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Methodology

Monitoring of Selected Persistent Organic Pollutants and Personal Care Products in the Sewage Sludge and their Bioremediation

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1. LITRATURE REVIEW

1.1. Sewage Sludge

1.1.1. What Is Sewage Sludge?

Sewage sludge defined as the solid or semi-solid waste material produced from waste water treatment plants (Wiśniowska et al. 2019; Fijalkowski et al. 2017). Sludge, which constitutes 1 % of wastewater entering the waste water plant, is digested anaerobically and dehydrated. Sewage sludge, at the outlet after mechanical drying, is made of approximately 80 % moisture and 20 % dry matter (Bonfiglioli et al., 2014).

Sewage sludge may substitute inorganic fertilizers because is rich in plant nutrients. However, the presence of potentially contaminants in sewage sludge often restricts it`s uses (Singh et al., 2012).

1.1.2. Properties and nutrient composition of sewage sludge

The properties of sewage sludge play an important part in their use for land application. It can be sorted into three categories: physical, chemical, and biological (Epstein, 2002). They are generally composed of organic compounds, macronutrients, a wide range of micronutrients, nonessential trace metals, organic pollutants, and microorganisms (Singh and Agrawal, 2008).

Table 1: Comparison of some basic agrochemical characteristics of sewage sludge from some different countries.

Properties	USA $^{1,8\&10}$	Czech Republic $^{2,7,9\&11}$	Spain ^{3}	India $^{4\&6}$	Thailand ^{5}
pH	5.6	7.1	8.6	7.1	6.82
Total organic matter %	50	62.7	43.4	23.2	19.8
Dry matter content %	--	24	--	--	20.5
Organic carbon %	23.2	25.4	--	25.8	--
Total nitrogen %	3.85	2.58	2.50	3.29	3.43
Total phosphorus %	0.59	2.66	1.06	1.23	--
Total potassium %	0.08	0.37	--	2.98	0.09

1) Hue and Ranjith (1994), 2) Hartman and Pohořelý (2006), 3) Fernando et al. (2002), 4) Solanki et al. (2016), 5) Parkpain et al. (1998), 6) Singh and Agrawal, (2008), 7) Mercl et al. (2018), 8) Lu et al. (2012), 9) Kolář et al., (2008) 10) Artiola and Pepper, (1992), 11) Adamcová et al. (2016), 12) (Pasda et al. 2006)

As described by Logan and Harrison (1995) Dewatered sewage sludge have a physical property of bulk density 0.59 mg/m^3, an average solids content of 62 %, particle density of 1.96 mg/m^3 and total porosity of 70 %. As well available water content was 27 % by volume (60 cm water tension, 1.5 MPa), which is good for crop production and improving physical property of the soil.

1.1.3. Widespread substances in sewage sludge

There are several papers focusing on substances which are serious threats to human health and ecosystem occurring in sewage sludge both chemicals (polycyclic aromatic hydrocarbons (PAHs), hydrocarbons; polychlorinated biphenyl (PCBs), perfluorinated surfactants (PFCs), personal care products (PCPs), pharmaceuticals (PhCs), benzotriazoles) and biologicals (*Legionella sp., Yersinia sp., Escherichia coli* sp.) (Fijalkowski et al. 2017).

A set of 45 samples of the sludge from wastewater factories in the Czech Republic found a sewage sludge contamination by POPs. Among all the determined substances only the value of the sum of polycyclic aromatic hydrocarbons (PAHs) content exceeded the threshold value given by the proposed EU directive (Vácha et al. 2005).

PCPs are also found in all sludge samples a study by Lee and Peart, with a concentration range from 7.1 to 239 ng/g on the dry weight basis (Lee and Peart 2002).

1.1.4. Possible Use of Sewage Sludge

Application of sewage sludge or compost blended with sludge to soil used for crop production is of great importance with regard to the supply of organic matter and nutrients, especially nitrogen and phosphorus (Mantovi et al., 2005). The higher availability of organic matter and nutrients also increases the activity of soil enzymes, soil microbial activity and soil microbial biomass growth (Singh and Agrawal, 2008).

