factor, which will be finally used.

For each soil, 5 g of wet weight soil were transferred into a 50 ml falcon tube, mixed with 45 ml of autoclaved tap water and vortexed for 1 min. Samples were further homogenized with ultrasonication for 2 minutes under 20 kHz ultrasound and vortexed again for 1 min each. Homogenized soil solutions were left for 1 min to sediment coarse particles in a beaker, and subsequently, the aliquot of ~30 ml was removed and distributed in 5 falcon tubes each for the estimation of centrifugation velocity optimum. Soil extracts were centrifuged at 0, 100, 250, 500 and 750 x g, respectively, for 10 min at room temperature (Uhlířová et al., 2003). Subsequently, 1 ml of each soil extract was serially diluted to obtain a proper cell density for bacterial counting. The proportion between centrifugation velocity and dilution factors are displayed in Table 2.

Table 2.: Proportion between dilution and centrifugation of soil extracts

Sample	Cetrifugation velocity [g]	Dilution factor
S-0	0	10^{-5}
S-100	100	10^{-4}
S-250	250	10^{-3}
S-500	500	10^{-2}
S-750	750	10^{-2}

4.5 Enumeration of total cell counts in soil extracts

The enumeration of Total cell counts in soil was done by microscopy observation using fluorescent dye 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI). When DAPI incorporates in cells, it connects to adenine and thymine bases in genetic material as such, that the cell's ability to absorb and emit light is much higher (Bloem, 1995). This method provides fast and general information about the abundance of prokaryotic cells in soil without any selection for particular bacterial phyla. This method provides relatively rough data since there is no activity of cells required to obtain fluorescence signals and thus even death cells can emit a signal.

4.5.1. **Assessing optimal dilution factor for enumerating Total cell counts in soils**

Before the main analysis of the manipulated soils, we have performed the enumeration of Total cell counts in Chernozem Haplic sample to determine the natural abundance of microorganisms. During this task, we assessed which dilution factor will provide the best resolution between the number of microorganisms that will be observed under a microscope and the number of residual particles that would cause noise during the microscopy, since the accurate purity of the sample is crucial. If the sample contains a higher amount of impurities, it can lead to distorted observations, and contrarily, more diluted the sample leads to a decrease of cell density below detection limits.

The DAPI staining solution was freshly prepared using 0.001 g of solid DAPI (Roth) and 10 ml of distilled water (final concentration1 mg ml^{-1}). To fix microbial cells, 1 ml of a soil extract sample was placed on the 0.22 µm polycarbonate filter using a vacuum manifold filtration apparatus. Subsequently, 50 µl of DAPI stain solution was added, the samples were incubated for 5 min and then staining solution was filtered out.

4.5.2. **Microscope analysis**

The filters with fixed cells were then covered with a drop of immersion oil, cover glass, and one cover drop of the immersion oil. Slides were put under the microscope Olympus BX-60 equipped with a source of fluorescence light (Olympus U-RFL-T). Microorganisms were visualized under 1000x magnification (10x ocular lens and x100 objective lens) upon using ultraviolet light (U-MWU filter, λex= 330-385 nm, λemis> 420 nm). The number of cells was counted at 50 microscope fields (d= 0.21 mm). Besides, stained cells were documented and edited using a digital camera (Olympus DP71) and software quick PHOTO MICRO 2.3.

The Total cell counts per gram of dry weight soil were calculated according to the formula below. The parameters of the used apparatus are considered in the formula as a calculation factor (considering the type of microscope, magnification, objective, and diameter of the filtration apparatus). The proportion of dw soil in samples can be seen in Table 3 in the supplement.

$$\frac{C * f * d}{F * s * V}$$

C..... Number of cells

f ..... Calculation factor, f=103628.72

d ..... Dilution factor

F ..... Number of fields

s ..... dw of soil

V.....Volume of extract used for microscopy (1 ml)

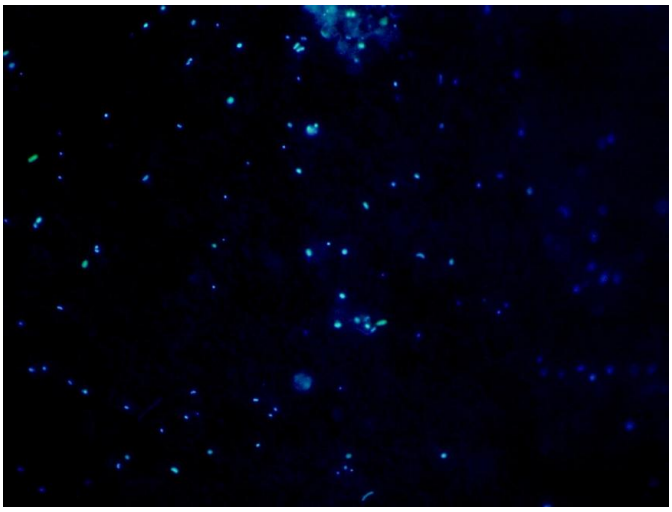

Figure 4.: Prokaryotic cells stained by DAPI stain under illumination with ultraviolet light

Results from these preliminary measurements are described in chapter 5.1.

4.5.3. **Determination of Total cell counts in manipulated soils**

The soil extracts and microscopic preparations were prepared according to the procedure described above (4.3) in triplicates. Soil samples from manipulative experiment were analysed for Enumeration of Total cell counts using centrifugation velocity 500 x g and dilution factor of 10^{-2}, which provided the best compromise between number of cells on filter and background noise signal coming from soil particles.

4.6 Enumeration of bacteria by Catalysed Reported Deposition- Fluorescence in situ hybridization (CARD- FISH) technique

The CARD-FISH technique is a valuable method for the determination of the abundance of particular groups of microorganisms in different types of samples. In comparison to the Enumeration of Total cell counts method, this technique provides an opportunity to focus on particular taxa of prokaryotes. A combination of the CARD-FISH and Enumeration of Total cell counts methods enables the comparison of the development of the population of a particular group of bacteria with the development of the population of the whole bacterial community. In this experiment, we have focused on *Bacteria* (EUB). An advantage of the CARD-FISH method is that the signal is

emitted only from probes hybridized to ribosomes specific to some or even single bacterial phylum (Daims et al., 1999).

