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Impacts of redox-mediator type on trace organic contaminants degradation by laccase: Degradation efficiency, laccase stability and effluent toxicity

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Authors

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Highlight

- TrOC removal performance of laccase - mediator systems is mediator dependent
- Non-phenolic compounds are more effectively removed by the N-OH type mediators
- Phenolics removal depend more on stability/redox potential of the mediator radicals
- Higher concentrations of mediators result in faster destabilisation of laccase
- Violuric acid degraded phenolic/non-phenolic TrOC w/out raising effluent toxicity

Impacts of redox-mediator type on laccase degradation of trace organic contaminants: Degradation efficiency, laccase stability and effluent toxicity

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Abstract

This study compares the effectiveness of seven redox-mediating compounds namely, 1-hydrozybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO), violuric acid (VA), syringaldehyde (SA), vanillin (VA), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), which follow distinct oxidation pathways, for the degradation of trace organic contaminants (TrOCs). These redox-mediators were investigated for improved degradation of four TrOCs, namely, oxybenzone, pentachlorophenol, naproxen and atrazine, which showed resistance to degradation by crude laccase harvested from the white-rot fungus *Pleurotus ostreatus*. A series of screening tests showed that ABTS, VA and HBT were the most efficient mediators for degradation of the selected TrOCs. ABTS and VA achieved the highest degradation of the phenolic compounds (*i.e.*, oxybenzone and pentachlorophenol), whereas the non-phenolic compounds (*i.e.*, naproxen and atrazine) were best removed using VA or HBT. This implies that the non-phenolic compounds are more effectively removed by the radical species generated by the N-OH type mediators (*i.e.*, VA and HBT), while removal of the phenolic compounds may depend more on the stability and the redox potential of the radicals generated from the mediator, irrespective of the type. Notably, enzyme stability was greatly affected by the N-OH type mediators but it was compensated by their rapid degradation capacity. Overall, VA and HBT (N-OH type) appear to be the best mediators for enhanced degradation of the selected compounds without causing significant toxicity in the effluent.

Keywords: Laccase degradation; Redox mediator; Trace organic contaminant; Effluent toxicity

1. Introduction

Laccase (EC 1.10.3.2) is a type 1 (blue) copper oxidase. It is widely distributed among white rot fungi, which are responsible for the degradation of complex organic polymeric lignins in nature (Yang et al., 2013). Laccase predominantly attacks the phenolic moieties in lignin, catalysing a one-electron oxidation via phenoxyl radicals. Laccase has been used in various industries such as textiles, pulp and paper, food manufacture and in remediation processes especially in the degradation of phenols and anilines (Hai et al., 2013; Witayakran and Ragauskas, 2009). In recent years, the removal of trace organic contaminants (TrOCs) from water and wastewater by laccase has received increasing attention. TrOCs are detected in various water bodies at low concentrations (*i.e.*, a few ng/L). Many TrOCs are ineffectively removed by the conventional wastewater treatment processes (Hai et al., 2014; Luo et al., 2014) and pose a threat to aquatic ecosystems and to the safety of drinking water resources.

The ability of laccase to degrade TrOCs has been shown for several subgroups such as industrial chemicals (Gros et al., 2014), anti-inflammatory drugs (Tran et al., 2010), antibiotics (Auriol et al., 2007; Weng et al., 2012), personal care products *e.g.*, UV filters (Garcia et al., 2011) and pesticides (Majeau et al., 2010). The oxidative efficiency of laccases depends on the redox potential difference between the reducing substrate and type 1 copper in laccase. Given the range of redox potentials that laccases from different fungi possess (0.17–0.80 V) (Cañas and Camarero, 2010), non-phenolic substrates are often not amenable to direct oxidation by laccase. The chemical structure of the substrate is also an important factor influencing its degradation by laccase. Particularly, the distribution of functional groups within the TrOC has a large influence on the efficiency of the oxidation process by laccase (Yang et al., 2013). There are two categories of functional groups: i) electron donating functional groups (EDGs) including hydroxyl and amine that are strong activating groups, meaning that compounds containing these groups are more susceptible to electrophilic attack; and ii) electron withdrawing functional groups (EWGs) such as nitro, halogen, carboxyl and amide groups that are strong deactivating groups making the oxidation of these compounds much slower and more complicated (Yang et al., 2013). Some TrOCs may not be directly oxidized by laccase. These include bulky compounds that cannot access the laccase-active site and those with a high redox potential. This limitation can be overcome by adding a small molecular weight redox-mediating substrate of laccase whereby highly reactive radicals generated due to oxidation of the mediator by laccase

can in turn degrade the target compound of interest. This mechanism is analogous to the natural lignocellulose biodegradation by white- and brown- rot fungi, which produce reactive oxygen species (*e.g.*, hydroxyl, peroxyl and hydroperoxyl radicals) to initiate the biodegradation of biopolymers found in wood (Hammel et al., 2002).

Three major mechanisms by which a mediator can oxidize a substrate have been reported in the literature: hydrogen atom transfer, electron transfer and ionic mechanisms (Astolfi et al., 2005). Mediators differ from each other in terms of optimal reaction conditions and in specificity towards a given target compound (Baiocco et al., 2003). Previous studies have focused on performance comparison of different laccase – mediator combinations for degradation of dyes (Khlifi et al., 2010; Mendoza et al., 2011) and TrOCs (Garcia et al., 2011; Jeon et al., 2008). However, critical aspects such as laccase stability and the treated effluent toxicity are often overlooked or not comprehensively covered. A study with such a focus would help identify the type and dose of mediators that improve TrOC removal while minimizing effluent toxicity and laccase inactivation.

This study aims to compare the effectiveness of seven selected redox-mediators, representing three different oxidative mechanisms, for enhancing the oxidation of four resistant TrOCs by laccase. The performance of laccase with different mediators was systematically compared particularly focusing on laccase stability, TrOC removal efficiency, and effluent toxicity to pinpoint the best mediator. A series of batch tests with crude laccase preparation from white-rot fungi *Pleurotus ostreatus* were used to evaluate the impact of mediator concentrations, types and reaction times.

2. Materials and methods

2.1 Crude laccase preparation

Erlenmeyer flasks (250 mL) containing 50 mL of malt extract at a concentration of 5 g L^{-1} were inoculated with the white-rot fungus *P. ostreatus* (ATCC 34675). The pH of the solution was adjusted to 4.5 and the culture incubated on a rotary shaker at 70 rpm and 28 °C for one week. The fungus secreted extracellular laccase into the media and this crude enzyme extract was separated from the biomass before storing in sterilized bottles at 4 °C.

