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

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Abstract:	In comparison to analytical tools, bioassays provide higher sensitivity and more complex evaluation of environmental samples and are indispensable tools for monitoring increasing in anthropogenic pollution. Nevertheless, the disadvantage in bioassays stems from the material variability used within the bioassays, and an interlaboratory adaptation does not usually lead to satisfactory test sensitivities. The aim of this study was to evaluate the influence of material variability on CXCL12 secretion by T47D cells, the outcome of an estrogenic activity bioassay, the CXCL-test. For this purpose, the cell line sources, sera suppliers, experimental and seeding media, and the amount of cell/well were tested. The multivariable linear model (MLM), employed as an innovative approach in this field for parameter evaluation, identified that all the tested parameters had significant effects. Knowledge of the contributions of each parameter has permitted step-by-step optimization. The most beneficial approach was seeding 20,000 cells/well directly in treatment medium and using DMEM for the treatment. Great differences in both basal and maximal cytokine secretions among the three tested cell lines and different impacts of each serum were also observed. Altogether, both these biologically based and highly variable inputs were additionally assessed by MLM and a subsequent two-step evaluation, which revealed a lower variability and satisfactory reproducibility of the test. This analysis showed that not only parameter and procedure optimization but also the evaluation methodology must be considered from the perspective of interlaboratory method adaptation. This overall methodology could be applied to all bioanalytical methods for fast multiparameter and accurate analysis.

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

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Abstract

In comparison to analytical tools, bioassays provide higher sensitivity and more complex evaluation of environmental samples and are indispensable tools for monitoring increasing in anthropogenic pollution. Nevertheless, the disadvantage in bioassays stems from the material variability used within the bioassays, and an interlaboratory adaptation does not usually lead to satisfactory test sensitivities. The aim of this study was to evaluate the influence of material variability on CXCL12 secretion by T47D cells, the outcome of an estrogenic activity bioassay, the CXCL-test. For this purpose, the cell line sources, sera suppliers, experimental and seeding media, and the amount of cell/well were tested.

The multivariable linear model (MLM), employed as an innovative approach in this field for parameter evaluation, identified that all the tested parameters had significant effects. Knowledge of the contributions of each parameter has permitted step-by-step optimization. The most beneficial approach was seeding 20,000 cells/well directly in treatment medium and using DMEM for the treatment. Great differences in both basal and maximal cytokine secretions among the three tested cell lines and different impacts of each serum were also observed. Altogether, both these biologically based and highly variable inputs were additionally assessed by MLM and a subsequent two-step evaluation, which revealed a lower variability and satisfactory reproducibility of the test. This analysis showed that not only parameter and procedure optimization but also the evaluation methodology must be considered from the perspective of interlaboratory method adaptation. This overall methodology could be applied to all bioanalytical methods for fast multiparameter and accurate analysis.

Keywords

Cytokine CXCL12/SDF1, T47D, multivariate linear model (MLM), interlaboratory method adaptation, material variability, estrogenic activity bioassay

Highlights

Material variability significantly influences CXCL12 secretion by T47D cells in the CXCL-test.
The MLM statistical method is an innovative approach for cell-based toxicological evaluation.
MLM and subsequent two-step normalization increases CXCL12 reproducibility.

1 Introduction

Endocrine-disrupting chemicals (EDCs) are natural or anthropogenic compounds that can mimic natural hormones or block natural hormone pathways (Kavlock et al. 1996). To date, almost 800 chemicals have been identified as EDCs (Karthikeyan et al. 2021), and most of them are either currently being used and are released continuously into the environment as a result or persist in environmental matrices due to past contamination. The majority of EDCs are considered micropollutants, which cause widespread trace contamination, usually in concentrations below the detection limits of the available analytical methods. Nonetheless, even low EDC contamination in the range of ng/l poses an ecotoxicological and human health risk (Wee and Aris 2017; Jackson et al. 2019), and the severity of EDC contamination has already been addressed in the European Union EDC screening program (Directive 2008/105/EC; Decision 2018/840/EU).

The development of sensitive and reliable methods is essential for EDC monitoring in the environment. While analytical methods assess the content of target chemicals, bioanalytical methods evaluate the biological effects of the whole mixture of pollutants in the sample. Moreover, chemical analyses provide lower sensitivity and only partially elucidate the biological effects evaluated by bioassays (Valitalo et al. 2016; Conley et al. 2017; Tousova et al. 2017).

The suitability and sensitivity of the current bioassays have been previously reviewed by several authors (Leusch et al. 2017; Li et al. 2020). However, even when the preconcentration of samples is taken into consideration, *in vitro* tests based on cell lines are among the most sensitive bioanalytical tools. The extensively used *in vitro* estrogenic activity bioassays employed in environmental sample evaluations are based on genetically modified estrogen-receptor-positive cells transfected with the estrogen response element sequence with an appropriate detection marker (Legler et al. 1999; Wilson et al. 2004; Leusch et al. 2017; Adam et al. 2020). Stably transfected cells provide both sensitivity and technical simplicity. High sensitivity was also proven with several alternative methods, such as immuno- or electrochemical assays (Li et al. 2020). Nevertheless, all these methods are focused only on one estrogenic pathway or on the detection of one estrogenic compound. The most sensitive bioassay that assesses complex cell response is the *in vitro* proliferation test, the E-screen (Soto et al. 1995; Leusch et al. 2017).

The CXCL-test, an *in vitro* bioassay also evaluating complex cell response to estrogenic compounds, was developed by Habauzit et al. (2010) as an alternative method to classical proliferation tests, and the sensitivity and time- and cost-effectiveness were improved later (Habauzit et al. 2017). The test is based on the quantification of secreted cytokine stromal cell-derived factor 1 (CXCL12 previously referred to as SDF1) in breast cancer cell lines after estrogenic stimulation. As the regulation of *CXCL12* transcription is controlled by multiple estrogen-related pathways (Boudot et al. 2011), the test provides overall information about the cell response. The CXCL-test revealed the endocrine-disrupting properties of several widely used pharmaceuticals and personal care products (Ezechias et al. 2016; Habauzit et al. 2017; Michalikova et al. 2019). Moreover, the test was also used as a tool for the development of a predictive mixture toxicity model (Ezechias and Cajthaml 2016), and its suitability for the evaluation of endocrine-disruptive activity of environmental samples was reviewed by Li et al. (2020).

The main disadvantage of bioassays stems from the use of highly variable biological materials, which makes reproducibility and interlaboratory protocol transfer difficult and usually does not lead to satisfactory sensitivity; therefore, the assay is sometimes abandoned. The aim of this study was to evaluate the effects of crucial bioassay parameters on the CXCL-test, a sensitive and complex *in vitro* bioassay, by a multivariate statistical methodology. Moreover, suggestions for the evaluation of the test performance and management of the result assessment were also determined. The tested experimental parameters were selected based on our previous experiments. The cell line source, medium used for cell seeding, experimental medium, serum supplier and cell density before and during the experiment were tested in a step-by-step experimental optimization procedure, and the multivariate linear model (MLM) was employed as a suitable statistical approach that allowed simultaneous assessment of the effects of multiple parameters and their interactions on an outcome. Therefore, the MLM was applied within this study to evaluate the influence of all the tested parameters on the secretion of CXCL12. From this knowledge, a new method of data interpretation was determined.

2 Materials and Methods

2.1 Chemicals

The 17β-Estradiol (E2, ≥98%) was purchased from Merck (Merck, Darmstadt, Germany), and ethanol (99.9%) was obtained from VWR (VWR, Prague, Czech Republic). All media and supplements from Gibco, Life Technologies, and Invitrogen brands were purchased from Thermo Fischer Scientific (Thermo Fischer Scientific, Waltham, USA). Charcoal-stripped fetal bovine serum (chsFBS) was obtained from 2 different suppliers, Merck and Biowest (Biowest, Nuaillé, France), and labelled in this study as chsFBS(Mer) and chsFBS(Bio).

2.2 Cell lines and cell maintenance

Three T47D breast carcinoma cell lines, T47D(ATCC) (directly purchased from American Type Culture Collection, ATCC® HTB-133™), T47D(IBT) (kindly donated by Dr. Truksa, Laboratory of Tumor Resistance, Czech Academy of Sciences), and T47D(IRSET) (kindly provided by Dr. Pakdel, Laboratory of Transcription, Environment and Cancer, Research Institute for Environmental and Occupational Health, France), were used.

All cell lines were routinely maintained in RPMI 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids solution (NEAA), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (PS); for details, see Table S1. The cells were cultured at 37 °C and 5% CO_2 in a humidified incubator. The culture medium was renewed every 3–4 days, and the cells were passaged by a trypsin (Gibco) treatment once per week.

2.3 Cell treatment

Estrogenic treatments were performed in 96-well plates (Nunc™, Thermo Fisher Scientific) according to Habauzit et al. (2017). Several protocol modifications were applied to investigate their influence on CXCL12 secretion. All experiments were performed with three T47D cell lines, T47D(ATCC), T47D(IBT), and T47D(IRSET). Two treatment media were tested: phenol red-free RPMI 1640 (Gibco) supplemented with 1% GlutaMAX™ (Life Technologies) and PS and phenol red-free DMEM (Gibco) supplemented with 1% NEAA, PS, 1 mM sodium pyruvate, and 4 mM glutamine. Both phenol red-free treatment media were completed with 2.5% chsFBS(Bio) or chsFBS(Mer). For details see Table S1. Additionally, different amounts of cells/well and different cell densities in the flasks before the experiment were tested.

The schedule of all A-C experiments is given in Fig. 1. The cells were seeded overnight (day 1). In Fig. 1A, the cells were seeded at a total amount of 20,000 cells/well in phenol red culture medium. As shown in Fig. 1B, the cells were seeded at 10,000 or 20,000 cells/well directly in treatment medium (phenol red-free RPMI or DMEM). Finally, the cell density in the flask before cell seeding was evaluated (Fig. 1C). Twenty thousand cells per well were directly seeded in phenol red-free DMEM treatment medium. Cells were issued from low (40% confluent) and high (80% confluent) cell densities in the initial flask.

When the cells were initially seeded in culture medium (Fig. 1A), they were double rinsing with Dulbecco's phosphate-buffered saline (DPBS). In all three setups, the medium was replaced by 200 µl of fresh treatment medium on day 2 and incubated for an additional 24 h. Thereafter, on day 3, the medium was removed and replaced by 150 µl of the treatment medium containing either E2 in the range of 2.5 10^{-12} – 1.0 10^{-8} M or ethanol (0.1% (v/v)). The cells were treated for 24 h, and the level of the secreted cytokine CXCL12 was quantified by enzyme-linked immunosorbent assay (ELISA) (day 4).

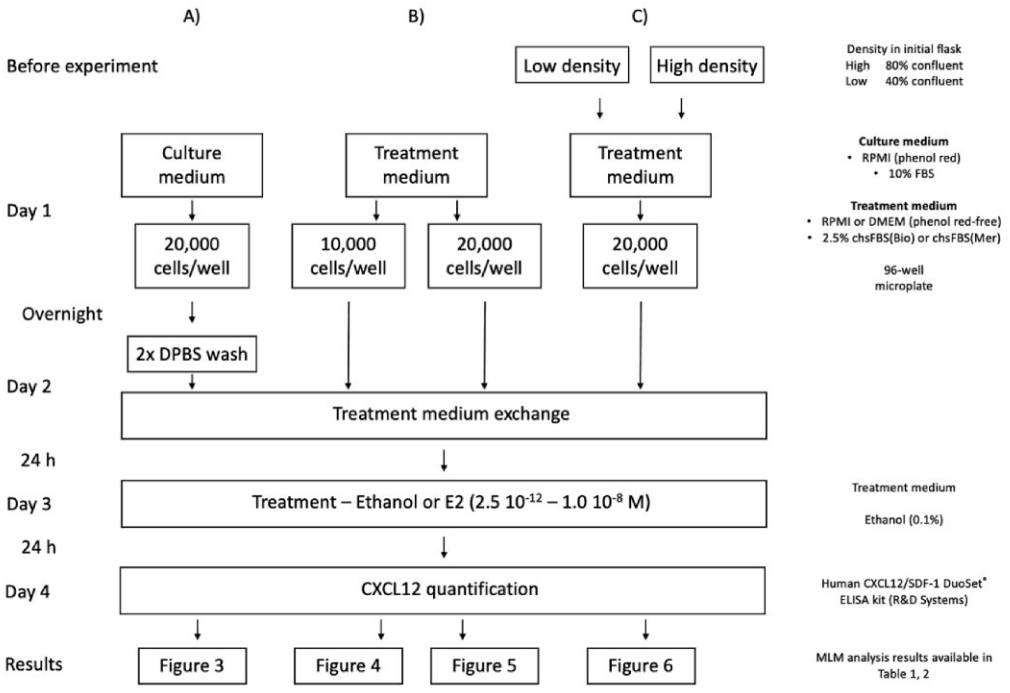


Fig. 1 Experimental setup schedule (A, B, C) for the CXCL-test performed with T47D(ATCC), T47D(IBT), and T47D(IRSET) cells. (A) The cells were seeded overnight in culture medium at a total amount of 20,000 cells/well. The next day, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and a new portion of treatment medium (RPMI or DMEM supplemented with charcoal-stripped fetal bovine serum (chsFBS(Bio) or chsFBS(Mer)) was

added and incubated for an additional 24 h. The cells were exposed to estradiol (E2) or ethanol in the respective treatment medium. (B) A total of 10,000 or 20,000 cells/well were directly seeded in treatment medium (RPMI or DMEM) supplemented with chsFBS(Bio) or chsFBS(Mer). The next day, the medium was exchanged, and 24 h later, the cells were treated with ethanol or E2. (C) Twenty thousand cells/well originating from flasks of two different cell densities (low or high) were seeded in DMEM supplemented with chsFBS(Bio) or chsFBS(Mer). Then, the medium was renewed, and the cells were treated with ethanol or E2. In all the experiments, the treatment was terminated after 24 h of incubation. Secreted CXCL12 was immediately quantified by an enzyme-linked immunosorbent assay (ELISA), and the results were evaluated by a multivariate linear model (MLM).

2.4 ELISA

The secreted cytokine CXCL12 was quantified by a human CXCL12/SDF-1 DuoSet® ELISA kit (R&D Systems, Minneapolis, USA). Quantification was performed according to the manufacturer's protocol, as previously described (Habauzit et al. 2010). The results of ELISA were assessed by measuring the absorbance at 450 and 570 nm with the Infinite M200 PRO microplate reader (Tecan, Switzerland).

2.5 Statistical and data analyses

Each experiment was analyzed separately using the MLM to test the respective effect of each experimental parameter and the E2 dose on the level of CXCL12 secretion. In experiment A, the effects of the cell line (reference = T47D(ATCC)), medium (reference = RPMI), serum (reference = chsFBS(Mer)) and E2 dose (reference = ethanol) were tested as the main fixed effects. Evaluated doses of E2 were $1.0\ 10^{-9}$ M, $1.5\ 10^{-10}$ M, $4.4\ 10^{-11}$ M, $2.8\ 10^{-11}$ M, $8.8\ 10^{-12}$ M, $2.5\ 10^{-12}$ M. To detect non-linearity in the E2 - CXCL12 secretion dose–response relationship and to estimate differential secretion at the specific E2 concentrations tested, the dose of E2 was considered a categorical variable. Moreover, the interactions among the cell line, dose of E2, medium, and serum were also evaluated in experiment A (data not shown).

In experiments B and C, as biological replicates were performed and mixed effect models were implemented, in which the sample (experimental/biological) incurred a random effect, to consider the repeated measures for each sample. Moreover, the additional E2 dose of $2.5\ 10^{-12}$ M was also evaluated. In experiment B, the analysis included not only the same fixed factors as in experiment A but also the "number of cells per well" (20,000 vs 10,000 as a reference). In experiment C, the "number of cells per well" was not included; the "cell density before

experiment" was considered instead (low vs high as a reference), and the medium effect was not taken into account. Interactions were also tested between the cell line and dose of E2 or between the cell line and serum (data not shown).

Furthermore, to reveal potential differences in the effect of the E2 dose and experimental parameters on CXCL12 secretion among the tested cell lines, the same analyses were applied to each cell line separately. The results of the analyses are presented as the difference in mean level of CXCL12 secretion expected for each level of the different experimental parameter and E2 dose compared to the reference category, and all other parameters remain equal.

2.6 Methodology for optimal analysis

The ethanol controls reflect each tested parameter. To identify only the estrogenic effect under each experimental condition, the corresponding secretion level in the ethanol control was subtracted from the secretion level in each replicate of E2 treatment. Then, to compare the final estrogenic effect, a second correction was performed. Each individual biological replicate was expressed as a percentage of CXCL12 secretion treated with $1.0\ 10^{-9}$ M E2, which was considered as 100%.

3 Results

3.1 Cell line source

T47D(IBT) and T47D(IRSET) cells were kindly provided by two laboratories (Czech Republic, France), while T47D(ATCC) cells were obtained directly from the original supplier, ATCC. The cell lines showed slightly different dynamics of growth during maintenance (data not shown) and different resistances to buffer rinsing (see below). The cell comparison under a phase-contrast microscope showed identical cell shapes and very similar cell sizes (Fig. 2).

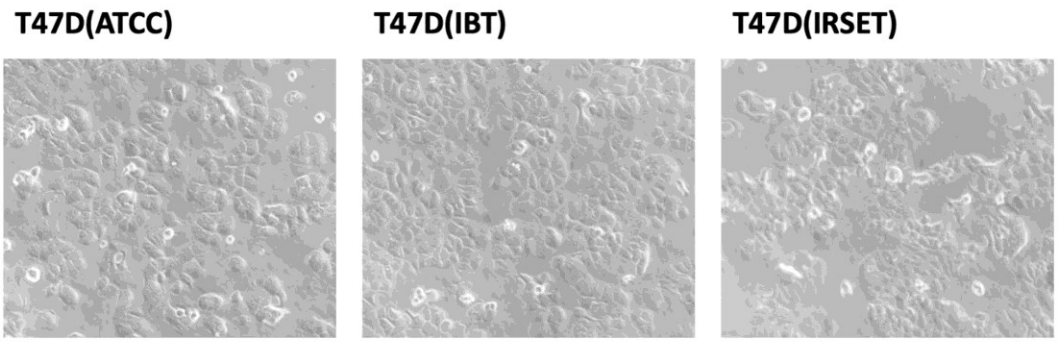


Fig. 2 Comparison of the T47D cell lines (ATCC, IBT, IRSET) originating from three sources. Cells of the 2nd passage were maintained in culture medium that was imaged by Olympus CK30 at a magnification of 200×.

As shown in Figs. 3, 4, 5, and 6, the cell lines demonstrated different levels of basal and maximal cytokine secretion. The lowest secreted level of CXCL12 was detected in T47D(ATCC) (basal 32–48 pg/ml, maximal 85–196 pg/ml); T47D(IBT) basally secreted 44–102 pg/ml of CXCL12 and maximally 210–332 pg/ml; and the highest secretion was detected in T47D(IRSET) (basal 103–217 pg/ml, maximal 280–412 pg/ml) when 20,000 cells/well were seeded in treatment medium (Fig. 5 and 6). Moreover, involvement of the cell line origin in CXCL12 secretion was deciphered by MLM analysis (Table 1). When the T47D(ATCC) cell line was considered as a reference (in table marked as 0 (ref)), the secretion of CXCL12 was significantly higher in the T47D(IBT) cell line by 9 to 110 pg/ml and in the T47D(IRSET) cell line by 76 to 150 pg/ml according to the experiment. The difference in mean CXCL12 secretion between T47D(ATCC) and T47D(IBT) cells was not significant in experiment C when parameters, such as the medium, were fixed, and the cell densities were tested before the experiment. Nevertheless, T47D(IRSET) was the cell line with the significantly highest secretion regardless of the experimental design. To obtain deeper insight and into CXCL12 secretion and understand it better, separate analyses were carried out for each cell line (Table 2). In general, technical parameters such as the medium, serum, amount of the cells/well, and cell density before the experiment mostly modified CXCL12 secretion in the same way, even though the magnitude (in pg/ml of the secreted CXCL12) and the statistical significance differed. For instance, the design in experiment C enabled a decrease in the detection limit and evidenced that the E2 treatment at 2.5 10^{-12} M induced a significant effect on CXCL12 secretion in T47D(ATCC) and T47D(IRSET) cells (Table 2).

3.2 Effects of the protocol and technical parameters on CXCL12 secretion

The MLM analyses confirmed that the protocol (medium used for the cell seeding or washing step) and technical parameters (cell line source, medium, serum, amount of the cells/well and cell density before the experiment) affected cytokine secretion. However, each cell line manifested their own levels of secretion and sensitivity to the parameters, and these were evaluated in two steps. First, the global effect of the experimental parameters was considered with all cell line sources together (Table 1). Second, the differences in cytokine secretion that were caused by the tested parameters in each cell line were examined (Table 2).

3.2.1 Cell seeding medium

In experiment A, the cells were seeded in culture medium, which contained estrogenic compounds (FBS hormones, phenol red). Therefore, a double buffer rinse was necessary before the medium was exchanged with the treatment medium. As previously mentioned, the microscope control revealed a different resistance to buffer washing in the tested cell lines. Indeed, during the washing step, the T47D(ATCC) and T47D(IBT) cells remained adhered to the surface, whereas the T47D(IRSET) cells were noticeably washed away. Subsequent tests of viability (see supplementary information) revealed up to approximately 50% loss of T47D(IRSET) cells when compared to those of T47D(ATCC) and T47D(IBT) cells (data not shown). Nonetheless, CXCL12 secretion by T47D(IRSET) cells was sufficient despite cell detachment (illustrative example Fig. 3); hence, cell detachment was not observed until viability tests were performed. Since the cell lines manifested different adherence properties and stability in the DPBS washing step, the cells were seeded directly in the respective treatment medium, and the rinsing step was eliminated in the following optimization tests, where no differences in the adherence properties were detected by the viability tests. Seeding in culture medium was not conducted in any other experiment. Moreover, the tested E2 concentration was subsequently decreased to determine the detection limit.

After adjusting for the effect of the other factors (medium, serum, replicate), it was apparent that T47D(ATCC) and T47D(IRSET) seemed to react in the same way. Unlike them, T47D(IBT) cells were more sensitive to the medium, DMEM increased the secretion by 165±30 pg/ml on average, and chsFBS(Bio) decreased the secretion (Table 2).

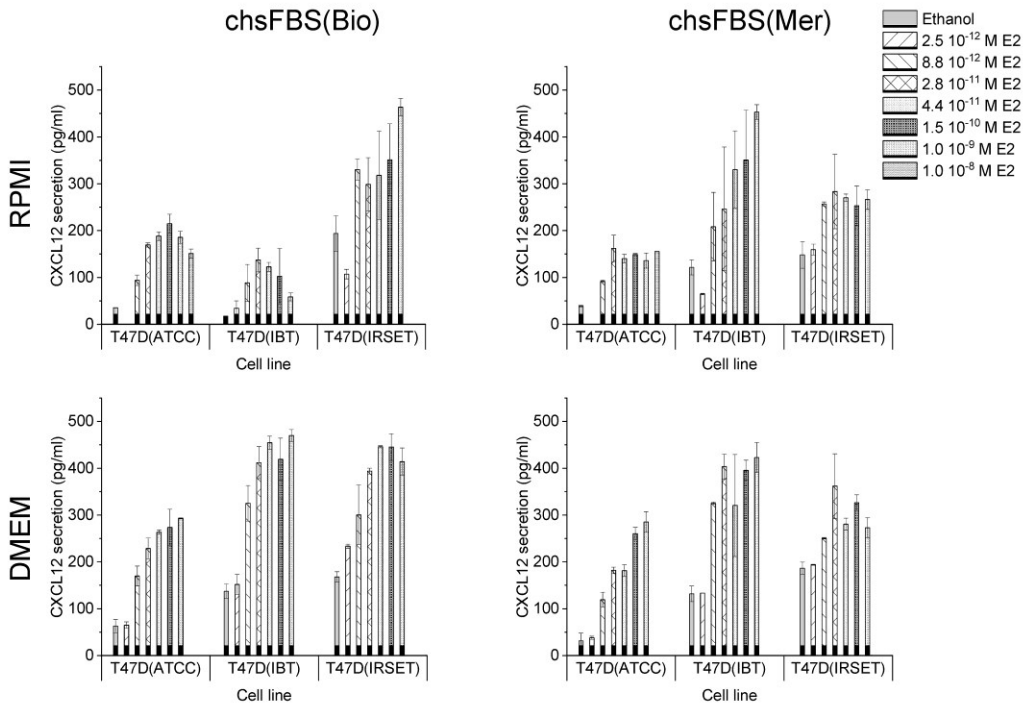


Fig. 3 Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after a 24-h exposure to estradiol (E2). Cells were seeded in cultivation medium and treated in two phenol red-free media (DMEM or RPMI) supplemented with two different charcoal-stripped sera (chsFBS(Bio) or chsFBS(Mer)).

3.2.2 The amount of cells/well

In general, basal CXCL12 secretion significantly increased by 119±7 pg/ml on average when the number of cells/well was raised from 10,000 to 20,000 (Table 1, experiment B). Even though the effect on each cell line differed, a dose–response relationship was confirmed for all three tested cell lines by MLM analysis (Table 2). When 10,000 cells/well was applied, both medium and serum were shown to affect the test sensitivity. As shown in Fig. 4, T47D(ATCC) cells treated in chsFBS(Mer)- or chsFBS(Bio)-supplemented RPMI did not secrete sufficient amount of cytokine to manifest an E2 dose–response effect. Similarly, DMEM supplemented with chsFBS(Mer) did not affect the effect in T47D(IRSET) cells. In conclusion, the amount of 10,000 cells/well was applicable only for T47D(IBT) and in some conditions for the T47D(IRSET) cell lines.

When the cells were seeded at 20,000 cells/well, an E2 dose–response effect was detected in all the tested cell lines (Fig. 5). As shown in Table 2 (experiment B), 39±4, 106±7, and 210±12

pg/ml of the secreted cytokine was manifested in T47D(ATCC), T47D(IBT), and T47D(IRSET), respectively. These modifications of basal secretion made the estrogenic effect of E2 unclear.

3.2.3 Medium used for the cell treatment

DMEM induced a statistically significant increase in CXCL12 secretion compared to that of RPMI, regardless of the medium used for cell seeding. As shown in Table 1 (experiments A and B), increases of 75±15 and 39±7 pg/ml were detected in culture medium-seeded and treatment medium-seeded experiments, respectively. As previously detailed, the cell lines differed in sensitivity to the medium used for the treatment.

Nonetheless, in both experiments (A, B) where the media were compared, significantly different levels of CXCL12 secretion were detected ($p < 0.05$). As shown in Table 2 (experiments A and B), DMEM increased cytokine secretion in all tested cell lines by 32±11, 165±30, and 34±16 pg/ml CXCL12 in culture medium-seeded T47D(ATCC), T47D(IBT) and T47D(IRSET) cells, respectively (experiment A). When the cells were seeded directly in treatment medium (experiment B), the secretion of CXCL12 increased in DMEM by 26±4 (T47D(ATCC)), 34±9 (T47D(IBT)), and 56±12 (T47D(IRSET)) pg/ml compared to RPMI. These effects on CXCL12 secretion were also clearly manifested in the ethanol control samples (Fig. 5).

