EMERGING CHALLENGES IN LANDFILL LEACHATE QUALITY AND ABILITY OF FUNGAL BIOREACTORS TO ADDRESS THEM

by

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ABSTRACT

AMIRHOSSEIN REZAEI ADARYANI. Emerging challenges in landfill leachate quality and ability of fungal bioreactors to address them. (Under the direction of DR. OLYA KEEN)

Landfill sites, that are the ultimate depository of solid waste stream in most societies, have the potential to generate leachate containing a complex mixture of contaminants of emerging concern (CECs) such as pharmaceutical compounds, plasticizers, and endocrine disrupting compounds, which can have negative effects on human and ecosystem health. More than 60% of the landfills in the U.S. discharge leachate to publicly owned treatment works (POTW), i.e. municipal wastewater treatment plants, because of its convenience and low cost. Apart from CECs, UV absorbing substances (UVAS) can cause major disruption to the effectiveness of wastewater treatment processes and often require pretreatment of leachate prior to discharge to POTW. Literature suggests that white-rot fungi are able to degrade both types of contaminants.

This work investigated the removal of a selection of CECs and UVAS in real landfill leachate with non-sterile aerobic bench scale reactors utilizing *Phanerochaete chrysosporium* in pellet form. Batch and column plug flow reactor were used in this study, and finally treated LL was evaluated for biotoxicity with activated sludge respiration inhibition test. Meanwhile, the leachate from four landfills with various closure status was tested for occurrence of CECs.

The study found that phenol, a regulated compound in LL, was consistently removed in both batch and plug flow reactors. Acetaminophen was removed significantly as well. Fungal reactors were not efficient in reducing UV absorbance while they were

effective in removal of carbon content up to about 40%. Landfill leachate characteristics in water quality parameters and CECs occurrence were variable among landfills of different closure status. While differences in some instances were statistically significant, they were not sufficient to justify different treatment approach to leachate based on closure status.

DEDICATION

I would like to dedicate this to the loving memory of my father. I would also like to dedicate this to my mother and sister, whose unconditional love and support enabled me to chase my dreams.

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CHAPTER 1: INTRODUCTION

Urbanization and wastewater effluent disposal to surface water cause prevalent contamination of freshwater resources with contaminants of emerging concern (CECs), such as pharmaceutical compounds (PhCs), plasticizers, and endocrine disrupting compounds (EDC). EDCs at certain doses can interrupt the endocrine system in mammals. These interferences can cause cancers, birth defects, and reproductive and developmental abnormalities. These chemicals survive municipal wastewater treatment and environmental processes post-discharge due to persistency. Occurrence of many classes of PhCs including antibiotics, diuretics, antihypertensives, anticonvulsants, and antidepressants are reported in source water.[1,2] It is established that CECs have negative effects on human and ecosystem health.[3] Occurrence of antibiotics at environmentally relevant concentrations can make variant functional shifts in composition of aquatic system's bacterial community. In a recent comprehensive national assessment by the Environmental Protection Agency (EPA) of 25 drinking water treatment plants, 148 compounds, most of which were CECs, were detected in the raw water at least once, and 121 were detected at least once in the finished drinking water. Some CECs get partially removed during treatment and hence are present in distributed potable water, even though at ng/L level, which causes sub lethal, long-term exposure, the effects of which are currently not well understood.[4–6]

The ultimate depository of assorted waste mixture from domestic, commercial, and industrial sources are landfills. Landfill sites have the potential to generate leachate containing a complex mixture of CECs. More than 60% of the landfills in the U.S. discharge leachate to publicly owned treatment works (POTW) because of its

convenience and low cost.[7–9] Landfill leachate can comprise various organics such as pharmaceuticals, personal care products, and other CECs as well as antibiotic resistant bacteria, due to the composition of landfill waste.[9–14] It is known that discharge of LL to POTWs for treatment, contributes to the load of CECs including prescription and non-prescription PhCs, although a recent study on three pairs of landfills and POTWs receiving LL with two POTW not accepting LL showed no apparent difference in their effluents.[14] Such studies are still limited and more research is needed to understand the indirect effects of LL on environment.

Since landfill leachate (LL) contains very diverse compounds like CECs and UV absorbing substances (UVAS), it requires proper treatment prior of discharge to POTW.[15,16] A survey of landfills (n=258) showed that about 29% have treatment (e.g., separation of solids, aeration, etc.) preceding discharge to POTWs, whereas 22% of the landfills were barred from disposing to POTWs for reasons like flow, chemical oxygen demand (COD), ammonia and sodium concentration. For removal of biodegradable organics from LL, conventional biological treatment is suitable and is often used. However, recalcitrant organic portions will remain. Generally, electrochemical, chemical, and physical treatments are more efficient for recalcitrant organics than biological treatments, but also more costly.[8,15,17] UVAS can block the UV disinfection process at POTWs that receive leachate. This could adversely affect the acceptance of LL in POTWs for treatment.[18] Therefore, on-site biological pretreatment of LL that could reduce the load of organics including BOD, UVAS and nutrients like ammonia on POTWs, while also addressing CECs, would be ideal.

1.1.Recalcitrant compounds in landfill leachate

A large number of CECs such as PhCs, phenols, flame retardants, etc., occur in US municipal LL with over 400 compounds in fresh LL.[9] It has been shown that many of these compounds have negative health and/or environmental impacts (e.g. endocrine disruption).[10] Initial studies put focus on toxic organic chemicals in LL including BTEX (benzene, toluene, ethylbenzene and xylenes), chlorinated hydrocarbons (e.g. trichloroethylene), polycyclic aromatic hydrocarbons (e.g. naphthalene) and organochlorine pesticides(e.g. dichlorobenzene).[19,20]

1.1.1.Phenols

A very important group of aromatic contaminants is phenols and derivatives. Phenol is a very common compound used as a disinfectant in various products. In various studies it was found frequently in LL at up to 1 mg/L.[9,16] In addition, phenol is among the organic compounds regulated for landfill effluent at 15 µg/L as monthly average for non-hazardous waste landfills.

Bisphenol A (BPA) is a phenolic compound used in production of most polycarbonate plastics, the majority of epoxy resins and thermal papers. Other bisphenols like bisphenol S (BPS) or bisphenol F (BPF) are alternatives to BPA in consumer products. [21,22] BPA has been frequently detected at high concentration of up to 6-17 mg/L in LL in the US.[9,16] Bisphenol A diglycidyl ether (BADGE), as a constituent of epoxy resins is another compound in this group.[23]

Another phenolic compound that is widely present in consumer products but is found less frequently in LL is the antimicrobial disinfectant, triclosan, detected at concentrations as high as 42.3 µg/L.[9]

The ubiquity of phenols and their derivatives, their toxicity at trace levels and more stringent environmental regulations in the future make it essential to develop processes for their removal from LL. Table 1 summarizes phenolic compound occurrence in LL.

Table 1.Phenols occurrence in fresh LL, United States

Phenolic compound	Range (μg/L)	Reference
Phenol	Maximum: 1,550; Median: 92.3	[9]
	Maximum:1,190; Median: 98.5	[16]
	0.6-1,200	[24]
BPA	Maximum: 6,380; Median:45.4	[9]
	Maximum: 17,200; Median: 45.4	[16]
	200-240	[24]
	516	[14]
4,4′-Bisphenol F	1280	[14]
Triclosan	Maximum: 42.3; Median: 8.9	[9]

1.1.2.Pharmaceuticals

Occurrence of numerous classes of PhCs and their degradates, both non-prescription and prescription, including antibiotics, antihistamines, analgesics, stimulants, diuretics, antihypertensives, anticonvulsants, steroid hormones, antidepressants etc. were detected in LL nationwide at measures ~10-10,000 ng/L. Among others, ibuprofen, lidocaine, amphetamine, carbamazepine, and carisoprodol were the ones found most frequently in LL.[9,10,16]

The exposure to mixtures of low concentrations of organic contaminants (<1000 ng/L), including CECs and many other organic compounds, has raised concerns because a variety of possible effects is likely even when the presence of each compound

at low concentrations is believed not to have an individual effect [25,26]. Table 2 condenses PhCs occurrence in LL.

Table 2.Pharmaceuticals occurrence in fresh LL, United States

Pharmaceutical	Range (ng/L)	Primary chemical use	Reference
17β-Estradiol	Maximum: 11; Median:7.18	Natural estrogen	9
Acetaminophen	Maximum: 333,000; Median:21,800	Analgesic	9
	Maximum: 42,600; Median:5,300		16
	Average: 7000; Maximum:64,000		10
	<400		14
Atenolol	<400	Beta-blocker	14
Metformin	<400	Antidiabetics	14
Ibuprofen	Maximum:705,000; Median:325,000	Analgesic	9
	Average:123,000; Maximum:256,000		10
Carbamazepine	Maximum:810; Median: 165	Anticonvulsant	16
	345		14
Lidocaine	Maximum:147,000; Median: 11,700	Local anesthetic	9
	Maximum: 47,900; Median: 5,080		16
	25,400		14
Amphetamine	Maximum: 7,320; Median: 424	Psychostimulant	9
	Maximum: 11,900; Median: 714		16
Carisoprodol	Maximum: 3,400; Median: 348	Muscle relaxant	9
	Maximum: 3,060; Median: 322		16
Trimethoprim	<400	Antibiotic	14

1.1.3.UV absorbing substances

The UVAS of leachate are comprised of hydrophobic humic substances (humic acids, HA, and fulvic acids, FA) and hydrophilic fraction. The formation of hydrophilic aliphatic carbons is by the breakdown of organic solid wastes in a landfill. Humic substances (HS), however, are formed by complex biological and chemical reactions through a humification process. The abundant presence of lignin in green waste, paper and paperboard, serves as a precursor for humic substances in landfills. Structurally, HS are heterogeneous macromolecules, with yellow to black hue, and are intrinsically acidic. Heterogeneity of HS in leachates are mainly known to be due to their progressive alteration over time. Leachate's high UV absorbance is attributed to different aromatic moieties (e.g., phenolic, benzoic, etc.) which absorb UV light.[15,18]

Recalcitrant organic matter is also challenging due to its high color, and its role in transport of hydrophobic organic contaminants (e.g. CECs) and heavy metals. When LL is co-treated at POTW, at volumetric contributions of LL ≥0.1%, transmittance at 254 nm (T_{254}) in wastewater effluents can fall below 65%, that is a typical guideline for effective UV disinfection. The recalcitrant (hydrophobic) fraction of dissolved organic nitrogen (DON) is highly colored and is positively correlated to T_{254} and COD of LL.[7] A number of studies show that LL could have substantial impacts on wastewater quality at rather low volumetric contribution through reduction in T_{254} and a rise in wastewater apparent color, DON, and dissolved organic carbon (DOC), which can interfere with UV disinfection.[7,18,27]

1.2.White-rot fungi

White-rot fungi (WRF) include both Ascomycota and Basidiomycota (eukaryotic). They typically grow as filaments (hyphae) and are common in nature, particularly abundant in forest ecosystems. WRF are heterotrophs that can secrete enzymes at the hyphal tips that allow them to decompose complex polymers into simple, soluble nutrients, which can then be absorbed through the wall and cell membrane. WRF are very efficient in their nitrogen use, and their oxidative enzymes are useful for bioremediation of contaminated matrix.[28,29] WRF growth is governed by various physical, chemical and biological parameters. Nutrients, water, air, temperature, pH, light, and the force of gravity comprise the physical/chemical influences. Any substrate available to a fungal specie can also carry other fungi and bacteria, all compete for space, nutrients, air, and water. In nature the individual factors do not work secluded, but instead can have synergistic or antagonistic effects.[30]

1.2.1.Enzymatic system of white-rot fungi

The most significant characteristic of WRF is their ability to degrade lignin in lignocellulosic substrates (e.g. wood) completely: WRFs have the strongest lignin modifying enzymes (LME) among other rare organisms identified to do this. Enzymes are protein substances that modify reaction activation energy and/or reaction rate without being present in the products of the reaction. The major LMEs are lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, versatile peroxidases, and other H_2O_2-generating oxidases. The LME system of WRF is extracellular, non-selective with very low specificity that makes it able to cleave the carbon-oxygen and carbon-carbon

bonds.[28,31,32] The potential of LME can be harvested and utilized to degrade persistent phenolic organic contaminants in LL.

It is remarkable, however, to recognize that lignin oxidation does not afford net energy gain to WRF, that translates to lignin not being a substrate in primary metabolism. It is decomposed during secondary metabolism to make access to wood polysaccharides that are sealed in hemicelluloses and cellulose (lignin-carbohydrate complexes), providing in this way a source of energy. This feature elucidates the main difference between bacteria and fungi when degrading xenobiotics. While recalcitrant pollutants usually are utilized as a nutritional carbon and/or nitrogen source for bacteria, often times an extra carbon and nitrogen source is necessary for primary metabolism of WRF. This cometabolism process is central to fungal remediation of contaminated matrices like LL.[33]

1.3.Fungal remediation of contaminants of emerging concern

Studies on the utilization of WRF and their enzymes for bioremediation of recalcitrant contaminants started over thirty years ago with demonstration that *Phanerochaete chrysosporium (PC)* degrades dioxins, polychlorinated biphenyls, and other chloroorganics.[34] A large literature body shows that WRF are among the most versatile of microbes in their ability to decompose organic pollutant with *PC* being the most extensively studied of the ligninolytic white-rot fungi.[33,34] Studies report biodegradation of various CECs such as 4-n-nonylphenol, bisphenol A, and 17a-ethinylestradiol by eight ligninolytic fungal strains (e.g. *Phanerochaete chrysosporium ME 446, Trametes versicolor 167/93* etc.). Some of these fungi degrade the contaminants successfully to below detection limit within a few days of cultivation.[35] The aerobic degradation of

carbamazepine by *Trametes versicolor* at near environmentally relevant concentrations of 50 µg/L was reported as 61% of the contaminant after 7 days.[36] WRF *Pleurotus ostreatus* was demonstrated to almost completely remove and transform lamotrigine (anticonvulsant) in liquid cultures at environmentally relevant concentrations (1 and 10 µg/L) within 20 days.[37] *Trametes versicolor* in a rotating biological contactor was used for treatment of wastewater spiked with 12 PhCs at 50 µg/L with 24 h of hydraulic retention time. Sulfamethoxazole, atenolol, caffeine, ibuprofen and sulpiride were eliminated with removal efficiencies ranging from 80-95%.[38] *PC* was evaluated in the elimination of tetracycline with 72.5% removal in 4 h.[39] In another study, metoprolol and metoprolol acid (i.e. metabolite) that are known to be recalcitrant to biodegradation in hospital wastewater were treated utilizing three fungi species, *Ganoderma lucidum, Trametes versicolor* and *Pleurotus ostreatus*. *Ganoderma lucidum* eliminated metoprolol up to 51% and its metabolite ~ 77%.[40] The WRF are suitable for degradation of antibiotics, because these compounds have no inhibitory effects on fungi. In fact, fungi were utilized for production of antibiotics like penicillin.[41]

The use of purified enzymes provides better control of the process, and higher rates of conversion of the target compound are possible. Several examples showed effectiveness at high rates for transformation after short reaction times of 10–60 min with single enzymes .[39,42] LiP was shown to remove a variety of persistent aromatic compounds comprising polycyclic aromatic and phenolic compounds. MnP that was isolated from *PC* has been observed to catalyze the oxidation of some monoaromatic phenols and aromatic dyes.[43] Fungal enzyme laccase was studied in batch tests using crude enzyme extracts for over 30 CECs, 13 of which, such as carbamazepine, ibuprofen, naproxen etc.,

degraded significantly. Some other molecules showed low or negligible degradation. The variation of enzymatic degradation efficiencies was attributed to the differences in chemical structure of the selected CECs.[44] The enzymes secreted from WRF can be applied for degradation of recalcitrant anticancer drugs.[45] Immobilization improved enzyme stability under environmental and denaturing conditions in the elimination of acetaminophen and naproxen.[39]

1.3.1.Fungal remediation of landfill leachate

To the best knowledge of the author, there are about 30 studies to date assessing remediation of LL via various fungal species, both attached or free form growth, and crude/filtered enzymes. All these studies measured water quality parameters over treatment time, and/or toxicity.[46] To the best knowledge of the author, no study applied WRF for remediation of CECs in LL. There are very limited studies concerning UVAS removal in LL[46], which shows the knowledge gap and the significance of this study. The following paragraph summarizes the results from the few general studies of LL treatment with WRF or its enzymes.

Abdullah et al. studied treatment of raw LL by WRF by immobilized mycelia of *Ganoderma austral* packed in a column, which removed up to 51% of COD and achieved 31% reduction in ammonia nitrogen in diluted leachate (50%).[47] Another study with the same WRF compared the use of free mycelia and immobilized mycelia for the removal of landfill leachate organics.[48] Free mycelia were able to remove leachate BOD_5 but not COD. Meanwhile after 4 weeks of treatment immobilized *Ganoderma austral* displayed 93.09% and 17.84% percentage removal of BOD_5 and COD, respectively. Next study used decolorization percentage as the measure of efficiency for 10 different fungal

species.[49] The best decolorization was achieved by *P. spadiceum MUT 1585, P. boydii MUT 721* and *MUT 1269* for wastewater effluent, as the raw LL did not allow active fungal growth. *Bjerkandera adusta MUT 2295* was utilized in a LL treatment, with co-substrate of glucose and cellulose, by which 63% COD removal achieved.[50]

1.3.2. Limitations of CECs degradation of by WRF

Fungal based systems for wastewater (or LL) treatment are not being applied commonly at industrial scale.[51] This could be due to some reasons as follows:

1) Nutrient addition requirement: Most experiments used malt extract or glucose-based spiked media (synthetic wastewater) and only a few studies could be found with real wastewater. The nutrient addition need in real wastewater treatments by WRF was recognized only after using real wastewater. Cruz-Morató et al. emphasized the need of glucose and ammonium tartrate addition for maintaining pelleted *T. versicolor* biological activity and enzymatic production in a fluidized bed bioreactor.[52,53]
2) Autochthonous microorganisms' competition: CECs removal has declined in some studies largely due to bacterial contamination and it has been identified as the main constraint of this system.[54–56] It is been demonstrated that bacteria impose substrate competitive pressure and this leads to the loss of fungal biomass, and destabilization of fungal enzymes.[57]
3) Necessity of high hydraulic retention times (HRT): Due to loss of extracellular enzymes and time-consuming degradation of some pollutants, fungal treatments usually require high HRT ranging 1–3 days. For comparison, HRT of conventional activated sludge process is a few hours.[44,58]

Some measures can be taken to overcome abovementioned constraints. First, we can favor fungal growth against rivals through operation of bioreactor at optimal pH of WRF (<5), partial renovating of the biomass, lowering carbon-to-nitrogen ratio to benefit fungal growth over bacterial growth, and lastly by immobilization of fungal mass. Second, by auto-immobilization of WRF, typically in the form of pellets, bacteria could get washed out.[51]

1.4.Bioassay-based toxicity tests for landfill leachate

Toxicity assessment of LL, both treated and untreated, is essential to monitor the impact of leachate discharges on the aquatic environment. Different trophic levels might be assessed as toxic compounds affect both producers and consumers present in aquatic environments.[59] A bioassay approach is valuable as it integrates the biological effects of all present compounds, taking into account aspects such as bioavailability, antagonism, or synergism. Thus, use of bioassays as screening tools to characterize contaminants in a range of environmental matrices like LLs has become a popular and powerful tool.[60] Diverse in vivo and in vitro methods are available to perform the toxicity assessments. Bacteria (*Vibrio fischeri*), microalgae (*Pseudokirchneriella subcapitata*), invertebrates (*Daphnia magna, Artemia salina*), fish (*Carassius auratus*), plants (*Vicia faba, Hordeum vulgare*) and mammalian cells (human peripheral blood *lymphocytes*) representing different trophic levels as test organisms have been selected by a number of researchers to evaluate the toxicity of LL through bioassays.[60–63]

1.4.1.Activated sludge respiration inhibition test

Respiration inhibition test using activated sludge have been successfully utilized in water sector to assess the toxicity of industrial influent or as an ecological effects test.[64]

The standard methods and results interpretation are published by "Organisation for Economic Co-operation and Development", OECD 209 (2010)[65], and "United States Environmental Protection Agency", EPA OCCSPP 850.3300 (2012)[64] among other institutions. The method basically weighs the test substance (e.g. LL) effect on microorganisms via respiration rate measurement under defined settings in contact with multiple concentrations of the test substance up to 3 hours. This method is cost effective and quick, with no more than basic instrument requirements, such as dissolved oxygen probe.[65] This method has not been reported for biotoxicity assay of LL[59] to the best knowledge of the author.

Previous works suggest the capability of WRF to degrade some of recalcitrant phenolic CECs in non-sterile fungal reactors which could be superior to largely bacterial processes. The present study investigated the removal of a selection of CECs and UVAS in real landfill leachate with non-sterile aerobic bench scale reactors utilizing *Phanerochaete chrysosporium* in pellet form. This investigation was performed to show the viability of fungal processes for up-scaling through the following experiments: Investigate the ability of PC to grow in undiluted and minimally augmented landfill leachate, and determine its operational requirements.

1) Determine the effectiveness of PC to degrade target contaminants (CECs and UVAS) in real landfill leachate in batch and plug-flow reactor configuration.
2) Compare the effectiveness of fungal processes to conventional aerobic and anaerobic bacterial processes for biodegradation of select biorecalcitrant compounds, and determine the effect of readily biodegradable carbon on the process.

3) Measure the effect of fungal treatment on biotoxicity of leachate.
4) Determine whether a landfill closure status affects the levels of CECs in leachate.

CHAPTER 2: MATERIALS AND METHODS

2.1.Fungal cultures

In this study two WRFs were used, *Ceriporiopsis Subvermispora (CS)* and *Phanerochaete chrysosporium (PC)*. *CS* was obtained from the research lab of Dr. Matthew Parrow from the UNC Charlotte Biology Department, and *PC* was purchased from ATCC® (24725™). These WRF were selected due to their potential to cultivate in LL and biodegrade the selected contaminants based on literature review. As shown in Section 3.1, the performance of *PC* outcompeted *CS* and therefore *CS* was dropped from further study at the initial stage. Biosafety Level One guidelines were followed while working with these fungi.

2.1.1.Solid medium

All the fungi in the present study were stored at room temperature on Difco™ yeast mold (YM) Agar:

- 3 g/L yeast extract
- 3 g/L malt extract
- 5 g/L peptone
- 10 g/L dextrose
- 20 g/L agar

Sub-cultures were made every two months and were used for inoculums of liquid cultures. These subcultures were either inoculated by transferring from an agar plate a small part of fungal growth with a loop or by transferring a 50 µL droplet of sterile water

contacted with previous pure culture agar plate and placing it in the center of a new agar plate.

2.1.2.Liquid medium

The liquid medium consisted of the same components of solid medium without the agar.

2.2.Inoculum preparation (auto-immobilization)

A mycelial suspension was made by placing four 1 cm diameter plugs from the pure culture fungus growing zone on agar plates into sterile YM broth in a 500 mL Erlenmeyer flask. This was incubated at 30 °C at constant agitation (135 rpm, r = 25 mm) for 4–5 days until a dense mycelial mass formed. Then, the mycelial mass was separated with a fine sieve from the culture medium, resuspended in an equal volume of a sterile saline solution (0.8% (w/v) NaCl) and then homogenized by the manual homogenizer at top speed for two minutes. Fungal pellets were obtained by inoculating 300 mL of YM broth with 40 mL of the mycelial suspension in a 1 L flask. The resulting pellet size was approximately 2 ± 1 mm in 3-4 days with the same incubation as in the previous step. After the pellet growth, the medium was withdrawn to use the fungal pellets in batch and plug flow bioreactors.

2.3.Growth of fungal strains

2.3.1.Maximum specific growth rate (μ_{max})

To evaluate the maximum specific growth rate of *PC,* the growth of mycelium in unlimited substrate (i.e. YM broth) conditions used. In triplicate, one milliliter of mycelial suspension was inoculated into half-full 125 mL flasks and incubated an

ambient temperature with constant agitation (135 rpm, r = 25 mm) for 10 days. For mass evaluation at the designed times (0, 1, 2, 3, 5 and 10 days), the content of each flask was filtered through GF/F filters and dried for a day at 60 ºC. Monod equation[66] was used to calculate $\mu_{max}(d^{-1})$:

$$\mu = \mu_{max}\left(\frac{S}{K_S+S}\right) \quad \text{Equation 1}$$

where:

- μ is the specific growth rate of the microorganism (d^{-1})
- μ_{max} is the maximum specific growth rate of the microorganism (d^{-1})
- S is the limiting concentration of the substrate for growth (mg/g)
- K_S is the half-velocity constant (mg/g)

The specific growth rate (μ) in its turn calculated as:

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \text{Equation 2}$$

where:

- X is the microorganism's concentration (mg/L)
- dX/dt is the growth rate

When the unlimited substrate is available ($S>>K_S$), μ_{max} would be equal to μ; after rearranging and integration, it leads to:

$$\mu_{max}.t = \ln\frac{X}{X_0} \quad \text{Equation 3}$$

Thereby, μ_{max} can be calculated as the slope of $\ln(X/X_0)$ against time.

2.3.2.Fungal growth in landfill leachate

Fungal growth rate data in contact with LL was obtained for both fungal strains, in duplicates at all steps. A 5 ×5 mm piece was taken from the edge of fungal mass from colonized YM broth. Then, it was placed in the center of an agar plate (multiple plates) with different compositions as detailed below. After inoculation, plates were sealed with

parafilm wrapping film and incubated at 27 ºC, 37 ºC or room temperature (20±2 ºC). Next, the colony radius of filamentous growth was measured daily to get the fungal growth rate for each fungal species in different conditions, until it covered the plate fully (D=85 mm) or stopped growing, Figure 1. The experiments were performed in three stages using the following types of plates:

- Type I: YM agar incorporated with 10% to 100% LL
- Type II: Agar with 100% LL
- Type III: Agar with 100% LL and 0.05-0.4% $MnSO_4$

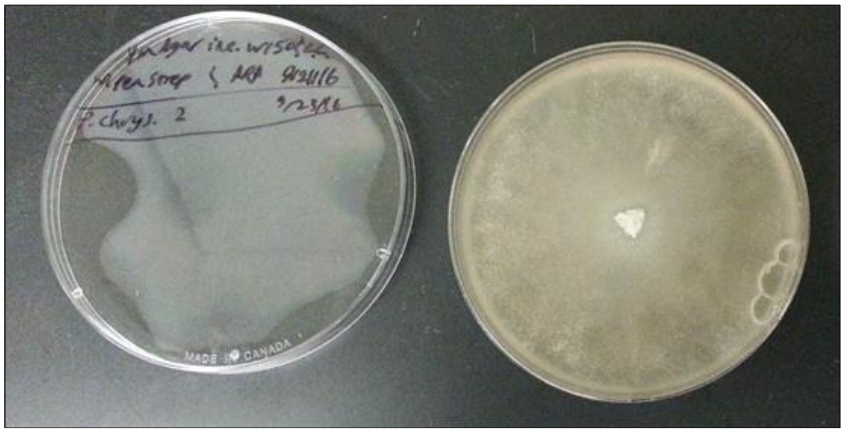


Figure 1. *PC* has covered a YM agar plate incorporated with LL

In order to block bacterial growth, Penicillin-Streptomycin (Gibco by Life Technologies, Grand Island, NY) was used at final concentration of 100 U/mL Penicillin and 100 µg/mL Streptomycin. This antibiotic was added to hot agar (~60 ºC) before pouring the plates. Also, the aliquots of this antibiotic were stored in separate 15 mL tubes at -20 ºC for each batch of agar preparation.

2.4.Landfill leachate

The raw LL used in this study for WRF treatment was obtained from a mixed cell landfill in North Carolina. Mixed cell landfills are defined by collection of LL from both

active and closed cell in a single sump (mixing). Leachate samples were stored in polyethylene containers refrigerated at 4 °C and filtered through glass fiber filter before use in reactors.

The fresh raw LL for PhCs detection study was collected from four other landfill sites in four consecutive seasons (2019-20) in North Carolina with various operation and closure status, Table 3. The LL from these cells were also used for absorbance reduction study.

Table 3. Landfill sites

Site	Description
1	Mixed cell
2	Active cell Closed cell, <5 years Closed cell, >10 years
3	Mixed cell
4	Active cell Closed cell, <5 years

2.5.Enzyme Assay

WRF produce one or more LMEs, but the most studied LMEs are LiP, MnP, and laccase. The LiP enzyme of *PC* was assayed using the dye Azur B method at various conditions with LL (10-100% LL). This assay is based on the oxidation of 32 µM Azure B dye by LiP enzyme in the presence of hydrogen peroxide, and absorbance measurement at 651 nm.[67] The concentration of H_2O_2 was measured using a modified triiodide method.[68] In this modified method volumes are scaled down for a 1.25 mL cuvette with water already incorporated into the adapted solutions A' and B'. This customized method measures H_2O_2 at concentrations up to 13 mg/L. Solution A', in 1 L of ultrapure water:

- 36.67 g potassium iodide

- 1.11 g sodium hydroxide
- 0.11 g ammonium molybdate tetrahydrate

Solution B' is 11.11 g potassium hydrogen phthalate (aka KHP) in 1 L of ultrapure water.

The final mixture is 0.563 mL of solution A', 0.563 mL of solution B' and 0.125 mL of sample added to the cuvette and mixed thoroughly.

MnP activity was directly measured by 2,6-Dimethoxyphenol (2,6-DMP) assay via observing the formation of Mn^{3+} tartarate complex during oxidation of 0.1 mM $MnSO_4$. The oxidation of 2,6-DMP is monitored using spectrophotometer at absorbance of 469 nm.[69]

2.6.High-performance liquid chromatography detection

This method was used for analysis of target contaminants in fungal bioreactor experiments where the analytes were added at sufficient concentrations to be detectable. The selected analytes (n=12) for this method were BPA, BPS, BPF, BADGE, phenol, triclosan, 17α-ethinylestradiol (EE2), ibuprofen, acetaminophen, doxycycline, trimethoprim and ciprofloxacin.

An analysis method has been set up for selected contaminants on Agilent 1100 High-performance liquid chromatography (HPLC) coupled to UV-Vis diode array detection (DAD) (Agilent Technologies, Santa Clara, CA, USA). The columns were Zorbax Eclipse plus C8 4.6x50mm with 5 µm particle and Poroshell 120 EC-C8, 4.6x50mm with 2.7 µm particle size, both by Agilent. The separation method used 0.1% formic acid as solvent A and acetonitrile as solvent B. The 20 min method had linear gradient, from

95%A:5%B to 100%B at 1 mL/min flow with 40-µL injection volume with the column held at 30 °C.

2.6.1.Size exclusion chromatography

An analysis method has been set up for size exclusion chromatography on Agilent 1100 HPLC using PL aquagel-OH 20, 7.5 x 300 mm column, with 8 µm particle size and molecular weight (MW) range 100–20,000 Da. The eluent flow rate was 0.4 mL/min with recipe as follows:

- 0.024 M sodium dihydrogen phosphate
- 0.0016 M sodium monohydrogen phosphate
- 0.025 M sodium sulphate

in ultrapure water.[18] The method duration was 45 min with the column held at 30 °C. Three polystyrene sulfonic acid sodium salt standards were used for calibration with mean MW of 32000, 17000 and 4300 Da (Sigma-Aldrich, St. Louis, MO, USA). The diode array detector of HPLC instrument was set at 254 nm and 400 nm. All the samples for HPLC methods were filtered by 0.45 µm nylon filter prior to injection.

2.7.Solid-Phase Extraction for examination of pharmaceuticals in leachate

Solid-phase extraction (SPE) was used for sample preparation in order to analyze for compounds of interest in a complex matrix like LL at trace levels (i.e. using mass spectrometry). Agilent BondElut ENV, PPL and Plexa 500 mg cartridges (Agilent Technologies, Santa Clara, CA), and Oasis hydrophilic-lipophilic-balanced (HLB) 500 mg cartridge (Waters Corporation, Milford, MA) for acidic, basic and neutral compounds were used for extracting the pharmaceuticals from leachate samples. All these cartridges

contain reversed-phase sorbent for a wide variety of compounds. Spiking with both internal and external standards (Table 4) at 1 μg/L (100 μL of 10 mg/L) in 100 mL of LL was necessary after the samples reached room temperature. The 100 mL sample was diluted by a factor of 10 -to 1L- with ultrapure water to prevent overloading of the media with humic compounds and losing analytes.