CARD-FISH technique combines principles of classic FISH methods with amplification of fluorescent signals by horseradish peroxidase. In classical FISH, cells are immobilized on the filter and fixed, then permeabilized, and incubated with the fluorescently labeled oligonucleotide probes. Oligonucleotide probes are hybridized to homologous regions of ribosomal RNA (rRNA) in the cell. The rest of the non-hybridized probes are washed out to reduce unspecific signals. When the hybridized probes are exposed to light it starts to emit a signal and labeled cells might be observed and enumerated (Ainsworth et al., 2006). Instead, the CARD-FISH method employs a covalently bound enzyme to an oligonucleotide probe. The detection than occurs by fluorescein-labeled substrate tyramid, providing an amplified signal via enzyme activity. The CARD-FISH method is thus very sensitive and can identify 94% of all cells in a sample (Eickhorst et al., 2008a).

4.6.1. **Preparation of the sample**

The 0.22 μm polycarbonate filters (GE Water & Process Technologies) were cut out to circles (d= 47 mm), autoclaved, and assembled into the filtration apparatus. Cells in the soil extract (preparation see chapter 4.3), were fixed with 200 μl formaldehyde (Penta, 38%) and incubated in apparatus for 10 minutes. Subsequently, the extract was filtered out and filters were stored at -20 °C in the dark till analyzed. As the following step, the filter was treated with 0.2 % low-melting agarose solution (Low-Melting agarose, Invitrogen-USA) dried at 35°C for 30 minutes and cleaned with 96% ethanol and dried on air (Pernthaler et al., 2002).

Next, cells attached to the filters were permeabilized. Dry filters were immersed in a lysozyme solution and incubated at 35°C for 60 minutes. The lysozyme solution (0.69 mM) was prepared with 100 mg lysozyme (Fluka), 1 ml 0.5M EDTA, 1 ml 1M Tris-Base(Biomol, TRIS-(hydroxymethyl)-amino methane,100%) dissolved in 8 ml MQ H2O and calibrated with HCl (pH=7.4). Subsequently, filters were incubated in achromopeptidase solution (0.2% vol.) under the same condition as before for 30 minutes. The achromopeptidase solution was prepared by dilution of 20 μl achromopeptidase (Sigma-Aldrich), 50μl 5M NaCl (Penta, 99.9%) and 100μl Tris-HCl up to 10 ml with MQ H2O. In the last step filters with permeabilized cells were

incubated at room temperature (RT) in 0.01M HCl for 10 minutes. Between the treatments, filters were washed for 1 minute in MQ water. Finally, filters were washed with 96% ethanol and dried (Sekar et al., 2003).

4.6.2. **Hybridization of probes, amplification of signal and staining with DAPI**

Each filter paper was cut on eight parts with a razor blade. These pieces were collected and incubated in a mixture of hybridization buffer and horseradish peroxidase-labeled oligonucleotide probe (Thermo Fisher Scientific) (1 µl ml^{-1}) for 2 hours at 35°C. The probe EUB 338 was designed to target in the majority of bacterial domains (97%) using a mixture of two oligonucleotide sequences (GCT GCC TCC and CGT AGG TGT, in 5´- 3´direction) investigated by (Amann et al., 1990) and (Daims et al., 1999) respectively. We attempted to enumerate as well the abundance of different groups of bacteria (Alphaproteobacteria, Gammaproteobacteria, Enterobacteria, and Flavobacteria) with taxon-specific probes to assess changes in diversity within bacterial communities, however, due to technical problems we did not complete these measurements.

Table 3.: Preparation of hybridization buffer used for EUB-338 oligonucleotide probe

Chemicals	Volume
5 M NaCl [ml]	3.6
1 M Tris-HCl [ml]	0.4
Dextran sulphate [g] (Sigma-Aldrich)	2.0
Formamide [ml] (Fluka, 99.5%)	11.0 [55%]
10% Blocking reag. [ml]	2.0
MQ H_2O [ml]	3.0

The 10% blocking reagent was formed by dilution of 10 g of Blocking reagent (Roche) in 100 ml of maleic acid buffer (50 ml 0.1 M maleic acid (Fluka) and 50 ml 0.15 M NaCl, pH= 7.5). After incubation in the hybridization buffer, the filters were incubated in a washing buffer, heated up for 10 minutes at 37°C. After the second incubation filters were dried slightly on the air (Sekar et al., 2003) (Eickhorst et al., 2008b) (Eickhorst et al., 2008a).

Table 4.: Preparation of washing buffer used for EUB-338 oligonucleotide probe

Chemicals	Volume [µl]
5 M NaCl [µl]	30
1 M Tris-Base [µl]	1000
0,5M EDTA [µl]	500
20% SDS [µl] (Fluka, 99,0%)	25
MQ H_2O	up to 50 ml

Subsequently, the filters were incubated in 1xPBS for 15 minutes at RT. The 100x H_2O_2 solution was prepared in the Eppendorf tube by mixing 5 µl 30% H_2O_2 with 1 ml 1xPBS. Simultaneously, an appropriate fresh amplification buffer was also prepared in advance. This buffer was composed of 2 µl Cyanine 3 (Perkin Elmer)/FITC (fluorescein isothiocyanate) tyramid, 10 µl 100x H_2O_2 (Sigma-Aldrich, 30%) and 1000 µl 10% amplification buffer (stock).

Table 5.: Chemical composition of amplification buffer used for EUB-338 oligonucleotide probe

Chemicals	Volume
Dextran sulphate [g]	4.0
5 M NaCl [ml]	16.0
20xPBS [ml]	2.0
10% Blocking reag. [ml]	0.4
MQ H_2O [ml]	up to 40 ml

Air-dried filters were placed in 1.5 ml Eppendorf tubes. The fresh amplification buffer was added in a volume of 400 µl. The Eppendorf tubes with filters were placed in the dark for 10 minutes. Then filters in tubes were washed with MQ H_2O and in 96% ethanol at RT and dried (Eickhorst et al., 2008b) (Eickhorst et al., 2008a).

Table 6.: Preparation of 10x and 20xPBS

Chemicals	10xPBS	20xPBS
NaCl [g]	4	8
KCl [g] (Penta.99.5%)	0.1	0.2
Na_2HPO_4 x $12H_2O$ [g] (Penta. 99.0%)	0.762	1.524
KH_2PO_4 [g] (Fluka. 99.5%)	0.1	0.1
MQ H_2O [ml]	50	50

Table 7.: Preparation of amplification buffer(stock) used for EUB-338 oligonucleotide probe

Chemicals	Volume
5M NaCl [ml]	16
Dextran sulphate [g]	4
20xPBS [ml]	2
10% Blocking reag. [ml]	0.4
MQ H_2O [ml]	17.6

After this treatment filters were immersed into the oil with DAPI stain (5 mg ml^{-1}). The stained filters were then placed on a glass plate and covered by a cover glass to observe and count visible cells (Bloem, 1995).