2.2 Trace organic contaminants and mediators

Four TrOCs namely oxybenzone, pentachlorophenol, atrazine and naproxen were selected based on their widespread occurrence in water and wastewater and their resistance to degradation by laccase in previous studies (Nguyen et al., 2014b; Yang et al., 2013). These compounds were selected to facilitate a systematic investigation of the effect of mediator addition. The physicochemical properties of these compounds are summarized in Supplementary Data Table S1.

A range of mediators, including 1-hydroxybenzotriazole (HBT), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), syringaldehyde (SA), (2,2,6,6-Tetramethylpiperidin-1-yl)oxy (TEMPO), Violuric acid (VA), Vanillin (VAN) and N-Hydroxyphthalimide (HPI) were used to compare the relative efficiency of mediators at aiding the removal of target contaminants. As shown in Table 1, these mediators follow three different mechanisms: hydrogen atom transfer (SA, HBT, VA, VAN and HPI), electron transfer (ABTS) and ionic mechanism (TEMPO).

[TABLE 1]

2.3 Experimental protocol

For the initial screening of the mediators, crude laccase extract (5 mL) with an initial activity of 52 ± 2 (*n=2*) $\mu M_{(DMP)}$ min^{-1} was added to 10 mL test tubes. Working solution, comprising the four TrOCs, was added to the test tubes containing crude enzyme to yield a final concentration of 500 μg L^{-1} of each compound. Selected mediators were added to the solutions separately to produce final mediator concentrations of 1 mM. Controls included TrOC solution in Milli-Q water and TrOC solution and enzyme solution without mediator. Tests were conducted in duplicate. Test tubes were sealed and incubated on a rotary shaker at 70 rpm and 25 °C for 24 h.

The three mediators that exhibited the greatest TrOC removal in the mediator screening experiments (*i.e.*, ABTS, VA and HBT) were selected for assessment of impact of incubation time (2, 4, 8 and 24 h) and mediator concentration (0.05, 0.1, 0.25, 0.5 and 1 mM). These tests were conducted in the same way as described above.

2.4 Analytical methods

Laccase activity was assayed by recording the change in absorbance (468 nm) due to oxidation of 2,6-dimethoxyl phenol (DMP) in the presence of sodium citrate (pH 4.5). Enzymatic activity was calculated using a molar extinction coefficient of 49.6 $(mM\ cm)^{-1}$ and expressed in $\mu M_{(DMP)}$ min^{-1}. The redox potential of the laccase solution before and after mediator addition was measured utilizing an oxidation-reduction potential meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia). Toxicity of untreated and treated media was analysed in duplicate by measuring inhibition of luminescence of naturally luminescent bacterium *Photobacterium leiognathi*, and expressed as relative Toxic Unit (rTU), the reciprocal of the IC_{20} value of the inhibition of luminescence *vs*. concentration curve (van de Merwe and Leusch, 2015). The concentration of the four TrOCs utilized in this study (*i.e.*, pentachlorophenol, oxybenzone, naproxen and atrazine) was measured by an HPLC–UV–vis detector system (Shimadzu, Japan) following a previously reported method (Nguyen et al., 2014a).

3. Results and discussion

3.1 Removal of TrOCs by laccase

Substrate degradation by laccase is limited by two factors, principally, the availability of strong EDG within its structure, and the redox potential of that particular type of laccase. Also, steric shielding from EWG may prevent electron abstraction from occurring. Hence, this section explains enzymatic degradation of the selected TrOCs based on their molecular structures and the presence of EDGs and EWGs (Yang et al., 2013). Results show low laccase-catalyzed degradation (15-23%) of the four TrOCs (Figure 1).

Among the tested TrOCs, oxybenzone was removed with the highest efficiency (23 ± 2%, (*n=2*)). Oxybenzone contains two EDGs, namely, methoxy and hydroxyl groups (Supplementary Data Table S1). However, the relatively low removal rates may be explained by the presence of carbonyl (an EWG) in its structure. Pentachlorophenol was also poorly removed by laccase (23 ± 8 %, (*n=2*)). The presence of five chlorine atoms in its ring creates a highly stable compound that is resistant to degradation by laccase. Even though pentachlorophenol contains the strong EDG hydroxyl, the steric effects of the chlorine groups prevent the hydroxyl group from gaining access to the active sites of the enzyme (Hai et al., 2011; Yang et al., 2013). Our observation regarding the low removal of oxybenzone and pentachlorophenol is in line with literature. For

example, Garcia et al. (2011) reported that laccase from *Trametes versicolor* could not oxidize oxybenzone. Jeon et al. (2008) observed no removal of pentachlorophenol by laccase derived from *Ganoderma lucidum*. In a contrasting study by Ullah et al. (2000), 60% removal of pentachlorophenol was observed by laccase from *Coriolus versicolor*. Ullah et al. (2000) did not report the redox potential of the laccase that they used, thus it could not be confirmed whether the laccase from *P. ostreatus* as used in this study (0.28 V) had a lower redox potential. However, it is likely that the difference in redox potential between the substrate and the specific laccase used was responsible for different levels of pentachlorophenol degradation.

The pesticide, atrazine, was also poorly removed, showing only 15 ± 6 %, ($n=2$) degradation. Even though atrazine contains methyl and amine EDGs, the presence of the strong EWG chlorine hinders laccase-catalyzed degradation (Nguyen et al., 2014a). The low removal of naproxen (15 ± 4 %) can be attributed to the presence of the EWG carboxyl, and the absence of any strong EDGs. The ether group present in this compound is a weak EDG, which makes naproxen a highly stable compound, able to resist enzymatic oxidation. For example, Marco-Urrea et al. (2010) also observed less than 10% removal of naproxen by laccase of *T.versicolor*.

Overall, the low removal of the tested TrOCs necessitates the addition of mediators in order to enhance their degradation. The enzyme activity remained virtually unchanged throughout the course of the experiment which demonstrates that these four contaminants do not have an adverse effect on the stability of the enzyme at the concentrations tested.

