University of South Bohemia
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Comparison of Irbesartan Microbial Transformation Processes in Pure Cultures and Consortia

Bachelor's thesis

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Annotation:

Pharmaceutical contamination of the environment affects living organism and poses risks for human health. Bioremediation is possible through various soil microorganisms. This thesis studies the removal of the pharmaceutical irbesartan by four bacterial strains, three fungal strains, and seven different consortia and identifies the obtained transformation products of irbesartan.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 11.05.2021 ………………………………..

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Abbreviations

WWTP	Wastewater treatment plant
EWW	Effluent wastewater
SW	Surface water
OD	Optical density
PCR	Polymerase chain reaction
TP	Transformation product
ASV	Amplicon sequence variant
LC-HRMS	Liquid chromatography coupled with high resolution mass spectrometry
MS-MS	Tandem mass spectrometry

1. Introduction

1.1 Pharmaceutical Contamination of the Environment

The extensive consumption of pharmaceuticals in recent years has resulted in their accumulation in the environment. Although many processes and regulations are employed to keep the contamination through pharmaceuticals to a minimum, substances still reach the environment through various pathways (Guedes-Alonso et al., 2020).

Pharmaceuticals are generally excreted or disposed into the sewage system from domestic, hospital or industrial waste and released into wastewater treatment systems (Naidu et al., 2016). Detected concentrations of pharmaceuticals in wastewaters in North-east Spain ranged from 21 - 730 µg L^{-1}. Highest concentrations were detected in hospital wastewaters, followed by veterinary hospital, university, and urban wastewaters (Lucas, Barceló, et al., 2016). However, pharmaceutical concentrations in the wastewater treatment system are subject to large seasonal variations, due to differences in seasonal consumption patterns of individual pharmaceuticals as well as increases during holiday or festival periods (Biel-Maeso et al., 2018).

The collected sewage is treated in wastewater treatment plants (WWTPs) in multiple ways, including various separation, oxidation, and filtration processes, which show varying results in the removal of pharmaceuticals. This is due to the fact, that efficiency in removal is affected by multiple factors, such as physiochemical properties of the pharmaceuticals, sorption, oxidation-reduction conditions, activated sludge age and other environmental conditions (Mackulak et al., 2019). Comparisons between natural WWTPs (i.e. constructed wetlands) and conventional WWTPs showed slightly better removal rates for the latter, however, all tested pharmaceuticals were still present in the treated wastewater with concentrations ranging from ng L^{-1} to µg L^{-1} (Guedes-Alonso et al., 2020). A study measuring the removal rates of the most abundant pharmaceuticals in WWTPs found that the majority of compounds exhibited removal efficiencies of less than 50 %, and only 5 compounds were efficiently eliminated (Biel-Maeso et al., 2018). Furthermore, many pharmaceuticals might accumulate in the solid phase of the wastewater treatment process, which is often transformed into biosolids (Ding et al., 2011).

Direct discharge of treated wastewater into water bodies or the irrigation of agricultural land with WWTPs effluents results in dissemination of pharmaceutical compounds into water and soil environments (Biel-Maeso et al., 2019; Ding et al., 2011). Another way of contamination

is through the application of biosolids on agricultural land, which is done due to their high content in organic matter, minerals, and nutrients, especially nitrogen and phosphor. However, this also allows pharmaceuticals to translocate into the soil and accumulate there with varying rates depending on the individual compound (García-Santiago et al., 2016). In the European Union, around 50 % of produced sewage sludge is used for land application, making it the main pathway for sewage sludge recovery (Collivignarelli et al., 2019). Approximately 17% and 32% of the Austrian and Czech Republican total sewage sludge production in 2014 were used in agriculture (Hudcová et al., 2019).

Another way in which pharmaceuticals are exposed to the environment, is through their use in the production of livestock. Pharmaceutical compounds reach the environment primarily through direct excretion of urine and dung and application of contaminated animal manure on land (CVMP, 2016). Furthermore, increased concentrations of pharmaceuticals have also been found around pharmaceutical production sites, since they reach the surrounding water bodies and soil through effluents from the factory (Fick et al., 2009). Pharmaceutical compounds may also reach the environment through improper disposal or through leakages from landfills (Boxall et al., 2012).

1.2 Effects of Pharmaceuticals on the Environment

Pharmaceuticals pose a high risk to ecosystems since they are designed to have biological effects even in very low concentrations, affecting both target and non-target species (Arnold et al., 2014). Many pharmaceutical compounds and their metabolites are not easily degradable and can persist in the environment for months up to years. Furthermore, some heavily used pharmaceuticals might be added continuously to the environment and can therefore show a pseudo-persistence (Barra Caracciolo et al., 2015). Additionally, pharmaceuticals are not only present in their original form but are often transformed or degraded into one or more transformation products by various environmental and wastewater treatment processes. These transformation products can possibly exhibit a higher toxicity and persistence than their parent compound and are much harder to detect and quantify (Boxall et al., 2012).

Pharmaceutical concentrations present in biosolids or treated wastewater can be taken up by various plant species (Bartrons & Peñuelas, 2017). The uptake depends on the physicochemical characteristics of the pharmaceuticals and the species of the plant. Root growth, shape and size of leaves, transpiration rates, and lipid contents all play an important role in the uptake of pharmaceuticals (Carter et al., 2014). Uptake might lead to inhibited

growth and death of the plant, as it was demonstrated in a study by Herklotz et al. (2010) using concentrations starting from 0.44 mg L^{-1} of the disinfectant triclosan.

Pharmaceutical compounds in the environment can also negatively affect wildlife populations. Animals lacking a similar metabolic, detoxification, or excretory system as humans might be especially susceptible to the main and side effects of the drugs, resulting in changes in their physical or reproductive cycles, which can have delayed negative effects on wildlife continuity (Arnold et al., 2014). For example, a near collapse of a vulture species population in Pakistan through renal failure was caused by exposure of the birds to diclofenac-treated animal carcasses (Oaks et al., 2004). A similar fate was observed in the fathead minnow population of a lake treated with estrogenic substances, which resulted in a feminization of the fish and subsequent near extinction of the species (Kidd et al., 2007). Due to the accumulation of pharmaceuticals in soils, the compounds can also be taken up by soil animals, such as earthworms. This is especially problematic since it can lead to the bottom-up effect, which describes the accumulation of environmental contaminants such as pharmaceuticals in animals at higher positions in the food chain (Carter et al., 2016). In a study by Kinney et al. (2009) over 25 anthropogenic contaminants including various pharmaceuticals, detergent metabolites, polycyclic aromatic hydrocarbons, pesticides, and biogenic sterols, were found in earthworms at concentrations up to 1 mg kg^{-1}.