4.6.3. **Microscope analysis of hybridized filters**

We have analyzed all specimens for signal developed by DAPI (U-MWU filter, λex=330-385 nm, λemis>420 nm) and signal that coming from Cyanine 3/ FTIC tyramid (U-MWB filter, λex=450-480 nm, λemis>520 nm). The counts of cells per gram of dry weight soil were determined in the same way as described in chapter 4.4.3.

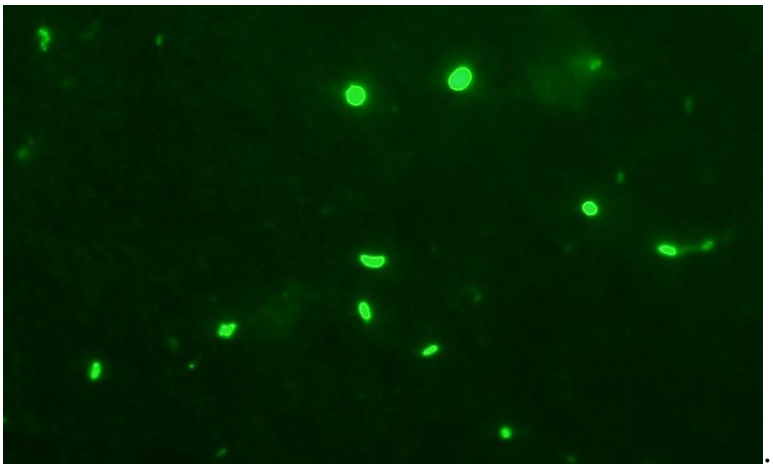

Figure 5.: Bacterial cells hybridized by horseradish peroxidase oligonucleotide using CARD-FISH technique. Signal was detected by fluorescein labelled substrate tyramid, activated by enzymatic activity of hybridized ribosomal probe

4.7 L-[^{14}C]-leucine incorporation

Incorporation of radiolabeled amino acids into microbial cells provides an efficient method for analysis of biosynthetic activity that directly reflects the growth of bacterial biomass and thus its significant decrease may refer to microbial stress (Uhlířová et al., 2003). We have measured a radiation signal that comes from L-[^{14}C]-leucine incorporated in microbial cells. This radiation signal is in direct proportion to the amount of leucine incorporated into protein, which refers to bacterial biomass that was formed in the soil in time of sampling.

The incorporation of L-[^{14}C]-leucine into proteins is an approach that is sensitive to the purity of the used soil extract, because of frequent adsorptions of L-[^{14}C]-leucine e molecule on organic matter in the solution (Bååth, 1992). Therefore, we have decided to use a similar extract as prepared for the DAPI staining method. The centrifugation velocity was kept at 500 x g, however, the dilution factor was changed from the original procedure (exact procedure see chapter 4.3) to 10^{-1}.

4.7.1. **Incorporation of L-[^{14}C]-leucine to microbial biomass**

Leucine incorporation was measured with the microcentrifugation method that was modified from (Bååth et al., 2001). At the beginning triplicate, 1.5 ml aliquots of the soil extract were transferred into 2 ml centrifugation tubes, which was followed by the addition of 4 µl of L-[^{14}C]-leucine (final concentration 800 nM, Perkin Elmer, Boston, U.S.A., specific activity 12.1 GBq mmol^{-1}). The samples treated with radiolabelled

leucine were incubated for 2 hours at RT. The incubation was terminated by the addition of 175 µl ice-cooled 50% trichloracetic acid (w/v, TCA) (Sigma-Aldrich, 99%). The solutions were subsequently homogenized and kept on ice for 50 minutes. In the case of control samples, the TCA was added before the radiolabelled L-[^{14}C]-leucine. Soil solutions in microtubes were three times centrifuged for 10 min at 14.000 rpm. The formed pellets were kept in the tubes, while the supernatants were removed. Pellets steps were washed after first and second centrifugation with 1.5 ml of ice-cold (w/v) 5% TCA and subsequently with 80 % ethanol. Pellets were after each washing step centrifuged for 1 min at 14.000 rpm. After the last centrifugation, the pellets in tubes were treated with 0.5 ml of 0.1 M NaOH (Penta, 98%) and boiled for 2 hours. The sample was transferred into 6 ml scintillation vials, supplemented with 4.5 ml of scintillation cocktail (Quicksafe A, Zinsser Analytic, Frankfurt, Germany) and the vials were assembled into liquid scintillation analyzer, where the radioactivity was determined using scintillation spectrometry (Tri-Carb 2900TR, Packard, U.S.A.). Results were corrected for blanks accounting for <5% of sample values. Bacterial production was expressed in units of µgC g^{-1} dw soil $hour^{-1}$ applying the conversion factor (CF) of 3.0 µgC $nmol^{-1}$ of incorporated leucine. This factor is used for the mathematical transformation of the amount of incorporated leucine to a real amount of biomass produced. This number was deduced from an average amount of leucine in proteins and the average amount of proteins in bacterial cells (Simon et al., 1989).

4.7.1. **Mathematical evaluation of data**

As the first step in data evaluation was determined the amount of Leucine incorporated per 1 l of soil extract in 1 hour. This value was calculated from the measured radiation intensity using the formula described below.

$$N = \frac{I}{I_0 * V} * d * \frac{1}{t}$$

N..... amount of incorporated Leucine, [N]= nmol $l^{-1}h^{-1}$

I.....radiation intensity, [I]= dpm, (dpm = Bq*60)

I_0.....standard radiation intensity, [I_0]= dpm $pmol^{-1}$

V ..... volume of soil extract, [V] = l

d .....dilution factor of soil extract

t..... time, [t]= h

The standard radiation intensity of Leucine is 728 dpm $pmol^{-1}$ and the volume of soil extract is considered to be 1.5 ml. The dilution factor for this calculation is 2. The particular time of incubation was 122-123 min.

As the second step in data evaluation, the amount of incorporated Leucine was recalculated to biomass (C) produced per 1 g of dw soil in particular time periods of experiment. For this calculation, the following formula was used.

$$C = N * 24 * C.F.* d.f.* \frac{100}{dw\ soil}$$

C..... biomass produced, [C]= µgC g^{-1} dw soil day^{-1}

N..... amount of incorporated Leucine, [N]= nmol l^{-1} h^{-1}

C.F. .. conversion factor

d .....dilution factor

dw soil ..... dry weight of soil [%]

A dilution factor used for this method was 10^{-1} according to the procedure described in chapter 4.4). Biomass production was calculated from the amount of incorporated Leucine with a conversion factor, 3.0 µgC $nmol^{-1}$ of leucine incorporated (Simon et al., 1989).

