Charles University, Faculty of Science
Institute for Environmental Studies

Doctoral study programme: Environmental Science

Summary of the Doctoral Thesis

Endocrine-disrupting properties and degradability of micropollutants with antimicrobial activity

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Prague, 2021

The dissertation thesis was funded by the Czech Science Foundation, grant numbers 17-15678Y and 20-14318Y.

The dissertation thesis was conducted in cooperation with the Institute of Microbiology of the Czech Academy of Sciences

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The presented work is based on the following publications:

Publication 1

Linhartová L, Costet N, Pakdel F, Cajthaml T, Habauzit D. Key parameter optimization and multivariable linear model evaluation of the *in vitro* estrogenic activity bioassay in T47D cell lines (CXCL-test). Cell Biology and Toxicology (submitted)

Publication 2

Michalíková K, Linhartová L, Ezechiáš M, Cajthaml T (2019). Assessment of agonistic and antagonistic properties of widely used oral care antimicrobial substances toward steroid estrogenic and androgenic receptors. Chemosphere 217: 534–541

Publication 3

Linhartová L, Michalíková K, Šrédlová K, Cajthaml T (2020). Biodegradability of dental care antimicrobial agents chlorhexidine and octenidine by ligninolytic fungi. Molecules 25: 400

Publication 4

Maryšková M, Linhartová L, Novotný V, Rysová M, Cajthaml T, Ševců A (2021). Laccase and horseradish peroxidase for green treatment of phenolic micropollutants in real drinking water and wastewater. Environmental Science and Pollution Research 28: 31566–31574

Abstract

The increasing pollution caused by compounds of anthropogenic origin can lead to harmful effects on human health and the environment. The majority of produced chemicals are continuously released into the environment, and restrictions are usually not employed until negative effects have already manifested. Recently, micropollutants have been given a lot of attention among researchers. These compounds are present in the environment at very low concentrations (ng–µg/l) and are transported over the globe through the hydrosphere. Micropollutants, including pesticides, pharmaceuticals, and personal care products, have a tendency to persist in the environment. Moreover, even trace concentrations of the compounds can have severe detrimental impacts. Many micropollutants can interfere with the natural functions of the endocrine system, which can result in the development of several types of cancer, decrease in fertility, or delayed puberty. The presence of endocrine disruptors in nature can eventually lead to the collapse of populations.

This thesis focuses on the study of the endocrine-disrupting effects and degradability of antimicrobial compounds, which are, besides other applications, widely used in oral care products. The emissions of these compounds are unregulated. The results demonstrated that none of nine tested compounds acted as an agonist of the estrogen and androgen receptors; nevertheless, five of the compounds exhibited antiandrogenic and/or antiestrogenic effects. The degradability of two selected antimicrobial compounds was studied using model ligninolytic fungi and their extracellular enzymatic apparatus. Only partial transformation of both compounds was detected. The limited degradation capability of this group of microorganisms, which were previously shown to transform a broad range of diverse chemicals, emphasizes the persistent nature of antimicrobial compounds.

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1 Introduction

The number of synthetized anthropogenic compounds is steadily increasing and almost 106,000 of them are used in the commercial sector. The fate of most of them is not monitored in the environment, even though the amount of manufactured chemicals is in the range of hundreds of millions of tons per year (ECHA 2021; Eurostat 2021).

Nowadays, the problems connected with new emerging pollutants, the so-called micropollutants, are being discussed. Micropollutants are present in the environment in very low concentrations in the range of ng–µg/l (ng–µg/g). However, the ecotoxicological and toxicological data show that even such low concentrations pose a risk for the environment and human health. Pesticides, industrial chemicals, steroid hormones, pharmaceuticals, and personal care products rank among micropollutants. The ecotoxicological aspects of antimicrobial compounds used in personal care products are not sufficiently characterized. Nevertheless, these substances are routinely used in many products such, as housekeeping products, toothpastes, and mouthwashes.

It was shown that antimicrobial compounds contribute to the antibiotic and antimicrobial resistance phenomenon (Shepherd et al. 2018; Mao et al. 2020). However, micropollutants can also affect the endocrine system homeostasis because the system is regulated by biologically active compounds (natural hormones) usually at the level of ng/l. No systematic data of the endocrine-disrupting properties of antimicrobial compounds used in personal care products, especially in toothpastes and mouthwashes, is available.