The overuse of antibiotics in human and veterinary medicine poses another well-studied risk accompanying contamination of ecosystems, as the extensive use of antibiotics in the live-stock production favours the development of antibiotic resistance of microorganisms. The developed resistance genes may spread between different organisms via horizontal gene transfer. The development of new antimicrobial agents is currently not able to keep pace with the increase in antibiotic resistance, which might lead to a potential future risk in ineffective treatment of many infectious diseases (Klatte et al., 2017).

All in all, the specific effects and risks pharmaceuticals may have on the environment and is habitants depend strongly on multiple different factors, including the technology of WWTPs, concentrations and persistence of the pharmaceutical, composition and rate of degradation through microorganisms, as well as environmental conditions, such as season, temperature, solar radiation, and properties of soil and water bodies (Mackulak et al., 2019).

1.2.1 Effects of Pharmaceuticals on Soil Microbial Communities

Soil microorganisms influence many vital soil processes and maintain soil health and quality (Cycoń et al., 2019). Some microbial species have the ability to utilize particular pharmaceuticals as a carbon source and are therefore able to degrade or transform them. However, degradation and remediation of contaminated soil is only possible if the microbial activity is not inhibited by the toxicity of pharmaceutical compounds (Barra Caracciolo et al., 2015).

Pharmaceuticals affect metabolic and community diversity of soil microorganisms by changing their ability to utilize different carbon sources and inhibit enzymatic activities. The degree of degradation and impact on microbes is strongly dependent on the individual pharmaceutical compound (Gielen et al., 2011). Overall, pharmaceuticals with similar physicochemical characteristics have similar effects on the diversity of microbial populations. Generally, non-steroid anti-inflammatory drugs were proven to be the least toxic, while antibiotics presented the highest toxicity (Pino-Otín et al., 2017).

Although non-steroid anti-inflammatory drugs normally show the least toxicity, high doses can negatively affect the activity level of soil enzymes, especially dehydrogenases. The largest decreases in soil microbial activity were found for ketoprofen, which lasted up to 30 days. Respiratory activity, ammonification, and nitrification rates of microbes for other pharmaceuticals, including naproxen and diclofenac, were not affected and even showed to be stimulated after an initial inhibitory effect (Cycon et al., 2016).

Due to their antimicrobial activity, antibiotics showed the greatest effect on microbial communities since they have the ability to significantly reduce activity of dehydrogenases and phosphatases. For example, the application of 1 µg kg^{-1} of tetracycline and 50 µg kg^{-1} of sulfamethazine resulted in short-term negative effect on enzyme activity (Barra Caracciolo et al., 2015; Cycoń et al., 2019). Similar results were also shown for various other antibiotics at concentrations from 1 - 200 mg kg^{-1} of soil. Chlortetracycline, oxytetracycline, and sulphonamide antibiotics decreased catabolic functions of soil microbial populations at concentrations of 1-90 mg kg^{-1}, however, these effects were observed to be only temporary (Cycoń et al., 2019; Pino-Otín et al., 2017). The specific effect and toxicity of antibiotics on microorganisms seems to be dependent on the antimicrobial activity of the compound, its concentration and time of exposure, as well as the physicochemical properties of the soil. The observed changes in microbial diversity may be induced through the ideath or inhibition of susceptible microorganisms and may therefore lead to an increase in resistant microorganisms or those capable of using antibiotics as a carbon source (Cycoń et al., 2019). However, not

only antibiotics can be toxic towards microbes. The ß-blocker nadolol, showed a significant decrease in the physiological diversity of microorganisms in soil at a concentration of 1.0 g kg^{-1} (Pino-Otín et al., 2017). The anti-hypertensive drug irbesartan was also shown to alter microbial composition in soil samples, mainly apparent in a decreased ratio in gram-positive to gram-negative bacteria and bacterial to fungal cultures (Frková et al., 2020).

Although, the exposure to pharmaceuticals may initially decrease metabolic activity and diversity of microbial communities, studies have found that long term exposure may cause some microbial species to adapt and to metabolize certain compounds. Microbial communities in soil under long-time exposure to pharmaceuticals through sewage irrigation were less stressed by an exposure to pharmaceutical compounds and were generally better able to decrease their effect on respiration and microbial growth (Gielen et al., 2011).

1.3 Pharmaceutical Degradation by Soil Microbial Communities

Soils are extremely susceptible for the accumulation of pharmaceuticals through the various pathways in which pharmaceuticals reach the environment. Pharmaceutical persistence and transport in the soil, however, is subject to multiple factors, including microbial activity, mineralogy and hydraulic properties of the soil, pH, temperature, moisture content, and other environmental conditions. Furthermore, physicochemical properties of the pharmaceutical compounds also influence the occurrence and degradation of pharmaceuticals (Biel-Maeso et al., 2018). For example, pharmaceuticals with a high octanol-water partition coefficient and a positive charge, may be more strongly bonded to the soil through hydrophobic interactions, and therefore less available for microbial degradation. On the other hand, negatively charged or neutral compounds mostly do not interact as strongly with the soil and are therefore easily degradable and transportable in deeper soil layers (Biel-Maeso et al., 2019). Indeed, exact assessment of the biodegradability of individual pharmaceuticals is difficult to prove since removal rates often vary in different studies. This might be explained through differences in soil composition, microbial activity, and other climate and environmental conditions (Grossberger et al., 2014). A study by Biel-Maeso et al. (2018) also found a seasonal dependence of microbial degradation, which was attributed to decreases in the activity of microorganisms and lower solar irradiance in winter months compared to the summer.
The most important factor affecting pharmaceutical persistence is the activity of microbes, since recovery from pharmaceutical contamination is only possible through the ability of microorganisms to degrade and transform various compounds via their metabolic or co-metabolic pathways (Barra Caracciolo et al., 2015).

Many bacterial genera, for example *Arthrobacter, Enterobacter,* and *Pseudomonas* have exhibited the ability to remove environmental contaminants, including various pharmaceuticals, and used them as a carbon source to obtain energy (Aissaoui et al., 2017). Co-metabolic systems have proven to be more effective than using the pharmaceutical as a single carbon source, since additional growth substrates can lead to the synthesis of cofactors, which are essential for co-metabolic degradation. Moreover, co-metabolic degradation might more closely resemble actual degradation pathways in the environment, since pharmaceutical contaminants often coexist with many other substances that can function as additional carbon, nitrogen, and energy sources (Domaradzka et al., 2015).

However, not only bacteria but also fungi have the potential to biodegrade environmental contaminants. In recent years, fungal treatment of waste waters has established itself as a promising technique for contaminant removal since fungi are able to grow fast, have a wide variety of non-specific extracellular enzyme systems suitable for the degradation of complex structures, and are able to do so in various pH-ranging and nutrient deficient environments (Lucas et al., 2016b). Specific performance of fungi cultures in wastewater treatment processes is dependent on various external factors, including physicochemical properties of the wastewater, such as temperature and pH, the presence of other contaminants, and the chemical properties of the pharmaceuticals (Yang et al., 2013). Nevertheless, studies utilizing stirred tank bioreactors with the white-rot fungi strain *Trametes versicolor* achieved a significant removal of over 80 % for most tested pharmaceuticals, including ibuprofen, naproxen, diclofenac, and sulfamethoxazole. These high degradation efficiencies are mostly due to the fungal extracellular lignin modifying enzymes (laccases) with the capability to degrade many complex structures, including lignin and other complex pharmaceutical and environmental contaminants (Asif et al., 2017).

