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Characterisation of electron beam irradiation-immobilised laccase for application in wastewater treatment

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Abstract

Laccase from *Phoma* sp. UHH 5-1-03 was cross-linked to polyvinylidene fluoride membranes by electron beam irradiation. Immobilised laccase displayed a higher stability than the non-immobilised enzyme with respect to typical wastewater temperatures, and pH at a range of 5 to 9. Batch tests addressed the removal of pharmaceutically active compounds (PhACs; applied as a mixture of acetaminophen, bezafibrate, indometacin, ketoprofen, mefenamic acid, and naproxen) by both immobilised and non-immobilised laccase in municipal wastewater. High removal rates (> 85%) of the most efficiently oxidised PhACs (acetaminophen and mefenamic acid) indicated a high efficiency of the immobilised laccase in wastewater. Continuous elimination of the aforementioned PhACs by the immobilised enzyme in a continuously operated diffusion basket reactor yielded a PhAC removal pattern qualitatively similar to those observed in batch tests. Clearly higher apparent V_{max} values and catalytic efficiencies (in terms of both $V_{max}/S_{0.5}$ as well as V_{max}/K_m values obtained from data fitting according to the Hill and the Michaelis-Menten model, respectively) observed for acetaminophen oxidation by the immobilised compared to the non-immobilised enzyme are in support of a considerably higher functional stability of the immobilised laccase especially in wastewater. The potential influence of acetaminophen on the removal of comparatively less laccase-oxidisable water pollutants such as the antimicrobial triclosan (TCS) was investigated. TCS was increasingly removed upon increasing the initial acetaminophen concentration in immobilised as well as non-immobilised laccase reaction systems until saturation became evident. Acetaminophen was consumed and not recycled during laccase reactions, which was accompanied by the formation of various acetaminophen-TCS cross-coupling products. Nevertheless, the simultaneous presence of acetaminophen (and potentially even more pollutant removal-enhancing laccase substrates) and more recalcitrant pollutants in wastewater represents an interesting option for the efficiency enhancement of enzyme-based wastewater treatment approaches.

Highlights

- Laccase was cross-linked to PVDF membranes by electron beam irradiation
- Immobilised laccase displayed a remarkable functional stability in real wastewater
- The batch-wise and continuous enzymatic removal of pharmaceuticals was demonstrated
- Pathways for acetaminophen and acetaminophen-triclosan conversions are proposed
- Enzymatic acetaminophen oxidation enhances triclosan removal via cross-coupling

Keywords

Acetaminophen; Laccase immobilisation; Micro-pollutants; Pharmaceutically active compounds; Transformation products; Wastewater

1. Introduction

A rapid industrial development and growing demands for various chemicals are accompanied by the continuous introduction of persistent and sometimes eco-toxic micro-pollutants (micropollutants) into aquatic systems, mainly from discharges of conventional wastewater treatment plants (WWTPs) (Luo et al., 2014). Due to the incomplete removal of micropollutants in conventional WWTPs, their toxicity potential, and potential long-term detrimental impacts even at the ng/L to the lower µg/L range, current challenges in developed countries relate to the development of advanced water treatment methods (Loos et al., 2013; Verlicchi et al., 2012). However, the application of advanced processes based on chemical oxidation, membrane filtration, or adsorption (e.g. by activated carbon) entails considerable costs (Loh et al., 2000). Whereas filtration and adsorption methods would require further treatment of the generated waste, chemical conversions of micropollutants could lead to undesirable by-products potentially being even more toxic than their parent compounds (Andreozzi et al., 2005; Gasser et al., 2014).

Enzymes as industrial biocatalysts offer promising advanced treatment methods, which potentially may overcome known drawbacks of conventional processes (Cabana et al., 2007a, b; Gasser et al., 2014). In recent years, oxidative enzymes such as laccases (EC 1.10.3.2; benzenediol: oxygen oxido reductase or phenol oxidase) have attracted considerable interest in this respect because of a relatively low substrate specificity and the usability of available air oxygen as an electron acceptor (Cabana et al., 2011). Laccase is a copper containing enzyme, which is able to oxidize a wide range of micropollutants including endocrine disrupting chemicals (EDCs), and pharmaceutically active compounds (PhACs) (Arca-Ramos et al., 2016; Gasser et al., 2014; Cabana et al., 2007a, b; Marco-Urrea et al., 2010). A remarkable characteristic of laccases relates to the possibility to enhance pollutant oxidation rates and expand the range of oxidisable compounds through laccase redox mediators. These are diffusible low-molecular-mass laccase substrates first being enzymatically oxidised to yield organic radicals, which subsequently oxidise further compounds in an abiotic manner (Jahangri et al., 2017). Ideally, redox mediators should

regenerate during pollutant oxidation thus becoming available for a next catalytic cycle. Such effects have been claimed for laccase oxidation systems involving the lignin-related phenolic syringaldehyde as a natural redox mediator and the pesticide dichlorophen as a target pollutant (Torres-Duarte et al., 2009). Contrary to such reports, presumed laccase redox mediators such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), acetosyringone and syringaldehyde have been found to be consumed instead of being recycled in other studies (Jahangiri et al., 2017; Margot et al., 2015).

Although frequently being considered for wastewater treatment, laccase freely suspended in real wastewater would undergo rapid denaturation and not provide long-term operational stability (Cabana et al., 2009b; Gasser et al., 2014). The use of enzymes in immobilised form is advancing in industrial and environmental applications, and has the potential to overcome shortcomings related to the use of free enzymes (Ba et al., 2014; Gasser et al., 2014; Touahar et al., 2014). Covalent enzyme binding to solid support materials has been reported to be the preferred laccase immobilisation method for wastewater treatment applications, and the corresponding biocatalysts are thought to be more stable than those obtained with other immobilisation techniques especially under the harsh conditions of real wastewaters (Gasser et al., 2014). However, “classical” covalent enzyme immobilisation methods are usually quite time-consuming and commonly involve coupling steps ranging from several hours up to about one day (Arca-Ramos et al., 2016; Cabana et al., 2009a; Hommes et al., 2012; Kumar et al., 2014; Zimmermann et al., 2011). They are additionally complicated due to the need for appropriate cross-linkers, and often involve numerous further time-consuming processing (e.g. surface modification, drying, washing) steps (Arca-Ramos et al., 2016; Cabana et al., 2009a; Hommes et al., 2012; Kumar et al., 2014; Zimmermann et al., 2011). Using commercial *Trametes versicolor* laccase (TvL), we have recently firstly demonstrated the general applicability of a very rapid, simple, and inexpensive one-step immobilisation procedure based on the electron beam (E-Beam) irradiation-induced covalent linking of the enzyme to commercial porous polyvinylidene fluoride (PVDF) membranes as the support material (Jahangiri et al., 2014). The laccase-containing membranes thus achieved are

advantageous with respect to long-term mechanical stability and easy separation from a reaction medium, and potentially hold promise for wastewater treatment approaches.

The present study aimed to establish and partly optimise a particularly rapid one-pot procedure for the immobilisation of laccase from the aquatic ascomycete *Phoma* sp. UHH 5-1-03 (Junghanns et al., 2008; Junghanns et al., 2009), based on E-Beam irradiation-induced cross-linking onto PVDF membranes as introduced above. Laccase from *Phoma* sp. was chosen because of its ability to oxidize substrates still at neutral to slightly alkaline pH values (Junghanns et al., 2009) relevant for wastewaters. However, the *Phoma* laccase was found to be quite sensitive towards glutaraldehyde used as a cross-linking agent in a previous study (Hommes et al., 2012). The E-Beam procedure allows enzyme immobilisation within the range of minutes (see sub-section 2.3 of the materials and methods section) instead of the several hours to days needed for conventional chemical enzyme coupling as described before. We further aimed to assess important characteristics of the immobilised laccase such as pH and thermal stability, reusability, and apparent kinetic parameters for micropollutant oxidation in comparison with the non-immobilised enzyme. For this, we have investigated the performance of laccase in either form in various aqueous matrices also including real wastewater. Enzymatic micropollutant removal was demonstrated using a cocktail of phenolic and non-phenolic PhACs in glass vial-based batch experiments, and a lab-scale perfusion basket reactor (BR) (Langford and Thomas, 2009). Among these PhACs, the phenolic acetaminophen was chosen as a model compound for comparing apparent kinetic parameters of PhAC oxidation by the immobilised and the free laccase in real wastewater and buffer. The formation of free acetaminophen radicals as primary products of acetaminophen oxidation by laccase (Lu et al., 2009) qualifies the compound as a potential laccase redox mediator, possibly enhancing the laccase-catalysed transformation of other MPs being more resistant towards laccase attack (Arca-Ramos et al., 2016; Touahar et al., 2014). We have therefore studied potential redox-mediating effects of acetaminophen in laccase reaction systems (Hachi et al., 2017) in more detail. In this regard, the influence of acetaminophen on both target pollutant removal and the formation of transformation products

was assessed. Triclosan (TCS), a persistent environmental contaminant with antimicrobial activity being comparatively slowly oxidised by the laccase from *Phoma* sp. (Hofmann and Schlosser, 2016; Jahangiri et al., 2017), was considered to be a suitable model target pollutant for these investigations. TCS concomitantly also offers previously established knowledge with respect to the mode of action and related transformation pathways of other compounds enhancing its laccase oxidation such as syringaldehyde (Jahangiri et al., 2017). Based on such data the present study also aimed to compare mechanisms underlying the effects observed with different presumable redox mediators, thereby further completing our picture about redox mediator functioning and the feasibility of related applications.

2. Materials and methods

2.1 Chemicals and other materials

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, purity ≥ 98%) was purchased from AppliChem (Darmstadt, Germany). Acetaminophen (≥ 99%), mefenamic acid (≥ 98%), bezafibrate (≥ 98%), naproxen (≥ 98.5%), ketoprofen (≥ 98%), indometacin (≥ 99%) and phosphate buffered saline (PBS, pH 7.0) were provided by Sigma-Aldrich (Saint-Louis, MO, USA). Triclosan (TCS ≥ 97%) was obtained from AppliChem. Oasis HLB 1 cc solid-phase extraction (SPE) cartridges with 30 mg sorbent were purchased from Waters (Eschborn, Germany). Hydrophobic PVDF membranes (Roti-PVDF, pore size 0.45 µm, thickness 125 µm) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Bicinchoninic acid (BCA) protein assay reagents A + B were provided by Pierce (Rockford, IL, USA). All chemicals were of analytical grade and used without further purification.

2.2 Laccase source

Extracellular laccase was derived from the aquatic ascomycete *Phoma* sp. strain UHH 5-1-03 (deposited as *Phoma* sp. DSM 22425 in the German Culture Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The isolation, identification and

maintenance of this fungus was previously described (Junghanns et al., 2008). For laccase production, *Phoma* sp. was cultivated in flasks (1 L) containing 300 ml of 2% (w/v) malt extract medium (pH 5.7) containing 50 µM $CuSO_4$ and 1 mM vanillic acid in addition, in order to stimulate laccase production (Junghanns et al., 2009). Each flask was inoculated with 6 ml of a mycelial suspension obtained as described before. Fungal cultures were incubated at 14 °C (a temperature previously found to be well suitable for growth and laccase production by this aquatic isolate; Junghanns et al., 2008; Junghanns et al., 2009) and 120 rpm up to 14 days (depending on the occurrence of maximum laccase activity) in the dark. Cell-free supernatants of fungal cultures were concentrated by ultrafiltration, using a 400-mL stirred cell (Model 8400, Merck Millipore, Billerica, MA, USA) and an Omega polyethersulfone membrane (10 kDa cut-off, Pall GmbH Life Sciences, Dreieich, Germany). Further laccase purification using hydrophobic interaction and gel filtration chromatography was carried out as previously described (Jahangiri et al., 2017; Junghanns et al., 2009).

2.3 Laccase immobilisation on PVDF membranes

A PVDF membrane disc (47 mm diameter) was first wetted with ethanol for 5 min, and afterwards washed with ultrapure water (three times for five min, respectively). Then, the membrane was immersed into a *Phoma* laccase solution (corresponding to protein concentrations indicated in the text, and in section 1.1 and Table S1 of the Supplementary Material) for 5 min. Thereafter, the membrane was taken out from the enzyme solution and irradiated by E-Beam with a dose of 150 kGy. Irradiation was performed at 160 kV and 10 mA in a N_2 atmosphere (O_2 concentration < 10 ppm) (Jahangiri et al., 2014). The absorbed dose was adjusted by the speed of the sample transporter (2 m/min) used to pass the sample through the irradiation chamber of the electron accelerator without a stop, corresponding to an irradiation time of about 1 s and an entire process duration of approximately 2 min. The irradiated membrane was rinsed (three times for 30 min, respectively) with 0.01 M phosphate buffered saline (PBS, pH 7.0). Finally, the membrane

196 was cutted into smaller discs (10 mm diameter) for further investigations, and stored in PBS
197 buffer (pH 7.0).
198
199 *2.4 Membrane characterisation*
200 The morphology of pristine and laccase-containing PVDF membranes was studied by
201 scanning electron microscopy (SEM; Ultra 55, Carl Zeiss SMT, Jena, Germany). Before
202 SEM, membranes were sputtered with a thin gold layer in order to avoid their charging.
203 The chemical composition of PVDF membrane surfaces before and after immobilisation of
204 *Phoma* laccase was analysed with X-ray photoelectron spectroscopy (XPS; AXIS Ultra,
205 Kratos Analytical, Manchester, England). The kinetic energy of the electrons was analysed
206 with a pass energy of 160 eV for the survey spectra and 40 eV for the energy resolved
207 spectra, respectively, as previously described (Jahangiri et al., 2014).
208 In order to determine the amount of *Phoma* laccase protein immobilised on the
209 membrane, the bicinchoninic acid (BCA) test (Smith et al., 1985) was used. As described in
210 Starke et al. (2013), membrane discs (10 mm diameter) were treated with BCA reagent for
211 25 min at 37 °C after E-beam irradiation and rinsing as described above. Thereafter, the
212 absorbance at 562 nm was recorded using a microtiter plate reader (Infinite M200; Tecan,
213 Crailsheim, Germany). For calibration, non-immobilised *Phoma* laccase corresponding to
214 protein concentrations of 5.00, 2.50, 1.25, 0.63, 0.31, and 0.16 µg mL^{-1} (as determined with
215 bovine serum albumin) was used.
216
217 *2.5 Laccase activity determination*
218 Free laccase activity was routinely monitored by following the oxidation of 2 mM 2,2-
219 azino-bis-(3-ethylbenzthiazoline 6-sulfonic acid) (ABTS) (McIlvaine, 1921) in McIlvaine buffer
220 at pH 4.0, using a microplate reader operated at 420 nm (Junghanns et al., 2008). One
221 enzymatic activity corresponds to the amount of enzyme that forms 1 µmol product per min.

