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Degradation of tetracyclines and sulfonamides by stevensite- and biochar-immobilized laccase systems and impact on residual antibiotic activity

Running title: Antibiotics removal by immobilized laccase

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Abstract

Background: Stevensite and biochar were investigated to covalently immobilize laccase from *Myceliophthora thermophila* (MtL) and *Pleurotus eryngii* (PeL) through the sequential application of aminopropyltriethoxysilane and glutaraldehyde. The immobilized preparations were tested to remove 3 tetracyclines and 6 sulfonamides at 0.1mM of each antibiotic. Degradation experiments were conducted both in the absence and in the presence (0.2mM) of ABTS, 1-hydroxybenzotriazol (HBT), syringaldehyde or violuric acid. The residual antibiotic activity was tested towards five bacterial species and a bacterial consortium from wastewater.

Results: Higher values of activity yields (74 and 70.3%) and catalytic capabilities (1426 and 1405 IU g^{-1}) were obtained with PeL on stevensite and biochar than with MtL. Stevensite enabled higher reusability and storage stability than biochar. Best removals of tetracyclines and sulfonamides were obtained with immobilized-laccase systems coupled to ABTS or syringaldehyde. Immobilized-laccase/ABTS systems removed 100% of tetracyclines while only chlortetracycline was completely removed in the presence of syringaldehyde. With ABTS, the most effectively removed sulfonamides were sulfathiazole and sulfadiazine (up to 100 and 54%), while syringaldehyde best supported the removal of sulfanilamide, sulfamethazine and sulfamethoxazole (up to 42,

45 and 46%, respectively). In some cases, an effective antibiotics removal led to either low or no residual antibiotic activity.

Conclusion: MtL and PeL were immobilized successfully on biochar and stevensite. The addition of either ABTS or syringaldehyde enhanced significant removals, up to 100%, of tetracyclines and sulfonamides by the immobilized laccase systems. Noteworthy, biochar-immobilized laccases/ABTS led to a complete suppression of the antibiotic activity of tetracyclines.

Keywords: antibiotics, wastewater treatment, water pollution, emergent organic pollutants, pharmaceutical compounds, ligninolytic enzymes.

Introduction

Antibiotics for human or veterinary purposes are consumed at high rates all around the world. For example, the total amount of antibiotics sold for veterinary purposes in the European Union amounted to 8298.7 t in 2015, the most used of which belonged to tetracyclines (2722.8 t), penicillins (2072.2 t) and sulfonamides (978.4 t).[1] Although tetracyclines (TCs) and sulfonamides (SAs) are mainly used in livestock management, they are still exploited in human medicine. To exemplify, TCs are employed in the treatment of a wide number of sexually transmitted diseases, gastritis, peptic ulcer and lower respiratory tract infections. The use of sulfonamides is mostly confined to sulfadiazine and sulfamethoxazole which are used to counteract *Nocardia* spp. infections, pneumonia caused by *Pneumocystis jirovecii* and enteritis by *Shigella* spp. [2] As a consequence, in spite of the downsizing in their medical use, very recent works report on the presence of both TCs and SAs in urban wastewater [3,4], hospital wastewater [5] and, ultimately, in rivers. [6,7] In fact, a high proportion of the given dose of antibiotic is excreted as parent compound from the body *via* urine [8] and will end up in conventional

wastewater treatment plants (WWTP) which are not designed to deal with pharmaceutical compounds. [3] Although the majority of veterinary drugs, instead, reach soils due to the consolidated procedure of land spreading of animal waste, the residual presence of both TCs and SAs in livestock WWTP has been reported. [9,10] Due to their reported inability of totally removing antibiotics and antibiotic-resistant genes from urban effluents, WWTP are deemed to be the major sources of environmental contamination of these compounds and diffusion of antibiotic resistant bacteria and related genes. [11] In addition to WWTP, antibiotic resistant bacteria and genes are found in soils irrigated with wastewater [12] and aquaculture environments. [13]

A variety of techniques have been proposed to prevent antibiotics spread, such as advanced oxidation and membrane processes, adsorption and combined methods. [14] The research on enzyme-catalyzed transformation methods for antibiotics and organic micropollutants have been explored in recent years. [15,16] In this respect, laccase (E.C. 1.10.3.2.; *para*-benzenediol:oxygen oxidoreductase), a copper-containing oxidase, appears to be very promising due to its low substrate specificity and its ability to bring about the mono-electronic oxidation of a wide array of aromatic compounds using molecular oxygen as the final electron acceptor. Consequently, due to its low substrate specificity, laccase has been successfully used for the oxidation of a wide variety of organic pollutants, such as pesticides, polycyclic aromatic hydrocarbons, dyes and pharmaceutical compounds, including antibiotics. [16–20]

Although some pollutants might be putative laccase substrates, they are not susceptible to direct oxidation by the enzyme, due to either steric hindrance hampering, their docking to the active site or due to their high redox potential. By mimicking nature, it is possible to overcome these limitations by using the so-called ‘redox mediators’, able to act as intermediate substrates (electron shuttles) between the enzyme and the target

molecule. [15,18,20] In this respect, Ding et al. [15] and Margot et al. [21] reported that antibiotics degradation by *Trametes versicolor* laccase was markedly improved in the presence of different synthetic redox mediators. The mediator:substrate molar ratio, i.e. [M]/[S] ratio, has been reported to be a critical factor in the removal of contaminants by laccase/mediator systems (LMS). [22] As for LMS-assisted degradation of tetracyclines and sulfonamides, [M]/[S] ratios ranging from 2 to around 25 [15,23] and from 1.1 to 11, [17,24–27] respectively, have been mostly used with the exceptions of two studies. [19,21] The use of high [M]/[S] ratios makes LMS of limited practical use due to both the mediator costs and to the fact that several mediators, including those of natural origin, are not devoid of toxicity. [28]

Low stability of laccase to potentially denaturing agents is another constraint to a large-scale use of this enzyme. However, its immobilization onto solid supports has been reported to increase stability properties and to enable enzyme reuse along successive oxidation cycles. [29–31] The most interesting method of laccase immobilization for industrial application is covalent binding. Silica-based supports and activated carbon have been mainly used for this purpose. [29,30] Among widely available inorganic supports, laccase has been covalently immobilized to natural smectites, such as montmorillonite, [32] and synthetic ones, such as laponite. [33] However, stevensite, a clay belonging to the smectite family with high ability to adsorb tetracycline antibiotics, [34] has not been investigated yet for this purpose. Another emerging material, the availability of which is increasing exponentially due to its variable uses, is the biochar, which is obtained by pyrolytic biomass conversion. Due to its physico-chemical characteristics, biochar has been recently recognized as a valuable support for enzyme immobilization. [35] So far, however, there are only two studies where functionalized biochar was used to immobilize laccase. [36,37] Another point that deserves attention

regards the source of laccase; in fact, with few exceptions, [26,27] the large majority of studies dealing with laccase-catalyzed oxidation have relied on *Trametes versicolor* laccase (TvL). [15–17,23,38–40]

Thus, the aims of this work were: i) to assess the adequacy of stevensite and biochar as immobilization supports of *Myceliophthora thermophila* (MtL) and *Pleurotus eryngii* (PeL) laccases, not yet used in antibiotics degradation, and the catalytic properties of the immobilized preparations ii) to determine the efficacy of these immobilized preparations in the removal of three tetracyclines (TCs) and six sulfonamides (SAs) from two synthetic effluents both in the presence and in the absence of four redox mediators and, finally, (iii) to assess the residual antibiotic activity in enzyme-treated samples. To our knowledge, this is first study reporting on the antibiotics-oxidizing and detoxyfying ability of PeL- and MtL-based immobilized systems.

2. Materials and Methods

2.1 Materials, chemicals and solvents

Two supports of different nature, mineral and carbonaceous, were investigated for their ability to covalently immobilize laccase. The mineral support was stevensite, a clay belonging to the smectite group, which was obtained from Tolsa S.A. (Spain). The carbonaceous material, instead, was holm oak (*Quercus ilex*) biochar which was supplied by Piroeco Bioenergy S.L. (Spain). The biochar was made by pyrolysis of holm oak pruning at 500 ºC. The BET surface areas of stevensite and biochar were 212 and 76 $m^2 g^{-1}$, respectively.

The three TCs, including oxytetracycline hydrochloride (OxTC, 97%), tetracycline hydrochloride (TET, 98%) and chlortetracycline hydrochloride (ClTC, 97%), were purchased from Sigma–Aldrich. SAs including sulfanilamide (SNA ≥98.0%),

sulfadiazine (SDZ, ≥99.0%), sulfathiazole (STZ, ≥99.0%), sulfapyridine (SP, ≥95.0%), sulfamethazine (SMZ, ≥99%), sulfamethoxazole (SMX, ≥99.0%) were purchased from Across Organics and Fisher. The redox mediators under study were 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazol (HBT), syringaldehyde and violuric acid (VA) (Sigma-Aldrich). The quality of water used for immobilization process, antibiotic oxidation assays and HLPC analysis was type I grade (Millipore). Trifluoroacetic acid (Scharlau), methanol (Scharlau) and acetonitrile (Panreac) were HPLC-grade. Ammonium acetate (Merck), acetic acid, potassium dihydrogen phosphate, potassium and sodium hydroxide (Panreac) and other chemicals were of analytical grade.

2.2 Laccase: source, partial purification and activity determination

Two laccase sources were used in this study. The former laccase was a liquid formulation from *Myceliophthora thermophila* (MtL) supplied by Novozymes (Denmark) and produced by submerged fermentation of genetically modified *Aspergillus* sp. The latter was obtained from *Pleurorus eryngii* (PeL) liquid cultures on tryptone soy broth (3%) at 28 ºC under orbital shaking (150 rpm) for 21 days. Both preparations were partially purified by ammonium sulfate precipitation (85% saturation) and subsequent centrifugation (11,000 x g, 30 min). The precipitate thus obtained was dissolved in potassium phosphate buffer 0.1 M pH 7.0, passed through a PD-10 desalting column (GE Healthcare) and then used for laccase immobilization process.

Activity of free and immobilized laccase was determined spectrophotometrically by following the oxidation of 2,6-dimetoxyphenol (DMOP) in 50 mM sodium acetate buffer (pH 5.0) at 477 nm (ε= 14600 M^{-1} cm^{-1}). One international unit (IU) was defined

as the amount of enzyme producing 1.0 μmol of product per minute under the assay conditions.