However, not only fungi from the phylum of Basidiomycota, but also fungi from the phylum Ascomycota are able to degrade environmental contaminants to a great extent, since they exhibit many of the same advantages for degradation of compounds as white-rot fungi. Fungi from this phylum contain many different lifestyles and morphologies and were often found to be the dominant phylum in activated sludge from WWTPs. This is probably due to their high adaptation to these unfavourable environments, which includes their capability to grow under slightly basic pH values and to chelate charged metals (Olicón-Hernández et al., 2017).

Paracetamol, a widely used antipyretic and analgesic drug, was shown to be degradable by bacteria of the genus *Stenotrophomonas* and *Pseudomonas,* whereby *Pseudomonas* was able

to degrade the pharmaceutical in concentrations up to 2.5 g L^{-1}, while *Stenotrophomonas* species degraded concentrations only up to 0.6 g L^{-1}. Higher concentrations of the drug inhibited the degradation completely, indicating that excess of the pharmaceutical would significantly hinder the growth of the bacteria species (L. Zhang et al., 2013).

The anti-inflammatory drug Naproxen was found to be easily degradable for concentrations of 100 µg L^{-1} under aerobic conditions by bacterial consortia found in water and soil (Barra Caracciolo et al., 2015). Naproxen mineralization was also found in agricultural soils with applied biosolids and was responsive to temperature and soil content (Topp et al., 2008). Observed half-lives (DT_{50} values) ranged widely from 3 to 69 days depending on the study and soil type (Lin & Gan, 2011; Monteiro & Boxall, 2009; Topp et al., 2008). Naproxen was also found to be readily biodegradable by some bacterial species, including a strain of the gram-positive bacteria *Planococcus*, which was able to decrease naproxen concentrations by 75 % and 86 % under co-metabolic conditions using glucose and phenol as additional carbon sources (Domaradzka et al., 2015). Ibuprofen was also found to be rapidly biodegradable, however, with varying half-lives over different studies ranging from less than 1 day to 15 days (Barra Caracciolo et al., 2015; Lin & Gan, 2011). Especially some bacterial strains of the genera *Sphingomonas* and *Sphingobium* were able to completely degrade 1 µg L^{-1} of the parent compound (Zhou et al., 2013). A study investigating the degradation of diclofenac in forest soil found that up to 1.0 g L^{-1} of the drug dissolved in a low salt medium were fully degraded in less than 10 days in some of the soil samples (Facey et al., 2018). A similar result was also obtained by Lin & Gan (2011), which found diclofenac (0.1 µg g^{-1}) to be degraded with a DT_{50} value of less than 5 days. Diclofenac is also one of the pharmaceuticals that is easily removed by filamentous fungi in WWTPs, which was documented by the complete removal of the pharmaceutical within 3 hours by the fungal strain *Trametes versicolor* (Dalecka et al., 2020). A study by Aissaoui et al. (2017) also found that diclofenac and ibuprofen were degradable by a bacterial consortia by 9 % and 23 % respectively, even when present in a mixture together with sulfamethoxazole. Three white-rot fungi strains (*Bjerkandera sp. R1*, *Bjerkandera adusta* and *Phanerochaete chrysosoporium*) were also able to completely degrade ibuprofen and naproxen within 24 hours (Rodarte-Morales et al., 2012). Similar results were also achieved with the fungal culture *Trametes versicolor*, which was found to completely remove several pharmaceuticals including ibuprofen, naproxen, and diclofenac after 48 hours (Tran et al., 2010).

The antiepileptic drug carbamazepine, which is known for its persistence in WWTPs, was found to degrade only slightly in different soil types with half-lives upwards of 50 days and

even exceeding 120 days in some soils (Grossberger et al., 2014; Li et al., 2013; Monteiro & Boxall, 2009). This seems to be partly confirmed in studies identifying specific bacterial strains with the potential to biodegrade carbamazepine. For example, the strains *Rhodococcus rhodochrous* was able to degrade only 15 % of the pharmaceutical in 28 days. Similarly, the fungi strain *Aspergillus niger,* belonging to the phylum of Ascomycota, was found to decrease carbamazepine concentrations only by 9 % over the period of 10 days under laboratory conditions (Gauthier et al., 2010). Similar persistence was also found for fluoxetine, which showed only slight biodegradation over a period of 60 days which might be due to their limited bioavailability in soils or persistence to microbial degradation (Grossberger et al., 2014; Monteiro & Boxall, 2009).

Environmental degradation of antibiotics depends on physicochemical properties and molecular structures of the individual compounds. Amoxicillin, for example, was found to be easily degradable with a half-life of less than one day. Antibiotics belonging to the group of sulphonamides also showed the ability to be degraded by some bacterial strains (Cycoń et al., 2019). Sulfamethoxazole, for example, was degraded by the bacterial strain *Rhodococcus rhodochrous* to an extent of 20 % over the course of 36 days (Gauthier et al., 2010). Fluoroquinolones, tetracyclines, and macrolides have a generally higher persistence in soil, with large half-lives ranging from 30 days to years. This might be due to their physicochemical properties which allows them to form stable residues through strong binding to soil components (Barra Caracciolo et al., 2015; Cycoń et al., 2019). Cefalexin, another widely used antibiotic, was found to be degradable by some bacterial strains of the genus *Pseudomonas*, which exhibited a resistance towards cefalexin. The antibiotic compound was removed by up to 92 % in 24 hours (Lin et al., 2015). Fungal treatment in WWTPs is a promising technology in the removal of antibiotics from wastewaters, since antibiotics mostly do not pose high stress on fungi cultures. Approximately 77 % of tested antibiotics belonging to seven different groups, including sulphonamides, fluoroquinolones, and macrolides, were removed after fungal treatment for 15 days compared to 49 % in conventional wastewater treatment processes (Lucas et al., 2016a).

Pharmaceuticals often co-occur with multiple other pharmaceutical as well as other environmental contaminants. Mixtures of pharmaceuticals may therefore decrease the degradation processes or increase the toxicity of compounds that do not show toxic effects on their own (Monteiro & Boxall, 2009). Furthermore, degradation of pharmaceuticals often results in the formation of one or more transformation products. These may inhibit more or

less of the bioactivity and persistence of their parent compound and often exhibit higher half-lives (Berkner & Thierbach, 2014; Gielen et al., 2011).