3.2.4 Treatment medium supplemented with charcoal-stripped serum

In fact, the serum, which was monitored in all the experiments, was shown to significantly affect cytokine secretion only in experiments B and C, where chsFBS(Bio) induced an increase in secretion (Table 1). Finally, the MLM analysis revealed a significant increase in cytokine secretion in all the tested cell lines in experiment C, where parameters such as the medium used for seeding and the treatment medium were fixed. As shown in Table 2 (experiment C), chsFBS(Bio) increased CXCL12 secretion by 36±10, 24±10, and 56±5 pg/ml in T47D(ATCC), T47D(IBT), and T47D(IRSET) cells, respectively.

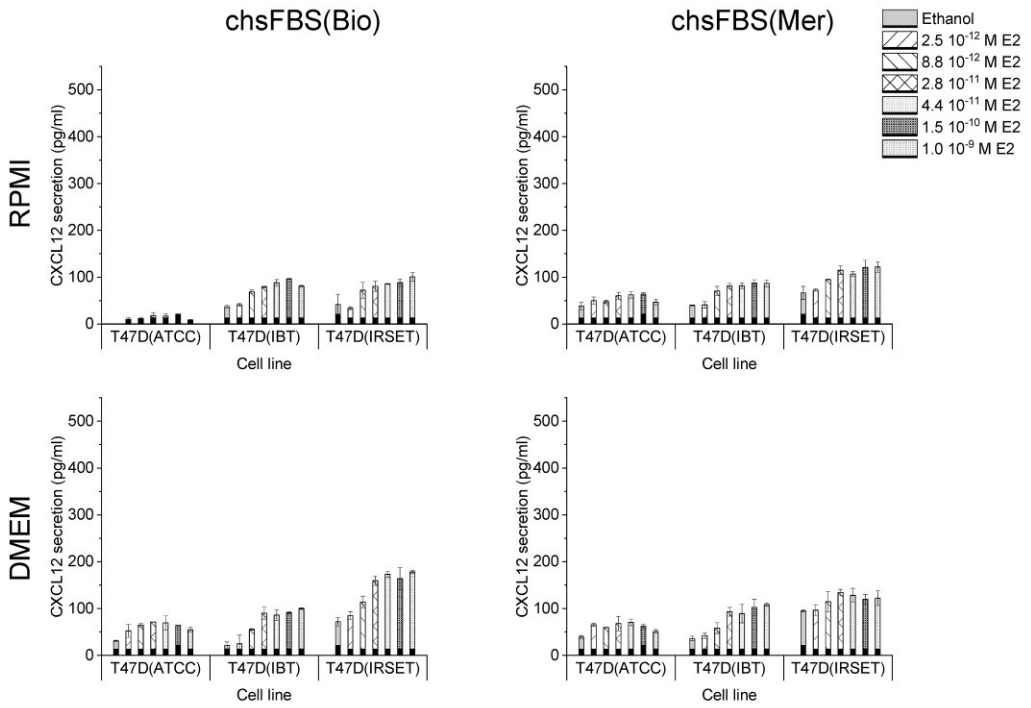


Fig. 4 Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after 24-h exposure to estradiol (E2). A total of 10,000 cells/well were seeded and treated in two phenol red-free media (RPMI or DMEM) supplemented with two different charcoal-stripped sera (chsFBS(Bio) or chsFBS(Mer)).

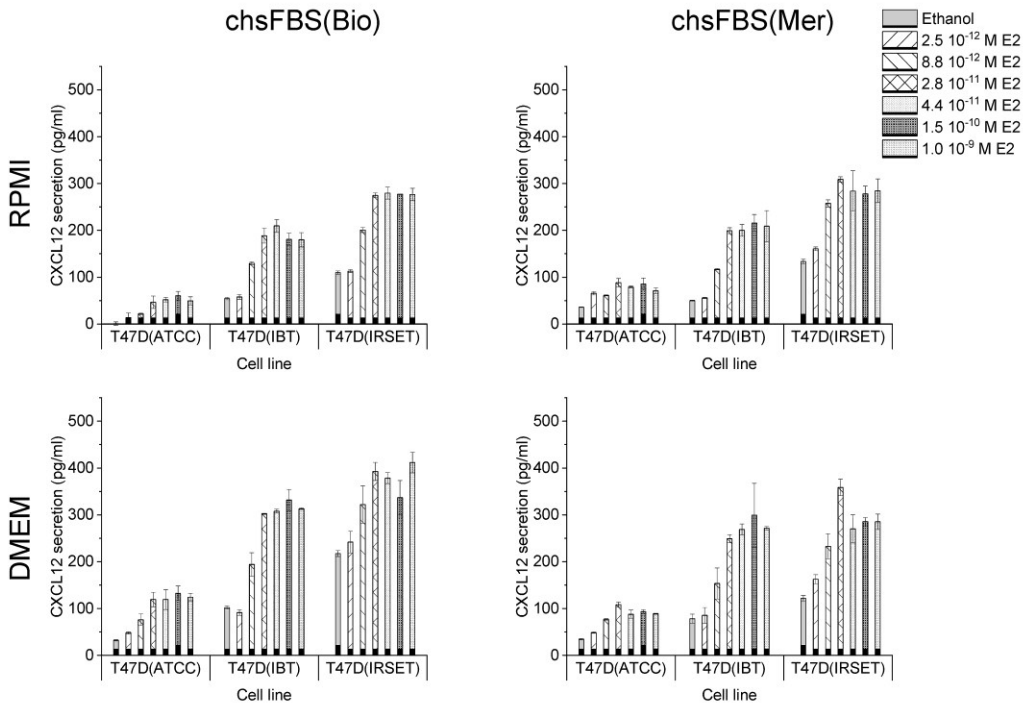


Fig. 5 Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after 24-h exposure to estradiol (E2). A total of 20,000 cells/well were seeded and treated in two phenol red-free media (RPMI or DMEM) supplemented with two different charcoal-stripped sera (chsFBS(Bio) or chsFBS(Mer)).

3.2.5 Cell density before the experiment

The treatment was performed only in DMEM, which was previously shown to increase the level of secreted CXCL12 and in this manner to improve the test sensitivity. As shown in Table 2, the density of the cells before the experiment affected cytokine secretion; densely cultivated cells secreted significantly lower amounts of the cytokine in all the tested cell lines. When an approximately eighty percent confluence was formed in the cultivation flask prior to the experiment, T47D(ATCC), T47D(IBT), and T47D(IRSET) cells exhibited significantly decreased secretion by 30±10, 44±10, and 19±5 pg/ml, respectively (Table 2, experiment C and Fig. 6).

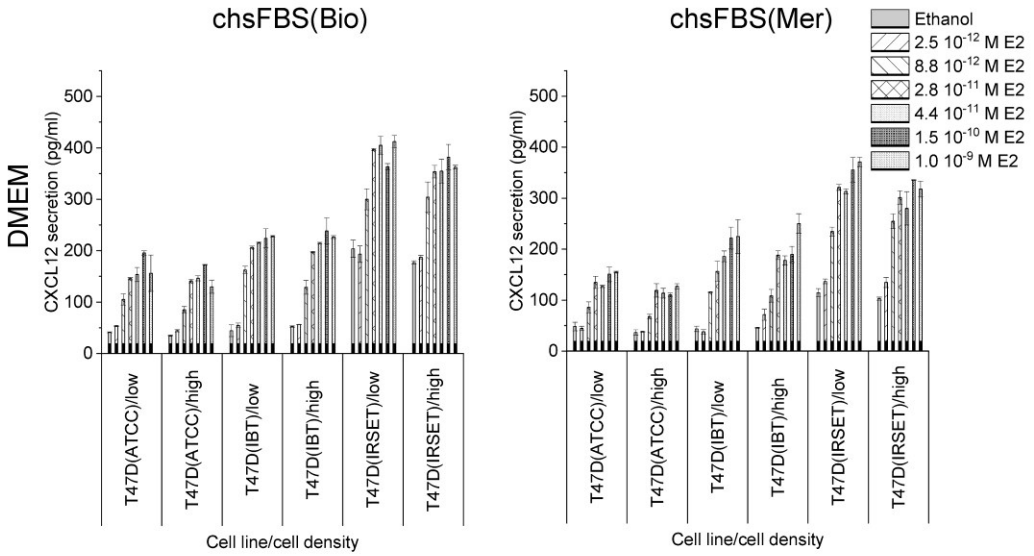


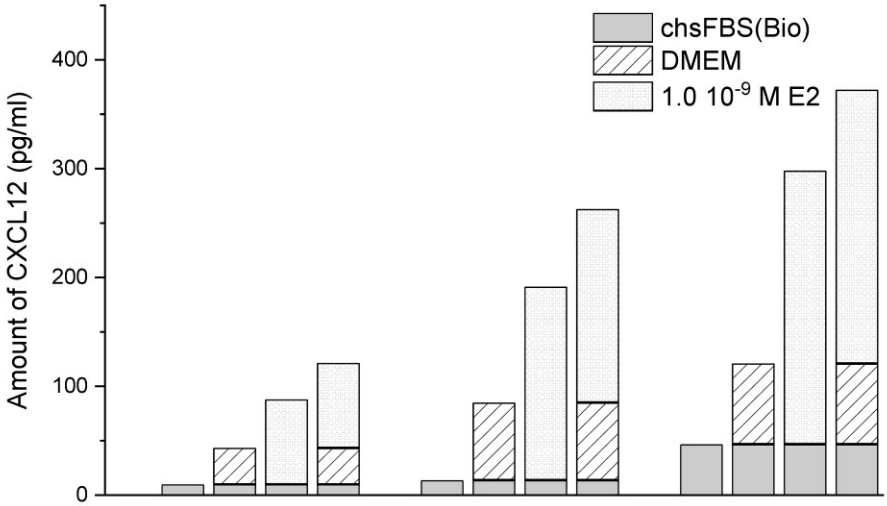
Fig. 6 Illustrative example of the secretion of the cytokine CXCL12 in three T47D cell lines (ATCC, IBT, IRSET) after 24-h exposure to estradiol (E2). All the cell lines were exposed to DMEM supplemented with two different sera (chsFBS(Bio) or chsFBS(Mer)). Cells were seeded from two different cell culture densities; high and low density in the initial flask represent ca. 80 and 40% confluent, respectively.

3.3 General Analyses Methodology

The MLM methodology allows the additive effects of each parameter to be assessed on the global variance of CXCL12 secretions with different combinations of experimental conditions. As illustrated in Fig. 7, the secretion observed in the ethanol controls reflects the mean secretion resulting from a particular combination of experimental conditions that generate different levels of secretion. For instance, the effect of serum was similar in T47D(ATCC) and T47D(IBT) cells, while T47D(IRSET) cells were more sensitive to the serum origin. The medium demonstrated a similar effect in T47D(IBT) and T47D(IRSET) cells, and the lowest response to the medium was observed in T47D(ATCC) cells. To identify only the estrogenic effect under each experimental condition, the corresponding secretion level in the ethanol control was subtracted from the secretion level in each replicate of E2 treatment.

Finally, all three cell lines and both sera were evaluated in the mean (and standard deviation) of all experiments performed in DMEM (Fig. 8a). The results are expressed as the mean of 5 to 6 independent experiments. As shown in Fig. 8a, the curves observed were quite different from one another. The secretion of each E2 concentration was individually corrected by the secretion of the respective ethanol control (Fig. 8b), and CXCL12 secretion was set to 0

(control) for the maximum concentration tested. This first correction showed that the T47D(IRSET) and T47D(IBT) curves were quite similar in shape and magnitude. However, T47D(ATCC) showed quite different magnitudes, and this magnitude was still dependent upon the serum (Fig. 8b). To compare the final estrogenic effect, the last correction was performed, and each individual biological replicate was expressed in percent of the secretion at 1.0 10^{-9} M E2, considered as 100% (Fig. 8c). This two-step operation showed that regardless of the cell line considered or the serum used, the estrogenic effect on secretion was equivalent.



chsFBS(Mer)	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
chsFBS(Bio)	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
RPMI	+	+	-	+	-	+	+	-	+	-	+	+	-	+	-
DMEM	-	-	+	-	+	-	-	+	-	+	-	-	+	-	+
1.0 10^{-9} M E2	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
cell line	T47D(ATCC)					T47D(IBT)					T47D(IRSET)				

Fig. 7 Illustration of the additional effect of the individual parameters on CXCL12 secretion in T47D(ATCC), T47D(IBT), and T47D(IRSET) cells. The amount of secreted CXCL12 was determined by a multivariate linear model for each parameter. The Merck-obtained charcoal-stripped fetal bovine serum (chsFBS(Mer)) and RPMI medium were considered references, whereas the effects of serum obtained from Biowest (chsFBS(Bio)) and DMEM were manifested in the columns with an effect of 1.0 10^{-9} M estradiol (E2).

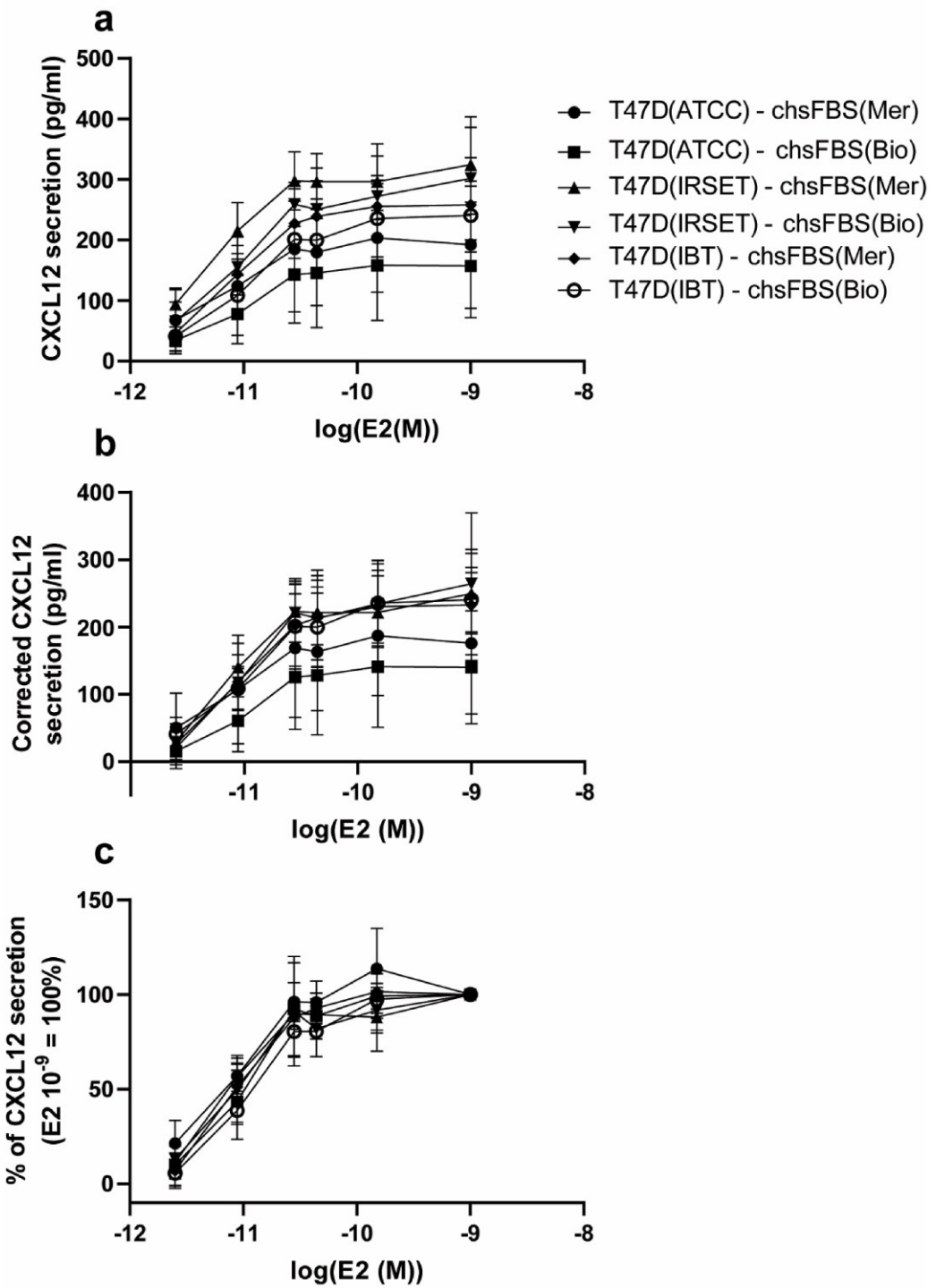


Fig. 8 CXCL12 secretion considered from all DMEM seeded and treated experiments. The influence of the three T47D cell lines (ATCC, IBT, IRSET) and two charcoal-stripped fetal bovine sera (chsFBS(Mer) and chsFBS(Bio)) on CXCL12 secretion was evaluated after 24-h exposure to estradiol (E2). The results are expressed as (A) the mean secretion, (B) the secretion of each E2 concentration corrected by the secretion of the respective ethanol control, and (C) the

individual biological replicate in percent of cytokine secretion at 1.0 10^{-9} M estradiol (E2), considered as 100%.

4 Discussion

Considering the wide diversity of material used among laboratories, interlaboratory protocol transfer requires an optimization procedure. Therefore, some of these parameters, such as cell line source, media used for cell seeding and treatment, serum supplier, amount of the cells and cell density before experiment, were evaluated. Moreover, the most appropriate protocol for endocrine disruption chemical evaluation, the CXCL-test, was proposed within this work. The involvement of each parameter in CXCL12 secretion was assessed during the three-step protocol optimization (Fig. 1) and subsequent MLM analyses, which were used for a global effect analysis and a stratified analysis according to the cell line.

The global MLM analysis (Table 1) revealed constant significant differences in CXCL12 secretion among the tested cell lines, even though the cell origin was initially the same; the original supplier (ATCC) and two laboratories (Czech Republic, France) purchased the cell line from ATCC (Habauzit et al. 2017; Tomkova et al. 2019). Identical cell shape did not indicate any diversity. However, the cell lines demonstrated immense differences in both basal and E2-stimulated CXCL12 secretion. Dissimilarities among the same cell lines have been discussed in recent decades (Resnicoff et al. 1987), especially in biomedical research. Advanced multi-omic analyses have discovered differences among the same commonly used cell lines (Kleensang et al. 2016; Liu et al. 2019), and suppliers require upgraded quality declaration (Lorsch et al. 2014). Moreover, Kleensang et al. (2016) demonstrated genetic variability even in the same ATCC frozen batch of the estrogen receptor-positive breast cancer cell line MCF-7, which is frequently used for estrogenic activity testing. In addition, starkly different estrogenic responses were identified between the same-batch cells in Kleensang's study. Reassuringly, E2 treatment of all the tested T47D cells induced an increase in CXCL12 secretion in a dose-dependent manner, which disproved the cell line dependency of the test and confirmed their suitability for the CXCL-test. However, the experiments and subsequent MLM analyses affirmed that the cell line as well as other parameters played an important role in this treatment.

In this work, each tested cell line required different optimal test conditions, and most of the tested parameters affected secretion differently according to the cell line. The global analysis did not allow us to observe the effect of parameters on the cell line individually. As formal testing of all the interactions between all the tested experimental conditions was not possible (there were too many parameters to estimate and interpret, inducing a lack of power), we focused on the interaction with the cell line and implemented a second MLM analysis stratified by cell line. This analysis allowed us to more precisely decipher the parameter impacts on the secretion in each cell line individually and the impact on new parameters studied on the secretion. The T47D(IRSET) cell line demonstrated suitability only with the treatment medium seeding design of the CXCL-test. The highest cytokine secretion (up to 412±12 pg/ml) was detected in T47D(IRSET) cells, and a positive effect for secretion was significantly identified when DMEM and chsFBS(Bio) were applied. Generally, cytokine secretion that is too high may also cause a problem with ELISA signal linearity, and overexpression of the cytokine in the assay should be resolved by additional supernatant dilution, as was previously described (Habauzit et al. 2010). In experiment A, T47D(IRSET) cells manifested equal or lower secretion than T47D(IBT) cells (e.g., DMEM, chsFBS(Mer)), which could be explained by the adherence properties of T47D(IRSET) cells. The lower secretion by T47D(IRSET) was not observed in any other experiment.

The T47D(IBT) cell line demonstrated sufficient E2-stimulated secretion, reaching 332±23 pg/ml (treatment medium-seeded experiments), in combination with quite low basal secretion in the controls. Compared to T47D(IRSET) and T47D(IBT), ATCC purchased cells (T47D(ATCC)) demonstrated the lowest CXCL12 secretion ability, which did not exceed 196±4 pg/ml in the treatment medium seeded experiments even in the favourable DMEM supplemented by chsFBS(Bio). However, low basal secretion enabled estrogenic treatment evaluation in the T47D(ATCC) cell line. In T47D(ATCC) cells, it was demonstrated that a decrease in basal secretion caused by unfavourable cell conditions could improve the test sensitivity in parameters such as fold of induction (FI). Nevertheless, the FI evaluation of the test sensitivity was considerably dependent on the test performance and on variability in the material. The material could increase basal secretion, but the maximal secretion was determined by the cell secretion ability. Therefore, the FI was not a convenient outcome to analyse the data obtained due to the variability of the ethanol controls in different conditions.

The number of cells per well (10,000 cells/well in a 96-well plate) was optimized in a preceding study by Habauzit et al. (2017) with T47D(IRSET) cells that secreted more CXCL12 than other tested cells evaluated within this work. In this study, the 10,000 cells/well did not provide a sufficient dose–response curve, especially in T47D(ATCC), where the amount of the secreted cytokine was in some cases also near the detection limit of the ELISA.

A marked effect of treatment medium was detected in all the experiments. DMEM significantly increased global CXCL12 secretion in all the tested cell lines compared to RPMI. Our results are in accordance with other studies where a medium impact on various cell outcomes was observed. Wu et al. (2009) showed that DMEM enhanced cell differentiation and induced the activity of important enzymes in periosteum-derived cells compared to that of RPMI medium. The composition of DMEM and RPMI (Table S2) shows that DMEM contains higher amounts of almost all the components in common, which stimulate cell metabolism. The medium was also shown to play a key role in the up- or downregulation of almost 9,000 genes in MDA-MB-231 cells, the breast carcinoma cell line, when the most common media (RPMI, MEM, and DMEM) involvement was studied (Kim et al. 2015).

The test was affected by not only the medium used for the treatment but also the seeding medium. When the cells were seeded in the culture medium, where the buffer washing step was needed, highly reduced adhesiveness was observed in the T47D(IRSET) cells. Direct seeding into the treatment medium was therefore applied in further experiments. Moreover, the benefit of cell preparation in steroid-free medium prior to the experiment was shown earlier. For instance, Wilson et al. (2004) recommend cultivation in steroid-free medium even one week before the experiment.

The serum was also shown to be mostly involved in cytokine secretion. A significant stimulating effect of chsFBS(Bio) was found in the experiments, where the cells were seeded in the treatment medium. Different impacts of serum were expected, as serum is the most lot-to-lot variable component of medium with variable mitotic and toxic properties. The composition of FBS is dependent on, e.g., different animal genetics, location of origin, animal feed, etc. Zheng et al. (2006) showed that high-abundance proteins were detected in comparable relative amounts among the sera of different suppliers. However, even lot-to-lot variability was observed in the content of growth factors. Moreover, when serum is applied in an estrogenic activity evaluation assay, charcoal-dextran treatment of serum is necessary to eliminate serum estrogens. Nevertheless, this additional procedure increases the lot-to-lot

variability. A great diversity and efficiency of several charcoal stripping procedures were demonstrated by Sikora et al. (2016). The final test efficiency and sensitivity could be strongly moderated by a small increase in basal cytokine secretion caused by a trace amount of hormone in chsFBS. This phenomenon is not rare. Experiences have shown us that an incomplete charcoal-dextran purification of serum even in the range of units of pg/ml E2 (declared in supplier's Certificate of Analysis) considerably increases basal secretion and markedly decreases test sensitivity.

The cell density in the initial flask before the experimental evaluation showed that a higher cell density significantly decreased cytokine secretion in all the tested cell lines. This result is concordant with the study by Kim et al. (2015), who proved there are diverse proteomic profiles of breast cancer cells at different cell densities. Moreover, decreased expression of CXCL12 in a growing tumor was proven earlier (Zhao et al. 2014). Nevertheless, the sensitivity of the CXCL-test was not modified because differences were observed in both control and E2-treated cells.

Two more parameters, technical and biological replicates, were included in the evaluation. While the technical replicates demonstrated equal cytokine secretion in the same treatment, the biological replicates provided statistically significant differences. This is not surprising, as for all optimization experiments, only two biological replicates were carried out. Nevertheless, the effects of the tested parameters were affirmed in both biological replicates. The optimization enabled us to show variability in the CXCL-test outcome when the original protocol was modified by interlaboratory material variability. Although some authors exhort strict protocol compliance (Kim et al. 2015), the current situation when great variability in cell lines is not fixed (Lorsch et al. 2014; Kleensang et al. 2016) and few methodology details are usually published does not allow us to follow their suggestion. The results show that some protocol modifications are required. However, the unavoidable variability in the biological material (chsFBS, cell line) allows only some general recommendations for estrogenic assay performance. The choice of serum without estrogen residues together with rich nutritive properties supporting the secretion in treatment increases the test sensitivity. Furthermore, a preliminary test of every new chsFBS lot is advised. Seeding directly to the treatment medium was shown to be strongly favourable in all the tested cell lines. Conformed confluence affected the secretion, but a clear benefit was not detected in any tested variant. Additionally,

the suitable number of cells/well was increased compared to the original study (Habauzit et al. 2017) to 20,000 cells/well, which suggests adjustment for this parameter if needed.

Finally, it was shown that the experimental conditions greatly affected the final results regarding the dose-response association of interest. The MLM statistical method offers real value for cell-based toxicological test evaluation. It provides a fast multiple parameter assessment, which simultaneously evaluates both fixed and random effects (when repeated experiments occur) and detects potential interactions among the monitored parameters. This statistical analytical methodology also permits the assessment of many parameters with a low number of replicates. A similar approach was used by deCastro and Neuberg (2007) in MCF-7 E-SCREEN assessment. A generalized linear mixed model was shown to represent an appropriate method to evaluate design variations such as plate-to-plate, well-to-well, and the interaction between plate-to-plate variation and the dose of E2. Nevertheless, only reproducibility in technical performance was considered in deCastro's study. To the best of our knowledge, this is the first paper evaluating the impact of a biological-based highly variable material such as different sera and different sources of cells using this statistical methodology.

The most common evaluation of toxicological tests based on a stimulated biological answer is the FI parameter, indicating how many times stronger the stimulated cell answer is in comparison to the appropriate control. However, this parameter could be considerably affected by differences in the basic protocol and the material variability, which was also proven within this work. Therefore, the test evaluation could be misinterpreted, and the test sensitivity could be undervalued. The most variable material based on biological origin (chsFBS and cell line) was finally evaluated in the general analysis, where all the experiments performed in DMEM were considered. First, the comparison of the means showed great differences among the six treatment variants (three cell lines, two sera). Nevertheless, among all possible interaction calculations, MLM did not permit evidence of any interaction among the parameters we tested. Because formal testing of all the interactions between all the tested experimental conditions was not possible (too many parameters to estimate and interpret, inducing a lack of power), we proposed a complementary analysis where only the test sensitivity (dose-response) was assessed after controlling for the effect of the combination of experimental parameters of each experiment. This normalization by the level of secretion in ethanol controls allows better control of the interactions between the experimental

conditions. This first step of the data normalization was not sufficient for comparing all the parameters. Indeed, the T47D(ATCC) curves demonstrated a lower magnitude than the other cell lines. This fact could be due to external factors of the remaining interaction. However, when a second normalization step where all these values were subsequently expressed in percent of E2 at the concentration of 1.0 10^{-9} M was carried out, all the curves became identical. This analysis revealed much lower variability among the tested cell lines and satisfactory reproducibility of the test. Step-by-step protocol (CXCL-test) optimization and subsequent MLM and "general" analyses enabled us to propose several suggestions applicable for interlaboratory transfer procedures and evaluation of the results.