2.3 Laccase immobilization

Prior to immobilization, the stevensite was activated by boiling in concentrated HNO_3 with continuous stirring for 1 h. The HNO_3 was removed by centrifugation (10,000 x g, 20 min) and the resulting pellet washed several times with distilled water until the pH of the supernatant reached 6.0. [41] Stevensite was then dried at 65 ºC overnight. Laccases from *M. termophila* (MtL) and *P. eryngii* (PeL) were immobilized on biochar (B_MtL or B_PeL, respectively) and stevensite (S_MtL or S_PeL, respectively) by a slight modification to the procedure described by Brandi et al. [29] Biochar or stevensite (200 mg) were aminopropylated with 5 mL of a 2% (v/v) solution of aminopropyltriethoxysilane (APTES) in acetone under stirring for 16 h. Excess of APTES was removed by centrifugation and the pellet underwent two centrifugation/washing cycles with 0.1 M potassium phosphate buffer at pH 7.0 (PB). The pellet was then suspended in 10 mL PB, containing 5% of glutaraldehyde (v/v), and stirred magnetically for 1 h. Excess of glutaraldehyde was removed by three cycles of centrifugation/washing with PB. The pellet was then suspended in 15 mL of PB, added with 400 IU of laccase, and stirred for 24 h at 5 ºC. Non-bound enzyme was removed by centrifugation/washing cycles with PB, until no more activity could be detected in the washings.

2.4 Partial characterization of immobilized laccase preparations

The activity yield (%) was determined as previously reported. [42] The catalytic capability was calculated by referring immobilized activity to unit mass of support. The effect of temperature on activity of free and immobilized laccase was determined at 40, 50, 55,

60 and 65 °C according to the method described above. To determine the effect of pH on the laccase activity, sodium acetate buffer pH 5.0 was replaced by 0.1 M McIlvaine buffer at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0. In order to assess the reusability of the immobilized preparations, 20 consecutive oxidative cycles were performed using 0.2 mM ABTS in 10 mM acetate buffer (AB). In particular, initial oxygen uptake was determined by a SA520 Clark oxygen electrode (Orion Instruments) and each cycle was allowed to proceed until all the substrate was oxidized. At the end of each cycle, the immobilized preparations (15 mg) were recovered by centrifugation (10000 x g, 10 min) and washed three times with AB and the procedure repeated with a fresh aliquot of substrate, as described previously. Reusability was inferred by percent of retained activity after 20 oxidation cycles. To determine the shelf-life, immobilized preparations were transferred to filter-sterilized 2,2,-dimethylsuccinate buffer (10 mM, pH 5.5) and residual activity determined after 3-month incubation at 4 °C.

2.5 Degradation of antibiotics by immobilized laccase

Antibiotics degradation by laccase from *M. thermophila* and *P.eryngii* immobilized on biochar and stevensite was carried out in 10 mL of sodium acetate buffer at pH 5.0 containing 0.1 mM of each TCs or SAs either in the presence or in the absence of redox mediator. Four redox mediators (i.e., ABTS, syringaldehyde, 1-hydroxybenzotriazol and violuric acid) were tested separately at a final concentration of 0.2 mM. In mediated reactions, the [M]/[S] ratios were set at 0.67 and 0.33 for TCs and SAs mixtures, respectively. Reactions were performed at 40 °C and initiated by the addition of 10 IU of immobilized laccase. Two types of controls were incubated in parallel, the former without the catalyst and the latter with the heat-denatured (120 min, 95 °C) catalyst. The former and the latter control were used in order to determine abiotic degradation and the

possible adsorption of TCs and SAs, on the immobilization supports, respectively. From here onwards, the resulting treatments are referred to as:

C: control without immobilized laccase

D: heat-denatured laccase after immobilization

L: immobilized laccase on stevensite or biochar

A: immobilized laccase + ABTS 0.2 mM

S: immobilized laccase + syringaldehyde 0.2 mM

H: immobilized laccase + HBT 0.2 mM

V: immobilized laccase + violuric acid 0.2 mM

Each treatment was carried out in triplicate and incubated for 0.25, 1, 2, 4, 8 and 24 h. At each set incubation time, an aliquot (1 mL) was collected, filtered through 0.45 μm Nylon syringe filter and residual antibiotics concentration determined immediately as described in subsection 2.6.

2.6 Analysis of antibiotics

The quantification of antibiotics was carried out by reversed-phase high performance liquid chromatography in an apparatus consisting of a 2695 Separation Module (Waters) coupled with a Waters 996 photodiode array detector (HPLC-PDA). Chromatographic separation of TCs was achieved with an Agilent Zorbax SB-C8 (250 x 4.6 mm, particle size 5 μm) column using a gradient elution program with trifluoroacetic acid 10 mM, acetonitrile and methanol according to manufacturer instructions (Table 1). The column temperature was set at 30 ºC. Chromatographic separation of SAs was achieved with a Phenomenex Luna C18 (250 mm × 4.6 mm; particle size 5 μm) column, using a

gradient elution program with 20 mM ammonium acetate in water adjusted to pH 4.50 with acetic acid and acetonitrile:methanol (1:1) [43] (Table 1). The column temperature was set at 40 ºC. The injection volume was 20 µL for both TCs and SAs analyses. The elution profiles were monitored at 355 and 270 nm for TCs and SAs, respectively. Antibiotics were identified on the basis of matching of both UV spectra (210 – 400 nm) and the retention times with those of commercially available standards (Sigma-Aldrich).

Calibration curves were drawn using 9 standard solutions the concentrations of which ranged from 0.250 to 50.0 mg L^{-1} for each antibiotic. The values of the coefficients of determination (R^2) of the calibration curves were greater than 0.999 with the only exception of SP (0.9983) (Table 2). Limits of detection (LOD) and limit of quantitation (LOQ) were calculated from Equations (1) and (2), respectively:

$$LOD = \frac{3 \cdot SD}{b} \qquad (1)$$

$$LOQ = \frac{10 \cdot SD}{b} \qquad (2)$$

where SD is the signal standard deviation of ten blank measurements and b is the linear coefficient (slope) of the calibration line. The LOD and LOQ of the TCs and SAs (Table 2) ranged from 3 to 32 µg L^{-1} and from 9 to 108 µg L^{-1}, respectively. These values of LOQ were able to assure a reliable determination of percentage of antibiotics degradation up to 99.75 % with respect to the initial concentration (0.1 mM).

2.7 Assay for bacterial growth inhibition

The residual antibiotic activity of the samples was evaluated by bacterial growth inhibition on three Gram-negative bacteria (*Escherichia coli*, *Salmonella thyphimirum* and *Klebsiella oxytoca*), two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and a bacterial consortium derived from the effluent from the

WWTP of the Autonomous University of Madrid. Each tested bacterial culture or 5.0 mL of wastewater were added separately to 500 mL of sterilized (121 °C, 20 min) tryptic soy broth (TSB) and incubated at 30 °C in the dark and under orbital shaking (120 rpm) for 24 h. The optical density at 600 nm (OD600) of the bacterial medium was adjusted to 0.100 by dilution with sterilized TSB. The samples obtained in the antibiotics degradation assay by immobilized laccase (1 mL) were mixed with TSB (4 mL) and incubated at 30 °C in the dark for 4 h. Growth controls, prepared by inoculating bacteria in 4 ml TSB added with 1 ml of 10 mM acetate buffer pH 5.0 in the absence of antibiotics, were incubated in parallel. The percentage of bacterial growth inhibition (GI%) was calculated by Equation (3):

$$GI\% = (1 - \frac{OD_{600s}}{OD_{600c}}) \cdot 100 \qquad (3)$$

where OD_{600S} and OD_{600c} are the optical densities of the sample and of the antibiotics-lacking control, respectively.

2.8 Statistical analysis

One-way analysis of variance was carried out after previously performing a Levene variance homogeneity test using the IBM SPSS Statistics v22 software package. To compare the differences between treatments, the Tukey or Games–Howell post hoc test (according to variance homogeneity) at $p < 0.05$ was used. Log-transformed removal of SAs data were also subjected to principal components analysis (PCA) by the use of the Simca-P 13.0 software (Umetrics, Umea, Sweden). The possible presence of either moderate or strong outliers in observations were assessed by the squared prediction errors and Hotelling (T^2) of t-scores, respectively. [44] At the variable level, variable

power (VP), defined as the explained standard deviation, was calculated by the equation (4):

$$VP = \frac{1 - SV_k}{SV_k^o} \qquad (4)$$

where SV_k is the residual standard deviation of the kth variable and SV^o_k is its initial standard deviation, which is equal to unity for all variables after soft scaling.

3 Results and discussion

3.1 Immobilized laccase systems: yields and some properties

Fig. 1A shows that the activity yield of the immobilization process of PeL on both stevensite and biochar was higher than 70% with the ensuing catalytic capabilities of the two immobilized systems amounting to 1426 and 1405 IU g^{-1} of dry support, respectively. MtL immobilization on stevensite and biochar, instead, led to activity yields lower than those obtained with PeL (i.e, 29.1 and 34.6%, respectively) (Fig. 1A). Two-way ANOVA showed that the activity yield was not affected by the support type variable (p=0.646) while the laccase source (p<0.0001) and the laccase source-support type interaction (p=0.029) were highly significant. Although MtL immobilization on stevensite and biochar was not highly efficient, the catalytic capabilities of the resulting preparations (i.e., 582 and 687 IU g^{-1} support) (Fig. 1A) can be deemed to be interesting on a comparative basis. [42,45,46] A potential advantage derived from the immobilization of biocatalysts is the possibility of their reuse upon their recovery. Thus, in order to assess their reusability, the percent of activity retained by each immobilized preparation after 20 consecutive oxidation cycles was determined, as shown in Figure 1B. Best results were obtained with S_MtL which retained around 85% of its initial activity. However, it is noteworthy, that the activity retention of all the immobilized systems exceeded 50%

of initial activity. Another parameter of practical significance for immobilized systems is their storage stability. Figure 1B shows that stevensite-immobilized PeL and MtL retained 68 and 73% of initial activity after 3-month storage at 4 °C; the same biochar-immobilized laccase preparations exhibited a lower stability than stevensite-immobilized ones (23 and 35%, respectively).

Fig. 2A shows that free MtL exhibited a broad pH optimum in the 5.5-6.5 range in agreement with literature data. [47] Its pH activity profile was shifted towards more acidic values upon immobilization on biochar with the highest activity being observed at pH 4.0 along with a marked decline in relative activity in the 4.5-7.0 range. Stevensite-immobilized laccase exhibited two distinct pH optima at 4.5 and 6.5. PeL exhibited a pH optimum 4.5 and its relative activity markedly declined at neutral pH. Irrespective of the support, immobilization led to a shift of the pH-activity profile towards more acidic values (Fig. 2B). In general, enzymes immobilization to charged supports has been shown to result in a displacement in the pH-activity profile and it has been explained on the basis of an uneven partitioning of H^+ and OH^- concentrations between the microenvironment of the immobilized system and the bulk phase. [48] With the exception of S_MtL, the displacement towards acidic values of the pH-activity curves might suggest a polycationic nature of the support. This hypothesis, however, might be likely for biochar but not for stevensite.