For activity determination of immobilised laccase, a previously published discontinuous assay based on recording of the oxidation of 2 mM ABTS in McIlvaine buffer (pH 4.0) was applied (Jahangiri et al., 2014).

2.6 Determination of catalytic properties of the free and immobilised laccase

The apparent Michaelis-Menton parameters K_m and V_{max} of immobilised (12 U L^{-1}) and free *Phoma* laccase (200 U L^{-1}) were determined for the laccase substrate ABTS (applied in a concentration range of 3.9 to 4000 µM) in McIlvaine buffer (pH 4.0) at room temperature. Triplicate assays were always performed. Apparent Michaelis-Menton kinetic parameters were calculated from the initial rates in ABTS oxidation upon non-linear regression of the obtained data (COD > 0.99), using the software OriginPro 9 (64 bit version; OriginLab Corp., Northampton, MA).

The pH stability of immobilised (10 U L^{-1}) and free *Phoma* laccase (200 U L^{-1}) was determined upon incubation in McIlvaine (pH values of 3.0, 5.0, and 7.0) and Britton-Robinson buffer (pH 9.0) at 4 ºC and 80 rpm over the time periods indicated in the text (at pH 7.0 the activity of free and immobilised laccase towards ABTS in Britton-Robinson buffer was found to be 94 and 96% of that in McIlvaine buffer, respectively, which was used to correct for the activities determined in Britton-Robinson buffer at pH 9.0). The laccase activity was determined with the routine ABTS assay at the indicated time points. Duplicate tests were always performed.

The temperature stability was investigated by incubating immobilised (10 U L^{-1}) and free laccase (160 U L^{-1}) in McIlvaine buffer (pH 4.0) at different temperatures (4 °C, 15 °C, 22 °C = room temperature, 35 °C, 45 °C, and 60 °C) and shaking at 80 rpm over the time periods indicated in the text. The residual enzyme activity was determined at the time points specified in the text, using the routine ABTS assay indicated above. Duplicate tests were always performed.

The reusability of the immobilised *Phoma* laccase (10 U L^{-1}) was assessed during 20 successive oxidation steps, using 2 mM ABTS as a substrate in McIlvaine buffer (pH 4.0) at

250 room temperature. After each ABTS oxidation step (always 6 min duration), membranes
251 containing immobilised laccase were thoroughly washed (5 times with 3 mL of distilled water
252 for 1 min, respectively) in order to remove all remaining ABTS and its oxidation products, and
253 subsequently used for the next ABTS oxidation step. Duplicate tests were always performed.
254
255 *2.7 Batch and continuous elimination of a mixture of PhACs*
256 Both immobilised and free *Phoma* laccase were employed in batch experiments
257 addressing the effect of either enzyme formulation on the removal of a cocktail of selected
258 PhACs. The PhACs solution consisted of acetaminophen, mefenamic acid, naproxen,
259 bezafibrate, indometacin and ketoprofen. The stock solution of each compound was
260 prepared individually at a concentration of 1 mM in 100% methanol. The aforementioned
261 PhACs were spiked to a final concentration of 10 µM each (corresponding to a final sum
262 concentration of PhACs of 60 µM and a final methanol concentration of 6%) into glass vials
263 containing 4 ml of one of the following solutions: i) original influent (pH 7.7) previously taken
264 from a municipal wastewater treatment plant (WWTP) located in Magog (QC, Canada), ii)
265 buffered influent (pH 7.0), composed of 80% (v/v) original influent and 20% of a 5-fold
266 concentrated (compared to the original composition) McIlvaine buffer solution (pH 7.0)
267 (McIlvaine, 1921), iii) original effluent (pH 8.0) from the WWTP, iv) buffered effluent (pH 7.0),
268 composed of 80% (v/v) original effluent and 20% of a 5-fold concentrated McIlvaine buffer
269 solution (pH 7.0), v) Milli Q ultrapure water (MQPW; 18.2 MΩ x cm at 25 °C, pH 7.0, TOC ≤
270 10 ppb), and vi) McIlvaine buffer (pH 7.0) according to the original formulation (McIlvaine,
271 1921). Further characteristics of wastewater form the Magog WWTP could be retrieved from
272 Table S1 of the Supplementary Material of reference Arca-Ramos et al. (2016)
273 (https://link.springer.com/article/10.1007%2Fs11356-016-6139-x#SupplementaryMaterial).
274 Prior to their use, WWTP influents and effluents were filtered through 0.45 µm filters to
275 remove particulate solids and microorganisms. Laccases were applied at 20 U L^{-1}
276 (immobilised enzyme) and 200 U L^{-1} (free enzyme) (See section 1.2 and Table S2 of the
277 Supplementary Material). Incubations were carried out at room temperature and 180 rpm for

278 24 h in the dark. Reaction mixtures containing heat-inactivated laccases served as controls.
279 In order to monitor the possible sorption of PhACs onto membranes as a potential cause of
280 compound removal, reaction mixtures containing enzyme-free membranes (i.e. membranes
281 previously subjected to a modified immobilisation procedure, where laccase had been
282 replaced by PBS buffer) were additionally employed. Samples (1000 µL) were taken as
283 indicated in the text and acidified (pH below 2.0) with 5% (v/v) formic acid. SPE using Oasis
284 HLB 1cc cartridges (Waters) was applied to extract PhACs from samples. SPE cartridges
285 were conditioned with 2 mL of methanol and equilibrated with 2 mL of MQPW (acidified to pH
286 2.0 with 1N HCl). Then, samples were gently passed through the cartridge in two successive
287 steps (500 µL, each) at one drop s^{-1}, respectively. Then, MQPW (1 mL; acidified to pH 2.0
288 with 1N HCl) followed by 2×0.5 mL methanol was used to elute the adsorbed PhACs at one
289 drop s^{-1}. Quantitative determination of PhACs by ultra-high performance liquid
290 chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) was carried out as
291 previously described (Touahar et al., 2014). The amounts of target PhACs already contained
292 in WWTP influent and effluent before spiking were determined prior to experiments, and are
293 compiled in Table S3 of the Supplementary Material.

294 A stirred tank reactor based on a metallic diffusion basket (300 mL working volume,
295 particle removal efficiency 99 % for 2.2 µm particles; (Cabana et al., 2009b; Kumar et al.,
296 2014) was used to study the elimination of selected PhACs by immobilised *Phoma* laccase in
297 a continuous mode. Prior to elimination experiments, the basket reactor (BR) was filled and
298 equilibrated by continuously feeding a mixture of acetaminophen, mefenamic acid,
299 bezafibrate, indometacin, naproxen, and ketoprofen (10 µM, each) in MQPW (pH 7.0) with
300 the help of a peristaltic pump (5 mL min^{-1} flow rate, corresponding to a hydraulic retention
301 time (HRT) of 1 h) for 6 h at room temperature. The BR was routinely agitated at 180 rpm
302 using three-bade propeller (25 mm diameter), which was located 2.5 cm above the BR
303 bottom. The reactor effluent was transferred through perfusion from the stainless basket to a
304 glass beaker. After filling and equilibrating the BR, immobilised laccase (16 U L^{-1}) was placed
305 inside the BR, which was then continuously operated for 6 h (corresponding to 6 consecutive

306 exchanges of the working volume of 300 mL) under the conditions described above.
307 Samples for PhAC analysis were taken from inside the BR at regular time intervals of 1 h (i.e.
308 always after a complete exchange of the working volume), and PhACs were analysed (see
309 sub-section 2.7). The removal of PhACs (%) was calculated relative to their respective initial
310 equilibrium concentrations (i.e. before adding the enzyme). The stability of the enzymatic
311 activity of the immobilised *Phoma* laccase was determined during operating the BR.
312
313 *2.8 Kinetic parameters of free and immobilised laccase toward acetaminophen*
314 Acetaminophen was chosen as a model compound for assessing and comparing apparent
315 catalytic parameters of immobilised and free *Phoma* laccase in both McIlvaine puffer (pH 7.0)
316 and municipal wastewater. Enzymatic reaction mixtures in buffer contained 23 and 20 U L^{-1}
317 of free and immobilised laccase from *Phoma* sp., respectively. Treated municipal wastewater
318 (effluent) was collected from the WWTP Rosental (Leipzig, Germany), and filtered
319 wastewater (0.45 µm) was used in enzymatic experiments without pH adjustment (original
320 pH 7.2). Further wastewater characteristics (always means from respective analyses over a
321 period of 21 months, starting from January 2016) are as follows: 24.9 mg L^{-1} chemical
322 oxygen demand, 3.2 mg L^{-1} biological oxygen demand, 9.4 mg L^{-1} total organic carbon, 8.8
323 mg L^{-1} total nitrogen bound, 0.23 mg L^{-1} ammonia (58% of all samples; <0.1 mg L^{-1} in 42% of
324 all samples), 6.6 mg L^{-1} nitrate, 0.04 mg L^{-1} nitrite (58% of all samples; <0.01 mg L^{-1} in 42%
325 of all samples), 0.4 mg L^{-1} total phosphorous, and 0.2 mg L^{-1} orthophosphate. Immobilised
326 laccase was employed at 24 U L^{-1} (i.e. similar to experiments conducted in McIlvaine buffer).
327 Since no acetaminophen degradation could be observed in real wastewater reaction
328 mixtures containing free laccase at 23 U L^{-1} (i.e. an amount being sufficient for
329 acetaminophen oxidation in McIlvaine buffer, see above), a higher enzyme amount of 200 U
330 L^{-1} was applied in these experiments (compare also Table S2 of the Supplementary
331 Material). Acetaminophen concentrations were always applied in a range of 15 to 2000 µM.
332 Corresponding mixtures containing heat-inactivated enzyme (instead of free active laccase)
333 and enzyme-free membranes (instead of immobilised active laccase) served as abiotic

controls. All experiments were performed in triplicate. Incubation was carried out at room temperature and 180 rpm for 24 h in the dark. Samples (0.5 mL) were taken from the acetaminophen-containing reaction mixtures before adding the respective enzymes, after 10 min following incubation in presence of enzymes, and after 1, 2, 4, 8 and 24 h of incubation. After addition of 0.5 mL of a mixture of 90% (v/v) methanol and 10% formic acid, samples were stored at -20 °C until analysis. Acetaminophen concentrations in samples were determined using UPLC analysis with diode array detection (see section 1.3 of the Supplementary Material). Data obtained from mixtures containing active laccase during the first 8 h of reaction were fitted to pseudo-first order removal kinetics (eq. 1) (Lu et al., 2009):

$$c_t = c_0 \times e^{-k't} \quad (1)$$

where c_t is the acetaminophen concentration at a given time (µM), k' represents the apparent first-order decay constant (h^{-1}) and t is the time of incubation in presence of acetaminophen (h). The corresponding coefficients of determination (COD) were always > 0.96 (> 0.95 and > 0.92 for 250 and 1000 µM acetaminophen treated with free laccase in buffer, respectively). Initial rates of acetaminophen removal were calculated by multiplying the respective C_0 and k' values (Hofmann and Schlosser, 2016).

Linear regression was applied to data obtained from control mixtures containing heat-inactivated enzyme (instead of free active laccase) and enzyme-free membranes (instead of immobilised active laccase) according to the following equation:

$$c_t = c_0 + r' \times t \quad (2)$$

where c_t is the acetaminophen concentration at a given time point (µM), c_0 refers to the initial acetaminophen concentration (µM), r' represents the apparent rate of acetaminophen removal (µM h^{-1}) and t is the time of incubation in the presence of acetaminophen (h). The corresponding COD values were always > 0.96 (except for acetaminophen concentrations of 31.25 and 15.625 µM treated with heat-inactivated free laccase in wastewater: COD > 82). The rates of enzymatic acetaminophen removal were calculated by substracting the removal rates observed for the controls from those obtained for corresponding reaction mixtures

containing active laccase. All kinetic calculations were performed using the software OriginPro 9 mentioned before.

For the subsequent identification of transformation products resulting from enzymatic oxidation of acetaminophen both, free and immobilised laccase-containing McIlvaine buffer-based reaction mixtures initially supplemented with 500 µM acetaminophen were incubated for 24 h as described above. Thereafter, LC-MS-QTOF analysis of the reaction mixtures was performed as described in section 1.4 of the Supplementary Material.

2.9 Targeting the possible function of acetaminophen as a laccase redox mediator

Triclosan (TCS) was used as a model target pollutant, in order to test whether acetaminophen could enhance its enzymatic removal through a redox-mediating or cross-coupling mechanism in laccase reactions. Enzymatic reaction mixtures in 5.5 ml McIlvaine buffer (pH 7.0) contained 50 µM TCS (added from a 1 mM methanolic stock solution, thus corresponding to a final methanol concentration in reaction mixtures of 5%), varying concentrations of acetaminophen (0, 50, 100, 250, and 500 µM; thus corresponding to molar acetaminophen : TCS ratios of 0:1, 1:1, 2:1, 5:1, and 10:1), and either immobilised (36 U L^{-1}) or free laccase from *Phoma* sp. (1454 U L^{-1}) (please refer to section 1.5 and Table S4 of the Supplementary Material for the determination of appropriate enzyme amounts for TCS degradation experiments). Corresponding mixtures containing heat-inactivated enzyme (instead of free active laccase) and enzyme-free membranes (instead of immobilised active laccase) served as abiotic controls. All experiments were carried out in triplicate. Incubations were carried out at room temperature and 180 rpm for 48 h in the dark. Samples (0.5 mL) were taken from the acetaminophen- and TCS-containing reaction mixtures before adding the respective enzymes, after 10 min following incubation in presence of enzymes, and at 1, 2, 4, 8, 24 and 48 h of incubation, and further processed for the determination of acetaminophen and TCS concentrations by UPLC coupled to photodiodearray detection (see section 1.3 of the Supplementary Material) as described above. The respective initial (maximal) removal rates of acetaminophen and TCS were determined via fitting of data of

389 compound concentration *versus* time plots as explained before, employing equation (1) for
390 mixtures containing active laccase and equation (2) for their corresponding control mixtures.
391 The corresponding COD values were always > 0.99 (except for 100 µM acetaminophen /
392 immobilised laccase: COD > 83).
393 For the subsequent identification of transformation products resulting from enzymatic
394 oxidation of acetaminophen and TCS both, free and immobilised laccase-containing reaction
395 mixtures initially supplemented with 100 µM acetaminophen and 50 µM TCS were incubated
396 for 24 h as described above. Thereafter, LC-MS-QTOF analysis of the reaction mixtures was
397 performed as described in section 1.4 of the Supplementary Material.
398
399 **3. Results and discussion**
400 *3.1 Immobilisation of the laccase from Phoma sp. and morphological and physico-chemical*
401 *characterisation of the laccase-containing membranes*
402 In initial orienting experiments immobilisation of the *Phoma* laccase by a previously
403 established procedure, which is based on E-Beam irradiation-induced cross-linking onto
404 PVDF membranes (Jahangiri et al., 2014), was carried out and compared for merely
405 ultrafiltration-concentrated laccase and enzyme derived from further purification by protein
406 chromatography. Very similar yields in terms of immobilised laccase activity were recovered
407 from membranes containing either of the two laccase preparations, and less activity got lost
408 for the ultrafiltration-concentrated compared to the further purified laccase during
409 immobilisation (Table S5 of the Supplementary Material). *Phoma* sp. previously has been
410 reported to produce only one laccase and peroxidase activities were not found (Junghanns et
411 al., 2009). Considering these facts and also taking economic considerations being of
412 relevance for practical applications into account, laccase concentrated by ultrafiltration but
413 not further purified by protein column chromatography was chosen for all following
414 immobilisation experiments.
415 The immobilisation procedure was optimised with respect to the influence of the protein
416 concentration of the applied laccase preparation on the laccase activity recovered in