Some studies showed that anaerobically digested biosolids applied to a silt loam soil at 11 rates ranging from 0 to 300 Mg ha^{-1} increased porosity, moisture retention, percentage of water-stable aggregates, and decreased bulk density significantly in the surface soils (0–15 cm) with biosolids application (Lu et al. 2012). Sludge addition improved consistently TOC, N, P and K content up to soils treated with 120 t ha -1year^{-1}(Hamdi et al. 2019).

1.2. Sewage Sludge Contamination

Wastewater sludge is a complex heterogeneous mixture of micro-organisms, undigested organics as cellulose, plant residues, oils, or fecal material, inorganic material, sand is a resource of organic matter, nitrogen, phosphorous, micronutrients and even heavy metals, bio-fuel, hydrogen, syngas, bio-oil, biodiesel, bio-plastics, bio-pesticides, proteins, enzymes, bio-fertilizers or volatile-acids (Fijalkowski et al. 2017) .

Contaminants of sewage sludge from both chemicals organic pollutants likes of (polyaromatic hydrocarbons (PAH), hydrocarbons; polychlorinated biphenyls (PCB), Perfluorinated Surfactants (PFCs), Personal Care Products (PCPs), and biologicals (pathogens such as bacteria, virus, fungui and parasites (Fijalkowski et al. 2017).

Research has shown that sewage sludge is a very efficient sorbent for all lipophilic contaminants that might be transported into the sewage system (Lidia et al. 2001).

1.2.1. Persistent Organic Pollutants

1.2.1.1. What are Persistent Organic Pollutants?

Persistent organic pollutants (POPs) are toxic organic compounds that are resistant to most of the degradation processes in the environment, and therefore they tend to persist in the environment, thus bioaccumulating in organisms and biomagnifying along the food chains and food webs in ecosystems (Zacharia 2019).

POP exposure may cause developmental defects, chronic illnesses, and death. Some are carcinogens. Many POPs are capable of endocrine disruptors within the reproductive system, the central nervous system, or the immune system (Zacharia, 2019).

Persistent organic pollutants (POPs) include but are not limited to: polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and difurans (PCDD/Fs), organochlorine pesticides (OCPs) and flame retardants (Karlaganis et al., 2001 ; Navarro et al. 2016).

1.2.1.2. Sources and Occurrence of Persistent Organic Pollutants

There are many ways that release POPs to the environment. POPs such as pesticides are released, as a result of plant protection treatment. Some other chemicals polychlorinated biphenyls (PCBs) have been used as oils, as dielectric and cooling fluids in capacitors and transformers, for wood preservation, etc., and are released into the environment as a result of spills and evaporation. A number of substances like dioxins/furans, polycyclic aromatic hydrocarbons (PAHs), and hexachlorobenzene are by-products of many industrial processes, mainly, thermal (fuel combustion and waste incineration, ferrous industry, coke and aluminum production, road transport, chemical synthesis of chlorinated substances, etc.) and are emitted directly into the air (Zacharia, 2019). PCBs are among the most widespread and recalcitrant contaminants and are present in abiotic and biotic environments throughout the world (Singh, 2006).

Additionally, some natural sources such as volcanic activities and vegetation fires are also responsible for releasing these pollutants into our ecosystem (El-Shahawi et al., 2010). Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment, especially in atmospheric particles, soils, sediments, and sewage sludges (Wikström et al., 1999).

1.2.1.3. Properties of Persistent Organic Pollutants

Persistent organic pollutants (POPs) can bioaccumulate, bioconcentrate and biomagnify because they are hydrophobic, persistent, toxic and have affinity for fat (Corsolini et al., 2005). POPs are mostly highly lipid soluble with semi-volatilities properties. Halogenated POPs are more resistance to the degradation reactions and these pollutants have abilities to associate with aerosols and can be transported from one place to another (Aceves and Grimalt, 1993; Kaupp and McLachlan, 2000).

Hydrophobic POPs have high lipid solubility (in which don`t dissolve readily in water and tend to lodge in fatty tissues and pass up the food chain in animal fat), which are responsible for their favorable bioaccumulation and longtime persistence (Darbre 2015;Wania and Mackay, 1996 ; Scheringer, 2009).