DMOP oxidation was more severely affected by the temperature in case of free MtL than PeL, the respective activation energies in the 40-50 °C range amounting to 38.60 and 7.61 kJ mol^{-1}. For free MtL, within the range under study (40-65 °C), activity increased as the temperature increased. S_MtL and B_MtL exhibited T optima at 50 and 60 °C, respectively, (Figure 2C) and their activation energies (i.e., 19.02 and 9.79 kJ

mol^{-1}) were lower than that of the free counterpart. S_PeL and B_PeL exhibited T optima at 55 and 50 °C, respectively and their relative activation energies in the 40-50°C range were unaffected for the former (7.56 kJ mol^{-1}) and increased for the latter (20.57 kJ mol^{-1}) with respect to the free counterpart.

3.2 Antibiotics removal by immobilized laccase

Laccase enzyme is an oxidoreductase able to oxidize aromatic compound like phenols and aromatic amines. [48] Although TCs and SAs were putative laccase substrates due to the presence of a hydroxyl and amino-groups directly attached to a benzene ring, respectively, the presence of either electron-withdrawing or bulky substituents associated with a high redox potential made these compounds poorly susceptible to laccase oxidation. [49] This limitation was overcome by adding redox mediators, acting as electron shuttles between laccase and the target molecule. This mechanism involves the oxidation of the mediator compounds by laccase thus resulting in the production of radical species endowed with higher diffusibility and oxidative capacity towards the substrate than the laccase itself. [18] In this way, laccases are able to oxidize compounds with a redox potential higher than 0.8 V and the direct interaction between the enzyme and the target substrate is no longer necessary. [18,20]

3.2.1 Tetracycline antibiotics removal

Time courses of TCs removal by immobilized PeL and MtL and relative incubation controls are shown in Figs. 3 and 4, respectively. Despite the surface modification of biochar and stevensite due to the immobilization process, adsorption phenomena on stevensite and, to a higher extent, to biochar were evident as it can be inferred from the removal kinetics of treatment D where the effluent was incubated with the heat-

denatured laccase systems. The adsorption process was strongly time-dependent and did not exceed 20% in the early 4 h of incubation. With regard to abiotic losses, instead, they amounted to less than 9% irrespective of the antibiotic in the early 8 h of the incubation. However, at the treatment´s end-point (24 h), average losses due to abiotic degradation of OxTC, TET and ClTC amounted to 19.3 ± 4.7, 22.3 ± 1.8 and 30.3±2.4%, respectively.

TCs removal in the absence of redox mediators was confined only to stevensite-immobilized PeL. In particular, the use of this catalyst led to significantly higher removal percentages than those observed with the respective heat-denatured control for OxTC (33.7 ± 1.0 vs. 27.0 ± 0.6, $p < 0.001$) and ClTC (54.7 ±0.6 vs. 37.3±0.6, $p < 0.001$). With regard to the ability of laccases to oxidize TCs in the absence of redox mediators, there are very contrasting results in the literature even with the same laccase source. On the one hand, free and immobilized *T. versicolor* laccase (TvL) were reported to degrade 30 and 56% TET (20 mg L^{-1}), respectively, in an enzymatic membrane reactor (EMR) operated in batch mode. [39] TvL was also shown to perform a 78% TET removal from a solution containing 100 mg L^{-1}. [40] Similarly, TET, ClTC, doxycycline and OxTC were significantly removed by crude TvL (16, 48, 34 and 14%, respectively) after 4 h incubation at an enzymatic load of 0.6 IU mL^{-1}. [23] On the other hand, in an EMR operated with TvL, Becker et al. [38] found that, among TCs, only doxycyline was slightly removed by immobilized TvL without mediators. In another work, purified TvL was reported to be unable to oxidize OxTC [50] and similar results were reported by Ding et al. [15] In addition to reaction conditions (i.e., pH, enzymatic load, immobilized catalyst, antibiotic concentration etc.), these largely variable results might be explained by the instability of these compounds as clearly shown by Ding et al. [15] and also confirmed in the present study. Magnetic cross-linked enzyme aggregates

(M-CLEAs) prepared for *Cerrena unicolor* laccase, were effective in degradation of TET and OxTC leading to removal extents around 60%; [19] in that study, however, a 20-fold higher enzymatic load (20 vs 1.0 IU mL^{-1}) and longer incubation times (48 vs. 24 h) than those of the present study were used.

With a sole exception, [19] there is a general agreement about the enhancement of laccase-triggered antibiotics degradation ensured by the addition of redox mediators. [16,21,23] However, in the present study, of the four tested mediators, only ABTS and syringaldehyde met this expectation.

Although the removal kinetics of TCs in the presence of ABTS was rather fast, it was clearly affected by the laccase source. After 15 min incubation, the TCs removal by MtL-based catalysts was between 84 and 100% while with PeL, they ranged between 11 and 64%. In the presence of the same mediator, after 4 h incubation, all the tested TCs were quantitatively removed from solution irrespective of both laccase source and immobilization support. At that incubation time, as already mentioned, both adsorption phenomena and abiotic degradation had been found to be negligible. Noteworthy, ABTS was the first synthetic compound which was reported to act as a laccase mediator in the oxidation of non-phenolic lignin model compounds. [51] Laccase-catalyzed oxidation of ABTS oxidation leads to a cation radical ($ABTS^{\bullet+}$) which might undergo to further oxidation to the dication ($ABTS^{2+}$); with this mediator, oxidation of non-phenolic aromatic compounds has been shown to occur *via* an electron transfer (ET) route. [52] The efficacy of syringaldehyde as a valuable redox mediator for laccase was demonstrated even earlier than ABTS; [53] since then, a variety of lignin-related compounds has been shown to act as laccase mediators and suggested to represent a cost-effective and sustainable alternative to synthetic mediators. [20] In the present study,

albeit less fast and effective than ABTS, syringaldehyde, led to interesting results and also in this case removal outcomes appeared to depend mainly on the laccase source. In particular, after 4 h, S_MtL and B_MtL led to ClTC and TET removals which were quantitative for the former and higher than 70% for the latter compound. At the same incubation time, the OxTC, concentration was halved by MtL-based immobilized systems and reduced by one third by PeL-based catalysts. After 24 h incubation, with syringaldehyde as the mediator, best results were obtained with B_MtL the use of which resulted in 84, 92 and 100% removals of OxTC, TET and ClTC, respectively. In summary, with these two mediators, MtL-based catalysts were generally more efficient in TCs removal than those based on PeL. Irrespective of the catalyst, the susceptibility to oxidation of target compounds was in the following decreasing order: ClTC > TET ≥ OxTC.

The other two redox active compounds tested in this study, namely HBT and VA, belong to the N-OH type mediators. They markedly differ each one another in terms of redox potentials and stabilities of their respective N-oxyl radical generated by laccase oxidation. [54] Although both compounds boosted the laccase-catalyzed removal of antibiotics, [15,23,26] their use, in the present study did not significantly enhance the oxidation of TCs by immobilized PeL or MtL. Although several factors might be invoked to explain the failure of these mediators, such as their high E° and decay into catalytically inactive forms, [55] the main reason with immobilized PeL appeared to be the early occurrence of enzyme inactivation. In fact, low amounts of residual laccase activity (5-15%) were detected in HBT- and VA-containing mixtures at early stages of incubation (2 h). In this respect, it is long known that the laccase-generated HBT and VA radicals can undergo chemical reactions with aromatic amino acid side chains thus leading to enzyme inactivation even though the extent of this phenomenon has been

shown to be dependent on the laccase source. [55] In case of MtL, instead, the high E° of HBT, which has been shown to amount to 1060 mV, [54] did not enable the enzyme to effectively oxidize HBT. In fact, MtL is reportedly a low redox potential enzyme (450 mV) and, as a consequence, the k_{cat} of this enzyme for HBT was 800-fold lower than that of ABTS. [55]

3.2.2 Sulfonamide antibiotics removal

Removal of SAs antibiotics by PeL and MtL immobilized on stevensite and biochar are shown in Figs. 5 - 8. SAs were highly stable in solution and their percent removals in control treatments ranged between 0.02 to 3% after 24 h of incubation. Even the extent of adsorption of SAs on modified stevensite and biochar was comparatively much lower than that observed for TCs and found to range between 4 and 12% and 4 and 14%, respectively, after 24 h incubation. As opposed to that observed for TCs, immobilized laccase systems were all able to perform partial SAs degradation in the absence of mediators. However, the solid support appeared to affect the number of SAs susceptible to enzymatic degradation. S_PeL was able to degrade STZ, SP, SMZ and SMX but not SNA and SDZ. In contrast B_PeL degraded SNA, SDZ, STZ, SP and SMX but not SMZ. In the case of MtL the effect of the solid support was even more evident than PeL. S_MtL degraded SNA, SDZ, STZ, SP and SMX but not SMZ while no antibiotics removal was evident upon incubation with B_MtL. Therefore, once again, the relevance of the solid support in the degradation of antibiotics by immobilized laccase was evident.

The use of all the redox mediators under study enhanced the ability of immobilized laccase to degrade SAs albeit to different extent depending on the mediator. Although the addition of either HBT or VA led to significant differences with respect to the L

treatments ($p < 0.05$), the amount of increase in SAs removal observed in their presence does not justify the use of these mediators. Similar results, involving a partial removal of SAs, by a TvL-HBT system were reported by Ding et al. [15] by using [S]/[M] ratios ranging from 2.5 to 10.3. Moreover, Rahmani et al. [17] reported that the improvement in the removal efficiency of two sulfonamides (STZ and SMX) by both immobilized and free TvL depended on HBT concentration and the effect of the mediator was evident as the [M]/[S] ratio was higher than 2.5. In another study, the use of VA enabled TvL to degrade efficiently SP and STZ and, in this case, the [M]/[S] ratio was around 5.0. [24] Thus, the failure of HBT and VA in promoting a significant increase in removal of SAs in the present study could have been dependent on the low [M]/[S] ratio (i.e., 0.33), which was chosen deliberately for the above mentioned economic and environmental considerations.

In contrast to HBT and VA, the use of ABTS and syringaldehyde resulted in marked improvement of SAs removal as already observed with TCs. Irrespective of the immobilized laccase system, the type of mediator exerted a marked effect on the SAs compounds which were preferentially removed. In particular, on the one hand, SDZ, STZ were removed to a significantly higher extent in ABTS-containing reaction mixtures than those added with syringaldehyde. Conversely, syringaldehyde was more effective than ABTS to support SNA, SMZ and SMX removals.