417 immobilised state (see section 1.1 and Table S1 of the Supplementary Material). As could be
418 expected, the immobilised laccase activity of the membranes in terms of absolute values
419 increased with increasing protein concentrations. An immobilisation yield (i.e. the
420 immobilised laccase activity recovered from the membranes relative to that initially applied in
421 the primary solution) of 58% obtained at the lowest laccase protein concentration applied
422 (3.4 mg/L; Table S1) is nearly twice as high as the highest immobilisation yield achieved
423 even after optimising chemical coupling of the *Phoma* laccase onto fumed silica
424 nanoparticles (about 33%; Hommes et al., 2012). This promising observation suggests a
425 high potential of the E-Beam immobilisation approach for further optimisation, also
426 considering a long incubation time of 24 h previously applied to immobilise the *Phoma*
427 laccase onto solid carriers by chemical means (Hommes et al., 2012) vs. less than one hour
428 needed for enzyme immobilisation within the present study (see sub-section 2.3 of the
429 materials and methods section). An inverse correlation observed between the immobilisation
430 yield and the amount of laccase used for immobilisation (in terms of the protein concentration
431 of the laccase preparation; Table S1) was also reported for conventional glutaraldehyde
432 cross-linking of laccase to solid support materials (Zimmerman et al., 2011). Increasing
433 losses of laccase activity after immobilisation with increasing protein concentrations of the
434 laccase preparations initially applied as recorded in our study (Table S1) could well be
435 explained by an increase in enzyme-inactivating reactions with increasing protein
436 concentrations. For instance, self-coupling and/or other inactivation reactions initiated by E-
437 beam irradiation-generated protein radicals may have become increasingly dominant over
438 the radical coupling of active enzyme to the membrane (Garman and Nave, 2009). A protein
439 concentration of 11 mg L^{-1} found to result in a maximum laccase activity of 29 mU per
440 membrane disc at an immobilisation yield of 22% (Table S1) was chosen as the routine
441 procedure for further experiments.

442 SEM imaging was applied for the characterisation of the morphology of enzyme-
443 containing PVDF membranes (Fig. 1). The original (i.e. enzyme-free) PVDF membrane used
444 as a reference is highly porous with an open sponge-like pore structure, as visible from

445 corresponding cross-section (Fig. 1A) and top view images (Fig. 1B). SEM did not yield any
446 indication for adverse morphological effects potentially resulting from laccase immobilisation,
447 and proved the preservation of the porous membrane structure also upon laccase
448 immobilisation (Fig. 1C). Also after employing laccase-containing membranes in
449 acetaminophen (applied at 1 mM) degradation experiments for 24 h, no changes in the pore
450 structure of the membrane could be observed (Fig. 1D). We therefore conclude that neither
451 the immobilisation procedure itself nor the subsequent application of laccase-containing
452 membranes in the degradation of PhACs cause adverse effects to the membrane
453 morphology, and PVDF membranes are well suited to serve as porous supports in
454 biocatalytic enzyme membrane reactors.

455 The immobilisation of the *Phoma* laccase on the PVDF membrane surface was confirmed
456 by XPS analysis (Table 1). The enzyme-free membrane mainly consisted of fluorine and
457 carbon (Table 1). A small amount of oxygen (0.34%) is attributable to water adsorbing to the
458 membrane surface, which could not completely be excluded. After E-Beam immobilisation of
459 the *Phoma* laccase a more than 11-fold increase in the oxygen content was observed (3.82%
460 after laccase immobilisation *versus* 0.34% in the absence of laccase; Table 1). Furthermore,
461 nitrogen was unambiguously detected upon laccase immobilisation at 1.33%, which was not
462 present in the absence of laccase. A clear decrease in the fluorine content of the membrane
463 surface observed after laccase immobilisation (Table 1) can be attributed to a tiny enzyme
464 layer covering the membrane surface.

Table 1

Atomic composition of laccase-containing and -free membranes as determined by XPS analysis.

Membrane type	**Elemental ratio (relative atom-%)**			
	F	**O**	**N**	**C**
PVDF Membrane (reference)	56.04	0.34	ND[a]	43.61
Immobilised *Phoma* laccase membrane	48.70	3.82	1.33	46.15

[a] ND: Not detectable.

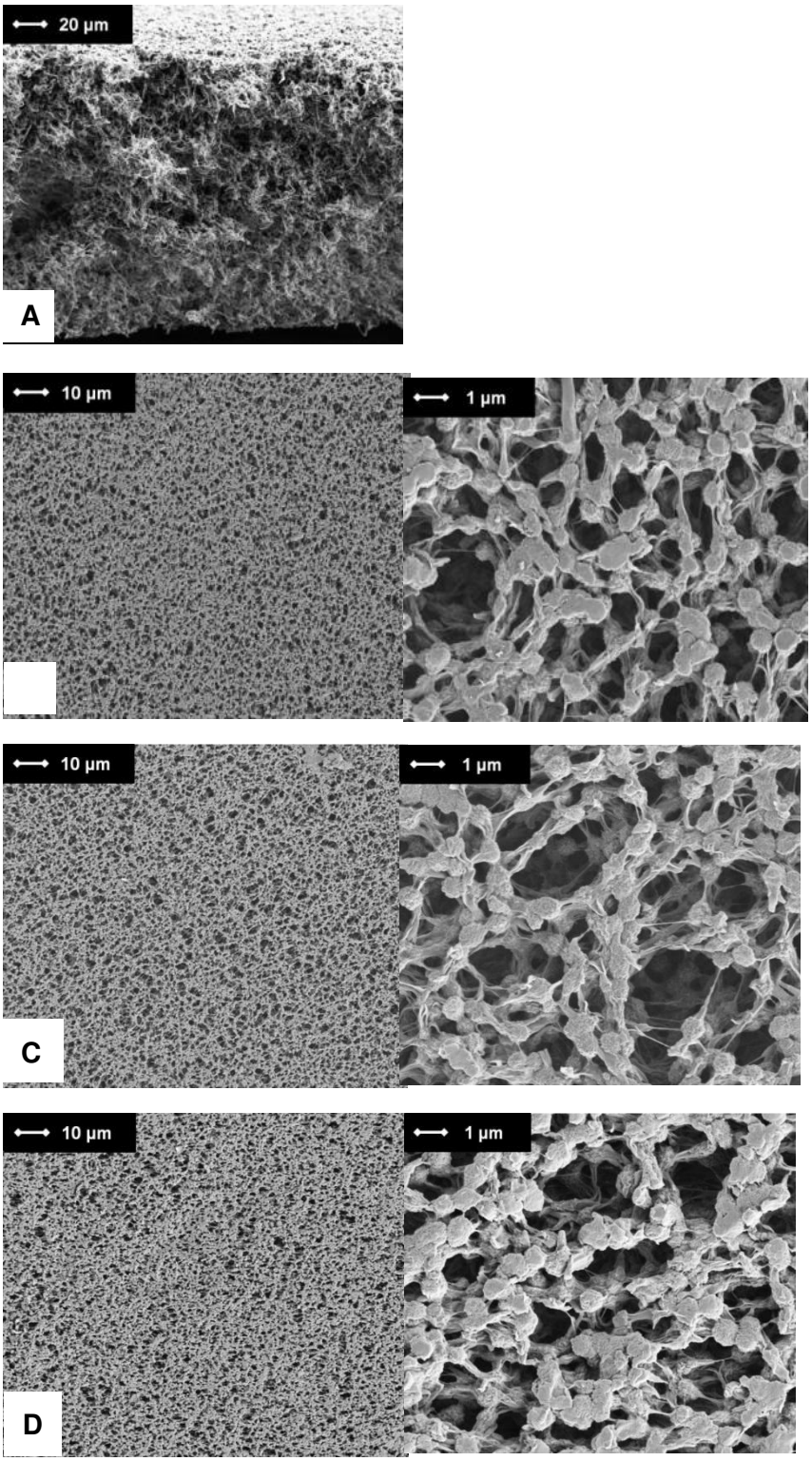


Fig. 1. SEM images of PVDF membranes. (A) Cross-section and (B) top view of the original enzyme-free membrane, (C) top view after E-Beam immobilisation of laccase, and (D) top view after employing the laccase-containing membrane in degradation of 1 mM acetaminophen for 24 h. Two different magnifications are shown for (B), (C), and (D), respectively.

479 The protein immobilised on the membrane surface was quantified using the BCA assay as
480 a complementary method. This assay allows the quantification of the immobilised laccase
481 protein amount throughout the complete membrane cross-section, while XPS just analyses
482 the surface. A value of 58.9 ± 4.9 µg (mean ± standard deviation for triplicate determinations)
483 of laccase protein per one membrane disc with a diameter of 1 cm (i.e. about 75 µg laccase
484 protein per cm^2) was obtained. Minor protein contamination not displaying laccase activity
485 (7.0 ± 1.0 µg per membrane disc) was detected for the enzyme-free membrane (membrane
486 subjected to a modified immobilisation procedure using PBS buffer instead of laccase) used
487 as a reference. Catalytic properties of the immobilised laccase are described in the following.
488
489 *3.2 Catalytic properties of free and immobilised laccase*
490 The apparent kinetic parameters for ABTS oxidation were assessed for both immobilised
491 and free laccase (Table 2). An about 1.6-fold higher K_m value of ABTS observed for the
492 immobilised compared to the free enzyme may indicate a lower immediate availability of this
493 substrate for the immobilised laccase, an effect frequently observed with immobilised
494 laccases and commonly caused by diffusion limitation (Arca-Ramos et al., 2016; Fernández-
495 Fernández et al., 2013; Gasser et al., 2014). In line with a limited substrate availability, ABTS
496 is quite hydrophilic (octanol-water partition coefficient log K_{ow} of 1.99; Margot et al., 2015)
497 whereas the PVDF membranes used for enzyme immobilisation are hydrophobic (Jahangiri
498 et al., 2014). The V_{max} value obtained with the free enzyme could not be fully reached with
499 the immobilised laccase even at 4 mM ABTS (i.e. about 64 times the K_m value), yielding a
500 catalytic efficiency (in terms of the V_{max} / K_m value) of the immobilised laccase of about half of
501 that of the free enzyme (Table 2). These observations strongly point to adverse effects of the
502 applied immobilisation procedure to a certain extent, e.g. causing a partial enzyme
503 inactivation or otherwise unfavourable protein conformational changes (Rekuć et al., 2010).
504 Similar relationships (i.e. an approximately 2-fold higher apparent K_m value and an about 2-
505 fold lower catalytic efficiency for ABTS oxidation by immobilised compared to free laccase)
506 were described for cross-linked laccase aggregates coupled to magnetic silica microbeads

using glutaraldehyde (Arca-Ramos et al., 2016). Even several times lower apparent enzyme affinities and catalytic efficiencies for ABTS oxidation by the immobilised compared to the free enzyme have been reported, e.g. for laccase chemically coupled to the diatomaceous earth support Celite® R-633 (Cabana et al., 2009a). Essentially comparable apparent enzyme affinities for ABTS as a substrate were found for laccase immobilised in the form of cross-linked enzyme aggregates (CLEAs) and free laccase (Cabana et al., 2007b). CLEAs may also offer higher specific activities (i.e. higher activity/volume or mass unit ratios) than enzymes immobilised on solid supports (Cabana et al., 2009b; Gasser et al., 2014), and sometimes also higher specific activities than free enzymes (Cabana et al., 2007b). However, they also suffer from certain drawbacks such as low mechanical stability and complicated procedures for their recovery from reaction media. For many CLEA applications, additional binding to a physical support may thus be required in order to improve the mechanical properties (Arca-Ramos et al., 2016).

Table 2

Apparent kinetic parameters of immobilised and free laccase for the oxidation of ABTS[a].

Laccase form	K_m **(μM)**	V_{max} **(U mg^{-1})**	V_{max} / K_m **(L h^{-1} mg^{-1} × 1000)**
Immobilised laccase	62.2 ± 12.5	1.4 ± 0.6	22
Free laccase	37.7 ± 7.1	1.7 ± 0.2	45

[a] Apparent K_m and V_{max} mean values ± standard errors arise from data fitting of means from triplicate experiments.

The pH is an important parameter influencing the stability of the enzymatic activity. The long-term storage stability of the immobilised and free laccase was compared at different pH values used for storage over the time periods shown in Fig. 2, and a temperature of 4 °C. As could be retrieved from Fig. 2, the activity of the immobilised laccase was clearly less decreasing than that of the free laccase within a pH range of 5 to 9. The immobilised laccase

was less stable compared to the free enzyme at pH 3, for reasons still remaining to be elucidated. A higher stability of the activity at pH 7 than at pH 5 observed in either case corroborates previous results (Junghanns et al., 2009). In summary it can be said that the immobilised *Phoma* laccase seems to be quite resistant to pH variations, which points to its potential applicability with regard to typical pH values of wastewaters (5-8) (Ba et al., 2014).

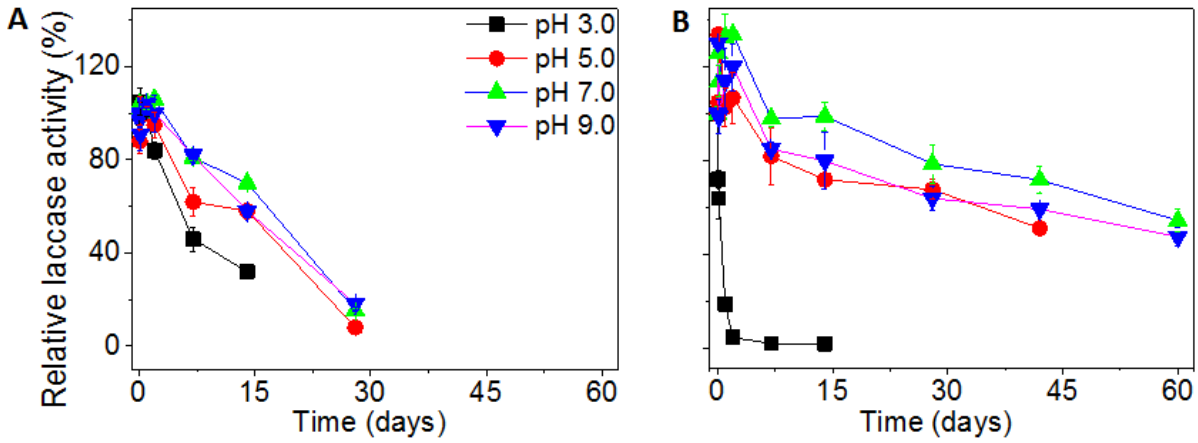


Fig. 2. Stability of free (A) and immobilised (B) laccase during incubation at different pH values over the indicated time periods. All values represent means ± absolute deviations (not visible if smaller than symbol size) from duplicate measurements.