3.2 Mediator screening

Redox mediators can act as an electron shuttle between laccase and the substrate in order to overcome steric hindrances and kinetic limitations of laccase. This mechanism involves the oxidation of the mediator compounds by laccase, which results in the production of radical species that possess higher oxidative capacity towards the substrate than the laccase itself. There are a number of variables that influence this process including molecular structure of the contaminant, mediator type and concentration, and the redox potential of the laccase. The type of mediator and its mechanism of action is known to have a significant impact on TrOC degradation (Kurniawati and Nicell, 2007). As noted in Section 2.2, there are three recognized degradation mechanisms, namely, hydrogen atom transfer, electron transfer and ionic mechanism. The mediators SA, HBT, HPI, VAN, and VA have all been shown to use hydrogen

atom transfer, whilst ABTS and TEMPO follow the electron transfer and ionic mechanisms, respectively.

Following mediator addition, oxybenzone and pentachlorophenol experienced the highest removal efficiencies, most probably because both these TrOCs are phenolic compounds. In comparison, atrazine and naproxen (which are both non-phenolics) experienced slightly lower removal rates (Figure 1) when mediators were added.

High oxybenzone removal (>70%) was achieved by three mediators, specifically, HBT, VA and ABTS. Among these, VA and ABTS were able to achieve almost complete removal of oxybenzone (98% and 96%, respectively). The high laccase-catalyzed degradation of oxybenzone due to ABTS addition agrees well with the findings of a study by Garcia et al. (2011) who, however, used laccase derived from *T. versicolor*. Notably, both VA and HBT reduce to aminoxyl radicals whereas ABTS reduces to $ABTS^{+\bullet}$ radicals. The distinct radicals produced by the laccase-catalyzed oxidation of ABTS and VA appear to have an important influence on the degradation of oxybenzone.

Pentachlorophenol was removed by around 75% by the aminoxyl radicals produced via oxidation of the mediators HBT and VA by laccase. However, the best removal of pentachlorophenol (83%) was achieved with ABTS. The observed high degradation of pentachlorophenol by laccase due to mediator dosing is in agreement with the findings of another study by Nguyen et al. (2014b), who, however, studied only HBT and used laccase from *T. versicolor*.

Atrazine degradation was best achieved with the addition of VA, which increased the removal efficiency from 24 to 73% upon mediator addition. In the case of atrazine, VA was discernibly far better than any other mediator. SA, HBT, VAN and ABTS were also capable of improving the degradation of atrazine, however, removal efficiencies ranged from only 38-50% when these mediators were added. Particularly, although HBT, HPI and VA are all N-OH type mediators, higher removal yields were achieved by the laccase -VA system. These results suggest that the affinity between the aminoxyl radicals from VA and atrazine play an important role in its degradation. The presence of different substituent groups in HBT, HPI and VA may have influenced the chemical properties of generated radicals (*e.g.*, polarity), consequently contributing to the difference in removal efficiency (Baiocco et al., 2003; d'Acunzo and Galli, 2003).

ABTS achieved the best removal (77%) of naproxen. This was closely followed by VA, which removed 63% of the initial concentration of naproxen. The N-OH type mediators, HBT and HPI as well as the methoxyphenol mediator, VAN, were all able to improve the efficiency of naproxen degradation by laccase, however, the removal rates were still very low (36-39%). The $ABTS^{+\bullet}$ radicals showed the greatest naproxen degradation potential, possibly due to the different mechanism of oxidation (*i.e.*, electron transfer). Laccase-mediated naproxen degradation has been studied previously by Lloret et al. (2013), who also observed that ABTS was the most efficient mediator compared to the N-OH type mediators HBT and SA. It is noted that the reactivity of mediators towards substrates in the laccase - mediator system varies depending on the functional groups in the substrates. For example, laccase-ABTS system is effective on benzyl alcohols but not on benzylic ether structures (Baiocco et al., 2003). On the other hand, HBT and VA are more efficient in degrading methyl veratryl ether (Camarero et al., 2005).

The initial screening of the mediators showed that the increase in TrOC degradation varied between mediator type, and that there was no distinct broad-spectrum mediator. Of the seven mediators tested, HBT, VA and ABTS achieved the highest removal of TrOCs.

[FIGURE 1]

In absence of any mediators, no loss in laccase activity was observed during the 24 h period of incubation. However, mediator addition to laccase solution caused some loss of laccase activity, and the degree of inhibition was different for different mediators (Figure 2). Interestingly, when TEMPO, HPI, SA or VAN was added separately, low removals of TrOCs were achieved, but the loss of laccase activity was also negligible (between 5-10 %). On the other hand, a significant loss in laccase activity was observed due to the addition of VA, HBT or ABTS, which in fact outperformed the other mediators in terms of TrOC degradation. The results from this study are in line with the study by Fillat et al. (2010) where HBT showed the highest laccase inactivation compared with the methoxyphenol type mediators, namely, SA, acetosyringone, P-coumaric acid and methyl syringate. Garcia et al. (2011) achieved 90% removal of oxybenzone by laccase - ABTS system with 64% loss of laccase activity, whereas laccase - P-coumaric acid system yielded 5% removal of oxybenzone with insignificant denaturation of laccase.

Increase in the redox potential of the reaction mixture is thought to be one of the reasons for the enhanced TrOC degradation by laccase-mediator systems. In this study, a mediator-specific increase in the redox potential of the laccase solution was observed after mediator addition (Figure 2). Among the N-OH type mediators HBT, HPI and VA, the highest increase in redox potential was achieved by VA, followed by HBT and HPI. This could explain the higher removal yields by laccase -VA system as noted earlier. However, it is noteworthy that improvement in TrOC removal did not always correlate with increase in redox potential. For example, SA and VA produced similar levels of redox potential increase, but VA achieved much better removal of TrOCs. Results here demonstrate that for an efficient laccase-mediator system, the redox potential is a contributing but not the sole factor. Overall, the mediators HBT, VA and ABTS that showed the greatest enhancement of TrOC degradation were used in subsequent investigations to further evaluate their effectiveness at different concentrations and reaction times.

[FIGURE 2]

3.3 TrOC removal by laccase – mediator system

3.3.1 Effect of mediator concentrations

Figure 3 depicts the relationship between mediator concentration and TrOC removal. It can be seen that TrOC removal increased with mediator concentration up until a threshold value, beyond which no significant enhancement in TrOC degradation occurred. The literature shows that this threshold value is dependent on factors such as mediator type, contaminant type and source of enzyme. For example, by increasing the concentration of HBT from 0.2 to 2 mM, Mizuno et al. (2009) achieved gradual increase in iso-butylparaben and n-butylparaben removal, but no further improvement was observed beyond 2 mM. By contrast, Lloret et al. (2010) obtained 40-80% removal of diclofenac with SA concentrations ranging from 0.1 to 0.5 mM, while complete removal was achieved at 1 mM.