Prior to SPE, samples were sequentially filtered by GF/F filter (Whatman, Piscataway, NJ), 0.45 μm and 0.22 μm nylon membranes (Agilent Technologies, Santa Clara, CA) using a standard vacuum flask filtration setup. All the glassware and GF/F filters were heat treated at 450 °C for four hours. The glassware was acid bathed overnight in 10% nitric acid and rinsed thoroughly by ultrapure water before baking. All other apparatuses that could not be cleaned in a furnace (e.g. filter funnel) were rinsed by 50 mL of methanol followed by two steps rinsing with ultrapure water.[70] Each nylon filter was prewashed with 500 mL ultrapure water to eliminate the release of nylon fibers into the sample.[71] Before extraction, 0.021 g of EDTA-Na_2 was added to filtered sample as a chelating agent.[72] Then, 6 N hydrochloric acid was added to adjust the pH of samples to ~3 at slow pace using 0.2 mL aliquots of acid while mixing slowly by magnet stirrer to reduce the (possible) foaming in a 1 L flask. The added acid volume was recorded to account for dilution. Whenever any more precipitant formed upon acidification, the sample was filtered through GF/F filter once more. The filtered samples were then extracted at 1 L with above mentioned SPE cartridges, SPE manifold and lines. The cartridges were washed with 5 mL HPLC-grade methanol followed by conditioning with 5 mL of HPLC-grade water, then the samples were loaded at approximately 5 mL/min.[73] Once the entire sample has passed through the cartridge, the cartridge was washed with

10 mL of HPLC grade water to remove the EDTA.[74] To remove any residual water, the cartridges were kept under 10 psi vacuum for 5 minutes. The adsorbed analytes then were eluted into disposable glass tubes using 2.5 mL of methanol and 2.5 mL of acetonitrile in sequence under gravity. To collect all the remaining solvent from cartridge, vacuum was applied for one more minute. After elution the extracts were evaporated under high purity grade nitrogen gas to reduce the volume to under 0.2 mL and then reconstituted to 1 mL using 20% acetonitrile, then kept in -20 °C.[6] Weight of each glass tube was recorded before and after elution for accurate reconstitution. The extract was filtered again with a prewashed 0.45 µm nylon membrane syringe filter (Agilent Technologies, Santa Clara, CA) and 0.2 µm cellulose acetate syringe filter (Advantec, Dublin, California). Right before analysis a 1:2 dilution of the extracted sample was prepared with 5% acetonitrile solution. The variation in LL quality can be seen in Figure 2.

Figure 2. Loading of LL samples on SPE cartridges

2.7.1.Solid-Phase Extraction for examination of biphenolic compounds in leachate

Agilent BondElut ENV 500 mg cartridge, and Oasis HLB 500 mg cartridges were used for extracting the phenolic compounds from leachate samples. The samples were

brought to room temperature and spiked with bisphenol standards at four calibration levels of 100, 10, 1 and 0.1 μg/L, plus 1 μg/L of Carbamazepine-D_{10} (Sigma-Aldrich, St. Louis, MO) as internal standard in 100 mL of LL. The rest of method was the same as described in Section 2.7.

2.7.2.Selected CECs for detection in leachate

The physicochemical properties of selected analytes in the study (n=13) along with three deuterated standards, are provided in Table 4.

Table 4. Physicochemical properties of analytes for detection in leachate [Reference: (https://pubchem.ncbi.nlm.nih.gov) and (https://comptox.epa.gov/dashboard)]

Compound	Chemical Formula	Molecular weight (g/mol)	pK_a	Solvent	Log P
17α-ethinyl estradiol (EE2)	$C_{18}H_{24}O_2$	296.41	10.33	Ethanol	3.67
Acetaminophen	$C_8H_9NO_2$	151.16	9.38	Methanol	0.46
Amoxicillin	$C_{16}H_{19}N_3O_5S$	365.4	3.2, 11.7	Methanol	0.87
Atenolol	$C_{14}H_{22}N_2O_3$	266.34	9.6	Methanol	0.16
Azithromycin	$C_{38}H_{72}N_2O_{12}$	748.99	8.74	Ethanol	4.02
Carbamazepine	$C_{15}H_{12}N_2O$	236.27	13.9	Ethanol	2.45
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.34	6.09, 8.74	Methanol	0.28
Doxycycline	$C_{22}H_{24}N_2O_8$	444.44	3.4, 7.7, 9.5	Methanol	-0.02
Gemfibrozil	$C_{15}H_{22}O_3$	250.33	4.5	Ethanol	3.4
Ibuprofen	$C_{13}H_{18}O_2$	206.28	4.91	Ethanol	3.97
Metformin	$C_4H_{11}N_5$	129.16	12.4	Methanol	-1.37
Ranitidine	$C_{13}H_{22}N_4O_3S$	314.4	2.3, 8.2	Water	0.27
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28	1.6, 5.7	Ethanol	0.89
Trimethoprim	$C_{14}H_{18}N_4O_3$	290.32	7.12	Methanol	0.91
Bisphenol A	$C_{15}H_{16}O_2$	228.29	9.6	Ethanol	3.32

Bisphenol S	$C_{12}H_{10}O_4S$	250.27	8.2	Ethanol	1.65
Triclosan	$C_{12}H_7Cl_3O_2$	289.54	7.9	Methanol	4.76
Carbamazepine-D10*	$C_{15}D_{10}H_2N_2O$	246.335	-	Methanol	-
Ibuprofen-D3*	$C_{13}H_{15}D_3O_2$	209.3	-	Methanol	-
Ciprofloxacin-D8*	$C_{17}D_8H_{10}FN_3O_3$	375.85	-	Methanol	-

* deuterated internal standard

2.8.Mass spectrometry

Analysis of the contaminants in LL samples was done using Agilent 6410 Triple Quadrupole liquid chromatography–mass spectrometry (TQLC-MS) (Agilent Technologies, Santa Clara, CA). In the mass spectrometry analytical method Agilent XBD-C18 column 4.6x50mm, with 1.8 μm particle size, was used (held at 50 °C) with 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B. Two positive ionization mode methods were developed and optimized for analysis of pharmaceuticals, as a few compounds (i.e. EE2, ibuprofen and ibuprofen-D3) had a better response at higher capillary voltage on TQ (Tables 5 and 6). One negative ionization mode method was developed and optimized for analysis of biphenolic compounds. The acquisition method was dynamic multiple reaction monitoring (DMRM) in all methods. All reagents and solvents were HPLC-grade. Tables 5, 7 and 9 show the timetable of gradient used. Tables 6, 8 and 10 show the DMRM scan segment of the methods. Agilent MassHunter software suite was used for data analysis.

Table 5. Timetable of eluent gradient for pharmaceuticals method No. 1(flow rate=0.8 mL/min)

Time (min)	A (%)	B (%)
0.25	95	5
10.2	5	95
13.2	0	100
13.4	95	5
16	95	5

post time: 3.5

Table 6. DMRM scan segments of pharmaceuticals method No. 1 (positive mode, Gas Temperature at 350°C, Capillary=2500 V) (Qual: Qualifier, Quant: Quantifier)

Compound	Precursor ion (m/z)	Product ion (m/z)	Ion type	Retention time (min)
Acetaminophen	152.1	65	Qual	1.99
Acetaminophen	152.07	110	Quant	
Acetaminophen-D4	156.1	114.1	Quant	
Acetaminophen-D4	156.1	69.1	Qual	
Amoxicillin	366.11	349.1	Qual	1.67
Amoxicillin	366.1	114	Quant	
Atenolol	267.2	74	Qual	2.04
Atenolol	267.17	145	Quant	
Azithromycin	749.5	83	Qual	5.6
Azithromycin	749.52	591.4	Quant	
Carbamazepine	237.1	193.1	Qual	7.18
Carbamazepine	237.1	194.1	Quant	
Carbamazepine-D10	247.2	202.2	Qual	7.13
Carbamazepine-D10	247.2	204.1	Quant	
Ciproflaxacin-D8	340.2	235.1	Quant	4.7
Ciproflaxacin-D8	340.2	296.2	Qual	
Ciprofloxacin	332.1	245.1	Qual	
Ciprofloxacin	332.14	288.2	Quant	
Doxycycline	445.16	428.2	Quant	5.87
Doxycycline	445.2	98.1	Qual	
Metformin	130.1	60.1	Quant	0.63
Metformin	130.11	71	Qual	
Ranitidine	315.15	176	Quant	2.07
Ranitidine	315.2	81	Qual	
Sulfamethoxazole	254.06	92	Quant	5.74
Sulfamethoxazole	254.1	65	Qual	
Trimethoprim	291.15	230.1	Quant	4.43
Trimethoprim	291.15	261.1	Qual	

Table 7. Timetable of eluent gradient for pharmaceuticals method No. 2 (flow rate=1 mL/min)

Time (min)	A (%)	B (%)
0.25	95	5
5.2	0	100
8.2	0	100
8.3	95	5
10	95	5

post time: 3.5

Table 8. DMRM scan segments of pharmaceuticals method No. 2 (positive mode, Gas Temperature at 350°C, Capillary=4000 V) (Qual: Qualifier, Quant: Quantifier)

Compound	Precursor ion (m/z)	Product ion (m/z)	Ion type	Retention time (min)
EE2	297.2	107.1	Quant	5.42
EE2	297.2	159.1	Qual	
Ibuprofen	207.1	133.9	Qual	5.98
Ibuprofen	207.14	161.1	Quant	
Ibuprofn-D3	210.2	136.9	Qual	
Ibuprofn-D3	210.2	164.1	Quant	

Table 9. Timetable of eluent gradient for biphenolic compounds method (flow rate=1 mL/min)

Time (min)	A (%)	B (%)
0.25	95	5
10.2	5	95
12.2	0	100
12.21	95	5
15	95	5
post time: 2		

Table 10. DMRM scan segments of biphenolic compounds method (Negative mode, Gas Temperature at 250°C, Capillary=2000 V) (Qual: Qualifier, Quant: Quantifier)

Compound	Precursor ion (m/z)	Product ion (m/z)	Ion type	Retention time (min)
BPA	227.1	212	Quant	7.02
BPA	227.1	133	Qual	
BPS	249	108	Quant	5.14
BPS	249	92	Qual	
Triclosan	286.9	35	Quant	9.76

2.9.Fungal bioreactors

Two types of reactors – completely mixed and plug flow – were chosen for LL treatment study in non-sterile condition. The same LL from a mixed-cell landfill (collected in fall 2018) was used for both types of reactors. One-liter flasks were used for completely mixed batch reactor. Plug flow column-shaped reactors (1 L, D= 2”, H≈20”)

were built with PVC material. Direct intake from the top of column through a PTFE pipe with a metal mesh (to filter out fungal mass) was used.

2.9.1.Selected analytes for bioreactor study

The selected contaminants of the study (n=12) and their spike levels were: BPA, BPS, BPF, and phenol, all spiked at 5 mg/L; BADGE spiked at 0.7 mg/L due to limited solubility; biocide triclosan and pharmaceuticals 17α-ethinylestradiol, ibuprofen, doxycycline, trimethoprim and acetaminophen, all spiked at 5 mg/L; antibiotic ciprofloxacin spiked at 2 mg/L due to its inhibitory effect. Since the spike solution consisted of over 70% organic solvents, 5 mL aliquots in test tubes were evaporated under nitrogen gas evaporator to a quarter of initial volume at 40 °C before addition to the reactors.

2.9.2.Abiotic sorption of CECs

Batch sorption tests were done for 21 days in 1 L flasks. Sampling (4 mL) was done 5 minutes after initial mixing of all the test contents and on days 1-4, 7, 14, and 21 (n=7) for analytes measurement. The flask 1 contents were 500 mL of LL with 10 mM sodium azide to inhibit fungal growth and enzymatic activity [75,76], fungal pellet biomass, and spike of the compounds on the list. Incubation was at room temperature, and the pH of LL was adjusted to 4.5 by 10 N sulfuric acid prior to the experiment after filtration through glass fiber filter. As described in section 3.1, this pH was selected because it was optimal in the preliminary study. Manganese sulfate was added at 0.1% to maintain the consistency with the biotic test. The flasks were covered by aluminum foil to avoid photodegradation (in a dark room), under mixing with low agitation and aeration at ~0.8 L/min, in triplicate. Aeration was applied through stainless steel tubes (without air stones)

to avoid undesirable fungal growth.[77,78] Flask 1 experiment was aimed for assessing sorption onto suspended biomass. The flask 2 contents were 500 mL LL with 10 mM sodium azide, and a spike of all listed compounds, with no biomass but otherwise the same conditions to flask 1 experiment. Flask 2 experiment was aimed to assess air stripping and possible glassware effects.[79] The difference of contaminant concentrations in flasks 1 and 2 represented sorption to biomass. Liquid loss due to evaporation was measured by weighing the flasks and was replenished to the initial mass of each flask (excluding volume loss due to prior samplings) by adding sterile DI water prior to sample collection or twice a week, whichever came earlier. Samples from the liquid phase in both flasks was collected and filtered through prewashed 0.45 μm nylon filters for further analyses with LC method. For the evaluation of dry weight of biomass, additional flasks were prepared and sacrificed: the content of these flasks was filtered through glass fiber filters, kept at 60 °C until dry and then weighted.[77]

2.9.3.Batch fungal bioreactor

Batch degradation tests were done for 21 days period in 1 L flasks. The flasks contents were 500 mL of LL, fungal pellet biomass, and a spike of the listed compounds. The fungal mass was aimed at 2-3 g/L, and the sacrificial mass is reported with every test in the results. Incubation was at room temperature, and the pH of LL was adjusted to 4.5 by 10 N sulfuric acid after filtration through glass fiber filter prior to the experiment. Other reactor conditions, sampling, sample preparation and water loss makeup were the same as in abiotic methods.

2.9.4.Plug flow reactor

The plug flow column reactor (Figure 3) was filled with 1 L of filtered LL spiked at the same levels as the batch reactors. Aeration applied through the inlet line at ~1 L/min. The recirculation rate was at 11 mL/min using peristaltic pump. Sampling was done at the same days as in batch bioreactor test. For this experiment, there was one biotic and one abiotic reactor with 10 mM sodium azide. Evaporation from the column was insignificant, as the system was virtually closed.

Figure 3. Plug flow column reactors (Left, biotic and right, abiotic)

2.9.5. Absorbance reduction of fungal bioreactors with various leachates

To evaluate the performance of fungal pellet bioreactor to reduce absorbance and UVAS, five different LL were tested for 21 days in 1 L flasks. All the LL samples used in this study were collected in December 2019 (excluding mixed cell LL) from three sites. Operation was at room temperature with aeration applied at ~1 L/min. The flasks contents were 500 mL LL and fungal pellet biomass for both biotic and abiotic reactors. Abiotic reactor contained 10 mM sodium azide as inhibitor. Liquid loss due to evaporation was insignificant as the column systems were closed and replenishment was not necessary.

2.9.6. Water quality parameters measurements for bioreactors

For each sample pH was measured either by pH 2-9 strips (VWR, Radnor, PA, USA) or H280G multimeter (HACH, Loveland, CO) depending on sample size. COD (HACH HR test) and TOC and TN (Shimadzu TOC-LCPN analyzer), transmittance at 254 nm, and UV-visible absorbance (Agilent Cary 60 Spectrophotometer) were measured for each sample. Ammonia nitrogen (ammonia HACH test TNT 832), total phosphate (HACH test, TNT low range) and BOD_5 were spot-checked.

2.10. Bioassay of leachate for toxicity

Activated sludge respiration inhibition test, as per "Organisation for Economic Co-operation and Development" OECD 209 [65] guidelines, was used to evaluate the toxicity of raw and treated leachate samples with a reduced number of dilutions and replications. The samples were stored in -20 ℃ prior to the experiment and were thawed to room temperature, filtered with GF/F filter to remove any suspended solids (e.g. fungal mass

residue) and adjusted to pH 7.5±0.5. A series of three dilutions, with a factor of 0.001, 0.01 and 0.1 of sample were used in duplicate. The test was done in the BOD bottles (300 mL) with YSI "Pro BOD IDS" dissolved oxygen (DO) probe (YSI Incorporated, Yellow Springs, OH). DO recording were in one-minute intervals until the value was near zero. The activated sludge (AS) was collected from Mallard Creek wastewater treatment plant and fed daily with synthetic sewage at 50mL/L, and tested daily for mixed liquor suspended solids (MLSS). Synthetic sewage contained:

- 16 g/L peptone
- 11 g/L vegetable extract
- 3 g/L urea
- 0.7 g/L sodium chloride
- 0.4 g/L calcium chloride dihydrate
- 0.2 g/L magnesium sulphate heptahydrate
- 2. 8 g/L anhydrous potassium monohdydrogen phosphate

Each test bottle contained 9.6 mL synthetic sewage, 150 mL AS, 0.3-30 mL sample (based on dilution factor) and DI water constituted the rest, Figure 4. The MLSS were kept at 3000 mg/L nominal concentration. If it was at a higher or lower concentration, the volume was adjusted with water accordingly. Blank (no LL sample, just DI) was in triplicates. Positive controls with an inhibitory substance of copper (II) sulfate were done at three concentrations 11.1, 19.2 and 34.5 mg/L in duplicates with rest of mixture same as blank. Both blank and positive control were done once at the start of the experiment. Linear DO results in the range of 2-7 mg/L were used for data analysis, per method.

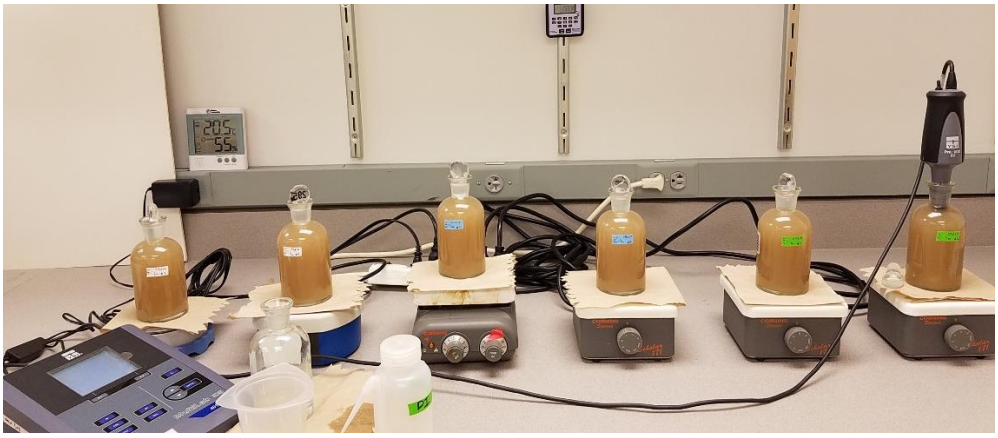


Figure 4. AS respiration inhibition test

2.11.Microbial uptake of selected pharmaceuticals

This part of the study aimed to examine the effect of food availability on the microbial uptake of selected biorecalcitrant PhCs in conventional aerobic and anaerobic bacterial processes and possible microbial community shift during biodegradation of PhCs over time.

2.11.1.Reactor operation and sampling

Six identical 1L anaerobic stirred bottle reactors with nitrogen gas in the headspace and six identical 1L aerated stirred tank reactors were prepared and operated at room temperature. The seed sludge was obtained from an anaerobic digester and an activated sludge tank. Before the experiment, the reactors were fed twice a week with primary clarifier effluent from a local WWTP. The experiments were done in three sets of two for each aerobic and anaerobic reactor consisting of non-spiked control set (I) to measure the background levels PhCs, next set spiked with PhCs and 1 g/L glucose (II), and the last set spiked with PhCs only (III). The PhCs spike consisted of 1 µg/L for each of ibuprofen, diclofenac, carbamazepine and doxycycline (Sigma Aldrich, St. Louis, MO) in DI water. From each reactor 500 mL were decanted and then refilled with primary effluent (I),

PhCs spike and glucose solution (II), and PhCs spike (III). For initial analysis 500 mL samples of primary effluent were prepared with spiked PhCs in triplicate. Reactors operated undisturbed for two days when half of the content of reactors collected and after five more days the rest of it were collected in clean amber bottle and stored at -20 °C. After collection, samples were filtered with glass fiber filters and 200 mg of EDTA was added to inhibit bacterial growth and to chelate metal ions. Culture samples (10 mL) were collected and stored in sterile containers at -80 °C beforehand, and after two and seven days of operation from all reactors.

2.11.2. Analytical procedures

The samples were extracted using Waters Oasis HLB cartridges and analyzed by Waters Acquity UPLC-Quattro Premier XE Mass Spectrometry by a second party at David H. Murdock Research Institute (DHMRI). The Waters TargetLynx Application Manager software package was used for data analysis.

2.12. Statistical analysis

Student t-test is used (with Microsoft Excel) for the following experimental data: a) normalized data on contaminants removal in fungal reactor, and removal comparison in biotic reactors versus abiotic controls b) PhCs detected in LL with various closure status. c) study on microbial uptake of pharmaceuticals. Means were considered as significantly different when the p-value was < 0.05.

Furthermore, one-factor analysis of variance (ANOVA) were used for calculation of limit of detection (LoD) of contaminants on HPLC method. LoD and LoQ were calculated by the relationship between the standard deviation (sd) and slope (S) of the

calibration curve. The LoD and LoQ were calculated by the equations in the following: LoD=(3.3xsd/S) and LoQ=(10xsd/ S).[80]

CHAPTER 3: RESULTS

3.1.Growth of fungal strains

3.1.1.Growth on solid media

The first fungi evaluated for growth in contact with LL, was *CS*. On YM agar plate incorporated with 10% LL and incubation at 27±1 °C, *CS* covered 85 mm plate after 19.5±3.54 days. No growth observed in higher concentration of LL. As it will be described later, the performance of *PC* outcompetes *CS* and therefore *CS* was dropped form study at the initial stage. The results provided hereafter, are exclusively obtained with *PC.*

On the other hand, *PC* showed growth on YM agar plates with up to 90% LL. Tables 11 and 12 show the *PC* growth at adjusted and unadjusted pH (~8) and at different temperatures, as described in 2.3.

Table 11. Growth of *PC* on type I plates at 27 ºC, adjusted pH at 6 (mean±sd)

LL (%)	10	20	30	40	50	100
Growth rate (mm/d)	17.0± 0.0	17.0± 0.0	12.1± 0.0	8.5± 0.0	6.8± 0.3	13.6±1.0 17.9±0.9[a]

[a] at 37 ºC

Table 12. Growth of *PC* on type I plates at 27 ºC, unadjusted pH (mean±sd)

LL (%)	50	60	70	80	90	100
Growth rate (mm/d)	17.0±2.5 28.3±0.0[a]	15.5±3.1	11.3±0.9	8.9±1.0	7.1±1.0	No growth

[a] at 37 ºC

As leachate content increased, *PC*'s growth was slower and started later, especially on plates with 80% and higher percentage of LL at 27 and 37°C. Contrarily, when pH was

adjusted to 6, the growth rate on Type I plates with 100% LL was comparable with the growth on lower LL percentage plates.

Initially, the growth of *PC* on Type II plates regardless of pH and temperature was unsuccessful. The dependence of WRF enzyme system on manganese was known previously for LiP and laccase.[43] Manganese sulfate addition at 0.1% helped *PC* greatly to grow on YM agar plates with 100% LL. On Type III plates, adjusted to pH 6 and with 0.1% $MnSO_4$, the growth rate was 1.5 mm/d at 27ºC and 4.5 mm/d at 37ºC, both showing partial coverage. The growth stopped after 14 days (observation continued for 10 more days). The growth of fungi was also successful at room temperature with type III plates as presented in Table 13. With manganese content over 0.05%, the growth rate decreased until there was no growth in 0.3% manganese or higher.

Table 13. Growth of *PC* on Type III Plates, adjusted pH at 6 and room temperature(mean±sd)

$MnSO_4$(%)	0.05	0.1	0.2	0.3	0.4
Growth rate (mm/d)	4.3± 3.67[a]	1.3± 0.24[a]	1± 0.35[a]	No growth	No growth

[a] partial coverage, growth stopped after 14 days

3.1.2.Maximum specific growth rate

The evaluation of μ_{max} as described in 2.3 was done in YM broth. The exponential growth part of the collected data from all replications (until day 5) as shown in Figure 5, resulted in maximum growth rate (μ_{max}) of 0.56±0.04 d^{-1}. A kinetics study on *PC* showed that the exponential growth would decline after 5 days, conforming the observation in this study.[81]

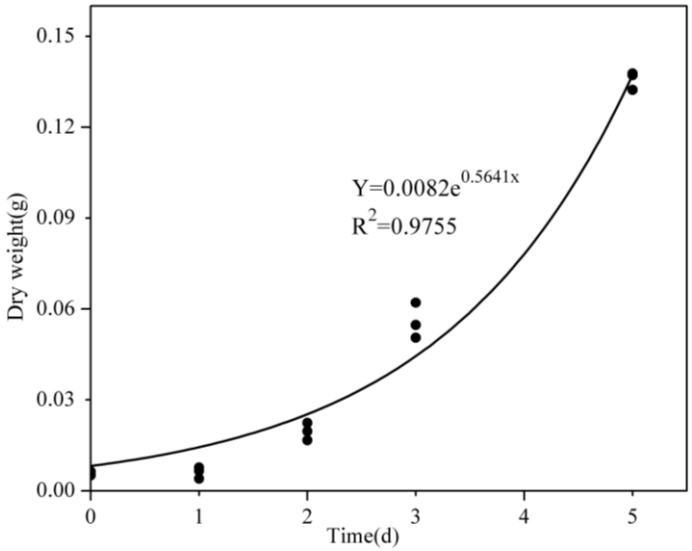


Figure 5. Dry weight of *PC* versus time in unlimited substrate (created by OriginPro 8)

The reported μ_{max} in the literature for other WRF strains, Table 14, are usually at lower range compared to *PC*, with the exception of widely utilized *Trametes versicolor*. Despite incubation at higher temperature and hence higher μ_{max} in another study [81], the observed value in this study is plausibly close to that value.

Table 14. Specific growth rates of similar WRF strains to *PC*

Fungal strain	μ_{max} (d^{-1})	Incubation temperature (°C)	Reference
Trametes pubescens	0.25	28	[82]
Trametes spp.	0.472	28	[83]
Trametes versicolor	0.94	22 ± 2	[84]
Pleurotus ostreatus	0.216	26	[77]
Bjerkandera adusta	0.215	26	[77]
Phanerochaete chrysosporium	0.64	32	[81]
Phanerochaete chrysosporium	0.564	30	This study

3.2. Enzyme Assay

The enzymatic activity of LiP in YM broth incorporated with LL samples inoculated with *PC* after 7 days and 14 days is presented in Table 15. At higher percentage of LL, the activity of LiP increased but not following a significant trend. After 14 days, the

enzyme activity at 5% LL doubled, while in most of higher concentration leachate samples it reduced from previous 7 days or fall below the detection limit. This analytical method, which relies on absorbance at 351 nm wavelength, was inefficient due to interferences from the absorbance of the complex matrix of LL, and therefore no further measurements were taken.

Table 15. LiP activity of *PC* (U/L, µmol/min/L), in YM broth incorporated with LL

LL (%)	5	10	15	20	25	30	35	40	45	50
Activity 7 days	0.0036	0.0173	0.0101	0.0133	0.0081	0.0136	0.0107	0.0138	0.013	0.0176
Activity 14 days	0.0072	0.0061	BDL [a]	0.0022	BDL [a]	0.0042	BDL [a]	BDL [a]	0.0002	0.0039

[a] below detection limit

The measured activities of LiP in LL at ambient temperature (~20°C) are significantly lower than in pure cultures grown at higher temperature. One of the initial studies on *PC* reported the LiP at 76 µmol/min/L.[32]

In conclusion, as *PC* showed satisfactory growth in undiluted LL at ambient temperature, with adjustment in pH and addition of manganese, it has the potential to be used in treatment of LL at full scale.

3.3. High-performance liquid chromatography detection

3.3.1.Detection limits for CECs in bioreactor studies in leachate

Peak areas of four-point calibration curves were used for determination of LoQ and LoD with ANOVA analysis. LoQ is LoD multiplied by a factor of 3.[80] In Table 16, the detection levels for Zorbax Eclipse Plus C8 column with the method described in 2.6 can be seen. Variability was observed among compounds with the highest response belonging

to BPA and the lowest to BADGE. Although, most of the analytes have a LoQ of around 0.5 mg/L.

Table 16. Detection levels of analytes on Zorbax Eclipse plus C8 column with HPLC-DAD

Compound	LoD (mg/L)	LoQ (mg/L)
EE2	0.15	0.46
Phenol	0.17	0.50
Ibuprofen	0.13	0.39
BADGE	0.19	0.57
BPA	0.05	0.16
BPF	0.15	0.46
Acetaminophen	0.11	0.34
Triclosan	0.14	0.42
BPS	0.06	0.19
Ciprofloxacin	0.18	0.54
Doxycycline	0.18	0.53

3.3.2.Size exclusion chromatography

The best standard points producing the highest R^2 were used to prepare the calibration curve (data not shown) of log(MW), (Da), versus retention time (t, min), that resulted in the following equation (R^2=0.9389):

$$MW = 10^{-(t-26.41)/2.7435} \quad \text{Equation 4}$$

Peaks of the SEC chromatograms were calculated back to the original MW via the Equation 4.

3.4. Landfill leachate

Sampled leachates collected during four seasons, were tested for aggregate parameters, absorbance, transmittance at 254 nm (T_{254}) and finally for PhCs. The pH of

collected samples was in the range of 7-8. Tables 17-21 provide general characteristics of LL samples from sites 1-4 along with their closure status. In Figures 6-12, absorbance scan of diluted (x10) LL samples are shown.

The organic content in LL samples from site 1 was very diverse with highest measured COD of 1317 mg/L. TOC also reflects the shift in the organic content. The strength of LL is dependent on the landfill's operation practices and especially the control on precipitation runoff. This means that after a heavy or a prolonged seasonal rainfall, the LL is expected to be diluted, as more water has percolated through the layers of landfill. There is a significant correlation between TN and T_{254} (P-value=0.0455). P-values were calculated as paired dataset with two-tails mode. There was a strong but not significant correlation between COD and T_{254} (P-value=0.0877), but not between TOC and T_{254} (P-value=0.1539). Dissolved organic carbon and dissolved organic nitrogen are able to contribute to T_{254}.[27] The stronger was the LL, the higher was the absorbance in the UV range, especially under 250 nm.

Table 17. Leachate samples characterization-Site 1

Description	Sample ID[a]	COD (mg/L)	TOC[b] (mg/L)	TN (mg/L)	T (254 nm) (%)[c]
Mixed cell	S1S1	729	134.9	342.9	51.5
	S1S2	-	349.6	380.5	11.0
	S1S3	1317	294.7	401.1	30.7
	S1S4	372	56.6	159.6	77.6
	S1S5	548	127.9	256.2	55.2
	S1S6	372	69.9	97.4	80.8
	Mean±SD	667.6± 391.9	172.3± 121.4	272.9± 124	51.1± 26.9

[a]ID's first part is site number and second part is sample number in collection date order (e.g. S1S1 = Site 1 Sample 1). [b]non-purgeable organic carbon; [c] diluted by factor of 10

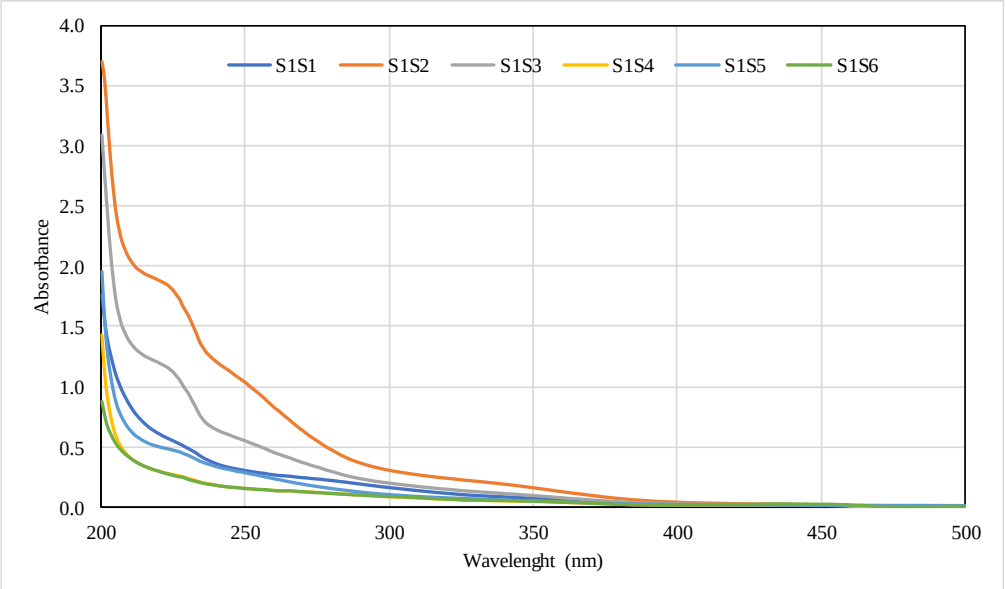


Figure 6. Absorbance scan-Site 1

Samples of LL from site 2 have variety in the strength -which translate to carbon or nitrogen content. Active cell samples ranging 292 to 1439 mg/L for COD, which could relate to the dilution effect of precipitation on landfill. COD and TN have relatively significant correlation with T_{254} with P-values of 0.0511 and 0.0644 respectively. Although this the relationship is not substantial for TOC (P-value=0.1896). The variability in UV transmittance can be seen.