1.4 Irbesartan and Its Use

Irbesartan is an anti-hypertensive drug used to decrease blood pressure and the progression of renal disease linked with hypertension and type-2-diabetes. It belongs to the group of angiotensin II receptor blockers, which selectively block AT receptors and therefore inhibit the renin angiotensin system, subsequentially widening blood vessels and lowering blood pressure (Gialama & Maniadakis, 2013). In 2018, over three million prescriptions of the drug were sold in the United States making it the 174th most commonly prescribed medication (ClinCalc DrugStats, 2020). Similar medications from the sartan group include valsartan, losartan, telmisartan, and candesartan (Muszalska et al., 2014). In the treatment of hypertension irbesartan is often used in a monotherapy or in combination with hydrochlorothiazide (Gialama & Maniadakis, 2013).

Irbesartan is also known under the names of Aprovel, Avapro, and Karvea and is usually taken orally in tablet form. Recommended daily doses start from 75 mg or 150 mg and can be increased to 300 mg for patients with extreme cases of hypertension (FDA, 2011). The highest concentration in the body is reached approximately 4 hours after intake of oral doses with 25-300 mg. Irbesartan and its metabolites are excreted via the biliary and renal pathways and have an elimination half-life averaging 11-15 hours (Gialama & Maniadakis, 2013).

Irbesartan has a molecular formula of $C_{25}H_{28}N_6O$ and a molecular weight of 428.5 g mol^{-1}. Its molecular structure can be seen in Figure 1. It is also known under the IUPAC name *2-butyl-3-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]-1,3-diazaspiro[4.4]non-1-en-4-one* and has a boiling point of 648.6 °C. It is practically insoluble in water, with a solubility of less than 1 mg mL^{-1}. However, it showed a slight solubility in alcohol and dichloromethane (NCBI, 2021). It has an octanol/water partition coefficient of 10.1 at a pH of 7.4 and is a nonpolar compound (FDA, 2011).

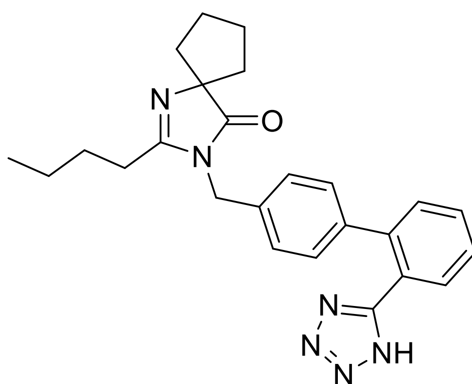


Figure 1: Molecular structure of irbesartan (Alexandre et al., 2004)

1.4.1 Transformation Products of Irbesartan

Analysis of irbesartan and its transformation and degradation products resulted in the identification of multiple compounds that were found to be formed through various processes, whether that being in wastewater treatment systems, under stress conditions or through activity of microbes (Figure 2).

Multiple transformation products (TPs) were found when analysing surface waters from a river in Eastern Spain. The TPs were identified as isomeric compounds resulting from hydrogenation and hydroxylation (447.2508 m/z (J)) (Boix et al., 2016). One of the TPs (J) appears to correspond to a TP also identified by Shah et al. (2010) and Alexandre et al. (2004) which was formed after exposing irbesartan to basic conditions and biodegradation through microbes, respectively. Four additional TPs were also observed after biotransformation of the pharmaceutical by active sludge from a WWTP in Amsterdam. Analysing with a UPLC-QTOF mass spectrometer showed that one of the TPs (443.2192 m/z) was an isomeric compound resulting from an oxidation. The other products were formed through dealkylation (387.1933 m/z), oxidation (459.2145 m/z), and subsequent hydroxylation (475.2094 m/z) of the irbesartan molecule (Boix et al., 2016). Three TPs (444.2274 m/z (K), 442.2117 m/z (I), 446.2430 m/z (J)) were also identified by Letzel et al. (2015) in an irbesartan degradation experiment involving effluents from a lab scale wastewater treatment system. One of the TPs (446.2430 m/z (J)) was also detected in environmental samples from the Ebrach, Regnitz, and Danube river in Germany (Letzel et al., 2015).

Testing the degradation of irbesartan under various stress conditions showed degradation under basic, acidic, and photo acidic conditions. The products (447.2503 m/z (J), 252.124 m/z

(C), 427.2241 m/z (D)) were formed to a degradation extent of 51.4 % under basic, 4.5 % under acidic, and 48.7% under photo acidic conditions (Shah et al., 2010).
Microbial degradation of irbesartan was studied by Alexandre et al. (2004), who used multiple bacterial and fungal species to generate five metabolites from the parent pharmaceutical. These resulted from hydrogenation (445.2715 m/z (A), calculated for $C_{25}H_{31}O_2N_6$: 447.2508), isomeric monohydroxylation of the cyclopentane ring (445.2352 m/z (E, F)), further hydrogenation of the monohydroxylated metabolite (G, H), and possible N-glycoside conjugation (B) of the parent compound. The degradation products A, B, E, and F were also found in the urine of humans given a 900 mg-dose of irbesartan (Chando et al., 1998).