In this study, oxybenzone was completely removed with the addition of either VA or ABTS at 1 mM. At the same dose of VA or ABTS, a slightly lower degradation (*i.e.*, maximum 80%) was achieved for the other phenolic compound pentachlorophenol. VA and ABTS were significantly

more effective than HBT at enhancing oxybenzone degradation regardless of concentration (Figure 3). On the other hand, the N-OH type mediators (*i.e.*, VA and HBT) were less effective at lower concentrations for pentachlorophenol degradation when compared to ABTS. However, at higher concentrations, all of the mediators performed similarly.

Atrazine was the most recalcitrant compound tested, with no clear relationship between mediator concentration and removal efficiency (Figure 3). This can be attributed to its molecular structure containing substituted isopropyl amine, ethylamine and chlorine groups. The arrangement of these groups around the triazine ring makes it highly resistant to biodegradation (Pereira et al., 2013). VA achieved the highest removal of atrazine (73%), while interestingly ABTS showed the lowest enhancement. Results here suggest that the radical species generated from VA have a much greater potential to overcome steric hindrances and solubility factors associated with these functional groups. HBT achieved the maximum removal efficiency (*i.e.*, 61%) at 0.1 mM, after which a decline was observed. This relationship is remarkably similar to that of VA which also happens to be an N-OH type mediator. The results may suggest that high removal efficiencies can be achieved at a comparatively lower mediator concentration when N-OH mediators are used to degrade atrazine.

HBT achieved the greatest naproxen removal of 80% at the highest mediator concentration (1 mM) used. In comparison, the performance of VA (another N-OH type mediator), which showed the highest removal of all other TrOCs, was lower for naproxen. HBT and ABTS were both very efficient even at an intermediate dose of 0.5 mM (72 and 75% removal, respectively).

Overall the radicals produced by the laccase-catalysed oxidation of ABTS appear to have a greater capacity to degrade oxybenzone, pentachlorophenol and naproxen, whereas degradation of atrazine seems to be conducted more efficiently by the aminoxyl radicals produced by the oxidation of VA. The oxidation mechanism of the ABTS radicals follow an electron transfer pathway (Table 1), which has been shown to be effective at oxidising high redox potential substrates (Kurniawati and Nicell, 2007).

It is noted that Figure 3 is based on data derived from duplicate experiments. A more rigorous statistical comparison would require a larger set of data. However, the available data indeed show the dependence of TrOC removal on mediator concentration, and therefore it could be used to point in the direction of a suitable mediator concentration in further testing.

The mediator type and concentration has a major impact on the practicality and feasibility of using laccase in TrOC degradation. The high cost of mediator compounds restrict the maximum dosage to an economically suitable concentration, thus, a trade-off between lower removal of TrOCs and lower concentration of mediator may be appropriate. Also, the toxicity of the effluent (see Section 3.4) due to mediator addition influences whether the mediator can be used in practical applications.

[FIGURE 3]

3.3.2 Effect of incubation time

A series of tests with incubation period varying from 2 – 24 h showed that TrOC degradation was rapid, with the majority of degradation occurring within the initial 2 h for all mediators (Figure 4). Degradation of TrOCs began to slow down over the next 6 hours, after which, degradation virtually ceased, and only minor improvements were observed in atrazine and pentachlorophenol removal. The rapid degradation at the beginning of the experiment indicates that the mediator was being rapidly oxidised by laccase, which led to a fast production of radical species. The cease of degradation within 8 h indicates that either laccase or the mediator had been affected. The literature suggests that the free radicals produced by the oxidation of mediator are able to destabilise the enzyme by reacting with the aromatic amino residues on the outer surfaces of the enzyme (Khlifi et al., 2010; Kurniawati and Nicell, 2007). Indeed the rapid degradation of contaminants coincided with a large drop in enzyme activity, indicating that the radical species may have been destabilizing the enzyme molecules (Figure 5). These results are in line with the study by Lloret et al. (2013) who also observed enzyme deactivation correlating with accelerated degradation rates of contaminants, diclofenac and naproxen, during the initial hours of the experiment. This aspect is further discussed in the next section.

[FIGURE 4]

3.3.3 Effect of mediator type and concentration on laccase stability

Enzyme activity was highly impacted by mediator addition (Figure 5). The N-OH type mediators HBT and VA both appeared to be detrimental to enzyme activity at all concentrations tested, although VA destabilised the enzyme faster than HBT. For example, the loss of laccase activity was 71% and *ca*. 100% for HBT and VA, respectively at 0.25 mM. The enzyme activity

reduction was lower for ABTS. Similarly, Lloret et al. (2013) observed that ABTS caused the least enzyme inactivation when ABTS, HBT and VA were compared for their enhancement in removal of naproxen and diclofenac. The difference in the rate of inactivation can be linked to the relative stability of the mediator radicals. Other studies have shown that enzyme stability also depends on the species of fungi used. For example, the mediator SA has been reported to deactivate the laccase from *Tramettes trogii* (Khlifi et al., 2010), while stabilising laccase from *Pycnoporus cinnabarinus* (Fillat et al., 2010).

As noted in Section 3.2.2, in accordance with the rapid TrOC removal, in this study laccase inactivation also occurred at a very early stage. Figure 5 shows that HBT, VA and ABTS destabilized laccase by 38, 66 and 70%, respectively within 2 h of incubation. This demonstrates that once radicals were generated from oxidation of mediators by laccase, they degraded TrOCs, but at the same time caused laccase deactivation. Although enzyme stability was greatly affected by the mediators, it was compensated by their rapid degradation capacity, eventually achieving significant TrOC degradation. However, the aspect of enzyme inactivation may be particularly important for establishing a continuous treatment process where long-term stability of laccase-mediator system is required. Thus, the selection of mediator type and concentration is important for the removal of TrOCs by laccase - mediator systems.