Bioanalytical methods provide an important tool to monitor the fate of endocrine disruptors in the environment. These methods are useful to complement the information about environmental contamination obtained the methods of analytical chemistry and can also be employed for pure substance activity testing. Moreover, bioanalytical methods are usually more sensitive; detection limits up

to three orders of magnitude lower in comparison to analytical methods were observed in several studies (Conley et al. 2017; Toušová et al. 2017).

Currently, no restrictions on the release of antimicrobial compounds into the environment are imposed and monitoring of their fate is not obligatory. Nevertheless, several studies revealed their presence in the environment (Shrivas and Wu 2007; Ostman et al. 2017), and no elimination from wastewater was observed (Lasek et al. 2019), only adsorption on the activated sludge (Keerthisinghe et al. 2019; Nguyen and Oh 2019).

Ligninolytic fungi have demonstrated a great ability to degrade a wide range of organic pollutants, including the recalcitrant and persistent polychlorinated biphenyls (Čvančarová et al. 2012), polycyclic aromatic hydrocarbons (Covino et al. 2010), as well as several micropollutants (Beck et al. 2018). Therefore, ligninolytic fungi (and their enzymatic apparatus) are considered model microorganisms in the studies of organic pollutant biodegradability.

The first part of this thesis is focused on the endocrine-disrupting properties of nine antimicrobial compounds used in oral hygiene products. The (anti)estrogenic and (anti)androgenic activities were examined *in vitro* using recombinant yeast assays and human cell line tests. Moreover, a human cell line estrogen activity test (CXCL-test) was optimized in this section. In the second part, the possibility of biodegradation of two selected antimicrobial compounds with endocrine-disrupting properties was evaluated using ligninolytic fungi and their extracellular enzymatic apparatus. The last part discusses the optimal activity conditions of two oxidoreductases promising for bioremediation applications. Furthermore, the ability to degrade the antimicrobial compound triclosan in a mixture with three other endocrine-disrupting compounds was explored in several matrices.

2 Aims of the study

The thesis is focused on the study of new emerging pollutants, particularly their endocrine-disrupting properties and degradability. Selected antimicrobial agents used in toothpastes and mouthwashes were studied.

The specific objectives are summarized below:

1. The optimization of a sensitive *in vitro* estrogenic activity bioassay employing the breast carcinoma cell lines T47D (CXCL-test).
2. The evaluation of (anti)estrogenic and (anti)androgenic properties of selected antimicrobial compounds used in toothpastes and mouthwashes.
3. The evaluation of the degradability of two selected antimicrobial compounds with endocrine-disrupting properties. Ligninolytic fungi and their extracellular enzyme apparatus were employed as model microorganisms for their excellent degradation abilities.
4. The assessment of the activity, stability, and optimal conditions of two enzymes (laccase and horseradish peroxidase) with a high degradation potential and their ability to degrade the antimicrobial compound triclosan and three other endocrine-disrupting compounds in several aqueous including wastewater.

3 Material and methods

The experimental methods are described in detail in the corresponding publications. A brief overview is presented below:

- The CXCL-test was performed in 96-well plates according to Habauzit et al. (2017). Several protocol modifications (the number of cells/well, seeding medium, density of the cells prior the experiment), biological parameters (T47D cell line source, serum supplier), and two different media (DMEM, RPMI 1640) were tested in 17β-estradiol treatment to investigate their influence on the secretion of cytokine CXCL12, the CXCL-test outcome. The cytokine was quantified by enzyme-linked immunosorbent assay (ELISA) and the multivariable linear model was employed for the evaluation of the parameter influence.
- The (anti)estrogenic and (anti)androgenic properties of nine antimicrobial compounds (chlorhexidine, octenidine, hexetidine, cetylpyridine, sanguinarine, thymol, limonene, menthol, and eucalyptol) were explored by both the recombinant yeast and human cell line assays. The tests with *Saccharomyces cerevisiae* BMAEREluc/ERα and *S. cerevisiae* BMAERE/AR were performed according to Leskinen et al. (2005). Human cell line assays – the CXCL-test (Habauzit et al. 2017) and AIZ-AR (Bartoňková et al. 2015) – were used to investigate the (anti)estrogenic and (anti)androgenic properties, respectively.
- Ligninolytic fungal strains *Pleurotus ostreatus* 3004 CCBAS 278 and *Irpex lacteus* 617/93 were used for 21-day *in vivo* degradation tests of two selected antimicrobial compounds. The initial concentrations of octenidine and chlorhexidine were adjusted to non-toxic doses of 2 μg/ml and 3 μg/ml, respectively. *In vitro* degradation experiments with octenidine