The amount of bacterial biomass produced in periods between sampling day was estimated with the following calculation approach.

$$C_p = \bar{x}(C_{t_2}; C_{t_1})x(t_2 - t_1)$$

C_p .....biomass produced in particular time period, [C_p]= µgC g^{-1} dw soil

C_{tn} .....biomass produced at any time point, [C_{tn}]= µgC g^{-1} dw soil day^{-1}

t_n .....time of incubation, [t]= day

4.8 Analysis of irbesartan concentrations in soil samples with laser diode thermal desorption with chemical ionization and tandem mass spectrometry detection method (LDTD/APCI-MS/MS)

In order to elucidate, if the irbesartan molecule undergoes any dissipation in soil, 5 g of soil from each soil sample (chapter 4.1.) were collected and stored at -20 °C and finally delivered to cooperating analytical laboratory, where the residual concentrations of the parent compound and its metabolites in all soils from the manipulative experiment were analyzed with LDTD/APCI-MS/MS method. This method was developed and described by colleagues Ing. Adam Bořík and doc. Mgr. Roman Grabic, Ph.D. at Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses in Vodňany (Borik et al., 2020).

4.9 Statistical evaluation of data

A full two factorial design was used to assess the effect of time after exposure to irbesartan and dose of irbesartan on the total cell counts and leucine incorporation. In the case of time factor the null hypothesis was stated to be:

The time of incubation has no effect on data obtained from methods assimilated within this thesis.

In the case irbesartan doses as a factor, the null hypothesis was stated in the following way:

There is no response of obtained data on the dose of irbesartan amended to manipulated soil.

The third null hypothesis considers the combination of two factors: There are no interactions between two factors affecting the obtained data.

On the other hand, one-way ANOVA was used to assess whether the irbesartan concentrations changed in time within each treatment.

The data obtained from Determination of Total cell count in manipulated soils and Radiolabeled L-[14C]-leucine incorporation (chapter 4.4.2 and 4.7 respectively) were analyzed with Two-way ANOVA (α=0.05) In the case to compare irbesartan concentrations in time within treated soils.

In addition to ANOVA analysis, the post hoc test (Tukey HSD test) was performed to elucidate pairwise comparisons. For this purpose, the Tukey HSD test was used. Data on Total cell counts, hybridized cells, and leucine incorporation were tested for the normality and homogeneity of variances, using Bartlett's test and Flinger-Killeen test, respectively (α=0.05). The data on leucine incorporation was normalized using decadic logarithm function (log (x+1) to meet requirements for parametric comparison.

5. Results

5.1 Bacterial abundance in soils using Enumeration of total cell counts method

5.1.1. **Preliminary quantitative analysis of Total cell counts in studied soil**

To find an optimal proportion between centrifugation velocity and dilution factor of soil extract the we did a quantitative analysis of Total cell counts in Chernozem Haplic (without irbesartan incorporation). The results of cell counts per field and calculated Total cell counts concerning the centrifugation velocity and dilution factor can be seen in Table 8.

Table 8.: Quantitative analysis of Total cell counts in soil samples under various proportions between centrifugation velocity and dilution factor. Fields labeled with grey color were excluded from total cell counts calculation: Means and standard deviations are shown (n=3).

Sample	Number of fields	Number of cells	Dilution factor	Total cell counts g^{-1} dw soil
S-0	6 (0)	5 (0)	10^{-4}	
S-100	7 (0)	4 (0)	10^{-3}	
S-250	18 (11)	54 (37)	10^{-3}	$3.31x10^{8}$ ($9.57x10^{7}$)
S-500	18 (9)	79 (37)	10^{-2}	$5.41x10^{7}$ ($2.86x10^{6}$)
S-750	12 (7)	68 (35)	10^{-2}	$7.56x10^{7}$ ($1.91x10^{7}$)
Average				**$1.54x10^{8}$**

Reliable results were found in samples where the centrifugation was applied and the supernatants were then diluted with lower dilution factor. The highest Total cell counts were found in both soils at centrifugation velocity 500 x g with consequent dilution factor 10^{-2}. In Chernozem soil extract we observed 79 cells at 18 fields.

Suitable results were also gained for samples centrifuged at 250 x g and 750 x g and then diluted with an appropriate dilution factor. Based on results from these three measurements it can be concluded that the concentration of prokaryotic cells in Chernozem soil is $1.54x10^{8}$ cell g^{-1} dw soil (calculation in Table 8).

Through this experience, we decided to use the centrifugation velocity of 500 x g and a dilution factor of 10^{-2} as a default approach for all subsequent analyses.

5.1.2. **Total cell counts in soils from manipulative experiment**

In this part of the work, we analyzed all samples from the manipulative experiment that were sampled destructively (see chapter 4.1). The soil extracts prepared from all 72 samples were analyzed for Total cell counts g^{-1} dry weight soil calculated as described in chapter 4.4.3. The results are given described in Supplementary Table 2.

To see the change of Total cell counts in time and the effect of irbesartan, the average Total cell counts g^{-1} dry weight soil from all samples were plotted against the time of incubation. This relation is given in Figure 6.

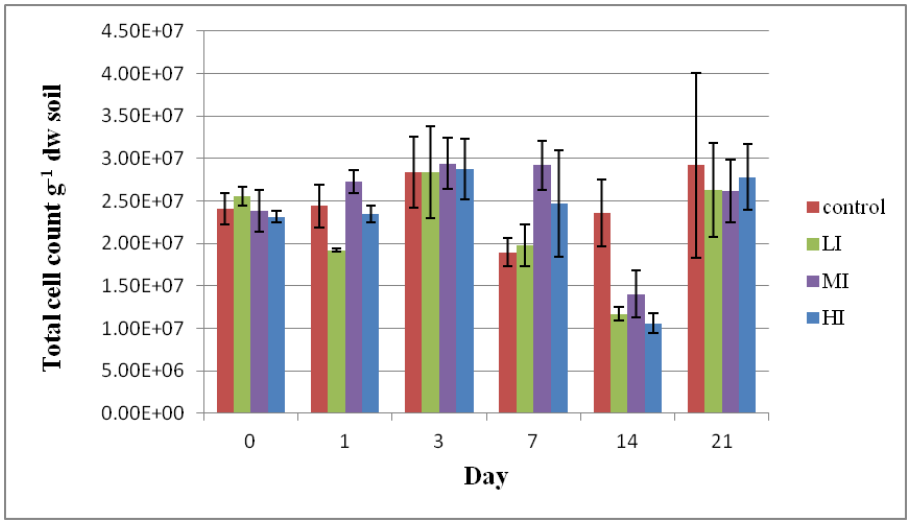

Figure 6.: Total cell counts in soil samples treated with different doses of irbesartan and untreated controls in the respective time. Means and standard deviations are presented (n=3). Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample

Based on the statistical analysis, the TCCs were found influenced by the time of incubation ($p< 0.001$). A different result was found in the case of the second null hypothesis stated in chapter 4.9. In this case, the TCCs in controls were observed to be analogous with values observed in LI, MI, and HI treatments ($p = 0.051$), which indicates that the irbesartan amendment does not affect TCCs. However there was no influence of irbesartan dose on Total cell counts detected, a slight interaction between both factors was observed to influence the TCCs in manipulated soils ($p= 0.01$). This finding was further analyzed with the Tukey test. The Tukey test revealed the TCCs in

controls to be significantly lower than TCCs in MI and HI treatments on day 7, however, the difference between controls and LI treatments was found negligible. A different situation was observed at day 14. At this time point, the TCCs in controls were found higher than in treated samples. At day 21 there has been found no significant difference among all the treatments and controls as can be seen in Figure 6.

5.2 Enumeration of living bacteria by CARD- FISH

One of the possibilities to estimate the effect of irbesartan amendments on the number of living bacterial cells offers the CARD-FISH technique. By using a oligonucleotide probe, targeting all bacteria (formerly *Eubacteria*), the most abundant group of soil prokaryotes, we were able to estimate the alternations in bacterial abundance. DAPI staining was performed as a control for the appropriate fixation of cells on the filter during the procedure. In this measurement, we focused on the three particular samples; controls from day 0 and control and HI treatment from day 14. At this time point, the Total cell counts determined in the previous measurement were found to be the lowest from the whole experiment. The Total cell counts g^{-1} dw soil were calculated in the same way as in the previous chapter (chapter 5.1). The results of this measurement are given in Table 9.

Table 9.: Total cell counts (DAPI staining) and counts living bacteria (CARD-FISH technique using EUB-338 probe) in selected soils and times. Abbreviations: Inc.- Incubation, TCC- Total cell count, B- living bacteria

Sample	Inc. time (day)	TCC g^{-1} dw soil	B g^{-1} dw soil
control	0	$9.23x10^6$	$8.55x10^6$
	0	$1.20x10^7$	$7.73x10^6$
	0	$7.34x10^6$	$9.15x10^6$
control	14	$9.54x10^6$	$7.30x10^6$
	14	$1.01x10^7$	$1.29x10^7$
	14	$1.05x10^7$	$9.17x10^6$
HI	14	$9.53x10^6$	$5.00x10^6$
	14	$1.35x10^7$	$9.46x10^6$
	14	$1.45x10^7$	$1.37x10^7$

The analysis of the results from this experiment showed differences between the count of cells evaluated as Total cell counts (TCC) and the count of living bacterial cells (B) observed using the CARD-FISH technique. The correlations between bacterial counts and Total cell counts in control and HI treatments are described with linear regression in Figure 7. The linear regression revealed the proportion of bacterial cell counts to the total cell counts observed in manipulated soils. The slope of the regression line was 0.8499 , however the reliability of regression was low.

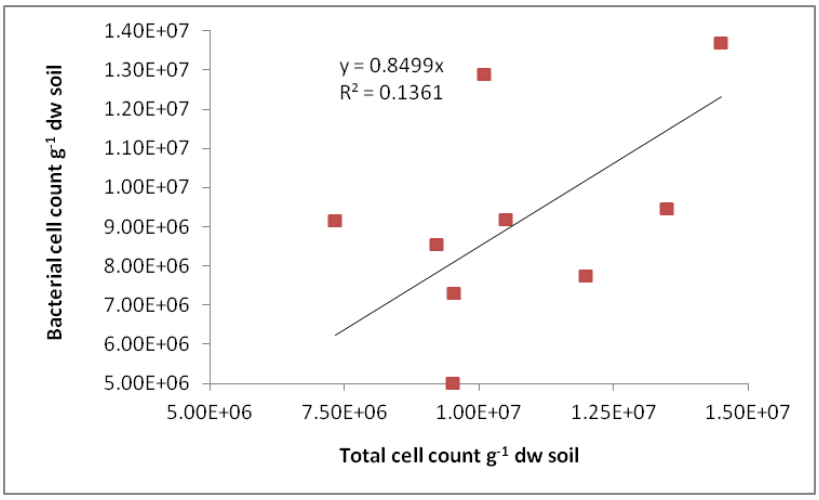

Figure 7.: Linear regression between total cells counts and Eubacterial cells found in control samples and HI treatments at the day 0 and day 14. Equations of linear regression and reliability coefficient are shown.

5.3 The effect of irbesartan amendments on soil bacterial biomass growth

The incorporation of L-[^{14}C]-leucine into bacterial biomass was used to evaluate the effect of irbesartan amendments on the soil bacterial activity (biomass production) in respective times of incubation. The amount of L-[^{14}C]-leucine incorporated per 1 l of soil solution per hour was calculated based on the measured radiation intensity as described in chapter 4.6.1. Means of the amounts can be seen in Supplementary Table 3 in the supplement.

The amount of incorporated L-[^{14}C]-leucine in manipulated soils at particular days is plotted in Figure 8. The incorporation of L-[^{14}C]-leucine into microbial biomass was assessed in the term of independency on incubation time and a dose of irbesartan amendment. In the case of correlation with a dose of irbesartan amendment in manipulated soils, the statistical analysis showed no interaction between incorporation

of L-[^{14}C]-leucine and dose of irbesartan amendment in manipulated soils ($p= 0.51$). While the dose of irbesartan amendment was found to have an influence on the incorporation of L-[^{14}C]-leucine, an opposite result was recorded for the length of incubation time, where the null hypothesis stated in chapter 4.9 was rejected ($p< 0.001$). The change in the incorporation of L-[^{14}C]-leucine was confirmed to be in association with the length of incubation time. The same information gave the analysis of the interaction between both factors and its possible effect on the incorporation of L-[^{14}C]-leucine. In this case, the alpha value was detected below the level of significance ($p= 0.02$). According to Figure 8, the most significant change in L-[^{14}C]-leucine was observed between day 7 and the last two measured times, day 14, and day 21. The Tukey test hasn't detected any significant differences in L-[^{14}C]-leucine incorporation at day 1, day 3, and day 7 except the decline in HI treatment at day 1. At day 14 the L-[^{14}C]-leucine incorporation was detected significantly lower for controls and LI treatment than for MI and HI treatments, while the values in these two compared pairs were found similar. Opposite information gave the Tukey test for day 21. In this case, the L-[^{14}C]-leucine incorporation was observed significantly higher in the case of controls than in all treatments. The values detected among the treatments appeared to be without any significant distinctions.