[FIGURE 5]

3.4 Effluent toxicity

[TABLE 2]

In this study, the laccase - mediator systems were assessed for their capacity to enhance TrOC removal efficiency. However, the overall quality of the treated effluent (*i.e.*, toxicity) is also important. A few recent studies have suggested that the radicals formed due to the oxidation of mediators can interact with the vitally important biomolecules and cause toxicity (Kim and Nicell, 2006; Nguyen et al., 2014b). Thus it is essential to evaluate the toxicity of the reaction mixture as it undergoes treatment. Of the three mediators screened based on their high TrOC degradation capacity, the N-OH type mediators *i.e.*, HBT and VA decreased the toxicity of the treated effluent, whereas ABTS increased. Compared to the toxicity levels of 3.4 ± 1.4 (*n=2*) and 4.3 rTu for separate solutions of laccase and the TrOCs, respectively, the toxicity of the treated effluent following HBT and VA addition was 2.1 ± 0.3 and 3.3 ± 2.1 rTU (*n=2*), respectively.

This suggests that treatment by HBT and VA did not produce significant amounts of toxic-by products and also that the N-OH type radicals generated caused negligible toxicity. This particular observation regarding the N-OH type mediators is consistent with that in a previous study (Nguyen et al., 2015), where performance of one of the N-OH type mediators used in this study *i.e.*, HBT was compared with SA. Notably, HBT and SA are structurally different (*i.e.*, N-OH type *vs*. methoxyphenol type), but both follow the hydrogen atom transfer mechanism to oxidize substrates (Table 1). Despite being a natural mediator, SA showed higher toxicity over the synthetic mediator HBT in the previous study (Nguyen et al., 2015). The results from the current study provides a critical clue to selection of mediators by revealing that another N-OH type natural mediator VA, which also follows the hydrogen atom transfer mechanism, enhances TrOC degradation without increasing effluent toxicity. The fact that VA is a natural mediator increases the practical significance of the current findings in terms of formulating an environmentally friendly and effective, yet economical process.

4. Conclusion

The results here confirm that the substrate spectrum of laccase can be broadened by the addition of redox-mediators, however, the performance of laccase - mediator systems is mediator dependent. Of the seven mediators investigated, HBT, VA and ABTS achieved the highest TrOC removal efficiencies. In particular, ABTS and VA achieved the highest degradation of the phenolic compounds, whereas the non-phenolic compounds were best removed using VA or HBT. This implies that the non-phenolic compounds are more efficiently removed by the N-OH type mediators (*i.e.*, VA and HBT), while removal of the phenolic compounds may depend more on the stability of the radicals and their redox potential. Laccase stability was greatly affected by mediator addition, with VA causing the greatest inactivation, followed by HBT and ABTS. This study also showed that higher concentrations of mediator (HBT and VA) result in faster destabilisation of enzyme, thus smaller doses of mediator are favoured. The benefit of using VA is, however, manifested in the fact that this natural mediator can improve the degradation of both phenolic and non-phenolic TrOCs and does not increase the toxicity of the treated solution - a concern which generally impedes the application of laccase – mediator systems.

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499

LIST OF FIGURES

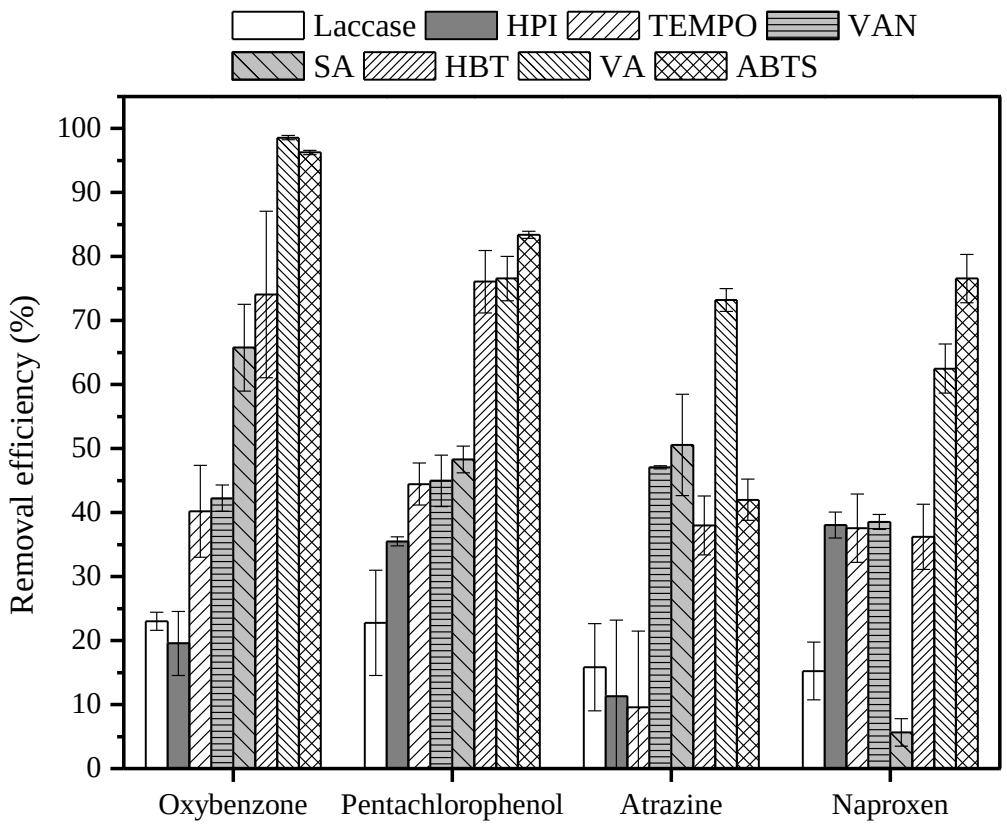


Figure 1: Removal efficiency (%) of selected TrOCs after 24 h treatment with laccase and mediators. Mediators were added separately at 1 mM concentration. The error bars represent the standard deviation of duplicate samples. HPI= N-hydroxyphthalimide; TEMPO=(2,2,6,6-tetramethylpiperidin-1-yl)oxy; VAN=vanillin; SA=syringaldehyde; VA=violuric acid; HBT=1-hydroxybenzotriazole (HBT); and ABTS=2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.