Experiments targeting the temperature stability of the activity of the immobilised compared to the free enzyme revealed a clearly higher temperature stability of the immobilised biocatalyst particularly at 4 and 15 °C (Fig. 3). Remarkably, about 48% of the initial activity of the immobilised enzyme was still retained after storage at 15 °C for 22 days, and approximately 43% of the initial activity could still be recovered after storage at 4 °C for 36 days. Enzyme immobilisation is generally known to provide protection from activity-decreasing protein denaturation and the related drastic conformational changes (Leontievsky et al., 2001; Mateo et al., 2007). The comparatively lower thermal stability of the immobilised *Phoma* laccase at 22 °C, which resembles that observed with the free laccase, and the generally low thermal stability of either laccase form above 22 °C is in line with previously published data and could be related to the seasonal predominance of rather low

temperatures in the river where the fungus was isolated (Junghanns et al., 2009). The high thermal resistance of the immobilised laccase below 22 °C well fits into a common temperature range of wastewaters in temperate regions of about 10 to 20 °C (Cirja et al., 2008).

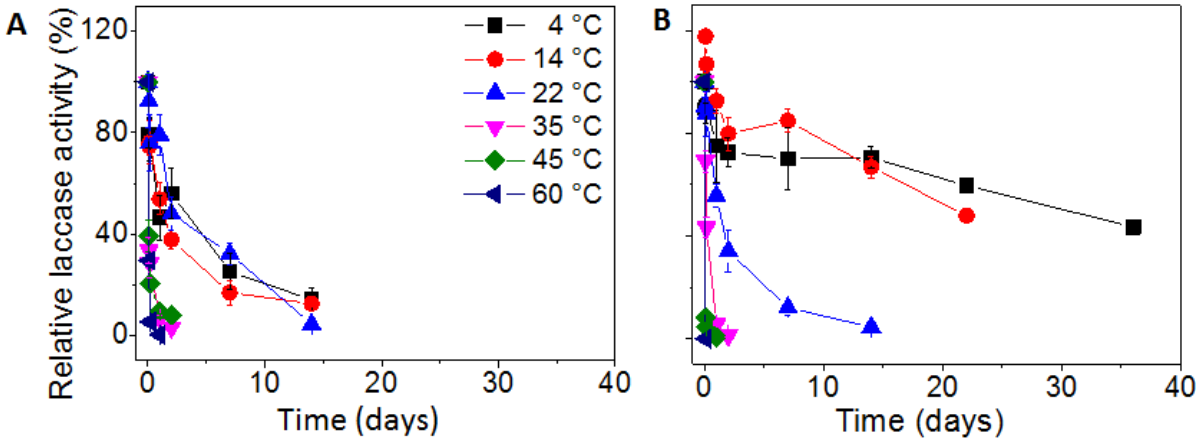


Fig. 3. Stability of free (A) and immobilised (B) laccase during incubation at different temperatures over the indicated time periods. All values represent means ± absolute deviations (not visible if smaller than symbol size) from duplicate measurements.

The reusability of an immobilised enzyme is an aspect governing its practical applicability from an economic viewpoint (Arca-Ramos et al., 2016). After 5 and 10 successive cycles of ABTS oxidation about 90 and 62% of the initial activity of the immobilised laccase could still be recovered. The half-life of the immobilised enzyme (in terms of 50% of its initial activity) was reached after 16 successive ABTS oxidation cycles, all together demonstrating a considerably high resistance towards potential enzyme inactivation and/or leakage as also reported by others (Kumar et al., 2014; Valle-Vigón and Fuertes, 2011; Wang et al., 2013). A further advantage of the applied immobilisation procedure is related to a high mechanical stability of the enzyme-containing membranes still after 20 successive ABTS oxidation cycles, and their easy separation from the reaction system, without any need for centrifugation (Ba et al., 2014) or magnetic field separation steps (Valle-Vigón and Fuertes, 2011; Wang et al., 2013).

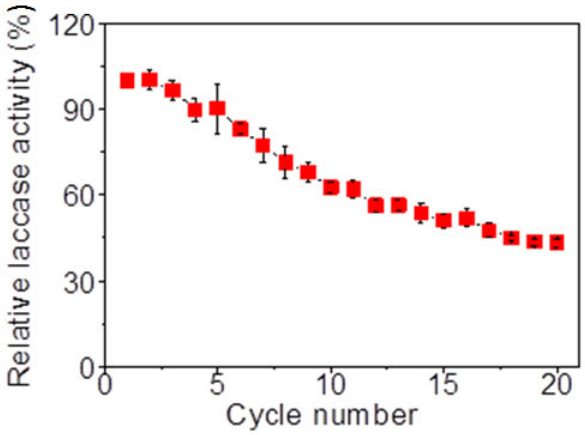


Fig. 4. Activity of the immobilised laccase during successive cycles of ABTS oxidation. All values represent means ± absolute deviations (not visible if smaller than symbol size) from duplicate determinations.

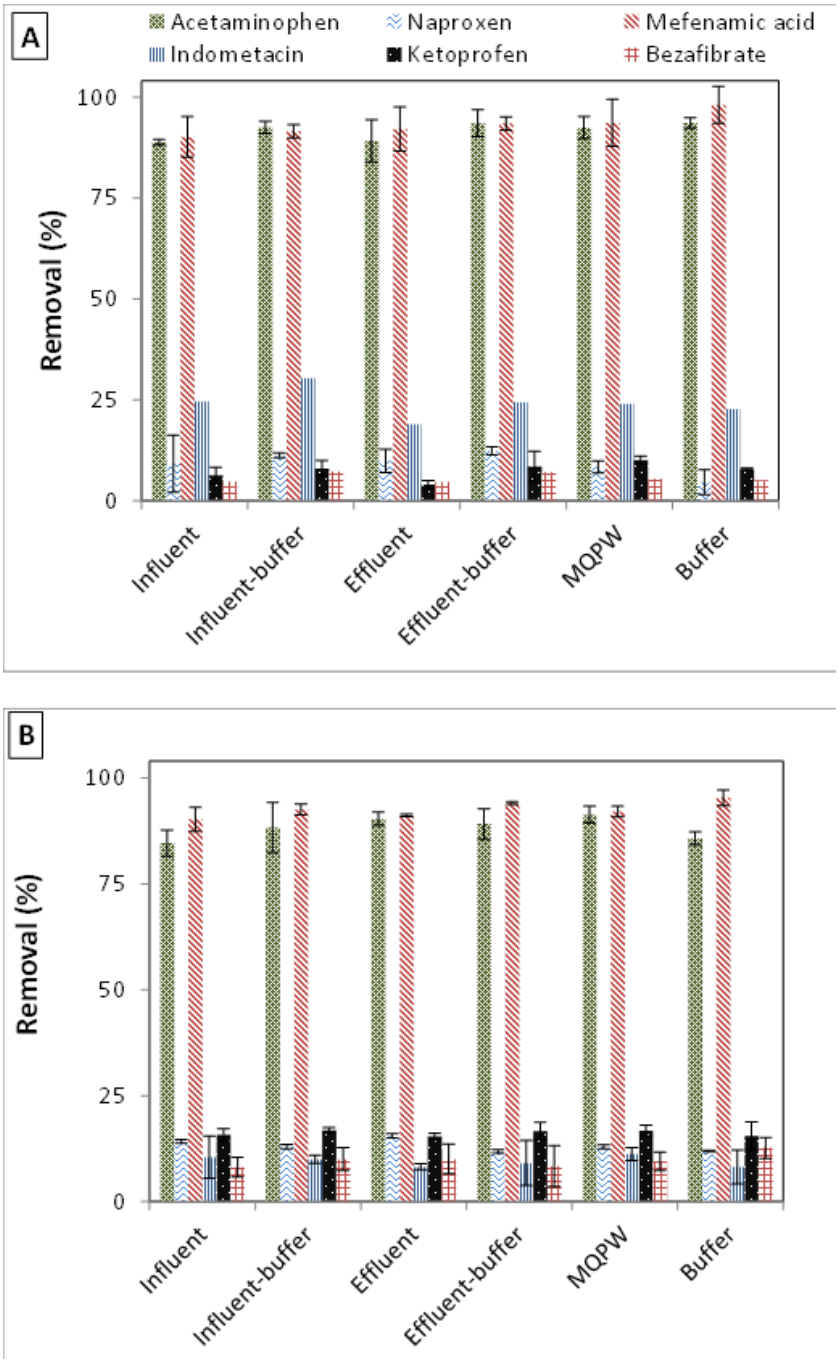
3.3 Batch and continuous elimination of PhACs applied in mixture

In order to ascertain the biodegradation potential of the immobilised laccase *versus* the free one in municipal wastewater matrices, a cocktail of phenolic and non-phenolic PhACs was designed. This mixture of PhACs was composed of the pain reliefers acetaminophen and mefenamic acid previously reported to represent laccase substrates (Arca-Ramos et al., 2016), indometacin (anti-pain/inflammation agent) and bezafibrate (lipid lowering agent) shown to be removed by laccase if applied together with other PhAcs in mixture (Arca-Ramos et al., 2016; Touahar et al., 2014), and ketoprofen (anti-pain/inflammation agent) and naproxen (arthritis pain reliever) not yet being reported to be directly laccase-degradable (Arca-Ramos et al., 2016). The selection of these PhACs was based on their prominent environmental occurrence in municipal and hospital wastewaters, and their toxicity potential to ecosystem and human health (Touahar et al., 2014). Preliminary experiments addressed the selection of appropriate amounts of immobilised and free laccase for PhACs removal. Original influent from the Magog WWTP (pH 8.0; see Table S3 of the Supplementary Material for background concentrations of target PhACs) was chosen as the wastewater matrix expected to provide the harshest conditions among the aqueous matrices tested. After spiking with the PhACs mixture mentioned before, varying amounts of immobilised and free

597 laccase were applied in elimination experiments, respectively (Table S2 of the
598 Supplementary Material). Generally, the amounts of immobilised laccase necessary to
599 achieve PhACs removal efficiencies comparable to those obtained with free laccase were
600 about one order of magnitude lower than those required in case of free enzyme. This effect
601 was most likely at least partly caused by a higher stability of the immobilised enzyme towards
602 the influent pH (8.0; please also refer to the higher stability of the immobilised compared to
603 the free laccase at pH 7.0 and above, as shown in Fig. 2), and/or further unfavourable
604 characteristics such as inhibitory compounds known to be present in wastewater (Arca-
605 Ramos et al., 2016; Lloret et al., 2013). Accordingly, 20 U L^{-1} of immobilised and 200 U L^{-1} of
606 free laccase were applied in further experiments comparing the removal of the six target
607 PhACs in various aquatic matrices such as influent and effluent of the Magog WWTP,
608 buffered influent and effluent, pure buffer (i.e. without wastewater), and MPQW (Fig. 5).
609 These different matrices were employed in order to test the applicability range of the
610 enzymatic oxidation systems with respect to differently (un)favourable reaction conditions.
611 In batch experiments with active laccases, quite high acetaminophen and mefenamic acid
612 removal rates (> 85%) being essentially comparable between the different aquatic matrices
613 employed and either laccase form despite the 10 times lower amount of immobilised laccase
614 (Fig. 5) indicate a high efficiency of the immobilised laccase even in real, unbuffered
615 wastewater. The electron-donating phenol and aniline moieties of acetaminophen and
616 mefenamic acid, respectively, are known to lower the redox potentials of these compounds
617 and consequently accelerate their oxidation by laccase (Kumar and Cabana, 2016; Margot et
618 al., 2013a; Nguyen et al., 2014a). Other target pollutants were less efficiently removed (19 -
619 30% of indometacin, 4 - 12% of naproxen, 4 - 10% of ketoprofen, and 4 - 7% of bezafibrate
620 by the free enzyme; 8 - 11% of indometacin, 11 - 16% of naproxen, around 15% of
621 ketoprofen, and 8 - 12% of bezafibrate by the immobilised laccase; Fig. 5). The removal of
622 PhACs observed in corresponding control experiments containing the hydrophobic laccase-
623 free membranes and heat-inactivated free laccase, respectively, was always < 4% (Fig. S1 of
624 the Supplementary Material); despite of non-negligible hydrophobicities of mefenamic acid

625 and bezafibrate (log D_{ow} at pH 7: 3.7 and 2.7, respectively; Margot et al., 2013b). The log D_{ow}
626 is a form of the octanol-water partition coefficient (log K_{ow}) accounting for the pH-dependent
627 dissociation or protonation of a compound, which at pH 7 ranges between 1.7 (ketoprofen
628 and naproxen) and 0.5 (acetaminophen) for the other, more hydrophilic PhACs tested in our
629 study (all log D_{ow} data taken from Margot et al., 2013b).

630 Previous studies demonstrated that ketoprofen and naproxen could not effectively be
631 degraded by laccases in a direct way if applied as single compounds (Lloret et al., 2010;
632 Marco-Urrea et al, 2010a, b). We are not aware of studies that have addressed the laccase-
633 catalysed oxidation of bezafibrate and indometacin upon their application as single
634 compounds. Weak indometacin removal (< 20%) applied as part of a mixture of PhACs was
635 observed using TvL (Arca-Ramos et al., 2016), whereas complete removal of this compound
636 from a mixture of PhACs by TvL under more favourable conditions was also reported (Tran
637 et al., 2010). Incomplete removal of bezafibrate (< 30%) employed as a part of a pollutant
638 cocktail was also previously indicated (Touahar et al., 2014). Chemical structure-reactivity
639 relationships of indometacin, naproxen, and ketoprofen have already been discussed in
640 detail before (Arca-Ramos et al., 2016). We therefore only briefly mention that electron-
641 donating groups (hydroxyl, amine, alkoxy, alkyl, and acyl groups) are generally susceptible to
642 oxidative attack, whereas electron-withdrawing groups (amide, carboxylic, halogen, and nitro
643 groups) decrease the reactivity of compounds (Arca-Ramos et al., 2016).



644

645 **Fig. 5.** Relative removal (i.e. in relation to the respective initial concentration) of PhACs after
646 24 h treatment using free (A, applied at 200 U L^{-1}) and immobilised laccase (B, applied at 20
647 U L^{-1}) in WWTP influent, influent-buffer, effluent, effluent-buffer, MQPW, and McIlvaine buffer
648 (pH 7.0). Bars represent means ± standard deviations from triplicate experiments.