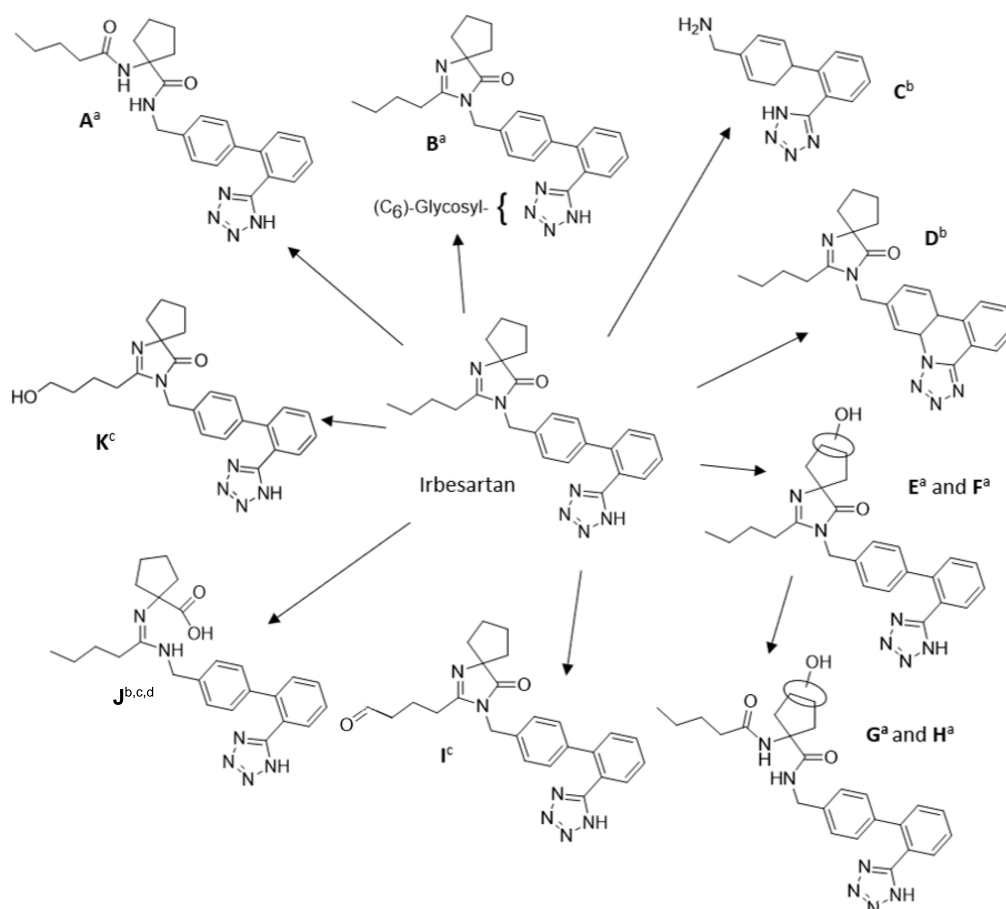


Figure 2: Known transformation products of irbesartan.
[a] (Alexandre et al., 2004), [b](Shah et al., 2010), [c](Letzel et al., 2015), [d](Boix et al., 2016)

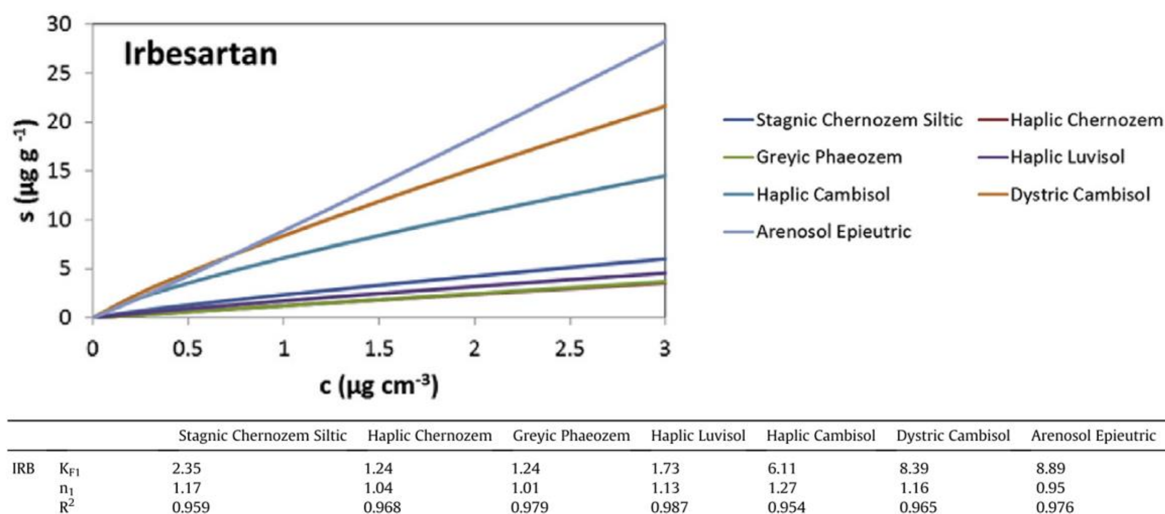
1.4.2 Concentrations of Irbesartan in WWTP Effluents and the Environment

Irbesartan is known to be relatively persistent to wastewater treatment processes and shows negative to very low removal rates of -42% to 6%. It was largely unaffected by hydraulic retention time and sludge temperature (Oosterhuis et al., 2013). Irbesartan was also one of the pharmaceuticals exhibiting negative removal (-81% to -41%) in a study assessing

concentrations of contaminants when passing through a pressure sewer pipe under anaerobic conditions. The authors attributed this to a possible reverse reaction of conjugates into the parent molecule (Jelic et al., 2015). Furthermore, only 81% of irbesartan is removed in advanced treatment techniques used for drinking water, including sand filtration, ozonation, and granulated activated carbon filtration (Huerta-Fontela et al., 2011).

Due to its persistence in wastewater treatment processes, high concentrations of irbesartan can be found in effluent wastewater (EWW) and surface water (SW). In a study by Boix et al. (2016) irbesartan was detected in 92 % of EWW and 39 % of SW samples in Eastern Spain. Significantly higher finding rates were observed in a study by Gracia-Lor et al. (2012), which identified irbesartan in 100 % of SW and EWW samples with median concentrations of 57 ng L^{-1} and 889 ng L^{-1}, respectively. Similar results were also obtained by a study of Oosterhuis et al. (2013) in surface waters in the Netherlands, which showed a recovery rate of 98 %. Irbesartan, valsartan, and caffeine were also the only three chemicals out of 44 tested that were present in all samples taken from multiple rivers in Slovenia (Klančar et al., 2018). Observation of irbesartan's TPs showed similar results with positive findings in more than 79 % of EWW samples analysed. Detection in surface waters were less frequent, however TPs were also identified in up to 22 % of the analysed samples (Boix et al., 2016). Other TPs were also detected in WWTP effluents at concentrations of up to 440 ng L^{-1} as well as in German rivers at concentrations between 50 to 90 ng L^{-1} (Letzel et al., 2015).
Removal rates of irbesartan in surface waters were lower compared to other pharmaceuticals, including ofloxacin, ibuprofen, and gemfibrozil, as only 10 % of irbesartan degraded after 65 days. A slightly better removal was shown in biotransformation studies using active sludge, where irbesartan exhibited a linear decay with approximately 25 – 30 % removal by 35 days (Boix et al., 2016). Irbesartan has the potential of accumulating in the aquatic food chain, however, only to a limited extend with no need for considerable regulation (Ong et al., 2018). Studies examining the sorption and degradation processes of irbesartan in different soil types have found that irbesartan had a relatively low sorption into loess and sediment soil, with 18 – 62 % of the compound remaining in solution (Klement et al., 2018). This is in accordance with a study by Martínez-Piernas et al. (2018), where concentrations were below 0.24 ng g^{-1} in greenhouse soil irrigated with reclaimed wastewater. Higher concentrations were observed in soilless perlite substrate, where values between 5.8 and 3.8 ng g^{-1} of irbesartan were detected (Martínez-Piernas et al., 2018).
Sorption of irbesartan in soil can be described by a Freundlich isotherm (Figure 3). Relationships between the Freundlich sorption parameters (K_F) and soil properties indicated

that sorption was highly dependent on soil pH (Klement et al., 2018). For irbesartan specifically, a positive correlation with soil hydrolytic acidity and a negative correlation with soil clay content and sorption complex saturation could be observed. This indicates that with increasing negatively charged sorption sites the negative charge of the pharmaceutical and therefore its repulsion toward the sorbent likely plays a more important role (Klement et al., 2018; Kodešová et al., 2020; Schmidtová et al., 2020).