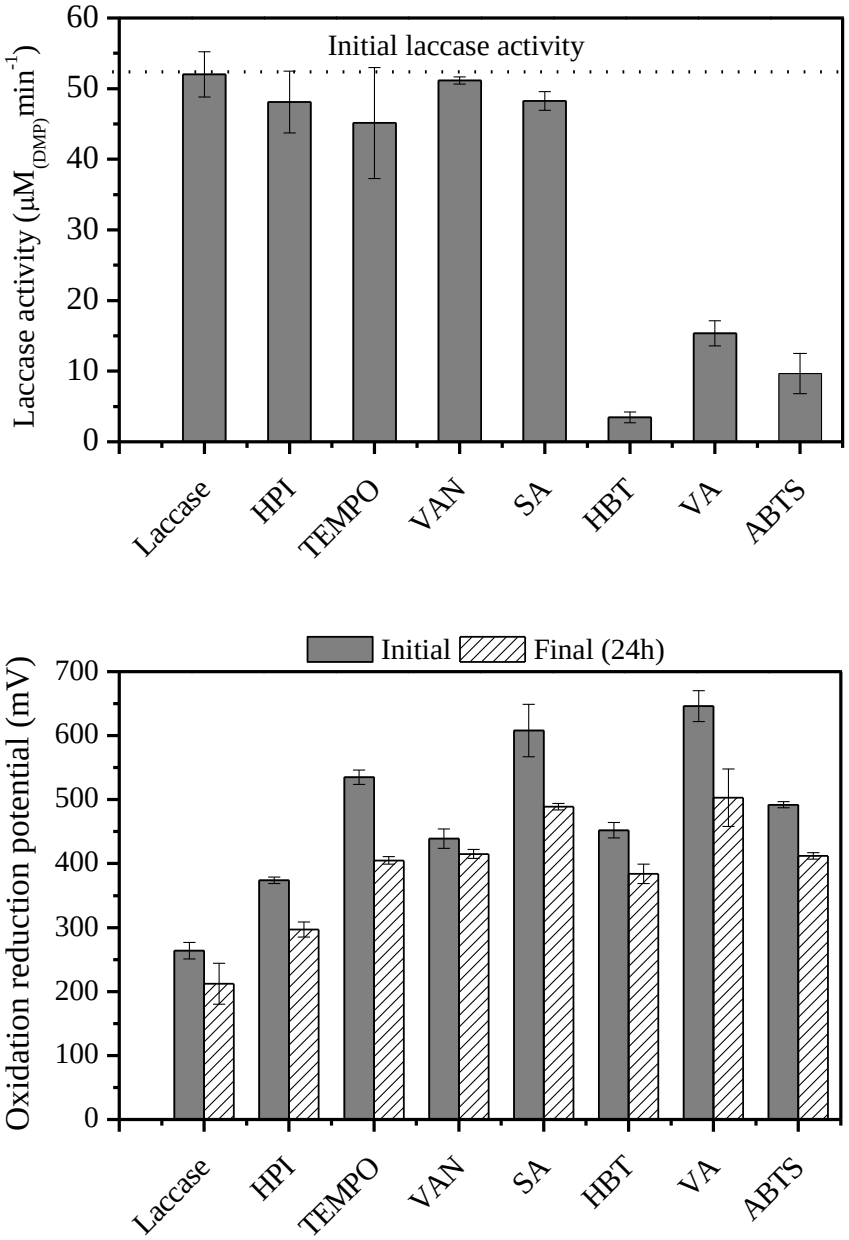


Figure 2: Impact of mediator addition on the oxidation reduction (redox) potential and the laccase activity. Mediators were added separately at 1 mM concentration. The error bars represent the standard deviation of duplicate samples.

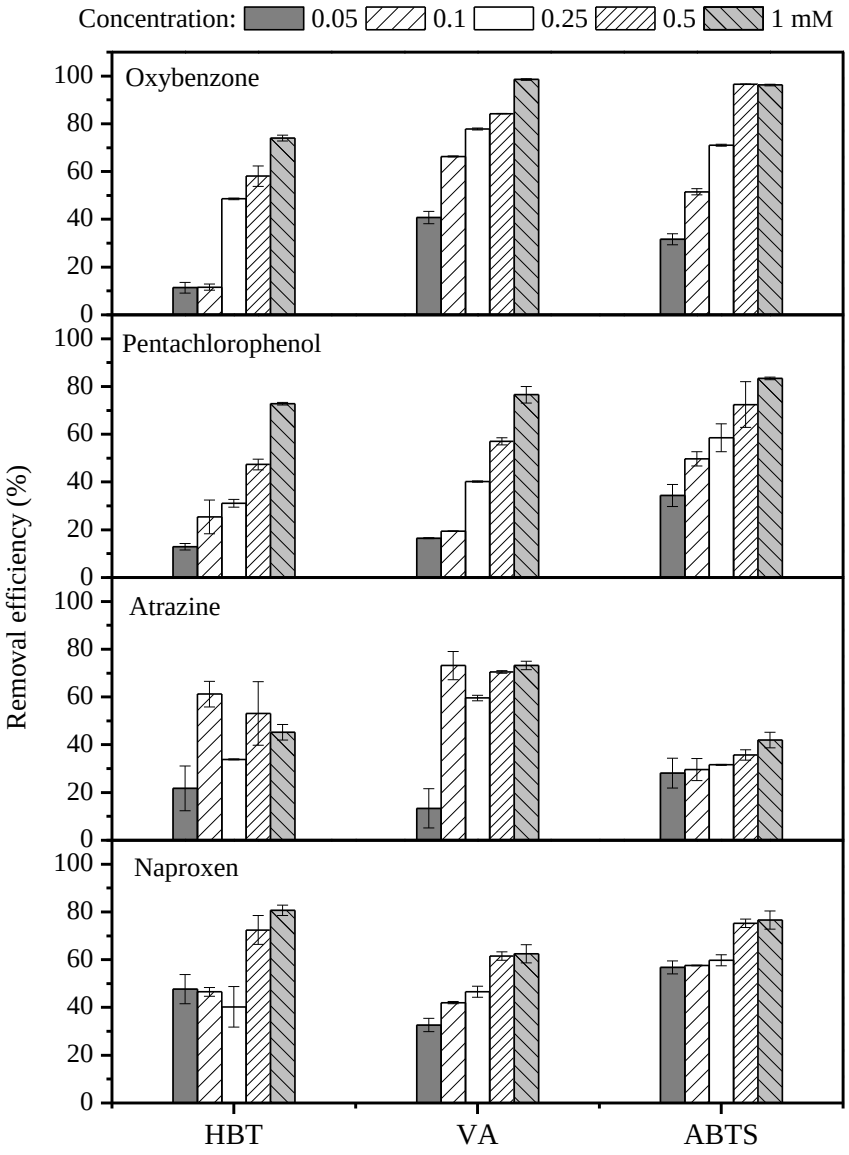


Figure 3: Effect of mediator concentrations on the TrOC removal. The error bars represent the standard deviation of duplicate samples.