649

650 The general applicability of the immobilised laccase for the continuous elimination of the
651 six selected target PhACs was demonstrated using a continuously operated BR. MQPW was
652 applied as the aqueous matrix in these experiments. At the applied hydraulic retention time

653 (HRT) of 1 h a fairly stable removal of the applied PhACs could be observed over the
654 investigated time period of 6 h, which in total corresponded to 6 exchanges of the working
655 volume of the BR (Fig. 6). The removal efficiency followed the rank order mefenamic acid
656 (about 28% removal on average) > acetaminophen (≈ 22%) > indometacin (≈ 20%) >
657 naproxen (≈ 13%) > bezafibrate (≈ 6%) > ketoprofen (≈ 2%), thus displaying a pattern
658 qualitatively similar to those observed in batch experiments (Fig. 6). The activity of the
659 immobilised laccase remained considerably stable, as monitored with the routine ABTS
660 oxidation assay. The initially applied activity of 16 U L^{-1} had only slightly decreased to 14 ±
661 1.6 (mean ± standard deviation from triplicate determinations) and then to 13.4 ± 0.8 U L^{-1}
662 (after four and six exchanges of the reactor volume, respectively. Similar high recoveries of
663 activities of immobilised laccases have previously been reported for similar experimental
664 settings (Cabana et al., 2009b; Demarche et al., 2012). Also, the potential of continuously
665 operated enzyme reactors for the efficient elimination of micropollutants has already
666 repeatedly been described before. At least 95% of the EDCs bisphenol A (BPA), nonylphenol
667 and TCS could be removed using a lab-scale BR containing immobilised laccase, which was
668 operated at a HRT of 325 min (Cabana et al., 2009b). Elimination of BPA by 90% was
669 achieved with immobilised laccase in a continuous stirred-tank membrane reactor at a HRT
670 of 1.85 h (Demarche et al., 2012). Using freely suspended laccases retained by ultrafiltration
671 membranes in continuously operated enzyme membrane reactors, estrone and estradiol
672 were eliminated by more than 80% at a HRT of 4 h (Lloret et al., 2012), and BPA and
673 diclofenac could be removed by more than 85 and 60%, respectively, at a HRT of 8 h
674 (Nguyen et al., 2014b). The removal of PhACs observed within the present study (below
675 30%; Fig. 6) cannot directly be compared with the higher micropollutant elimination rates
676 reported in the aforementioned studies, due to different laccase sources, amounts and
677 immobilisation methods, reactor configurations and operation conditions, chemical nature of
678 micropollutants and their loads, aqueous matrices, and HRTs that were applied. It remains to
679 be elucidated whether and to which extent an increase in the elimination of PhACs upon

increasing the contact time between biocatalyst and pollutant by increasing the HRT of our system could be achieved.

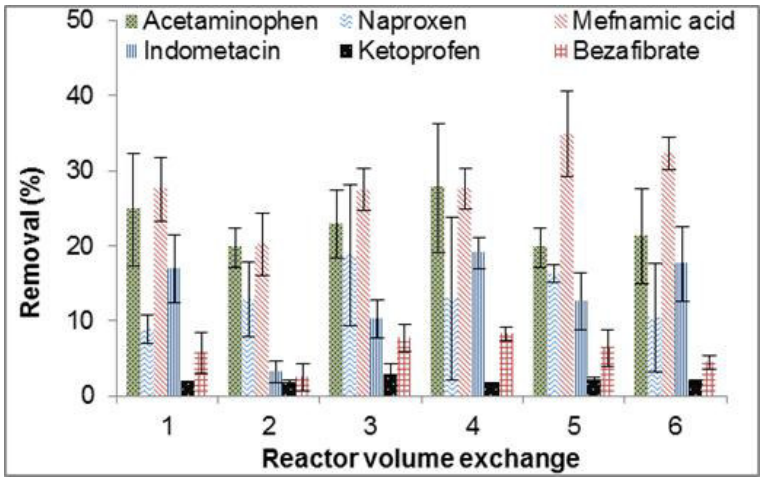


Fig. 6. Relative removal (i.e. in relation to the respective initial concentration) of PhACs during continuous treatment of PhAC-spiked MPQW in a BR containing immobilised laccase initially applied at 16 U L^{-1}. Bars represent means ± standard deviations from triplicate determinations.

Our aforementioned results and also those of previous studies cited below suggest that radicals derived from easily laccase-oxidisable PhACs could in turn oxidise compounds which are less or not at all susceptible to laccase attack, thus enhancing or enabling the oxidative removal of comparatively more recalcitrant environmental contaminants (Arca-Ramos et al., 2016; Haroune et al., 2014; Touahar et al., 2014). We have therefore addressed the laccase-catalysed oxidation of the PhAC representative acetaminophen and its potential influence on the elimination of less oxidisable water pollutants (Hachi et al., 2017) in more detail in sub-sections 3.4 – 3.6 below.

3.4 Apparent kinetic parameters of free and immobilised laccase toward acetaminophen

The oxidation of acetaminophen, which was applied at varying concentrations, by free and immobilised laccase was investigated in McIlvaine buffer (pH 7.0) and real wastewater collected from a municipal WWTP (see sub-section 2.8 of the materials and methods for

more details). Laccase amounts of 20 and 23 U L^{-1} for the immobilised and free laccase, respectively, were sufficient for the determination of apparent kinetic parameters of acetaminophen oxidation in buffer. However, no acetaminophen degradation was observed in real wastewater reaction mixtures containing free laccase at 23 U L^{-1}, most likely due to a considerably lower stability of the free compared to the immobilised enzyme, and resembling observations already made in the context of degradation experiments applying PhACs in mixture (compare sub-section 3.3 above). Therefore, free laccase in real wastewater was applied at 200 U L^{-1}, i.e. an amount already successfully employed for the PhAC mixture (Fig. 5). Immobilised laccase was applicable without problems in real wastewater at 24 U L^{-1}.

Application of the Hill model overall yielded better fits for acetaminophen oxidation by both free and immobilised laccase (COD always ≥ 0.99; related acetaminophen oxidation *versus* concentration plots are exemplified in Fig. S2 of the Supplementary Material) than employing Michaelis-Menten kinetics (COD range of 0.97 to > 0.99). The corresponding apparent kinetic parameters (i.e. the oxidation rate half-saturating substrate concentrations $S_{0.5}$ and K_m for the Hill and the Michaelis-Menten model, respectively; together with the respective V_{max} values) are compiled in Table 3. Better fits with the Hill than with the Michaelis-Menten model were already described for the laccase-catalysed oxidation of the synthetic dye Acid Blue 62 (Abu62) (Junghanns et al., 2009). Like for Abu62 in the aforementioned study, we consider the involvement of radicals formed during primary oxidation of acetaminophen by laccase rather than cooperative enzyme kinetics as a potential reason. Once formed, such radicals could in turn also abiotically oxidise parent acetaminophen, thereby increasing the overall oxidation rate.

Higher apparent $S_{0.5}$ and K_m values observed for acetaminophen oxidation by the immobilised compared to the free laccase in buffer (Table 3) resembles the results obtained with ABTS as a laccase substrate (Table 2), and suggests a limited immediate availability also of acetaminophen for the immobilised enzyme. The high hydrophilicity of acetaminophen (log D_{ow} at pH 7: 0.5; (Margot et al., 2013b)) and the hydrophobicity of PVDF membranes used for enzyme immobilisation (Jahangiri et al., 2014) would be in line with

730 such an effect. A lower K_m value for acetaminophen oxidation (about 99 µM) by immobilised
731 compared to free laccase (about 204 µM) in aqueous solution was reported for laccase from
732 *Lentinus polychrous* immobilised in hydrophilic barium alginate, and an enhancement of the
733 substrate affinity by immobilisation was suggested (Ratanapongleka and Punbut, 2017).
734 About 2.7- and 1.6-fold higher $S_{0.5}$ and K_m values, respectively, for acetaminophen oxidation
735 of the free than of the immobilised laccase in wastewater (Table 3) may indicate
736 unfavourable conformational changes of the enzyme protein caused by certain inhibitory
737 inorganic ions known to be a part of the wastewater matrix (Arca-Ramos et al., 2016; Lloret
738 et al., 2013; Zimmermann et al., 2011). Such alterations may have lowered the substrate
739 affinity particularly of the free enzyme under wastewater conditions. A potential partial
740 prevention from such adverse effects due to immobilisation would also be in line with the
741 observed higher stability of the immobilised compared to the free enzyme in the pH range of
742 wastewaters (Fig. 2). Higher $S_{0.5}$ and K_m values for acetaminophen oxidation were obtained
743 with both the immobilised and free enzyme in wastewater compared to buffer, respectively
744 (Table 3). These observations could be explained by wastewater-related and substrate
745 affinity-lowering conformational changes as already mentioned before, and/or the presence
746 of additional organic compounds potentially competing for the enzyme in wastewater (Arca-
747 Ramos et al., 2016). Lower V_{max} values in wastewater compared to buffer found to be
748 especially pronounced for the free laccase (likely lacking protection by immobilisation) and
749 observed with both the Hill and the Michaelis-Menten model (Table 3), were likely to be
750 caused by inhibitory compounds present in wastewater (Arca-Ramos et al., 2016; Lloret et
751 al., 2013). About 12- and 3-fold higher apparent V_{max} values (Hill model) and approximately
752 31- and 2-fold higher catalytic efficiencies (in terms of $V_{max}/S_{0.5}$ values) of the immobilised
753 than the free enzyme in wastewater and buffer, respectively (Table 3), are likely attributable
754 to a considerably higher functional stability of the immobilised laccase. The enzyme
755 immobilisation may have restricted enzyme inactivation and perhaps also substrate affinity
756 losses expected to be particularly drastic for the free laccase in wastewater, as could also be

757 inferred from previously published data (Arca-Ramos et al., 2016; Corvini and Shahgaldian,
758 2010).

759 No removal of acetaminophen observed in the respective controls (see Fig. S3 of the
760 Supplementary Material) is in line with data reported by (Lu and Huang, 2009), and further
761 corroborate our results obtained with PhACs as described above (no remarkable sorption of
762 PhACs to membranes; Fig. S1 of the Supplementary Material). Photocatalysis was reported
763 to play a moderate role in transformation of acetaminophen (Yamamoto et al., 2009) and
764 may have contributed to removal of the compound from biocatalyst-free controls in previous
765 studies (Ba et al., 2014), but could be ruled out as a cause of PhACs within the present
766 study.

767

768 **Table 3**

769 Apparent kinetic parameters for the oxidation of acetaminophen by free and immobilised laccase as derived from application of the Hill (COD
770 always ≥ 0.99) and the Michaelis-Menten model (COD range of 0.97 to > 0.99)[a].

Laccase form and aqueous matrix	Hill model			Michaelis-Menten model		
	$S_{0.5}$ (µM)	V_{max} (nmol h^{-1} U^{-1})[b]	$V_{max}/S_{0.5}$ (mL h^{-1} U^{-1})[b]	K_m (µM)	V_{max} (nmol h^{-1} U^{-1})[b]	V_{max}/K_m (mL h^{-1} U^{-1})[b]
Immobilised laccase in buffer	642 ± 96	7681 ± 559	11.96	990 ± 142	9385 ± 648	9.48
Immobilised laccase in wastewater	705 ± 30	4321 ± 121	6.13	2650 ± 1194	9210 ± 2714	3.48
Free laccase in buffer	569 ± 162	2703 ± 363	4.75	849 ± 176	3235 ± 307	3.81
Free laccase in wastewater	1895 ± 563	372 ± 67	0.20	4285 ± 912	607 ± 96	0.14

771 [a] Apparent $S_{0.5}$, K_m, and V_{max} values ± corresponding standard errors arise from data fitting of means from triplicate experiments.

772 [b] For comparability, V_{max} values were based on ABTS units (U; i.e. laccase amounts in terms of activity as determined with the routine ABTS
773 assay). The validity of this approach was deduced from a previous study (Lu et al., 2009), demonstrating a linear dependency between the
774 observed acetaminophen oxidation rate and the applied laccase activity within an activity range comparable to that of the present study.

775 *3.5 Acetaminophen as a potential laccase redox mediator*

776 Radicals derived from enzymatic attack on easily oxidisable, phenolic PhACs such as
777 acetaminophen have previously been suggested to interact with more resistant PhACs, thus
778 enhancing their transformation (Arca-Ramos et al., 2016; Hachi et al., 2017; Kumar and
779 Cabana, 2016; Touahar et al., 2014). We have accordingly addressed potential redox-
780 mediating effects of acetaminophen on other environmental pollutants in more detail,
781 investigating its influence on both pollutant removal (this sub-section) and the formation of
782 transformation products (next sub-section). We considered the biocide and suspected EDC
783 TCS, which is comparatively slowly to moderately oxidised by laccases in a direct manner
784 (Cabana et al., 2007a; Hofmann and Schlosser, 2016) and hereby predominantly yields di-
785 and trimers (Jahangiri et al., 2017), to be an appropriate model target pollutant in these
786 experiments. TCS, which was attacked by the immobilised and free laccase in the absence
787 of acetaminophen only to a limited extent, was found to be increasingly removed upon
788 increasing the initial acetaminophen concentration present in the respective reaction systems
789 (Fig. 7A). Like the previously investigated presumable laccase redox mediators ABTS,
790 acetosyringone and syringaldehyde (Jahangiri et al., 2017; Margot et al., 2015),
791 acetaminophen was consumed and not recycled during the laccase reactions as also
792 supported by our investigations targeting TCS-acetaminophen oxidation products formed
793 during laccase reactions (see next sub-section). The enzymatic acetaminophen oxidation
794 has thus governed TCS removal as would be expected from its application at concentrations
795 of up to 500 µM, not exceeding its apparent oxidation rate half-saturating concentrations
796 shown in Table 3. The TCS removal tended to become saturated with increasing
797 acetaminophen concentrations applied and hence consumed (Fig. 7A), as has already been
798 observed for effects of increasing ABTS concentrations on the removal of the herbicide
799 isoproturon (Margot et al., 2015). A likely explanation given by Margot et al. (2015) is that
800 with an increasing redox mediator concentration self-coupling of the rapidly formed redox
801 mediator radicals may increasingly become dominant over reactions between redox mediator
802 radicals and the target pollutant. The observed influences of increasing acetaminophen

concentrations on TCS removal (Fig. 7A) would also be expected if the rapidly produced acetaminophen radicals would increasingly react with previously formed oxidation products of the acetaminophen-TCS reaction laccase system instead of parent TCS. Such reactions would result in increasing molar ratios acetaminophen consumed : TCS removed with increasing molar acetaminophen : TCS ratios initially applied, as is evident from Fig. 7B. In the next sub-section we provide evidence for the prominence of several acetaminophen-TCS oligomerisation products; which are in support of reactions of acetaminophen radicals with other already formed oxidation products rather than with parent TCS as suggested just above. Higher ratios of acetaminophen consumed : TCS removed observed with the immobilised compared to the free laccase (Fig. 7) could be explained by a close vicinity of newly produced acetaminophen radicals and already formed coupling products possibly caused by the porous matrix of the membranes used for laccase immobilisation. Such effects could possibly favour reactions between acetaminophen radicals and already formed coupling products to a higher extend in case of the immobilised compared to the free laccase.