5 Conclusion

The CXCL-test, one of only a few *in vitro* bioassays for estrogenic activity that are not based on a genetically modified organism, was optimized, and the impact of variable material used in the test, the test procedure and the data interpretation were evaluated within this work to facilitate interlaboratory protocol transfer.

All the tested parameters demonstrated a significant impact on CXCL12 secretion, the outcome parameter of the test. The step-by-step optimization allowed us to formulate the most suitable protocol for the CXCL-test performance. The T47D(IBT) cell line was considered the most suitable cell line for further experiments. However, the variability of biological parameters, such as the cell line or serum, makes the definite recommendation of a particular serum or cell line inapplicable, further demonstrating the necessity for the optimization, for instance, of each new batch of serum. Nonetheless, the remaining parameter optimization clearly showed that the seeding of the 20,000 cell/well directly in the phenol red-free treatment medium and DMEM used as an exposure medium were the most suitable parameters for the CXCL-test.

Moreover, not only the impact of the material variability and the methodology procedure should be considered in the interlaboratory protocol adaptation but also the way data interpretation affects the final test results. For instance, the commonly used evaluation parameter FI was influenced by the material variability, and an innovative approach of the test assessment was determined. MLM analyses and two-step normalization decreased the variability and increased the reproducibility of the CXCL-test results. This statistical method

provides an exploration of the factors involved in EDC evaluation and could be applied, for instance, in the assessment of the mixture of molecules (e.g., synergetic antagonism evaluation). These general requirements should permit us to compare all results obtained even from different laboratories and could be generalizable to other bioassays usually used for EDC evaluation.

Declarations:

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Conflicts of interests

The authors declare that they have no conflict of interest.

Authors' contribution statements

Conceptualization: [Denis Habauzit], [Tomas Cajthaml]; Methodology: [Denis Habauzit], [Lucie Linhartova]; Formal analysis and investigation: [Lucie Linhartova], [Denis Habauzit], [Nathalie Costet]; Writing - original draft preparation: [Lucie Linhartova], [Denis Habauzit]; Writing - review and editing: [Lucie Linhartova], [Denis Habauzit], [Nathalie Costet], [Farzad Pakdel], [Tomas Cajthaml]; Funding acquisition: [Tomas Cajthaml]; Resources: [Tomas Cajthaml], [Farzad Pakdel]; Supervision: [Denis Habauzit], [Tomas Cajthaml]

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Table 1 CXCL12 secretion changes in experiments A-C (Fig. 1), where three T47D cell lines (ATCC, IBT, IRSET), two media (RPMI, DMEM), two charcoal-stripped fetal bovine sera (chsFBS, chsFBS(Bio) or chsFBS(Mer)), number of cells/well and different cell density before experiment were tested with or without estradiol (E2) treatments. The data were analyzed by a multivariate linear model (MLM).

		MLM analysis		
		Experiment A	Experiment B	Experiment C
Tested parameter		Added value in pg/ml (*p*-value)		
Cell line	T47D(ATCC)	0 (ref)	0 (ref)	0 (ref)
	T47D(IBT)	110±24 (****)	83±11 (****)	9±10 (ns)
	T47D(IRSET)	145±24 (****)	150±8 (****)	76±10 (****)
Medium	RPMI	0 (ref)	0 (ref)	na
	DMEM	75±15 (****)	39±7 (****)	
Serum	chsFBS(Mer)	0 (ref)	0 (ref)	0 (ref)
	chsFBS(Bio)	22±14 (ns)	17±7 (*)	39±8 (****)
E2	Ethanol	0 (ref)	0 (ref)	0 (ref)
	1.0 10^{-9} M	195±25 (****)	108±13 (****)	221±16 (****)
	1.5 10^{-10} M	186±25 (****)	108±13 (****)	215±16 (****)
	4.4 10^{-11} M	174±25 (****)	97±13 (****)	194±16 (****)
	2.8 10^{-11} M	163±25 (****)	94±13 (****)	195±16 (****)
	8.8 10^{-12} M	100±25 (****)	47±13 (***)	115±16 (****)
	2.5 10^{-12} M	na	18±15 (ns)	28±16 (ns)
Cells/well	10,000	na	0 (ref)	na
	20,000		119±7 (****)	
Cell density	High	na	na	0 (ref)
	Low			31±8 (***)
Replicate	Experimental	9±6 (ns)	1±1 (ns)	2±2 (ns)
	Biological	11±24 (ns)	60±17 (***)	22±8 (**)

na = not analysed; ns = not significant; ref = reference; $p>0.05$ ns; $p\leq0.05$ *; $p\leq0.01$ **; $p\leq0.001$ ***; $p\leq0.0001$ ****

Table 2 CXCL12 secretion changes in three T47D cell lines (ATCC, IBT, IRSET) in experiments A–C, where two media (RPMI, DMEM), two charcoal-stripped fetal bovine sera (chsFBS, chsFBS(Bio) or chsFBS(Mer)), number of cells/well and different cell density before experiment were tested in estradiol (E2) treatment. The data were analyzed by a multivariate linear model (MLM).

		MLM analysis								
		Experiment A			Experiment B			Experiment C		
		T47D(ATCC)	T47D(IBT)	T47D(IRSET)	T47D(ATCC)	T47D(IBT)	T47D(IRSET)	T47D(ATCC)	T47D(IBT)	T47D(IRSET)
Tested parameter		pg/ml (*p*-value)								
Medium	RPMI	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	na	na	na
	DMEM	32±11 (**)	165±30 (****)	34±16 (*)	26± 4 (****)	34±9 (***)	56±12 (****)			
Serum	chsFBS(Mer)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	chsFBS(Bio)	49±9 (****)	-80±30 (*)	81±16 (****)	2±4 (ns)	8±7 (ns)	39±12 (**)	36±10 (***)	24±10 (*)	56±5 (****)
E2	Ethanol	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)	0 (ref)
	1.0 10^{-9} M	170±17 (****)	249±53 (****)	180±27 (****)	47±8 (****)	120±13 (****)	160±23 (****)	189±18 (****)	232±18 (****)	242±8 (****)
	1.5 10^{-10} M	178±17 (****)	215±53 (***)	170±27 (****)	55±8 (****)	118±13 (****)	154±23 (****)	200±18 (****)	221±18 (****)	228±8 (****)
	4.4 10^{-11} M	166±17 (****)	205±53 (***)	155±27 (****)	43±8 (****)	109±13 (****)	141±23 (****)	177±18 (****)	195±18 (****)	211±8 (****)
	2.8 10^{-11} M	142±17 (****)	198±53 (**)	160±27 (****)	44±8 (****)	103±13 (****)	136±22 (****)	179±18 (****)	192±18 (****)	215±8 (****)
	8.8 10^{-12} M	70±17 (****)	135±53 (*)	110±27 (***)	19±8 (*)	54±13 (****)	67±22 (**)	105±18 (****)	107±18 (****)	132±8 (****)
	2.5 10^{-12} M	na	na	na	20±10 (ns)	9±13 (ns)	38±29 (ns)	37±18 (*)	22±18 (ns)	26±8 (**)
Cells/well	10,000	na	na	na	0 (ref)	0 (ref)	0 (ref)	na	na	na
	20,000				39±4 (****)	106±7 (****)	210±12 (****)			
Cell density	High	na	na	na	na	na	na	0 (ref)	0 (ref)	0 (ref)
	Low							30±10 (**)	44±10 (****)	19±5 (****)
Replicate	Experimental	7±6 (ns)	29±14 (ns)	17±11 (ns)	2±1 (ns)	6±2 (**)	1±3 (ns)	4±3 (ns)	4±2 (ns)	1±3 (ns)
	Biological	33±12 (**)	na	na	0±5 (ns)	42±9 (****)	34±13 (*)	115±10 (****)	40±10 (****)	90±5 (****)

na = not analysed; ns = not significant; ref = reference; $p>0.05$ ns; $p\leq0.05$ *; $p\leq0.01$ **; $p\leq0.001$ ***; $p\leq0.0001$ ****

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

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Viability test methodology

The cell viability was assessed according to the Boaru *et al.'s* protocol (Boaru et al. 2006) and slightly modified. Briefly, the cells were rinsed once by 200 µl of Dulbecco's phosphate–buffered saline (DPBS). Then 100 µl of Hanks' balanced salt solution (HBSS, Gibco) containing two fluorescent dyes: AlamarBlue™ Cell viability reagent (AB, 1.25%, Invitrogen, USA) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, 1 µM, Invitrogen) was incubated with the cells for 90 minutes at 37 °C and 5% CO_2 in humidified incubator. The fluorescence was determined by Infinite M200 PRO microplate reader (Tecan). The excitation/emission wavelengths for AB and CFDA-AM assay were 532/590 nm and 485/535 nm, respectively.

Table S1: Media composition

	Medium Composition	Supplier, Catalogue number
Culture medium	RPMI 1640	Gibco, 21875
	10% FBS	Gibco, 10270
	1% NEAA	Gibco, 11140
	1% Sodium Pyruvate	Gibco, 11360
	1% PS	Gibco, 15140
Treatment medium (RPMI)	RPMI 1640	Gibco, 32404
	2.5% chsFBS	Merck, F6765 Biowest, S181F
	GlutaMAX™	Gibco, 35050
	1% PS	Gibco, 15140
Treatment medium (DMEM)	DMEM	Gibco, 31053
	2.5% chsFBS	Merck, F6765 Biowest, S181F
	1% NEAA	Gibco, 11140
	1% Sodium Pyruvate	Gibco, 11360
	1% Glutamine	Gibco, 25030
	1% PS	Gibco, 15140
Viability medium	HBSS	Gibco, 14175
	1.25% AB	Invitrogen, DAL1025
	1 µM CFDA	Invitrogen, C1354
Rinsing buffer	DPBS	Gibco, 14190

FBS = fetal bovine serum, NEAA = non-essential amino acids, PS = Penicillin-Streptomycin, chsFBS = charcoal-stripped FBS, HBSS = Hanks' balanced salt solution, AB = AlamarBlue™ Cell viability reagent, CFDA = 5-carboxyfluorescein diacetate acetoxymethyl ester, DPBS = Dulbecco's phosphate-buffered saline

Table S2: Comparation of commercial phenol red-free RPMI 1640 (Gibco, Catalogue number 32404) and DMEM (Gibco, Catalogue number 31053), which were used for treatment media preparation.

Component	Concentration [mM]	
	RPMI 1640	DMEM
Glycine	0.133	0.400
L-Arginine	1.149	0.398
L-Cystine	0.083	0.201
L-Isoleucine	0.382	0.802
L-Leucine	0.382	0.802
L-Lysine	0.274	0.798
L-Methionine	0.101	0.201
L-Phenylalanine	0.091	0.400
L-Serine	0.286	0.400
L-Threonine	0.168	0.798
L-Tyrosine	0.110	0.398
L-Valine	0.171	0.803
L-Tryptophan	0.025	0.078
L-Histidine	0.097	0.200
L-Asparagine	0.379	-
L-Aspartic acid	0.150	-
L-Glutamic acid	0.136	-
L-Hydroxyproline	0.153	-
L-Proline	0.174	-
Choline chloride	0.021	0.029
D-Calcium pantothenate	0.001	0.008
Folic Acid	0.002	0.009
Niacinamide	0.008	0.033
Pyridoxine hydrochloride	0.005	0.019
Riboflavin	0.001	0.001
Thiamine hydrochloride	0.003	0.012
i-Inositol	0.194	0.040
Biotin	0.001	-
Para-Aminobenzoic acid	0.007	-
Vitamin B12	0.000	-
Calcium nitrate	0.424	-
Calcium chloride	-	1.802
Magnesium sulfate	0.407	0.814
Potassium chloride	5.333	5.333
Sodium bicarbonate	23.810	44
Sodium chloride	103	110
Sodium phosphate dibasic	5.63	-
Sodium phosphate monobasic	-	0.91
Ferric nitrate	-	0.00025
D-Glucose	11.1	25
Glutathione (reduced)	0.003	-

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Assessment of agonistic and antagonistic properties of widely used oral care antimicrobial substances toward steroid estrogenic and androgenic receptors

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HIGHLIGHTS

- Steroid receptor interactions with oral care product ingredients were investigated.
- Novel cell line AIZ-AR was employed to assess androgen receptor-binding activities.
- Endocrine disruption potential was indicated for some antimicrobial agents.
- Antiestro/androgenic properties were proven by human cell lines and yeast assays.
- Hazard quotients were calculated and suggest potential risk to the environment.

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ABSTRACT

Personal care product consumption has increased in the last decades. A typical representative ingredient, i.e., triclosan, was identified in the scientific literature as an endocrine disruptor, and its use is restricted in several applications. Oral hygiene formulations contain various compounds, including synthetic phenol derivatives, quaternary ammonium compounds (QACs), various amides and amines, or natural essential oils containing terpenes. The aim of this paper was to explore possible endocrine-disrupting effects of these most-used compounds. For this purpose, two different assays based on recombinant yeast (BMAEREluc/ERα; BMAEREluc/AR) and human cell lines (T47D; AIZ-AR) were employed to investigate the agonistic and antagonistic properties of these compounds on human estrogen and androgen receptors. The results showed that none of the compounds were indicated as agonists of the steroid receptors. However, octenidine (OCT, QAC-like) and hexadecylpyridinium (HDP, QAC) were able to completely inhibit both androgenic (IC_{50} OCT = 0.84 μM; IC_{50} HDP = 1.66 μM) and estrogenic (IC_{50} OCT = 0.50 μM; IC_{50} HDP = 1.64 μM) signaling pathways in a dose-dependent manner. Additionally, chlorhexidine was found to inhibit the 17β-estradiol response, with a similar IC_{50} (2.9 μM). In contrast, the natural terpenes thymol and menthol were found to be competitive antagonists of the receptors; however, their IC_{50} values were higher (by orders of magnitude). We tried to estimate the risk associated with the presence of these compounds in environmental matrices by calculating hazard quotients (HQs), and the calculated HQs were found to be close to or greater than 1 only when predicted environmental concentrations were used for surface waters.

1. Introduction

Antimicrobial agents contained in oral hygiene products (toothpastes and mouthwashes) help to control or reduce gingivitis, plaque, tooth decay and *halitosis* (bad breath) by eliminating infectious pathogens (Tan et al., 2002; Claffey, 2003; Latimer et al., 2015; Malhotra et al., 2016; Welk et al., 2016; Marchetti et al., 2017).

Abbreviations: 17β-estradiol, (E2); chlorhexidine, (CHX); dihydrotestosterone, (DHT); dimethyl sulfoxide, (DMSO); eucalyptol, (EUC); hexadecylpyridinium chloride, HDP); hexetidine, (HEX); human breast cancer cell line, (T47D); limonene, (LIM); menthol, (MEN); octenidine, (OCT); quaternary ammonium compounds, (QACs); sanguinarine, (SAN); stably transfected human reporter cell line, (AIZ-AR); stroma cell-derived factor 1, (SDF1 also referred as CXCL12); thymol, (THM); triclosan, (TCS); U.S. Food and Drug Administration, (FDA).

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In general, their influence on human health is believed to be mainly beneficial, and according to sales statistics, home dental care in order to maintain acceptable health standards is becoming a much more common practice. Toothpaste was the third leading health and personal care product (PCP) in the United States in 2017. It is also expected that 211 million of Americans will use mouthwash/dental rinse in 2020, suggesting a 10% increase in dental rinse consumers in the US within the period 2011–2020 (Statista, 2018).

Antiseptics and disinfectants, such as phenol and its derivatives, quaternary ammonium compounds (QACs), biguanides, amidines, and essential oils, are the main and most commonly used antimicrobial compounds to provide the therapeutic effect (Tan et al., 2002; Claffey, 2003). In addition, these compounds also might be found in soaps, hand washes, surface disinfectants, repellents or general cosmetic products, where they might also serve as preservatives, slight anesthetics and/or fragrances (Bakkali et al., 2008; de Sousa, 2011). They possess diverse modes of action for killing or inhibiting the growth of microorganisms such as bacteria, viruses and/or fungi (Tan et al., 2002; Koburger et al., 2010). In contrast to antibiotics, which are usually effective at low concentrations and against certain types of organisms, concentrations of the antimicrobial additives higher than the minimum inhibitory concentration or minimum bactericidal concentration are typically applied (White and McDermott, 2001).

In general, acute toxicity effects of existing or new antiseptics/disinfectants used in mouthwashes and toothpastes against various pathogens are well documented (Ellis, 2010; Koburger et al., 2010; Welk et al., 2016). Much less is known about their adverse health effect on humans or environmental impact after long-term exposure (e.g., endocrine disruption). Their extensive consumption in everyday household activities and their substantial increase in production worldwide could suggest possible contamination of aquatic ecosystems and bioaccumulation in biota.

The global market of antiseptics and disinfectants reached 6.4 billion USD in 2015, and a compound annual growth rate of 5.2% is predicted through 2022. Currently, a steady rate across the US and European markets is observed, while emerging markets (e.g., China, India, Africa, Brazil) show a slightly faster increase (BCC Research, 2017). An even higher profit is expected in the global essential oil market, which is expected to reach USD 11.67 billion by 2022 (Grand View Research, 2018).

Triclosan (TCS) is a frequent subject of investigation because of its persistence in the environment (Coogan et al., 2007; Kinney et al., 2008) and its association with hormonal activities (Veldhoen et al., 2006; Ahn et al., 2008). It has also been found in human urine, plasma and breast milk (Adolfsson-Erici et al., 2002; Azzouz et al., 2016). Chuanchuen et al. (2001) described cross-resistance between TCS and antibiotics. Irritant and allergenic properties of various antiseptics including TCS were reviewed by Lachapelle et al. (Lachapelle, 2014). Notably, the U.S. Food and Drug Administration (FDA) banned phenol and widely used phenol derivatives (including TCS) from over-the-counter consumer antiseptic products (FDA, 2016). As a result, the phenol and derivatives market will grow at a lesser rate (1.5%) through 2022 than was expected in a report from 2015 (7.6%) (BCC Research, 2017).

Adverse effects of TCS on humans and wildlife are evident. However, it is still permissible to use TCS in toothpastes, cosmetics, clothes, toys, and other products. Therefore, human exposure to TCS remains. Moreover, detailed data about the fate of antimicrobial alternatives used in toothpastes, mouthwashes and elsewhere are missing. Biguanide, chlorhexidine (CHX) and QAC, hexadecylpyridinium chloride (HDP), which are frequently used in mouthwashes, already have been detected in sewage sludge and wastewater effluents at ng/g and ng/L levels, respectively (Ostman et al., 2017). There are also demands for natural substances to be used in oral care hygiene. Essential oils are chemically or biologically synthesized and have been already detected in industrial waters and wastewaters, as well as surface waters (Escalas et al., 2003; Catanzaro et al., 2011; Klaschka et al., 2013). With respect to potential adverse effects of newly emerging pollutants, *in vitro* assays employing human cell lines and recombinant yeast assays are commonly used to assess interference of environmental pollutants with various types of receptors (Wang et al., 2012; Ezechias et al., 2012, 2016).

In this paper, nine selected antimicrobial compounds that are frequently used in oral care hygiene products, possessing various chemical structures, namely, HDP, octenidine (OCT), hexetidine (HEX), and sanguinarine (SAN) – associated with quaternary ammonium moiety; the biguanide CHX; and the terpenes limonene (LIM), menthol (MEN), thymol (THM), and eucalyptol (EUC), were systematically tested for their interactions with sex hormone steroid receptors. A stably transfected human reporter cell line containing endogenous human receptor (AIZ-AR) (Bartonkova et al., 2015) was employed to assess the transcriptional activity of the human androgen receptor. The T47D breast carcinoma cell line, using an altered secretion of cytokine CXCL12/SDF1 (Habauzit et al., 2010), allowed the determination of estrogen receptor binding activities. Additionally, recombinant yeast assays with the BMAEREluc/ERα and BMAEREluc/AR strains (Leskinen et al., 2005) also possessing human estrogen receptor and androgen receptor were carried out to provide complementary data related to endocrine disruption potential of the studied compounds.

2. Methods

2.1. Chemicals

CHX (≥99.5%), HDP monohydrate (99–102%), MEN (≥99%), 17β-estradiol (E2, ≥98%), 4,5α-dihydrotestosterone (DHT, ≥99%) were obtained from Sigma-Aldrich (Germany). OCT dihydrochloride (98%), THM (≥98%), LIM (97%), and 1,8-cineole (EUC, 99%) were obtained from Alfa Aesar (USA). HEX (mixture of stereoisomers) was obtained from Santa Cruz Biotechnology (USA). SAN chloride (≥98%) was obtained from the Cayman Chemical Company (USA). The tested compounds were diluted in dimethyl sulfoxide (DMSO, ≥99.9%, Sigma-Aldrich), while ethanol (96%, Penta, Czech Republic) and acetic acid (99%, Lachner, Czech Republic) were used for the dilution of the standards. D-luciferin was obtained from Biotech a.s. (Czech Republic).

2.2. Yeast strains and cell lines

The recombinant bioluminescent yeast strains *Saccharomyces cerevisiae* BMAEREluc/ERα, *S. cerevisiae* BMAERE/AR and *S. cerevisiae* BMA64luc were introduced by Leskinen (Leskinen et al., 2005) and were treated accordingly.

The human T47D breast carcinoma cell line was kindly donated by Dr. Truksa, Laboratory of Tumor Resistance, Czech Academy of Sciences. The cells were cultivated in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and the antibiotics penicillin, streptomycin, amphotericin and gentamicin (Invitrogen). The cell line was maintained at 37 °C in 5% CO_2, and the cells were subcultured twice a week.

The transgenic reporter gene cell line AIZ-AR (Bartonkova et al., 2015) was kindly provided by professor Dvorak, Department of Cell Biology and Genetics, Faculty of Science, Palacký University Olomouc, Czech Republic. The AIZ-AR cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 4 mM L-glutamine and 1 mM sodium pyruvate and the antibiotics penicillin

and streptomycin (Invitrogen). The cells were maintained at 37 °C and 5% CO_2, and they were subcultured twice a week. The growing medium was supplemented with the selection marker hygromycin B (0.5 mg/ml, Invitrogen) once per week.

2.3. Yeast assay

Recombinant strains of *S. cerevisiae* were used to assess the estrogen- and androgen-like activities of the tested compounds. The protocol by Leskinen (Leskinen et al., 2005) was applied and slightly modified as described previously (Ezechias et al., 2016). In brief, the strains *S. cerevisiae* BMAEREluc/ERα and *S. cerevisiae* BMAERE/AR produce luciferase extracellularly after stimulation of the estrogen and androgen receptors, respectively. During the exponential phase, the yeast strain was incubated for 2.5 h with the tested compounds and 3% DMSO (v/v) in the presence (antagonist mode) or in the absence (agonistic mode) of either E2 or DHT. After the incubation, the culture was shaken shortly, 100 µl of 1 mM D-luciferin solution was added, and the sample was immediately measured by a Lumino-M90a luminometer (ZD Dolní Újezd, Czech Republic). The integration time for luminescence detection was 60 s. All samples were tested in three parallels with the BMA64luc toxicity-control strain.

2.4. T47D-CXCL12 test and ELISA assay

The T47D cell line was employed to examine the transcriptional activity of estrogen receptor in the presence of the target chemicals. The assay was performed according to Habauzit et al. (2010). Briefly, the cells were plated in 24-well, with 7×10^4 cells per a well in phenol red-free RPMI 1640 medium with 5% charcoal-stripped FBS (Sigma-Aldrich) without antibiotics. The cells were treated with the tested compounds added in ethanol at concentrations given in Table 1. The final concentration of ethanol did not exceed 0.2% (v/v), and all of the samples were assessed in triplicate. Control samples containing only E2 (EC_{80}) in ethanol and only ethanol 0.1% (v/v) were also assayed on each plate. The secretion of CXCL12 was determined as a response of the cells to the presence of the estrogen receptor agonist. A full logistic curve for E2 was recorded, in which 9.18 pM of E2 reached 80% of the maximal response. For the antiestrogenic assay, the solution of E2 corresponding to EC_{80} was incubated with the tested compounds, and variations in the secretion of CXCL12 were monitored. Culture supernatants of each triplicate were pooled after 2-day exposition and were immediately determined by the enzyme-linked immunosorbent assay (ELISA). A Quantikine kit (R&D Systems, USA) following the manufacturer's instructions was employed for the ELISA test. The absorbance at 450 and 570 nm was monitored using a 96-well microplate reader (Infinite Pro, Tecan, Switzerland).

2.5. AIZ-AR reporter gene assay

A stably transfected gene reporter cell line AIZ-AR was used to evaluate the transcriptional activity of the androgen receptor. The assay was performed in 96-well cell culture microplates (Nunc, Thermo Scientific, USA) as described by Bartonkova (Bartonkova et al., 2015). The cells were incubated for 16 h in RPMI 1640 medium without antibiotics and fortified with 10% charcoal-stripped FBS (Sigma-Aldrich) instead of FBS, and afterward, they were exposed to the tested compounds dissolved in ethanol (maximal concentration in the wells did not exceed 0.2% v/v). The exposure was performed either in the presence or absence of DHT as an antagonistic or agonistic mode of the assay, respectively. After 24 h of incubation, the medium was removed, and the cells were lysed with lysis buffer (Promega, USA). The luciferase activity was measured on a luminometer, GloMax (Promega).

2.6. Toxicity tests

A viability criterion for the validity of the agonistic and antagonistic tests with the human cell lines and yeast recombinant strains was a minimum of 80% survival of the yeasts or human cells. The yeast toxicity assessment was performed with the control BMA64luc toxicity-control strain. To exclude toxic effects of the antimicrobial compounds toward the human cell lines (T47D, AIZ-AR), a trypan blue exclusion test of cell viability and an alamarBlue® toxicity test were used.

The trypan blue test is based on visualization of the dead cells by the dye. The cells were trypsinized, redissolved in the respective exposure medium and mixed with 0.4% trypan blue stain according to the manufacturer's protocol (Invitrogen). The amount of living cells was assessed with an automatic cell counter (Invitrogen).

The alamarBlue® toxicity test is based on the living cells ability to reduce resazurin to resorufin. The test was performed according to Lammel et al. (2013). Briefly, 96-well microplates were inoculated with the cell lines at a density of 5×10^4 per well. After a 24-h stabilization period, the cells were treated with the tested compounds and the solvents for 24 h. After the incubation, the cells were washed with phosphate-buffered saline (DPBS, Invitrogen) and incubated for 30 min with 1.25% (v/v) alamarBlue® (Invitrogen) in a phenol red-free and serum-free medium. Resorufin was measured spectrophotometrically at 532/590 nm (Infinite 200 Pro, Tecan).