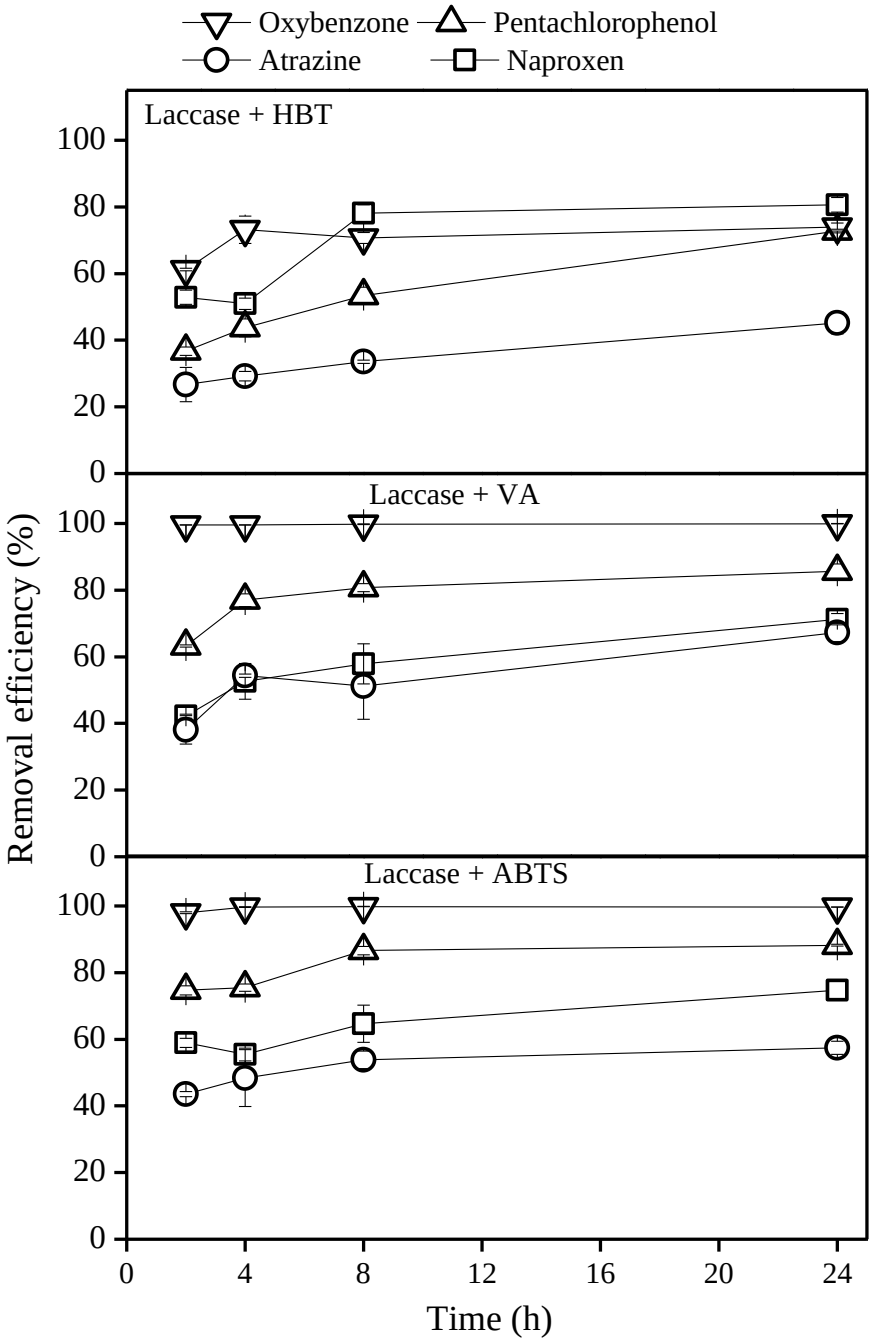


Figure 4: Impact of reaction time on TrOC removal efficiency of laccase and mediators. The error bars represent standard deviation of duplicate samples.