To gain further insights into the impact of the treatments on SAs, PCA analysis was conducted on log-transformed percent removals of each SA in treatments involving the most effective redox mediators (i.e, ABTS and syringaldehyde) at the endpoint of the incubation (24 h). No strong outliers among scores were found since none of the observations fell outside of the confidence region delimited by the Hotelling's ellipse

(Fig 9a). The first component that explained 52.6% of total variability led to a clear-cut segregation of laccase/mediator systems on the basis of the mediator, as shown in Fig 9a. Along the second component, explaining 26% of total variance, the majority of treatments were separated on the basis of the laccase source with the only exception of the biochar-immobilized MtL/ABTS mediator system that clustered with PeL-based treatments in the lower quadrants. The most influential variables that drove separation along the first principal component were STZ, SDZ and SNA. As a matter of fact, irrespective of both support type and laccase source, STZ and SDZ were more significantly removed in ABTS-containing reaction mixtures than those relying on syringaldehyde as the mediator while an opposite outcome was evident for SNA which, in fact, was located in the upper right quadrant. SMZ and SP, instead, were the most influential variables along the second component. With only exception the aforementioned biochar-immobilized MtL/ABTS mediator system, MtL-based systems were generally more effective in removing SMZ and SP than those relying on PeL, regardless of both mediator and support. Among the tested variables, only SMX was not adequately explained by the model as it can be inferred from is low VP value (Fig. 9b).

ABTS and syringaldehyde radicals derived from laccase oxidation were able to oxidize the aromatic ring of the sulfonamide group, as it was made evident from SNA oxidation in the presence of these redox mediators. The different susceptibility of SAs to treatments by immobilized laccase with and without redox mediators highlights the role of the substituents linked to the sulfonamide group (H_2N-phe-SO_2-R) for the oxidation process. In the majority of cases, the susceptibility to oxidation of SNA was lower than other sulfonamides and this suggests clearly that ABTS and syringaldehyde radicals were able to react with the group(s) linked to sulfonamide moiety. The clearest case was the markedly higher and faster removal of STZ (100% removal) than that of SNA (18-

26% removal) in the presence of ABTS (Fig. 5-8). However, to gain further insights into these results, HPLC-mass spectrometry analysis of reaction products will be necessary. In this respect, Margot et al. [21] and Shi et al. [27] identified several oxidation products formed during the degradation of SAs by laccase/ABTS and laccase/syringaldehyde mediator systems. For the latter system, dimeric coupling products of SAs with either syringaldehyde or 2,6-dimethoxy-1,4-benzoquinone, a typical product of syringaldehyde oxidation, were found. For the former, in addition to ABTS dead-end products, some unspecified SMX-derived fragments were found. The gradual consumption of redox mediators due to the concomitant formation of both degradation products of the redox mediators themselves and of dimers between these redox mediators and antibiotics might explain the incomplete removal of TCs and SAs (Fig. 3-8).

3.3 Assessment of residual antibiotic activity

The biodegradation of the antibiotics does not necessarily lead to either elimination or strong reduction of the antimicrobial activity since their breakdown products might retain significant antibiotic activity. [38] Therefore, the chemical analyses were integrated by determining the residual antibiotic activity of TCs- and SAs-containing mixtures that had been treated with the most effective laccase-mediator systems, ABTS and syringaldehyde. Results are summarized in Table 3 and compared with those of the relative incubation controls.

The two synthetic wastewater containing either TCs or SAs inhibited the tested bacterial species to different extents. In particular, the growth of the five bacterial species and bacterial consortium derived from a WWTP were totally inhibited by the TCs mixture added with heat-denatured laccase at start and after 24 h incubation. Conversely, the

SAs mixture did not exert any inhibition towards *E. coli, S. aureus* and *E. faecalis* but inhibited *S. thyphimirum*, *K. oytoca* and the bacterial consortium only partially.

Chemical analyses had indicated that the use of immobilized laccase coupled to either ABTS or syringaldehyde enabled the attainment of high TCs removals (100 and 69 - 100% respectively) (Figs. 3 and 4). As a matter of fact, biochar-immobilized laccases coupled to ABTS were able to remove completely the antibiotic residual activity for all the bacteria tested. However, treatments with stevensite-immobilized laccases led to residual antibiotic activity towards *E. coli* (7 and 9.2% for MtL and PeL respectively) and *S. typhimurium* (8.3 and 11.5% for PeL and MtL respectively) despite the complete removal of TCs. Therefore, the degradation intermediates of TCs could retain antibiotic activity for some bacteria. In particular, this phenomenon was clearly observed for TCs that had undergone treatment with immobilized laccase coupled to syringaldehyde. The growth inhibition of *E. coli, S. typhimurium, S. aureus* and WWTP bacteria exerted by laccase/syringaldehyde-treated TCs solutions did not differ from respective incubation controls. Treatment with the same laccase-mediator system led to a lower antibiotic activity towards *K. oxytoca, E. faecalis* than that exerted by 24-h-old TCs incubation controls. Becker et al. [38] reported negligible bacterial growth for antibiotics treated by laccase coupled to syringaldehyde despite the effective removal of antibiotics detected in chemical analysis.

The assessment of the impact of the laccase treatments on the residual antibiotic activity of the SAs mixture was only partial since three out of the five bacterial species, namely *E. coli*, *S. aureus* and *E. faecalis*, were not inhibited at all by this mixture. With regard to the remaining species, stevensite-immobilized PeL and MtL coupled to ABTS or syringaldehyde completely removed the growth inhibition of *K. oxytoca*. As for *S.*

typhimurium, the low growth inhibition (around 17%) observed in 24-h-old incubation controls was not significantly removed by the enzyme treatments. Conversely, in the case of the bacterial consortium from WWTP, all the treatments tested led to either a complete suppression of the antibiotic activity or to a negligible growth inhibition (Table 3).

4 Conclusions

P. eryngii and *M. thermophila* laccases were immobilized successfully on biochar and stevensite. These supports showed similar abilities to immobilize laccase albeit stevensite-based catalysts exhibited higher reusability and storage stability properties than biochar-based ones. Although antibiotics adsorption on modified stevensite and biochar was observed, especially for tetracyclines, the main removal mechanism was the mediator-assisted laccase oxidation. Among the tested mediators, the addition of either ABTS or syringaldehyde promoted significant removals of tetracyclines and, to a lesser extent, of sulfonamides by the immobilized laccase systems. However, the former mediator was better than the latter in terms of removal kinetics, extents of removal and detoxification. In particular, combination of ABTS with biochar-immobilized laccases led to a complete suppression of the antibiotic activity of the tetracycline mixture. Although syringaldehyde turned out to be less effective as compared to ABTS, its 45-fold lower bulk price (i.e., 0.1-0.3 US$/kg) makes its potential large-scale use more feasible than that of ABTS provided that low [M]/[S] ratios be used.

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Table 1: Gradient elution program of mobile phases for the separation of tetracyclines and sulfonamides antibiotics by HPLC-PDA.

Elution gradient for Tetracyclines				
Time (min)	Flow (mL min^{-1})	TFA (%)	ACN (%)	MeOH (%)
0.0	1.5	95	4	1
7.5	1.5	70	24	6
13.5	1.5	65	28	7
15.0	1.5	95	4	1
Elution gradient for Sulfonamides				
Time (min)	Flow (mL min^{-1})	NH_4Ac (%)	ACN:MeOH (1:1) (%)	
0.0	0.9	85	15	
17.0	0.9	71	29	
23.0	0.9	67	33	
26.0	0.9	67	33	
30.0	0.9	85	15	

TFA: trifluoroacetic acid 10 mM in water; ACN: acetonitrile; MeOH: methanol; NH_4Ac: Amonium acetate 20 mM in water adjusted at pH 4.50 with acetic acid.

Table 2: retention time, coefficients of determination (R^2) of the calibration curves, limits of detection (LOD) and limits of quantitation (LOQ) of the HPLC-PDA methods for tetracyclines and sulfonamides.

	Retention time (min)	R^2	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)
Tetracyclines				
OxTC	9.48	0.9994	17	58
TET	10.23	0.9993	32	108
ClTC	12.85	0.9998	20	67
Sulfonamides				
SNA	5.23	0.9994	8	27
SDZ	9.75	0.9994	3	9
STZ	10.80	0.9998	11	35
SP	12.04	0.9983	4	12
SMZ	16.82	0.9999	21	71
SMX	21.37	0.9992	12	39

Table 3: Growth inhibition (%) of five bacterial species and a bacterial consortium (BC) from real wastewater treatment plant in the presence of five-fold diluted incubation controls of either the tetracycline or the sulfonamide mixture (TCs and SAs, respectively) at start (IC 0 h) and after 24 h (IC 24 h) and in the same diluted mixture incubated for 24 h with immobilized laccase from *M. thermophila* (MtL) or *P. eryngii* (PeL) on stevensite (S) or biochar (B) coupled to ABTS (A) or syringaldehyde (S). Data are the mean of three replicates ± standard deviation. Different superscript letters indicate significant differences among treatments ($p < 0.05$).