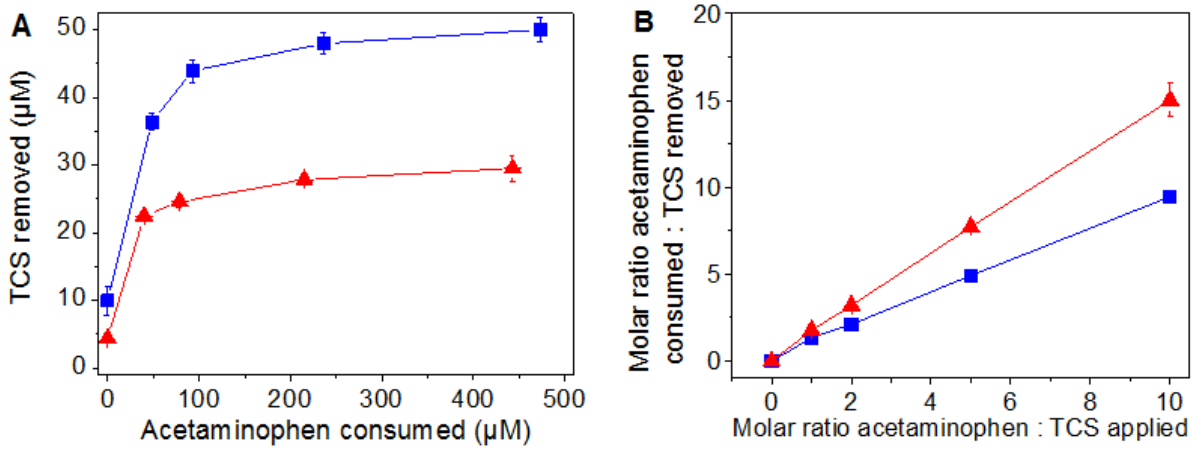


Fig 7. TCS removed *versus* acetaminophen consumed at different molar acetaminophen : TCS ratios initially applied (range of 0:1 to 10:1) (A), and molar ratios acetaminophen consumed : TCS removed in dependence on the corresponding molar acetaminophen : TCS ratios initially applied (B) as observed with immobilised (red triangles and lines) and free

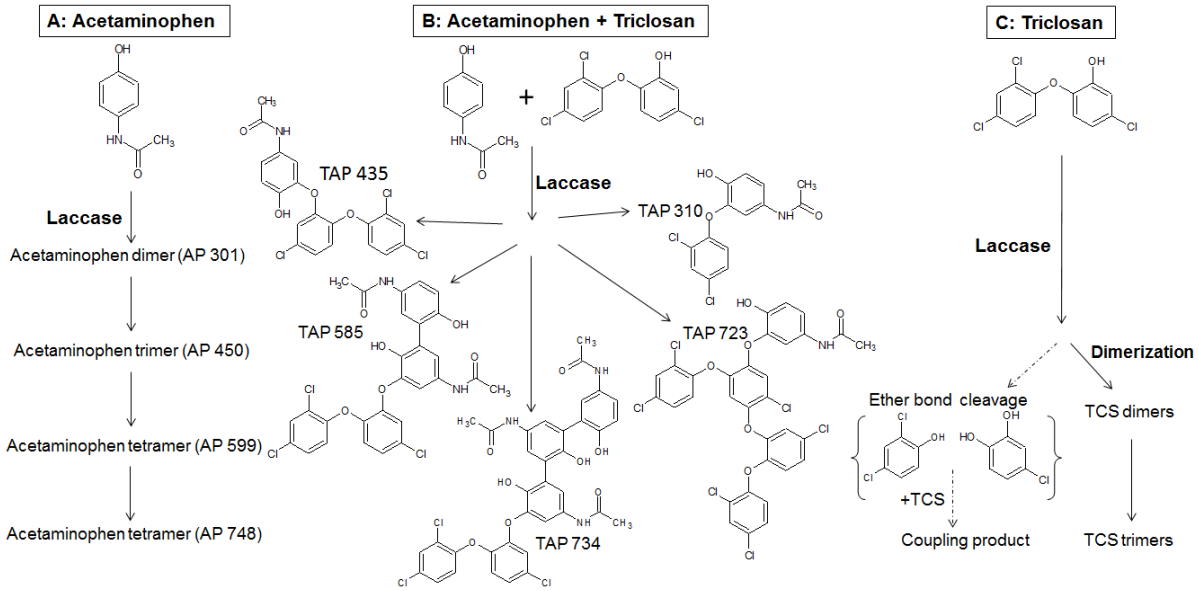
laccase (blue squares and lines) after 8 h of reaction. Symbols represent means ± standard deviations from triplicate experiments.

3.6 Acetaminophen and triclosan-acetaminophen oxidation products formed during laccase reactions

LC-MS-QTOF analysis was employed to analyse acetaminophen oxidation products in reaction mixtures containing either immobilised or free laccase. With either laccase form compounds with molecular ions at *m/z* values of 301.1194 (designated as AP 301 in Fig. 8, and in Fig. S4 and Table S6 of the Supplementary Material), 450.1653 (AP 450), 599.2133 (AP 599), and 748.2642 (AP 748) were detected (Fig. 8, Fig. S4, Table S6). These products represent di- to pentamers arising from oxidative coupling of acetaminophen (molecular ion at *m/z* 152.0724; Table S6) and follow a general molecular mass pattern of $nM_0-2(n-1)$ (n corresponds to the number of coupled molecules and M_0 represents the molecular mass of parent acetaminophen), as has already been described before (Ba et al., 2014; Lu et al., 2009). Their appearance in the form of different isomer species was also observed by other authors (Lu and Huang, 2009) and would be expected from laccase-initiated radical coupling processes. Such coupling products are believed to be biologically inactive (Lu et al., 2009), hereby remarkably differing from the well-known toxicity of the common acetaminophen metabolite N-acetyl-*p*-benzoquinone imine (Bender et al., 2004). N-acetyl-*p*-benzoquinone imine was not observed within the present study. However, we cannot exclude its intermediate formation e.g. via disproportionation of N-acetyl-*p*-semiquinone imine, the primary one-electron oxidation product of acetaminophen anticipated for laccase catalysis. N-acetyl-*p*-benzoquinone imine formation via the aforementioned reaction mechanism has been reported for the one-electron oxidation of acetaminophen by horseradish peroxidase, even though acetaminophen polymerisation was found to be predominant (Potter and Hinson, 1987).

In the additional presence of TCS, the same cross-coupling products of acetaminophen and TCS were found for both the immobilised and free laccase. An *m/z* value of 310.0035 of

852 the molecular ion of compound TAP 310 (Table S6, Fig. 8) implies coupling of
853 acetaminophen and dichlorophenol, with the latter arising from laccase-mediated ether bond
854 cleavage of TCS (Jahangiri et al., 2017). Coupling of acetaminophen and parent TCS is
855 indicated by the detection of product TAP 435 (*m/z* 435.9895, Table S6; Fig. 8). Two more
856 products (TAP 585 with *m/z* 585.038, and TAP 734 with *m/z* 734.086, Table S6; Fig. 8)
857 seemingly represent coupling products of one TCS and two and three acetaminophen
858 molecules, respectively. Compound TAP 723 (*m/z* 723.9241, Table S6; Fig. 8) was most
859 likely formed from coupling of acetaminophen to a TCS dimer (TP 572 in (Jahangiri et al.,
860 2017)). Compounds TAP 585 and TAP 734, and likely also TAP 723 thus advocate for
861 reactions between acetaminophen radicals and already formed products (instead of parent
862 TCS) as already suggested in the previous sub-section. Several isomers were detected for
863 each of the acetaminophen-TCS coupling products but TAP 310 (Table S6). It remains to be
864 elucidated if and to which extent acetaminophen-TCS coupling products would still be
865 biologically active. A clearly diminished antibacterial activity after laccase treatment of TCS in
866 presence of syringaldehyde, which results in the formation of syringaldehyde-TCS coupling
867 products but also products stemming from either syringaldehyde or TCS, has been
868 demonstrated before (Jahangiri et al., 2017). No products solely involving either
869 acetaminophen or TCS could be detected upon the simultaneous employment of
870 acetaminophen and TCS in laccase reaction mixtures, possibly due to too low concentrations
871 of such products under the applied reaction conditions. Similarly, acetaminophen self-
872 coupling was found to be decreased in the additional presence of natural organic matter
873 (NOM) at the expense of acetaminophen cross-coupling with NOM in laccase reaction
874 systems (Lu and Huang, 2009). The proposed pathways for the laccase-catalysed
875 transformations of acetaminophen (if applied alone), acetaminophen and TCS (if applied in
876 mixture), and TCS (if applied alone) are summarised in Fig. 8.
877



878

879 **Fig. 8**. Proposed pathways of (A) acetaminophen (if applied as a single compound), (B) TCS
880 and acetaminophen (if applied in mixture), and (C) TCS transformation (if applied as a single
881 compound; modified from (Jahangiri et al., 2017)) by both immobilized and free *Phoma*
882 laccase. Labelling of chemical structures refers to that applied in Table S5 of the
883 Supplementary Material. AP denotes acetaminophen products, and TAP denotes coupling
884 products involving both triclosan and acetaminophen. The accompanying numbering
885 indicates the respective mass of the corresponding molecular ion. For TAP 435 and TAP 723
886 the likely predominant isomeric structure is shown, respectively.

887

888 **4. Conclusions**

889 In this study, a novel robust biocatalyst with a remarkable functional stability also under the
890 harsh conditions of real wastewater has been presented. Its potential suitability for both,
891 discontinuous as well as continuous treatment of mixtures of typical PhACs found in
892 wastewaters could be demonstrated. In line with previous studies targeting syringaldehyde
893 as a presumable laccase redox mediator, also the PhAC acetaminophen turned out to be
894 consumed during laccase reactions. Acetaminophen thus does not represent a “true” laccase
895 redox mediator in the sense of being recycled during target pollutant oxidation. Nevertheless,
896 the simultaneous presence of comparatively easily laccase-oxidisable compounds (e.g.
897 acetaminophen) and much more recalcitrant pollutants in wastewater represents an

interesting option for the efficiency enhancement of enzyme-based wastewater treatment approaches. Such a strategy would also enable to avoid previously reported toxicity issues related to exogenously added laccase redox mediators. However, its feasibility under the minute concentrations of PhACs and other micropollutants in real wastewaters still needs to be elucidated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at…

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Figure captions

Fig. 1. SEM images of PVDF membranes. (A) Cross-section and (B) top view of the original enzyme-free membrane, (C) top view after E-Beam immobilisation of laccase, and (D) top view after employing the laccase-containing membrane in degradation of 1 mM acetaminophen for 24 h. Two different magnifications are shown for (B), (C), and (D), respectively.

Fig. 2. Stability of free (A) and immobilised (B) laccase during incubation at different pH values over the indicated time periods. All values represent means ± absolute deviations (not visible if smaller than symbol size) from duplicate measurements.

Fig. 3. Stability of free (A) and immobilised (B) laccase during incubation at different temperatures over the indicated time periods. All values represent means ± absolute deviations (not visible if smaller than symbol size) from duplicate measurements.

Fig. 4. Activity of the immobilised laccase during successive cycles of ABTS oxidation. All values represent means ± absolute deviations (not visible if smaller than symbol size) from duplicate determinations.

Fig. 5. Relative removal (i.e. in relation to the respective initial concentration) of PhACs after 24 h treatment using free (A, applied at 200 U L^{-1}) and immobilised laccase (B, applied at 20 U L^{-1}) in WWTP influent, influent-buffer, effluent, effluent-buffer, MQPW, and McIlvaine buffer (pH 7.0). Bars represent means ± standard deviations from triplicate experiments.

Fig. 6. Relative removal (i.e. in relation to the respective initial concentration) of PhACs during continuous treatment of PhAC-spiked MPQW in a BR containing immobilised laccase initially applied at 16 U L^{-1}. Bars represent means ± standard deviations from triplicate determinations.

Fig 7. TCS removed *versus* acetaminophen consumed at different molar acetaminophen : TCS ratios initially applied (range of 0:1 to 10:1) (A), and molar ratios acetaminophen consumed : TCS removed in dependence on the corresponding molar acetaminophen : TCS ratios initially applied (B) as observed with immobilised (red triangles and lines) and free laccase (blue squares and lines) after 8 h of reaction. Symbols represent means ± standard deviations from triplicate experiments.

Fig. 8. Proposed pathways of (A) acetaminophen (if applied as a single compound), (B) TCS and acetaminophen (if applied in mixture), and (C) TCS transformation (if applied as a single compound; modified from (Jahangiri et al., 2017)) by both immobilized and free *Phoma* laccase. Labelling of chemical structures refers to that applied in Table S5 of the Supplementary Material. AP denotes acetaminophen products, and TAP denotes coupling products involving both triclosan and acetaminophen. The accompanying numbering indicates the respective mass of the corresponding molecular ion. For TAP 435 and TAP 723 the likely predominant isomeric structure is shown, respectively.