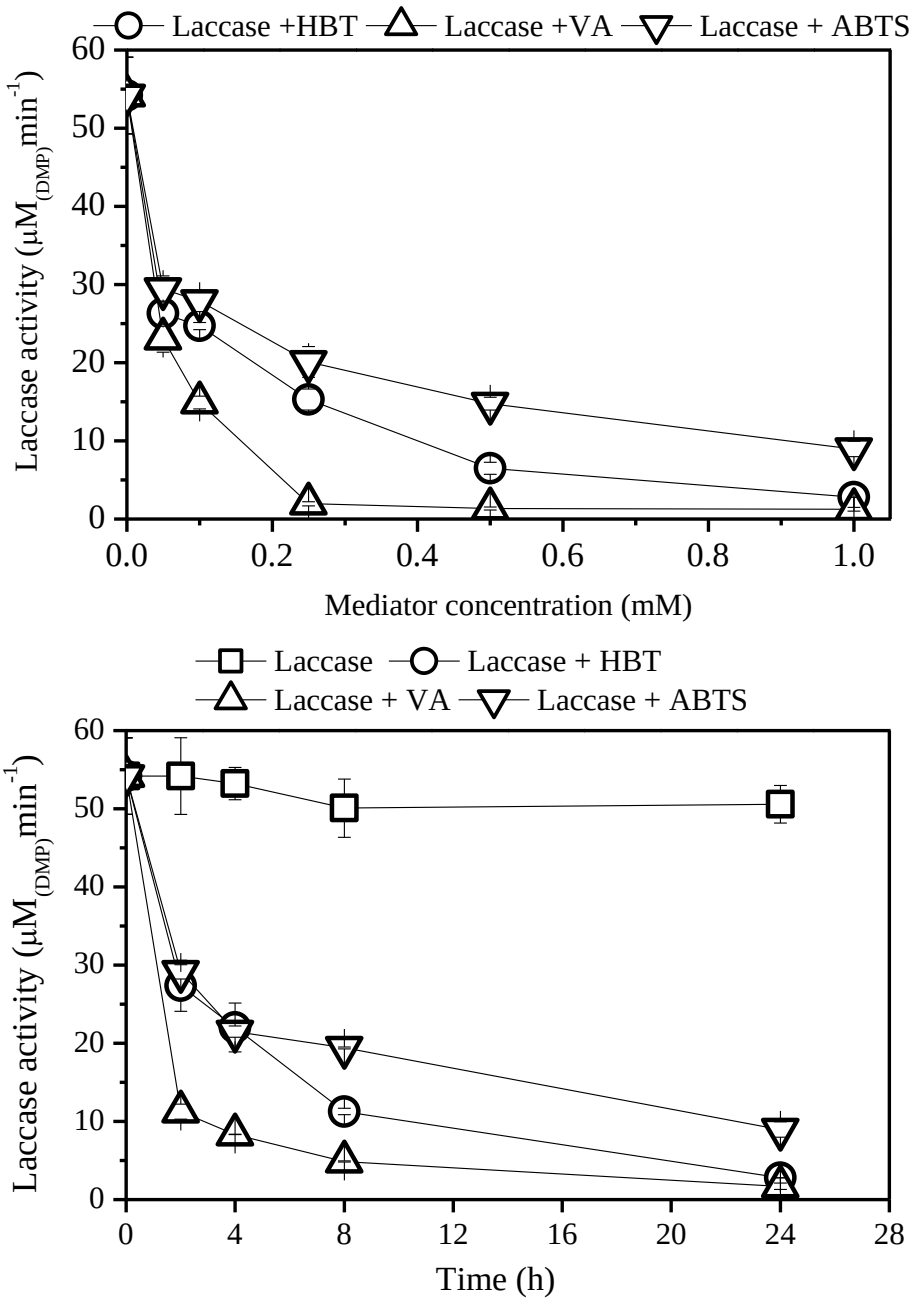
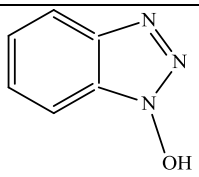
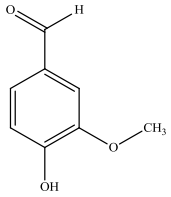
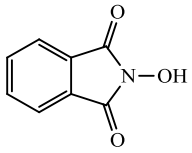
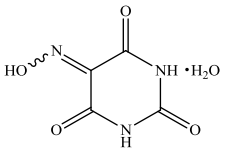
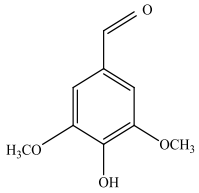
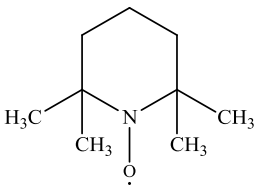
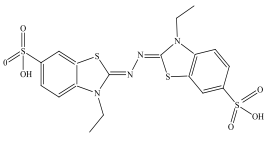


Figure 5: Effect of reaction time and mediator concentrations on the laccase stability. The error bars represent the standard deviation of duplicate samples.

LIST OF TABLES

Table 1: Physicochemical properties of the redox-mediators

Redox mediator	Type of mediator	Free radical generated	Oxidation mechanism	Natural/synthetic	Chemical structure
1-hydroxybenzotriazole (HBT)	N-OH	=N-O· Aminoxyl	HAT	Synthetic	
Vanillin (VAN)	$C_6H_4(OH)(OCH_3)$	C_6H_5O· Phenoxyl	HAT	Synthetic	
N-hydroxyphthalimide (HPI)	N-OH	=N-O· Aminoxyl	HAT	Synthetic	
Violuric acid (VA)	N-OH	=N-O· Aminoxyl	HAT	Natural	
Syringaldehyde (SA)	$C_6H_4(OH)(OCH_3)$	C_6H_5O· Phenoxyl	HAT	Natural	
2,2,6,6-tetramethylpiperidinyloxyl (TEMPO)	N-O·	N=O Oxoammonium	Ionic	Synthetic	
2,2-azino-nis(3-ethylbenzothiazoline-6-sulfnoic acid (ABTS)	ABTS	ABTS+• ABTS++	ET	Synthetic	

HAT = hydrogen atom transfer, ET = electron transfer and, Ionic = Ionic oxidation

Table 2: Comparison of toxicity following laccase treatment of TrOCs with different mediators. Mediators were added separately at a concentration of 1 mM. The limit of detection of the toxicity assay was 10% inhibition of luminescence (*i.e.*, 1 rTU).

Reaction mixture	**Toxicity (rTU) (*n* =2)**
Laccase	3.4 ± 1.4
TrOCs	4.3
TrOCs + Laccase + ABTS	8.8 ± 5.2
TrOCs + Laccase + HBT	2.1 ± 0.3
TrOCs + Laccase + VA	3.3 ± 2.1

Impacts of redox-mediator type on laccase degradation of trace organic contaminants: Degradation efficiency, laccase stability and effluent toxicity

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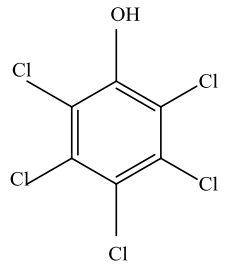
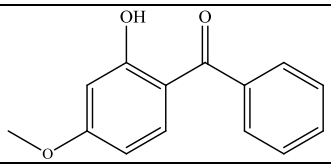
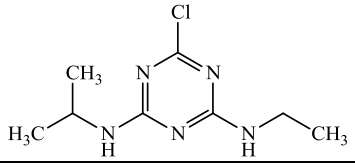
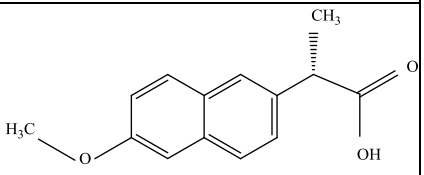
Table S1: Physicochemical properties of the selected micropollutants

Compound (formula) (CASRN)	Molecular weight (g/mol)	Functional groups		Chemical structure
		EDG	EWG	
Pentachlorophenol (C_6HCl_5O) (87-86-5)	266.34	Hydroxyl	Chlorine	
Oxybenzone ($C_{14}H_{12}O_3$) (131-57-7)	228.24	Hydroxyl	Carbonyl	
Atrazine ($C_8H_{14}ClN_5$) (1912-24-9)	215.68	Methyl and amine	Chlorine	
Naproxen ($C_{14}H_{14}O_3$) (22204-53-1)	230.26		Carboxyl	

[a] Source: SciFinder database: https://origin-scifinder.cas.org