	Growth inhibition† (%) on TCs					
	E. coli	*S. typhimurium*	*K. oxytoca*	*S. aureus*	*E. faecalis*	BC
IC 0 h‡	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
IC 24 h‡	99.5 ± 0.8^{a}	40.5 ± 1.5^{b}	97.6 ± 0.5^{a}	69.0 ± 0.5^{b}	57.3 ± 1.0^{b}	99.2 ± 1.3^{a}
S PeL_A	9.2 ± 12^{b}	8.2 ± 2.2^{c}	-32.2 ± 3.5^{f}	-41.5 ± 19^{cd}	-2.6 ± 1.6^{cd}	-63.2 ± 17^{d}
S PeL_S	92.3 ± 0.5^{a}	39.1 ± 3.1^{b}	1.8 ± 5.9^{c}	72.8 ± 3.9^{b}	8.5 ± 3.6^{c}	70.7 ± 2.3^{ab}
B PeL_A	-13.3 ± 4.9^{c}	-9.9 ± 0.8^{d}	-43.3 ± 8.0^{f}	-60.5 ± 6.2^{d}	-24.1 ± 9.8^{e}	-342.9 ± 24^{f}
B PeL_S	95.1 ± 2.9^{a}	35.5 ± 1.9^{b}	-19.4 ± 2.3^{de}	65.9 ± 4.3^{b}	-2.5 ± 16^{cd}	26.3 ± 14^{c}
S MtL_A	7.0 ± 2.0^{b}	11.5 ± 1.5^{c}	-14.9 ± 5.1^{d}	-43.2 ± 8.5^{cd}	-1.2 ± 3.7^{cd}	-61.7 ± 8.5^{d}
S MtL_S	98.7 ± 2.0^{a}	33.9 ± 2.8^{b}	5.9 ± 4.4^{bc}	73.3 ± 3.9^{b}	10.1 ± 3.7^{c}	54.9 ± 8.1^{bc}
B MtL_A	-31.0 ± 12^{c}	-15.3 ± 5.8^{d}	-31.6 ± 3.2^{ef}	-23.6 ± 5.0^{c}	-14.3 ± 7.8^{de}	-137.6 ± 12^{e}
B MtL_S	87.7 ± 4.1^{a}	33.8 ± 0.8^{b}	16.6 ± 2.8^{b}	64.6 ± 2.0^{b}	7.6 ± 2.0^{c}	69.2 ± 11^{ab}
	Growth inhibition† (%) on SAs					
	E. coli	*S. typhimurium*	*K. oxytoca*	*S. aureus*	*E. faecalis*	BC
IC 0 h‡	6.0 ± 6.4^{R}	$17.6\pm2.9^{n.s.}$	32.4 ± 2.6^{a}	7.8 ± 13^{R}	13.0 ± 2.6^{R}	31.6 ± 6.9^{a}
IC 24 h‡	-38.5 ± 3.0	16.8 ± 5.7	12.9 ± 5.5^{b}	-25.2 ± 5.8	1.1 ± 3.8	9.0 ± 7.3^{ab}
S PeL_A	-20.4 ± 8.5	16.1 ± 1.5	-12.5 ± 4.3^{c}	-37.4 ± 6.2	-3.5 ± 3.5	-51.9 ± 13^{de}
S PeL_S	-13.7 ± 7.2	14.1 ± 1.5	-21.1 ± 7.1^{cd}	-51.0 ± 14	-7.7 ± 1.5	-8.3 ± 21^{bc}
B PeL_A	-26.5 ± 3.1	13.8 ± 4.8	-22.0 ± 3.9^{cd}	-29.6 ± 1.1	-12.1 ± 2.1	-131.6 ± 12^{f}
B PeL_S	-10.8 ± 7.4	8.1 ± 6.5	-26.9 ± 3.7^{d}	-38.5 ± 4.4	-19.1 ± 7.0	-78.2 ± 21^{e}
S MtL_A	-19.4 ± 9.8	14.2 ± 2.4	-15.8 ± 3.2^{cd}	-46.6 ± 5.8	0.9 ± 2.0	-31.6 ± 9.4^{cd}
S MtL_S	-24.5 ± 2.7	13.9 ± 1.8	-16.8 ± 2.0^{cd}	-40.5 ± 3.4	3.9 ± 2.7	-55.6 ± 8.1^{de}
B MtL_A	-14.7 ± 8.3	11.4 ± 3.4	2.7 ± 3.0^{b}	-48.5 ± 2.8	-3.9 ± 1.5	-27.8 ± 7.9^{cd}
B MtL_S	-7.5 ± 5.5	12.6 ± 2.4	4.1 ± 2.4^{b}	-30.9 ± 2.7	-7.4 ± 1.4	3.0 ± 8.1^{abc}

† Inhibition was referred to a growth control obtained by replacing the TCs or SAs with distilled water; R, resistant bacteria towards the tested antibiotics mixture; n.s., not significant differences among treatments ($p > 0.05$); ‡ Within each group of antibiotics, data obtained with incubation controls containing either ABTS or S did not significantly differ each one another. For this reason, they were averaged.

Figure Captions

Figure 1: (A) Activity yields (%) and catalytic capabilities (IU g^{-1}) of the immobilization process of *P. eryngii* (Pe) and *M. thermophila* (Mt) laccases on stevensite (S) and biochar; (B) Reusability defined as percent residual activity after 20 consecutive oxidative cycles of 0.2 mM ABTS solutions and shelf-life after 3 months storage at 4 °C and pH 5.5. Data are the mean ± standard deviation of 3 replicates.

Figure 2: Effect of pH (A and B) and temperature (C and D) on activity of free and immobilized *P. eryngii* (Pe) and *M. thermophila* (Mt) laccases on stevensite (S) and biochar (B). Data are the mean ± standard deviation of 3 replicates.

Figure 3: Time courses of removal (%) of oxytetracycline (OxTC), tetracycline (TET) and chlortetracycline (ClTC) by *P. eryngii* immobilized laccase systems on stevensite (S_PeL) or biochar (B_PeL) during 24 h of incubation at 40 ºC and pH 5.0. Tested treatments were: C: control, D: heat-denatured immobilized laccase, L: immobilized laccase, A: L+ABTS, H: L+HBT, S: L+syringaldehyde, V: L+ violuric acid.

Figure 4: Time courses of removal (%) of oxytetracycline (OxTC), tetracycline (TET) and chlortetracycline (ClTC) by *M. thermophila* immobilized laccase systems on stevensite (S_MtL) or biochar (B_MtL) during 24 h of incubation at 40 ºC and pH 5.0. Tested treatments were: C: control, D: heat-denatured immobilized laccase L: immobilized laccase, A: L+ABTS, H: L+HBT, S: L+syringaldehyde, V: L+ violuric acid.

Figure 5: Time courses of removal (%) of sulfanilamide (SNA), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SP), sulfamethazine (SMZ), sulfamethoxazole (SMX) by *P. eryngii* immobilized laccase systems on stevensite (S_PeL) during 24 h of incubation at 40 ºC and pH 5.0. Tested treatments were: C: control, D: heat-denatured immobilized laccase, L: immobilized laccase, A: L+ABTS, H: L+HBT, S: L+syringaldehyde, V: L+ violuric acid.

Figure 6: Time courses of removal (%) of sulfanilamide (SNA), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SP), sulfamethazine (SMZ), sulfamethoxazole (SMX) by *P. eryngii* immobilized laccase systems on biochar (B_PeL) during 24 h of incubation at 40 ºC and pH 5.0. Tested treatments were: C: control, D: heat-denatured immobilized laccase, L: immobilized laccase, A: L+ABTS, H: L+HBT, S: L+syringaldehyde, V: L+ violuric acid.

Figure 7: Time courses of removal (%) of sulfanilamide (SNA), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SP), sulfamethazine (SMZ), sulfamethoxazole (SMX) by immobilized *M. thermophila* laccase systems on stevensite (S_MtL) during 24 h of incubation at 40 ºC and pH 5.0. Tested treatments were: C: control, D: heat-denatured immobilized laccase, L: immobilized laccase, A: L+ABTS, H: L+HBT, S: L+syringaldehyde, V: L+ violuric acid.

Figure 8: Time courses of removal (%) of sulfanilamide (SNA), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SP), sulfamethazine (SMZ), sulfamethoxazole (SMX) by immobilized *M. thermophila* laccase systems on biochar (B_MtL) during 24 h of incubation at 40 ºC and pH 5.0. Tested treatments were: C: control, D: heat-denatured immobilized laccase, L: immobilized laccase, A: L+ABTS, H: L+HBT, S: L+syringaldehyde, V: L+ violuric acid.

Figure 9: Principal components analysis of log-transformed SAs removal data showing scores plot (A) of the different immobilized laccase/mediator systems and variable loading plots (B). Treatments have been coded as follows: A and S denotes the type of mediator (ABTS and syringaldehyde, respectively); Mt and Pe denote the laccase source (*Myceliophthora thermophila* and *Pleurotus eryngii*, respectively); St and Bc denote the support (stevensite and biochar, respectively). Filled squares and hollow circles denote PeL and MtL systems, respectively. The values of each variable power (sulfanilamide (SNA), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SP), sulfamethazine (SMZ), sulfamethoxazole (SMX)), calculated according to Equation (1), are reported between round brackets close to the variable label. Percent variability explained by each principal component is shown between round brackets after axis caption.

Figure 1:

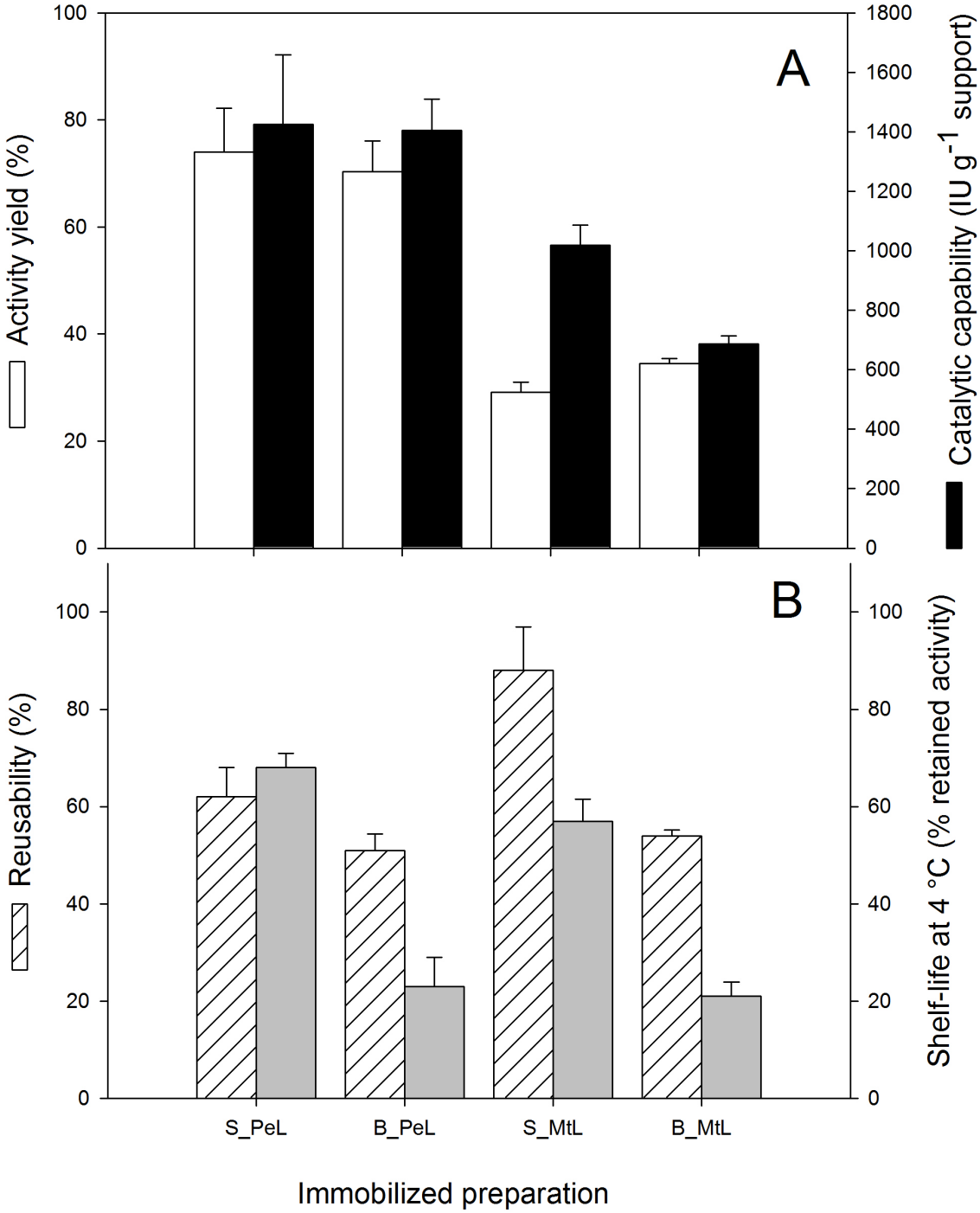

A
B
Activity yield (%)
Catalytic capability (IU g-1 support)
Reusability (%)
Shelf-life at 4 °C (% retained activity)
S_PeL
B_PeL
S_MtL
B_MtL
Immobilized preparation