Figure 1

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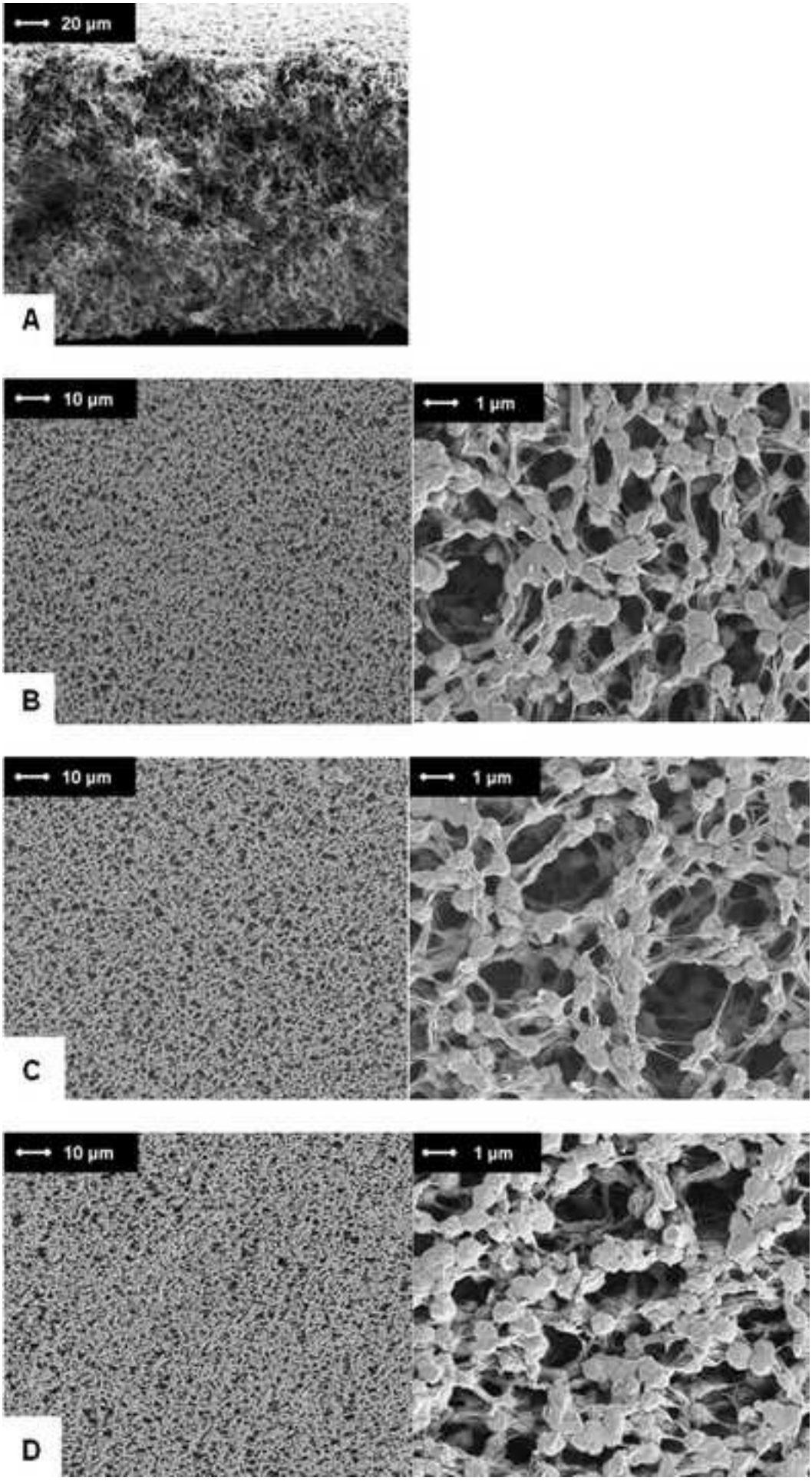

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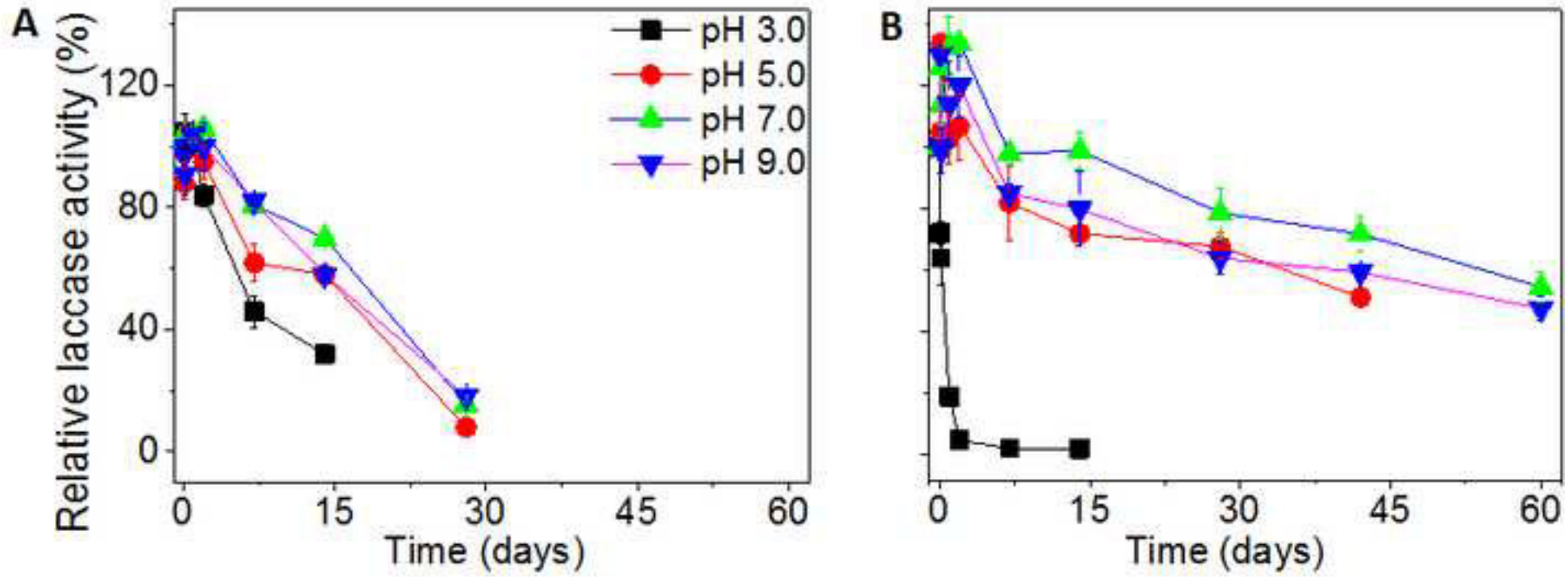

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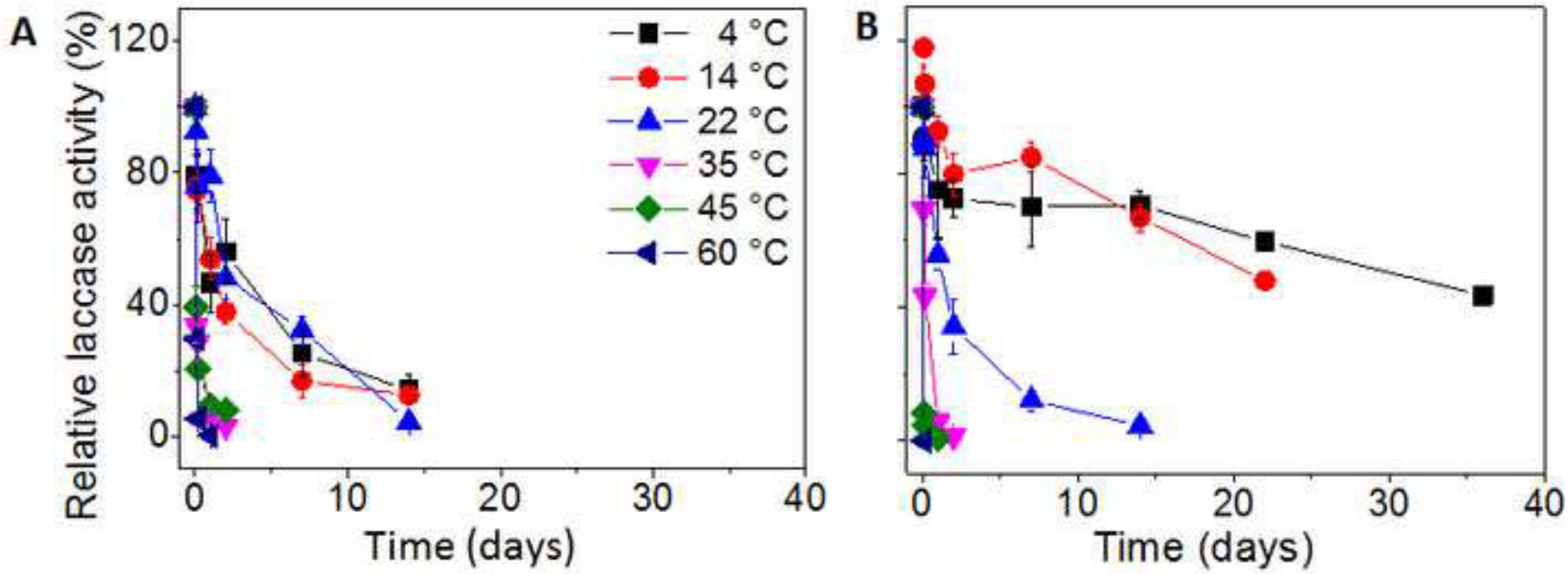

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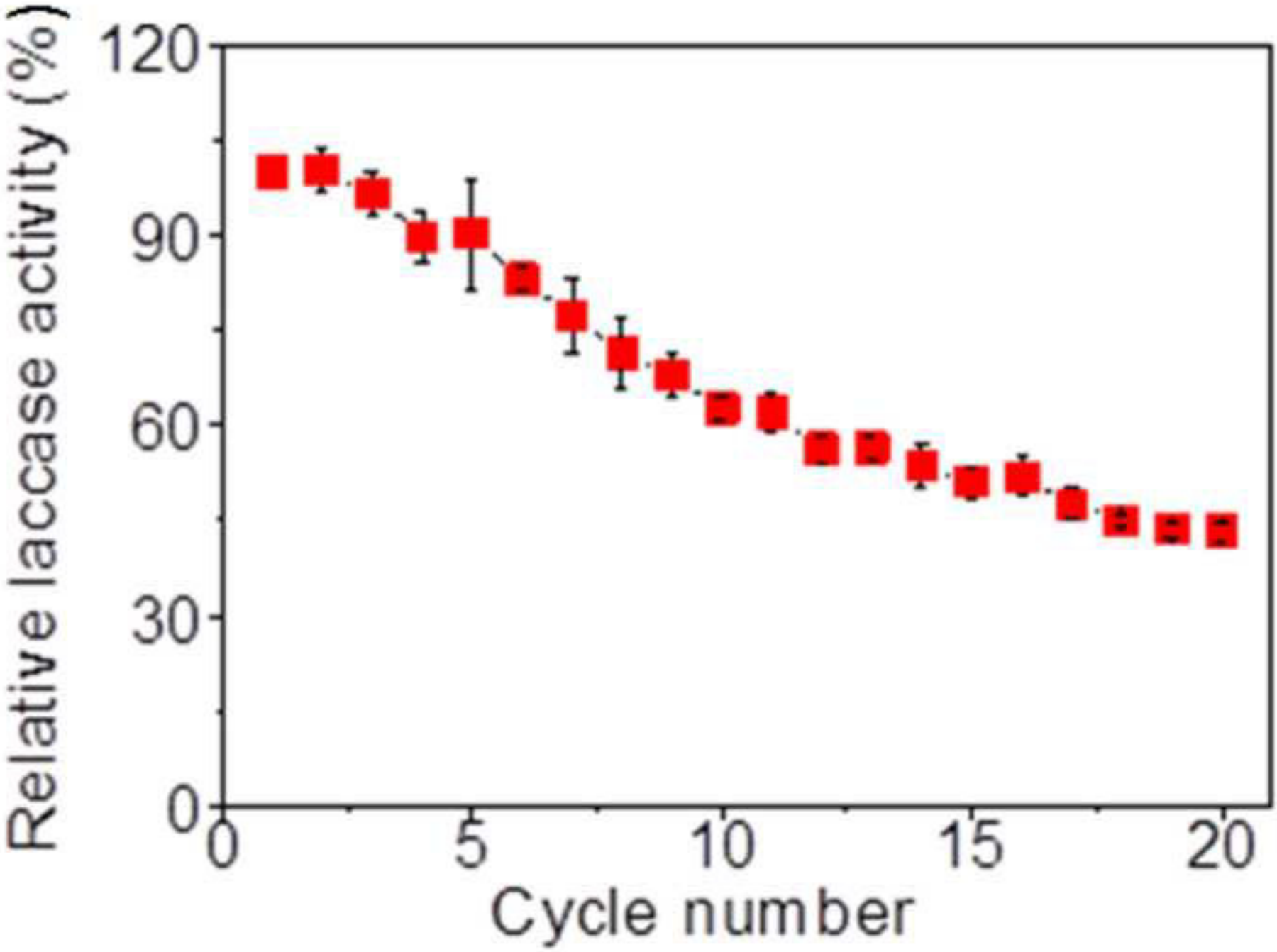

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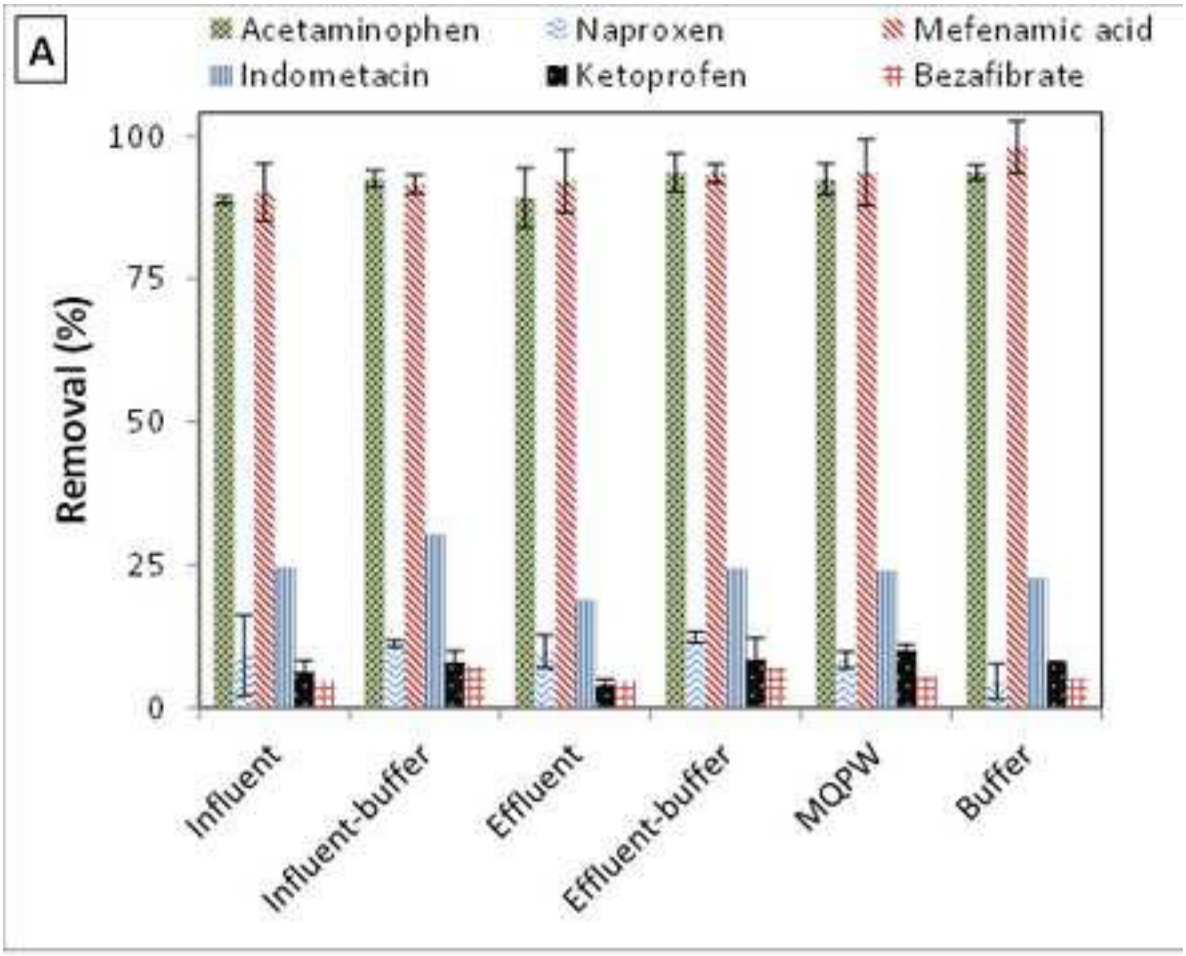