1.2.1.4. Concerns of Persistent Organic Pollutants

Persistent organic pollutants (POPs) bio-accumulate into food chain (animals and human beings) through oral, inhalation and dermal (ingestion and contact) ; leading to various notorious health hazards and environmental effects (Alharbi et al., 2018). Almost everyone has POPs in his/her body. The exposure of these pollutants creates various serious health problems such as hormone disruption, cancer, cardiovascular diseases, obesity, reproductive and neurological ailments, learning disabilities and diabetes. Besides, these pollutants develop defects in women embryo too (Scheringer, 2009).

1.2.2. Personal Care Products

1.2.2.1. What are Personal Care Products?

Personal care products (PCPs) are biologically active compounds that includes a large number of synthetic chemicals used in everyday products such as soaps, lotions, toothpaste, fragrances, cosmetics and sunscreens (Brausch and Rand, 2011; Comerton et al., 2009). Among the main PCPs triclosan and triclocarban antimicrobial compounds used in soap, toothpaste, and other consumer products. Triclosan is also used as a biocide in sportswear, footwear, carpets, plastic toys, and kitchenware (Canosa et al., 2007). Another group of PCPs are UV filters and parabens (Peck 2006).

Synthetic musks (SMs), known as a class of synthetic fragrance additives, are widely used in a number of personal care and household products (e.g. skin care lotions, fabric softeners, perfumes, shampoos, and detergents) (Reiner et al., 2007). As economical substitutes to natural counterparts, SMs can be classified into four main groups, namely nitro, polycyclic, macro cyclic and alicyclic musks (Homem et al., 2015).

1.2.2.2. Sources and Occurrence of Personal Care Products

Typical sources and pathways of personal care products to wastewater treatment plants are industrial wastes, hospital wastes and domestic wastes coming through sewerage system. After treatment wastewater treatment plants also release these contaminants to the Environment (ground water, soil zone and surface water) as wastewater effluent and sewage sludge (Ravi et al., 2016).

Personal care products (PCPs) are released to the environment mainly from anthropogenic sources (Montes et al, 2017). So, this PCPs enter into the environment from different sources such as: (i) Effluents from wastewater treatment plants; (ii) Leakage form septic tanks or landfill sites; (iii) Surface water run-off; (iv) Direct discharge into waters (Caliman and Gavrilescu, 2009). As the global human population and percentage of the population living in high-density urban areas continue to increase, PCPs contamination of ecosystems is expected to increase substantially (Richmond et al. 2017).

1.2.2.3. Properties of Personal Care Products

Parabens have a potential of bio-accumulation in the skin, especially after a number of applications. They are white, odorless, fine crystalline substances which are effective preservatives that work well in a wide pH-range from 4.5 - 7.5. (Garner et al 2014). Unlike wastewater, paraben levels in sludge remain stable over time (Hussein et al. 2007; Haman et al. 2015). In certain species UV filters have a strong tendency towards bioaccumulation in their fatty acids. UV filters are stable toward bio-and photo degradation. (Juliano et al. 2017). Some UV filters show lipophilic characteristics, and are insoluble in water (Giokas et al. 2007; Durand et al. 2009). Triclosan also have a potential to bioaccumulate in higher organisms (DeLorenzo et al. 2008).

1.2.2.4. Concerns of Personal Care Products

Personal care products (PCPs) have raised significant concerns in recent years for their persistent input and potential threat to ecological environment and human health (Liu and Wong, 2013). A large number of PCPs are bioactive compounds which can directly affect non target organisms. Chronic effect of PCPs exposure to these non-target terrestrial and aquatic organisms can induce biological changes over long periods of time, sometimes for an entire community of organisms over several generations (Dey et al. 2019).

Triclosan have impacts on soil macro-organisms, especially earthworms (Eisenia foetida), are of particular interest because earthworms live in soil, and most of their diet consists of soil (Pannu et al 2012).

While endocrine disruptors of PCPs likes of parabens inhibit the activity of naturally occurring hormones, others might induce unprecedented physiological changes like damage to reproduction mechanism (Dey et al., 2019; Kabir et al 2015).

1.2.3. Behavior of Contaminants During Composting

Because of their physical and chemical properties, POPs can be either degraded through the microbial activity that develops during composting or be sorbed onto waste organic matter (OM) (Lashermes et al., 2010).