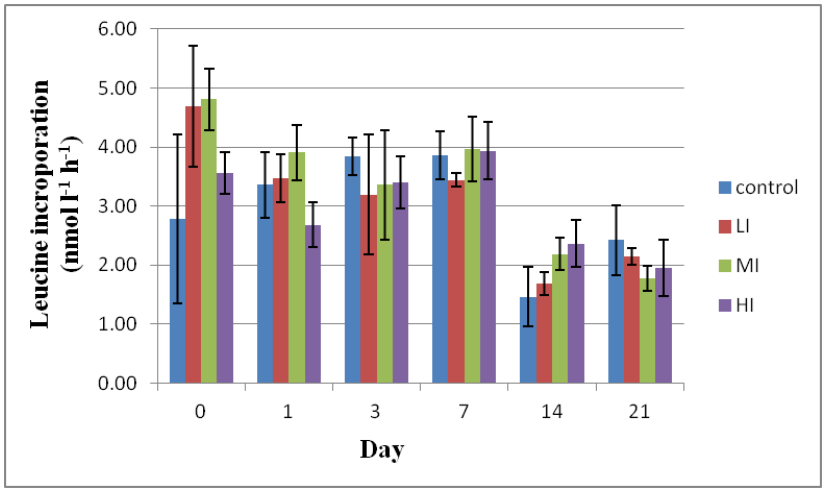

Figure 8.: Graphical representation of the average amount of leucine incorporated during the incubation time determined per 1 l of soil extract. Means and standard deviations are presented (n=3). Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample.

The biomass (C) produced per 1 g of dw soil was estimated from the amount of incorporated L-[^{14}C]-leucine, as described in chapter 4.6.2. using a conversion factor determined by (Simon et al., 1989). The biomass produced at particular periods is shown in Table 10.

Table 10.: The bacterial biomass production at particular time of the experiment calculated based on the amount of leucine incorporated, the estimation of biomass produced in periods between sampling days, and the accumulation of biomass in the soil during the experiment. Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample.

Bacterial biomass production (μgC g^{-1} dry soil day^{-1})				
Time (day)	control	LI	MI	HI
0	2.58	4.37	4.48	3.32
1	3.14	3.23	3.64	2.50
3	3.55	2.99	3.12	3.15
7	3.57	3.16	3.62	3.63
14	1.35	1.56	2.01	2.19
21	2.21	1.92	1.60	1.78
Bacterial biomass production in time periods (μgC g^{-1} dry soil)				
Time period	control	LI	MI	HI
0-1	2.86	3.80	4.06	2.91
1-3	6.69	6.22	6.76	5.66
3-7	14.25	12.30	13.47	13.58
7-14	17.23	16.51	19.72	20.38
14-21	12.45	12.19	12.66	13.90
Accumulation of bacterial biomass in particular time (μgC g^{-1} dry soil)				
Time (day)	control	LI	MI	HI
0	0.00	0.00	0.00	0.00
1	2.86	3.80	4.06	2.91
3	9.55	10.02	10.82	8.57
7	23.80	22.32	20.23	19.23
14	41.03	38.83	44.01	42.52
21	53.49	51.02	56.66	56.42

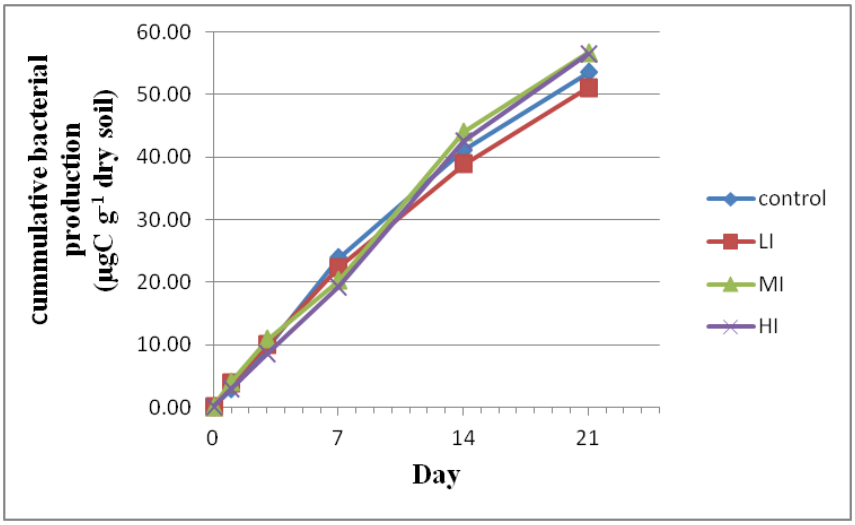

Figure 9.: Accumulation of bacteria biomass estimated by leucine incorporation in soil samples in time. Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample

The accumulation of bacterial biomass in time was calculated based on the data from Table 12. The accumulation of bacterial biomass upon particular types of treatment is shown in Figure 9. The biomass produced in soil by soil bacteria was estimated from the amount of L-[^{14}C]-leucine incorporated in the soil. The results revealed almost identical values regardless treatments, so there is no further statistical evaluation required.

5.4 Analysis of irbesartan concentrations in soil samples with laser diode thermal desorption with chemical ionization and tandem mass spectrometry detection method (LDTD/APCI-MS/MS)

The concentrations of irbesartan molecules in soil samples were detected with LDTD/APCI-MS/MS method. In our work we treated 100 g of soil with 6 ml of autoclaved tap water containing no irbesartan and contaminated with a low, moderate and high dose of irbesartan (1.23 µg g^{-1} dw soil, 12.3 µg g^{-1} dw soil and 123 µg g^{-1} dw soil respectively). Data were provided by Ing. Adam Bořík and doc. Mgr. Roman Grabic, Ph.D. from the analytical laboratory at Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses in Vodňany (Borik et al., 2020). The average concentrations of irbesartan detected in samples are described in Supplementary Table 4.

The concentrations of irbesartan in all types of samples changed from their original value. To see the development of these concentrations at a time scale, the values from Supplementary Table 4 were plotted against time. This plot is depicted in Figure 10. Figure 10 showed concentrations of irbesartan measured at particular time intervals from day 0. The highest concentrations were found in soil samples that were treated with a high dose of irbesartan. The concentration in the remaining two types of samples (low dose and a moderate dose of irbesartan) was determined to be smaller approximately by a factor of 10 and 100 respectively in comparison to the HI treatments. The ANOVA analysis confirmed a significant decrease of irbesartan concentration only in the case of HI treatment ($p= 0.005$), while the concentrations in LI and MI treatments were found not affected by the time of incubation ($p= 1.5$, $p= 0.13$ respectively). The analysis of data from HI treatment with the Tukey test has revealed a significant decline in the concentration of irbesartan at day 14.