Table 18. Leachate samples characterization-Site 2

Description	Sample ID	COD (mg/L)	TOC[a] (mg/L)	TN (mg/L)	T (254 nm) (%)[b]
Active cell	S2S1	1012	236.1	403.4	19.0
	S2S2	1439	284.1	455.3	23.6
	S2S3	731	182.6	250.7	42.4
	S2S4	530	100.9	193.6	58.7
	S2S5	316	78.7	178.2	71.9
	S2S6	292	48.3	94.1	81.4
	Mean±SD	720.0± 443.8	155.1± 94.0	262.6± 139.5	49.5± 25.5
Closed cell, <5 years	S2S7	144	43.7	20.5	71.2
	S2S8	1171	166.9	389.8	55.4
	S2S9	856	200.1	417.8	54.3
	S2S10	559	115.7	314.4	59.9
	S2S11	620	132.1	330	65.6

	S2S12	528	125.4	305.6	56.6
	Mean±SD	646.3± 344.6	130.7± 52.8	296.4± 142.2	60.5± 6.6
Closed cell, >10 years	S2S13	734	167	386.6	56.7
	S2S14	365	42.2	32.1	70.6
	S2S15	208	52.7	38.2	64.3
	S2S16	145	28	39.4	90.2
	S2S17	132	40.8	61.7	87.4
	S2S18	84	19.1	6.7	89.3
	Mean±SD	278.0± 243.7	58.3± 54.5	94.1± 144.4	76.4± 14.5

[a] non-purgeable organic carbon; [b] diluted by factor of 10

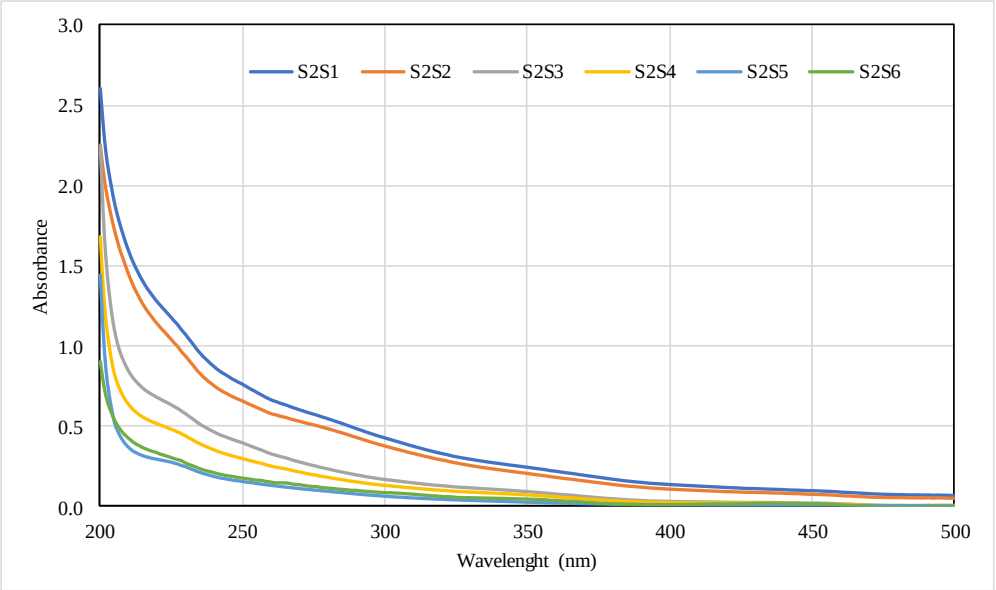


Figure 7. Absorbance scan-Site 2, active cell

In the closed cell (<5 years), samples had a wide range in measured parameters, and yet not as wide as active cell of this site. The correlation between T_{254} and COD, TOC and TN are significant, as shown respectively by P-values of 0.0048, 0.0061 and 0.0002. This shows one of very few consistent correlations among mentioned parameters in all LL samples sets. This could be because of stability of the closed landfill cell, despite its variability. Decomposition of waste in landfill will continue after closure (i.e. placement of final cover), but its rate will decrease until it eventually becomes insignificant. This

phenomenon is reflected by reduction is landfill gas production (and change in its composition) and LL concentration and volume.[24] UV scans, as illustrated in Figure 8, did not show as much change among samples as leachate from the open cell.

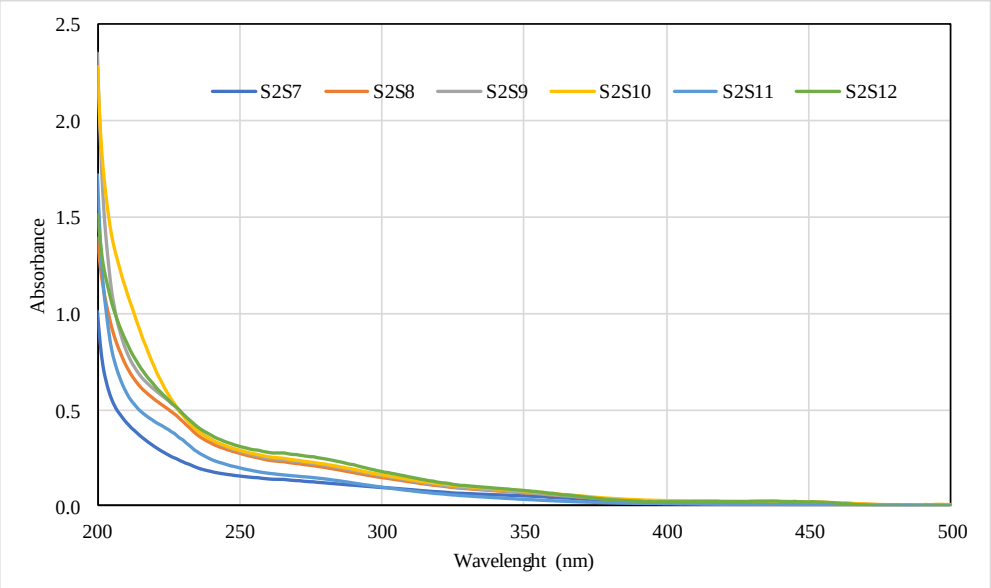


Figure 8. Absorbance scan-Site 2, Closed cell (<5 years)

In the other cell of site 2, closed cell (>10 years), the carbon and nitrogen content of LL samples were lower. This is expected in landfills when over 10 years has passed from their closure, when they reached stability. Although depending on the refuse constitution and moisture content the long-term stabilization could take 20 to 50 years.[24] The correlation between T_{254} and, TOC and TN are statistically significant with P-values of respectively 0.0152 and 0.0132. Contrarily COD is not showing significant relation (P-value=0.1139). The low absorbance observed in this cell (and overall low strength) could be the result of the collection well, being open to the atmosphere and close to the surface. Therefore, rain water, directly or indirectly through runoff, contributes to its dilution.

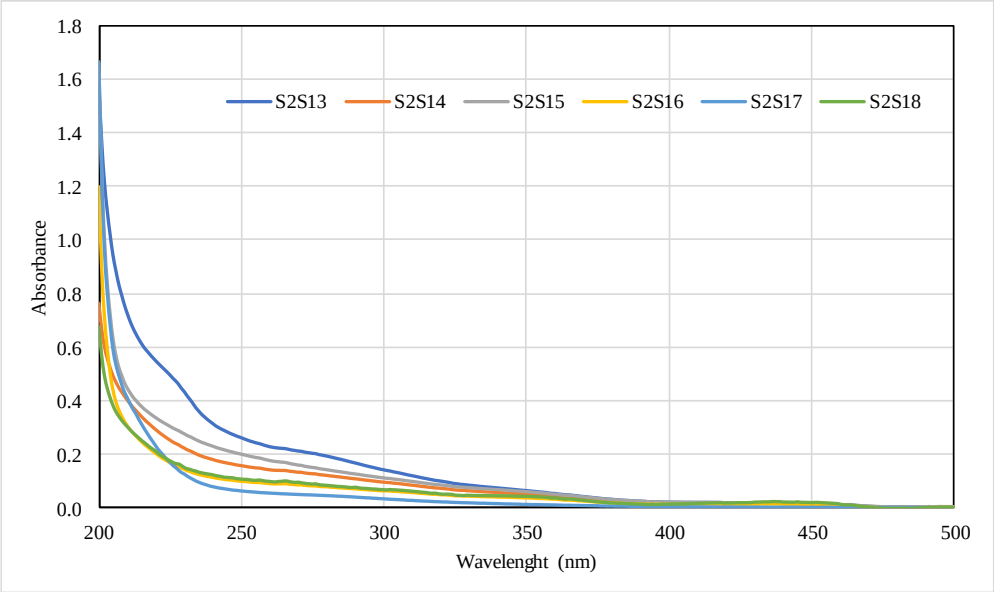


Figure 9. Absorbance scan-Site 2, Closed cell, >10 years

Samples from site 3, are low strength in general but still show the variability among limited samples. COD has a range of 109 to 479 mg/L, while TOC range is 32.4 to 64. The correlation between T_{254} and TN is the only significant parameter with P-value of 0.0102. COD and TOC has not any significant relation with T_{254} with P-value of 0.1953 and 0.1145. Despite being a mixed cell, the LL showed relatively low UV absorbance (high transmittance).

Table 19. Leachate samples characterization-Site 3

Description	Sample ID	COD (mg/L)	TOC[a] (mg/L)	TN (mg/L)	T (254 nm) (%)[b]
Mixed cell	S3S1	109	32.4	61.9	77.1
	S3S2	479	64	152	69.4
	S3S3	281	60.7	153.8	68.5
	Mean±SD	289.7± 185.2	52.4± 17.4	122.6± 52.5	71.7±4.7

[a] non-purgeable organic carbon; [b] diluted by factor of 10

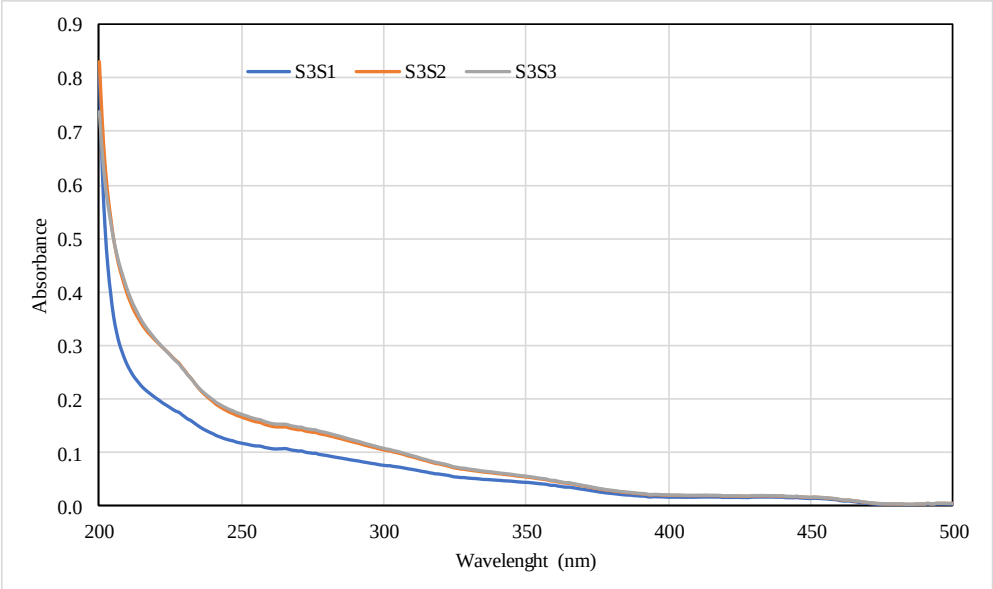


Figure 10. Absorbance scan-Site 3

In the active cell of site 4, the TOC was measured regularly that ranges 276 to 1419 mg/L. None of TOC and TN showed significant correlation with $T_{254,}$ as shown by P-value of 0.1249 and 0.1842. Some variation was observable in the UV scan, but 3 out of 4 LL samples had similar absorbance.

Table 20. Leachate samples characterization-Site 4

Description	Sample ID	COD (mg/L)	TOC[a] (mg/L)	TN (mg/L)	T (254 nm) (%)[b]
Active cell	S4S1	-	1419	200.6	51.0
	S4S2	-	1297	222.9	44.4
	S4S3	-	1319	299.3	51.3
	S4S4	1053	276.2	83.9	78.6
	Mean±SD	-	1077.8± 537.0	201.7± 89.2	56.3± 15.2
Closed cell, <5 years	S4S5	1125	264.6	302.9	56.0
	S4S6	1302	298.7	372.5	52.3
	S4S7	1044	229.2	344.7	54.9
	S4S8	1289	255.8	358.2	90.2
	S4S9	924	213.2	331.2	64.7
	Mean±SD	1136.8± 161.6	252.3± 33.1	341.9± 26.7	63.6±15.6

[a] non-purgeable organic carbon; [b] diluted by factor of 10

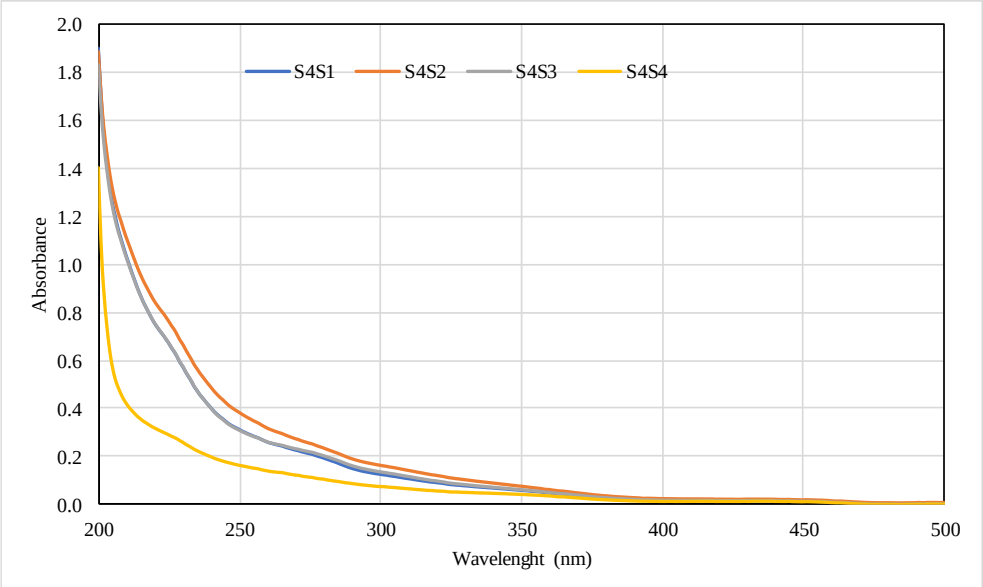


Figure 11. Absorbance scan-Site 4, active cell

The closed cell of site 4 had consistent carbon and nitrogen content. COD ranged from 924 to 1302 mg/L, while TOC was in the range of 213.2 to 298.7 mg/L. All measured parameters had significant statistical correlation with T_{254} with P-values of 0.00131 for COD, 0.0034 for TOC and 0.00019 for TN. The absorbance scan showed lower values compared to the active cell.

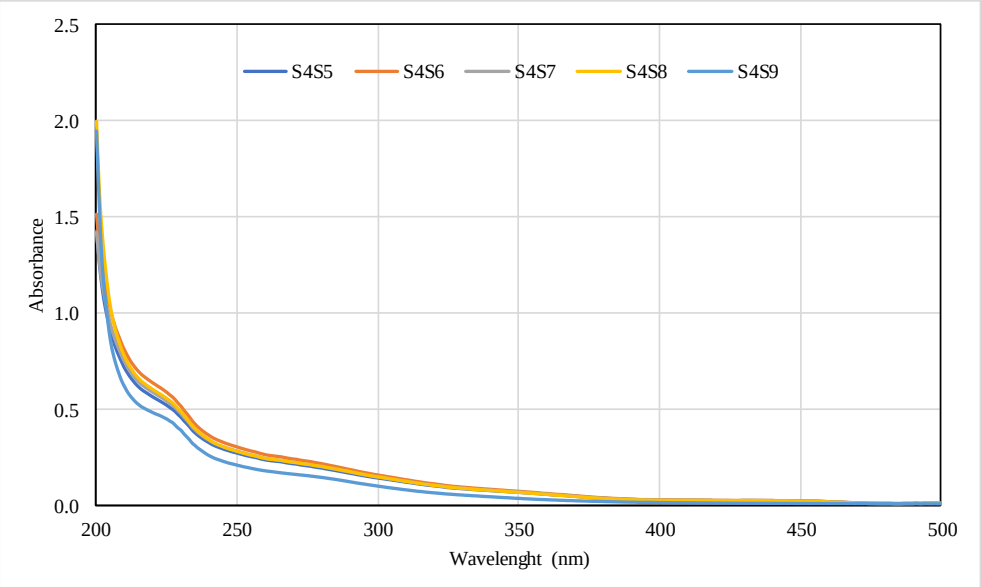


Figure 12. Absorbance scan-Site 4, Closed cell (<5 years)

Ammonia nitrogen was measured for two sets of samples from different sites, and ranged from 80.4 to 184.8 mg/L for active cells and from 37.4 to 335 mg/L for a closed cell. Due to lack of sufficient data, any correlation cannot be established between this parameter and T_{254}.

Table 21. Ammonia nitrogen in a batch of LL samples

Description	Sample ID	NH_3-N (mg/L)
Mixed cell	S1S5	159.6
	S1S6	248
Active cell	S2S4	184.8
	S2S5	172
Closed cell, <5 years	S2S10	300
	S2S11	343
Closed cell, >10 years	S2S16	37.4
	S2S17	55.3
Active cell	S4S4	80.4
Closed cell, <5 years	S4S8	335
	S4S9	317

Table 22 provides statistical analysis of all LL in samples in four categories. In all types of LL (n=26), other than closed cell (>10 years) significant correlation observed

between COD and T_{254}. On the other hand, TOC has significant association with T_{254} in all categories of LL (n=27), with exception of mixed cell. All types of samples have significant correlation between TN and T_{254} (n=36). Active cell and Closed cell (<5 years) (n=21) showed significant association among TOC, COD and TN with T_{254}. The lack of statistical connection among parameters with T_{254} is presumably because of high variability in the dataset and a relatively small number of samples.

Table 22. Statistical analysis of raw LL samples; P-values vs. T_{254}

Description	COD	TOC	TN
Mixed cell	0.0114	0.1416	0.0103
Active cell	0.0052	0.0294	0.0023
Closed cell, <5 years	0.00003	0.00038	0.00001
Closed cell, >10 years	0.1139	0.0152	0.0132

As shown in Figure 13, Closed cell (<5 years) LL samples, had the highest COD, TN and NH_3-N among other categories of samples. Lowest transmittance at 254nm, belonged to active cell samples, while the least strong leachate samples from closed cell (>10 years) had the highest transmittance.

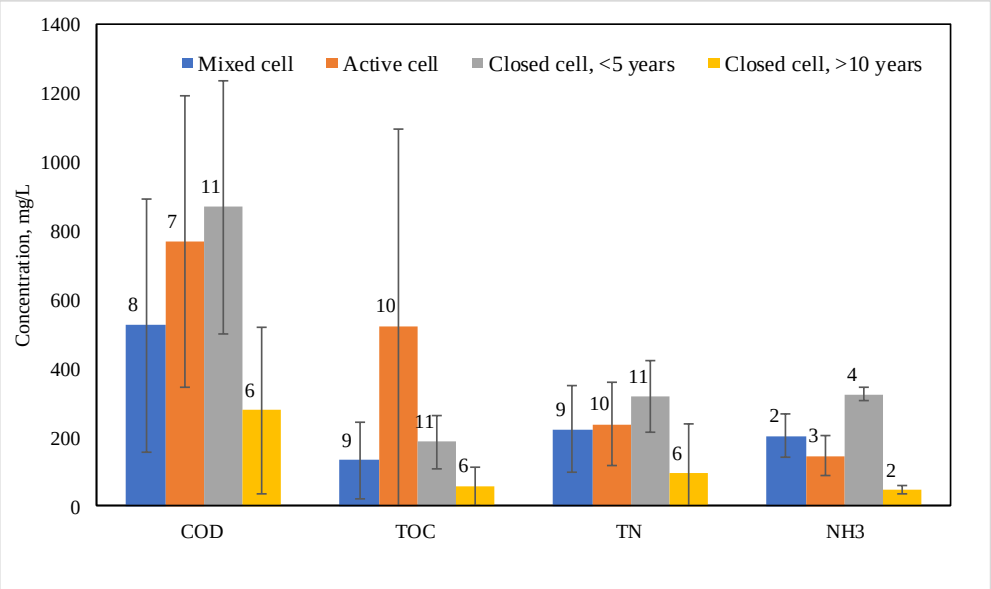


Figure 13. Landfill leachate characteristics (Mean values with SD and sample counts)

The low T_{254} of raw leachates for mixed cell, active cell and closed cell (<5 years) at x10 dilution reconfirmed previous studies reporting that direct discharge of leachate to municipal wastewater treatment plants has a negative impact on effluent transmittance when typical T_{254} needs to be above 65% for UV disinfection purpose as illustrated in Figure 14. This impact have been observed when LL constitute as low as 0.01% of volumetric stream municipal wastewater.[85]

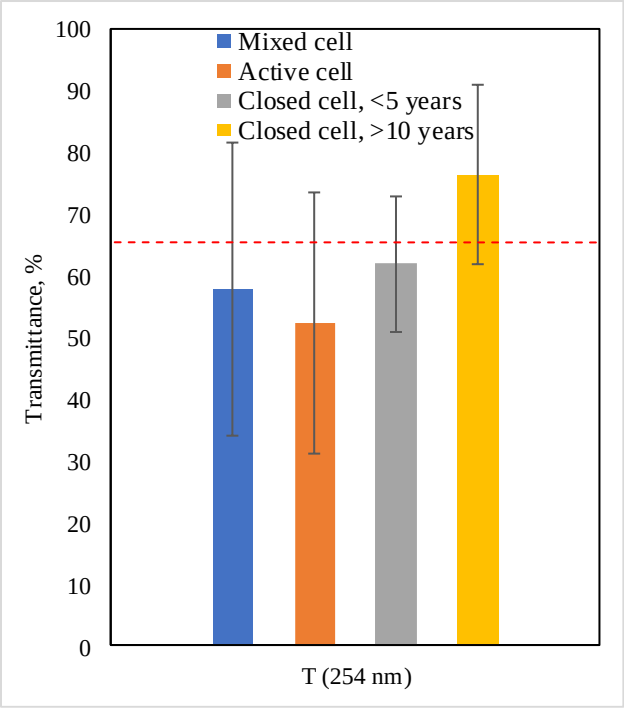


Figure 14. Transmittance of leachate samples, x10 diluted (Mean values with SD; cutoff line at 65%)

3.5.Contaminants of emerging concern in leachate

Mass spectrometry methods were used to analyze the occurrence of CEC in LL as described in section 2.8. Table 23 provides LoQ of the compounds analyzed in landfill leachate in this part of study. As shown, it ranges from 10 ng/L for half of the compounds to 10 µg/L for BPA.

Table 23. LoQ of analytes with mass spectrometry methods

Compound	LoQ (ng/L)
Acetaminophen	10
Amoxicillin	100
Atenolol	10
Azithromycin	100
Bisphenol A	10,000
Bisphenol S	100
Carbamazepine	10

Carbamazepine-D10	10
Ciprofloxacin	10
Ciprofloxacin-D8	10
Doxycycline	10
EE2	1,000
Ibuprofen	100
Ibuprofen-D3	100
Metformin	10
Ranitidine	10
Sulfamethoxazole	10
Triclosan	100
Trimethoprim	10

The measured concentrations are presented as normalized level to the concentration present in the open cells in three sets of samples collected. The main purpose was to examine the shift in occurrence and concentrations of analytes after closure of landfill cells. Another reason for comparison of normalized concentrations was because the calculations for each of the three sample sets were based on different calibration curves. Set I, as can be seen in Figure 15, provided the PhCs data from summer 2019 samples (n=21). Figure 16 shows set II, that is PhCs data from winter 2019, and spring/ summer 2020 (n=15). Figure 17, shows combined set for bisphenols data from all collected samples (2019-20) (n=36). Student t-test used in this part of the study, was two tailed with unequal variance and 95% confidence level.

SPE-extracted calibration curve was used in quantitation of set I. In this set, AZT with a very high LoQ at 1 µg/L was not detected in any sample. Despite this, there is still possibility of its presence in LL at levels below LoQ, as commonly PhCs are present in

wastewater and leachate at below 1 µg/L. CIP, RAN and TMP were consistently not detectable in all samples even though LoQ was 100 ng/L. GEM was not efficiently detectable due to low retention time, and therefore was omitted from the study. The rest of the compounds were acceptable to report as shown in Figure 15. CBZ had the highest occurrence in all types of landfill cells, and a large variation in concentration. The statistical analysis showed significant difference in concentrations ($p=0.047$) between closed cell LL with the rest of LL types. IBP and DOX were present in all types of landfill with some variability but close to normalized level of open cell. ACT, ATN and EE2 showed statistical significance (p-values were 0.037, 0.032 and 0.02 respectively) between open (and mixed) cells and closed cells. They all had comparable level in both open and open/mixed cells. AMX occurred in all types of landfill cells but more noteworthy in open cells. ACT has a fast biodegradation rate[86] that could justify its absence in closed-cell landfills. AMX is known to be unstable in the environmental and degrades through hydrolysis. [87] Although its presence in older landfills is hard to explain as a result. ATN was reported to be degradable to high extent, but not as high as ACT in POTW. Therefore, its occurrence in all landfill cells is reasonable as it will take a long time to degrade completely.[86] CBZ is known to escape through most of biological treatments due to its biological persistence. Although there is a variation in its concentration, but higher concentration in LL from landfills with higher age could be a sign of conjugation/deconjugation processes (and low adsorption) occurring that resulted in higher levels.[86] DOX is also reported to be biodegradable in AS systems, but as it is non-polar, therefore it has affinity for adsorption.[88] This might be the reason it was detected in closed cells with higher ages as well as open cells. EE2 as a synthetic

hormone was detected in the environment along with natural steroid hormones such as estradiol, all of which are well biodegradable over time.[89] As EE2 occurred at higher concentration in open cell leachate, this could mean that it was degraded in a closed landfill cell. IBP, similar to ACT is among the highly consumed PhCs globally and found abundantly in the environment. The removal efficiency of IBP is high, but it is not completely biodegradable [89] and was detected in all landfill cells.

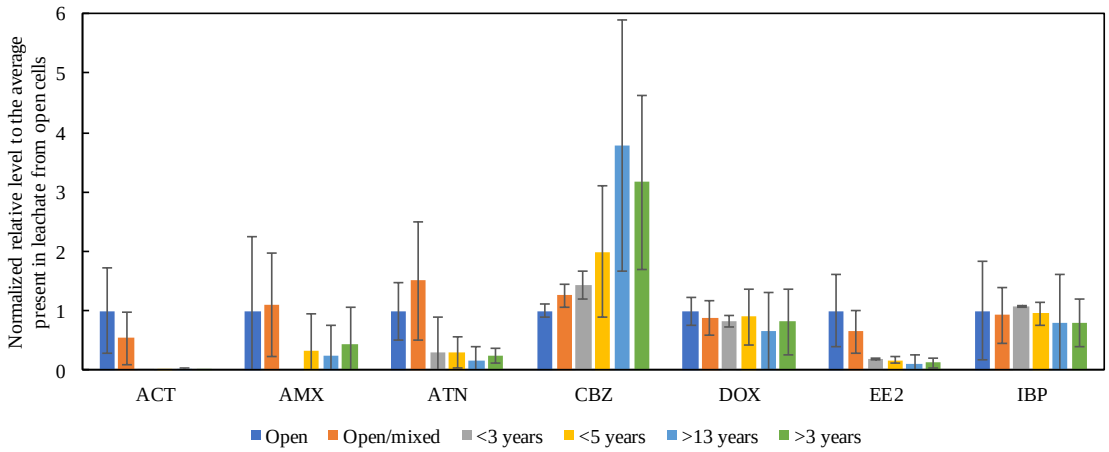


Figure 15. Set I, PhCs in landfill leachate

As illustrated in Figure 16, set II had four more compounds compared to set I, but less variety in concentration in different types of samples. Both CIP and SUL were below detection limits in all analyzed samples, the same as for set I. AMX had the highest difference in concentrations in open cells vs. closed/mixed, although with large deviation (excluding open cells). DOX and IBP were statistically significantly different between closed cells and the non-closed ones with p-values of 0.039 and 0.052. DOX had similar levels in all types of cells, but higher in closed cells. The opposite is true for IBP, with higher occurrence in open cells. ACT was mainly present in non-closed cells, but with considerable variation. CBZ was present in all types of LL but higher in >13 years closed

cell LL. AZT was not detected in >13 years, while present in the rest of cells. ATN, MET, RAN, TMP and EE2 occurred in all types of cells.

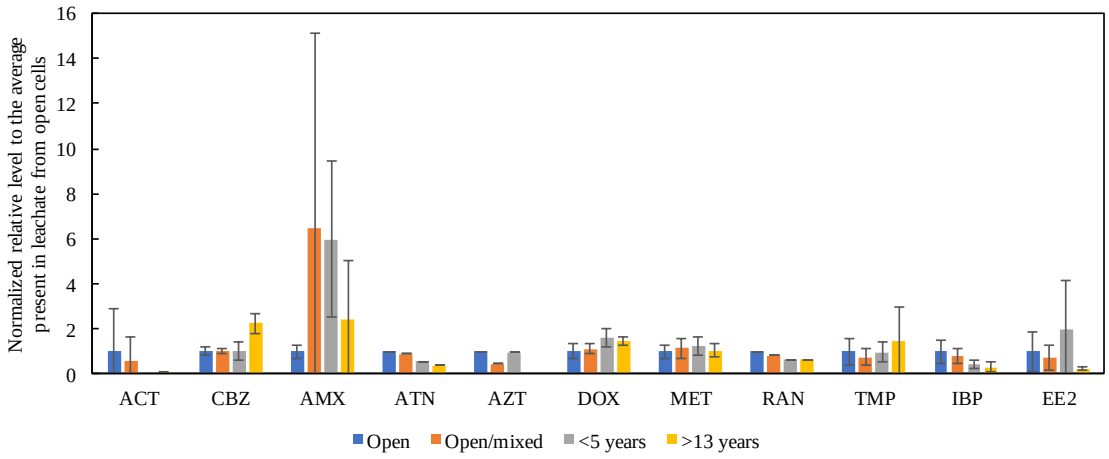


Figure 16. Set II, PhCs in landfill leachate

The comparison among the two sets, showed that all analytes in set I also exist in set II. Similar trends were observed in occurrence of ACT, CBZ, ATN and EE2 in both sets. Among national and local studies on LL, this study reports the occurrence of antibiotics AMX, AZT and DOX for the first time in both open and closed cell landfills, in either of sets.