2.7. Data analysis

In order to distinguish whether or not antagonism of compounds is competitive in nature, we applied the Schild analysis (Arunlakshana and Schild, 1959; Schild, 1949). This method involves the construction of two or more dose-response curves for agonist in the absence and presence of a fixed concentration of the antagonist. When the antagonists compete for the binding site of the receptor, we should observe: (1) parallel shifts to the right in the dose–response curves and (2) no decrease in the maximum response that can be achieved. However, clear deviation from parallel shifts in the dose–response curves and significant depression of the maximum response indicate that the antagonism is not competitive (Wyllie and Chen, 2007; Ezechias and Cajthaml, 2018a).

All results are expressed as the mean ± standard deviation. The dose-response data were fitted using four-parameter logistic curves and the inflection points of the curves represent the IC_{50} concentrations that induce half of the maximum effects (Ezechias and Cajthaml, 2018b). All statistical analyses and calculations were performed with OriginPro 8.5 (OriginLab, USA) software.

The hazard quotients (HQs) method was used to estimate the environmental toxicological risk related to the selected compounds. The HQ for each individual compound was calculated according to EU guidelines (European Commission, 2003), as a ratio between environmental concentrations of the antimicrobial agents with detectable endocrine activities derived from the human cell lines assays and predicted no-effect concentrations (PNECs). PNECs were estimated using determined IC_{50} values from the dose-response curves and after correction by an assessment factor of 1000 (European Commission, 2003).

Table 1
The IC_{50} values of the tested antimicrobial compounds and concentrations ranges applied in the human cell lines and recombinant yeast assays. All the experimental results are expressed as the mean ± standard deviation. The number of figures and decimal places were used according to the validity of the data.

Antiseptic compound/ Type of compound	Typical therapeutic concentration %(w/v)/μM	Tested concentration range or highest tested concentration (μM)				IC_{50} of antiestrogenic effect (μM)		IC_{50} of antiandrogenic effect (μM)	
		T47D-CXCL12 assay	Estrogenic yeast assay	AIZ-AR assay	Androgenic yeast assay	T47D-CXCL12 assay	Estrogenic yeast assay	AIZ-AR assay	Androgenic yeast assay
CHX	0.1–1	0.10–10	54	5.0	54	2.9 ± 0.2	ND	ND	ND
Biguanide	2300–20000								
OCT	0.1–2	0.016–1.6	0.45–270	0.08–1.6	0.0024–24	0.50 ± 0.04	1.4 ± 0.1	0.84 ± 0.01	4.4 ± 0.2
QAC-like	1800–36000								
HEX	0.1	3.0	20	3.0	20	ND	ND	ND	ND
QAC-like	2900								
HDP	0.07	0.014–2.9	0.82–490	0.14–2.8	1.3–38	1.64 ± 0.07	9.7 ± 0.9	1.66 ± 0.06	11.0 ± 0.2
QAC	2300–20000								
SAN	0.01	–	75	5.4	75	–	ND	ND	ND
QAC	300								
THM	0.06	1.6–500	30–1300	700	6.0–300	177 ± 39	237 ± 16	ND	73 ± 9
terpene	4300								
LIM	2.5–15	–	170	12	170	–	ND	ND	ND
terpene	180–1100								
MEN	0.04	–	3800	600	68–1000	–	ND	ND	571 ± 35
terpene	2700								
EUC	0.09	–	300	320	300	–	ND	ND	ND
terpene	6000								

3. Results

3.1. Estrogenic recombinant yeast assay

The results obtained from the recombinant *S. cerevisiae* BMAEREluc/ERα yeast assay showed that none of the measured compounds could trigger estrogenic effects even at the highest concentration levels used. On the other hand, three of the investigated compounds, OCT, HDP and THM, exhibited significant antiestrogenic effects. The observed effects of these substances were concentration-dependent, and almost entire dose-response inhibition curves were constructed. Higher concentrations of the compounds were toxic for the employed yeast strain, and therefore, these values could not be used. The observed dose-dependent curves are displayed in Fig. 1, and the respective IC_{50} values including the used concentrations ranges are shown in Table 1. Noteworthy, the most potent antiestrogenic effect was observed for OCT when this compound was followed by HDP and THM.

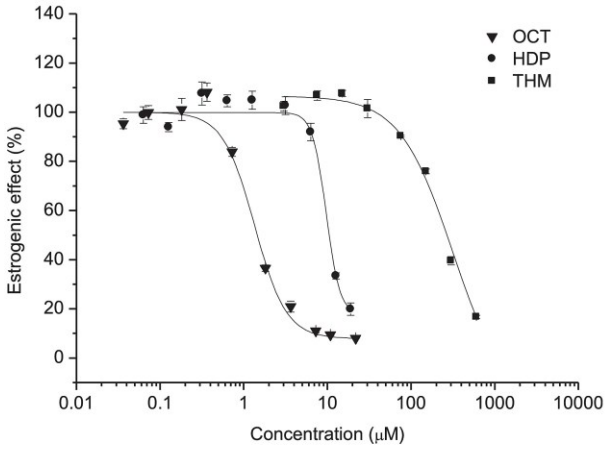

Fig. 1. Antiestrogenic activity of octenidine (OCT), hexadecylpyridinium (HDP) and thymol (THM) determined by the recombinant BMAEREluc/ERα yeast assay. 100% represents no inhibition of the 17β-estradiol response. The Y-error bars represent the standard deviations (n = 3).

3.2. T47D-CXCL12 test

The human cell line T47D was also used to confirm the estrogenic and antiestrogenic activities of the selected antimicrobial compounds. As well as in the case of this assay, we were not able to detect any estrogenic response of the test caused by the set of the compounds. However, we recorded an antiestrogenic effect of OCT, HDP, and THM, and additionally one of CHX. The dose-dependent manner of the compounds is visualized in Fig. 2, and the results are summarized in Table 1. Additionally, in this case, OCT was the most potent compound, followed by HDP, CHX and THM.

3.3. Androgenic recombinant yeast assay

We obtained very similar results using the *S. cerevisiae* BMAERE/AR, which enables measurement of the androgenic and antiandrogenic properties of organic compounds. None of the compounds exhibited detectable androgenic properties. However, OCT, HDP, THM, and MEN were indicated as antagonists of the androgenic receptor, and the respective dose-response curves were recorded (Fig. 3). The resulting IC_{50} values and the respective ranges of concentrations applied in the assay are shown in Table 1.

3.4. AIZ-AR assay

Two of the compounds also exhibited antiandrogenic properties, in the case of the AIZ-AR test employing human tissue cells. We were able to record dose-dependent antagonistic curves of OCT and HDP (Fig. 4 and Table 1) when higher concentrations of THM and MEN were already toxic for the assay.

3.5. Schild analysis

The Schild analysis was performed using OCT, HDP, THM and CHX. The concentrations of the used antagonists produced at least 50% inhibition of the respective inhibition curves. The details are provided in the supplementary material. The results of the Schild

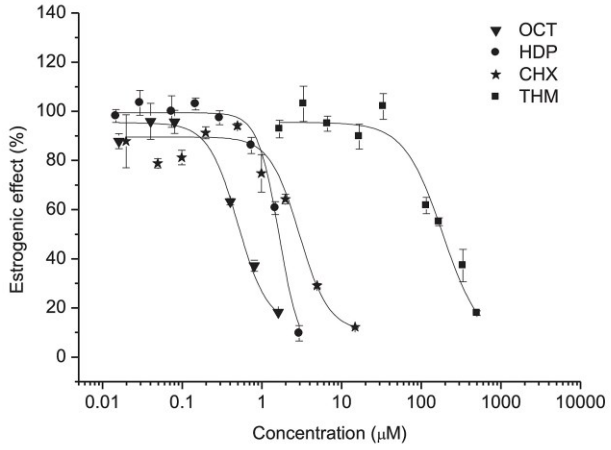

Fig. 2. Antiestrogenic activity of octenidine (OCT), hexadecylpyridinium (HDP), chlorhexidine (CHX), and thymol (THM) determined by the T47D-CXCL12 test. 100% represents no inhibition of the 17β-estradiol response. The Y-error bars represent the standard deviations (n = 3).

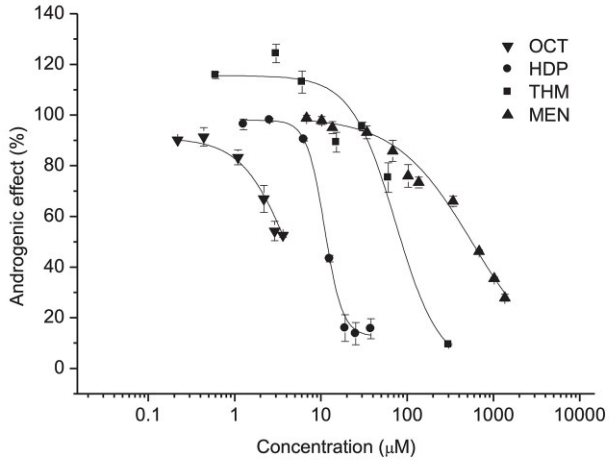

Fig. 3. Antiandrogenic activity of octenidine (OCT), hexadecylpyridinium (HDP), thymol (THM) and menthol (MEN) determined by the recombinant BMAEREluc/AR yeast assay. 100% represents no inhibition of dihydrotestosterone. The Y-error bars represent the standard deviations (n = 3).

analysis indicated, that THM and MEN competed with the natural ligands for the binding sites of the receptor and therefore the antagonism is competitive in nature. THM shifted the dose-response curve of the agonist and did not decrease the maxima in both estrogenic and androgenic yeast assays (Figs. S1 and S2). The same situation occurred for MEN measured by the androgenic recombinant yeast assay. On the other hand, OCT, HDP and CHX acted in a non-competitive manner. These compounds caused a significant decrease in the maximum responses in both estrogenic and androgenic assays and did not shift the EC_{50} of the agonist curves (Figs. S1 – S3).

3.6. Hazard quotients

With respect to the detected antiestrogenic and antiandrogenic properties of the compounds, we tried to estimate the HQs according to the European Commission (2003). The environmental

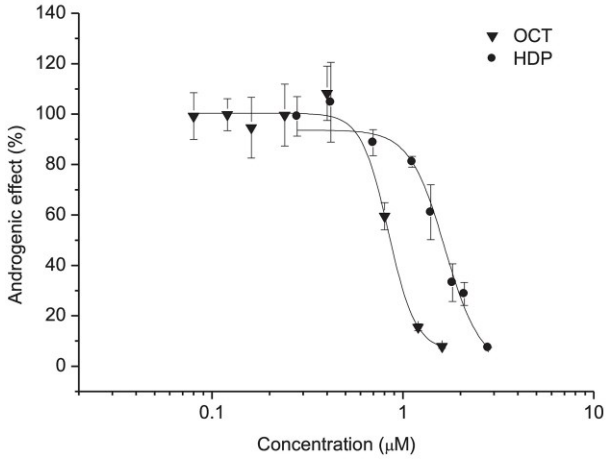

Fig. 4. Antiandrogenic activity of octenidine (OCT), hexadecylpyridinium (HDP) determined by the luminescence AIZ-AR cell line test. 100% represents no inhibition of dihydrotestosterone. The Y-error bars represent the standard deviations (n = 3).

concentrations detected in wastewater treatment plant (WWTP) effluents and predicted environmental concentrations (PECs) in surface waters and the HQs of antiestrogenic and antiandrogenic effects are shown in Table 2. The HQ values for MEN and THM, which possessed only weak inhibition activities, were close to zero in all cases, and these data were thus omitted form Table 2 to make the data summary more straightforward.

4. Discussion

In this study we focused on a group of antimicrobial compounds used in mouthwashes and toothpastes. According to our results, five of the nine tested compounds could be identified as inhibitors of steroid receptor signaling pathways (Table 1). The employed *in vitro* assays with human cell lines or recombinant yeasts are widely used tools to measure and demonstrate the estrogenic or androgenic activity of various compounds (Svobodova and Cajthaml, 2010; Orton et al., 2011; Ezechias et al., 2016). Using these assays, we observed that none of the oral care antimicrobial compounds have the ability to trigger estrogenic or androgenic effects. On the other hand, we observed a significant inhibitory effect of some of the compounds towards the estrogen or androgen receptors.

The receptor antagonistic effect was revealed for THM and MEN. Shifts in the dose-response curve of the agonist and no changes in the maximum responses were observed while applying the Schild analysis (Figs. S1 and S2), that suggested the direct interaction with the binding sites of the receptors. Noteworthy, the THM antagonistic effect was not observed in the case of the AIZ-AR human cell line, which is probably due to sensitivity of the assay when higher applied concentrations were toxic to the cell line. The same explanation is applicable for MEN when its antagonistic properties were recorded only in the case *S. cerevisiae* BMAERE/AR test at high concentrations, resulting in an IC_{50} equal to 571 μM.

We observed another situation in the case of CHX. This compound was able to inhibit the estrogen signal only when the human cell line was employed; however, the yeast recombinant test did not indicate any effect. According to the Schild analysis, CHX acted as a non-competitive antagonist. The non-competitive nature of the antagonism could explain the differences between the recombinant yeast and human cell line assays. In fact, signaling pathways in

Table 2
Hazard quotients (HQs) calculated according to the European Commission (2003) using environmental concentrations[a] in wastewater treatment plant (WWTP) effluents (maximum values) and predicted environmental concentrations[b] in surface waters.

	Effluent of WWTP[a] μg/L	Reference	Surface water[b] μg/L	Reference	PNEC μg/L		HQ Effluent of WWTP		HQ Surface water	
					Antiestrogenic	Antiandrogenic	Antiestrogenic	Antiandrogenic	Antiestrogenic	Antiandrogenic
HDP	0.005	Ostman et al. (2017)	2.76	EFSA (2012)	0.50	0.51	0.01	0.01	5.53	5.46
CHX	0.031	Ostman et al. (2017)	0.35	Environment Canada (2013)	1.48	–	0.02	–	0.23	–
OCT	–		0.10	FGK (2001)	0.28	0.46	–	–	0.36	0.22

these assays slightly differ and CHX as a non-competitive antagonist seems to interact only with the human cell line cascade. The differences between yeast-based and mammalian *in vitro* assays regarding ligand potencies have been already reported in the literature. Impermeability of the yeast cells, the absence of cell-specific factors, yeast-strain-specific differences in metabolism, modulation of bioavailability by protein binding are among the most frequently discussed (Lyttle et al., 1992; Arnold et al., 1996; Gray et al., 1997; et al., 1999; Baker, 2001). Sensitivity of the yeast assays is generally an order of magnitude lower in comparison with mammalian *in vitro* assays (Zacharewski, 1997; Legler et al., 2002). On the other hand, yeasts cells are more resistant to environmental contaminants and thus more suitable for measurement of environmental samples characterized by complex matrices (Leskinen et al., 2005). The yeast-cell limitations mentioned above as well as lower sensitivity of the yeast assay might also explain our observation. Moreover, multiply charged character of CHX might have considerable impact on its bioavailability. Of course, the discrepancy between the results of the assays emphasizes the need for further research on the widely used oral care antimicrobial compound CHX in this direction.

OCT and HDP caused a significant decrease in the maximum responses of both estrogenic and androgenic assays (Figs. S1 and S2). Therefore, the Schild analysis indirectly proposed an inhibitory effect on transcriptional activity of the steroid receptors. Endocrine disrupting activities of these antimicrobial compounds are rarely described in the literature. To the best of our knowledge, this is the first report about the inhibitory effect of OCT towards steroid receptor signaling pathways. In addition, OCT demonstrated the most potent inhibition properties by both assays among the compounds under the study.

The observed HDP antiestrogenic properties are in accordance with a study of Datta et al. (2017), which confirmed inhibition of estrogen receptor by means of a luciferase-based assay using recombinant human breast carcinoma cells (VM7Luc4E2). The authors (Datta et al., 2017) also hypothesized a mechanistic relationship between the disruption of mitochondrial integrity and antiestrogenic activity of some QAC, since early biosynthesis of steroid hormones occurs in the mitochondrial matrix (Felty and Roy, 2005). A similar mechanism, *i.e.* inhibition of steroid hormone synthesis due to mitochondrial matrix disruption, might be also assumed for OCT and CHX. These compounds are positively charged at physiological conditions and due to this they can bind to the negatively-charged sites of biological membranes causing destabilization and interferences with osmosis. As a consequence, consumption of mitochondrial O_2 and ATP synthesis is compromised. (Szostak et al., 2018; Hidalgo and Dominguez, 2001). Indeed, an additional study has to be carried out to elucidate the mechanism of indirect effect of particular QAC on steroid receptors in detail.

Human receptor-binding properties of terpenes were studied in the context of essential oils used as culinary herbs and spices (Bartonkova and Dvorak, 2018b, a). It was indicated that essential oils of caraway and spearmint, for which LIM is a major constituent, inhibit the aryl hydrocarbon receptor (AhR). An opposite weak agonistic effect was observed with oil of dill, which has a high LIM content. Interestingly, LIM itself did not exhibit any affinity to the AhR receptor (Bartonkova and Dvorak, 2018b). Likewise, EUC was a potent AhR agonist only as a part of bay leaf oil, but EUC itself was considered inactive. Essential oils containing MEN as a major constituent were found to be AhR-inactive. The inconsistent results described for the individual compounds and for the mixtures suggest additional effects of other constituents. Such a phenomenon also might be assumed for antimicrobial compounds that exist in PCPs in combination with various other chemicals. Noteworthy, complex mixtures of hormone-disrupting compounds are typically detected in the environment. Therefore research in the field of mixture toxicology using novel mathematical models represents an important direction for understanding the overall effects of newly emerging pollutants (Ezechias and Cajthaml, 2016, 2018b).

Bartonkova et al. (Bartonkova and Dvorak, 2018b) also concluded that THM, a main component of thyme and oregano essential oils, is responsible for partial activation of AhR. We identified THM as being responsible for the inhibition of both steroid receptors (androgen and estrogen). In addition, Chen et al. (2007) observed inhibition of the transcriptional activity of testosterone caused by THM in a cell-based human androgen receptor-mediated bioassay. All of these findings support the theory proposing a relationship between AhR and estrogen receptor signaling pathways (Matthews and Gustafsson, 2006). Besides interaction of xenobiotics with other receptor systems such as AhR leading to cross-talks, there are several other mechanisms that can cause endocrine disruption. These mechanisms include, for example inhibition of steroid hormone synthesis or metabolism and interactions with transport proteins (Gillesby and Zacharewski, 1998). For instance, *in vitro* antiestrogenic activity of QACs benzalkonium chloride and HDP mediated by mitochondrial disruption was described by Datta et al. (2017). Azole fungicides were showed to inhibit cytochrome P450 aromatase, which catalyzes biosynthesis of estrogens from androgens (Egbuta et al., 2014). Surfactants with alkylphenol moiety 4-nonylphenol and 4-tertoctylphenol were identified as potent ligands of human plasma sex-hormone binding globulin (hSHBG). These compounds can displace endogenous sex steroid hormones from hSHBG binding sites and disrupt the androgen-to-estrogen balance (Dechaud et al., 1999). The effect of quaternary benzo[c]phenanthridine alkaloids sanguinarine and chelerythrine on the AhR signaling pathway were comprehensively studied by Dvorak et al. (2006). The activity evaluated by means of rat hepatoma cell line H4IIE.luc was not affected by the studied compounds. The findings of their study favored rather inactivity or modest inhibitory effect of these compounds on AhR signaling pathways. This might for instance involve protein kinases, which are also one of the quaternary benzo[c]phenanthridine alkaloids targets (Wang et al., 1997).

The global and frequent use of these antimicrobial compounds is accompanied by their high production. If we consider our target

group of the chemicals, terpenes belong among the most-produced PCP ingredients. According to the European Chemicals Agency (ECHA, 2018a, b, c), 10–100 thousand tons of MEN per year (ECHA, 2018a) are manufactured by or imported to the EU. Not naturally occurring, HDP is the substance with the highest production rate (100–1000 tons per year (ECHA, 2018b). The lowest production (up to ten tones) is recorded for OCT (ECHA, 2018c), which is rather new on the market, and it is preferably used in clinical practice so far (Obermeier et al., 2015). However, the advantages of OCT over the other traditional compounds used in mouthwashes are already discussed in the literature (Malhotra et al., 2016; Welk et al., 2016).

After the quantification of the so far unreported adverse effects of the oral hygiene antimicrobial compounds under the present study, HQs were calculated using environmental concentrations and PNEC values. PNECs were derived from the results of the human cell lines toxicity data described in this manuscript, representing acute toxicity. The IC_{50} values derived from the human cell lines assays (Table 1) representing the worst-case situation for the respective assays were divided by an assessment factor 1000 to calculate the PNECs. Either maximum environmental concentrations (MECs) or PECs available in the literature were used for the risk assessment expressed by HQs. In fact, uniform input values (measured or predicted concentration) would be appropriate for the HQ calculation to compare relevance between surface waters and WWTP effluents. Nevertheless, we did not find such data for the combinations of the studied compounds in the literature. Our results indicate that the studied compounds showing endocrine-disrupting properties likely pose no risk in WWTP effluent (Table 2). In these cases, in which we used published MECs, all of the HQs were close to 0. However, for the surface water environment we had to use more restrictive PEC values, and the HQs of CHX and OCT were in the intervals of 0.1 and 1, where the risk is usually considered as low but potential (European Commission, 2003). In the case of HDP, HQs greater than 1 were recorded. The results show that HDP is of environmental concern, and further investigation to provide more reliable data on its fate and behavior should be carried out.

5. Conclusion

The results of this study show that seemingly safe antimicrobial compounds used in oral care hygiene can represent a certain toxicological and environmental risk. The data document that antimicrobial agents most frequently contained in oral hygiene products can interact with the human estrogen and androgen receptors. The results of these receptor-binding properties were proven with two different assays, and especially synthetic QAC-like and QAC represented by OCT and HDP, respectively, were indicated to be able to inhibit both the androgenic and estrogenic signaling pathways in a dose-dependent manner. Apart from this, biguanide CHX was able to inhibit the estrogen signal only, and its direct interaction with human estrogen receptor is not clear. Natural terpenes, represented by THM and MEN, were found to be much weaker competitive inhibitors (in order of magnitude) of these receptors in comparison with non-competitive inhibitors OCT and HDP.

In addition, we tried to estimate the risk associated with presence of the compounds in environmental matrices by calculating HQs. Nevertheless, the data about their presence in the environment are limited, and when we used real detected concentrations in WWTP effluents, the HQs values were low, suggesting no risk. Despite the calculated HQs were found close to or greater than 1 only when predicted PEC values for surface waters were used, it is expected that the production and consumption of these compounds will increase in the next years. Accordingly, these results emphasize a need for the systematic monitoring of these compounds in the environment, including that of their fate and degradation processes, to eliminate their concentrations in the environment.

Considering that these compounds almost gradually replaced triclosan as a proved endocrine disruptor in oral hygiene products, the situation does not seem better for the future. These results also pose questions regarding their use in mixtures and possible additional effects of the compounds on humans and, generally, on ecosystems. Vice versa, the results showing dramatically different antagonistic potencies of the individual compounds can serve as a manual for “greener chemistry” improved formulation of personal care hygiene products.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.11.006.

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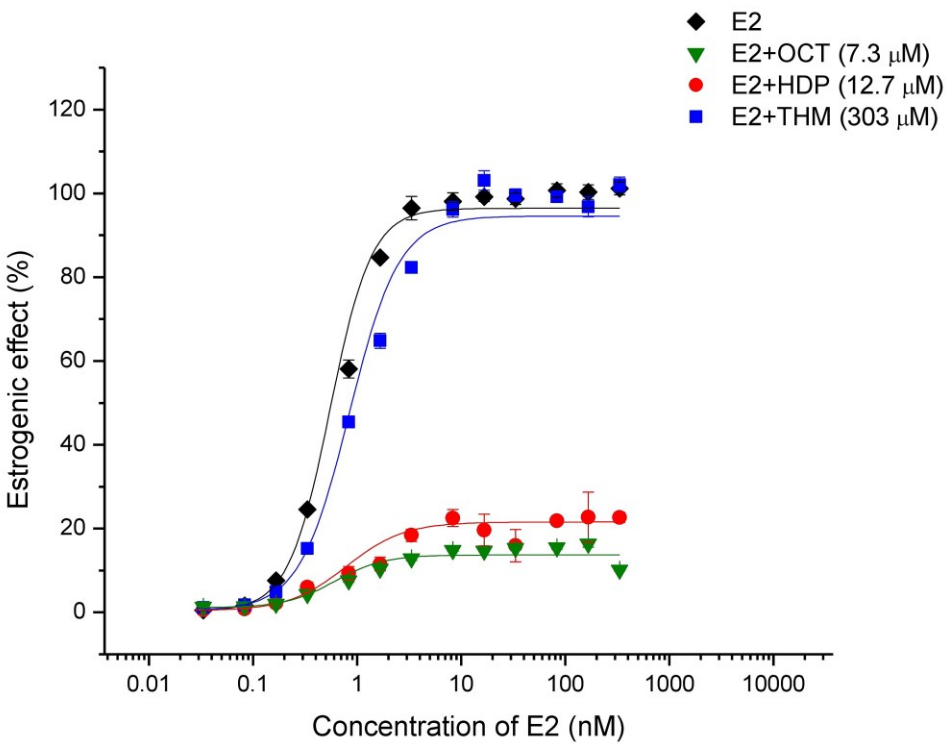

Figure S1 The Schild analysis using the yeast estrogenic assay; Dose-response curves of 17β-estradiol (E2) in the presence and absence of fixed concentrations of antagonists: octenidine (OCT), hexadecylpyridinium (HDP) and thymol (THM). The concentrations of the used antagonists produce at least 50% inhibition (IC_{50}) of the respective inhibition curves. Compounds OCT and HDP significantly reduce the maximum effect of E2. The nominal EC_{50} for curves are 0.55 ± 0.01 nM, 0.61 ± 0.10 nM, 0.79 ± 0.15 nM and 0.84 ± 0.05 nM for E2, E2+OCT, E2+HDP and E2+THM, respectively. The Y-error bars represent the standard deviations (n = 3).