(5 and 50 μg/ml) and chlorhexidine (5 and 50 μg/ml) were performed with concentrated extracellular liquids of *P. ostreatus* (rich in laccase, final activity of 120 U/l) and *I. lacteus* (rich in manganese-dependent peroxidase, final activity of 60 U/l). The quantitative analysis and qualitative analysis (metabolite identification) were performed using high-performance liquid chromatography equipped with a UV detector (HPLC/UV) and liquid chromatography–mass spectrometry (LC–MS), respectively.

- The activities of two enzymes, laccase from *Trametes versicolor* and peroxidase from *Armoracia rusticana* (horseradish), were compared in different experimental conditions (pH, matrix, H_2O_2 concentration in the case of the peroxidase). The optimal conditions were applied in a degradation study of triclosan, diclophenac, 17α-ethynylestradiol, and bisphenol A in several matrices (pure McIlvaine's buffers of pH 3 and 7, deionized water, tap water, tap water supplemented by 2.5% McIlvaine's buffer of pH 3, wastewater, and wastewater supplemented by 2.5% McIlvaine's buffer of pH 3 and 7). The quantitative analysis was performed using HPLC-UV, and the estrogenic activity before and after *in vitro* degradation was evaluated using *S. cerevisiae* BMAEREluc/ERα (Leskinen et al. 2005).

4 Results and discussion

4.1 Key parameter optimization and multivariable linear model evaluation of the *in vitro* estrogenic activity bioassay in T47D cell lines (CXCL-test) (publication 1)

The CXCL-test is based on the quantification of estrogen-stimulated secretion of cytokine CXCL12 in the breast carcinoma cell line T47D. Therefore, the test is suitable for estrogenic compound identification or detection. The assay provides more complex cell answer in comparison to transfected cell lines. Nevertheless, the stability of the cell line is not controlled by any selectable marker (usually used in the transfected cell lines), and the test results could therefore be inconsistent. The test was optimized, and the most suitable protocol was proposed. The multivariable linear model revealed that all the tested parameters, the seeding and experimental medium, serum, cell density before the experiment, and exact protocol execution significantly affected the cytokine secretion. Moreover, great differences in the minimal and maximal cytokine secretion were found among three tested T47D cell lines obtained from the original supplier and two laboratories. It was demonstrated that not only the tested parameters but also the test evaluation affected the test results.

4.2 Assessment of agonistic and antagonistic properties of widely used oral care antimicrobial substances toward steroid estrogenic and androgenic receptors (publication 2)

The yeast estrogen activity assay (*S. cerevisiae* BMAEREluc/ERα) revealed the antiestrogenic properties of cetylpyridine, octenidine, and thymol, and were also confirmed by the human cell line CXCL-test. Moreover, chlorhexidine was identified as an antiestrogen by the CXCL-test. Octenidine and cetylpyridine were identified as antiandrogens using the cell line AIZ-AR. The yeast assay (*S. cerevisiae* BMAERE/AR) confirmed these results; furthermore, the antiandrogenic effect of thymol and menthol were

revealed using this assay. None of the tested compounds were agonists of estrogenic or androgenic receptors. No effects were observed with hexetidine, limonene, sanguinarine, and eucalyptol.