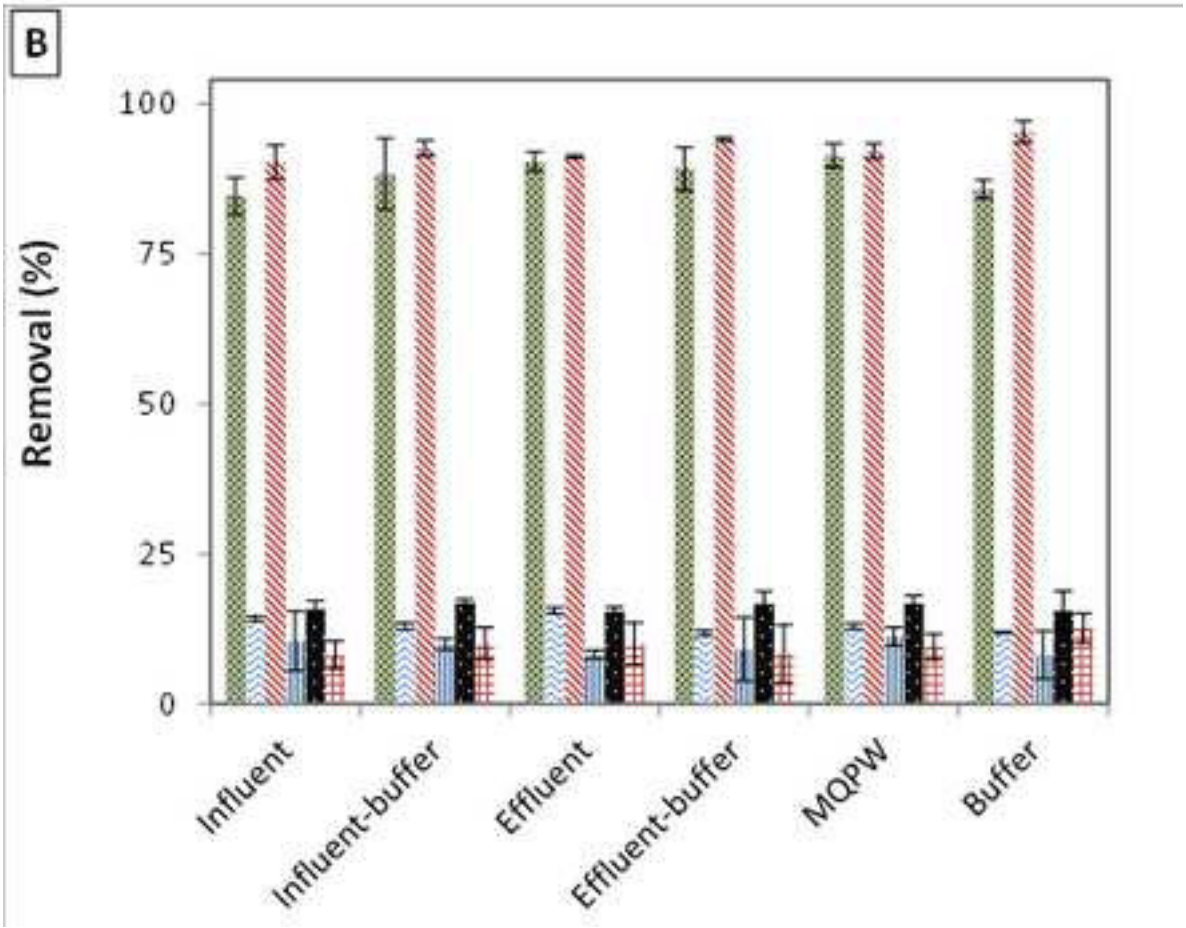

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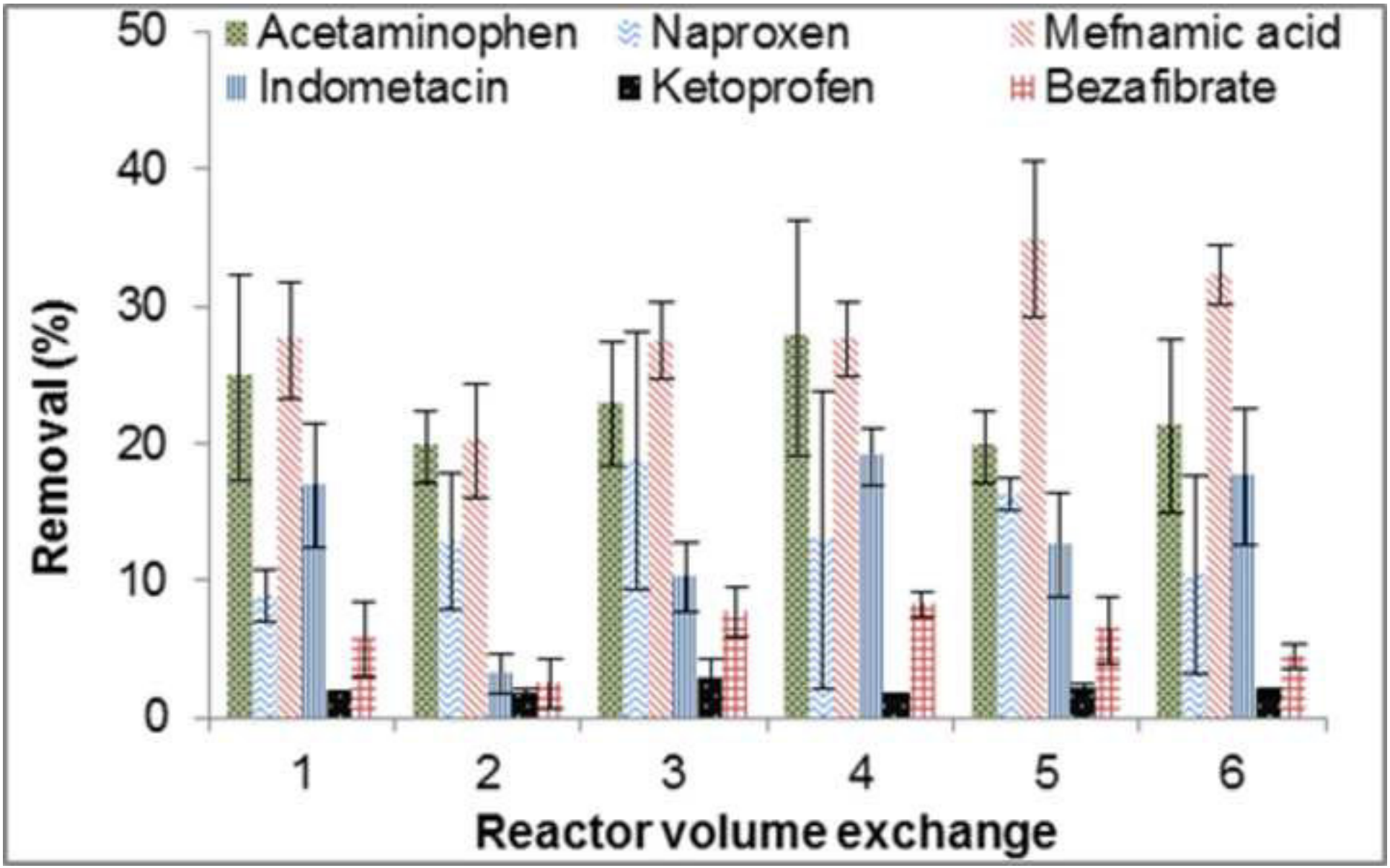

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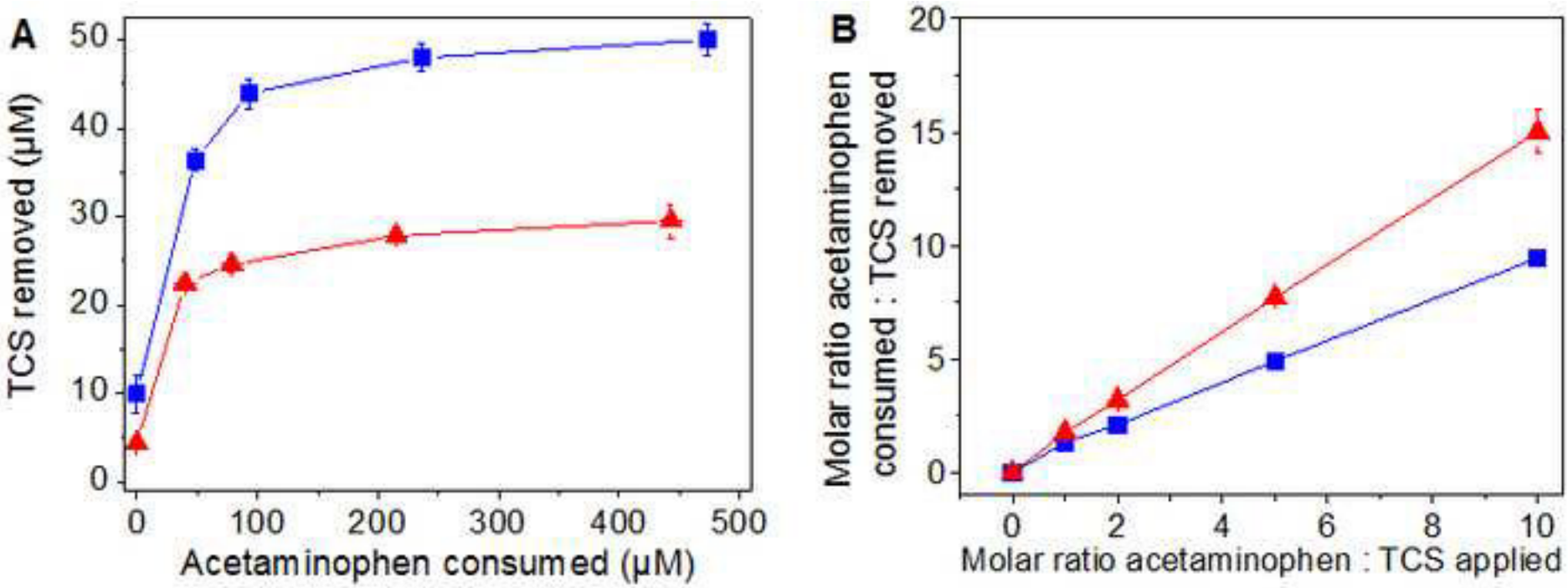

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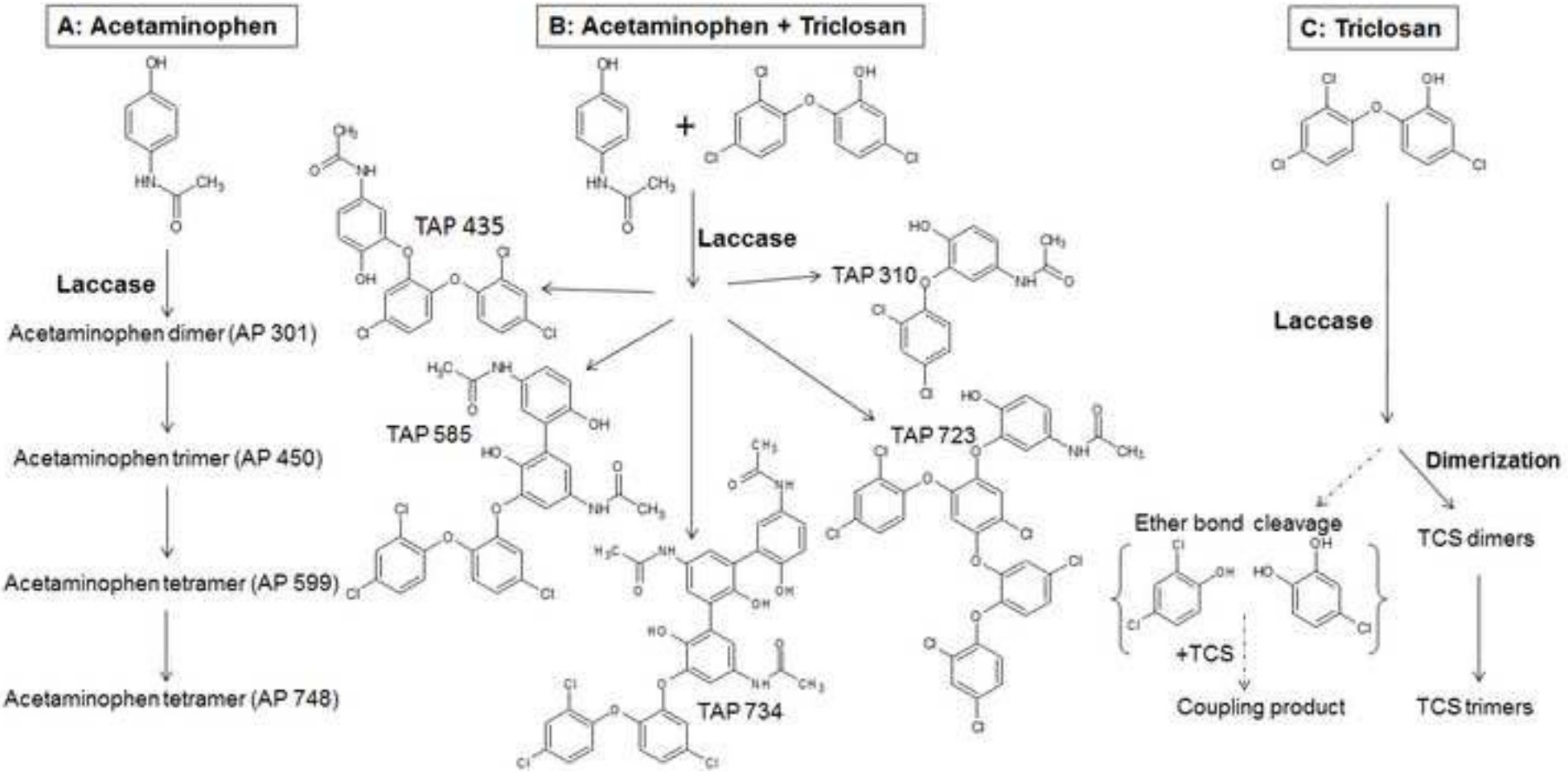

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