The combined set (Figure 17) showed the occurrence of BPA and BPS in landfill leachate. Triclosan was always below the LoQ of 100 ng/L (Table 23) in extracted samples. BPA presence was similar in all landfill cells and it was the highest in landfills with longest age of post closure. BPS occurred statistically significantly higher ($p=0.0004$) in non-closed landfill cells (open or mixed). This was the most substantial age dependent relation within all data sets of LL samples. BPA in LL has been reported in US at 516 µg/L [14] and 45.4 µg/L[16] (median), both of which have been analyzed with a gas chromatography-mass spectrometry method. No attempted measurement in LL for BPS

were found in the literature, but for the purpose of comparison, in the raw municipal wastewater this compound was found at 31.2 ng/L.[90] The relative prevalence of the two bisphenols can be explained, as BPA has been in use for decades as a core ingredient of many plastics and consumer products until recently[22], and plastic material in the solid waste will slowly release it over time. On the other hand, BPS as a replacement for BPA, especially in thermal receipt paper has been introduced recently.[91]

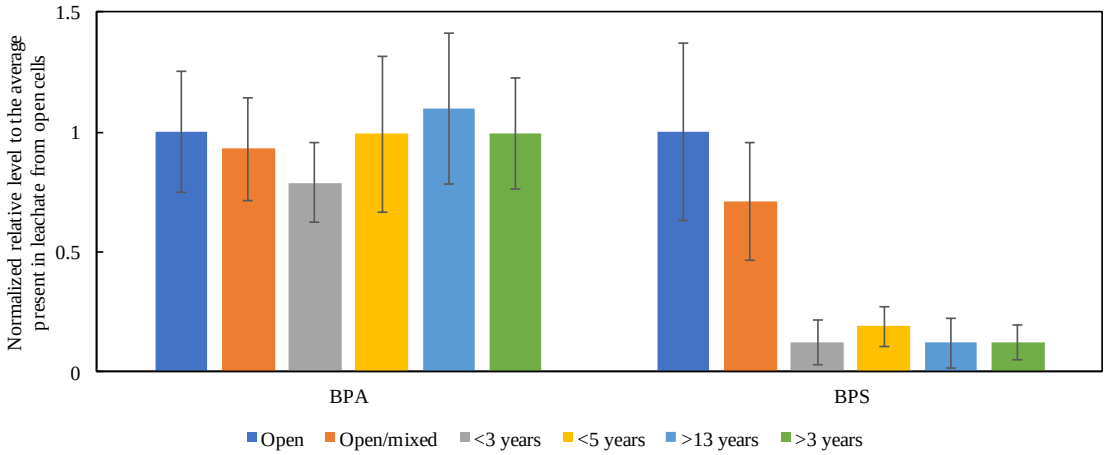


Figure 17. Combined set bisphenols in landfill leachate

In general, national level studies[12,16] showed that concentrations of CECs are significantly greater in open cell LL. Although the results from our study agree with this statement for ACT, ATN, EE2 (set I), and DOX and IBP (set II) and BPS, it contradicts it for CBZ (set I). Nevertheless, occurrence of CECs was again demonstrated by this study, with samples from landfills with different ages and closure status over one year of sampling. The presence of the CECs in LL shows their contribution to presence of CEC in environment, depending on their fate[16], and potential harm to water bodies and humans if not contained and treated properly.[14,16]

3.6.Batch fungal reactor

As the batch fungal experiments have been done two times, as described in 2.9, they will be referred as Study I and II. The main difference between the two studies was that the second one (i.e. study II) had lower spike levels, and sodium azide was used as inhibitor for abiotic part of study instead of autoclaving.

3.6.1.Study I

Study I of batch fungal reactors was done in duplicates for 21 days. Biotic fungal mass was 0.574 g in the sacrificed reactor while abiotic fungal mass was 0.649 g, that is 13% more than the biotic one. Figure 18 shows the CECs concentrations over the course of operation in biotic and abiotic reactors. Phenol was the first compound removed in biotic reactor within two days, while its concentration virtually did not change in abiotic reactors during operation. This agrees with a study which showed removal of phenol at 1mM in immobilized *Trametes pubescens* fed by glucose within a day [82]. ACT was removed by about 50% in the biotic reactors, completely removed from abiotic reactors with fungal mass, and remained the same in another set of abiotic reactors without fungal mass. We hypothesize that sorption on fungal mass is the reason for its removal. IBP is the only compound that was fully removed from all reactors but faster (9 days) in biotic reactor compared to 12 and 21 days in abiotic with and without fungal mass. It seems that biodegradation process occurred in biotic reactor while adsorption was contributing to removal of IBP in abiotic reactors due to non-polarity of the compound.[92] At low concentrations (<2.5 µg/L) both ACT and IBP were removed in non-sterile urban sewage with *Trametes versicolor* batch fluidized bed bioreactor.[53] BPA was removed similarly in biotic and abiotic reactors with no fungal mass, but showed sorption and desorption in

abiotic reactors with fungal mass. Majority of successful studies on removal bisphenols were using enzymes extracted from WRF immobilized on various organic or inorganic media, and direct enzymatic treatment.[93–97] CIP was not changed during the test period in any of the reactors. A study with *Trametes versicolor* incubated in malt extract broth removed more than 90% of CIP (at 2 mg/L) after 7 days.[98] Significant statistical difference ($p<0.05$) observed in this study between biotic and abiotic without fungi reactors for removal of phenol, BPA, BPF, ACT and CIP.

There was inconsistency in the chromatograms of three type of reactors which prevents the detection of all spiked analytes. For example, appearance of an unknown but significant peak blocked detection of TMP in abiotic reactor with fungal mass.

By day 6, the pH in biotic reactors raised from initial value of 4.5 to neutral pH. Although this pH stabilization in abiotic reactors with fungal mass was similarly observed within 8 days from start of the test, only a slight increase to pH 5 was observed over the course of experiment in abiotic reactors without fungi.

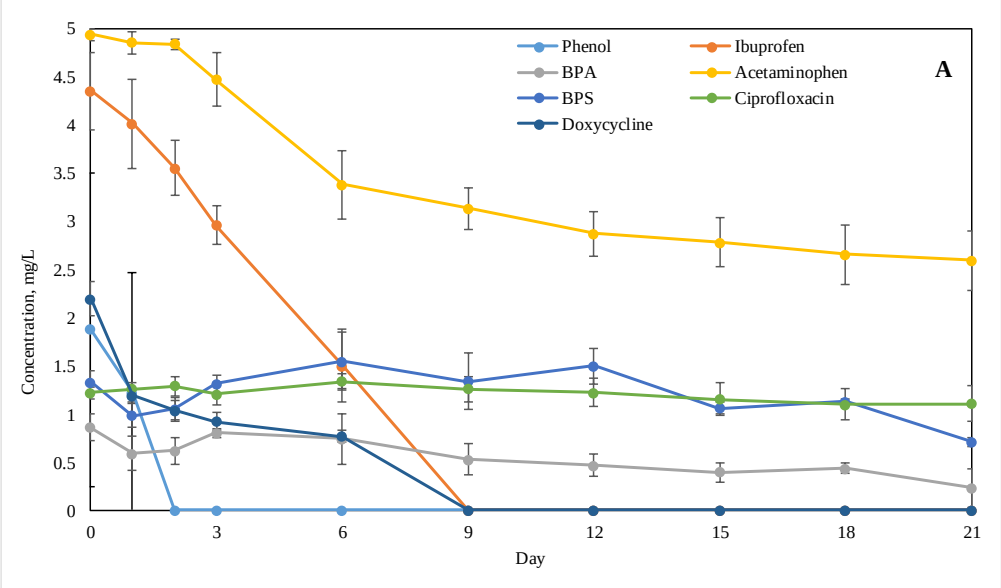
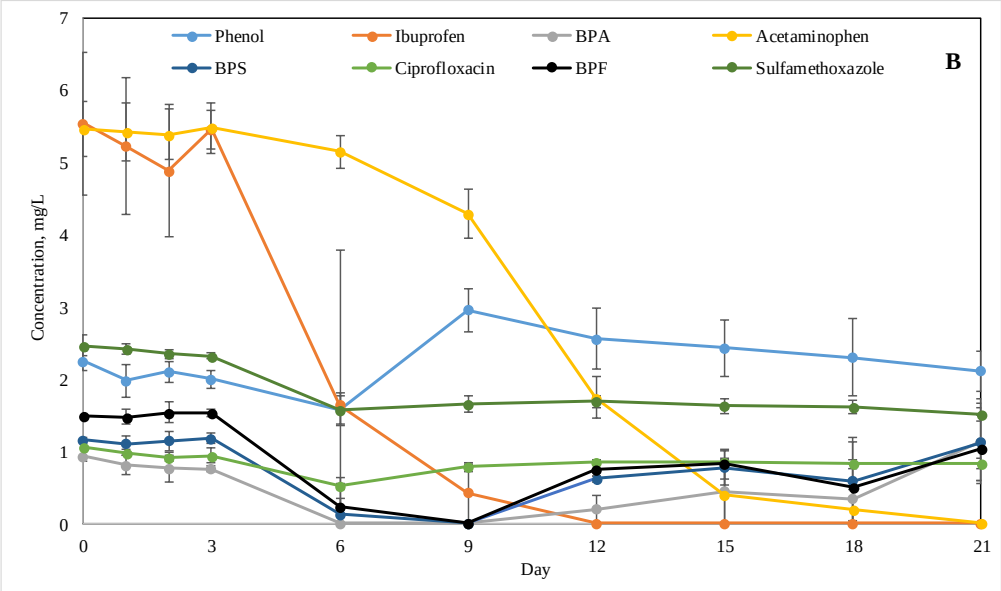


Figure 18. Contaminants removal in fungal batch reactors(mean±SD); Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)



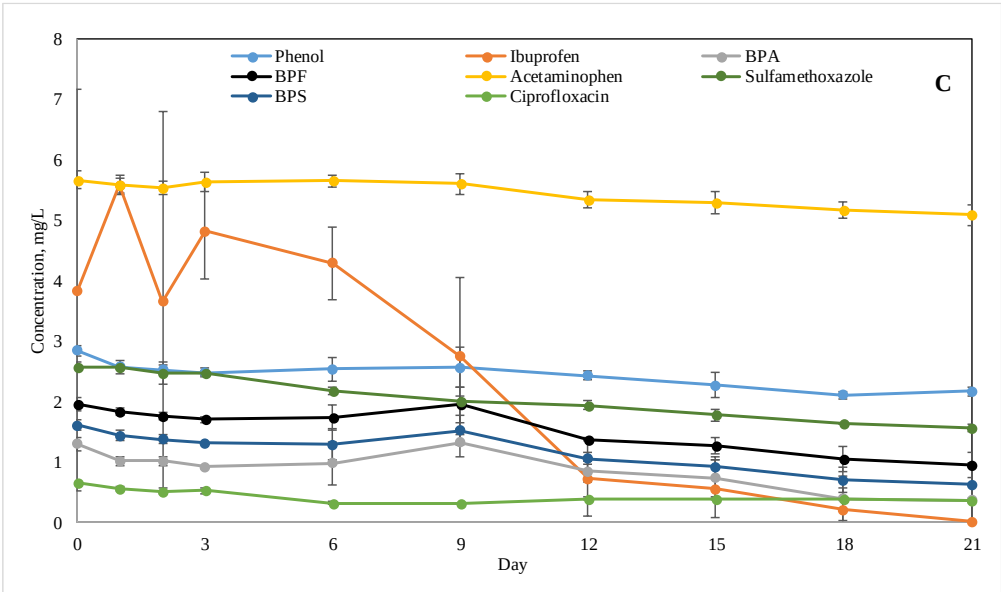


Figure 18, cont.

Both COD and TOC declined about 65% for the first three days and did not significantly change afterwards in biotic reactor as shown in Figure 19. TP had a dip after day one, but increased subsequently to over four times of initial level until end of experiment, while TN showed a minute rise on the first day but remained stable

thereafter. In abiotic reactors with and without fungal mass, around 60% reduction in TOC observed, while TN persisted virtually the same. Similar trend also can be seen for COD with over 60% and 45 % drop during the experiment time for abiotic with and without fungal mass respectively. We hypothesized that the part of the loss of carbon content in reactors are due to release of volatile organic compounds (VOCs) from LL (the reactors and both abiotic controls were aerated). TP generally showed decrease until day 6, but increased to tumble back again after day 15 to about initial level in abiotic with fungal mass and to 1.2 mg/L in abiotic without fungal mass. Although at lower concentrations, non-sterile fluidized bed reactor in municipal sewage occulated with *Trametes versicolor* reported significant removal of TP and ammonia nitrogen.[99] A study on LL treatment with *Aspergillus niger* and *Cladosporium herbarum* in presence of glucose had showed production and accumulation of nitrite, nitrate and orthophosphate in the reactor.[100] As the fungal-containing reactors (biotic and heat-treated abiotic) in this study displayed elevated concentration of TP to over 20 mg/L(in dissolved from as samples were filtered), despite abiotic without fungi remained lower than 5 mg/L, release of phosphates and organic phosphorus from the fungal biomass is the likely explanation.

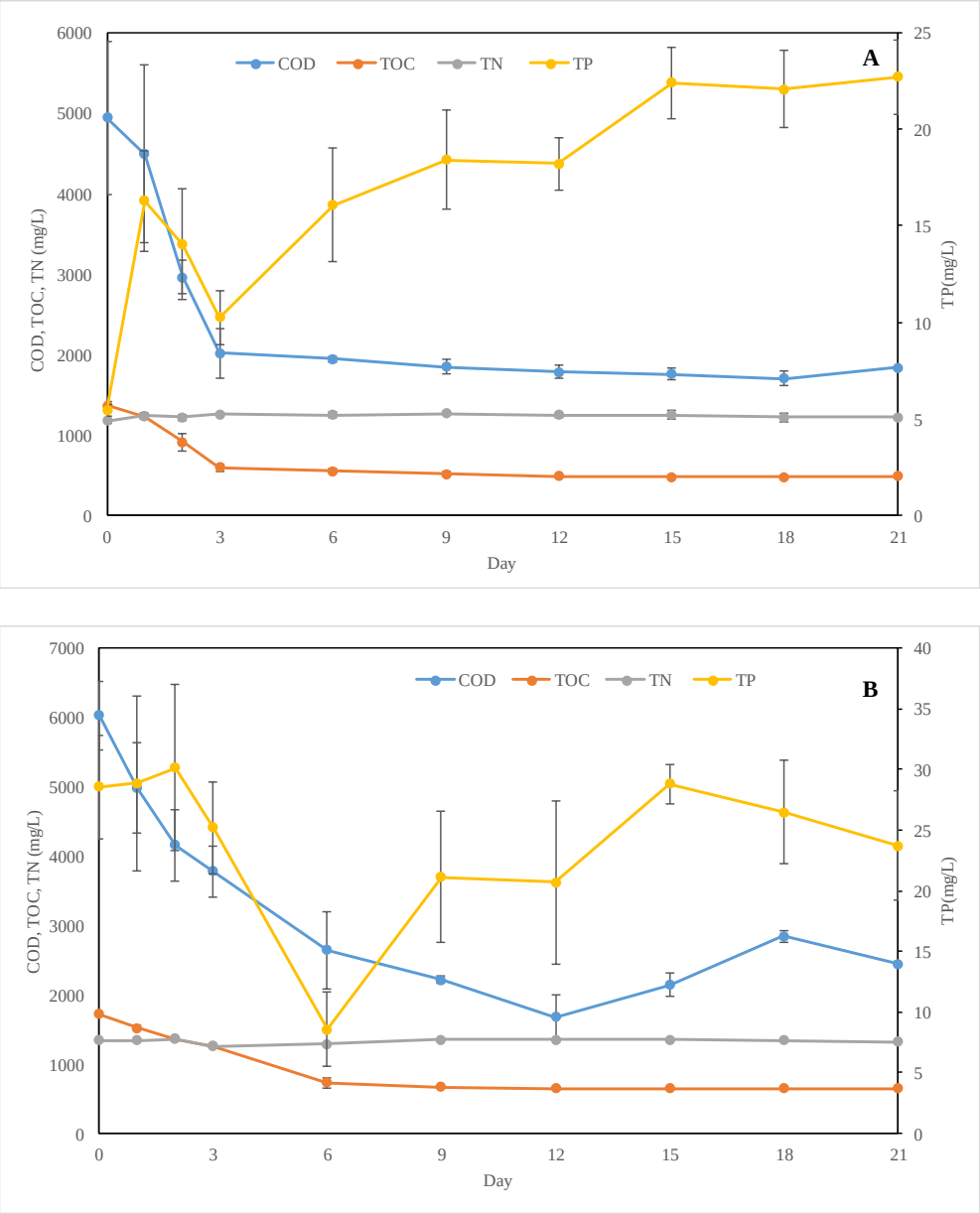


Figure 19. Aggregate parameters in fungal batch reactors(mean±SD); Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

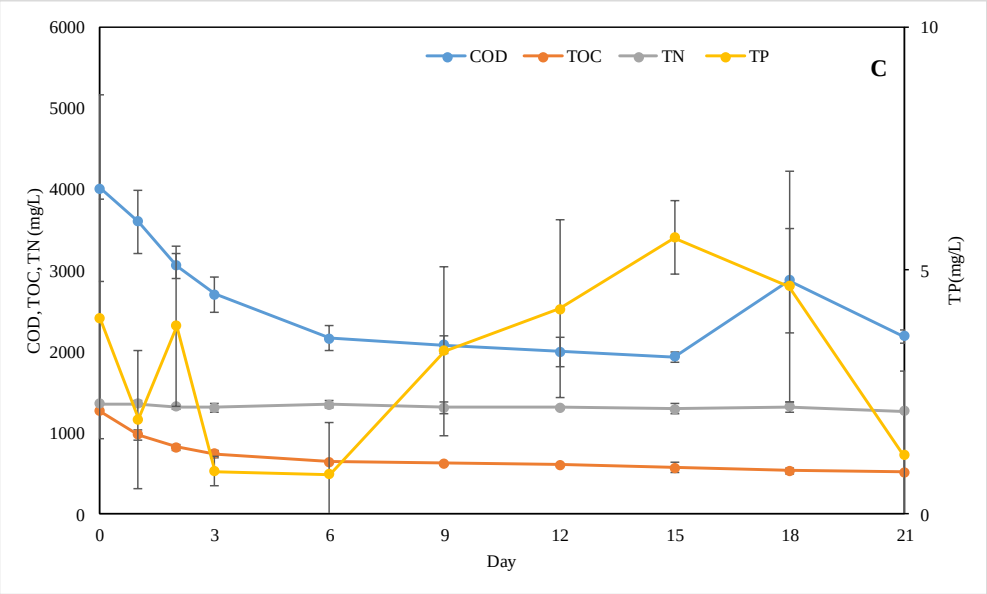
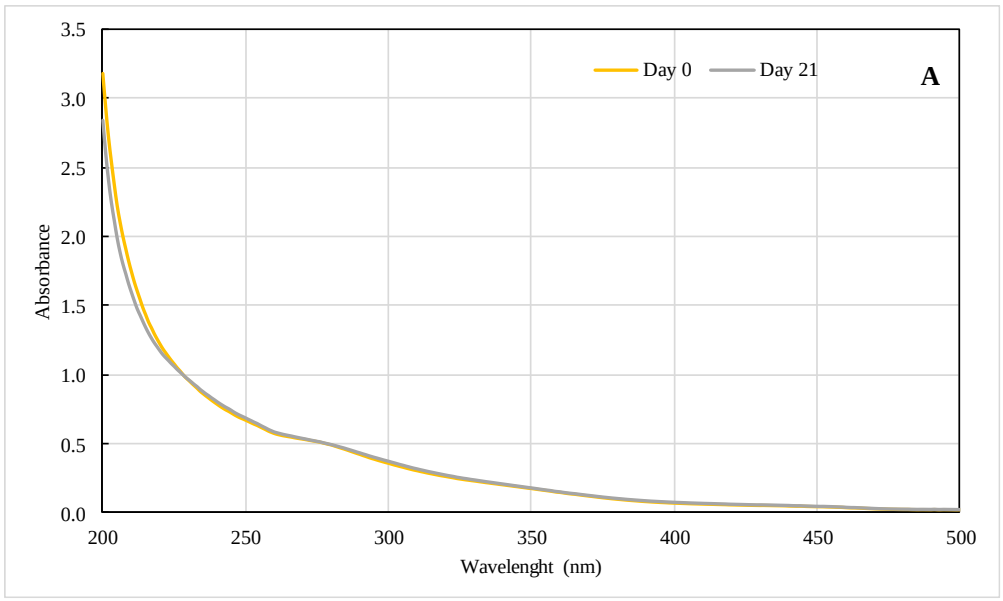


Figure 19, cont.

Figure 20 shows the absorbance scan of biotic and abiotic reactors at the start and the end of the experiment. The curve in biotic reactor shows a smooth trend and almost no reduction is absorbance over the 21-day course of the experiment. Both abiotic reactors had a peak around 240 nm before and after experiment. It is possible that heat-treatment changed part of humic substances in the LL which created this absorbance peak in abiotic reactors.



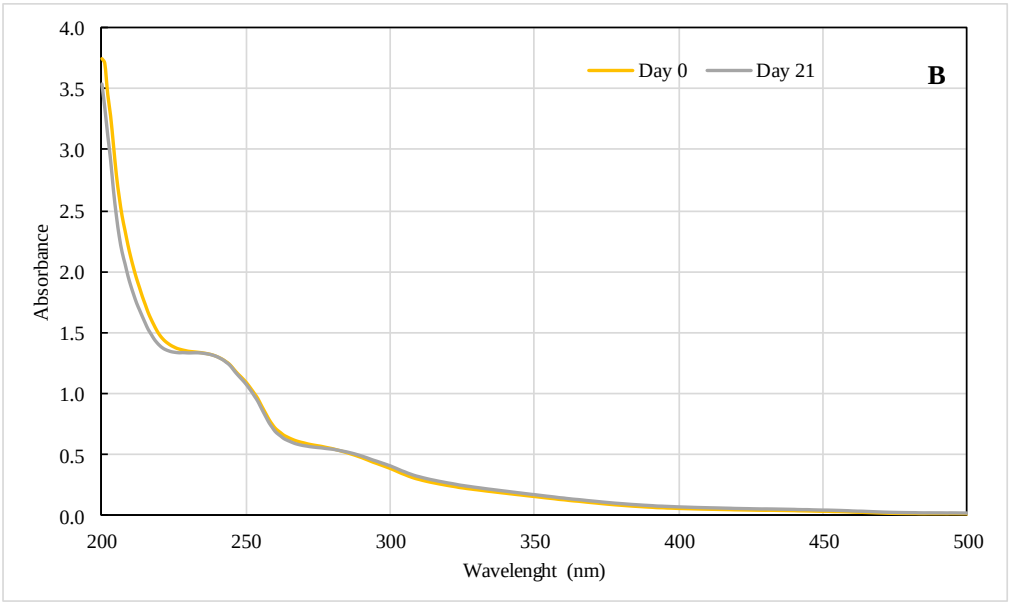


Figure 20. Absorbance (mean, x20 diluted) of reactors at day 0 and 21; Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

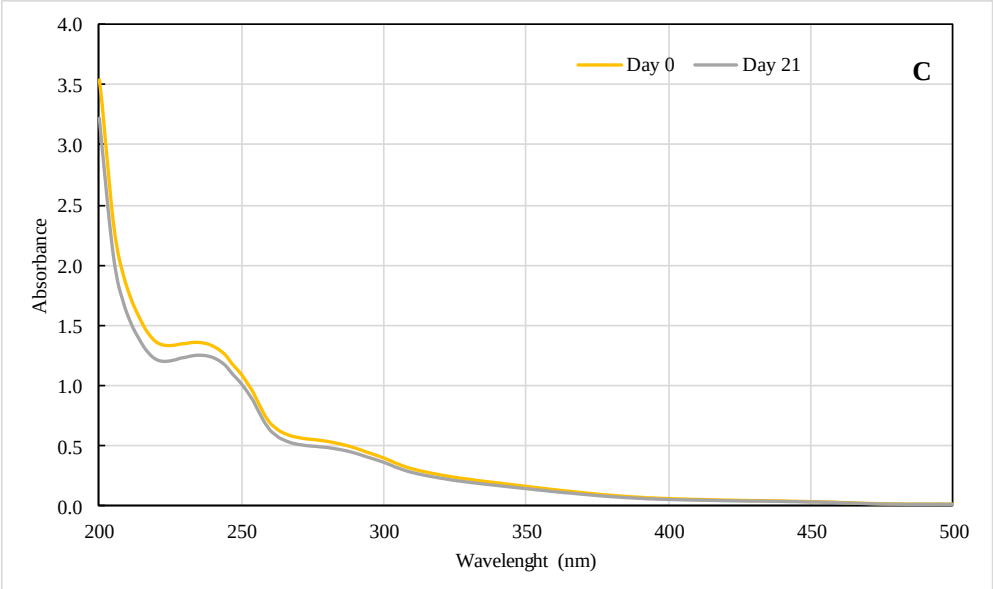


Figure 20, cont.

3.6.2.Study II

The fungal mass of this study was 1.1225 g. Figure 21, illustrates removal of selected contaminants in biotic and abiotic batch fungal reactors. Phenol was the only compound removed completely from the biotic reactor within two days, but remained virtually constant in the abiotic reactors. Phenol's complete removal within a day at 1mM in immobilized *Trametes pubescens* fed by glucose was previously reported.[82] Bioaugmentation of AS batch system with *PC* for treatment of phenol wastewater showed improvement in the performance up to 80% higher removal compared to control AS reactor.[101]

DOX were not detectable after day 3 in biotic reactors and after day 7 in abiotic reactors while no significant change was observed for BPA among all reactors. Margins of error (i.e. SD) in the measurements for ACT and BPF were significant that no conclusion can be drawn about the removal of these two compounds. Microbial removal

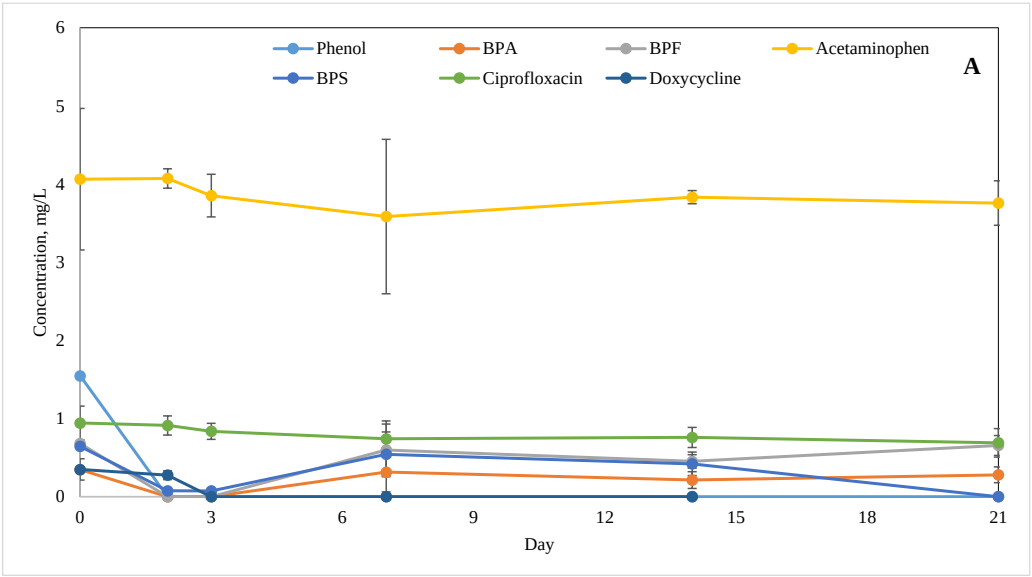
of other analytes was not considerable. ACT concentration was virtually constant in abiotic reactors without fungal mass, while about 20% reduction observed possibly by abiotic sorption onto fungi. ACT and IBP at low concentrations (<2.5 µg/L) were previously reported removed in non-sterile urban sewage with *Trametes versicolor* batch fluidized bed bioreactor.[53]

No significant removal was observed for spiked bisphenols in biotic reactors. Majority of successful studies on removal bisphenols were through enzymes extracted from WRF immobilized on various organic or inorganic media, and direct enzymatic treatment.[93–97] Unsuccessful removal of BPA at high concentration (<10 ppm) with *PC* was reported after 14 days of incubation in malt extract-glucose medium.[35]

CIP removal was approximately 20% in biotic reactors, around 40% in abiotic reactors with fungi, but remained virtually constant in abiotic reactor without fungi. *Trametes versicolor* incubated in malt extract broth, removed more than 90% of CIP (at 2 mg/L) after 7 days as reported by Prieto et. al [98]. Poor removal of CIP was reported overall with *Trametes versicolor* regardless of operation conditions in another study.[102]

Statistical analysis demonstrated significant difference between biotic and abiotic reactors without fungi for phenol, ACT, CIP and IBP ($p<0.05$). Other than IBP, the rest of analytes show results consistent with study I (3.7.1).

WRF removes contaminants by bioadsorption and biotransformation processes (e.g. biomineralization and biodegradation) facilitated by both extracellular and intracellular enzymatic systems [103], it is not easy to distinguish which process would participate in the removal of each analyte.



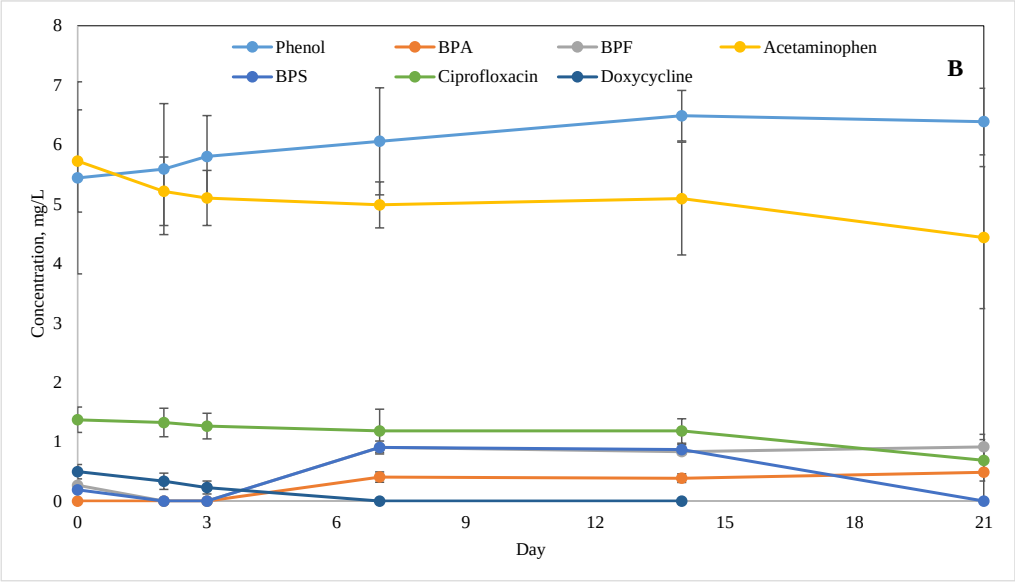


Figure 21. Contaminants removal in fungal batch reactors(mean±SD); Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

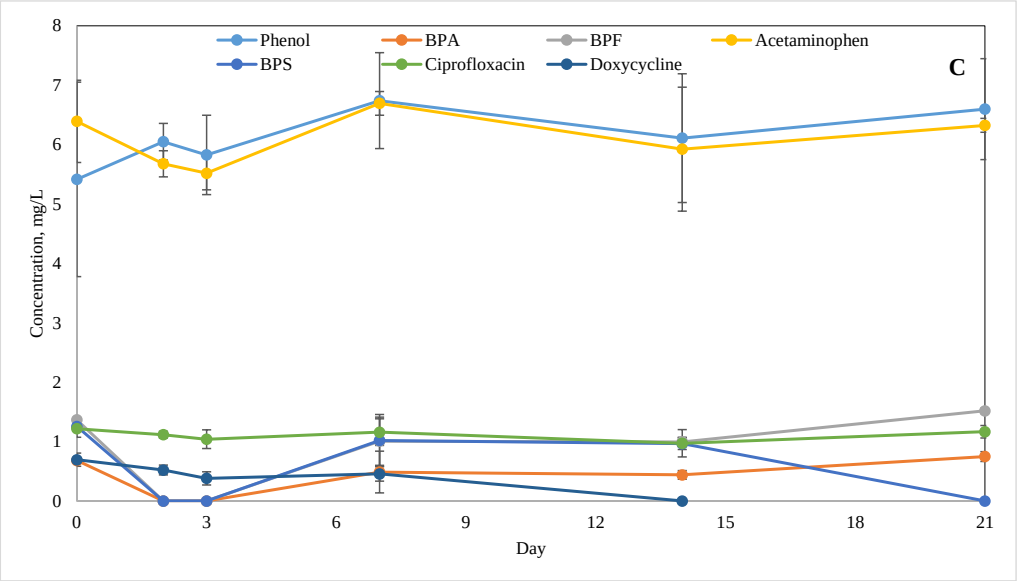
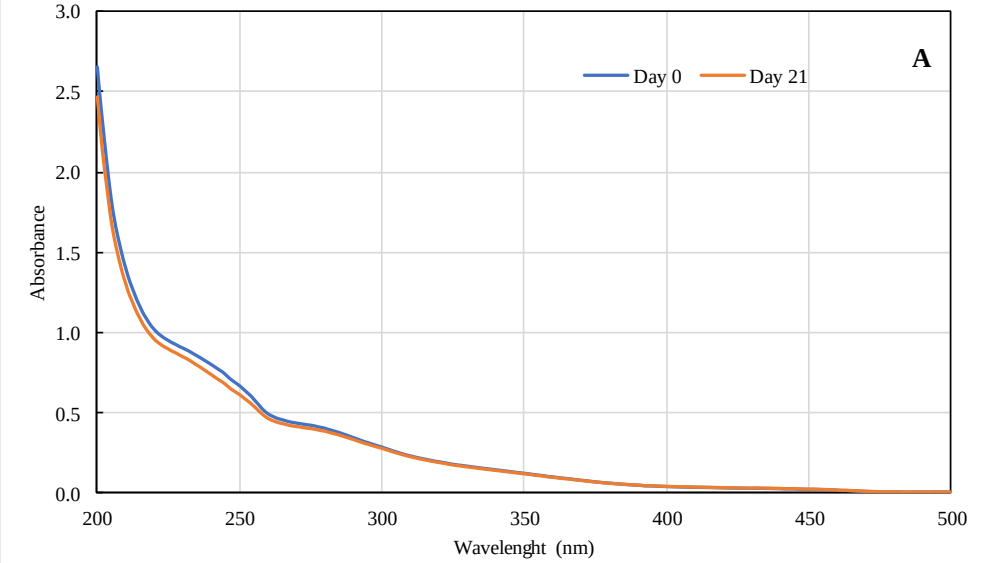


Figure 21, cont.