		Stagnic Chernozem Siltic	Haplic Chernozem	Greyic Phaeozem	Haplic Luvisol	Haplic Cambisol	Dystric Cambisol	Arenosol Epieutric
IRB	K_{F1}	2.35	1.24	1.24	1.73	6.11	8.39	8.89
	n_1	1.17	1.04	1.01	1.13	1.27	1.16	0.95
	R^2	0.959	0.968	0.979	0.987	0.954	0.965	0.976

Figure 3: Freundlich sorption isotherms and the parameters K_{F1} ($cm^{3/n}$ $\mu g^{1-1/n}$ g^{-1}), n_1, and the coefficient of determination, R^2 for irbesartan and different soil types (Klement et al., 2018).

Degradation of irbesartan was shown to be approximately 41 % to 78 % depending on the soil type after 13 days and approximately 80 % after two months (Figure 4) (Frková et al., 2020). Similar results were obtained in a different study by Kodešová et al. (2020), in which irbesartan was shown to have a dissipation half-life of approximately 24 days across seven different soil types. A slightly increased half-life could be detected if irbesartan was present in combination with other pharmaceuticals including carbamazepine, sulfamethoxazole, citalopram, fexofenadine, and clindamycin. The authors attributed this to the impact of the antibiotics (sulfamethoxazole and clindamycin) applied in the pharmaceutical mixture on the soil microbial communities (Frková et al., 2020; Kodešová et al., 2020). The same study also showed that persistence of irbesartan decreased with increasing microbial biomass and total phospholipid fatty acid content (Kodešová et al., 2020).

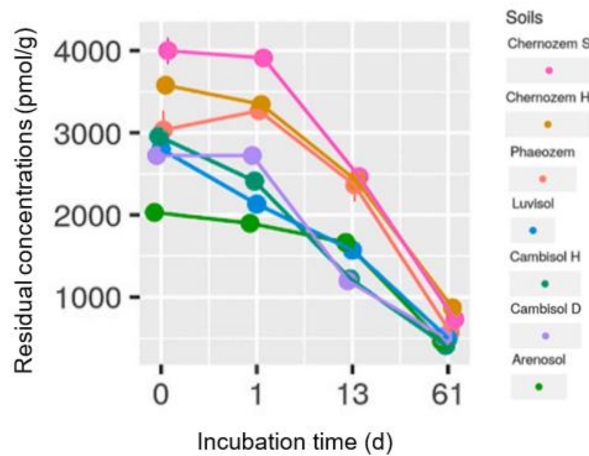


Figure 4: Residual concentrations of irbesartan in different soil types (Frková et al., 2020).

Irbesartan's influence on soil microbial communities was examined in a study by Frková et al. (2020), which showed a strong increase in biomass of bacteria and protozoa in all soil types amended with irbesartan after 13 days. Shifts in microbial community composition were reflected by changes of the ratio of bacteria to fungi and gram-positive to gram-negative bacteria. For irbesartan, a general decrease in the ratio in gram-positive to gram-negative bacteria could be observed, most pronounced in Luvisol. Apart from Stagnic Chernozem Siltic, a decrease in the ratio in bacterial to fungal cultures was also visible in all soil types, indicating pronounced reorganisation of microbial composition. The shifts were most evident on day 13 and mostly persisted in the long term (61 days) (Frková et al., 2020).

These findings indicated that contamination of the environment with irbesartan proposes several risks and environmental implications. The high stability of the molecule and the low removal rates of in wastewater treatment plants indicate that potentially high amounts of irbesartan may persist in the environment. Additionally, the low sorption of the pharmaceutical in some soils such as loess, increases its potential to translocate to surface and ground water and potentially contaminate drinking water (Klement et al., 2018). Further, as studies have indicated irbesartan concentration of up to 1 $\mu g\ g^{-1}$ have the ability to severely alter microbial growth and species composition (Frková et al., 2020) and might be able to be taken up into crops, posing potential risks for human health (Martínez-Piernas et al., 2018).

2. Aim of the Work

This bachelor's thesis aims to analyse and compare the transformation of the anti-hypertensive drug irbesartan by selected fungal and bacterial strains as well as bacterial consortia.

In detail this bachelor's thesis focuses on:

- Optimising cultivation conditions for degradation experiments using fungi and bacterial consortia
- Characterising bacterial consortia composition by next generation sequencing and 16S rRNA metagenomics analysis
- Comparing the effects of incubation period and media on the composition of the bacterial consortia over the course of the degradation experiment
- Determination and comparison of the transformation of irbesartan by pure bacterial strains, pure fungal strains, and bacterial consortia
- Identification of novel microbial transformation products of irbesartan

This work is a continuation of the work done by Anna Košinová (2019), focussing on four selected bacterial strains as well as assessing the transformation potential of fungal cultures and bacterial consortia. Further, a focus is laid on the transformation timeline and optimization of the cultivation period and growth conditions of fungal cultures and bacterial consortia.

3. Material and Methods

3.1 Media preparation

3.1.1 Solid media

M2 and R2A solid media was used for the inoculation of individual bacterial strains in petri dishes. The R2A solid agar medium contained 15.2 g L^{-1} of R2A agar (Merck KGaA, Germany) to which an additional 2.8 g L^{-1} of agar were added.

The M2 solid medium contained:

M2 Solid Medium	
Malt extract	10 g/L
Yeast extract	4 g/L
Glucose	4 g/L
Agar	20 g/L

The pH of the media was adjusted to 7.3. The media was subsequently sterilized through autoclaving and then poured into sterile petri dishes in a laminar flow box (TelStar, Biostar).

3.1.2 Minimal M9 Media

The liquid minimal M9 media were used for the transformation experiment of irbesartan involving individual bacterial strains and consortia.

The minimal M9 medium contained:

M9 Minimal Medium	
NH_4Cl	1 g/L
Na_2HOP_4 * 12 H_2O	15.137 g/L
KH_2PO_4	3 g/L
NaCl	0.5 g/L
1 M $MgSO_4$	2 mL/L

The media was prepared with distilled water and 5 M and 1 M NaOH were used to adjust the pH to 7.4. The $MgSO_4$ solution and glucose solutions were sterilized by filtering through a

0.2 µm syringe filter (VWR INTERNATIONAL, Cellulose Acetate membrane, catalog. No. 514-0061). Additionally, 20 mL L^{-1} of filtered glucose solution were added to each flask as a carbon source. Sterilization of the media was ensured through autoclaving at 120 °C for 90 min and any additions were carried out in a laminar flow box (TelStar, Biostar).

3.1.3 Liquid R2A Media

The R2A liquid media was used to pre-inoculate the consortia strains and establish initial growth, since this media showed high growth rates and degradation abilities of the inoculated bacteria in a study by Zhou et al. (2013).