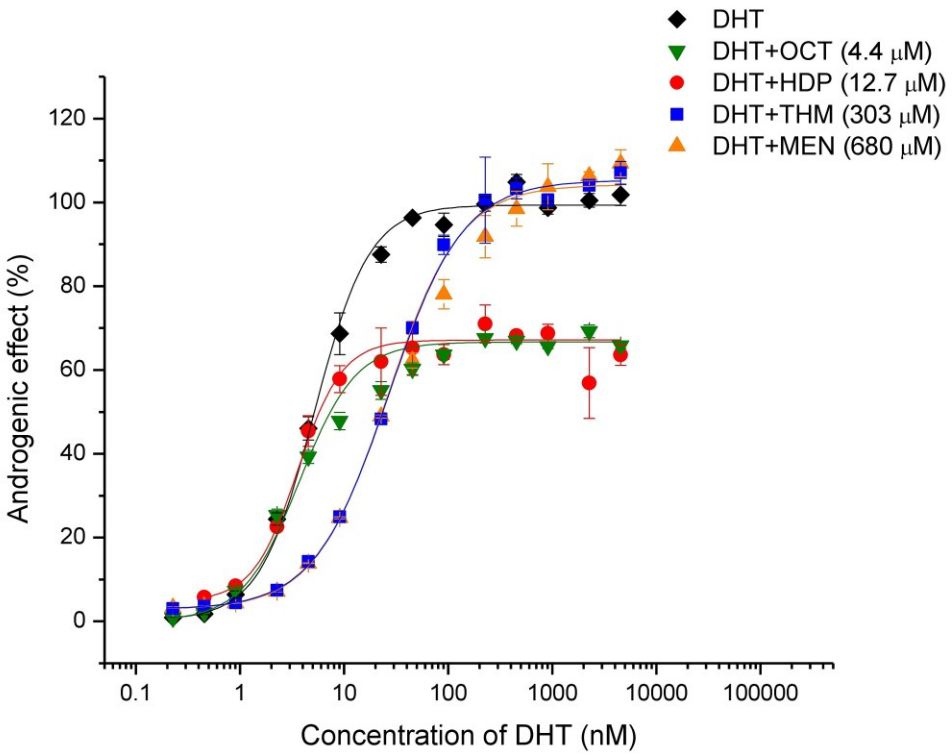

Figure S2 The Schild analysis using the yeast androgenic assay; Dose-response curves of dihydrotestosterone (DHT) in the presence and absence of fixed concentrations of antagonists: octenidine (OCT), hexadecylpyridinium (HDP) and thymol (THM). The concentrations of the used antagonists produce at least 50% inhibition (IC_{50}) of the respective inhibition curves. Compounds OCT and HDP significantly reduce the maximum effect of DHT. The nominal EC_{50} for curves are 5.17 ± 0.43 nM, 3.71 ± 0.40 nM, 3.58 ± 0.16 nM, 26.0 ± 0.9 nM and 26.8 ± 1.4 nM for DHT, DHT+OCT, DHT+HDP, DHT+THM and DTH+MEN, respectively. The Y-error bars represent the standard deviations (n = 3).

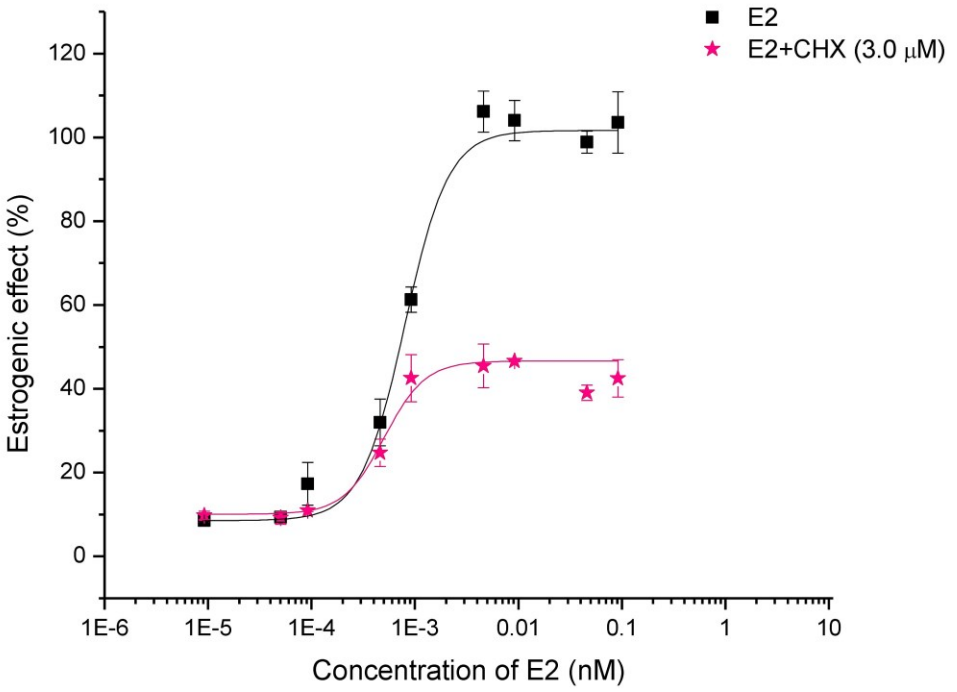

Figure S3 The Schild analysis using the T47D-CXCL12 assay; Dose-response curves of 17β-estradiol (E2) in the presence and absence of fixed concentration of chlorhexidine (CHX). The concentrations of the used antagonists produce at least 50% inhibition (IC_{50}) of the respective inhibition curves. The nominal EC_{50} for curves are 0.80 ± 0.06 pM and 0.50 ± 0.16 pM for E2 and E2+CHX, respectively. CHX significantly reduce the maximum effect of E2. The Y-error bars represent the standard deviations (n = 3).

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Article

Biodegradability of Dental Care Antimicrobial Agents Chlorhexidine and Octenidine by Ligninolytic Fungi

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Abstract: Chlorhexidine (CHX) and octenidine (OCT), antimicrobial compounds used in oral care products (toothpastes and mouthwashes), were recently revealed to interfere with human sex hormone receptor pathways. Experiments employing model organisms—white-rot fungi *Irpex lacteus* and *Pleurotus ostreatus*—were carried out in order to investigate the biodegradability of these endocrine-disrupting compounds and the capability of the fungi and their extracellular enzyme apparatuses to biodegrade CHX and OCT. Up to 70% ± 6% of CHX was eliminated in comparison with a heat-killed control after 21 days of in vivo incubation. An additional in vitro experiment confirmed manganese-dependent peroxidase and laccase are partially responsible for the removal of CHX. Up to 48% ± 7% of OCT was removed in the same in vivo experiment, but the strong sorption of OCT on fungal biomass prevented a clear evaluation of the involvement of the fungi or extracellular enzymes. On the other hand, metabolites indicating the enzymatic transformation of both CHX and OCT were detected and their chemical structures were proposed by means of liquid chromatography–mass spectrometry. Complete biodegradation by the ligninolytic fungi was not achieved for any of the studied analytes, which emphasizes their recalcitrant character with low possibility to be removed from the environment.

Keywords: chlorhexidine; dental hygiene; laccase; manganese-dependent peroxidase; octenidine; ligninolytic fungi; personal care products; quaternary ammonium compounds; recalcitrant pollutant

1. Introduction

Antiseptics and disinfectants, whose worldwide consumption is increasing year by year, rank among the most intensively studied trace organic contaminants [1]. The wide range of their usage in daily urban activities, including dental hygiene, contributes to the continual release of these biologically active compounds into the environment in amounts that lack any control or restrictions. Quaternary ammonium compounds (QACs), such as hexadecylpyridinium chloride (HDP) and octenidine (OCT), phenolic derivatives (e.g., triclosan, TCS), and biguadines (e.g., chlorhexidine, CHX) belong among frequently used antiseptic compounds in toothpastes and mouthwashes and some of them were already detected in wastewater effluents [2,3].

The water solubility of these micropollutants has the potential to cause widespread contamination, usually at very low concentration levels (pg–µg/L). Due to the fact that wastewater treatment plant (WWTP) technologies are usually not designed for their removal, most disinfectants are not being eliminated [4,5]. Several studies demonstrated that antiseptics and disinfectants were released from WWTPs [2,6] and their ecotoxicological impact was observed. The increasing concentrations of

disinfectants have caused changes in microbial communities in polluted rivers [7], sediments [8], as well as in activated sludge [9]. Microbial resistance and/or cross-resistance with antibiotics was already described for some disinfectants [4,10,11]. Michalíková et al. [12] revealed antiestrogenic and/or antiandrogenic properties of dental care antiseptics CHX, OCT, and also HDP. The fate of these compounds in the environment is not yet fully understood. For instance, the removal of CHX and HDP in WWTPs was studied by several authors [9,13–16] with the same general conclusion that these compounds are not biodegraded during wastewater treatment processes. The removal of OCT has not been investigated so far. Moreover, the stability under different physical and chemical conditions and resistance to hydrolysis has been presented as a benefit of this novel disinfectant [17].

Various anthropogenic pollutants persist in the environment and scientific interest is generally aimed at the impacts on organisms and human health and also mechanistic studies of their degradability. Besides physicochemical degradation, a lot of attention is focused on biodegradation studies. In particular, microorganisms possess a wide range of metabolic pathways that have been shown to be very effective in the decomposition of pollutants.

Ligninolytic (white-rot) fungi are excellent model degraders, well-known for their efficiency in the decomposition of a broad range of xenobiotics, even those resistant to bacterial breakdown or generally hardly biodegradable, e.g., polychlorinated biphenyls [18,19], polycyclic aromatic hydrocarbons [20,21], chlorobenzoic acids [22], explosives [23], and also the most toxic organic pollutant known so far—2,3,7,8-tetrachlorodibenzodioxin [24]. The degradation ability of ligninolytic fungi to decompose xenobiotics has also been documented for dyes, several endocrine disruptors, pharmaceuticals (including antibiotics), plasticizers, UV filters, etc. [25–28].

Ligninolytic fungi possess a unique extracellular enzyme apparatus with low substrate specificity naturally targeted toward the degradation of the aromatic moieties of lignin. These extracellular enzymes, such as lignin peroxidase, manganese-dependent peroxidase (MnP), and the phenol oxidase laccase (Lac), catalyze nonspecific one-electron radical oxidations and have been shown to transform a wide range of organic pollutants. TCS, a chlorinated antimicrobial compound, was shown to be degraded in vitro by ligninolytic enzymes [29,30]. Baborová et al. [31] demonstrated the transformation of polycyclic aromatic hydrocarbons by MnP from *Irpex lacteus* and proved the formation of oxidized metabolites and aromatic ring cleavage. Extracellular enzymes are assumed to be responsible for the main degradation ability of ligninolytic fungi. Nevertheless, several authors have proven the role of the intracellular cytochrome P-450 of ligninolytic fungi in the transformations, e.g., with the synthetic hormone 17α-ethynilestradiol [28] and chlorobenzoic acids [32].

The aim of this work was to investigate the biodegradability of CHX and OCT, which are used in oral care products and were identified in our previous research as endocrine disruptors, by two model ligninolytic fungal strains, *I. lacteus* and *Pleurotus ostreatus*, i.e., species with proven biodegradation capabilities. To the best of our knowledge, no biodegradation study of these disinfectants by ligninolytic fungi has ever been carried out. The biodegradability of CHX and OCT was studied in vivo with fungal cultures grown in a liquid medium, as well as in vitro with concentrated extracellular liquids rich in ligninolytic enzymes. The samples were evaluated for the presence of metabolites, which is rarely described in the literature.

2. Results and Discussion

2.1. In Vivo CHX and OCT Transformation

Both CHX and OCT are designed to act against microorganisms. Thus, the highest nontoxic concentrations of both analytes towards the fungal strains were investigated as the first step of the assessment. Each compound was added in dimethyl sulfoxide (DMSO, final concentration 0.5% of DMSO) to the medium with the fungal suspension, and the weight of the biomass was compared to a control culture (0.5% DMSO) after 7 and 14 days (data not shown). The final concentrations of 3 and

2 μg/mL of CHX and OCT, respectively, were established as nontoxic concentrations for the growth of the studied fungal cultures.

The relationship between the presence of extracellular enzymes and degradation rates is often monitored in order to explain a part of the complex in vivo degradation mechanisms [33]. Hence, the most abundant enzymes were monitored throughout the entire in vivo experiment to observe possible relationships.

2.1.1. Chlorhexidine

MnP was the major extracellular enzyme in the culture of *I. lacteus* and was produced continuously with the activity level of 5 ± 1 U/L during the whole 21-day experiment. Even though *I. lacteus* was found to also produce extracellular Lac [33], the enzyme activity measured in the culture medium was usually low [34,35], which corresponds with our findings. The dominant enzyme in the culture of *P. ostreatus* was Lac with the initial activity of 33 ± 4 U/L, which decreased about five times after three weeks of cultivation (see Table S1 for details).

After 21 days of static in vivo cultivation of *I. lacteus* and *P. ostreatus* in a liquid medium, the residual amounts of CHX were 30% ± 6% and 43% ± 9%, respectively, in comparison with the respective heat-killed controls, HKCs (Figure 1a, left axis). The extraction recovery (the ratio of the concentration of the analyte determined in the HKC versus in the abiotic control − AC) indicated that 99% ± 2% and 100% ± 2% of CHX was extracted from the fungal cultures of *I. lacteus* and *P. ostreatus*, respectively (Figure 1a, right axis).

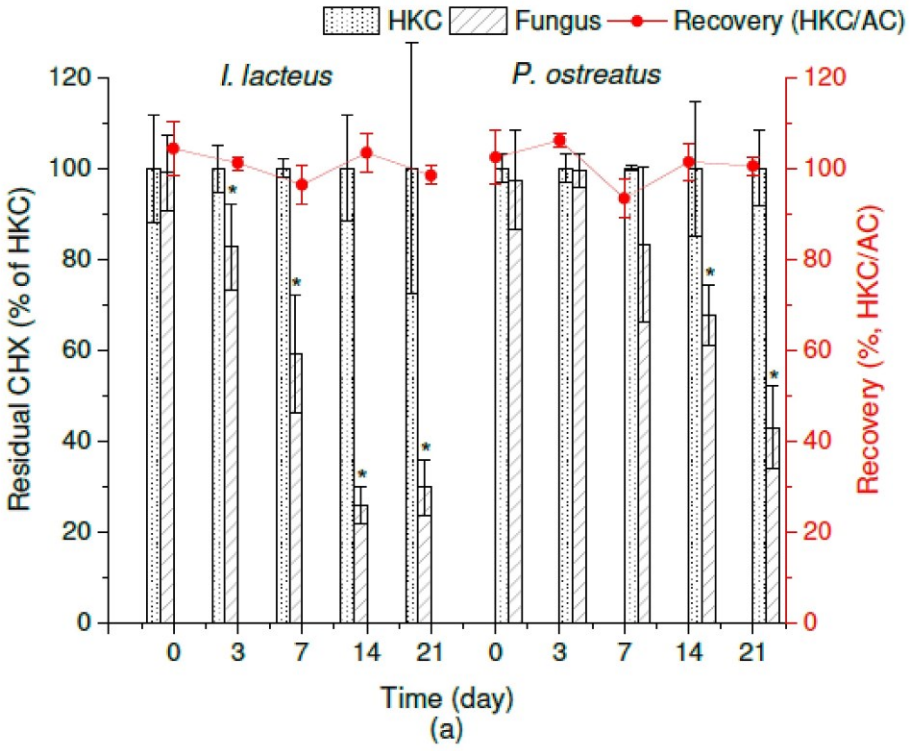


Figure 1. *Cont.*

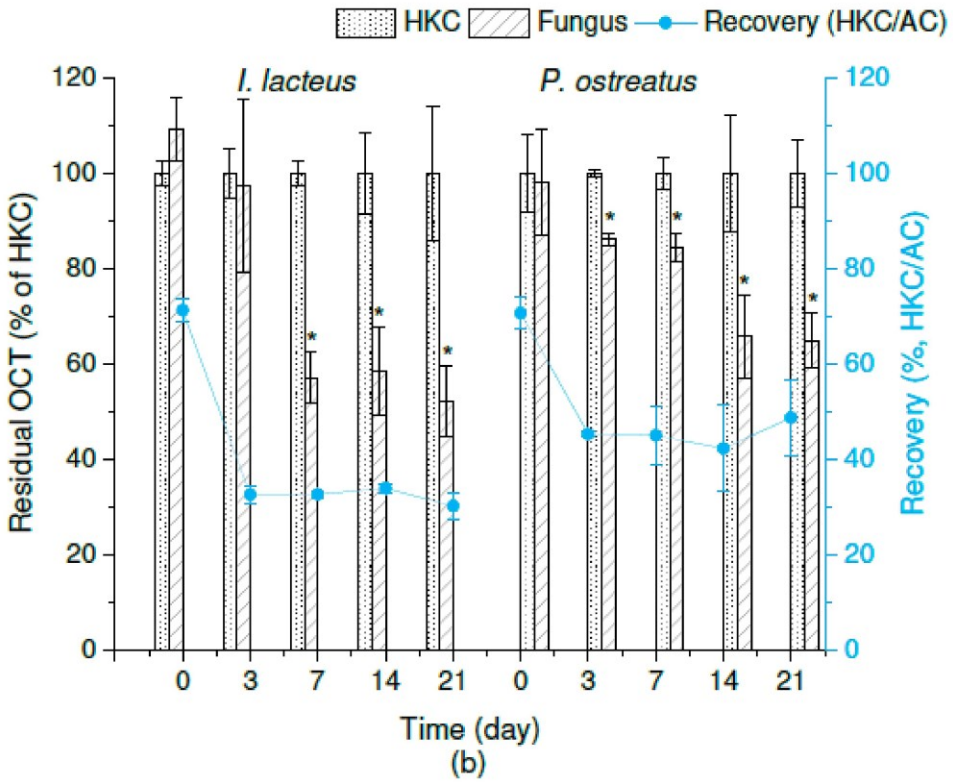


Figure 1. Residual concentration of (**a**) chlorhexidine (CHX) and (**b**) octenidine (OCT) after the 21-day in vivo degradation by *I. lacteus* and *P. ostreatus* related to the respective heat-killed controls (HKCs). The red (CHX) and blue (OCT) line graphs show extraction recovery during the experiment expressed as the HKC and abiotic control (AC) ratio. Error bars represent standard deviation (n = 3). The asterisk marks a significant difference between the individual harvesting day and the control group—0 d (post hoc Dunnett's Test).

Notably, the extraction solvent, 0.8% formic acid (FA) in 20% acetonitrile (ACN, *v*/*v*), together with sonication at elevated temperature improved CHX recovery from both fungal cultures by a factor of 1.4 (compared to the value obtained without any extraction). This result proposes the disruption of the ionic binding of the analyte to the negatively charged sites of the fungal cultures. The enhancement of the CHX recovery in acidic conditions are in accordance with the observation of Havlíková et al. [36].

A strong sorption phenomenon on the biomass of an activated sludge studied for its potential to remove CHX was observed under laboratory conditions [9]. The authors concluded that biosorption was mainly responsible for the CHX elimination. Similarly, a study of the mass balance of CHX in a WWTP revealed its 98% removal from the wastewater. In this specific case, the sorption to the sludge was the only mechanism of elimination and the authors highlighted the lack of CHX degradation [13]. The bioaccumulation of CHX in the lipids of both diatoms and bacteria in river biofilm communities was also observed and a subsequent stable isotope analysis indicated the absence of CHX mineralization [37,38]. Fortunato et al. [6] performed a degradation study with several adapted bacterial strains that were characterized and isolated from water samples in urban regions. CHX was found to be the most resistant and toxic compound in comparison with TCS and benzalkonium chloride. These observations emphasize the recalcitrant character of CHX and the low possibility of its removal from the environment. Microbial degradation of CHX was accomplished by bacterial isolates from an activated sludge in a study by Tanaka [39]. An 80% reduction of CHX antimicrobial activity

was achieved and the structure where pyruvate is bound to the CHX molecule was proposed as a less active intermediate.

2.1.2. Octenidine

In the case of OCT, the residual concentration of the analyte reached values of 52% ± 7% and 65% ± 6% in comparison with the HKC after 21-day cultivation in the cultures of *I. lacteus* and *P. ostreatus*, respectively (Figure 1b, left axis). The activities of the enzymes were comparable with the values reached in the experiment with CHX. MnP activity (2.6 ± 0.8 U/L) remained nearly constant until the termination of the experiment with *I. lacteus*, while the initial activity of Lac of *P. ostreatus* (31.5 ± 0.5 U/L) was suppressed approximately 10 times after the 21-day degradation of OCT.

A comparison of the extraction yields of OCT in the AC and HKC revealed massive sorption of OCT on the biomass. The sorption of OCT was already evident at the beginning of the experiment (day 0)—it was possible to extract only about 70% of the analyte from the biomass, in both cultures (Figure 1b, right axis). At the end of the experiment, 70% ± 3% and 51% ± 8% of OCT was adsorbed in the cultures of *I. lacteus* and *P. ostreatus*, respectively. The analyte did not release from the fungal mycelium, even after the ultrasound-assisted extraction at elevated temperature in the acidified polar organic solution. Despite our best effort to achieve better extraction yields, it is not clear whether the extracellular enzymes of *I. lacteus* and *P. ostreatus* were responsible for the biodegradation of OCT in vivo or if the sorption was the only reason for its removal. Differences between HKC and live biomass (ANOVA, $p < 0.05$) favor the enzymatic transformation. However, the changes in the sorption capacity of the HKC samples caused by autoclaving might also play a role in the data interpretation. Unfortunately, information about the fate of OCT in the environment is insufficient so far. Nevertheless, the adsorption of different QAC on particulate matter is often discussed in the literature [2,14]. The characteristics of OCT, namely the positive charge in physiological conditions and the strong binding to the negatively charged sites of biological membranes [17,40], indicate that the interaction with the mycelium will be similar.

2.2. Extracellular In Vitro Transformation

Detectable activities of the extracellular enzymes MnP and Lac were recorded during the in vivo transformation experiments with *I. lacteus* and *P. ostreatus*, respectively (see Table S2 for details). Transformation associated with the activities of MnP and Lac was further investigated employing a concentrated extracellular liquid of the eight-day-old malt extract-glucose (MEG) culture of *I. lacteus* supplemented with Mn^{2+} and a hydrogen peroxide-generating system and the eight-day-old MEG culture of *P. ostreatus*, respectively. The initial activities of MnP and Lac in the reaction mixture were 60 U/L and 120 U/L, respectively, and they did not decrease below 25% of their initial value during the whole 192-h experiment.

2.2.1. Chlorhexidine

The residual amounts of CHX related to the HKC after in vitro incubation with MnP and Lac were 59% ± 2% and 72% ± 2%, respectively (Figure 2, left axis). Significant removal of CHX was recorded after 4 h in the case of MnP and after 24 h in samples enriched by Lac (ANOVA, $p < 0.05$). The percentage of the extraction recovery reached 94% ± 3% on average (Figure 2, right axis).

Several studies have documented that the degradation of various recalcitrant pollutants by ligninolytic enzymes is usually very fast (in the range of hours) and mostly up to 100% effective [20,41,42]. Our results show that MnP and Lac were able to catalyze only 41% ± 2% and 28% ± 2% of the transformation of CHX after 192 h, respectively. Interestingly, even though the activities of the enzymes were higher than in the case of the in vivo experiment, the degradation achieved with the whole fungal culture was faster. The presence of enzymes bound to the mycelia (e.g., Lac), which were not harvested for the in vitro enzyme experiment and thus were available only in the in vivo incubation (where they were unaccounted for by the enzyme activity assays), could be a possible explanation [28].

In conclusion, the in vitro results suggest both mechanisms—sorption and biotransformation—might be involved in the removal of CHX.

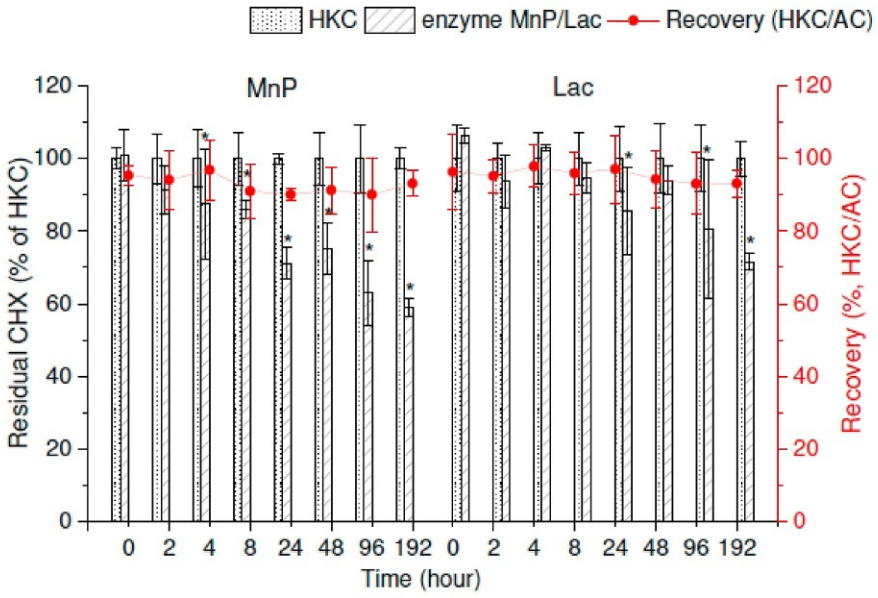


Figure 2. In vitro degradation of chlorhexidine (CHX) in concentrated extracellular liquids of *I. lacteus* (manganese-dependent peroxidase, MnP) and *P. ostreatus* (laccase, Lac). Initial concentration of CHX was 5 µg/mL in both experiments. The red line graphs show the recovery of CHX extraction during the experiment expressed as the heat-killed control (HKC) and abiotic control (AC) ratio. Error bars represent standard deviation (n = 3). The asterisk marks a significant difference between the individual harvesting day and the control group—0 d (post-hoc Dunnett's Test).

2.2.2. Octenidine

The removal of OCT was not recorded until the 96th hour of incubation in the case of MnP (ANOVA, $p < 0.05$). No decrease of the initial amount of OCT in time was observed with samples containing Lac after 192 h, ANOVA, $p > 0.05$ (see Figure 3 for details). Due to the sorption of the analyte observable for the HKC and the high variability of the data, the participation of MnP and Lac in OCT biodegradation is disputable. It is also important to note that the crude extracellular liquid is a complex matrix containing concentrated proteins and the involvement of other extracellular enzymes not considered in the activity assays might be another possible explanation for the removal of OCT. We argue that the slight decrease in the residual concentration relates rather to the sorption (~20%). This experiment did not support the theory that extracellular enzymes are responsible for the removal of OCT, but points toward the adsorption mechanism.

Total removal was achieved for neither CHX nor OCT under any conditions and the biotransformation of CHX in vitro was considered slow and less efficient in comparison with previous studies dealing with the biodegradation of recalcitrant pollutants by white-rot fungi. For instance, *P. ostreatus* was found to decompose nearly 100% of a mixture of polychlorinated biphenyls in MEG and low-nitrogen mineral medium after 42 days of incubation [18]. An excellent degradation rate (≥88%) of endocrine-disrupting compounds as well as the suppression of estrogenic activity were accomplished with various ligninolytic fungal strains in 14 days [26]. In addition, Muzikář et al. [22] demonstrated that selected ligninolytic fungal strains are powerful degraders of chlorobenzoic acids under both model liquid conditions and in contaminated soil—the chlorobenzoic acids reached 85%–99% degradation within 60 days.