In biotic reactors and abiotic reactors without fungi, COD removals was similar at about 40%, while in abiotic with fungi it was only 12%. Therefore, biotic removal of carbon content was not significant.

The change in the absorbance scan in biotic and abiotic reactors is shown in Figure 22. The absorbance in biotic reactor was reduced by up to 10% (7.5% at 254 nm), while the absorbance for both abiotic reactors increased by the end of experiment. When comparing day 21 absorbance of biotic reactors with abiotic ones (Figure 22, D), the former is around 25% lower than the latter and the difference is significant. This change was visually observable.



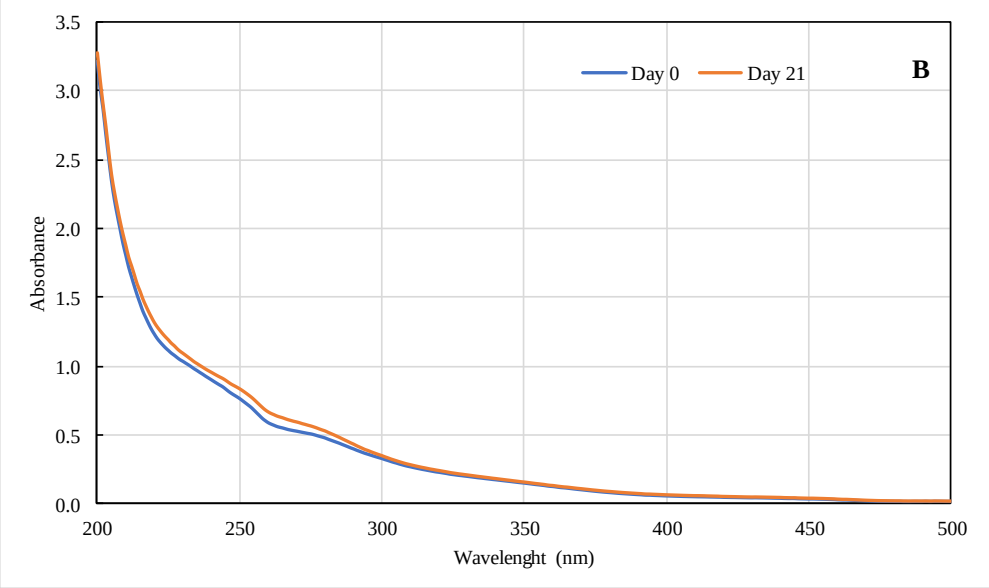
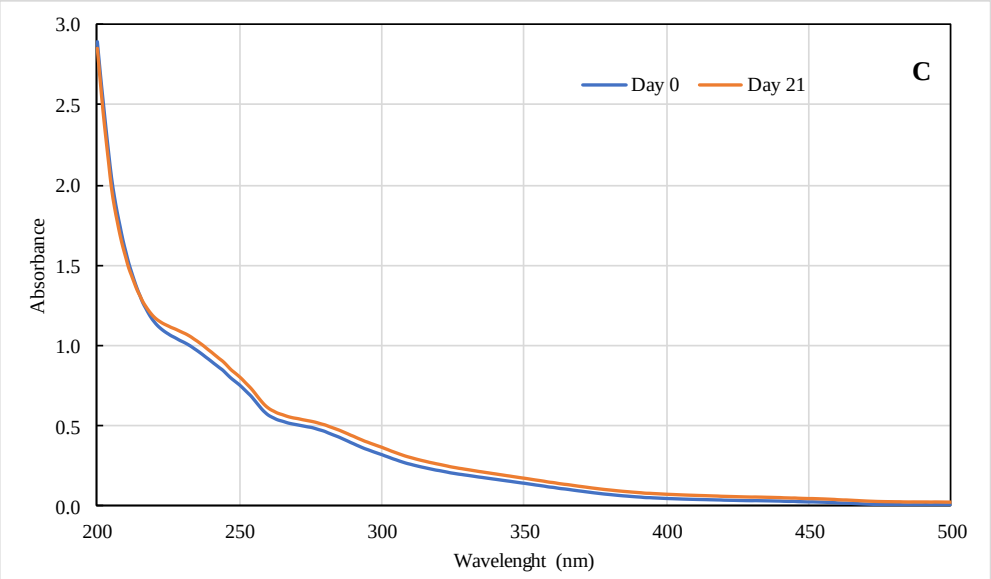


Figure 22. Absorbance (mean, x20 diluted) of reactors at day 0 and 21; Biotic (A), abiotic with fungal mass (B), abiotic without fungal mass (C), Biotic vs. abiotics at day 21 (D)



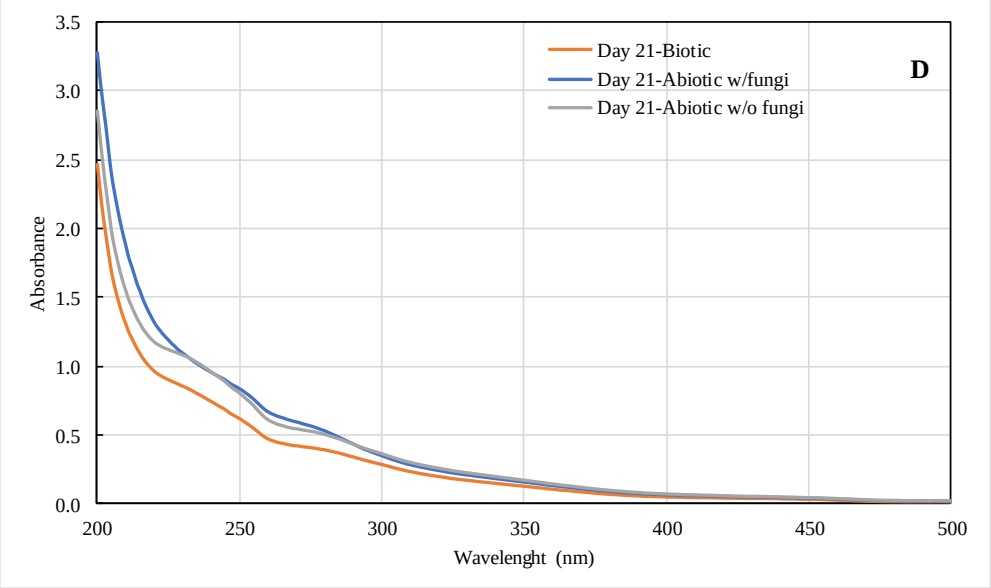


Figure 22, cont.

In biotic reactors, the pH increased to 5.5 over the operation period from the initial pH of 4.5. In all abiotic reactors, the stabilization of pH to 7 occurred within 2 days from the start of the experiment.

3.6.3.Study on fungal bioreactors with various leachates for absorbance reduction

In this part of the study the results from operation of five bioreactors (no replication) with various LL from different sites is provided. The fungal mass in the sacrificed reactor of this study was 0.74 g.

In fungal batch reactor with leachate from site 1 (S1) (Figure 23), reduction in COD and TOC were 24.5% and 52% respectively, while TN remained virtually constant during the experiment. In abiotic with fungi, increase observed in all parameters due to degradation of fungal mass, while all parameters did not change significantly in abiotic without fungi except 15% reduction in TN. Statistical analysis between biotic reactor and abiotic without fungi showed almost significant difference for TOC removal (p=0.0733). Reduction in carbon content was significant in the first day and slowed down afterwards.

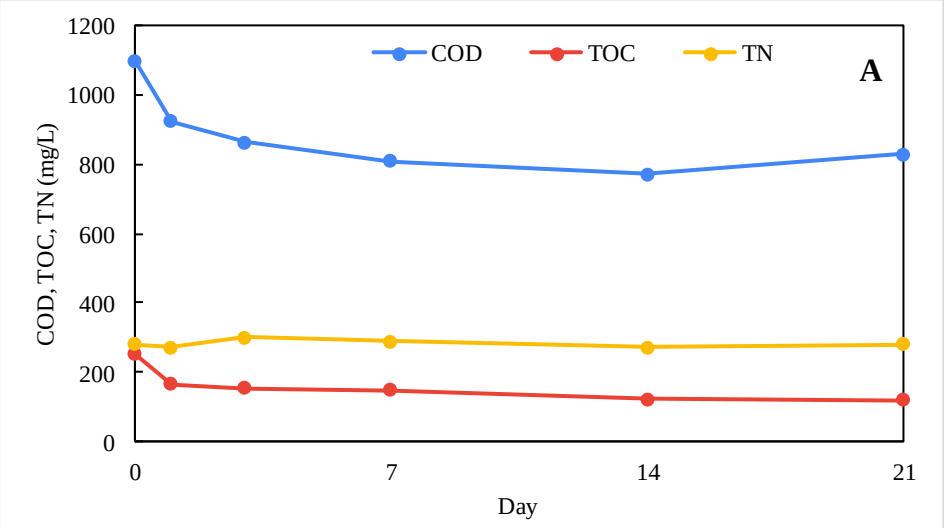
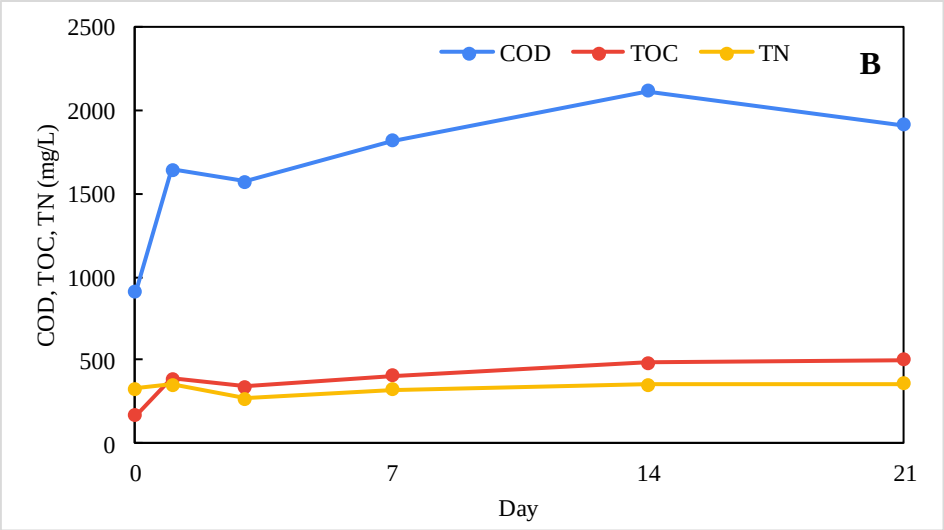


Figure 23. Aggregate parameters in fungal batch reactors for S1 leachate; Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)



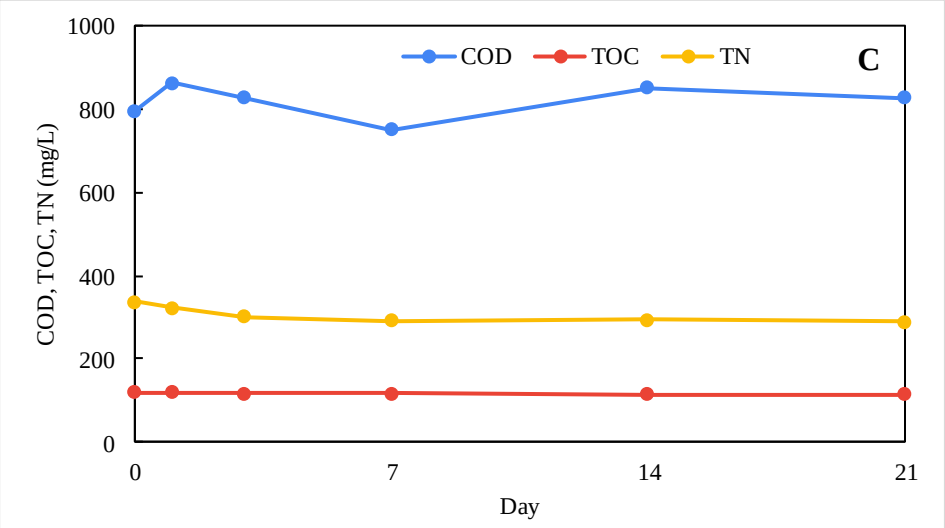


Figure 23, cont.

LL from site 2 active cell (S2A) treated in fungal batch reactors (Figure 24), showed 19.5% and 36.5% reduction in COD and TOC while 19% increase in TN. The trend was the same for abiotic reactors, as S1 sample, with 14% increase in TN for abiotic without fungi and significant increase of all parameters in abiotic with fungi. Both of carbon content parameters (COD and TOC) showed significant difference ($p<0.05$) in statistical analysis, in comparison of biotic and abiotic without fungi reactors.

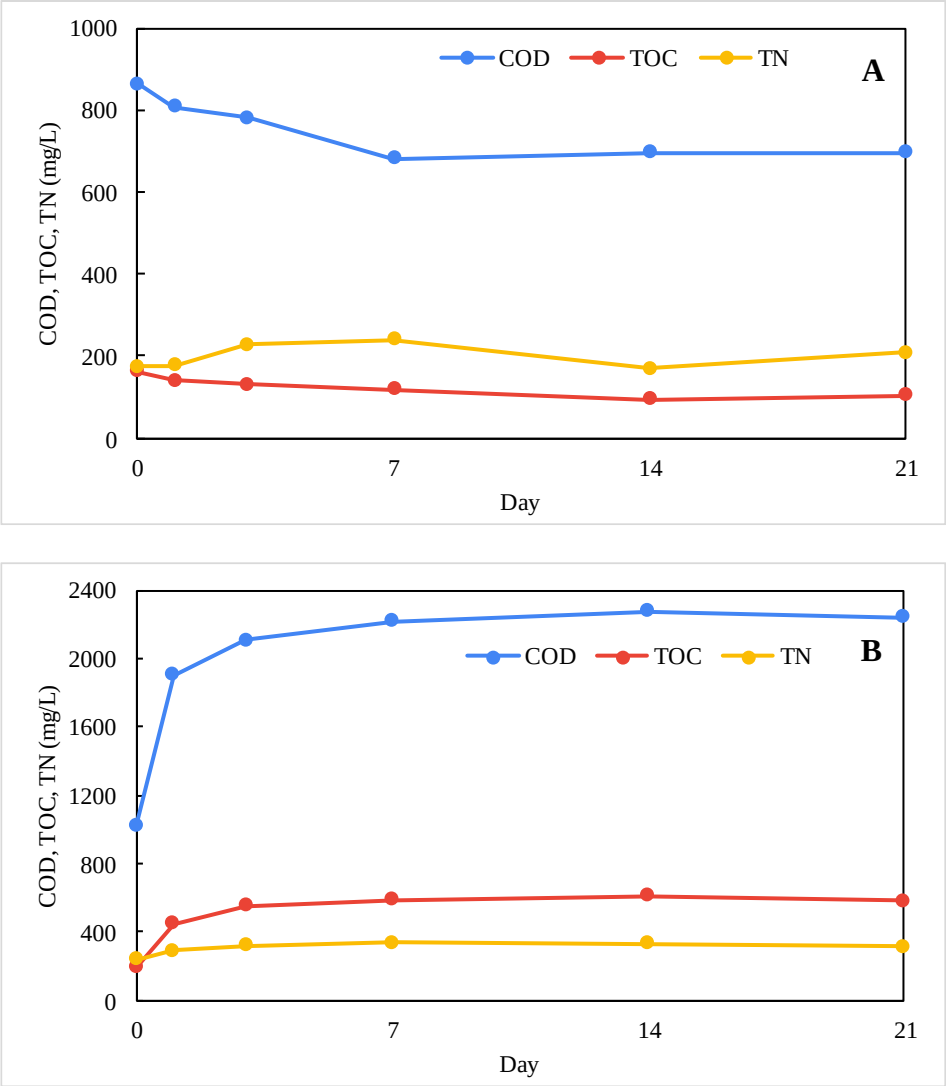


Figure 24. Aggregate parameters in fungal batch reactors for S2A leachate; Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

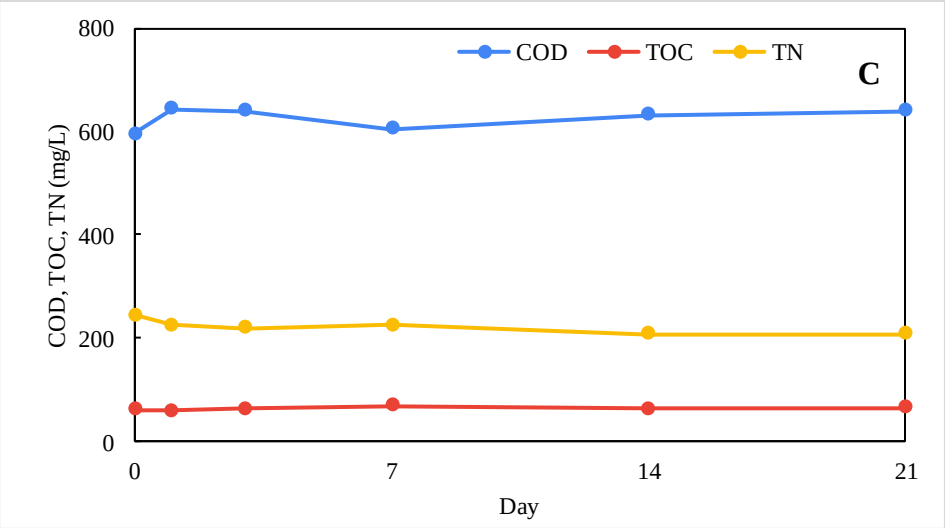


Figure 24, cont.

In fungal batch reactor with leachate from site 2 closed cell (S2C) (Figure 25), 24.5% and 41% decline was observed for COD and TOC while TN had a minute increase. Significant increase in all parameters in abiotic with fungal mass is similar to other reactors of same type, whereas no substantial change happened in abiotic without fungi. Only TOC showed significant statistical variance ($p<0.05$) between biotic and abiotic without fungi reactors.

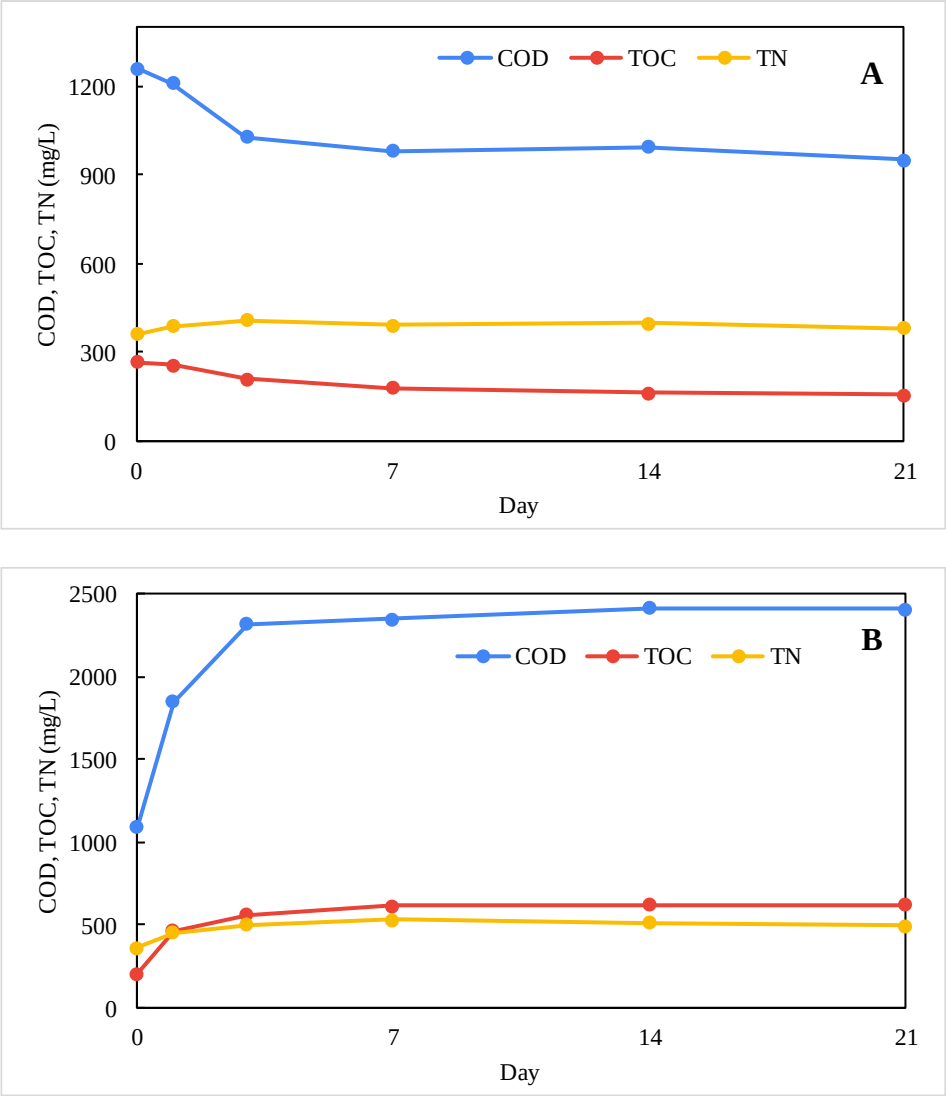


Figure 25. Aggregate parameters in fungal batch reactors for S2C leachate; Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

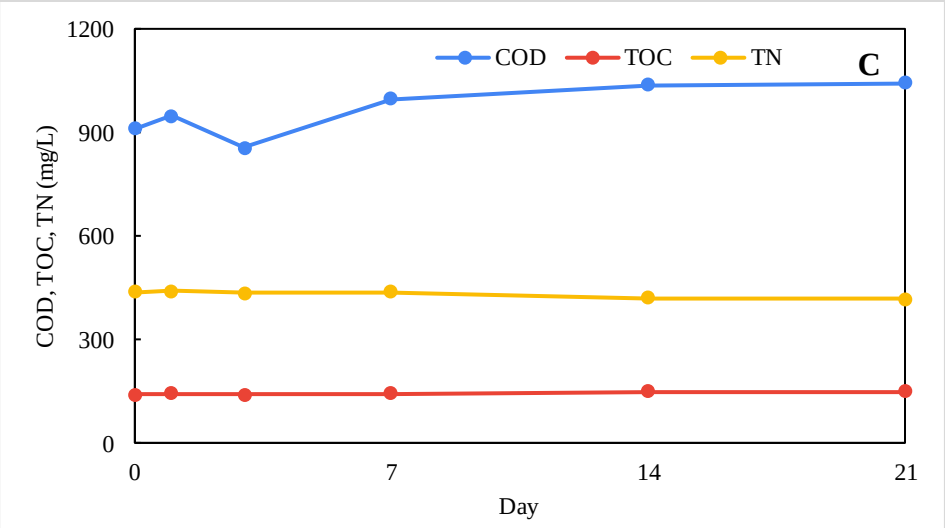


Figure 25, cont.

Leachate from mixed cell landfill, treated in fungal batch reactors (Figure 26) had 21.5%, 32% and 7% reduction respectively in COD, TOC and TN. In the abiotic reactor without fungi, same decrease in TN was observed and no notable change in the rest of the parameters. In the abiotic reactor with fungi, significant increase in all parameters has been observed. None of aggregate parameters showed significant difference between biotic and abiotic without fungal mass reactors.

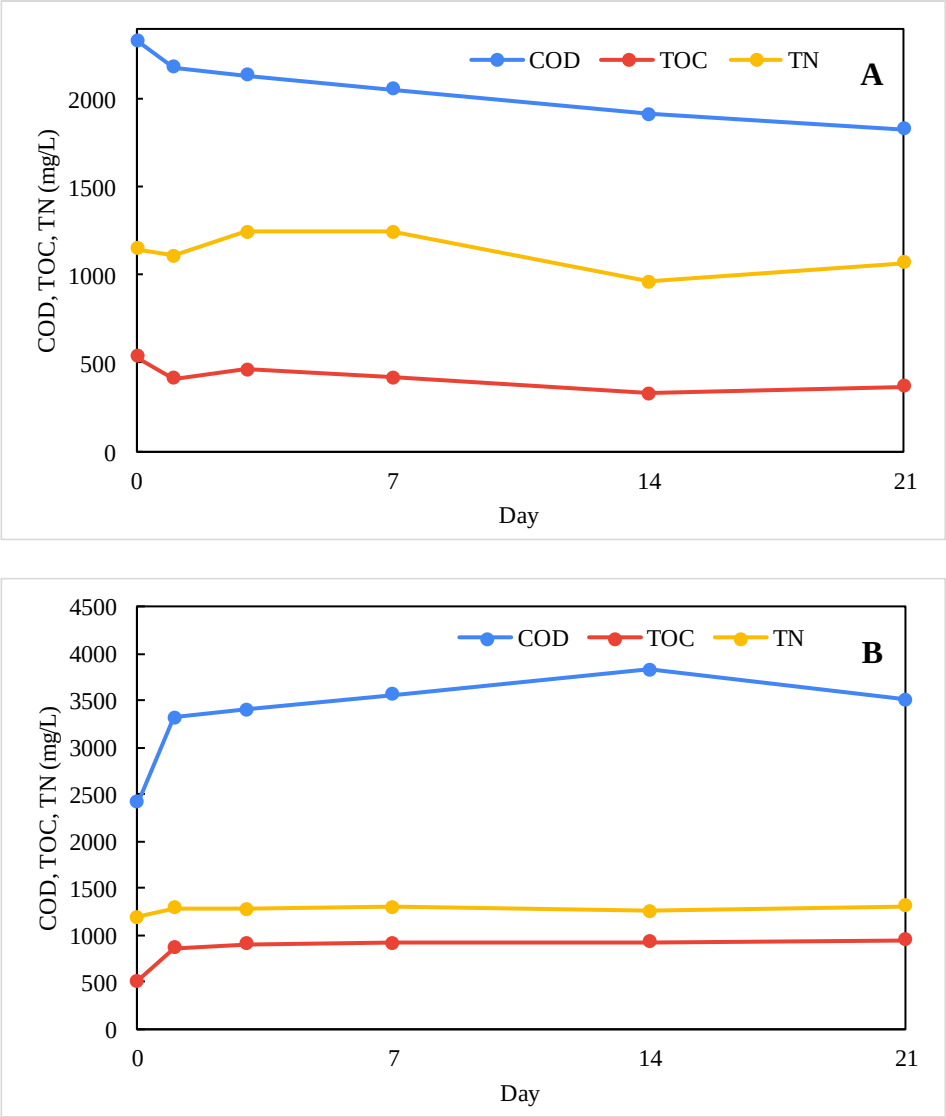


Figure 26. Aggregate parameters in fungal batch reactors for mixed cell leachate; Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

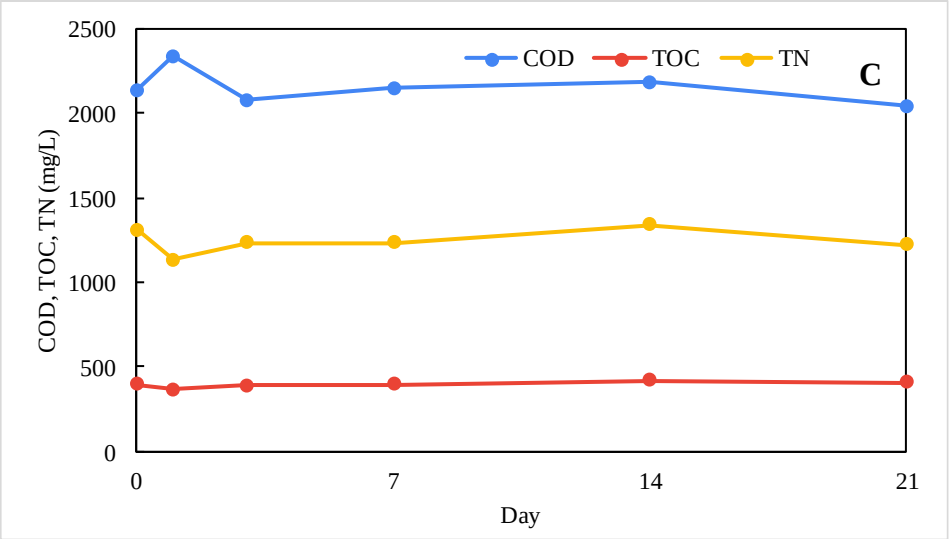


Figure 26, cont.

In fungal batch reactor with leachate from site 4 closed cell (S4C) (Figure 27), 37.5% and 47.5% decrease in COD and TOC was observed, with no considerable change in TN. Abiotic reactor with fungal mass, had a significant rise in all parameters, while abiotic without fungi showed 10% and 17% reduction in COD and TOC. There was no statistically significant difference between the biotic reactor and abiotic reactor without fungal mass in aggregate parameters.