Several studies have reported the dissipation of POPs and PCPs during composting, including that of polycyclic aromatic hydrocarbons (PAHs) (Košnář et al., 2019), pesticides (Vischetti et al., 2008), sodium linear alkylbenzene sulfonate (LAS) and 4-n-nonylphenol (NP) (Pakou et al., 2009) and personal care products likes of diclofenac, metformin and triclosan (Haiba and Nei 2018).

The PCBs in contaminated soil had composted and resulted up to a 40% loss of PCBs (Michel et al., 2001). As Košnář and co. also stated higher PAH removal was observed, up to 84.5% at the end of composting during their 240 days experiment (Košnář et al. 2019).

1.2.4. Adsorption of Contaminants by Plants

The pathway by which organic pollutants enter the vegetation is a function of chemical and physical properties of each pollutant, such as hydrophobicity, water solubility, and vapour pressure, as well as environmental conditions, such as temperature, organic content of the soil, and plant species (Hellström 2004).

Transport in plants takes place in the two-vessel systems xylem and phloem. In the xylem fibrous cells provide support, parenchyma cells storage area for starch and fat, and in the main cells, tracheary elements are responsible for most of the water transport in the plant (McFarlane 1995).

Adsorption of naphthalene by roots of two plant species (tall fescue, *Festuca arundinacea* Schreber. and alfa-alfa, *Medicago sativa* L.) with different lipid content has been quantified in a greenhouse experiment indicating that lipid content is a controlling factor in adsorption of naphthalene onto plant roots (Schwab et al., 1998).

Kacálková and Tlustoš (2011) also tested plants which were planted on a contaminated field site, as a result the highest phenanthrene concentration was found in above ground biomass of sunflower and the highest concentration of pyrene, in maize roots.

1.3. Removal of Contaminants From Sewage Sludge Through Bioremediation

1.3.1. What is Bioremediation?

The term "bioremediation" refers to all biochemical reactions starting with natural attenuation including all biotic and abiotic processes used to minimize contaminant levels. This practice uses various biological agents such as bacteria, algae, yeast, fungi, and enzymes to degrade environmental pollutants, as well higher plants up to some extent. Bioremediation is considered as one of the safe, clean, cost-effective, and eco-friendly technologies for decontaminating sites which are contaminated with varied range of pollutants (Yadav et al., 2019).

1.3.2. Types of Bioremediation

Bioremediation approaches are generally classified as *in situ* or *ex situ*. In which, *in situ* bioremediation involves treating the polluted material at the site while *ex situ* involves the removal of the polluted material to be treated elsewhere (Megharaj et al., 2011). Bioremediation can occur naturally on its own (natural attenuation or intrinsic bioremediation) or can be spurred via addition of amendments s for the enhancement of bioavailability of contaminants within the medium (biostimulation). There are different types of techniques under bioremediation processes such as biostimulation, bioattenuation, bioaugmentation, bioventing, bioleaching, bioreactor, composting (Yadav et al., 2019). The suitability of a particular bioremediation technology is determined by several factors, such as site conditions, indigenous population of microorganism, and the type, quantity and toxicity of pollutant chemical species present (Megharaj et al., 2011).

Composting is a biodegradation process of a mixture of substrates carried out by a microbial community composed of various populations in aerobic conditions in the solid state (Insam and de Bertoldi, 2007). As well, vermicomposting is a biotechnological process in which organic waste is converted into nutrient-rich vermicompost by using earthworms (Ali et al., 2015).

Phytoremediation is the use of vegetation for *in situ* treatment of contaminated soils, sediments, and water (Dietz and Schnoor, 2001). Generally, phytoremediation of contaminants by a plant involves the following steps: uptake, translocation, transformation, compartmentalization, and sometimes mineralization. Factors affecting the uptake, distribution and transformation of organic compounds by a plant are mainly related to the physical and chemical properties of the compound (e.g. water solubility, molecular weight, octanol-water partition coefficient), as well as environmental conditions (e.g. temperature, pH, organic matter, and soil moisture content) and plant characteristics (e.g. root system, enzymes) (Truu et al. 2015).

1.3.3. Advantages and Limitations of Bioremediation

Advantages by Alvarez and Illman (2005) Iosob et al. (2016) and (Azubuike et al. 2016).