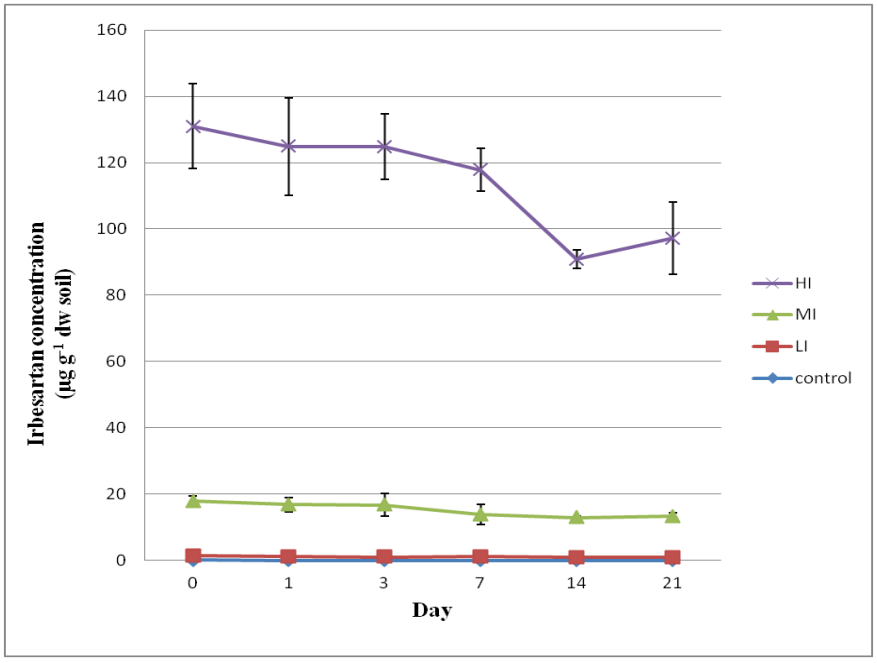

Figure 10.: Development of irbesartan concentration in particular soil extract samples in time. Means and standard deviations are shown (n=3). Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample.

6. Discussion

Within this thesis, we tested the interaction of bacterial abundance and biomass production with various amendments of irbesartan in Chernozem Haplic. For this purpose, we employed the Enumeration of Total cell counts, CARD-FISH technique, and L-[^{14}C]-leucine incorporation methods.

Based on the statistical analysis of our results we found no direct influence of the irbesartan amendments on the bacterial abundance in Chernozem Haplic. The data showed negligible differences among untreated control and soils treated with irbesartan within the first week of exposure as well as at the end of the experiment (day 21). The only exception was the decline in bacterial abundance at day 14 (Figure 6). Since the decline was observed for all treatments and controls, the reasonable explanation might be the depletion of available nutrients in soils, which caused a decrease in bacterial abundance (Richardson et al., 2011). Despite the decline in bacterial abundance at day 14 was observed in all types of samples, we found a significantly deeper decline in LI and MI treatments than in controls. The deepest decline was observed in soil treated with the highest dose of irbesartan. The relative abundance of bacterial phyla in arable soils has been described to be stable in time upon no external disruptions of the ecosystem (Ding et al., 2014). According to this finding, the decline in bacterial abundance can be a consequence of irbesartan presence in soils amended with irbesartan.

Based on these experiences, we wanted to test the living bacterial abundance in controls and HI treatments at day 0 and in controls and HI treatments at day 14 using the CARD-FISH technique. The results revealed a change in the bacterial abundance in manipulated soil treated between day 0 and day 14. The estimated slope (slope= 0.84) (Figure 7) indicates that approximately 84% of bacteria cells in soils manipulated with irbesartan were metabolically active. Thus it can be concluded that the bacteria activity was affected by the irbesartan amendment in HI treatments, which represents the irbesartan contamination in a concentration above the environmental levels. The concentration of PhACs in soil has been described as the fundamental factor for their toxicity in the environment (Bartrons et al., 2017).

We found similar results when we observed metabolic activity of soil bacteria. The changes in biomass production were observed to be related to the incubation time. The most significant decline in biomass production occurred at day 14 and day 21, while the data from the first week of measurements (from day 0 to day 7) showed constant values as can be seen in Figure 8. A reasonable explanation for this finding might be a depletion of nutrients required for biomass production (organic carbon, nitrogen, and phosphorous), which have had a strong influence on the amount of leucine incorporated in bacterial biomass previously (Aldén et al., 2001). Since we preceded the incubation in a closed system with no nutrient supply, this finding gives a reasonable explanation of the decline we have observed. The biomass production at day 14 declined to approximately one-half of the values detected at day 7.

The only differences in biomass production among the treatments were observed at day 14. At day 14 the biomass production was significantly higher in MI and HI treatments than in controls and LI treatments. The explanation for increased L-[^{14}C]-leucine incorporation in those treatments might be that various bacterial phyla assimilate leucine in their proteins at different rates. According to the findings of (Cottrell et al., 2003), the leucine incorporation in bacterial proteins is unequal among the bacterial phyla or even classes. The irbesartan amendment might caused a decline in the relative abundance of some bacterial members with a low contribution to the total amount of leucine incorporated. The decline reduced natural competition within bacterial communities and provides easier access to leucine. Unfortunately, this idea cannot be proved, since we lack more complex evidence about the relative abundance of specific bacterial taxa in manipulated soils.

Nevertheless, at day 21, we observed the recovery in bacterial biomass production in treated soils, since the values reached the same values as untreated control again. This can be explained by the recovery of sensitive bacterial members after 14 days of exposure. The accumulation of bacterial biomass in soil was estimated for the whole time period (21 days, Figure 9) based on the biomass production determined for each incubation time. Despite we observed different biomass production at day 14, the biomass production was estimated completely same for control as well as for all treatments for the whole time period, which has shown the irrelevance of dose of irbesartan amendments for bacterial biomass production.

The results of LDTD/APCI-MS/MS analysis detected the concentrations of irbesartan in treated soils close to concentrations that were expected right after amendments in Chernozem Haplic (day 0, Figure 10, chapter 4.1). In untreated controls the irbesartan concentration was below the detection limit and was neglected. Despite the Cherzomen Haplic contains a significant amount of clay and organic material, due to its neutral pH (Vysloužilova et al., 2016) more than 80% of irbesartan molecules stay in non-ionic form because the sorption coefficient of such molecules on the surface of soil particles is very low (Klement et al., 2018). This fact implies that the majority of irbesartan molecules were likely available to microorganisms along the whole incubation time.