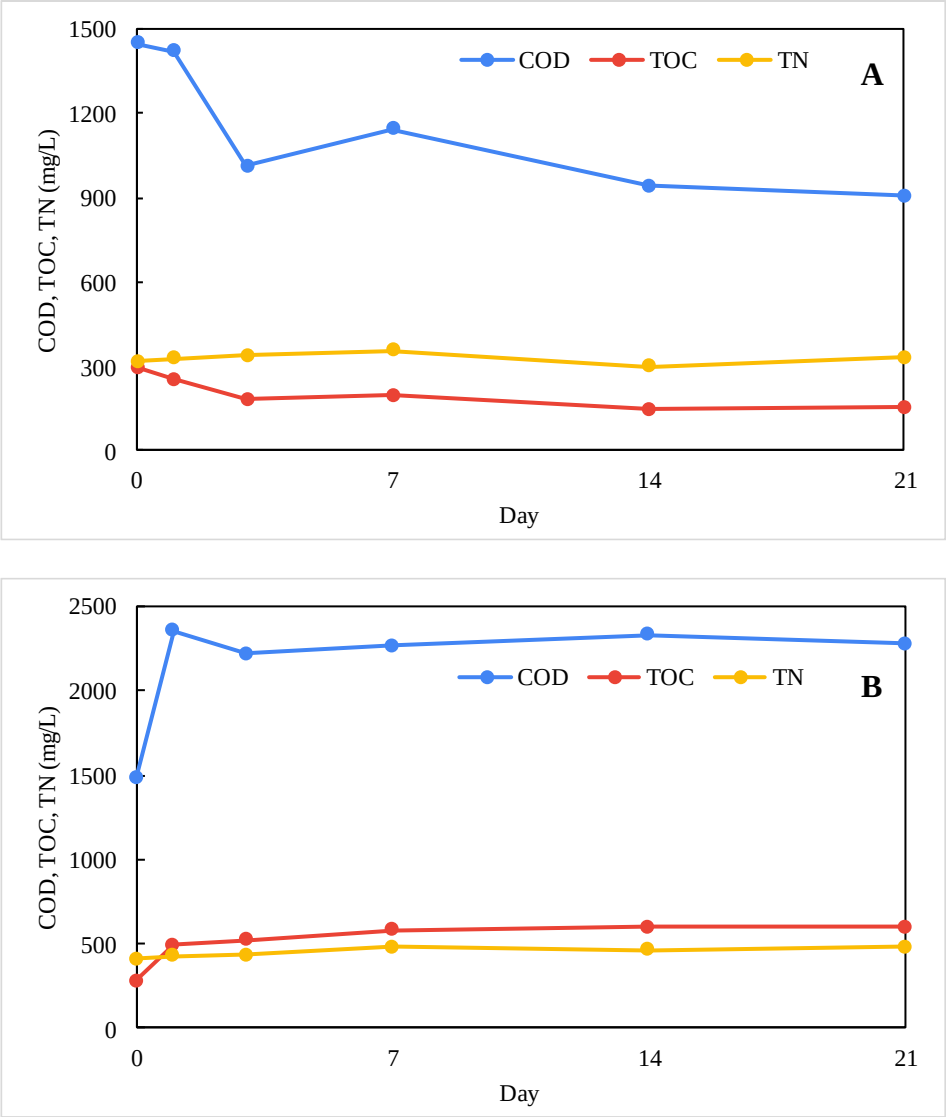


Figure 27. Aggregate parameters in fungal batch reactors for S4C leachate; Biotic(A), abiotic with fungal mass (B), abiotic without fungal mass (C)

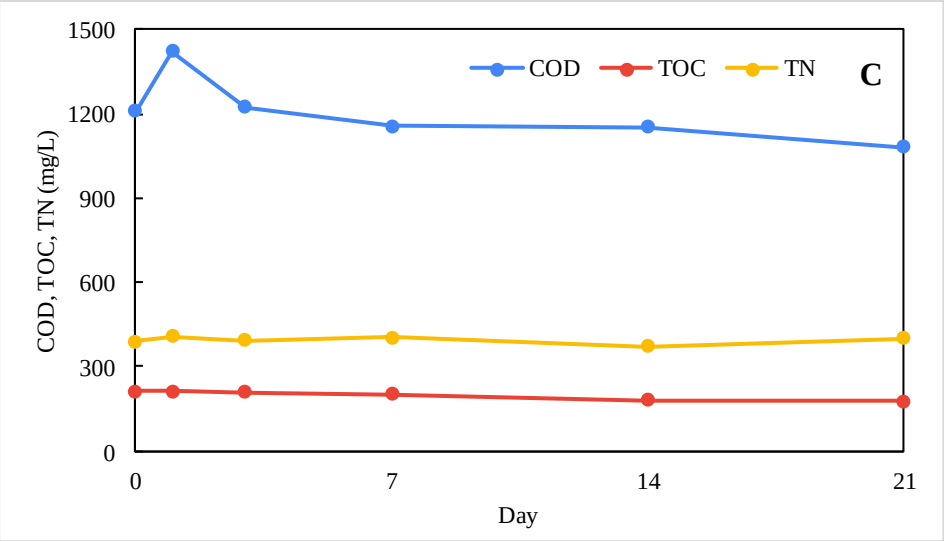


Figure 27, cont.

In conclusion three of the biotic leachate treatment reactors - S1, S2A and SC- showed to have significant difference in removal of TOC compared to abiotic reactors without fungi.

Although the pH in the reactors was adjusted to approximately 5 at the start of the experiment, on day 3 for biotic and on day 1 in abiotic reactors, the measured pH was neutral. The pH remained stable for the rest of the experiment in all reactors with different LL.

Immobilized *Ganoderma australe* reportedly removed up to 22.8% or 32% of the COD depending on the landfill leachate characteristics.[47,104] This is comparable with COD removal of 19.5-37.5% in fungal batch reactor in this part of the study.

Figures 28 to 32 illustrate absorbance scan of LL samples in biotic and abiotic reactors for day 0 and 21, end of experiment.

For S1 LL, absorbance decreased insignificantly from Day 0 to Day 21 after fungal treatment compared to the abiotic reactor without fungi (Figure 28). On the other hand,

abiotic with fungi showed an increase, as expected, due to degradation of fungal mass. When absorbance on the last of day of the experiment is compared, the biotic reactor showed increase in absorbance vs. abiotic reactor without fungi. In other words, biotic reactor showed negative efficiency.

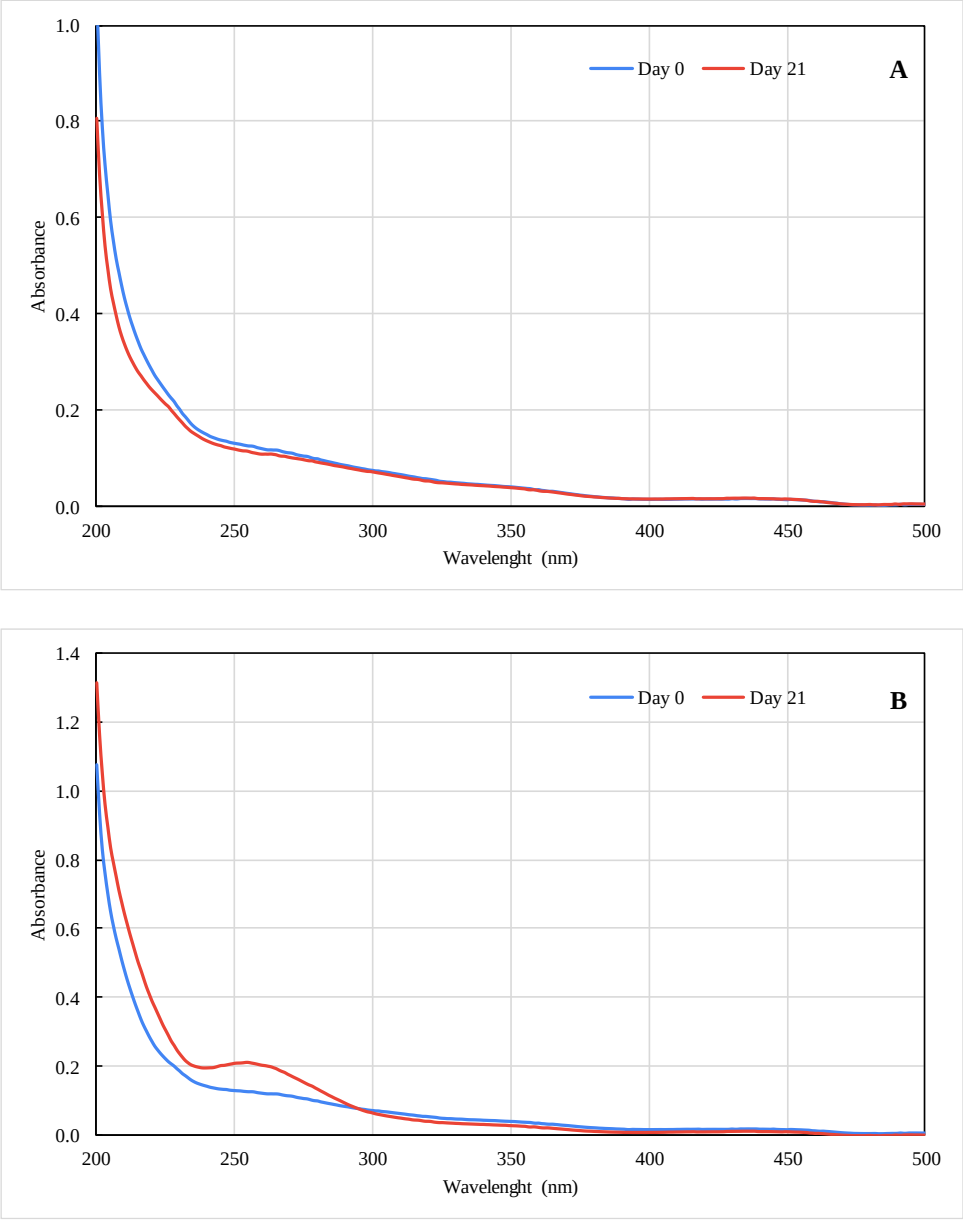
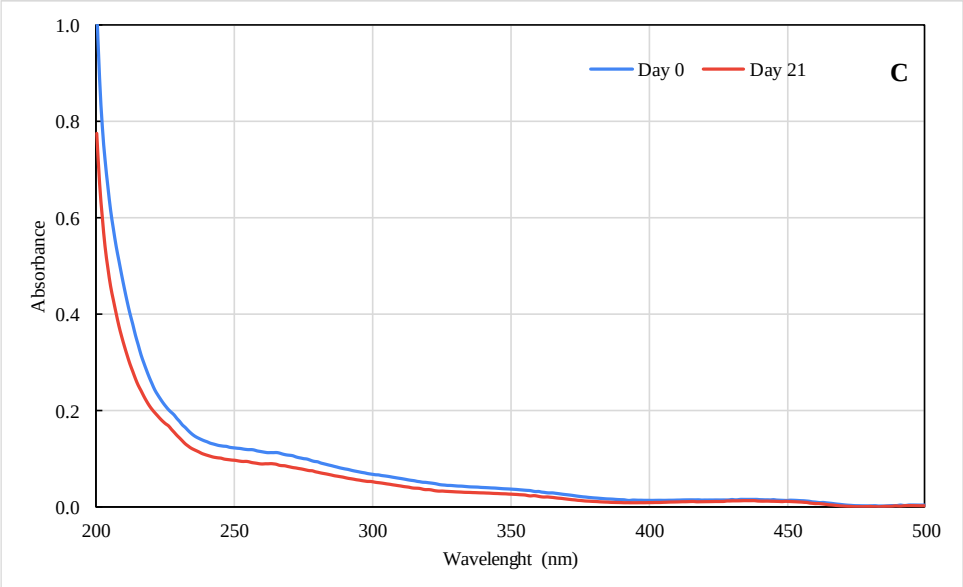


Figure 28. Absorbance (x20 diluted) of reactors for S1 leachate; Biotic (A), abiotic with fungal mass (B), abiotic without fungal mass (C), Biotic vs. abiotics at day 21 (D)



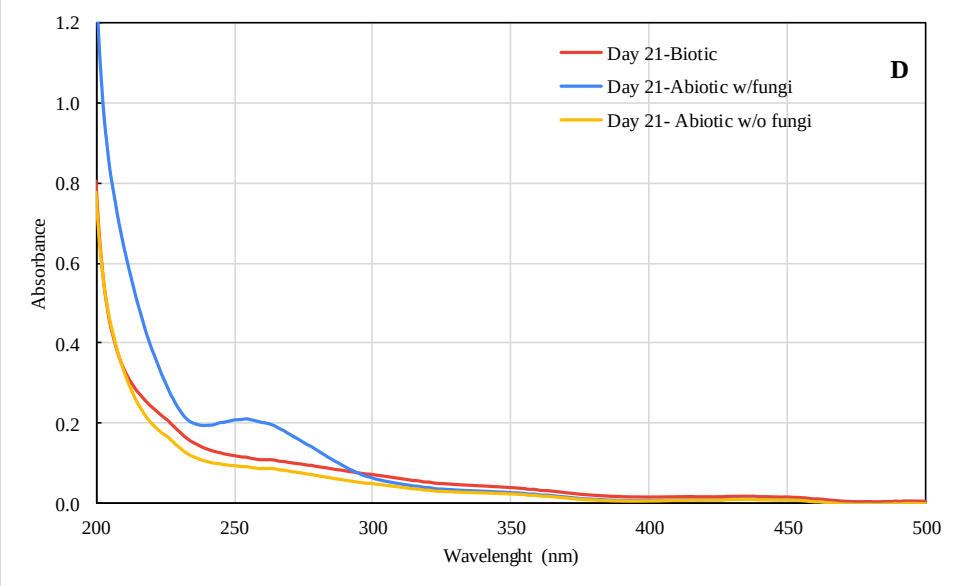
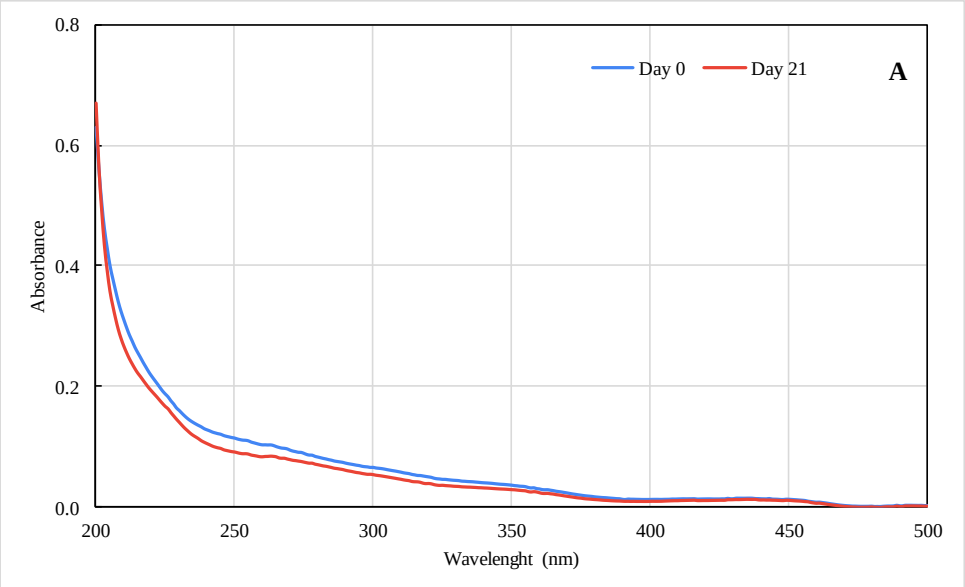


Figure 28, cont.

For S2A LL, absorbance was reduced in the biotic reactor similar to abiotic reactor without fungal mass (Figure 29). Abiotic reactor with fungi showed a significant increase in absorbance especially around 250 nm. Comparing absorbance on the last day of the experiment, biotic reactor showed noticeable increase compare to abiotic reactor without fungi, just as with the first sample.



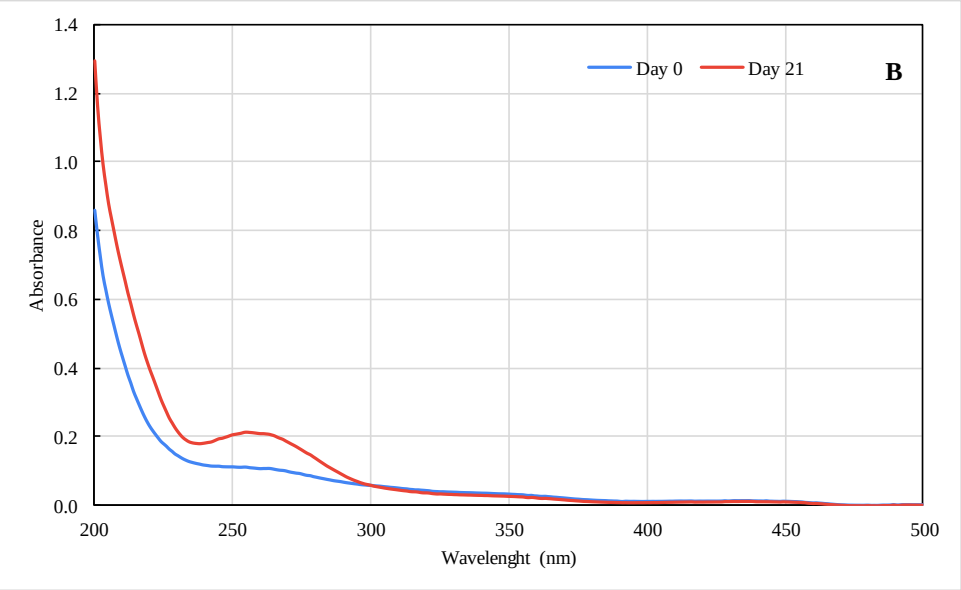
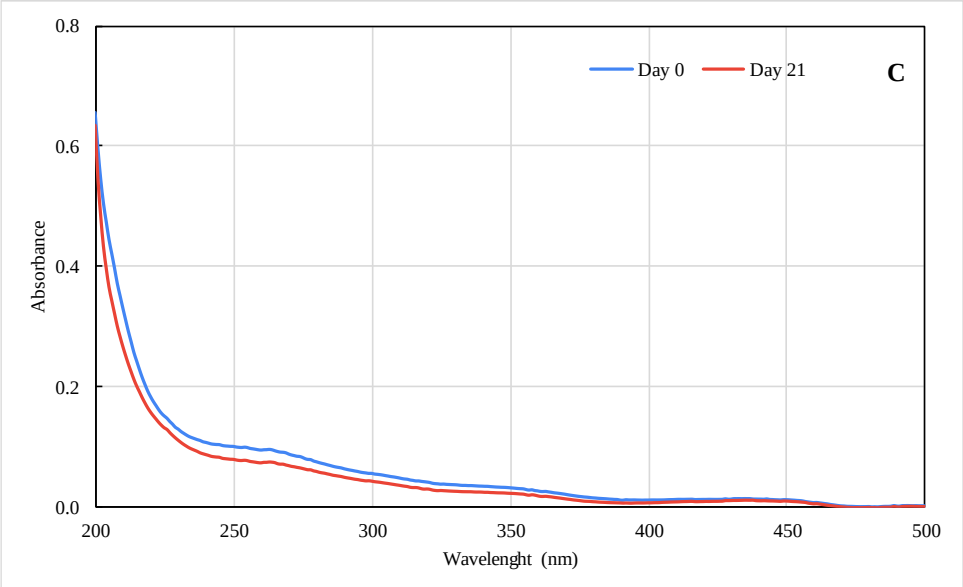


Figure 29. Absorbance (x20 diluted) of reactors for S2A leachate; Biotic (A), abiotic with fungal mass (B), abiotic without fungal mass (C), Biotic vs. abiotics at day 21 (D)



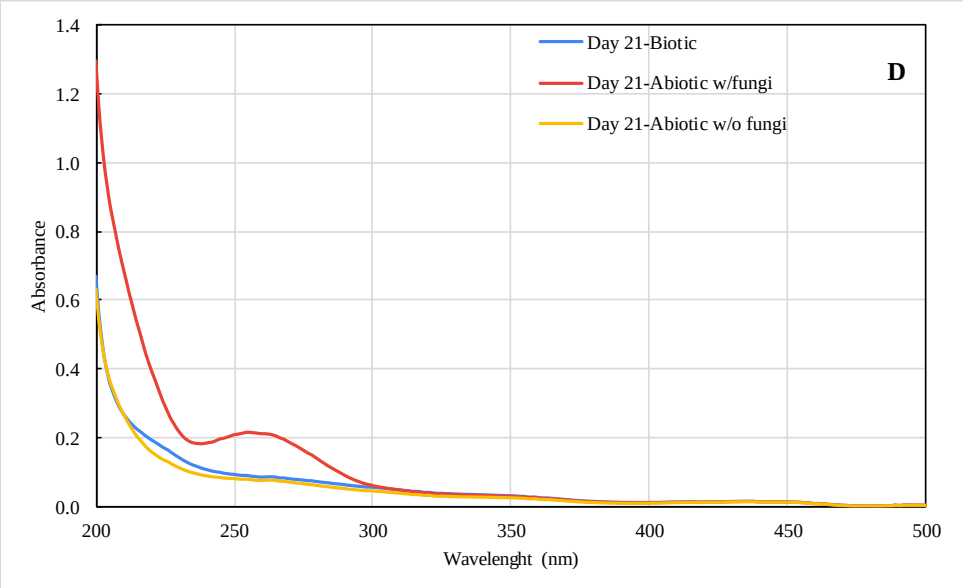
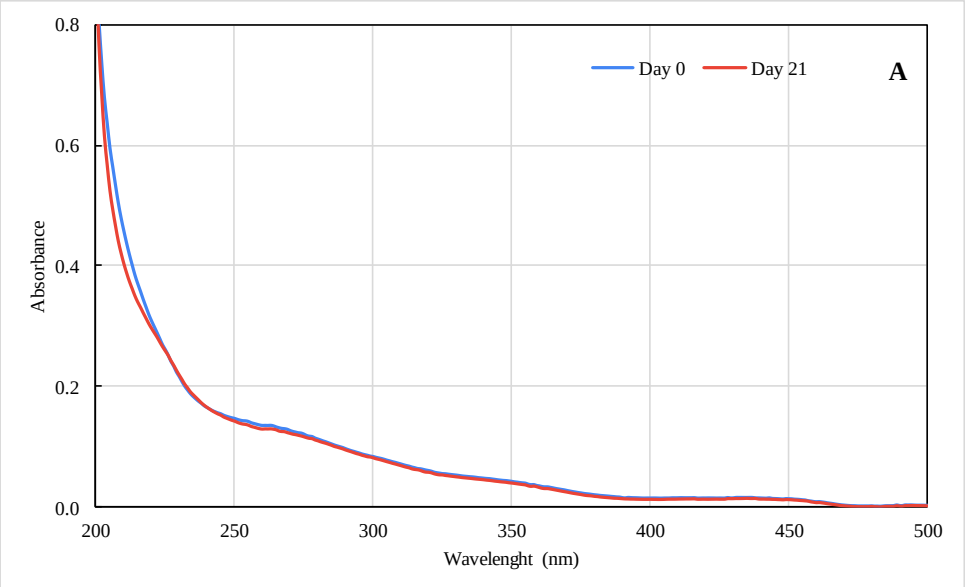


Figure 29, cont.

Next LL sample is S2C, in which biotic reactor did not show significant change in absorbance whereas abiotic without fungi had limited reduction (Figure 30). Abiotic with fungi, as usual, showed notable increase over the course of experiment. Absorbance scans were essentially the same when comparing the results of biotic reactor and abiotic reactor without fungi at the end of experiment.



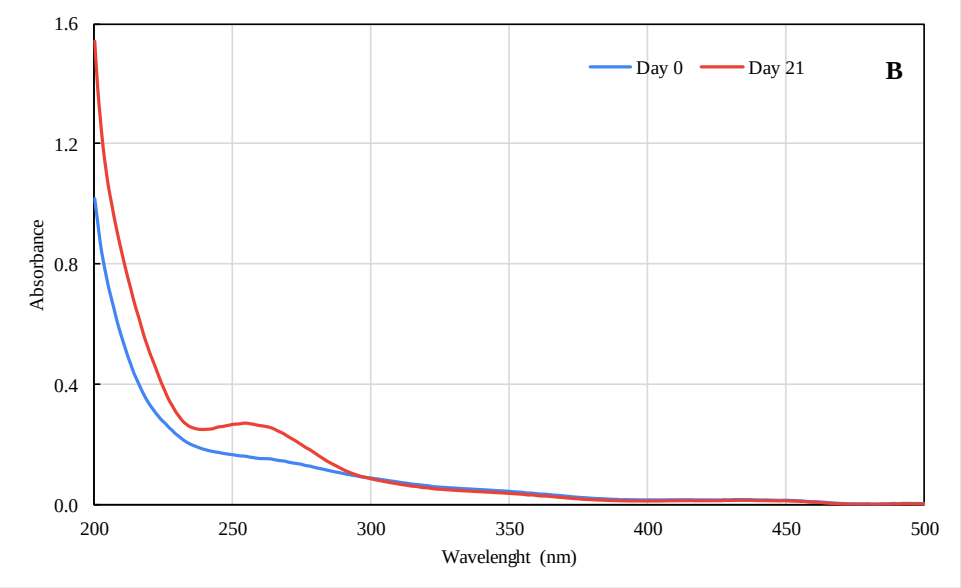
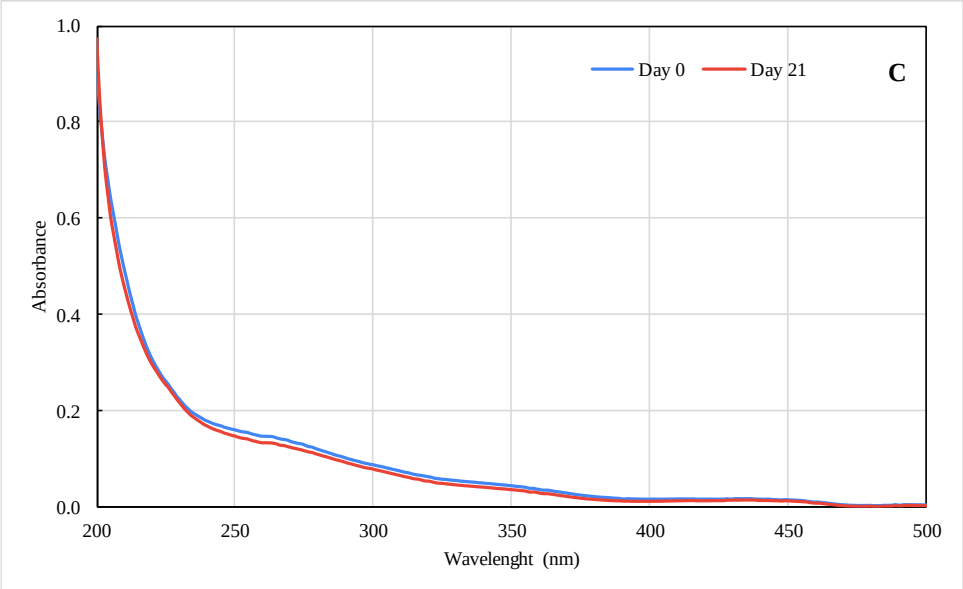


Figure 30. Absorbance (x20 diluted) of reactors for S2C leachate; Biotic (A), abiotic with fungal mass (B), abiotic without fungal mass (C), Biotic vs. abiotics at day 21 (D)



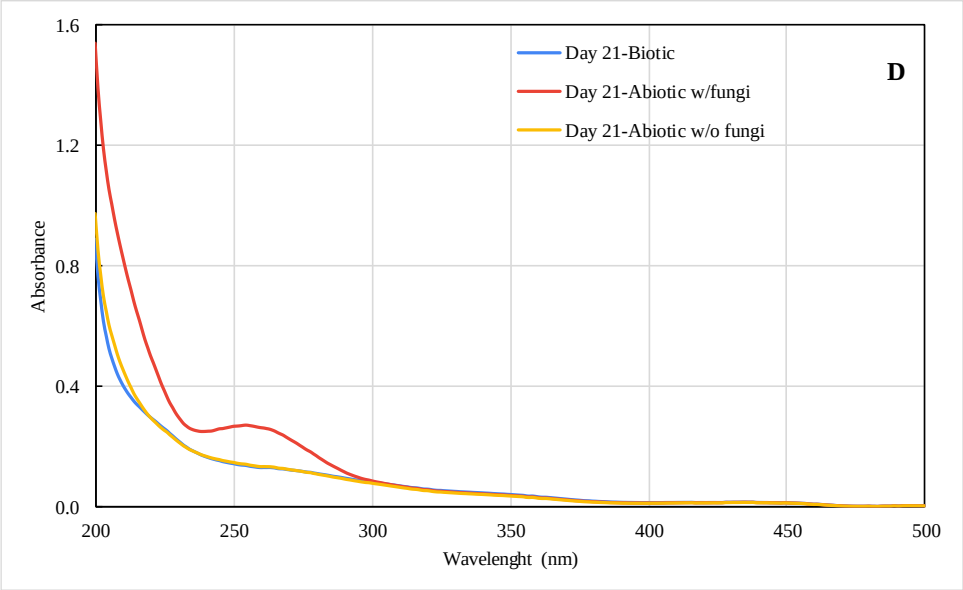


Figure 30, cont.

When looking at the most humic leachate sample of the set, with highest molecular weight LL sample (3.6.4) among other ones (mixed cell LL), biotic reactor showed noticeable reduction of absorbance while abiotic without fungi was stable over the course of experiment (Figure 31). Increase in absorbance in abiotic with fungal mass has been observed similar to the other reactors of same type. At the last day of the test, absorbance

of biotic reactor was significantly lower than that of abiotic without fungi (17.6% at 254nm) especially at 270 nm or lower.