4.3 Biodegradability of dental care antimicrobial agents chlorhexidine and octenidine by ligninolytic fungi (publication 3)

The degradability of octenidine and chlorhexidine was evaluated using the ligninolytic fungi *I. lacteus* a *P. ostreatus* (*in vivo*) and their extracellular enzymatic apparatus (*in vitro*). Substantial adsorption of octenidine on the fungal mycelium was detected *in vivo*. Nevertheless, a decrease in octenidine concentration in the range of 35–48% compared to the control was achieved. The amount of chlorhexidine decreased by 57–70% in the 21-day *in vivo* experiment. No sorption of this antimicrobial agent was detected. The initial amount of chlorhexidine decreased by 41 and 28% when extracellular enzymatic apparatus of *I. lacteus* (rich in manganese-dependent peroxidase) and *P. ostreatus* (rich in laccase) were employed, respectively. Octenidine concentration decreased significantly *in vitro* only with *I. lacteus* (by 23%). The involvement of the extracellular enzymatic apparatus of ligninolytic fungi was affirmed by the detection of transformation products of both octenidine and chlorhexidine.

4.4 Laccase and horseradish peroxidase for green treatment of phenolic micropollutants in real drinking water and wastewater (publication 4)

The activity and degradation potential of two promising oxidoreductases were tested in different reaction conditions and various aqueous matrices. The antimicrobial compound triclosan and three other endocrine-disrupting compounds were effectively degraded by both laccase from *T. versicolor* and horseradish peroxidase, even when the activities of the enzymes were undetectable by standard enzymatic activity methods. Up to 100 and 83% degradation was observed in deionized water and tap water, respectively. The degradation of the studied compounds was

suppressed in wastewater, where the degradation did not exceed 11%. Nevertheless, the addition of 2.5% McIlvaine's buffer into the wastewater enhanced the degradation efficiency of both enzymes considerably (up to 93%).

5 Conclusions

CXCL-test, the sensitive *in vitro* estrogenic activity bioassay with the T47D cell line, was optimized, and key parameters were evaluated using a multivariable linear model. Antiestrogenic and/or antiandrogenic properties of chlorhexidine, octenidine, cetylpyridine, thymol, and menthol were identified using assays based on recombinant yeast and human cell lines including the CXCL-test. No antiestrogenic or antiandrogenic effects were observed with hexetidine, limonene, sanguinarine, and eucalyptol. None of the tested compounds exhibited estrogenic or androgenic activities. The ligninolytic enzyme laccase and horseradish peroxidase proved high degradation capacity even in unfavorable conditions, including environmental matrices. However, only a partial transformation of octenidine and chlorhexidine by ligninolytic fungi and their extracellular enzymatic apparatus was detected, which shows the recalcitrant character of both the tested antimicrobial compounds.

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List of the author's scientific publications and conference contributions

Publications:

Maryšková M, Linhartová L, Novotný V, Rysová M, Cajthaml T, Ševců A (2021) Laccase and horseradish peroxidase for green treatment of phenolic micropollutants in real drinking water and wastewater. Environmental Science and Pollution Research 28: 31566–31574

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Conference contributions:

- 25th Interdisciplinary Toxicology Conference (TOXCON 2020), 3. – 5. 9. 2020, Czech Republic
  Poster: Key parameter evaluation of endocrine-disruption bioassay using T47D cell lines (CXCL-test)
- 11. PGS conference, 5. – 6. 11. 2019, Czech Republic
  Oral presentation: Study of new types of micropollutants
- 55th congress of European Societies of Toxicology (EUROTOX 2019), 7. – 11. 9. 2019, Finland
  Poster: Toxicity and degradability of widely used personal care products
- 10. PGS conference, 1. – 2. 11. 2018, Czech Republic
  Oral presentation: Study of new types of micropollutants
- 7th European Bioremediation Conference (EBC-VII) and the 11th International Society for Environmental Biotechnology conference (ISEB 2018), 25. – 28. 6. 2018, Greece
  Poster: Antimicrobial agents in personal care products cause antiestrogenic effect
- 9. PGS conference, 14. – 15. 10. 2017, Czech Republic
  Oral presentation: Study of new types of micropollutants
- 17th European Congress on Biotechnology, 3. – 6. 7. 2016, Poland
  Poster: Degradation of Endocrine Disruptors in Wastewater by *Pleurotus ostreatus* Fungal Batch Bioreactor