Figure 2:

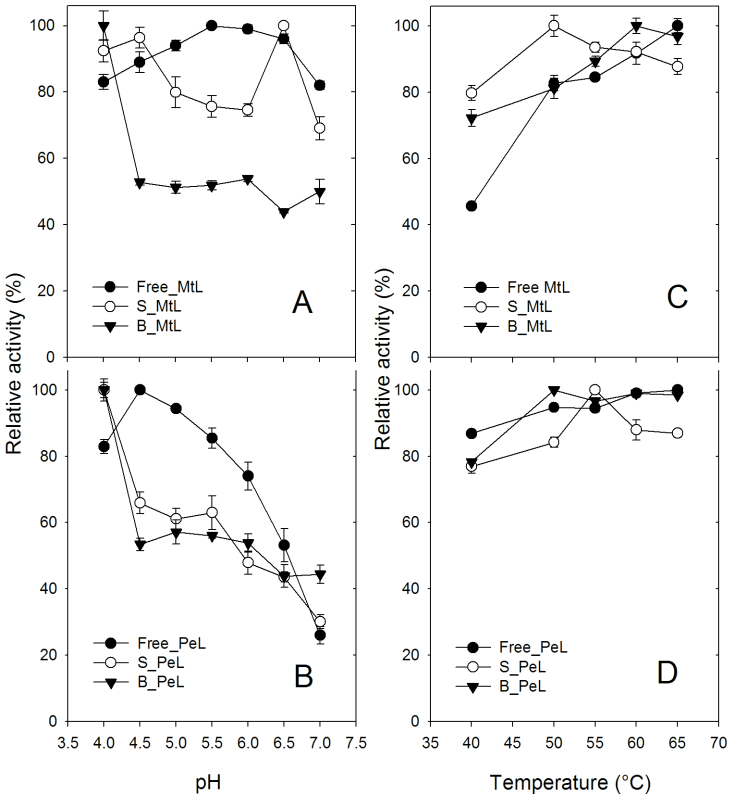

Relative activity (%)
Free_MtL
S_MtL
B_MtL
A
Free_PeL
S_PeL
B_PeL
B
pH
Free MtL
S_MtL
B_MtL
C
Free_PeL
S_PeL
B_PeL
D
Temperature (°C)

Figure 3:

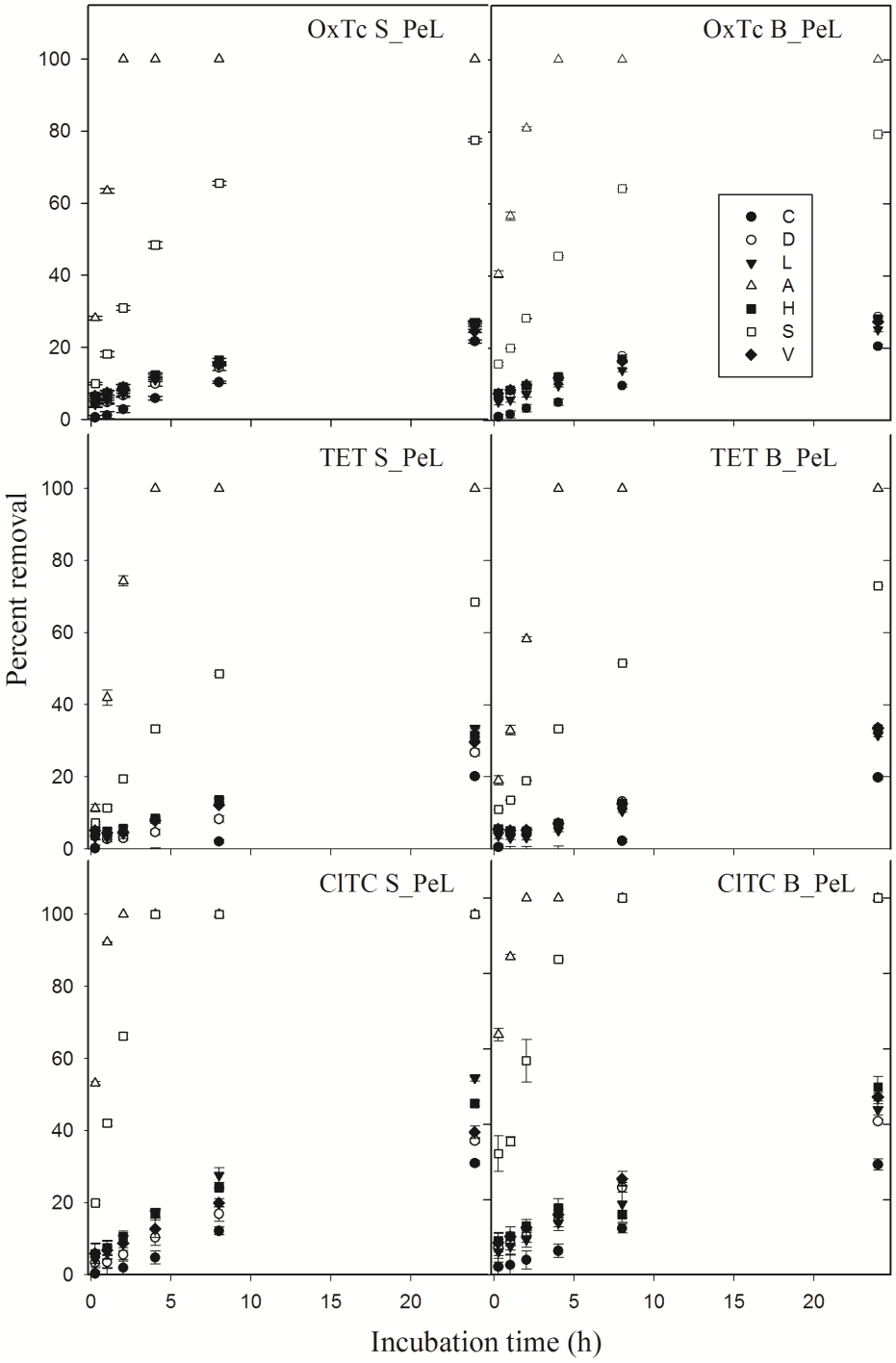

OxTc S_PeL
OxTc B_PeL
TET S_PeL
TET B_PeL
ClTC S_PeL
ClTC B_PeL
C
D
L
A
H
S
V
Percent removal
Incubation time (h)
0
20
40
60
80
100
0
5
10
15
20

Figure 4:

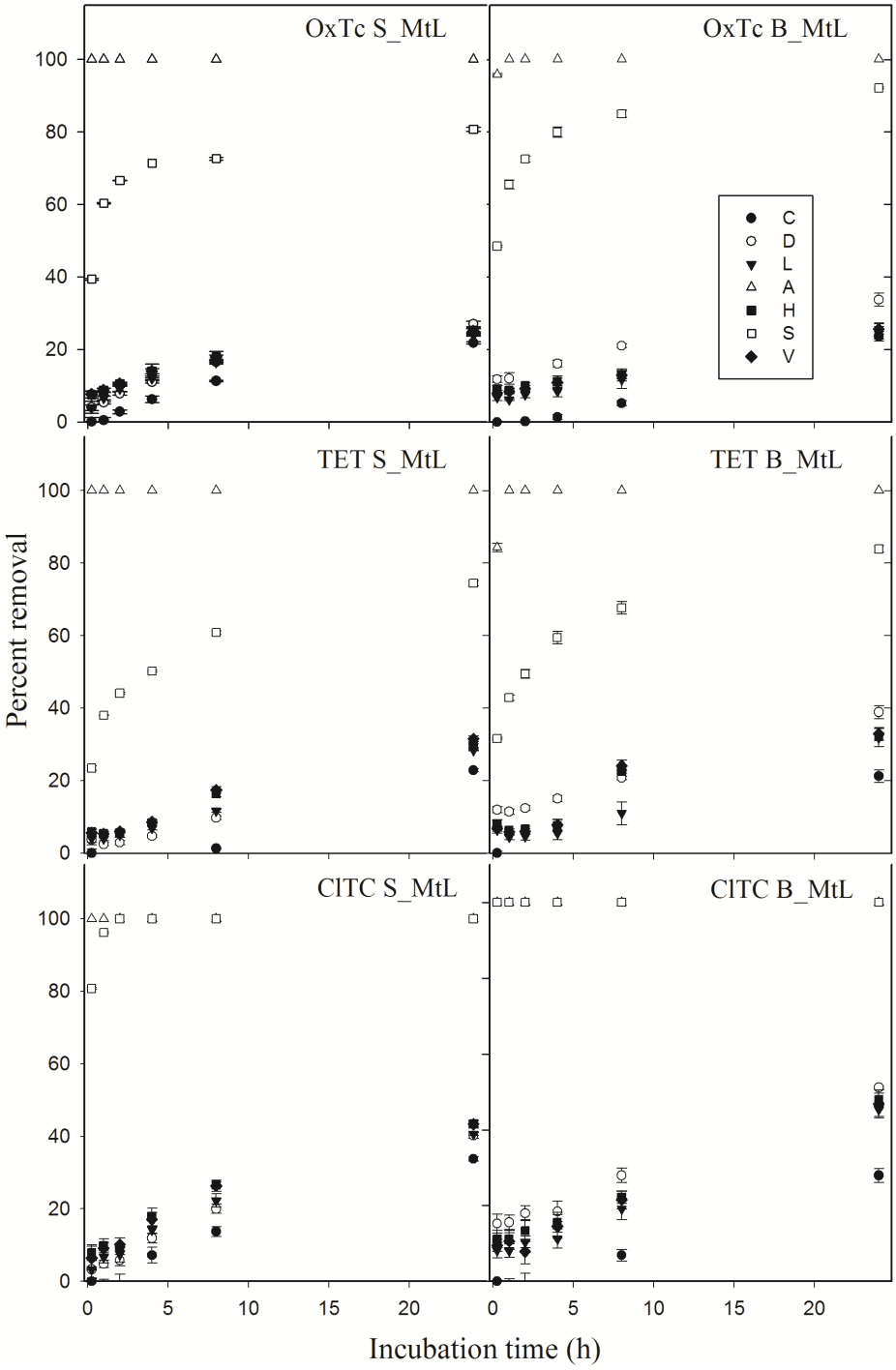

OxTc S_MtL
OxTc B_MtL
TET S_MtL
TET B_MtL
ClTC S_MtL
ClTC B_MtL
C
D
L
A
H
S
V
Percent removal
Incubation time (h)
0
20
40
60
80
100
0
5
10
15
20