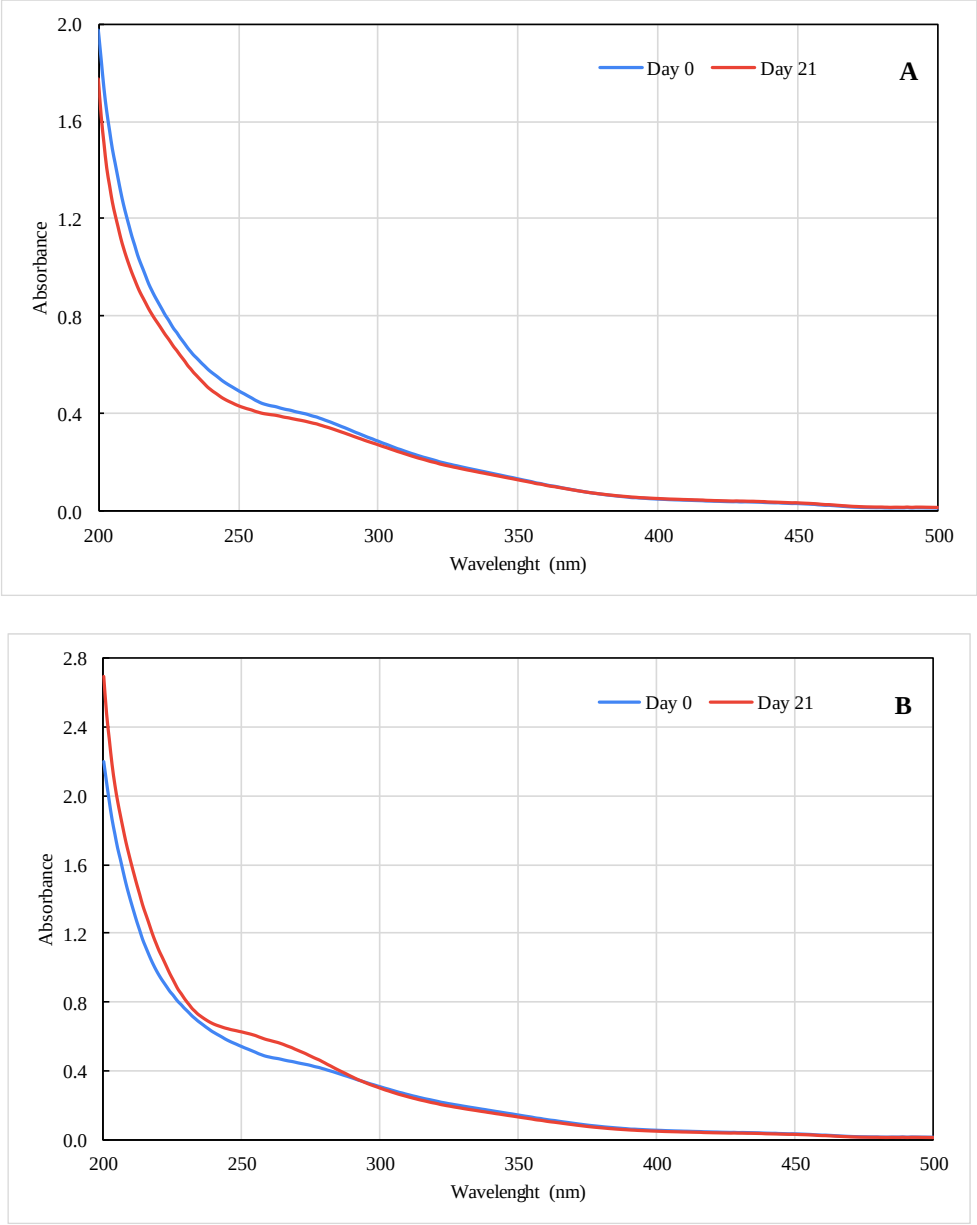
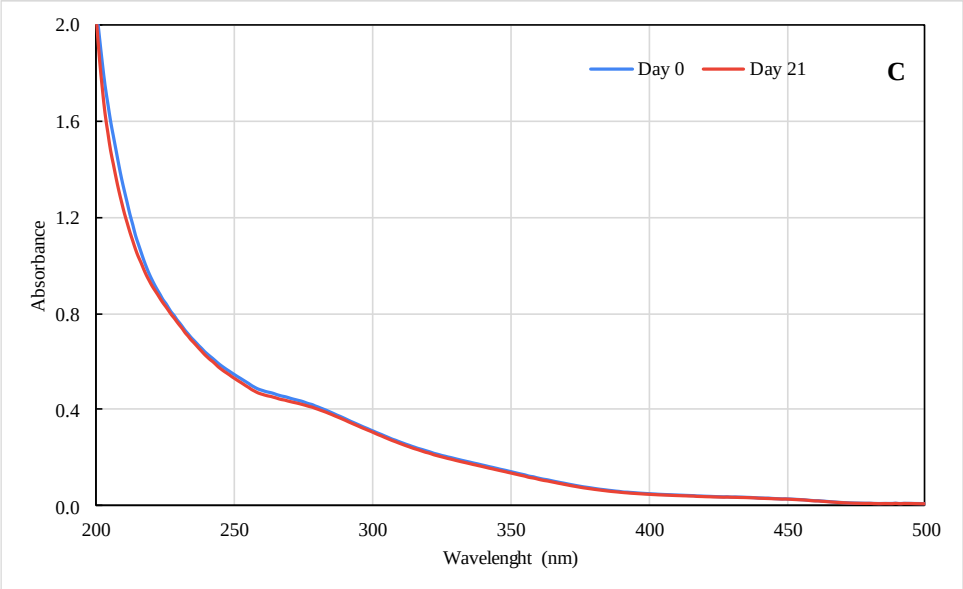


Figure 31. Absorbance (x20 diluted) of reactors for mixed cell leachate; Biotic (A), abiotic with fungal mass (B), abiotic without fungal mass (C), Biotic vs. abiotics at day 21 (D)



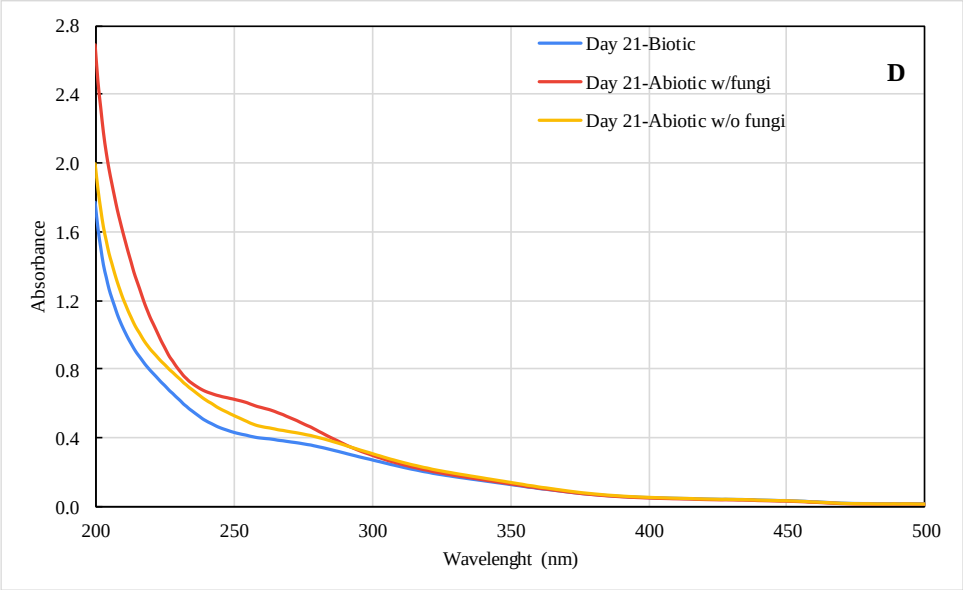
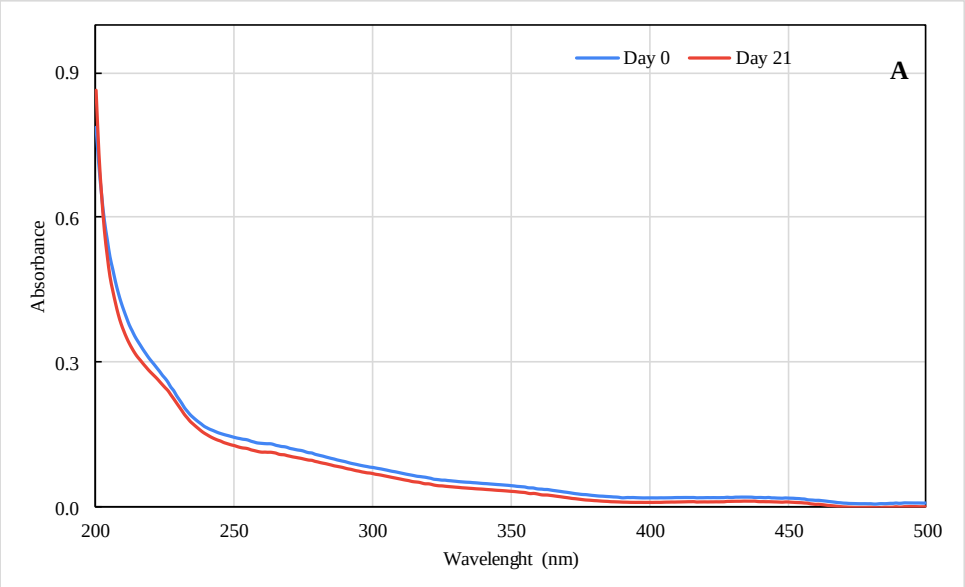


Figure 31, cont.

Figure 32 of S4C LL depicts that the abiotic reactor without fungi had more absorbance decrease than the biotic reactor, although the difference between the two reactors was not significant. Abiotic reactor with fungal mass showed the usual increase in absorbance during the experiment. Nevertheless, at the end of the test, virtually no difference was observable between biotic and abiotic without fungal mass reactors.



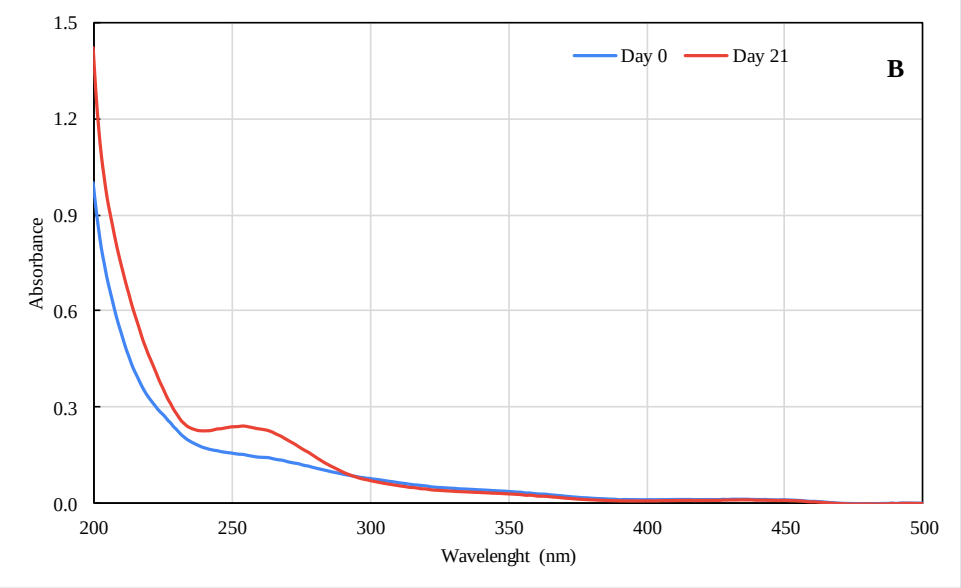
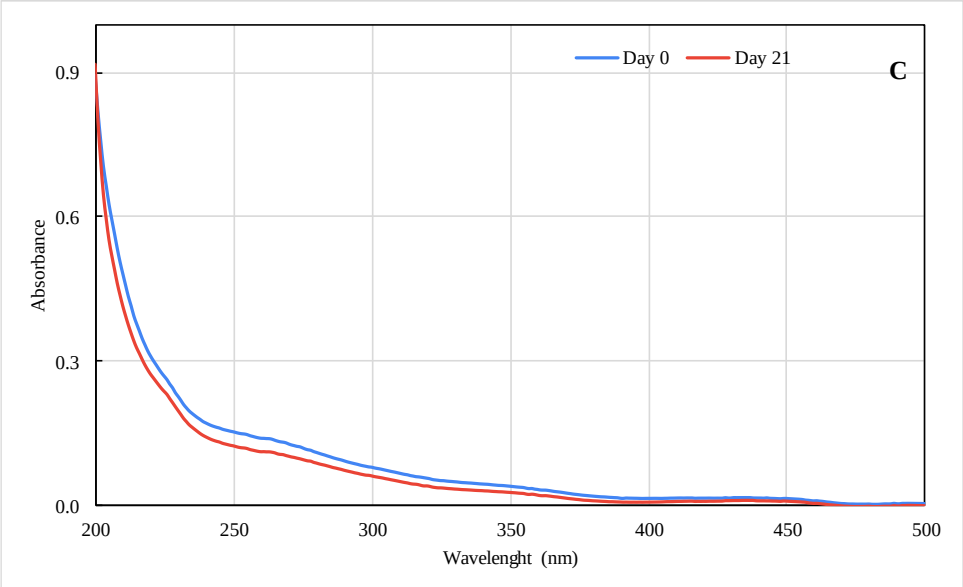


Figure 32. Absorbance (x20 diluted) of reactors for S4C leachate; Biotic (A), abiotic with fungal mass (B), abiotic without fungal mass (C), Biotic vs. abiotics at day 21 (D)



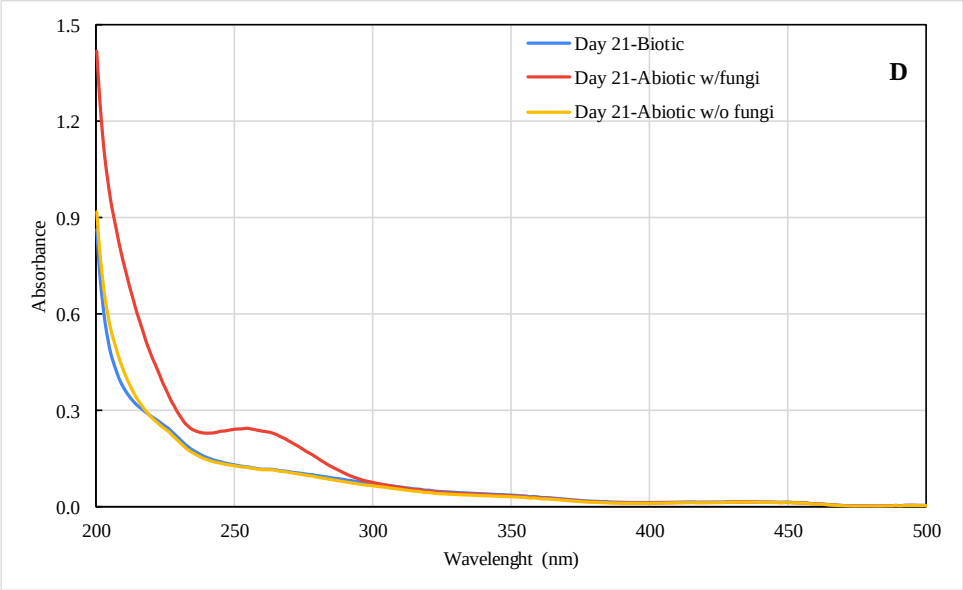


Figure 32, cont.

Overall, absorbance reduction in fungal batch reactors was significant only in the humic LL sample (i.e. mixed cell LL) among five tested LL samples. A study on various mature and young leachates, also showed that biological pretreatment was not efficient in reducing absorbance at 254nm, compared to COD or TOC. This ineffectiveness is likely

related to the incapability of biological treatment to break down recalcitrant organic substances.[105] In other words, removed organics had low UV absorbance.

3.6.4.SEC analysis

Figure 33 illustrates molecular size distribution in five raw landfill leachates that were used in this study (3.7.3). Mixed cell LL had much higher absorbance at the same dilution level compared to other leachates.

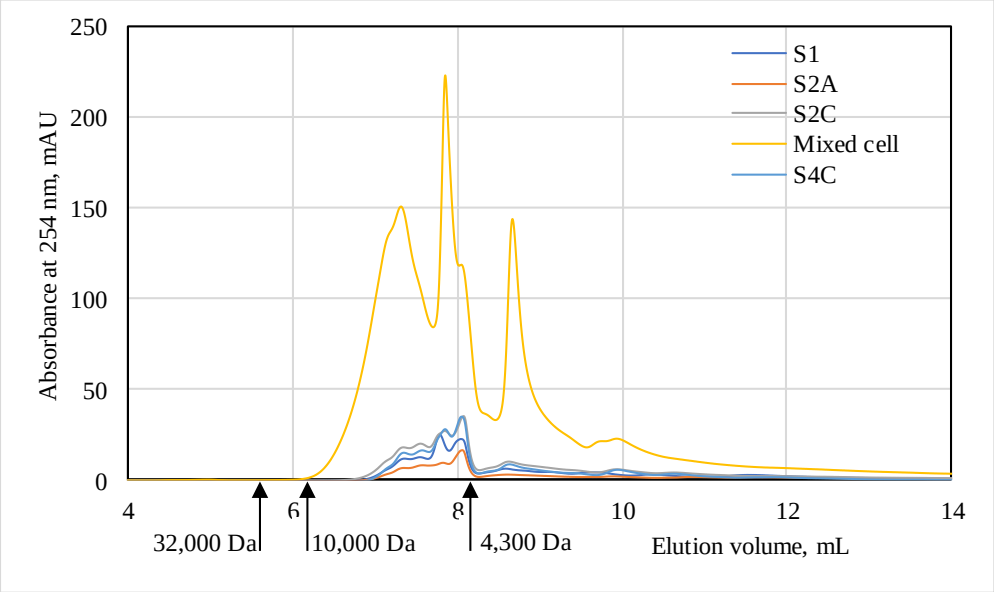


Figure 33. Molecular size distribution in five raw landfill leachates

Figures 34 through 38, provide a comparison between raw LL samples and treated in batch fungal reactor after 21 days. All samples except mixed cell LL, show a slight increase in the absorbance after treatment. Mixed cell LL on the other hand, had a minute reduction at the large MW peak (MW=74000 Da, extrapolated) and about 50% drop at (MW=380 Da, extrapolated).

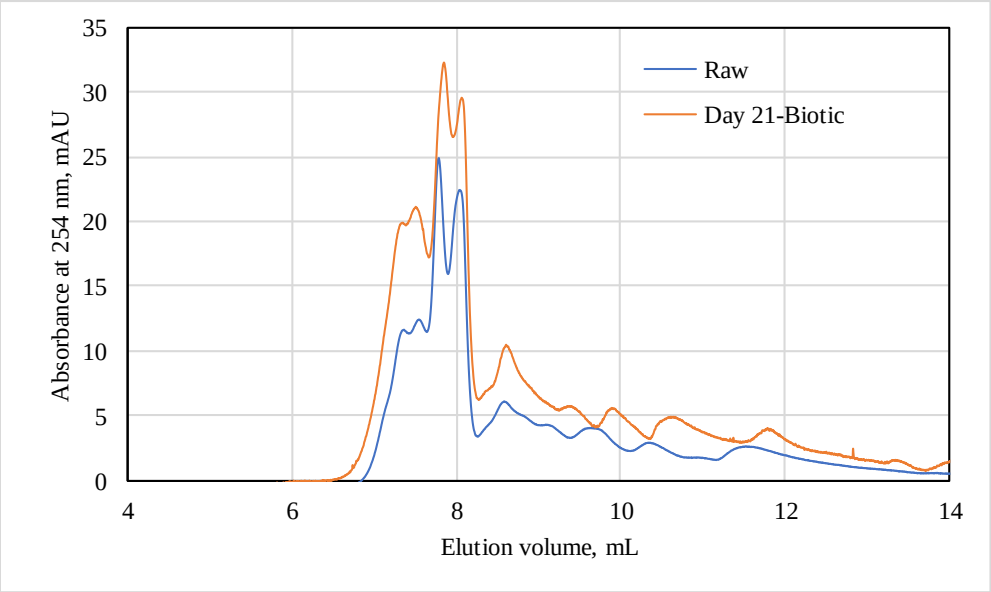


Figure 34. Molecular size distribution in raw and treated S1 leachate

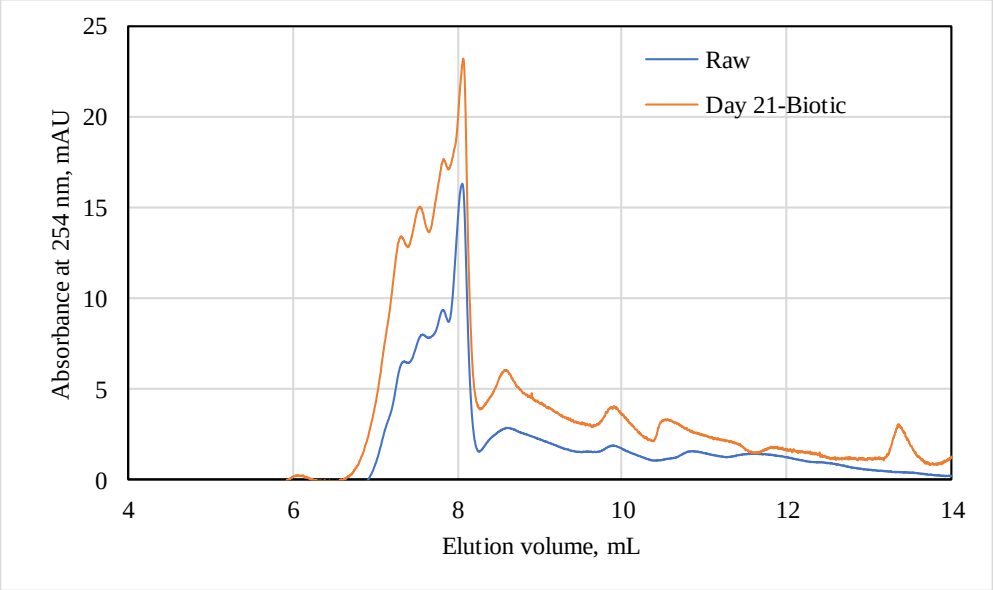


Figure 35. Molecular size distribution in raw and treated S2A leachate

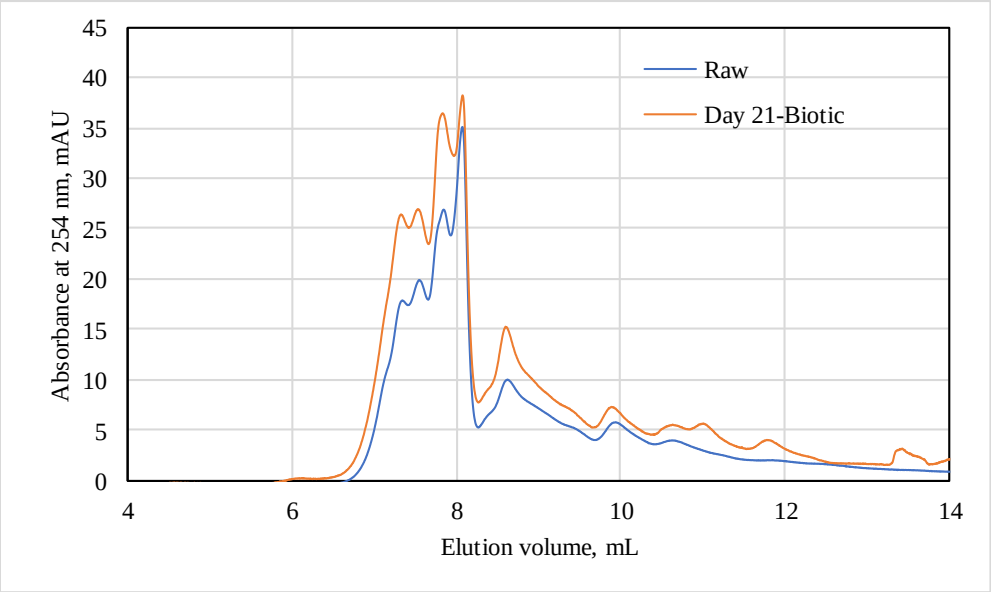


Figure 36. Molecular size distribution in raw and treated S2C leachate

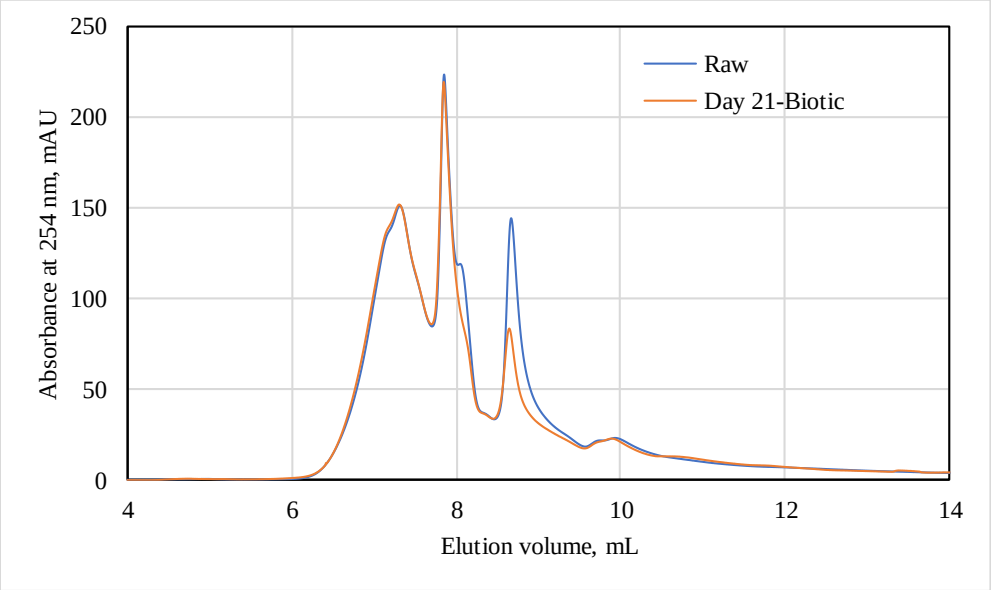


Figure 37. Molecular size distribution in raw and treated mixed cell leachate

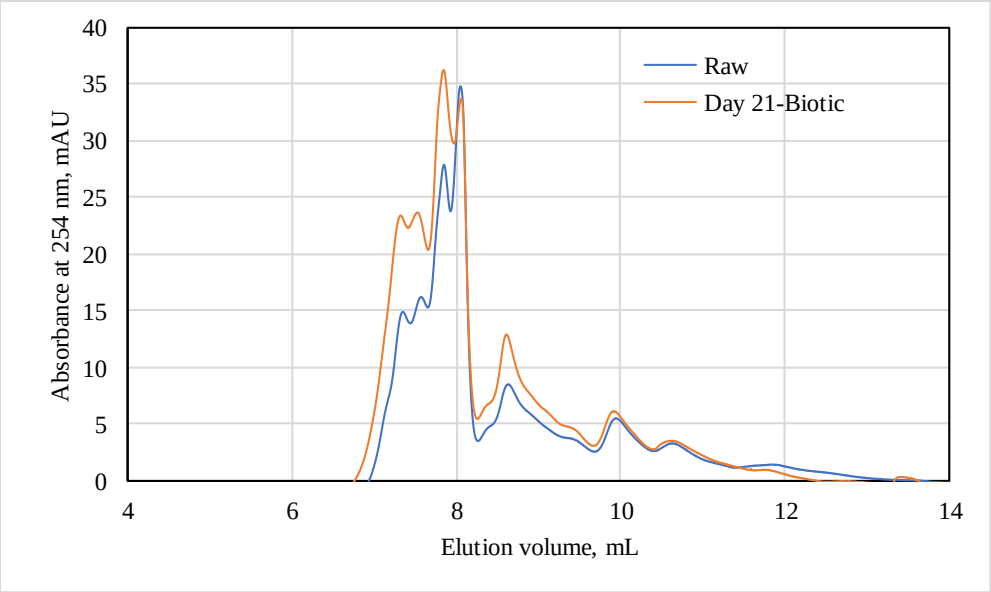


Figure 38. Molecular size distribution in raw and treated S4C leachate

As provided in Table 24, all the samples after treatment had increase in weight-average molecular weight (M_w) and polydispersity (d).[106] Mixed cell leachate has a d value over 2, which means in this sample dissolved organic matters (DOM) molecules consist of wide range of molecular weights (varied over orders of magnitude) and size fractions.[107] The rest of samples on contrast have relatively narrow size fraction. These samples also cluster together at lower M_w compared to mixed cell LL.

Table 24. Weight -average molecular weight (Da) and polydispersity

Sample	Raw leachate		Treated leachate	
	M_w	d	M_w	d
S1	571	1.57	670	1.75
S2A	558	1.54	682	1.76
S2C	626	1.68	731	1.87
Mixed cell	1088	2.25	1140	2.27
S4C	542	1.51	651	1.65

3.7.Plug flow fungal bioreactor

In this section the results from the plug flow column reactor are presented. In this part of the study two reactors were used, biotic and abiotic with fungi, without replication. The LL used was the same as batch reactor studies (I and II) for consistency (mixed cell, humic LL). The fungal mass of the sacrificial reactor was 2.12 g (in one liter) initially. On day 10 fungal mass was replenished at 0.37g for both reactors. The pH in the biotic reactor reached neutral over 15 days of operation, while in abiotic with fungi it only took 6 days to increase to neutral pH from initial pH of 4.5.

Figure 39 provides aggregate parameters of biotic and abiotic reactors over 15 days of operation. The biotic reactor showed 35.7% and 32.7% reduction is COD and TOC respectively while, 9% of increase in TN. The replenishment of fungal mass at day 10 did not improve the removal of carbon content but rather contributed to a miniscule increase in TN. The abiotic reactor with fungal mass depicted significant increase in all parameters, particularly in TOC (34.6%).

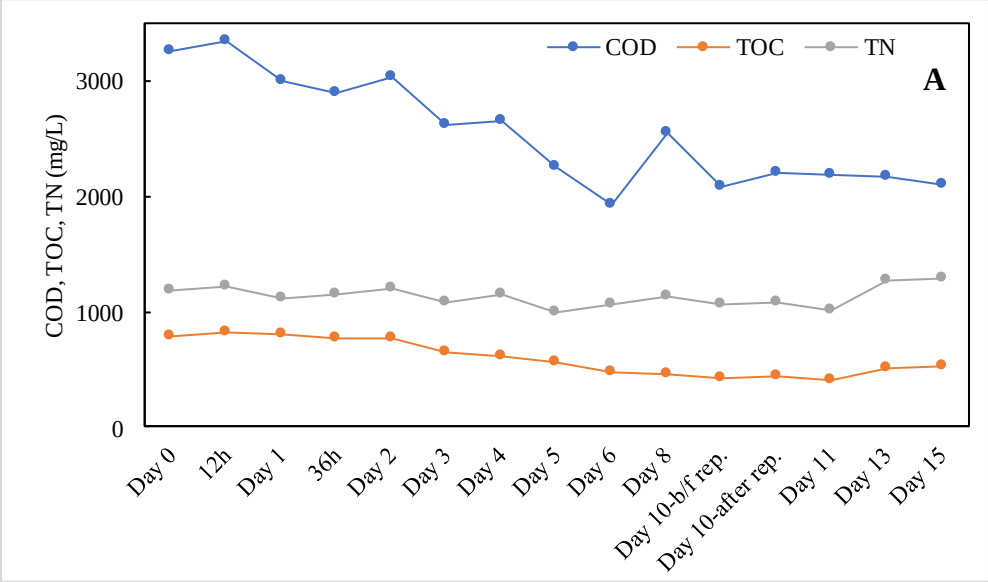


Figure 39. Aggregate parameters in fungal column reactors; Biotic (A), abiotic with fungal mass (B)

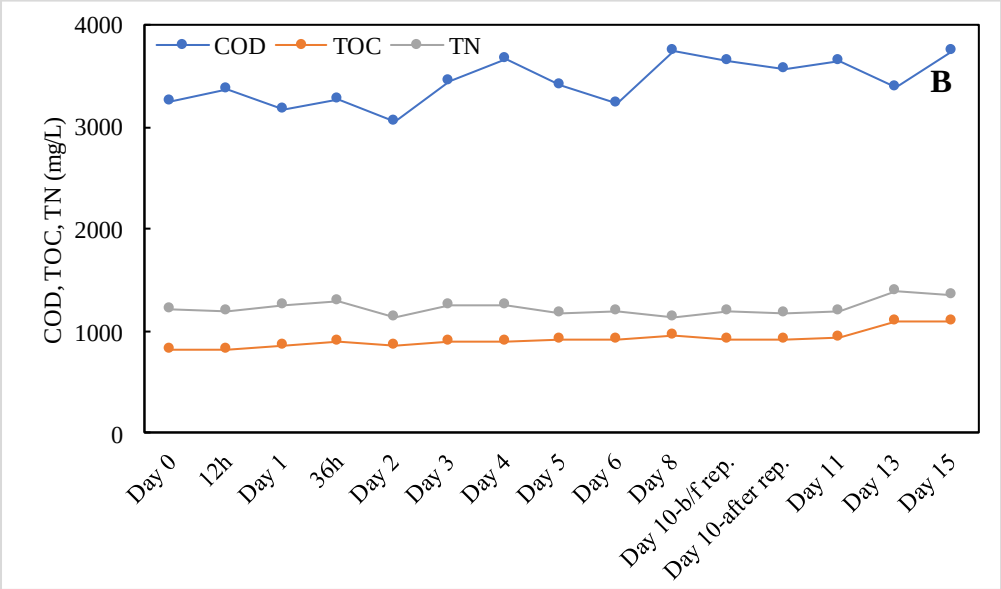


Figure 39, cont.

Statistical analysis showed significant difference ($p<0.05$) between all aggregate parameters in Figure 39.

Figure 40 illustrates the level of spiked analytes over the operation period of biotic and abiotic with fungi reactors. Phenol was the first compound that was removed in the biotic reactor within 36 hours, whereas it remained unchanged in the abiotic reactor. Detection of IBP has been blocked by a large “ghost peak” after day 4 in the biotic reactor, but the trend was towards decrease in IBP until then, with about 40% of removal by day 4. The same incident happened in abiotic reactor for IBP detection, although removal was lower at about 25% by day 4. ACT was removed at 75% in the biotic reactor slowly over time, while only 33% was removed in the abiotic reactor mainly at the last few days of operation, probably due to fungal mass replenishment. BPA was removed most probably via sorption on fungal mass but was released back after 11 days in the biotic reactor, while it remained adsorbed in the abiotic one. This pattern of adsorption-desorption for

bisphenols is consistent with what was observed in fungal batch reactor studies. CIP was removed the same in both reactors around 40% most likely via sorption. The rest of spiked contaminants behaved similarly in both biotic and abiotic reactors and were removed completely by sorption within 13 to 15 days.