The R2A liquid media contained:

R2A Medium	
Yeast extract	0.5 g/L
Proteose peptone	0.5 g/L
Glucose	0.5 g/L
Casein acid	0.5 g/L
Starch	0.5 g/L
Sodium pyruvate	0.3 g/L
K_2HPO_4	0.3 g/L
$MgSO_4$ * 7 H_2O	0.5 g/L

The pH of the R2A media was adjusted to 7.2 and the media was sterilized through autoclaving at 120°C for approximately 90 min.

3.1.4 LB Media

To identify individual bacterial strains in the consortia, the consortia was cultivated in LB media and M9 minimal media with a final concentration of 2 mM glucose as a carbon source (for composition see above).

The LB medium contained:

LB Medium	
Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	7.5 g/L

The pH was adjusted to 7.4 and 7 for the M9 minimal media and the LB media, respectively. The media was sterilized through autoclaving at 120°C for 90 min.

3.1.5 Modified NCIMB medium no. 206

For the irbesartan transformation experiments involving fungal cultures minimal media NCIMB was used.

The minimal modified NCIMB medium no. 206 contained:

Minimal Medium NCIMB no. 206	
K_2HPO_4	0.5 g/L
KH_2PO_4	0.04 g/L
NaCl	0.1 g/L
$CaCl_2 * H_2O$	0.002 g/L
$(NH_4)_2SO_4$	0.2 g/L
$MgSO_4 * 7H_2O$	0.02 g/L
$FeSO_4$	0.001 g/L

The media was prepared with distilled water and sterilized through autoclaving at 120°C for 90 min. A filtered glucose solution was added as a carbon source to the media.

3.2 Microbial Strains and Pharmaceuticals

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Bacteria and fungi were isolated from soil microcosms amended with irbesartan (S10 and S100 treatments), while bacterial consortia (7 distinct consortia) were captured from treatment S-10 only, since this concentration resembles most closely those found in the environment (Martínez-Piernas et al., 2018). To refresh microorganisms, microbial glycerol stocks (15% glycerol), stored at – 76 °C (Deep freezer Panasonic) were used.

Fungal strains were cultivated on Potato Dextrose agar (PDA; Himedia Laboratories, USA) enriched with 25 µg L^{-1} of chloramphenicol and 50 mg L^{-1} of irbesartan. Unique fungal strains were isolated and after inoculation cultivated in Potato Dextrose Broth (PDB; Himedia Laboratories, USA). After a 5-day growth period the DNA was isolated using the Nucleo Spin Soil kit (Macherey Nagel, Germany). The isolated DNA underwent PCR amplification and sequencing (SEQme, s. r. o., Czech Republic) (Lorenc et al., in prep). Identification of the

sequences was done using the BLAST Sequence Analysis Tool (Altschul et al., 1990). Out of 16 isolated and identified fungal strains, three fungal strains were selected based on their possible potential to degrade irbesartan.

The used pharmaceutical irbesartan (CAS: 138402-11-6) was obtained from TCI (Japan) with over 98% analytical grade purity. It was filtered, dissolved in pure DMSO at a concentration of 10 mg mL^{-1} and stored in the fridge.

3.3 Microbial Transformation of Irbesartan

This section is not shown completely in the shortened version.

3.3.4 Sampling Procedure

On each sampling day 2.5 mL of liquid from each sample flask were extracted and transferred through disposable filters into 2 mL sample tubes. The filters used for fungal cultures contained 0.45 µm RC membranes (Chromafil® RC-45/15 MS, Macherey-Nagel GmbH & Co. KG, Germany) and filters for bacterial cultures contained 0.2 µm RC membranes (Chromafil® RC-20/15 MS, Macherey-Nagel GmbH & Co. KG, Germany). The samples were then stored at -20°C to await further analysis through liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) at the Faculty of Fishery and Water Protection in Vodňany.

Sampling of the media from the flasks was done in a laminar flow box (TelStar, Biostar) to ensure sterility. The filtration was done in a non-sterile environment since contamination at this stage was insignificant for further analysis. The weight of each flask was taken before and after sampling, in order to measure evaporation of the liquid.

3.4 Community Composition of Bacterial Consortia

3.4.1 Inoculation of the Bacterial Consortia

This section is not shown in the shortened version.

3.4.2 DNA Extraction and Isolation

For extraction of the genomic DNA from microorganisms, NucleoSpin® Microbial DNA kit was used (Macherey-Nagel GmbH & Co. KG, Germany). The procedure was performed according to the protocol for bacterial DNA isolation. Cells were harvested from 1 mL of the bacterial culture and subsequently resuspended in 100 µL elution buffer. The cell suspension

was then transferred into a NuceloSpin® Bead Tube Type B and MG buffer and Proteinase K (14 -22 mg mL^{-1}) were added. The tube was then agitated on a swing mill (Vortex Genie and adaptor for 2 mL tubes, Scientific Industries, USA) for approximately 12 min. After adding an additional 600 µL of MG buffer and centrifuging, the supernatant was transferred into a DNA spin column with a collection tube. The collection tube was centrifuged at 11 000 RPM and the flowthrough was discarded. The membrane was then washed in multiple steps using buffer BW and B5 and centrifuged after each washing step at 11 000 RPM for 30 sec. Lastly, the purified DNA was collected twice in 100 µL elution buffer. The isolated DNA was then stored at -20°C. The quality and quantity of DNA (A_{260}, A_{280}, A_{260}/A_{280}) was measured using NanoDrop (Thermo Fisher Scientific).

3.4.3 16S rDNA Amplification by PCR

The genomic DNA was diluted in the ratio 1:80 to prevent PCR inhibition and underwent amplification through Polymerase Chain Reaction (PCR). The procedure was performed according to the protocol for 16S Illumina Amplicon Protocol by the Earth Microbiome Project (Caporaso et al., 2018). The PCR reaction mixture consisted of 1 µL of isolated genomic DNA, 24 µl of MasterMix and a final concentration of 0.3 µM each of 16S rRNA forward and reverse primers. The MasterMix contained a final concentration of 1x Fast Start PCR Master (Roche Diagnostics, Switzerland), 0.6 mg mL^{-1} bovine serum albumin (BSA), and 9.3 µL of MilliQ water. The forward primer used was 515F_mod_CS1 (5' - GTGCCAGCMGCCGCGGTAA - 3') and the reverse primer used was 806R_mod_CS2 (5' - GGACTACHVGGGTWTCTAAT - 3'), both carrying adapters (CS1 and CS2) for ligation of sample-specific barcodes during the library preparation step. The primers bind to complementary sequences in the 16S rRNA gene, which is used as a phylogenetic marker and can therefore identify individual bacterial taxa (Caporaso et al., 2011).

The PCR started with an initial denaturation at 95°C for 5 min, followed by 28 cycles of a denaturation stage at 94°C for 45 sec, annealing stage at 50 °C for 45 sec, and an extension stage at 72°C for 45 sec. The final extension step was carried out at 72°C for 10 min.