The concentration of irbesartan in LI and MI treatments was found constant along with the whole experiment, however, in HI treatments we observed a decline in irbesartan concentration between day 7 and day 14, which correlates with the lowest bacterial abundance detected at the same day. The irbesartan was found degradable by some bacterial phyla. As an example, the actinomycete *Lentzea pudingi* might be given, which has been found to have a major contribution to irbesartan degradation between soil bacteria in laboratory experiments with pure cultures (Košinová, 2019). The most common microbial metabolites of irbesartan degradation are hydroxy- modified metabolites and N-glycoside irbesartan conjugates(Alexandre et al., 2004). The decline in irbesartan concentration thus can be a consequence of biological (microbial) degradation of irbesartan molecules. The biological degradation of PhACs is expected to be the dominant way of their transformation in soil (Biel-Maeso et al., 2019). Although some PhACs are biologically degradable, this process is sometimes associated with a decline in bacterial abundance in soil. This behavior has been described in the case of analgesics and antipyretics drugs, detected in soil in concentrations above the environmental levels. Upon these conditions, the biodegradation of pharmaceuticals has been observed to be in association with the decline in bacterial abundance in soil (Barra Caracciolo et al., 2015). The decline at day 14 might be a consequence of the incorporation of irbesartan in cells of zymogenous bacteria, representing the major part of the soil microbial community (Conn, 1948), as an alternative source of organic carbon when the nutrients and organic carbon in soil were depleted. In our experiment the methylated metabolites were detected by (Grabic et al., 2019), however, no complete degradation has been observed. We expected methylated metabolites to be

products of microbial transformation of irbesartan in soil, however, we did not have any evidence to prove it yet.

7. Conclusion

The microbial community of Chernozem field soil was studied for its response to irbesartan amendments in terms of bacterial abundance and biomass production within three weeks. Based on the analysis of soil samples amended with irbesartan we have found no consistent impact of irbesartan on bacterial abundance and biomass production, regardless of the concentration of the contaminant. The bacterial community has been found affected by the irbesartan amendment within a week's time interval in terms of bacterial abundance. However this disturbance has appeared to be significant, it can be fully attributed to irbesartan pollution only in the case of high dose amendment (123 μg g^{-1} dw soil), which is a way higher value than actual irbesartan environmental concentrations. During the experiment we have observed methylated metabolites of irbesartan in soils, which are expected to be products of biological transformation, however, we lack the data to validate it.

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Supplementary information

Table 1.: The proportion of dry weight soil (%) in Chernozem haplic sample and manipulated soils: Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample

Dry weight soil (%)				
Chernozem haplic sample				
85.25				
Manipulated samples				
Time	control	LI	MI	HI
0	77.70	77.10	77.17	77.05
1	77.03	77.37	77.24	77.30
3	77.88	76.95	77.63	77.58
7	77.83	78.40	78.68	77.96
14	77.92	77.85	78.35	77.73
21	79.13	80.26	79.73	79.10

Table 2.: Total cell counts in soil samples treated with different doses of irbesartan and untreated controls in respective time. Means and standard deviations are shown (n= 3): Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample

Day	control	LI	MI	HI
0	$2.41x10^7$ ($1.88x10^6$)	$2.56x10^7$ ($1.11x10^6$)	$2.38x10^7$ ($2.42x10^6$)	$2.32x10^7$ ($7.23x10^5$)
1	$2.44x10^7$ ($2.50x10^6$)	$1.92x10^7$ ($2.08x10^5$)	$2.72x10^7$ ($1.35x10^6$)	$2.34x10^7$ ($9.87x10^5$)
3	$2.84x10^7$ ($4.16x10^6$)	$2.84x10^7$ ($5.37x10^6$)	$2.94x10^7$ ($3.00x10^6$)	$2.87x10^7$ ($3.57x10^6$)
7	$1.90x10^7$ ($1.66x10^6$)	$1.98x10^7$ ($2.44x10^6$)	$2.92x10^7$ ($2.84x10^6$)	$2.47x10^7$ ($6.30x10^6$)
14	$2.36x10^7$ ($3.95x10^6$)	$1.17x10^7$ ($8.00x10^5$)	$1.41x10^7$ ($2.72x10^6$)	$1.06x10^7$ ($1.15x10^6$)
21	$2.92x10^7$ ($1.09x10^7$	$2.63x10^7$ ($5.56x10^6$)	$2.62x10^7$ ($3.70x10^6$)	$2.78x10^7$ ($3.88x10^6$)

Table 3.: Amount of L-[^{14}C]-leucine incorporated in soil extracts at particular time points. Means and standard deviations are shown (n= 3). Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample

L-[^{14}C]-leucine incorporation [nmol l^{-1} h^{-1}]				
Day	control	LI	MI	HI
0	2.79 (1.43)	4.68 (1.03)	4.80 (0.51)	3.56 (0.35)
1	3.36 (0.56)	3.47 (0.41)	3.90 (0.47)	2.68 (0.38)
3	3.84 (0.32)	3.20 (1.01)	3.36 (0.92	3.40 (0.44)
7	3.86 (0.41)	3.44 (0.11)	3.96 (0.55)	3.93 (0.49)
14	1.46 (0.50)	1.69 (0.19)	2.19 (0.28)	2.36 (0.39)
21	2.43 (0.59)	2.15 (0.14)	1.78 (0.22)	1.96 (0.48)

Table 4.: Concentrations of irbesartan in manipulated soils detected with LDTD/APCI-MS/MS method. Means and standard deviation are shown (n= 3). Abbreviations: LI- low dose of irbesartan, MI- moderate dose of irbesartan, HI- high dose of irbesartan, control – untreated sample

Irbesartan concentration [μg g^{-1}]				
Time	Control	LI	MI	HI
0	0 (0.00)	1.27 (0.17)	16.52 (1.51)	113.17 (12.83)
1	0 (0.00)	1.06 (0.25)	15.72 (2.09)	108.11 (14.70)
3	0 (0.00)	1.01 (0.25)	15.72 (3.36)	108.11 (9.97)
7	0 (0.00)	1.06 (0.21)	12.69 (2.99)	104.06 (6.45)
14	0 (0.00)	0.93 (0.14)	11.98 (0.33)	77.98 (2.77)
21	0.003 (0.00)	0.84 (0.19)	12 (0.97)	84 (10.88)