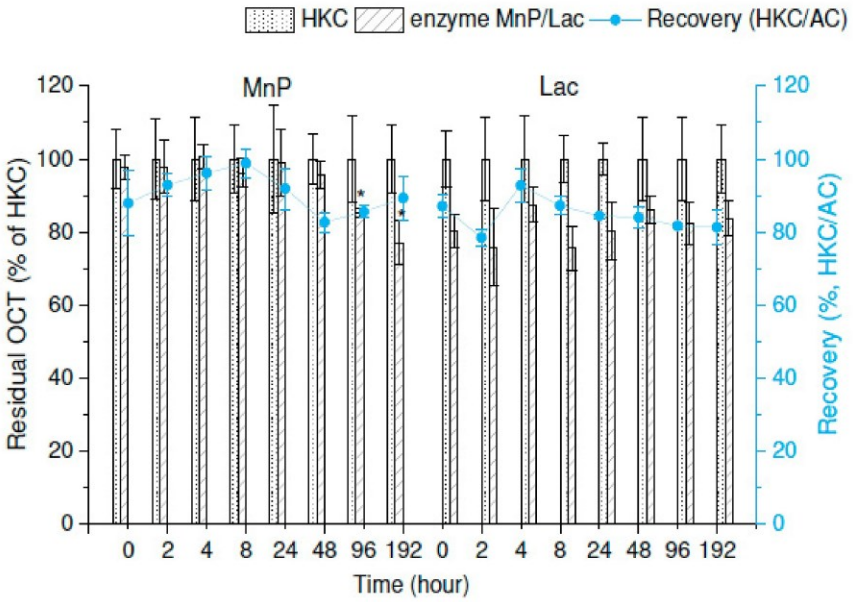


Figure 3. In vitro degradation of octenidine (OCT) in concentrated extracellular liquids of *I. lacteus* (manganese-dependent peroxidase, MnP) and *P. ostreatus* (laccase, Lac). Initial concentration of OCT was 5 µg/mL in both experiments. The blue line graphs show the recovery of OCT extraction during the experiment expressed as the heat-killed control (HKC) and abiotic control (AC) ratio. Error bars represent standard deviation (n = 3). The asterisk marks a significant difference between the individual harvesting day and the control group—0 d (post-hoc Dunnett's Test).

2.3. Identification of Metabolites

In vivo experiments did not reveal any transformation products. Detectable amounts of metabolites were recorded only during the in vitro tests carried out with both enzymes. Figures 4 and 5 show the LC-UV chromatograms obtained during the 8-day experiment spiked with 50 µg/mL of CHX and OCT, respectively (for the sake of clarity only the experiment with one of the enzymes—MnP—is displayed because the same products were observed). All peaks, especially those that did not appear in the control samples, were carefully assessed by nontargeted LC-MS analysis.

2.3.1. Chlorhexidine

An additional peak with the retention time (R_t) 5.3 min, whose area increased within the degradation experiment, was recorded in the chromatogram (Figure 4). A similar profile of the UV absorption spectra (λ_{max} = 260 nm) suggested a relationship with CHX (R_t = 4.8 min).

LC-MS analysis (full-scan mode) revealed the major m/z 258.2 $[M + 2H]^{2+}$ and 515.2 $[M + H]^{+}$ for the peak with the R_t = 5.3 min. Detailed characteristics of the ESI^{+} mass spectra of CHX and its transformation product, the comparison of their m/z values with the theoretical ones, as well as the suggested structures of the metabolites, are given in Table 1. The specific isotopic pattern of the CHX metabolite (ions 258.2; 259.0; 260.0 and 515.2; 517.1; 519.1) indicated the existence of multiple chlorine atoms in the molecule. According to the presented structure (Table 1, R_t 5.3 min), oxidation and simultaneous dehydrogenation seemed to be involved in the CHX transformation. Hydroxylation is a common reaction facilitated by ligninolytic enzymes possessing low substrate specificity [43]. Dehydrogenation was described for the degradation of steroid compounds by *P. ostreatus* [28]. Our observation suggests multiple dehydrogenation of the alkyl chain of CHX. Product ion spectra of $[M + 2H]^{2+}$ and $[M + H]^{+}$ obtained by fragmentation in a linear ion trap and the description of the main fragments are proposed in Figure S1.

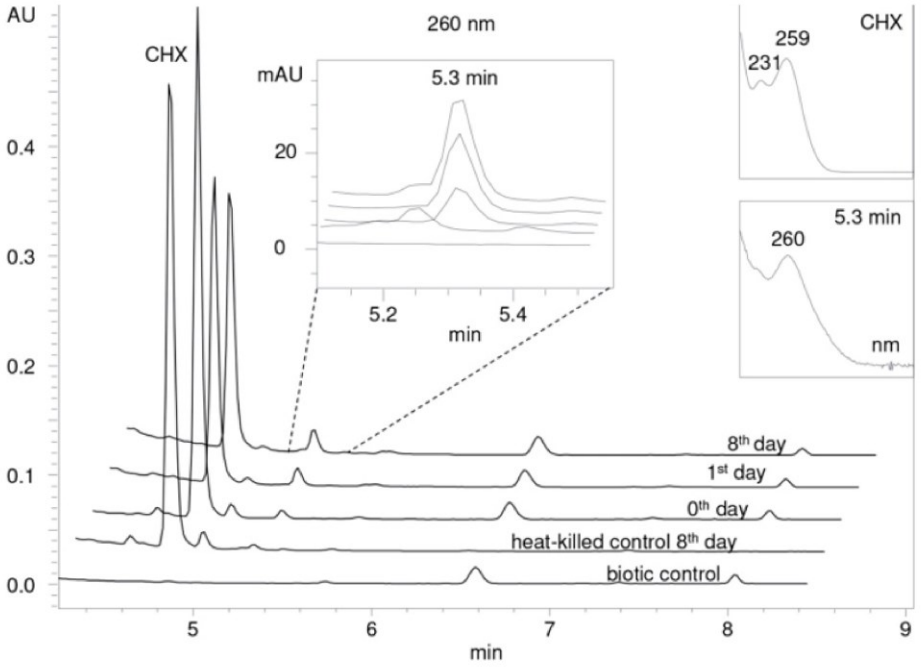


Figure 4. LC-UV plots of degradation samples acquired during in vitro chlorhexidine (CHX) degradation in the concentrated extracellular liquid of *I. lacteus* supplemented with Mn^{2+} and H_2O_2. The UV absorption spectra of specific peaks are given in the insets.

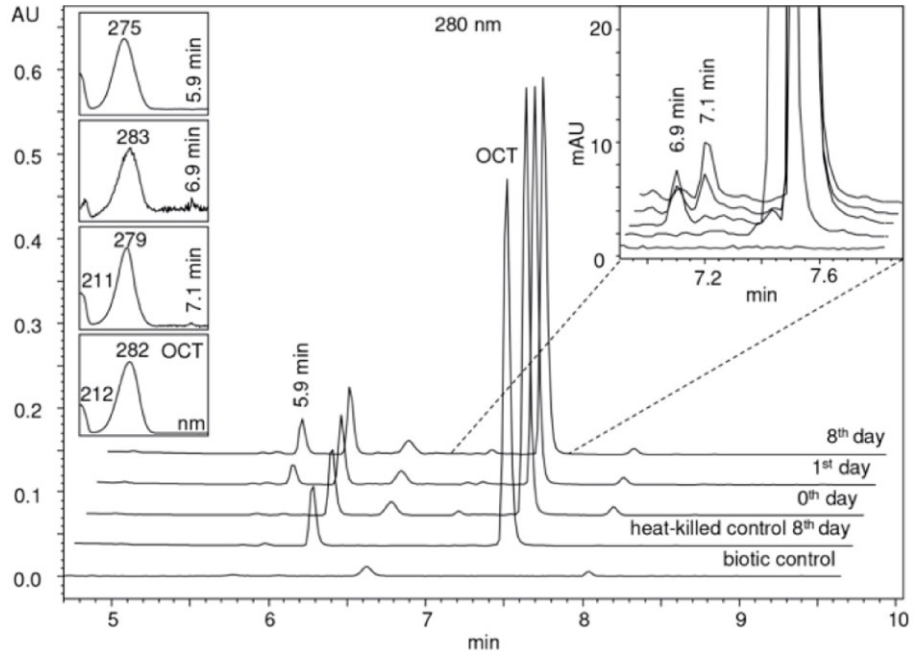
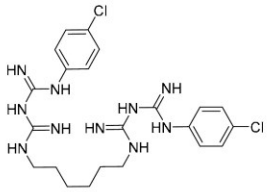
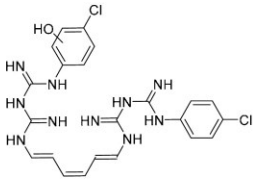
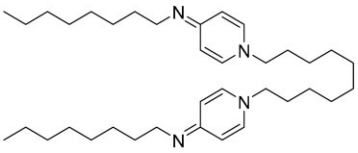
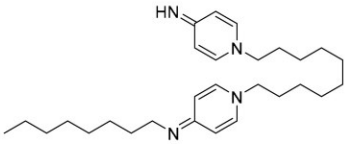
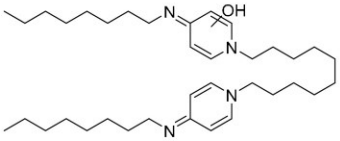
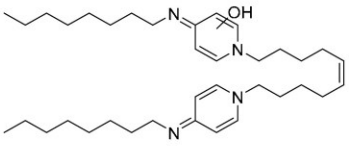


Figure 5. LC-UV plots of degradation samples acquired during in vitro octenidine (OCT) degradation in the concentrated extracellular liquid of *I. lacteus* supplemented with Mn^{2+} and H_2O_2. The UV absorption spectra of specific peaks are given in the insets.

Table 1. Characterization of the detected chlorhexidine (CHX) and octenidine (OCT) metabolites by nontargeted LC-MS.

R_t [min]	Suggested Structure	Theoretical Mass (Monois.) *m/z*	Mass Spectra Characteristics (ESI+) *m/z* (Intensity, %)
CHX 4.8		505.2105 253.1089	253.2 (100); 254.1 (98); 255.1 (29); 505.2 (31); 507.1 (22); 509.1 (5); 336.1 (20); 338.2 (7); 319 (14)
5.3		515.1584 258.0829	258.2 (100); 259.0 (70); 260.0 (13); 515.2 (59.7); 517.1 (44); 519.1 (9.7); 346.0 (6.2); 348.1 (4.9); 498.0 (5.4); 500.1 (3.7); 502.1 (1)
OCT 7.5		551.5047 275.7524	276.3 (100); 551.4 (17.6); 345.4 (1.6)
5.9		439.3795 220.1934	220.2 (100); 439.4 (13.7); 345.3 (3.1)
6.9		567.4996 284.2534	284.2 (100); 230.3 (459) (46.7); 488.2 (4.2); 459.5 (2.7); 567.3 (2.1); 276.2 (1)
7.1		565.4840 283.2455	283.2 (100); 565.3 (5.4); 275.3 (4.6); 269.3 (3.9); 220.7 (1.5)

Several authors tracked CHX degradation by bacterial isolates *(Pseudomonas* sp.) from activated sludge [15,16,44]. As a result, Tanaka et al. [39] proposed two ways of CHX transformation. The first is a direct degradation to *p*-chlorophenylurea (PCPU) and *p*-chloroaniline (PCA). The second pathway assumes an intermediate in which pyruvate is bound to the CHX molecule (*m/z* 531 $[M + H]^+$). PCA and PCPU are also known products of the hydrolysis of CHX under acidic or alkaline conditions [45,46]. We did not observe any detectable amount of PCA or PCPU in the degradation samples.

2.3.2. Octenidine

Three additional peaks appeared at R_t 5.9, 6.9, and 7.1 min (Figure 5). The detailed mass spectral information of all observed OCT transformation products together with the comparison of the proposed chemical structures of OCT metabolites and their theoretical *m/z* values are summarized in Table 1. The product assigned with the R_t = 5.9 min, distinguished by LC-MS as the *m/z* 220.2 $[M + 2H]^{2+}$,

corresponds to the loss of the octyl moiety from the OCT structure. The amount of this transformation product clearly increased within incubation time (Figure 5). Notably, detectable amounts were also recorded in the HKC and AC samples but their abundancies remained constant. This phenomenon can be explained as a physicochemical degradation of OCT that might be accelerated in the presence of ligninolytic enzymes. The peaks with the R_t = 6.9 min and R_t = 7.1 min occurred only in trace amounts and their baseline resolution was not achieved. Interestingly, one of these metabolites (R_t = 6.9 min) started to disappear after several hours, while the amount of the second compound (R_t = 7.1 min) increased after 24 h of incubation, indicating a less stable intermediate. A hydroxylated and hydroxylated/dehydrogenated structure was proposed for major ions 284.2 $[M + 2H]^{2+}$ and 283.2 $[M + 2H]^{2+}$ assessed by LC-MS, respectively (see Table 1 for details). Product ion spectra of all the transformation products and the description of their fragmentation products are displayed in Figures S2–S5.

The specific example of QAC degradation by ligninolytic fungi is lacking in the literature. On the other hand, the potential of the monooxygenases and dioxygenases of *Rhodobacter* spp. to biotransform HDP, a structurally similar compound, via dealkylation and/or benzene ring oxidation was hypothesized in the study of Nguyen et. al. [14]. Three main mechanisms of hydroxylation were described for the biotransformation of QACs in aerobic conditions [4], but the fate of these compounds under anaerobic conditions (e.g., aquatic sediments where they are usually adsorbed) remains unknown.

3. Materials and Methods

3.1. Chemicals

CHX (99.5%) was obtained from Merck (Darmstadt, Germany). OCT dihydrochloride (98%) was obtained from Alfa Aesar (Ward Hill, MA, USA). The tested compounds were diluted in DMSO (99.9%, Merck). Malt extract broth (Oxoid, Basingstoke, UK) and glucose (Penta, Prague, Czech Republic) were used for the MEG medium for fungal cultivation. ACN (HPLC grade and LC-MS grade, VWR, Prague, Czech Republic), milli-Q water prepared by the Direct-Q® water purification system (18.2 MΩ·cm, Merck), FA (98%, Penta, Czech Republic), FA (LC-MS grade, Honeywell, Charlotte, NC, USA), and trifluoroacetic acid (TFA ≥ 99%, Merck) were used for quantitative analyses.

3.2. Cultivation of Organisms and Degradation Tests

3.2.1. Fungal Cultivation

Ligninolytic fungal strains *P. ostreatus* 3004 CCBAS 278 and *I. lacteus* 617/93 were obtained from the Culture Collection of Basidiomycetes of the Czech Academy of Sciences (Prague, Czech Republic). One week-grown fungi were maintained on MEG (0.5% malt extract broth, 1% glucose) agar plate and stored at 4 °C. One week before the degradation tests, fungal inocula containing 3 mycelial plugs (0.7 mm Ø) of *I. lacteus* and *P. ostreatus* were grown in 20 mL of the MEG medium in 250 mL Erlenmeyer flasks (ErF) under static conditions for 5 and 7 days, respectively. The inocula were homogenized by Ultra-Turrax T 25 (IKA, Staufen, Germany).

3.2.2. In Vivo Transformation

Aliquots (1 mL) of the homogenized fungal suspension from Section 3.2.1. were used to inoculate static cultures (20 mL of the MEG medium, 250 mL ErF). In vivo cultures were contaminated with the highest nontoxic concentration of CHX (3 μg/mL in 0.5% DMSO) or OCT (2 μg/mL in 0.5% DMSO) three days after inoculation, all in triplicate. Biotic controls (BC, fungal culture with 0.5% DMSO) and HKCs were prepared in parallel. HKCs were grown for the same amount of time as the respective fungal cultures and subsequently inactivated by sterilization (121 °C, 30 min). After that, they were contaminated and incubated for the same amount of time as the active cultures. The samples and

corresponding controls were harvested after 0, 3, 7, 14, and 21 days of cultivation (static conditions, at 28 °C in the dark) and then a quantitative analysis was performed (see Section 3.4.1).

3.2.3. In Vitro Transformation with Concentrated Extracellular Liquids

Extracellular enzymes produced by the studied fungal strains were used for in vitro transformation tests. *I. lacteus* and *P. ostreatus* were grown in the MEG medium (20 mL in 250 mL ErF) for 8 days. The cultures were filtered through a 0.22 µm cellulose membrane (Whatman, Little Chalfont, UK). The targeted enzymes were concentrated 100-fold using 10 kDa cut-off membrane (Whatman) at 4 °C. The crude concentrated extracellular liquids were used for in vitro transformation experiments. The experiment with MnP from the *I. lacteus* strain was performed in 2 mL reaction mixtures containing MnP at initial activity of 60 U/L, 50 mM malonate buffer (pH 4.5), 1 mM $MnSO_4$, H_2O_2-generating system (30 mM glucose, 60 U/L of glucose oxidase), and 5 µg/mL of CHX or OCT, respectively. In the case of the experiment with Lac from the *P. ostreatus* strain, the reaction mixtures contained Lac with initial activity of 120 U/L, 60 mM acetate buffer (pH 5.0), and 5 µg/mL of either CHX or OCT. All samples were prepared in triplicate. ACs were prepared with distilled water instead of the enzyme. The enzyme was inactivated (100 °C, 30 min) to prepare corresponding HKCs; BCs contained 5% DMSO instead of the analytes. The samples and the respective controls were incubated for 0, 2, 4, 8, 24, 48, 96, and 192 h at 28 °C in the dark, shaken at 80 rpm on a rotary shaker. All samples were then extracted and the residual concentration of the analytes was determined (Section 3.4.1).

3.3. Enzyme Activities

Extracellular ligninolytic enzymes were measured during the cultivation, enzyme preparations for the in vitro experiments, and all degradation experiments. Lac was determined by the 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (Merck) oxidation test [47]. MnP and manganese-independent peroxidase were assessed with 2,6-dimethoxyphenol (Merck) as the substrate [48]. One enzyme unit produced 1 µmol of the reaction product per minute under the reaction conditions.

3.4. Chemical Analyses

3.4.1. Quantitative Analyses

In vivo transformation samples as well as the controls were homogenized by the Ultra-Turrax T 25 and the fungal suspension was sonicated with 20 mL of an extraction solution containing 0.8% FA in 20% ACN (*v*/*v*) at 70 °C for 45 min. The extracted suspension was centrifuged (4136× *g*, 15 min) and the supernatant was measured by LC-UV.

In vitro transformation samples were added with 2 mL of the extraction solution and sonicated at 70 °C for 45 min. The extracts were centrifuged (6000× *g*, 10 min) and the supernatant was analyzed by LC-UV.

The Waters Alliance 2695 LC system (Waters, Milford, MA, USA) equipped with a diode-array detector (Waters 2996) was used for the analysis. The analytes were separated on the XBridge C18 (4.6 × 150 mm, 3.5 µm) column (Waters). The mobile phase consisted of 100% ACN (A) and 10% ACN with 0.1% TFA (*v*/*v*) (B), the flow rate was 0.8 mL/min, and gradient elution was applied (min/% of A): 0/20, 8–10/95, 15/20. Column temperature was set at 35 °C, sample injection was 10 µL. Detection wavelengths for CHX and OCT were 260 nm and 280 nm, respectively.

3.4.2. Identification of Metabolites

The metabolites in all transformation samples were investigated by LC-MS analysis using the NexeraXR ultra-high performance liquid chromatograph (Shimadzu, Kyoto, Japan) coupled via electrospray ionization (ESI) to the QTrap 4500 mass spectrometer (Sciex, Framingham, MA, USA). The Analyst 1.6.3. software was used for data evaluation. Separation was achieved on the Cortecs T3 C18 column (150 mm × 3 mm, 2.7 µm) at 0.4 mL/min using a mobile phase composed of 0.1% FA in

water (A) and ACN (B). The linear gradient was as follows (min/% of A): 0/10, 8/70, 9–12/100, 12–15/20. The mass spectrometer operated in the positive mode. Curtain gas, ion spray voltage, vaporizer temperature, ion source gas 1, and ion source gas 2 were set at 30 psi, 5.5 kV, 450 °C, 40 psi, and 50 psi, respectively. Full scan analysis in the mass range of 100–600 *m*/*z* was used for metabolite identification. Enhanced product ion, tandem mass spectrometry, and enhanced resolution scans with alternated collision energies and mass ranges were used to study the metabolites in more detail.

3.5. Statistical Analysis

The one-way analyses of variance (ANOVA) and subsequent Dunnett's tests were run to identify the pairs with significant differences. The differences were considered significant at $p < 0.05$. Microsoft excel 2016 (Redmond, WA, USA) was used for data handling.

4. Conclusions

Our results indicate that the metabolic transformation of CHX might be associated with the activity of extracellular enzymes of ligninolytic fungi, MnP and Lac, but only slow removal of the pollutant was observed under the model conditions. Moreover, the mechanism elucidating the metabolism of OCT remains unexplained since the majority of the analyte was adsorbed to the mycelial matter. For the first time, metabolites indicating enzymatic transformation of both CHX and OCT were detected and their chemical structures were proposed. However, complete biodegradation by the ligninolytic fungi was not achieved for any of the studied analytes, which emphasizes their recalcitrant character. Taken together with the substantial increase in the production and their worldwide consumption during everyday household activities, there is little prospect of CHX and OCT being removed from the environment. Activated sludge and agricultural lands are expected to be the environments impacted by these compounds in real circumstances. The conclusions of this study highlight that biodegradation studies of newly developed and extensively consumed synthetic compounds are crucial to predict their fate in the environment.

Supplementary Materials: The following are available online, Table S1: Activity of manganese-dependent peroxidase (MnP) from *I. lacteus* and laccase (Lac) from *P. ostreatus* during in vivo degradation of octenidine (OCT) and chlorhexidine (CHX); Table S2: Activity of manganese-dependent peroxidase (MnP) from *I. lacteus* and laccase (Lac) from *P. ostreatus* during in vitro degradation of octenidine (OCT) and chlorhexidine (CHX); Figure S1: Product ion spectra and suggested fragments of (a) *m*/*z* 515.2 $[M + H]^+$ and (b) *m*/*z* 258.2 $[M + 2H]^{2+}$; Figure S2: (a) mass spectrum of the peak with Rt = 5.9 min, *m*/*z* 439.4 $[M + H]^+$ (b) product ion spectra and suggested fragments of *m*/*z* 439.4 $[M + H]^+$; Figure S3: Product ion spectra and suggested fragments of (a) *m*/*z* 567.5 $[M + H]^+$ and (b) *m*/*z* 284.3 $[M + 2H]^{2+}$; Figure S4: (a) mass spectrum of the peak with R_t = 7.1 min, *m*/*z* 283.2 $[M + H]^+$ (b) product ion spectra and suggested fragments of *m*/*z* 565.5 $[M + H]^+$; Figure S5: Product ion spectra and suggested fragments of (a) *m*/*z* 565.5 $[M + H]^+$ and (b) *m*/*z* 283.3 $[M + 2H]^{2+}$.

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Sample Availability: Samples of the compounds CHX and OCT are available from the authors.

Supplementary Data

Biodegradation of dental care antimicrobial agents chlorhexidine and octenidine by ligninolytic fungi

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The list of supporting information:

Table S1: Activity of manganese-dependent peroxidase (MnP) from *I. lacteus* and laccase (Lac) from *P. ostreatus* during *in vivo* degradation of octenidine (OCT) and chlorhexidine (CHX).

Table S2: Activity of manganese-dependent peroxidase (MnP) from *I. lacteus* and laccase (Lac) from *P. ostreatus* during *in vitro* degradation of octenidine (OCT) and chlorhexidine (CHX).

Figure S1: Product ion spectra and suggested fragments of (a) *m*/*z* 515.2 $[M+H]^+$ and (b) *m*/*z* 258.2 $[M+2H]^{2+}$.

Figure S2: (a) mass spectrum of the peak with R_t = 5.9 min, m/z 439.4 $[M+H]^+$ (b) product ion spectra and suggested fragments of m/z 439.4 $[M+H]^+$.

Figure S3: Product ion spectra and suggested fragments of (a) *m*/*z* 567.5 $[M+H]^+$ and (b) *m*/*z* 284.3 $[M+2H]^{2+}$.

Figure S4: (a) mass spectrum of the peak with R_t = 7.1 min, *m*/*z* 283.2 $[M+H]^+$ (b) product ion spectra and suggested fragments of *m*/*z* 565.5 $[M+H]^+$.

Figure S5: Product ion spectra and suggested fragments of (a) *m*/*z* 565.5 $[M+H]^+$ and (b) *m*/*z* 283.3 $[M+2H]^{2+}$.

Table S1. Activity of manganese-dependent peroxidase (MnP) from *I. lacteus* and laccase (Lac) from *P. ostreatus* during *in vivo* degradation of octenidine (OCT) and chlorhexidine (CHX).

Degradation	OCT		CHX	
Time	MnP (U/l)	Lac (U/l)	MnP (U/l)	Lac (U/l)
0 d	3.6 ± 0.4	31.5 ± 0.5	4.6 ± 0.5	33 ± 4
3 d	3.0 ± 0.4	17.2 ± 0.5	6.2 ± 0.5	15 ± 3
7 d	1.5 ± 0.5	14.1 ± 0.1	5.7 ± 0.3	14.1 ± 0.7
14 d	2.5 ± 0.9	10.8 ± 0.1	6.3 ± 0.5	9.1 ± 0.4
21 d	2.5 ± 0.5	1.8 ± 0	4 ± 1	5.1 ± 0.1

Data are means ± SD (n=3).

Table S2. Activity of manganese-dependent peroxidase (MnP) from *I. lacteus* and laccase (Lac) from *P. ostreatus* during *in vitro* degradation of octenidine (OCT) and chlorhexidine (CHX).

Degradation	OCT		CHX	
Time	MnP (U/l)	Lac (U/l)	MnP (U/l)	Lac (U/l)
0 h	60 ± 1	120 ± 2	60 ± 2	120 ± 7
2 h	58 ± 2	119 ± 3	59 ± 4	118 ± 5
4 h	57 ± 2	118 ± 8	57 ± 5	116 ± 5
8 h	58 ± 2	105 ± 12	57 ± 3	110 ± 8
24 h	58 ± 8	79 ± 1	56 ± 5	86 ± 7
48 h	52 ± 4	55 ± 2	54 ± 3	62 ± 3
96 h	30 ± 5	47 ± 1	32 ± 7	42 ± 5
192 h	17 ± 2	33 ± 0	21 ± 4	31 ± 4

Data are means ± SD (n=3).