Figure 5:

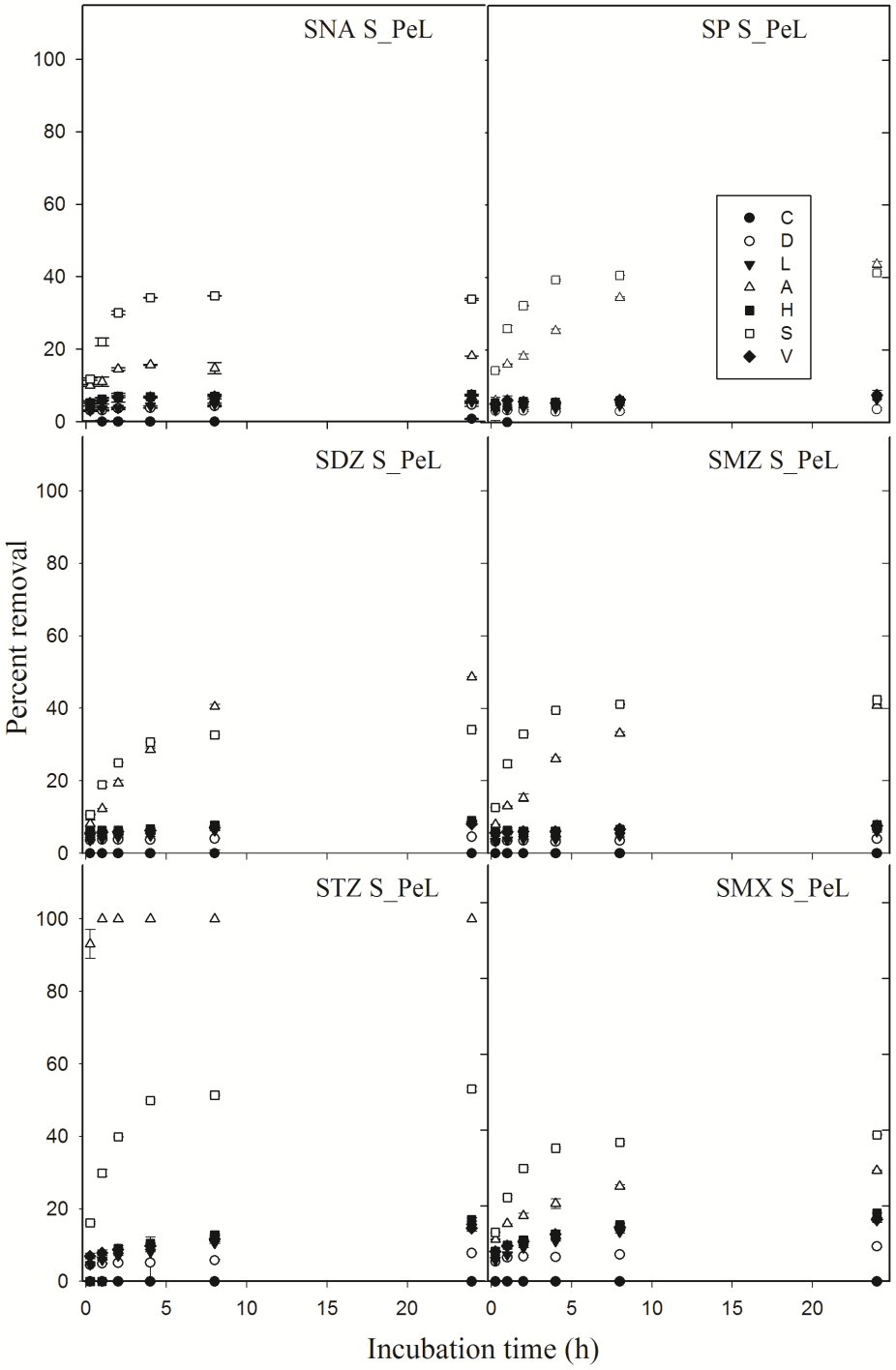

SNA S_PeL
SP S_PeL
SDZ S_PeL
SMZ S_PeL
STZ S_PeL
SMX S_PeL
C
D
L
A
H
S
V
Percent removal
Incubation time (h)
0
20
40
60
80
100
0
5
10
15
20

Figure 6:

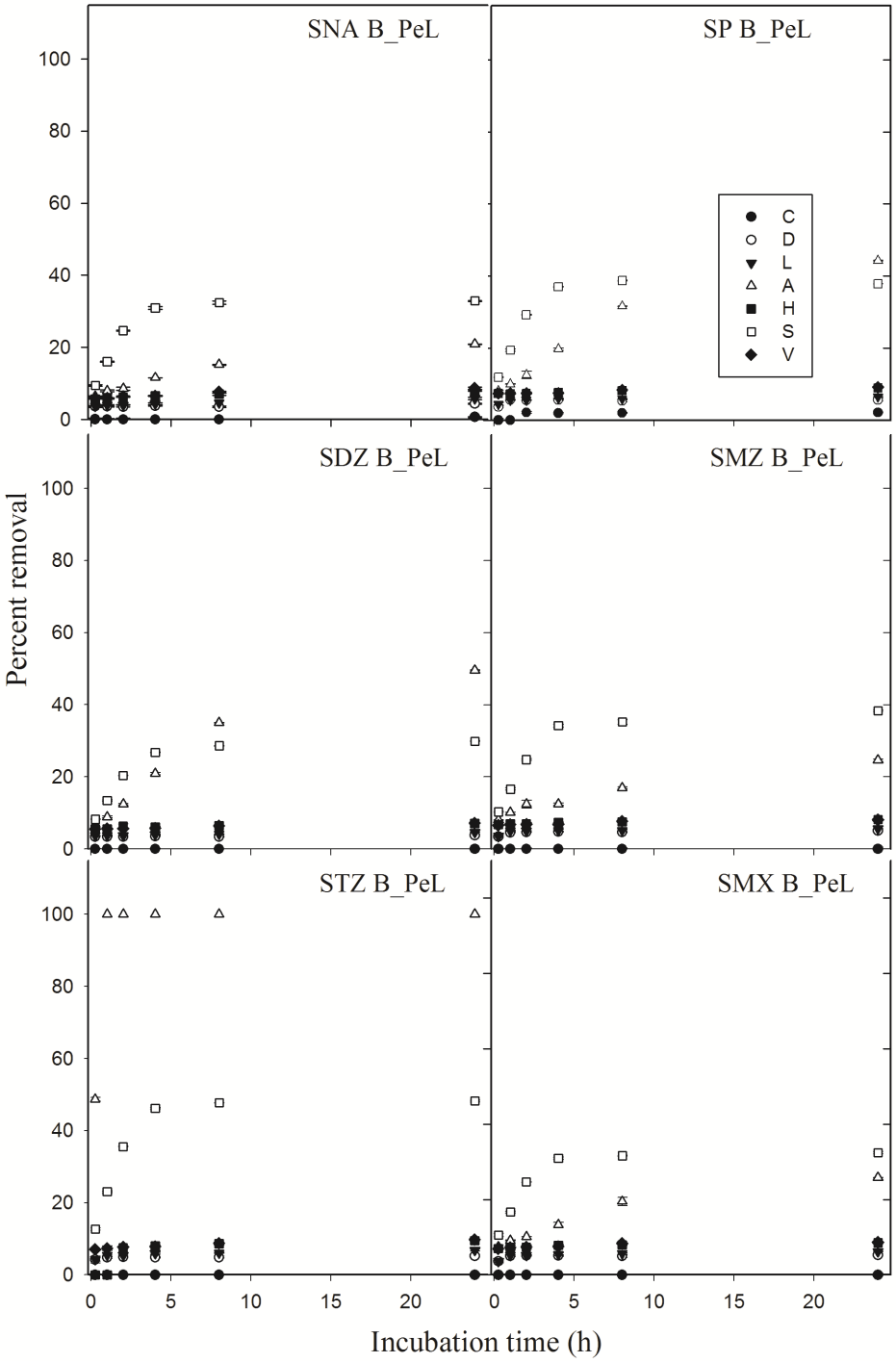

SNA B_PeL
SP B_PeL
SDZ B_PeL
SMZ B_PeL
STZ B_PeL
SMX B_PeL
C
D
L
A
H
S
V
Percent removal
Incubation time (h)
0
20
40
60
80
100
0
5
10
15
20

Figure 7:

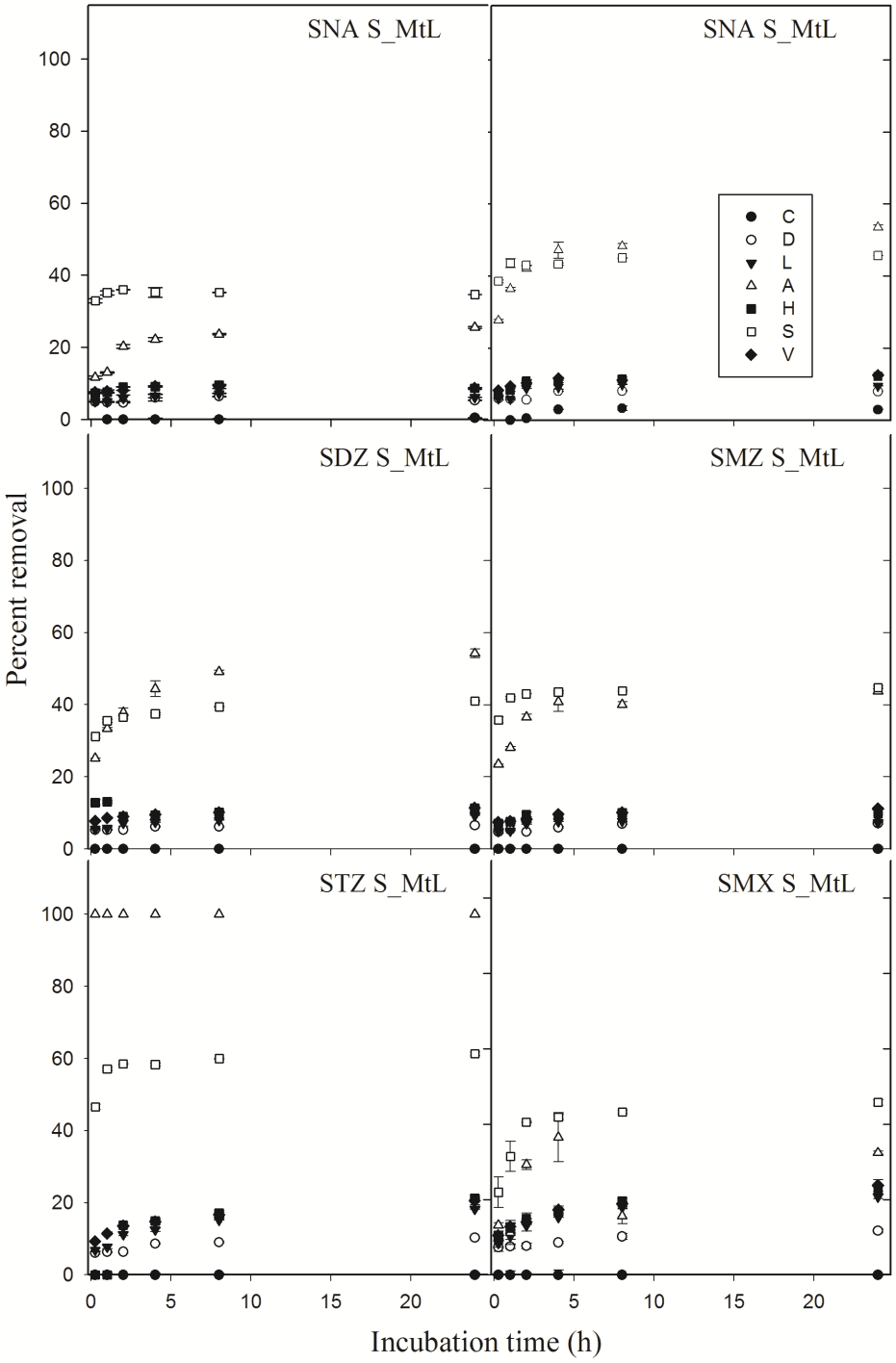

SNA S_MtL
SNA S_MtL
SDZ S_MtL
SMZ S_MtL
STZ S_MtL
SMX S_MtL
C
D
L
A
H
S
V
Percent removal
Incubation time (h)
0
20
40
60
80
100
0
5
10
15
20

Figure 8:

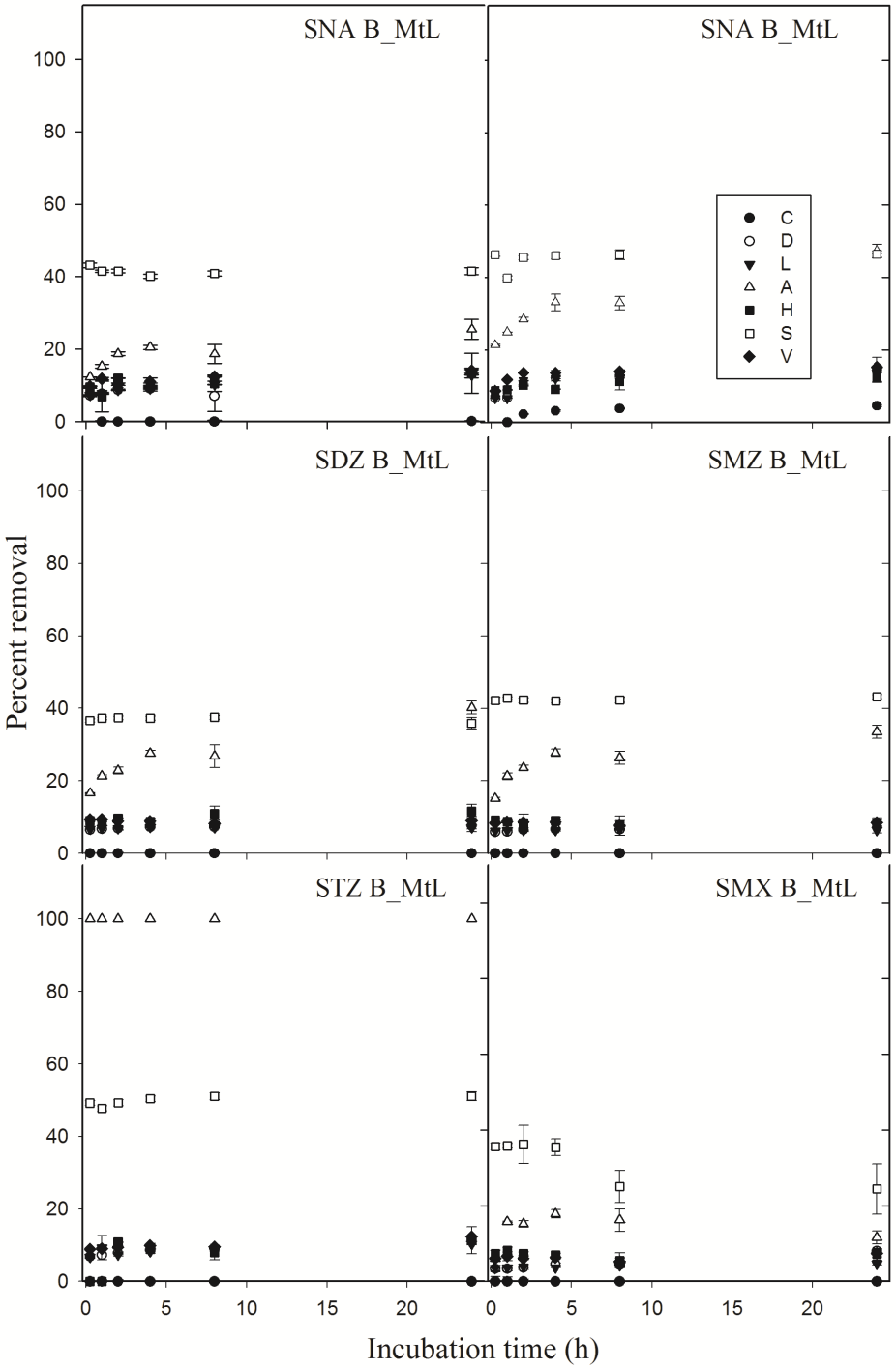
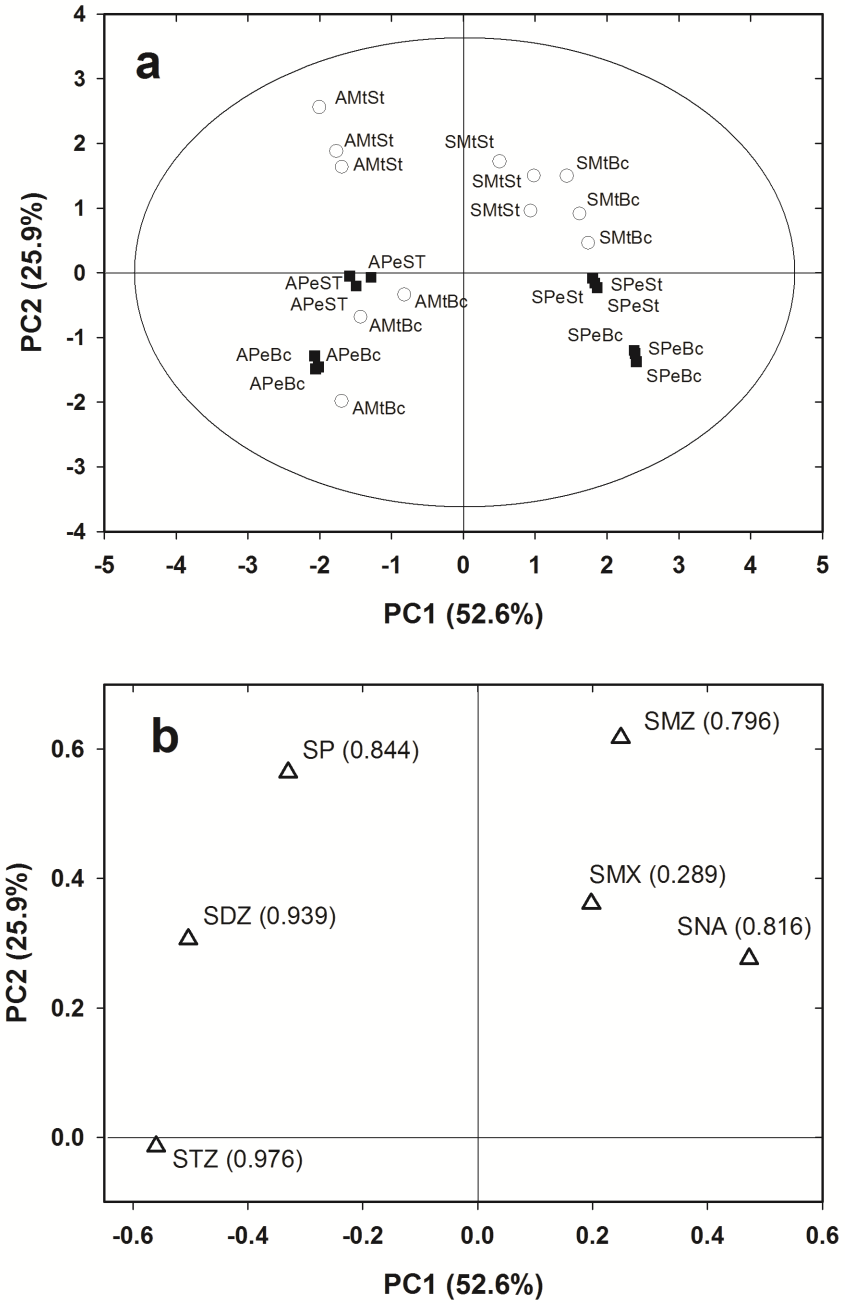

SNA B_MtL
SNA B_MtL
SDZ B_MtL
SMZ B_MtL
STZ B_MtL
SMX B_MtL
C
D
L
A
H
S
V
Percent removal
Incubation time (h)
0
20
40
60
80
100
0
5
10
15
20

Figure 9:


a
AMtSt
AMtSt
AMtSt
SMtSt
SMtSt
SMtSt
SMtBc
SMtBc
SMtBc
APeST
APeST
APeST
AMtBc
AMtBc
SPeSt
SPeSt
SPeSt
SPeBc
SPeBc
SPeBc
APeBc
APeBc
APeBc
AMtBc
PC2 (25.9%)
PC1 (52.6%)
b
SP (0.844)
SMZ (0.796)
SMX (0.289)
SDZ (0.939)
SNA (0.816)
STZ (0.976)
PC2 (25.9%)
PC1 (52.6%)