- ✓ Cleanup occurs *in situ*, which eliminates hazardous waste transportation and liability costs.
- ✓ Organic hazardous wastes can be destroyed (e.g., converted to H_2O, CO_2, and mineral salts) rather than transferred from one phase to another, thus eliminating long-term liability.
- ✓ Relies on natural biodegradation processes that can be faster and cheaper.
- ✓ Can be made directly on the site and ecosystem disruption is minimal
- ✓ Minimum land and environmental disturbance.
- ✓ Treating polluted substances at the site of pollution
- ✓ Does not generate waste
- ✓ Are self-sustaining
- ✓ Environmentally sound with public acceptance. Can be used in conjunction with (or as a follow up to) other treatment technologies.
- ✓ May be combined with other technologies
- ✓ It is accompanied by little or no disturbance to soil structure

Disadvantages by Alvarez and Illman (2005) and (Iosob et al. 2016).

- ✓ Certain wastes are not eliminated by biological processes.
- ✓ Is not going well on clay soils and compacted soils
- ✓ It may require extensive monitoring.
- ✓ Requirements for success and removal efficiency may vary considerably from one site to another.
- ✓ Some contaminants can be present at high concentrations that inhibit microorganism growth.
- ✓ Process of bioremediation lasts much longer than other treatments
- ✓ There is a risk for accumulation of toxic biodegradation products.
- ✓ greater efforts are needed in education and training to win public acceptance for its monitored natural attunation

2. HYPOTHESES

1) Sewage sludge from wastewater treatment plant can accumulate a high amount of persistent organic pollutants and personal care products.

2) Composting and vermicomposting can stabilize and remediate and persistent organic pollutants and personal care products from sewage sludge.

3) Ligninolytic-fungi can increase the biodegradation efficiency of contaminated sewage sludge with composting and vermicomposting.

4) Plants can have the ability to extract, accumulate, immobilize and degrade organic contaminants from sewage sludge amended soils.

3. OBJECTIVES

1. Monitoring of selected persistent organic pollutants and personal care products in sewage sludge from wastewater treatment plant.
2. Bioremediation of selected persistent organic pollutants and personal care products from sewage sludge using composting and vermicomposting.
3. Composting and vermicomposting of sewage sludge using a ligninolytic fungi to enhance the biodegradation of selected persistent organic pollutants and personal care products.
4. Phytoremediation of selected persistent organic pollutants and personal care products in sewage sludge amended soils.

4. METHODOLOGY

During the start of the study prior to establishment of experiments, the sewage sludge will be collected from waste water treatment plants in Czech Republic. At least 20 sludge samples will be sampled during a year long. The selected wastewater factories will be situated in areas of district towns, towns with an increased percentage of industry and in the areas of middle and small settlements. At least 0.3 m^3 sludge is needed for the experiment. Homogenized and dried sewage sludge samples will be characterized by the main physico-chemical properties and further examined for selected persistent organic pollutants (POPs) and personal care products (PCPs). The extraction of POPs and PCPs will be done with an ultrasonic bath. Compound determination and quantification will be performed using GC/MS/MS or HPLC/MS/MS. Laboratory experiment will be conducted to evaluate the biodegradation of organic pollutants through the composting and vermicomposting of sewage sludge solely and also in the mixture of substrates containing selected ligninolytic fungi. The long-term composting and vermicomposting will be done in laboratory scale. The composting will be done 80L plastic open top drum laboratory fermenters, in which the composting will be a mixture of sewage sludge, pellets (straw), fresh grass, or livestock manure. The resulted compost and vermicompost will be used in further phytoremediation experiments with six treatments and four replications. Pot experiments with plants will be conducted to evaluate the ability of plants to extract, accumulate and immobilize/degrade pollutants from soil amended by contaminated raw, composted and vermincomposted sewage sludge. Size of the pot will be 6 L polypropylene pots: with open top, 21 cm; base, 18 cm; height, 20 cm. The plant will be maize, in which to be planted just three seedlings per pot. The influence of substrates with ligninolytic fungi to increase the biodegradation efficiency of contaminated sewage sludge in soil will be also studied. The soil will be collected from long term trial sites. The selected POPs and PCPs will be measured in the sewage sludge amended soil during the phytoremediation as well in shoots and roots of plants. The studied bioremediation approaches will be compared by the removal of POPs and PCPs in relation to the selected enzymatic activities, ergosterol content and microbial community's dynamics during the experiments.