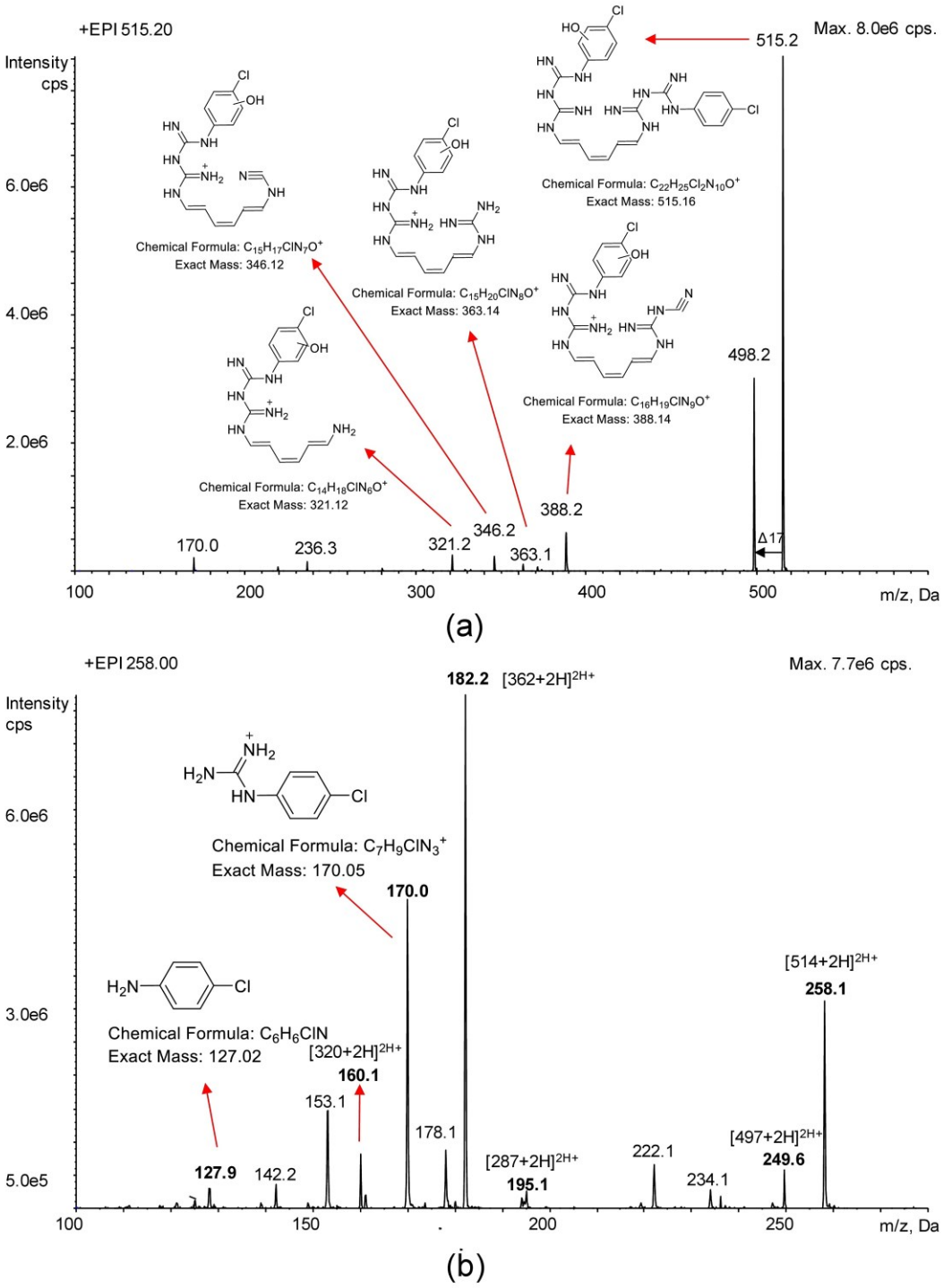


Figure S1: Product ion spectra and suggested fragments of (a) *m/z* 515.2 $[M+H]^+$ and (b) *m/z* 258.2 $[M+2H]^{2+}$.

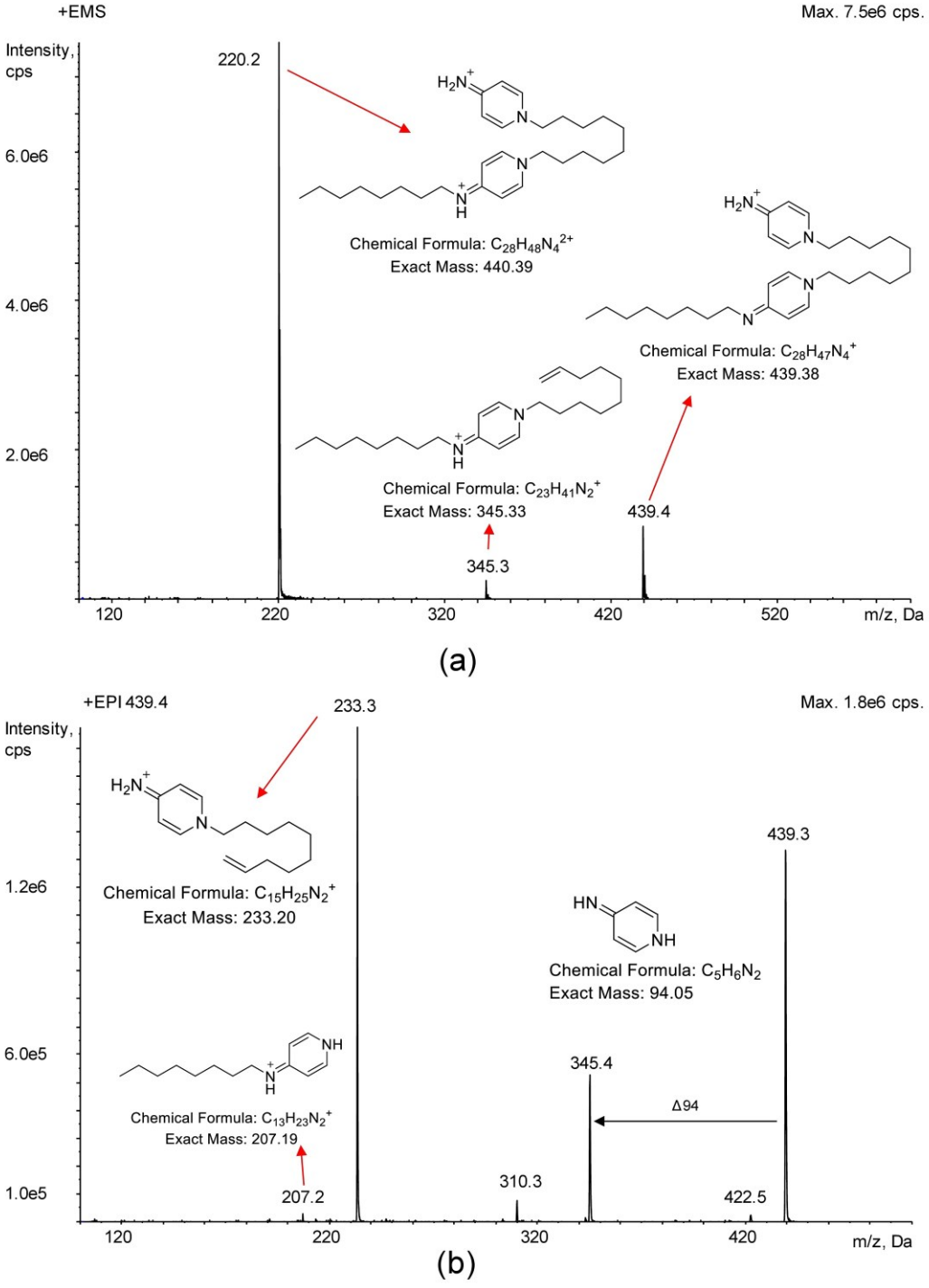
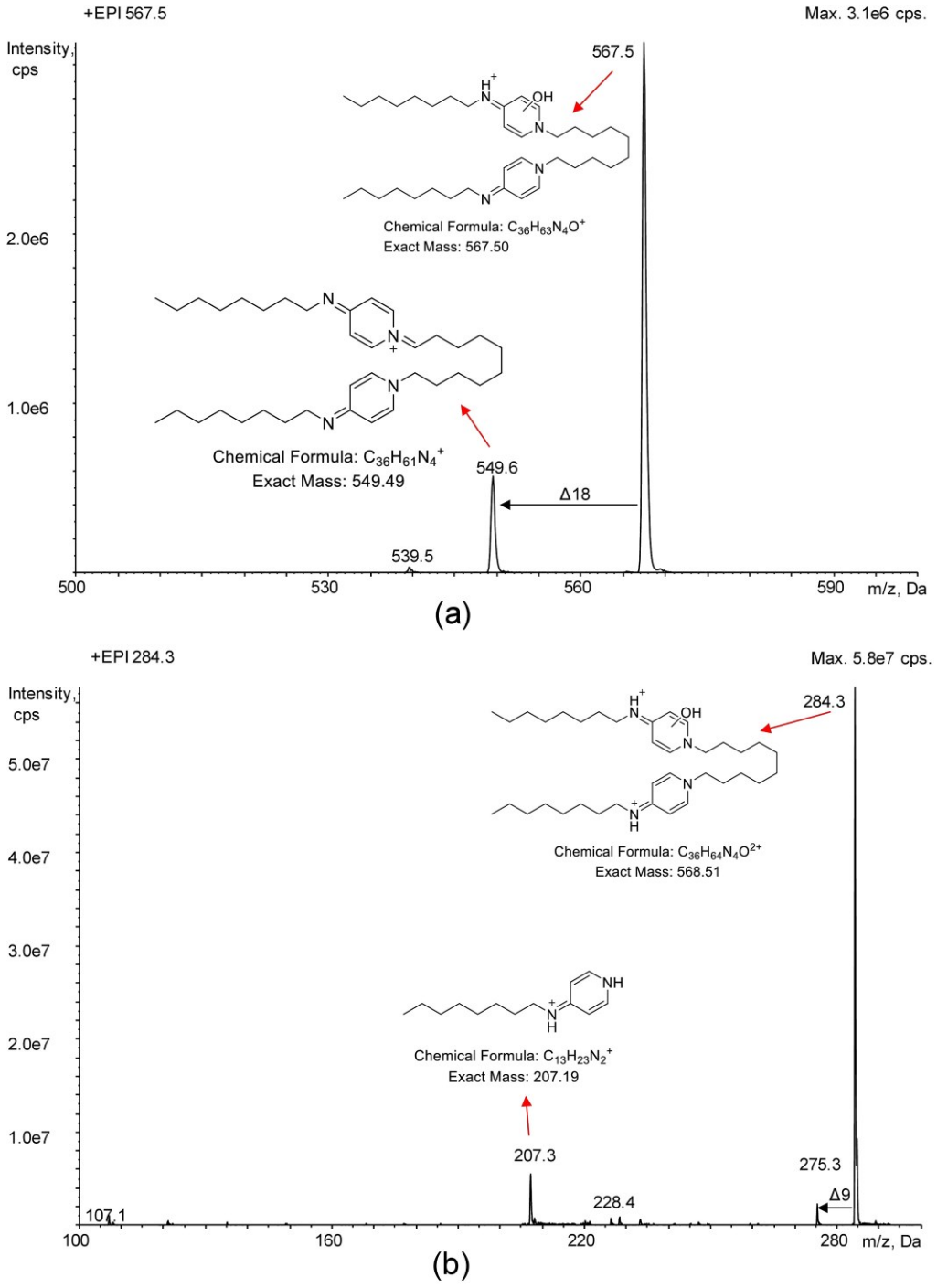


Figure S2: (a) mass spectrum of the peak with R_t = 5.9 min, *m/z* 439.4 $[M+H]^+$ (b) product ion spectra and suggested fragments of *m/z* 439.4 $[M+H]^+$.



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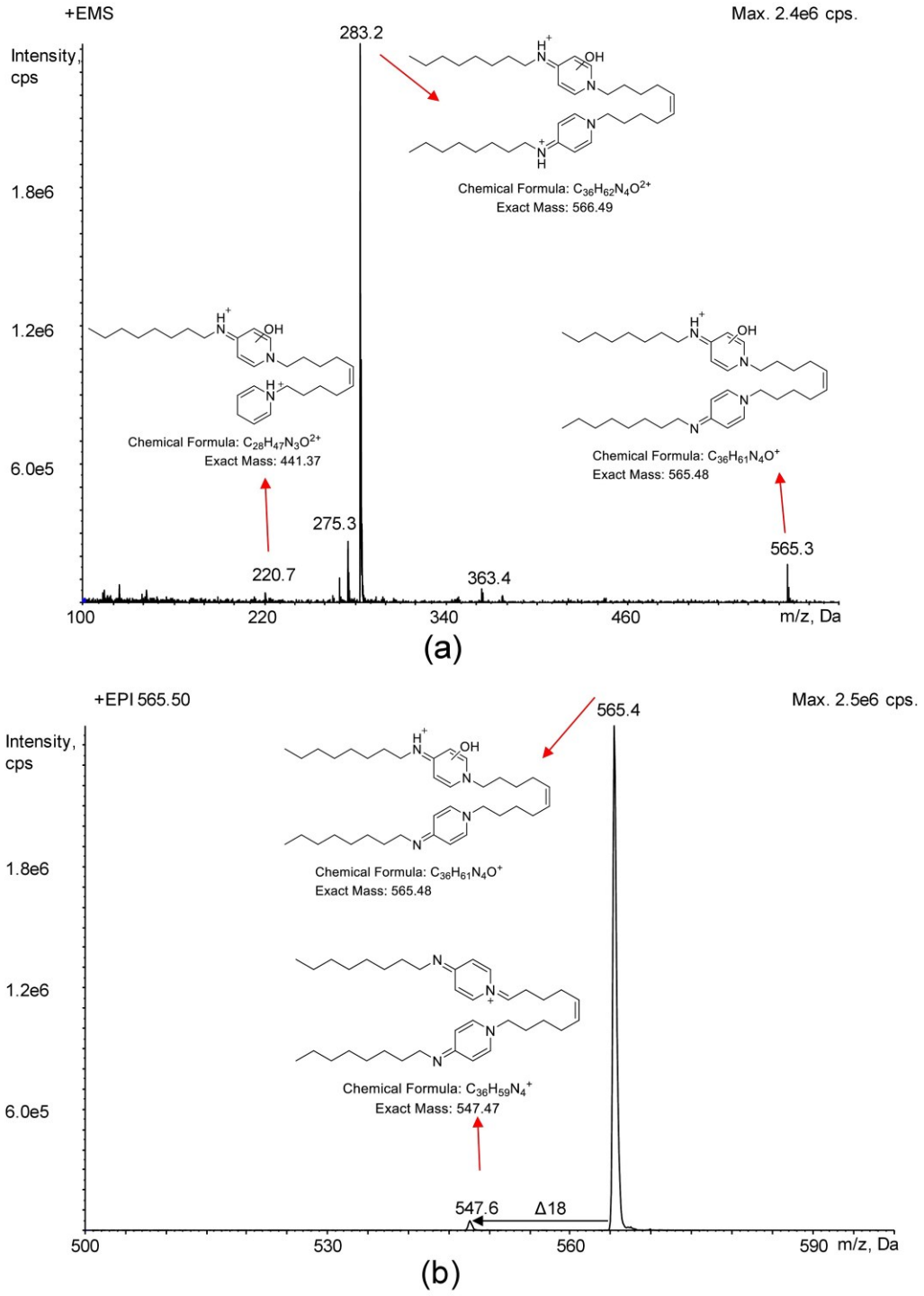

Figure S4: (a) mass spectrum of the peak with R_t = 7.1 min, *m*/*z* 283.2 $[M+H]^+$ (b) product ion spectra and suggested fragments of *m*/*z* 565.5 $[M+H]^+$.

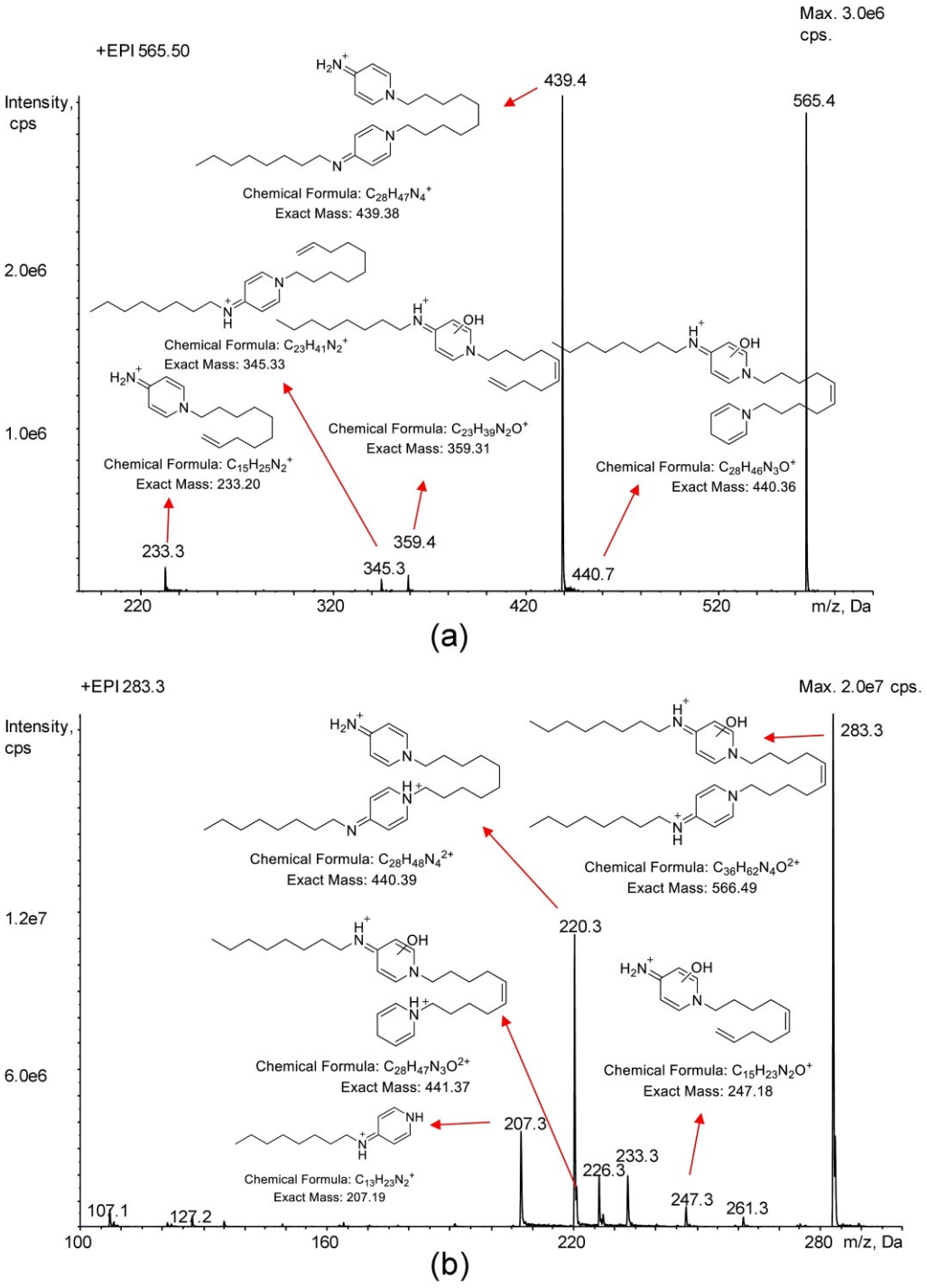

Figure S5: Product ion spectra and suggested fragments of (a) *m/z* 565.5 $[M+H]^+$ and (b) *m/z* 283.3 $[M+2H]^{2+}$.

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RESEARCH ARTICLE

Laccase and horseradish peroxidase for green treatment of phenolic micropollutants in real drinking water and wastewater

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Abstract
Biologically active micropollutants that contain diverse phenolic/aromatic structures are regularly present in wastewater effluents and are even found in drinking water. Advanced green technologies utilizing immobilized laccase and/or peroxidase, which target these micropollutants directly, may provide a reasonable alternative to standard treatments. Nevertheless, the use of these enzymes is associated with several issues that may prevent their application, such as the low activity of laccase at neutral and basic pH or the necessity of hydrogen peroxide addition as a co-substrate for peroxidases. In this study, the activity of laccase from *Trametes versicolor* and horseradish peroxidase was evaluated across a range of commonly used substrates (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine, and guaiacol). Moreover, conditions for their optimal performance were explored along with an assessment of whether these conditions accurately reflect the effectivity of both enzymes in the degradation of a mixture of bisphenol A, 17α-ethinylestradiol, triclosan, and diclofenac in tap drinking water and secondary wastewater effluent. Laccase and horseradish peroxidase showed optimal activity at strongly acidic pH if ABTS was used as a substrate. Correspondingly, the activities of both enzymes detected using ABTS in real waters were significantly enhanced by adding approximately 2.5% (v/v) of McIlvaine's buffer. Degradation of a mixture of micropollutants in wastewater with 2.5% McIlvaine's buffer (pH 7) resulted in a substantial decrease in estrogenic activity. Low degradation efficiency of micropollutants by laccase was observed in pure McIlvaine's buffer of pH 3 and 7, compared with efficient degradation in tap water of pH 7.5 without buffer. This study clearly shows that enzyme activity needs to be evaluated on micropollutants in real waters as the assessment of optimal conditions based on commonly used substrates in pure buffer or deionized water can be misleading.

Keywords Anthropogenic pollution · Biodegradation · Enzymatic treatment · Endocrine-disrupting chemicals · Ecotoxicity · Wastewater treatment

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Introduction

Wastewater and drinking water treatment plants have to deal with increasing amounts of emerging micropollutants that contain phenolic/aromatic structures, occur at very low concentrations (from μg/L to below ng/L), are highly persistent, and have negative effects on both the environment and human health. These chemicals mainly originate from pesticides, pharmaceuticals, cosmetics, flame retardants, perfumes, waterproofing agents, plasticizers, and insulating foams (Kim and Zoh 2016; Margot et al. 2015).

One group of such micropollutants, endocrine-disrupting chemicals (EDCs), interferes with the vertebrate (including human) endocrine system by imitating or blocking the effect of natural hormones. In particular, bisphenol A (BPA), 17α-

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ethinylestradiol (EE2), triclosan (TCS), and diclofenac (DCF) are widely recognized and commonly found in wastewater treatment plants and river systems (Diamanti-Kandarakis et al. 2009; WHO 2015).

Green catalysis processes that include oxidation by oxidoreductase enzymes represent a prospective approach for the removal of these micropollutants from drinking water and wastewater (Martinkova et al. 2016; Maryskova et al. 2019; Zhang et al. 2020). Oxidoreductases, which occur widely within microbes, plants, and animals, catalyze the transfer of electrons from one molecule to another, resulting in oxidation or reduction depending on whether the enzyme is an electron donor or acceptor (Toone 2010). At present, the most promising oxidoreductases for water treatment are laccases and peroxidases (Grelska and Noszczyńska 2020; Xiao et al. 2020).

Laccases are multifaceted, copper-based biocatalysts capable of oxidizing phenolic, polyphenolic, and aniline compounds and even some inorganic compounds. This ability has promoted their use in biotechnological processes aimed at degrading chemicals produced in the paper, textile, and petrochemical industries (Agrawal et al. 2018; Khakshoor et al. 2020; Kupski et al. 2019). Laccases are mostly obtained from white rot fungi (*Trametes versicolor*, *Pleurotus ostreatus*) or plants (*Toxicodendron vernicifluum*); however, the heterologous expression in bacteria (*Bacillus* sp., *Escherichia coli*), yeast (*Saccharomyces cerevisiae*), and other fungi (*Aspergillus* sp., *Penicillium canescens*) is being explored to produce pure enzymes of higher quality (Antošová and Sychrová 2016; Strong and Claus 2011).

Peroxidases are heme-containing enzymes that utilize hydrogen peroxide or organic hydroperoxides as a co-substrate to catalyze the oxidation of a broad range of organic and inorganic compounds (Hamid and Khalil ur 2009; Sakuyama et al. 2003). Horseradish peroxidase (HRP) in particular is widely used in biochemistry for enzyme immunoassays but is now being applied in novel fields such as the synthesis of organic and polymer chemicals or wastewater treatment (Chauhan and Kang 2018; Shanmugapriya et al. 2019).

The catalytic activity of an enzyme depends on its biological source, its purity, and environmental conditions (Chauhan et al. 2017). While simple assays for the quantification of enzymatic activity exist, the results depend strongly on the concentration of the enzyme, the substrate, the relative proportion of the enzyme and substrate, and most importantly, the catalytic reaction conditions (e.g., water content, temperature, and pH). The vast majority of current studies are focused on establishing the best experimental conditions to achieve the highest catalytic effect against single micropollutants, usually in deionized water (DIW) or pure buffers. Consequently, the outcomes of such studies are not really applicable as regards real treatment environments. Wastewater effluents, as an illustrative example, often contain elevated concentrations of micropollutants, together with many organic and inorganic substances that hinder the biocatalytic effect (Margot et al. 2015; Strong and Claus 2011).

In this study, we investigated the biocatalytic ability of two oxidoreductase enzymes, laccase from *T. versicolor* and HRP, for degradation of a mixture of BPA, EE2, TCS, and DCF in a range of different media, including tap drinking water and secondary wastewater effluent, with and without the addition of McIlvaine's buffer. The estrogenic activity of treated wastewater samples was determined to prove that the products of biocatalysis no longer possess endocrine disrupting capability. Moreover, three commonly used commercial substrates, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), syringaldazine (SYR), and guaiacol (GUA), were compared to select the most suitable for determination of enzyme activity in real waters. In this way, it was possible to directly compare actual biodegradation capacity of laccase and HRP in different matrices, including tap water and wastewater effluent.

Material And Methods

Material

Laccase from *T. versicolor* (E.C. 1.10.3.2; ≥ 10 U/mg; powder) and peroxidase from *Armoracia rusticana* (E.C. 1.11.1.7; ~ 150 U/mg; lyophilized powder) were used to prepare stock solutions by dissolving 2 mg of enzyme powder in 1 mL of McIlvaine's buffer. SYR (99%), ABTS (> 98%), GUA (≥ 98.0%), BPA (≥ 99%), EE2 (≥ 98%), TCS (analytical standard), DCF (analytical standard), and dimethyl sulfoxide (DMSO, ≥ 99.9%) were purchased from Sigma-Aldrich (USA), while D-luciferin (≥ 99%) was obtained from Biotech (Czech Republic). Acetonitrile and methanol (HPLC for Gradient Analysis) were purchased from Thermo Fisher Scientific (USA).

Enzyme activity analysis

The catalytic activity of laccase and HRP, assessed by ABTS oxidation, was measured at room temperature according to Hassani et al. (2013), using a BioTech Synergy HTX microplate reader (BioTech Instruments Inc., USA) set to 420 nm ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$). The laccase activity assay was carried out in 160 μL of McIlvaine's buffer (pH 3) with 20 μL of the stock laccase solution. The catalytic reaction was then started by addition of 20 μL of 0.5 mM ABTS (Ardao et al. 2015; Maryskova et al. 2016; Ramirez-Cavazos et al. 2014). All reaction mixtures with HRP (140 μL of McIlvaine's buffer and 20 μL of stock enzyme solution) were completed with a corresponding amount (20 μL) of 5% hydrogen peroxide

(enzyme solution to hydrogen peroxide = 1:1, v/v; evaluated as the optimal concentration based on preliminary experiments) and the catalytic reaction started by addition of 20 μL of 0.5 mM ABTS.

Catalytic activity of laccase and HRP assessed by SYR oxidation was performed in a similar manner to that by ABTS oxidation (SYR concentration = 0.5 mM), except that the absorbance peak was set at 526 nm (ε = 65 mM^{-1} cm^{-1}). Likewise, catalytic activity of laccase and HRP assessed by GUA oxidation (GUA concentration = 0.5 mM) was measured at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) under the same conditions outlined above.

Effect of pH on enzymatic activity

The influence of pH on enzyme catalytic activity was determined in McIlvaine's buffer at pH 3, 4, 5, 6, 7, and 8 at 25 °C using ABTS as the substrate. The pH range used was the maximum available by mixing 0.1 M citric acid and 0.2 M disodium phosphate. Prior to determination, the enzymes were pre-incubated in buffer of the required pH for 24 h.

Effect of hydrogen peroxide concentration on HRP activity

Six hydrogen peroxide solutions of different concentrations (0.5, 1, 2.5, 5, 7.5, and 10%) were prepared and used for the assessment of the catalytic activity of HRP using ABTS in McIlvaine's buffer (pH 3), as described above. The effect of concentration was evaluated from four replicates of activity measurements using one-factor analysis of variance (ANOVA).

Enzymatic activity of laccase and HRP in different water samples

Samples of ultrafiltrated secondary wastewater were obtained from a medium-sized wastewater treatment plant (site name classified) and drinking water from a household tap in the biological laboratory between January and March 2019. The enzymatic activity of laccase and HRP was measured using ABTS as described above, except real water samples replaced the McIlvaine's buffer. The experiment was performed in four replicates, and detected enzymatic activities were compared to activities in McIlvaine's buffer at pH 3 and DIW. A similar approach was used to study the influence of buffer infusion, with McIlvaine's buffer (pH 3) being added at concentrations ranging from 0 to 20% (v/v) to tap water and wastewater.