The yield of the PCR product was then visualized using horizontal gel electrophoresis in 2 % agarose gel. The gels were loaded with 5 µL DNA and 1 µL loading dye (Thermo Fisher Scientific, USA) and run at 110 V for 45 min. The gel was then stained using ethidium bromide and documented using UV light in an Azure c280 (Azure Biosystems).

3.4.4 Sequencing Capture, Processing, and Analysis

The library preparation, pooling, cleaning, and pair-end sequencing was performed on an MiSeq Illumina instrument in DNA Sequence facility at the Research Resources Center at the University of Illinois in Chicago, Massachusetts, USA.

The DADA2 pipeline in R (Callahan et al., 2016) was used for sequence processing. Good quality profiles were obtained for forward and reverse reads, and the reads were trimmed at 130 bp. After filtering the sequences were subject to DADA2's error rate algorithm and plotting of possible base transitions as a function of quality indicated good correlation of the observed error rates to the expected error rates under the nominal definition of the Q-value. After core sequence-variant interference was carried out, which tested whether individual sequences with given error rates are too abundant to be explained by sequencing errors, the paired end reads were merged if they had at least a 20-nucleotide overlap. Needlemann-Wunsch global alignment of each sequence was performed to check and remove bimeric chimeras (Callahan et al., 2016). Taxonomy of obtained amplicon sequences variants (ASV) was assigned using the SILVA species assignment package (version 132, Callahan, 2018).

3.5 Correction of Irbesartan Concentration for Evaporation Loss

Since evaporation of the media resulted in higher measured concentrations of irbesartan through LC in some cases, corrections for the irbesartan concentration were necessary. Evaporation was measured through weighing each flask before and after each sampling, as well as on day 0 and the empty flask (exact weight losses through evaporation can be seen in the Appendix). In case day 0 was not measured (bacteria and consortia biodegradation experiments) the values of day 0 were inferred using a line of best fit from values of the first three days as these most closely represented evaporation conditions on day 0.

The normalized concentration of irbesartan considering the losses through evaporation was calculated according to an approach also used by Gauthier et al. (2010).

The used equations were:

$$C_{evap} = \frac{C(t) * V(t)}{V(t_0)} \tag{1}$$

$$V(t) = V(t_0) + \sum_0^t V_{evap}(t) \tag{2}$$

$$V(t_0) = \frac{M(t_0) - M_{empty}}{\rho} \tag{3}$$

$$V_{evap}(t) = \frac{M_{after}(t-1) - M_{before}(t)}{\rho} \quad (4)$$

C_{evap}	Concentration of irbesartan corrected for evaporation loss, defined by (1)
$C(t)$	Concentration of irbesartan measured by LC-HRMS
$V(t)$	Volume in the flask at day t, defined by (2)
$V(t_0)$	Volume in the flask at day 0, defined by (3)
$M(t_0)$	Mass of the flask at day 0
M_{empty}	Mass of the empty flask
$M_{before}(t)$	Mass of the flask at day t before sampling
$M_{after}(t)$	Mass of the flask at day t after sampling
$V_{evap}(t)$	Volume of evaporation, defined by (4)
ρ	Density (assumed to be 1 g mL^{-1}, since most of the media was water)

3.6 Statistical Analysis

One-way ANOVA at a significance level of $p < 0.05$ was used to assess whether the irbesartan concentration differed between the abiotic control and different microorganisms in a particular experiment. Additionally, pairwise comparison was performed using Post-Hoc Tukey's test. Normality and homogeneity of variances of irbesartan concentration was tested using Bartlett's, Fligner-Killeen's, and Leven's test. If nonparametric data was indicated the Kruskal-Wallis test ($\alpha = 0.05$) was used, followed by the Dunn's test for pairwise comparison (Dinno, 2015). When using the Dunn's test the p-values were adjusted according to Holm's adjustment (Holm, 1979). To check for outliers in the evaporation data Rosner's and Grubb's Test for Outliers were used. All statistical procedures were done using RStudio (https://rstudio.com/) or Origin Pro (https://www.originlab.com/).

Statistical analysis of the metagenomic data of the bacterial consortia was analysed using Primer-e 7 software (https://www.primer-e.com/). Standardisation by total and a fourth root transformation of the ASV abundance data was carried out and homogeneity of dispersion was tested using Leven's test. A dissimilarity matrix using Bray-Curtis similarity was used as a basis for non-metric multidimensional scaling (NMDS) analysis. NMDS plotting and further analysis was done in RStudio using the metaMDS (Faith et al., 1987). The similarity percentage function (SIMPER) was used to assess primary responsibility of taxa on the observed dissimilarity between consortia composition (Warton et al., 2012). Three-way

permutational multivariate analysis of variance (PERMANOVA) testing with a significance level of $p < 0.05$ was used to determine differences between consortia and treatment groups. The bacterial consortia were treated as a random variable since repetition of the experiments with the same bacterial consortia cannot be ensured. Additionally, T-Tests with a significance level of $p < 0.05$ were carried out to examine differences within groups.

4. Results

The results are not shown in the shortened version.

5. Discussion

5.1 Irbesartan Transformation Experiments

The decrease in residual irbesartan concentration in the abiotic control samples can possibly be explained through physicochemical degradation processes, especially caused through photolysis, oxidation, hydrolysis, and thermal stress (Shah et al., 2010). Under aqueous conditions at 25°C and a pH of 7.4, which corresponds approximately to temperature and pH value of the M9 medium throughout the biotransformation experiment, the half-life of decomposition of irbesartan was determined to be approximately 28 h (Mbah, 2004).

However, the transformation experiments indicated that even though irbesartan is subject to physicochemical degradation in abiotic conditions, inoculation with some microbial species significantly decreased irbesartan concentrations, indicating their potential to transform and degrade the pharmaceutical.

The results obtained in this thesis indicate that the selected bacterial and fungal species are able to grow in soils contaminated with irbesartan. These results could be useful for further studies analysing the degradation of pharmaceutical compounds found in soil and might be applicable for the specific degradation and removal of irbesartan from contaminated soil or in wastewater treatment systems in biomanipulation experiments. Further research is needed to assess the role of bacterial consortia in the transformation process of irbesartan, as well as determine the possible effects the pharmaceutical has on the composition and abundance of the bacterial species found in the consortia. Additionally, confirmation of the degradation potential of the selected microorganisms in soil environment should be carried out. Moreover, further experiments are needed to determine the exact structure and position of functional groups in the newly detected transformation products of irbesartan as well as assess their toxicity and potential effects on microbial communities.

The remaining discussion is not shown in the shortened version.

6. Conclusion

In order to characterize the removal and transformation processes of the pharmaceutical irbesartan by microbial species, the co-metabolic transformation potential of four selected bacterial strains and seven different bacterial consortia was studied through inoculation of the microorganisms in liquid cultivation media amended with 10 µg mL^{-1} of irbesartan. Irbesartan concentrations and transformation products were monitored over a period of 10 or 21 days using LC-HRMS.

The remaining conclusion is not shown in the shortened version.

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