4.1. Analytical procedures

I. Extraction of POPs

The extraction of POPs in samples will be done with the ultrasonic extraction (US EPA 2007). A dry and homogenized sample will be weighed into a flask and *n*-hexane/acetone extraction mixture, and surrogate solutions will be added into each flask. The samples will be extracted with the ultrasonic extraction system. The samples will be ultra-sonicated for 30 min and repeated two times. Extracts will be cleaned with silica cartridges and concentrated to 1.0 ml. The internal solutions will be added to each extract before further analysis. Blanks will be prepared following the same procedure without adding the sample. Calibration curve will be at 10-1000 ng/ml. The extracts will be analyzed using gas chromatography equipped with mass spectrometry detection (GC/MS/MS) (Košnář et al., 2018).

II. Analysis of POPs Using GC-MS Analysis

Analysis of POPs will be done according to US EPA Method 3550C (2015). Based on US EPA Method (8270D, 2014) the analysis of POPs will be carried out on an Agilent GC–MS/MS. The separation of POPs will be carried out using a GC/MS capillary column. Pure helium (6.0, Linde, Czech Republic) will be used as the carrier gas at a constant ramped flow rate of 1.0 mL/min. The extracts will be injected under the pulsed split less condition mode (1 μL, 1 min, purge flow 70 mL/min at 0.75 min), pulsed pressure 25.0 PSI and keep at an initial temperature of 300 °C. The temperature of mass selective detector will be 300 °C and the quadrupole will be at 180 °C as was described by Košnář et al. (2018).

III. Extraction Procedures for PCPs

After spiking 1 g of sewage sludge dry matter with deuterated PCPs, 20 mL ethanol and about 30 mg diethyl ammonium diethyl-dithiocarbamate as complexing agent will be added. Extraction will be carried out on a rotation-shaker for 150 min followed by ultrasonic bath extraction for 3 min. Twenty millilitres of sodiumacetate buffer (pH = 3.4) and 20 mL n-hexane will be added. The mixture will be extracted again for 150 min with a rotation-shaker, followed by ultrasonic bath extraction for 3 min. After centrifugation at 3000 rpm the hexane layer will be separated. The extraction will be repeated with 5 mL n-hexane. The combined n-hexane extracts will be concentrated under a gentle stream of nitrogen to 5 mL. The extract will be applied to an aluminium oxide clean up column. The elution will be done with 35 mL n-hexane/ethyl acetate (90/10). The eluate will be concentrated and isotope labelled compounds as injection standard will be added and the extract will be filled up to a final volume of 1 mL (Clara et al., 2011) .

IV. Analysis of PCPs Using GC/MS

The GC–MS analysis (Agilent GC/MS/MS System) will be carried out on a GC/MS capillary column (30 m _ 0.25 mm id, 0.25 lm film thickness) using the selected ion monitoring mode. Following temperature program will be employed: temperature increase from 60 to 120 °C with a temperature gradient of 6 °C min^{-1}, holding 120 °C for 1 min, further temperature increase from 120 to 330 °C with a temperature gradient of 10 °C min^{-1} and held for 3 min at 330 °C. Injection temperature will be at 260 °C and transfer line temperature will be set to 330 °C, source temperature to 255 °C. A 1 μL aliquot will be injected splitless into the GC–MS/MS system. Basic validation will be done. Limits of quantification (LOQ) as well as limits of detection (LOD) will be calculated and will be dependent on sample matrix and volume, blank contamination as well as recovery ratios of the added deuterated surrogate standard. LOD and LOQ will be calculated by multiplying those basic validation values with the enrichment factors and the recoveries of the analytes (Clara et al. 2011).

5. WORK PLAN

		2019	2020				2021				2022				2023				Remarks
			Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	
1	Submission and approval of methodology	▨																	
2	Experiment 1		▨	▨	▨														
3	Publication of first article										▨	▨							
4	Experiment 2					▨	▨	▨											
5	Publication of second article												▨	▨					
6	Experiment 3								▨	▨	▨								
7	Publication of third article														▨	▨			
8	Experiment 4											▨	▨	▨					
9	Publication of fourth article																▨	▨	
10	State Doctoral Examination												▨						
11	Deadline of dissertation																	▨	

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