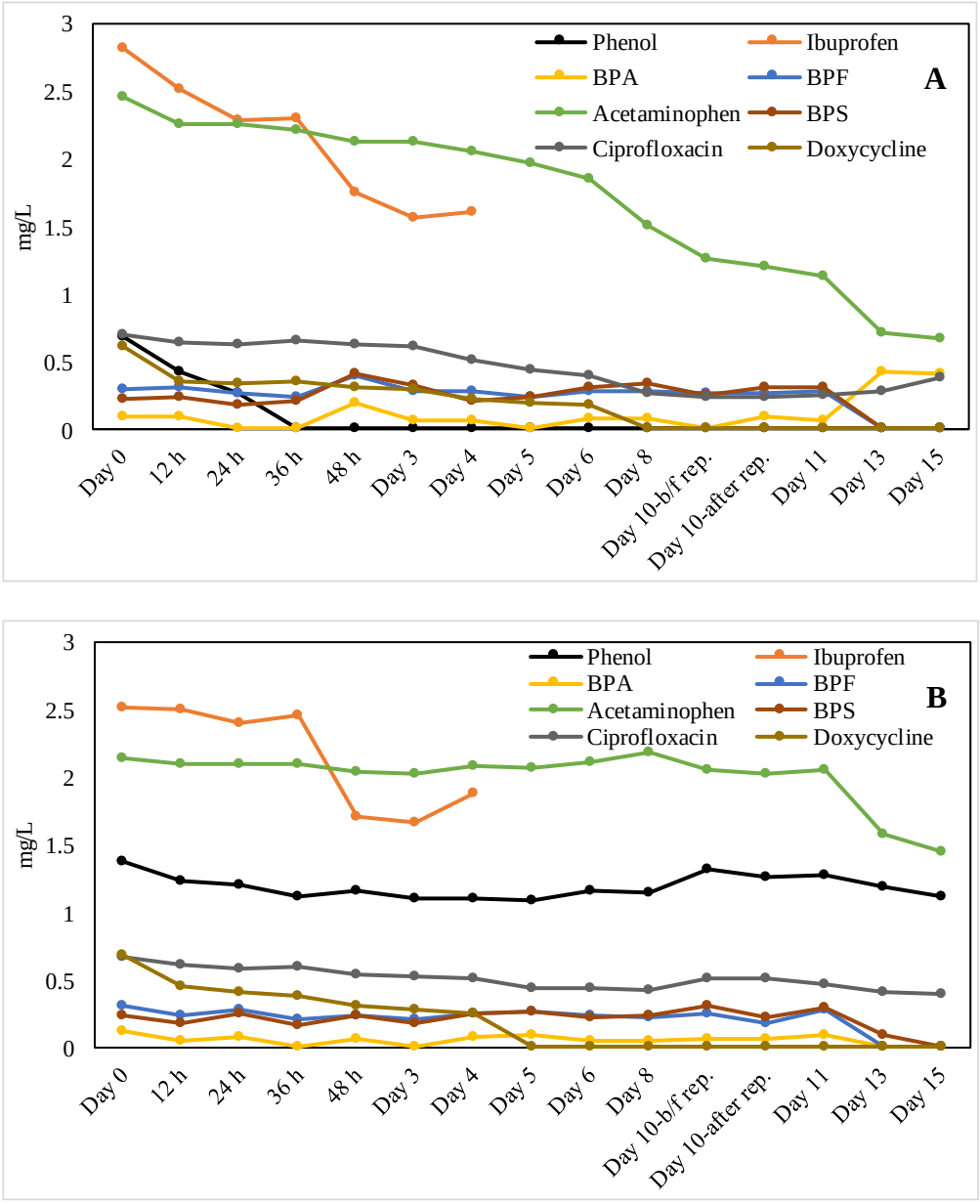


Figure 40. Contaminants removal in fungal plug flow reactors; Biotic(A), abiotic with fungal mass (B)

Excluding phenol, no other compound showed significant decrease ($p<0.05$) in the statistical analysis between biotic and abiotic reactors. ACT removal was relatively significant with p-value of 0.092. The contribution of adsorption onto fungal mass to overall removal, as explained by Lucas et. al, [108] varies among different fungus strains and PhCs (7% on average), although it should be taken into account as it still contributes to the removal and comparable to adsorption onto activated sludge.

Figure 41, provides absorbance scan of biotic and abiotic reactors at day 0 and 15. The biotic reactor did not show a significant change in absorbance over 15 days of operation, while as expected absorbance increased in the abiotic reactor with fungi.

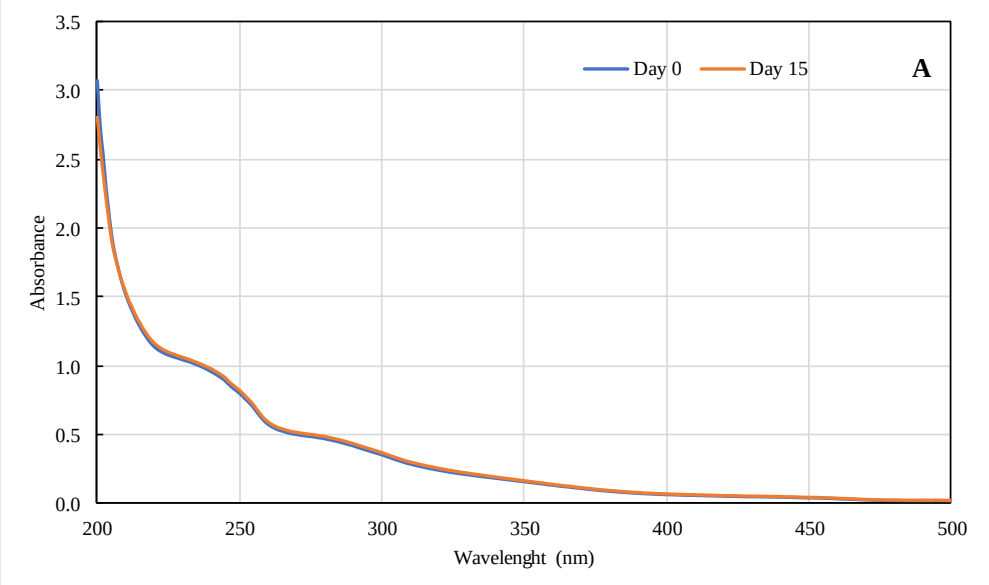


Figure 41. Absorbance (x20 diluted) in fungal plug flow reactors; Biotic (A), abiotic with fungal mass (B)

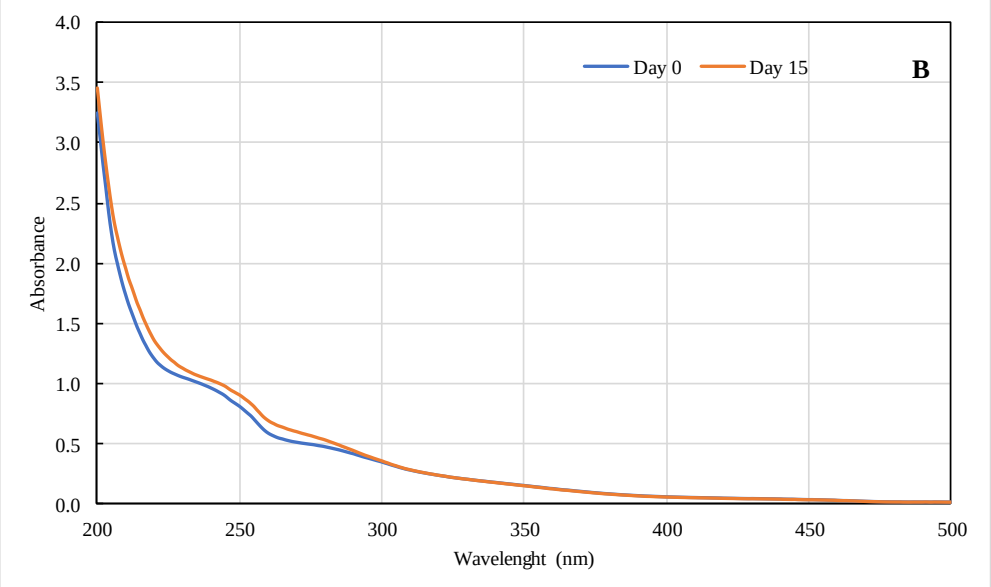


Figure 41, cont.

SEC analysis of biotic samples have been done for raw and treated LL. As illustrated in Figure 42, a shift in the peaks happened after treatment. Specifically, the highest peak in raw LL with MW at 24000 Da decreased in treated LL and formed a larger peak around 650 Da. This shows the capacity of fungal plug flow treatment to break down big and recalcitrant organic molecules into smaller, more bioavailable ones.

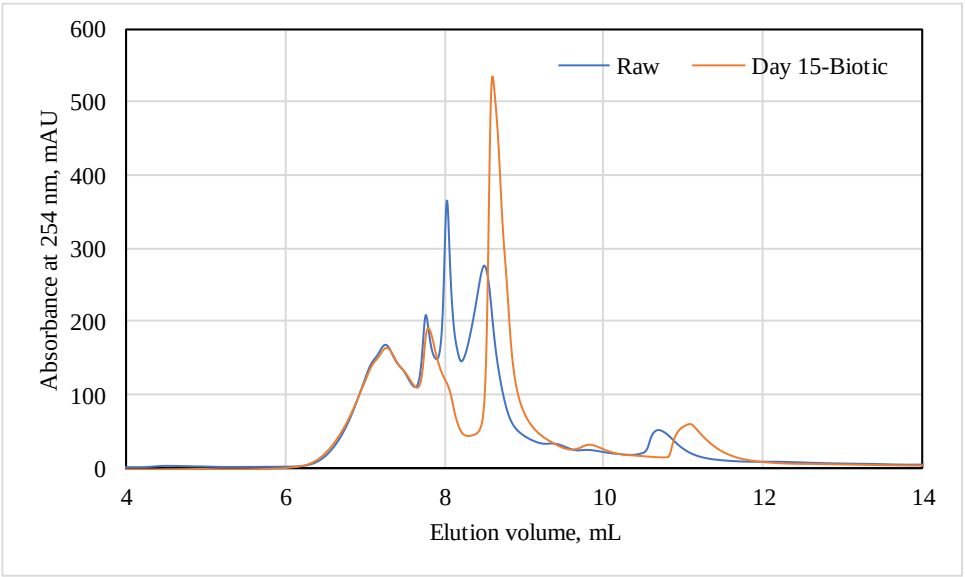


Figure 42. Molecular size distribution of raw and treated leachate in column reactor

3.8.Bioassay of leachate for toxicity

D. magna bioassay was originally used for assessing LL toxicity, but due to complexities in cultivation of this organism and experience deficiency, a decision was made to opt for activated sludge respiration inhibition test.

3.8.1.Activated sludge respiration inhibition test

Obtained respiration rate of blank (triplicate) and positive control of copper(II) sulfate (duplicate) is provided in Table 25.

Table 25. Respiration rates of blank and positive control (mean with SD)

Control type	Respiration rate (mg/L.h)	R^2, average
Blank	0.051±0.002	0.9884
Positive control (11.1ppm)	0.033±0.002	0.9795
Positive control (19.2ppm)	0.012±0.002	0.97
Positive control (34.5ppm)	0.005±0.001	0.972

Percentage of inhibition in respiration rate is the measure used for representation of inhibitory properties of the leachate samples before and after fungal treatment. Inhibition percentage of raw and treated LL at three dilutions (1/1000, 1/100, 1/10) from absorbance reduction study (section 3.7.3) is presented in Figure 43.

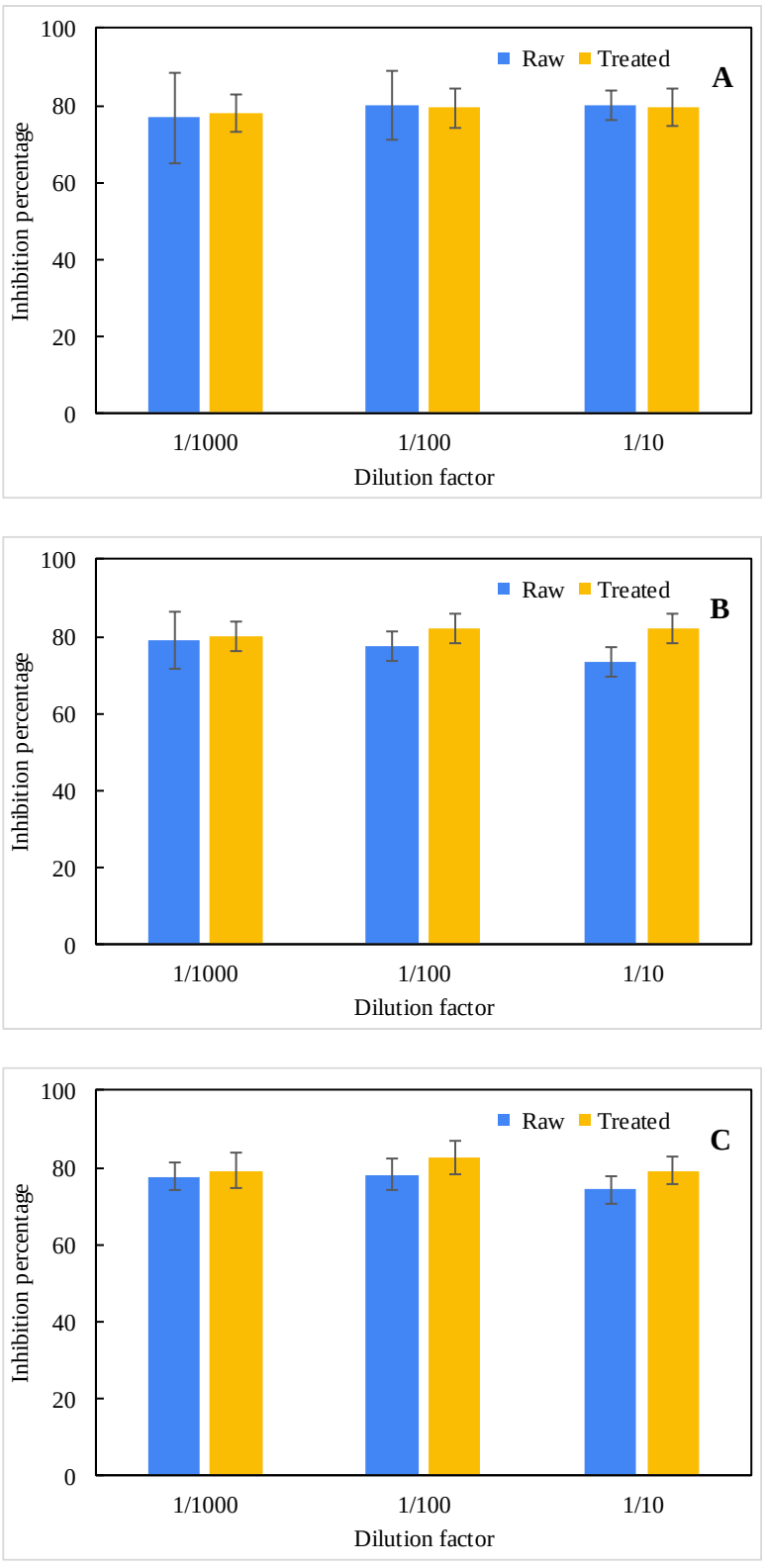
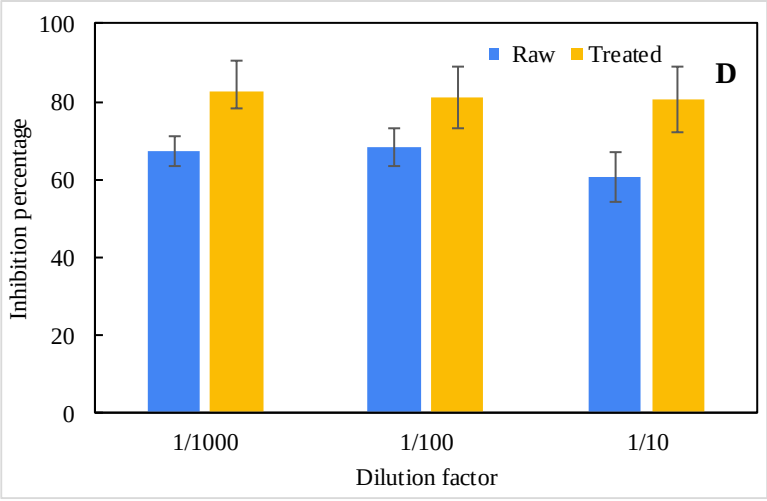


Figure 43. Percentage of inhibition of raw and treated LL samples in fungal batch reactor; S1 (A), S2A (B), S2C (C), Mixed cell (D) and S4C (E)



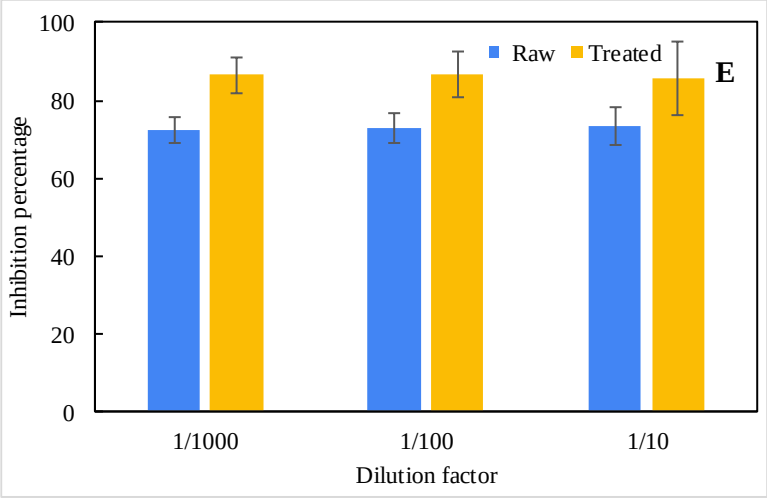


Figure 43, cont.

Sample A was the only LL which did not show change in inhibition after treatment. As can be seen in Figure 43, the rest of samples exhibit slightly higher inhibition at higher concentration of LL (1/10 dilution factor). Although because inhibition of raw LL samples is not consistent with the dilution factor, no conclusion can be made. There could be two explanations for this phenomenon. First, the tested raw leachates in the given setting had no toxic effect on activated sludge, but contrarily at higher concentration acted as food source and therefor showing less inhibition. On the other hand, treated leachate lost readily biodegradable fraction of its organics, hence displaying higher

inhibition. Second, the intrinsic sensitivity of activated sludge varied over the time that tests were in progress (about 10 days) and thus showing different inhibition responses.

Activated sludge process is used frequently for treatment of landfill leachate or toxicity removal.[109,110] To the best knowledge of the author and as stated by Thomas et. al [59] activated sludge inhibition test has not been applied for biotoxicity measurement of LL. This is despite the two main advantages of this test: it can quickly be done in three hours and the required equipment is cost efficient.

3.9.Microbial uptake of selected pharmaceuticals

The main goal of this part of study was to assess the efficiency of basic aerobic and anaerobic predominantly bacterial processes on the removal of both recalcitrant and biodegradable PhCs as compared to fungal bioreactors.

As discussed in the methods section, the analysis of the samples was done by a third-party lab. The reported LoQ of pharmaceuticals in the study is presented in Table 26.

Table 26. LoQ of analytes in this study

	Carbamazepine	Diclofenac	Doxycycline	Ibuprofen
LoQ (ng/L)	39.1	39.1	19.5	39.1

Figures 44 to 47, present the concentration of the four analytes in the aerobic and anaerobic microbial reactors in three sets of reactors: PhC-spiked with glucose (additional feed), PhC-spiked without glucose (in deionized water, DI) and non-spiked control.

The initial spiked concentration of influent for CBZ, DCF, DOX, and IBP were 513.0±82.9, 212.2±72.2, 803.3±47.0 and 492.3±253.2 ng/L respectively. Aerobic removal of DCF and IBP were about 95% in 7 days in both sets of spiked reactors with

added glucose and in DI, while CBZ degradation was about 50%. Most of these removals occurred within first three days of experiment. In anaerobic reactors DOX removal was almost the same as aerobic at 95%, while CBZ concentration decreased about 66% anaerobically for both sets of spiked with added glucose and DI. Although, DCF eliminated fully in aerobic reactors, the anaerobic removal efficiency was 51% and 73% for spiked with added glucose and DI sets respectively. For both anaerobic sets a two-fold increase in IBP was observed after 3 days, while same increase was observed for PhC-spiked with added glucose sets at the end of the experiment. DOX, IBP, DCF and CBZ removal was reported at 99%, 99%, 50% and <25% in conventional activated sludge which is the same as DOX and IBP removal in this study, but was virtually half of the elimination for the other two as compared to this study.[88,111,112] In anaerobic reactors, DOX was shown to be removed at 61% [113] which is less than of 95% observed removal. CBZ remained constant or showed negative removal in anaerobic digestion in prior studies [111], while 66% elimination was observed on average for both sets of spiked reactors in this study. Under anaerobic conditions DCF removal was limited at 22% in previous reports [112] while in this study 51% reduction for spiked reactors with added glucose was measured, and higher removal of 73% was observed with spiked DI. Negative removal of IBP in anaerobic reactor, has been reported previously which could be due to deconjugation. Most of excreted IBP from microbial cells is in conjugated or metabolized form, and not from full degradation.[112] Statistical analysis, comparing spiked reactors with glucose versus spiked in DI showed no significant correlation ($p>0.05$) in either aerobic or anaerobic reactors for any of analytes over the course of the test.

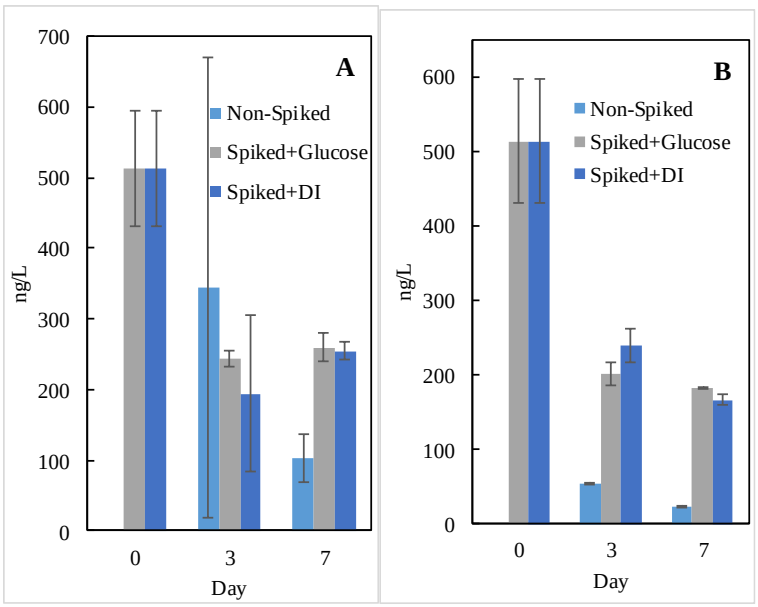


Figure 44. Carbamazepine concentration (mean with SD); Aerobic (A) and anaerobic (B)

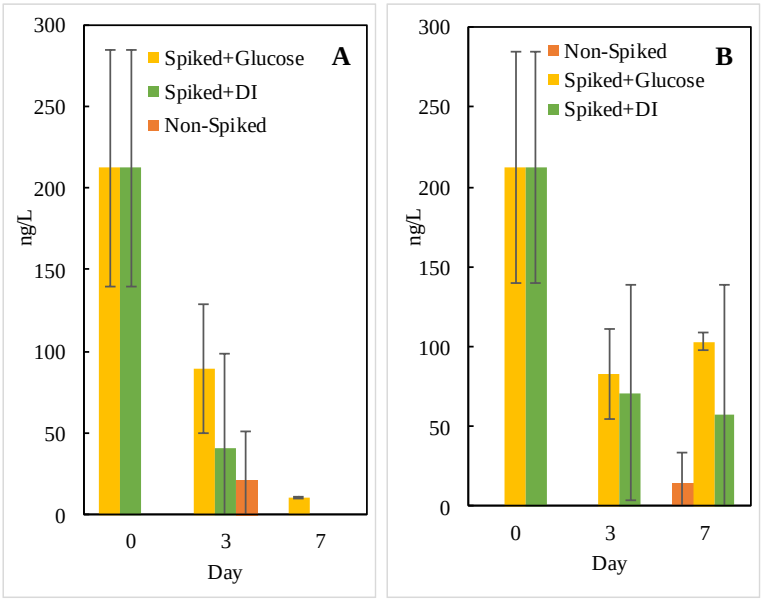


Figure 45. Diclofenac concentration (mean with SD); Aerobic (A) and anaerobic (B)

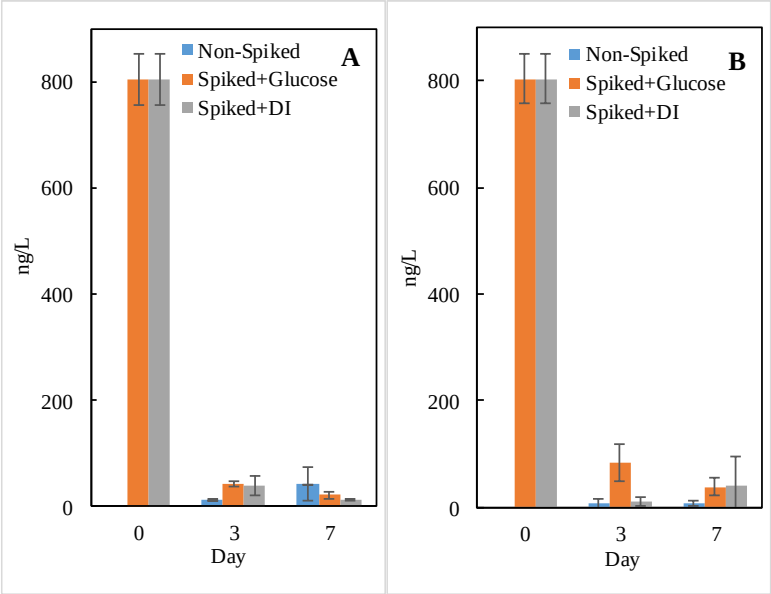


Figure 46. Doxycycline concentration (mean with SD); Aerobic (A) and anaerobic (B)

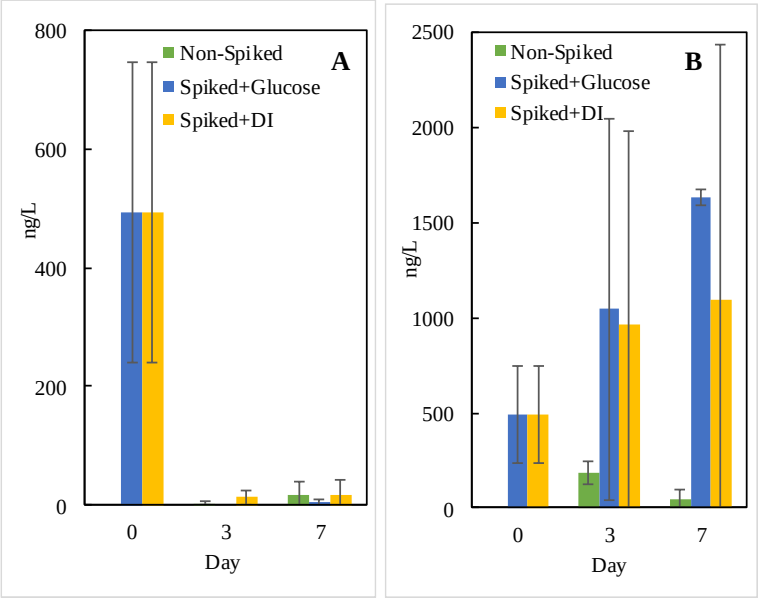


Figure 47. Ibuprofen concentration (mean with SD); Aerobic (A) and anaerobic (B)

CHAPTER 4: CONCLUSIONS

The main goal of the present study was to demonstrate applicability of fungal reactors for simultaneous removal of CECs and UVAS. Overall, *PC* showed that it can be grown in undiluted leachate with minimal operational investments, specifically lowering the pH of leachate to 4.5 and adding supplemental manganese at 0.05%. However, the enzyme activity in leachate was lower compared to the activity in uninhibited growth in a nutrient broth as reported in literature. The performance of the reactors showed plausible reduction in levels of a few CECs and humic substances in some leachates, with more detailed summary in the sections below.

4.1.Fungal bioreactors

The selected WRF, *PC*, showed satisfactory growth in presence of LL at ambient temperature, pH of 4.5 and micronutrient (i.e. manganese) availability at 0.05%. The maximum specific growth rate of *PC* was 0.56 d^{-1} at 30 °C.

In fungal batch and plug flow reactors, phenol as a regulated compound in LL, at a spiked concentration higher than regularly reported levels in municipal LL, has been completely removed in two days or less. Such level could be toxic to aquatic organisms and to bacterial processes (e.g. AS). This is a significant finding as this is the first study to demonstrate such efficient phenol removal by *PC*, which holds a potential for full-scale applications. ACT was another significantly removed compound up to 75% in both batch and plug flow bioreactors. Although biodegradation of CIP was significant in batch reactors, the plug flow reactors were not displaying the same results; therefore, the results

were inconclusive. A consistent pattern of adsorption-desorption was observed for bisphenols in all bioreactors.

The bioreactors showed a substantial reduction in carbon content with about 40% reduction in COD, 33% as TOC, slight change in TN but considerable accumulation of orthophosphate or organic phosphorous (in batch bioreactors) and increase of pH from acidic to neutral in the course of experiment. A maximum of 25% reduction in UV absorbance was observed in batch reactors, while no significant change was detected in the plug flow reactor. SEC results for the plug flow reactor showed a significant shift from higher to lower molecular size organic matter.

The comparison between five batch bioreactors for reduction of UVAS, showed the fungal reactors were not efficient in reducing UV absorbance while they were effective in removal of carbon content.

Despite the biotoxicity results not showing significant toxicity removal from leachate during treatment, only a slight suppression of microbial respiration by leachate was observed in general. The activated sludge respiration inhibition test was found to be applicable for biotoxicity evaluation of LL.

In conclusion, fungal bioreactors showed to be effective in degrading a few CECs and reducing UVAS in very humic LL, but they have limitation such as long reaction time, the continuous need for addition of micronutrient and pH control. In non-sterile full-scale operating fungal reactor in, stabilization of pH and micronutrient, refurbishment (and waste) of fungal mass and probably providing extra feed (e.g. ligninolytic or carbohydrate based), should carefully be done. All these measures are necessary to keep biomass viable, and the competing microorganisms minimal. The amount of fungal mass

in the reactor is a key factor in the ultimate efficiency of reactor. Considering the heterogeneity of LL, achieving a steady-state reactor with satisfactory effluent could be challenging. Nevertheless, fungal bioreactor results showed this process is promising for up scaling it as a form of pretreatment for LL before releasing to POTWs, especially for treatment of phenol.

4.2.Predominantly bacterial aerobic and anaerobic reactors

The aerobic reactors showed high performance at 95% in removal of DCF, IBP and DOX, and moderate efficiency of 50% for removal of CBZ. In the anaerobic reactors CBZ was removed better compared to the aerobic reactors, at 66%. DCF was moderately removed in the anaerobic reactors, 51-73%, and similar removal for DOX was observed compared to the aerobic reactors. No significant effect was observed with addition of glucose. Overall, the aerobic reactors had a better performance in removal of selected CECs than anaerobic reactors. Also, conventional microbial processes (both aerobic and anaerobic) appeared to be superior for CEC removal than fungal bioreactors, based on the select set of compounds analyzed.

4.3.Landfill leachate

Among all categories of samples, closed cell (<5 years) LL samples had the highest COD, TN and NH_3-N. Lowest and highest transmittance at 254 nm, 50% and 75% (mean), belonged to active cell and closed cell (>10 years) samples respectively. Significant correlation between T_{254} and TN was observed in all types of samples. A

significant correlation was observed in some of the landfills/cells between COD and TOC with T_{254}, excluding closed cell (>10 years) and mixed cell LL samples.

In this investigation ACT, CBZ, AMX, ATN, DOX, MET, RAN, TMP, IBP, EE2, BPA and BPS were detected in non-hazardous waste landfill leachates with various closure status. ACT, ATN and BPS were consistently detected at lower concentrations in closed cell LL. There was no significant difference as a function of the landfill closure status in the concentrations of the rest of the analytes. The results indicate that contamination of leachate from deposited waste continues for a long period of time, and leachate would need treatment for CECs likely for the lifetime of the landfill.

4.4.Future perspective

The mitigation of any form of pollution with WRF and its enzymes have been of high research interest for the last few decades. Despite the promising outcomes from a large number of studies, there is no known full-scale facility utilizing such processes. The current available biological technologies are not always the best, technically or economically. While the concerns regarding the emerging contaminants in numerous waste streams is rising, the establishment of state-of-the-art WRF-based technologies require partnerships among research groups, water and waste management industry professionals, and local and federal authorities.

Knowledge gaps that exist on the path to up size the reactor should be addressed with a regression analysis and experimental design method; pH, temperature, retention time of biomass, substrate and micronutrient requirement etc. in operation of a reactor and challenges regarding reliable mycelium production should collectively be addressed. The optimization of all these factors is required to make fungal bioreactors competitive to

traditional technologies. Identification of transformation products, due to WRF different metabolism, may be necessary.

Other alternatives that are not well studied but may be promising are as follows:

- Augmentation of traditional processes with WRF to boost removal of particular contaminants (e.g. CECs)
- Application of WRF reactors as side stream treatment along with conventional processes
- Utilizing waste streams with ligninolytic properties, or high carbon and nitrogen content, to help feed the WRF reactor or mycelium production
- Beneficial use of waste fungal biomass for food or bioenergy, due to its generally high protein content

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