Degradation of endocrine-disrupting micropollutants

Degradation efficiency of laccase and HRP was determined as a decrease in the concentration of micropollutants over 20 h of incubation at 25 °C. First, 0.1 U of the respective enzyme was added to glass vials containing 5 mL of a mixture of BPA, EE2, TCS, and DCF (10 mg/L of each compound). Incubation in McIlvaine's buffer (pH 3 and 7) was tested to evaluate the effect of pH on the degradation of the micropollutants studied. DIW, tap water, wastewater, tap water amended with 2.5% (v/v) of buffer at pH 7, and wastewater amended with 2.5% (v/v) of buffer at pH 3 or 7 were tested using the same experimental set up. In the case of samples with HRP, 700 μL of the EDC mixture was replaced with the same volume of 1% hydrogen peroxide, the concentration having been determined from preliminary measurements. After 20 h incubation, 100 μL of 10% sodium azide was added, thereby preventing further degradation of the EDCs (Ardao et al. 2015). Each experiment was performed in triplicate, and the results are presented as the mean value ± standard deviation.

BPA, EE2, TCS, and DCF degradation by laccase and HRP were measured following the protocol described in our previous study (Maryskova et al. 2019) using a Dionex Ultimate 3000 high pressure liquid chromatograph (Thermo Fisher Scientific, USA).

Estrogenic activity

Estrogenic activity was determined in secondary wastewater following degradation of the micropollutant mixture in samples containing 2.5% (v/v) McIlvaine's buffer (pH 7) using genetically modified yeast extracellularly producing luciferase after estrogen receptor stimulation. Samples without enzymes served as a control. The samples were diluted 100 to 12,800 fold with DMSO (final assay concentration of DMSO was 3%), and the estrogenic yeast assay was performed according to Leskinen et al. (Leskinen et al. 2005), with a slight modification as described in Ezechias et al. (2016). Briefly, the strains *Saccharomyces cerevisiae* BMAEREluc/ERα and *S. cerevisiae* BMA64luc (control strain) were incubated with the samples (9:1) for 2.5 h (30 °C, 200 rpm on a rotary shaker) in white 96-well plates at a total volume of 100 μL. After incubation, 50 μL of 1 mM D-luciferin solution was added and luminescence measured using a GloMax luminometer (Promega, USA). Estradiol equivalent was determined using the dose–response curve of 17β-estradiol, which was incubated simultaneously.

Results and discussion

Optimal pH for oxidoreductase catalysis

Commercial *T. versicolor* laccase showed highest catalytic activity toward ABTS, SYR, and GUA at pH 3, 4, and 4.5, respectively. For all substrates, therefore, optimal pH ranged between 3 and 5.5, indicating that laccase shows highest activity at more acidic pH levels (Fig. S1a). According to the literature, the pH optimum of laccases isolated from different

strains is between 1.8 and 4.4 when using ABTS as the substrate, between 4.8 and 8.2 when using SYR (Kolomytseva et al. 2017), and between 4.0 and 6.0 when using GUA (Abd El Monssef et al. 2016; Holker et al. 2002). Several studies have suggested that a buffer of pH 3 is the most efficient for ABTS oxidation (Barrios-Estrada et al. 2018; Gonzalez-Coronel et al. 2017; Iimura et al. 2018). In comparison, HRP was most active at a more neutral pH of between 6.5 and 8 for all substrates except ABTS, where the optimal pH was 3.5 (Fig. S1b). In other studies, buffers at pH 5 (Pellicer and Gomez-Lopez 2017) and 6 (Cai et al. 2018; Jiang and Penner 2015) have been used for ABTS oxidation, with a similar pH being used for SYR (de Souza et al. 2007) and GUA (Tsikrika et al. 2018) peroxidase catalysis.

Both laccase and HRP showed high affinity towards ABTS and SYR; however, the use of an organic solvent is required for SYR because of its low water solubility, which represents a considerable disadvantage. Compared to SYR and GUA, ABTS is safe to handle, highly soluble in water, and the solution is more suitable for long-term storage. As such, ABTS was chosen for further experimentation as the most suitable for both enzymes.

Optimal hydrogen peroxide concentration for catalytic activity of HRP

No single optimal hydrogen peroxide concentration was recorded between 0.5 and 10% when measured in McIlvaine's buffer of pH 3 (Fig. S2; one-way ANOVA ($F(5,18) = 1.87$, $p = 0.1496$). In previous studies, both the concentration of hydrogen peroxide used and the type and concentration of substrate differ significantly. In principal, very low concentrations are required for assays of 0.0085–3% (Chauhan and Kang 2018; Wang et al. 2017), which corresponds with the 1% concentration used in our study.

Catalytic enzyme activity in real waters

The catalytic activities of laccase and HRP measured in tap water, wastewater, McIlvaine's buffer of pH 3, and DIW, using ABTS as the substrate, differed strongly (Table 1), presumably because of differences in pH and water composition (Table 2). Both laccase and HRP achieved highest activity in buffer of pH 3 when ABTS was used. In DIW, activity dropped to approximately 20%, most probably due to higher pH (pH 7.50) and the absence of beneficial ions. Though both tap water (pH 7.45) and wastewater (pH 8.30) samples had pH levels similar to DIW, the main factor affecting catalytic activity was the presence of inhibiting ions. It has previously been reported that water content, and especially the presence of inorganic salts (i.e., those containing divalent and trivalent cations or halides), has a negative impact on the catalytic activity of laccase (Chapple et al. 2019; Raseda et al. 2014).

Table 1 Catalytic activities of laccase from *T. versicolor* (TV) and horseradish peroxidase (HRP) in pure McIlvaine's buffer (pH 3), deionized water (DIW), tap water (TAP), and wastewater (WASTE) using ABTS as a substrate ($n = 4$)

Laccase from TV Relative activity (%)		HRP Relative activity (%)	
pH 3	100 ± 4	pH 3	100 ± 10
DIW	29.9 ± 2.5	DIW	17.4 ± 3.5
TAP	0.39 ± 0.01	TAP	0.18 ± 0.12
WASTE	0.04 ± 0.01	WASTE	0.21 ± 0.14

As expected, the most unfavorable type of water for laccase activity was wastewater, which has the highest ionic concentration (Table 2). Measurement of HRP activity proved more complicated than for laccase as hydrogen peroxide consumption increased significantly with increasing water pollution and ion concentration, resulting in a shortening of the linear part of kinetic activity measurement. As such, the data may be burdened with error. However, the specific inhibition effects of each salt were not explored further in this study.

A significant increase in catalytic activity was observed with 2.5% (v/v) of McIlvaine's buffer (pH 3) in the case of laccase (Fig. 1) and with 2.5–5% (v/v) of buffer in the case of HRP (Fig. 2). Both enzymes showed highest activity in tap

Table 2 Chemical analysis of real water samples. TAP stands for tap water, and WASTE represents filtered secondary wastewater

mg/L	TAP	WASTE
Fluoride	< 0.05	0.16
Chloride	1.6	213
Nitrate	0.66	9.5
Nitrite	< 0.05	< 0.05
Sulfate	0.66	9.5
TOC	1.6	6.2
Ag	< 0.001	0.0019
Al	0.033	0.035
Be	< 0.001	< 0.001
Ca	37.6	127
Co	< 0.002	< 0.002
Cr	< 0.001	< 0.001
Cu	0.01	< 0.001
Fe	0.02	0.003
K	0.35	74.8
Mg	0.897	21.3
Mn	0.003	< 0.001
Na	2.59	119
Ni	0.004	0.002
Pb	< 0.01	< 0.01
V	< 0.01	< 0.01
Zn	0.094	0.014
pH	7.45	8.30

water, where the addition of buffer caused a larger pH decrease (pH 4.03 in tap water and pH 5.20 in wastewater). Lower enzymatic activity in wastewater compared to tap water of the same pH could have been caused by the presence of inhibiting ions. The highest peak of catalytic activity for both laccase and HRP, using ABTS as the substrate, was detected at pH < 4. Adjustment of pH has previously been discussed as a necessary step for wastewater treatment, with the optimal pH for removal of phenolic compounds being reported as ranging between pH 4.5 and 6 (Asadgol et al. 2014; Zdarta et al. 2019).

Degradation of endocrine-disrupting micropollutants

In general, the best degradation efficiencies of both enzymes were observed in conditions that did not correspond to the proposed optimal conditions (Fig. S1). Highest laccase activity was detected at an acidic pH with all three commercial substrates (Fig. S1a); however, the 20 h degradation experiment showed better degradation of BPA and EE2 in McIlvaine's buffer of pH 7 than in the same buffer at pH 3 (Fig. 3a). No difference was observed in the case of DCF, and only TCS degraded better in McIlvaine's buffer of pH 3.

As has already been mentioned, in the case of HRP, an acidic pH resulted in highest activity for ABTS only; highest activity for SYR and GUA being determined at neutral pH (Fig. S1b). The degradation experiments showed HRP to be approximately six times more efficient in McIlvaine's buffer of pH 7 compared with pH 3 when the sum of the micropollutants was considered. Neutral pH was favorable for degradation of all tested analytes by HRP (Fig. 3b).

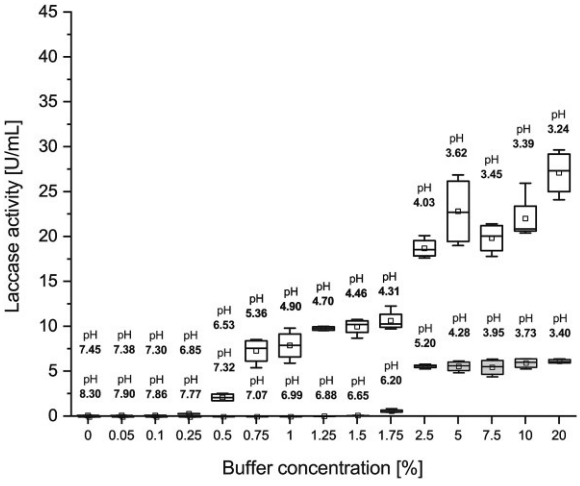

Fig. 1 Catalytic activity of laccase from *T. versicolor* in tap water (white) and wastewater (gray) supplemented by McIlvaine's buffer (pH 3) and determined using ABTS substrate. Whiskers represent minimum and maximum values; the rectangle encompasses the interquartile range, with denoted median (horizontal line) and mean (square) values ($n = 4$)

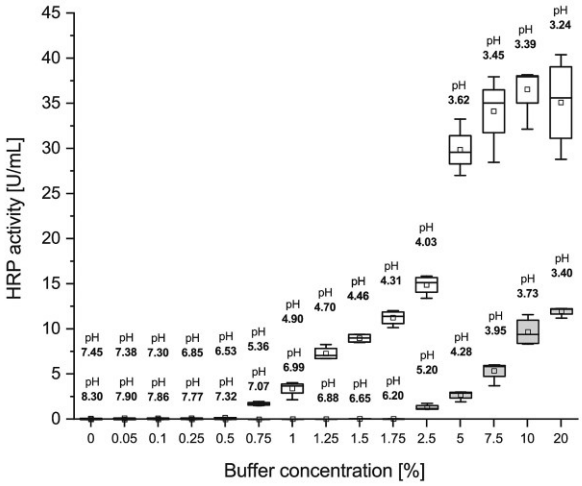

Fig. 2 Catalytic activity of horseradish peroxidase (HRP) in tap water (white) and wastewater (gray) supplemented by McIlvaine's buffer (pH 3) and determined using ABTS substrate. Whiskers represent minimum and maximum values; the rectangle encompasses the interquartile range, with denoted median (horizontal line) and mean (square) values ($n = 4$)

In order to determine the degradation ability of both enzymes under real environmental conditions, additional 20 h degradation experiments were performed in different water matrices comprising DIW, tap water, tap water amended with 2.5% (v/v) of McIlvaine's buffer of pH 7, ultrafiltrated wastewater, and ultrafiltrated wastewater amended with 2.5% (v/v) of McIlvaine's buffer of pH 3 or 7. As shown in Fig. 4a, the best degradation efficiency of BPA and EE2 by laccase was achieved in DIW, where 91 and 100% of the initial amount was degraded, respectively, with lower degradation levels observed for DCF and TCS. Similar results were observed with HRP, where BPA and EE2 showed best degradation in DIW (91 and 100%, respectively), while DFC and TCS degradation results were lower (Fig. 4b).

In tap water, the degradation of BPA, EE2, DCF, and TCS by laccase reached 44, 68, 42, and 61%, respectively, and 83, 75, 49, and 56%, respectively, in the case of HRP. Overall, enzyme degradation efficiency was not improved by amendment of tap water by McIlvaine's buffer, with the exception of BPA degradation by laccase and TCS degradation by HRP (Fig. 4; Student's *t*-test, $p = 0.05$).

Lowest degradation efficiency by both laccase and HRP was recorded in untreated ultrafiltrated wastewater, where no more than 7 and 11% of EE2, respectively, was eliminated after 20 h. In contrast with tap water, however, the addition of 2.5% (v/v) of McIlvaine's buffer of pH 3 or 7 to wastewater enhanced the degradation efficiency of both enzymes markedly. As shown in Fig. 4a, laccase degradation of BPA, EE2, and DCF was significantly better when supplemented by McIlvaine's buffer of pH 7, compared with pH 3, whereas no significant difference was observed for TCS when comparing the two amended samples (Student's *t*-test, $p = 0.05$).

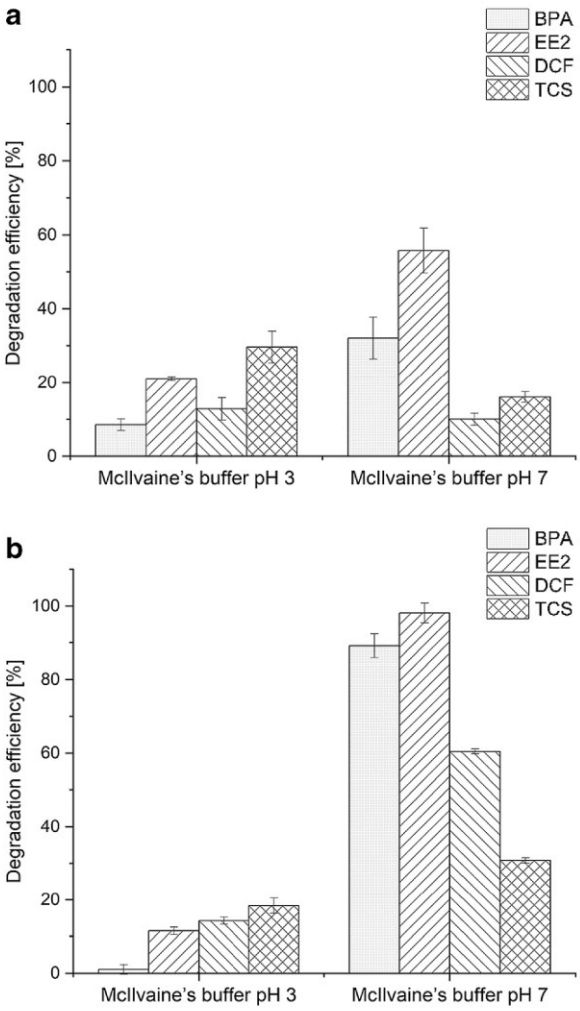

Fig. 3 Elimination of a mixture of micropollutants (10 mg/L of each)—bisphenol A (BPA), 17α-ethinylestradiol (EE2), diclofenac (DCF), and triclosan (TCS)—in pure McIlvaine's buffer (pH 3 and 7) after 20 h by **a** laccase from *T. versicolor* (20 U/L) and **b** horseradish peroxidase (20 U/L) ($n = 3$)

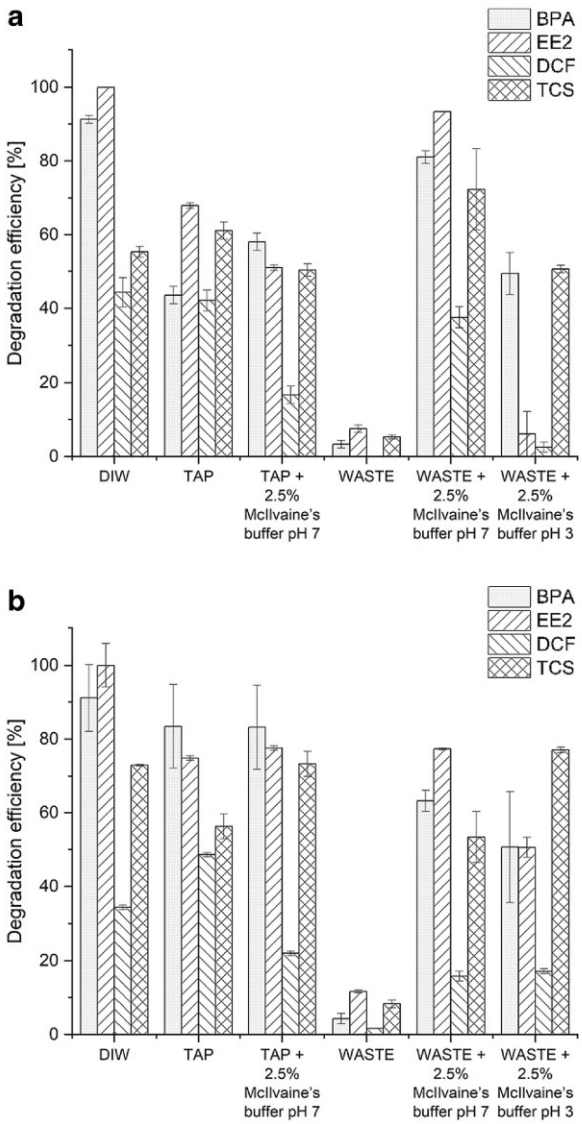

Fig. 4 Elimination of a mixture of micropollutants (10 mg/L of each)—bisphenol A (BPA), 17α-ethinylestradiol (EE2), diclofenac (DCF), and triclosan (TCS)—in deionized water (DIW), tap water (TAP), wastewater (WASTE), and tap or wastewater supplemented by 2.5% (v/v) McIlvaine's buffer (pH 3 or pH 7) after 20 h by **a** laccase from *T. versicolor* (20 U/L) and **b** horseradish peroxidase (20 U/L) ($n = 3$)

Degradation of BPA, EE2, DCF, and TCS in wastewater supplemented by McIlvaine's buffer of pH 7 reached 81, 93, 38, and 72%, respectively. Stronger effect of McIlvaine's buffer of pH 7 could be explained by its higher concentration of hydrogen phosphate anions comparing to the same buffer of pH 3. Based on Hofmeister series, these anions are able to stabilize enzymes (Okur et al. 2017; Zhang and Cremer 2006) and thus enable their activity in unfavorable environment such as secondary wastewater.

Degradation of EE2 by HRP in wastewater was higher with the addition of buffer of pH 7, while TCS degradation was improved by enrichment with McIlvaine's buffer of pH 3 (Fig. 4b; Student's *t*-test, $p < 0.05$). No significant differences were observed for BPA and DCF degradation by HRP with the addition of McIlvaine's buffer of either pH level (Student's *t*-test, $p > 0.05$).

Comparison of our results with previous studies is difficult as the authors used different activity assays, different concentrations of chemicals, or performed the tests at different pH levels or temperatures. Hongyan et al. (2019), for example, reported rapid degradation of BPA but only in an optimal buffer system, while Olajuyigbe et al. (2019) compared free and immobilized laccase under conditions that cannot be

Table 3 Elimination of estrogenic activity (EEQ, estradiol equivalent) by the laccase from *T. versicolor* (TV) and horseradish peroxidase (HRP) in wastewater samples containing 2.5% (v/v) McIlvaine's buffer (pH 7) after 20 h of degradation ($n = 3$)

	Residual EEQ concentration (mg/L)	Residual concentration (%)
Control	10.0 ± 0.2	100 ± 2
Laccase from TV	0.89 ± 0.05	8.8 ± 0.5
HRP	1.982 ± 0.004	19.74 ± 0.04

compared with real waters. Garcia-Morales et al. (2015) used a 100 U/L laccase cocktail to eliminate BPA, EE2, TCS, and nonylphenol (10 mg/L) in a buffer of pH 5, with over 90% of the micropollutants degraded within 5 h. In our study, however, we employed oxidoreductases at a much lower activity level (ca. 20 U/L) to eliminate a similar concentration of micropollutants, which explains the lower degradation efficiencies observed. Most importantly, our experiments were performed using real waters, representing significant progress in the study of water treatment using oxidoreductases.

The benefits of oxidoreductase treatment were also confirmed by the estrogenic assay (Table 3). Degradation of micropollutants in wastewater containing 2.5% McIlvaine's buffer at pH 7, which best corresponded to the conditions of real wastewater treatment plant effluents, showed a massive decrease in estrogenic activity. After 20 h of degradation, both laccase and HRP were able to decrease estrogenic activity by over 91 and 80%, respectively. These findings demonstrate that the degradation products were not estrogenic. The decrease in estrogenic activity observed corresponded to a decrease in EE2 concentration, the main estrogenic compound in the mixture. These results are in agreement with previous studies in which laccase proved able to lower estrogenic activity (Cajthaml 2015; Kresinova et al. 2017; Kresinova et al. 2012).

Conclusions

This study highlighted the necessity of testing catalytic activity of *T. versicolor* laccase and HRP on EDCs in real waters. Relying solely on commercial substrates and presumed optimal pH conditions can easily lead to faulty decisions. As an example, highest laccase activity has commonly been reported (including this study) in acidic conditions (pH 2–5) when determined using ABTS, SYR, or GUA. However, our results clearly show that degradation efficiency on a mixture of micropollutants was better in DIW and tap water of neutral to slightly basic pH than in more commonly used pure buffer solutions of acidic pH.

Wastewater effluent is an extremely unfavorable environment for degradation of EDCs; nevertheless, biocatalytic efficiency was greatly increased by the addition of 2.5% (v/v) McIlvaine's buffer. The main function of the buffer was most probably in providing beneficial anions that stabilized enzymes and thus enabled their activity. Laccase and HRP were both shown to be robust and universal in application. Though HRP was more efficient in the elimination of EDCs under the defined conditions (McIlvaine's buffer of pH 7), laccase, the more reasonable choice for degradation of EDCs as it does not require hydrogen peroxide as a co-substrate, exhibited the best degradation capability in wastewater effluent supplemented by McIlvaine's buffer (pH 7), where it was able to reduce estrogenic activity by 91%.

The efficient biocatalytic activity of laccase under real treatment environments, as shown in this study, should encourage prospective studies leading to greener water treatment technologies.

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Author contribution MM, AS, and TC designed the study. MM performed most of the experiments and wrote the manuscript. LL determined estrogenic activity, prepared the figures, and participated on writing and finalization of the manuscript. VN carried out quantitative analyses of micropollutants. MR participated on the enzyme activity experiments. All authors read and approved the final manuscript.

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Data availability The datasets used and analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

Competing interests The authors declare that they have no competing interests.

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Supplementary information

Environmental Science and Pollution Research

Laccase and horseradish peroxidase for green treatment of phenolic micropollutants in real drinking water and wastewaters in the Czech Republic

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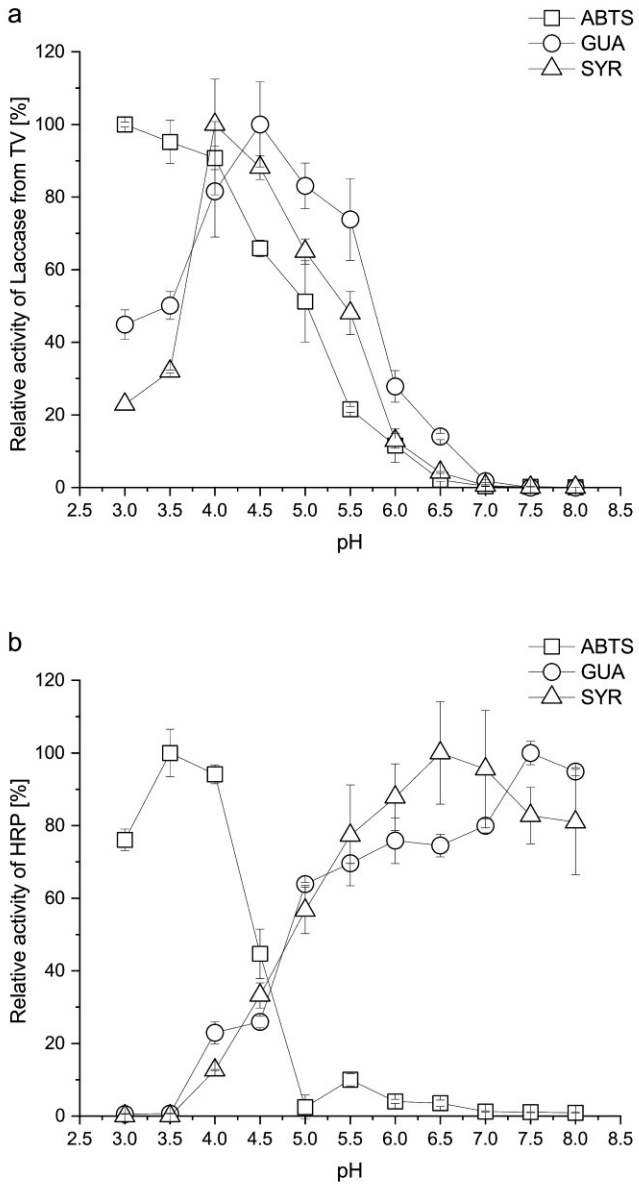

Fig. S1 Activity of (a) laccase from *Trametes versicolor* (TV) and (b) horseradish peroxidase (HRP) at different pH levels using substrates ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), SYR (syringaldazine), and GUA (guaiacol); (n = 3)

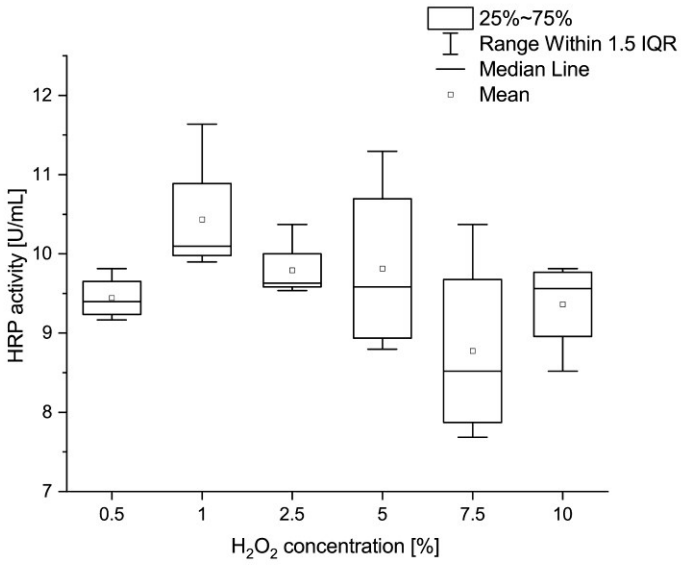

Fig. S2 Activity of horseradish peroxidase (HRP) with different H_2O_2 concentrations. Whiskers represent minimum and maximum values, the rectangle encompasses the interquartile range, with denoted median (horizontal line) and mean (square) values; (n = 4)